INSTITUT INTERNATIONAL DE CHIMIE SOLVAY

ONZIEME CONSEIL DE CHIMIE

tenu à l'Université Libre de Bruxelles
du 1er au 6 juin 1959

LES NUCLEOPROTEINES

INTERSCIENCE PUBLISHERS, INC.
250, fifth avenue
NEW-YORK, N.Y., U.S.A.

R. STOOPS Editeur
76-78, Coudenberg
BRUXELLES, BELGIQUE

INSTITUT INTERNATIONAL DE CHIMIE SOLVAY

Fondé à l'initiative de Monsieur E. Solvay

le 1er mai 1913

Le onzième Conseil de Chimie Solvay
a rendu hommage

à la mémoire du
Professeur H.J. Backer

et

à la mémoire du
Professeur K. V. Linderström-Lang
Docteur Honoris Causa de l'Université Libre de Bruxelles

respectivement ancien membre et membre du Comité Scientifique
où leur collaboration avait été hautement appréciée

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LE RÔLE BIOLOGIQUE DES ACIDES NUCLÉIQUES

par **J. BRACHET**
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I. INTRODUCTION

Il ne fait pas de doute que l'intérêt que l'on porte actuellement aux acides nucléiques a eu pour point de départ leur importance biologique : on a, en effet, de très fortes raisons de croire qu'ils jouent un rôle fondamental dans deux phénomènes d'une importance capitale en biologie : la transmission des caractères héréditaires et la synthèse des protéines. Les faits expérimentaux qui ont conduit à cette double conviction sont extrêmement nombreux et variés : tout ce que nous pourrons faire, dans le présent rapport, sera de résumer, de la façon la plus succincte possible, les principaux arguments en faveur d'une intervention directe des acides nucléiques dans la transmission des caractères héréditaires et dans les synthèses protéiques.

Après une brève esquisse historique, nous examinerons successivement le rôle génétique des acides désoxyribonucléique (ADN) et ribonucléique (ARN), leur intervention dans la synthèse de protéines spécifiques et, enfin, les relations existant entre l'ADN, l'ARN et les protéines au sein de la cellule vivante.

II. BREF RAPPEL HISTORIQUE

La confusion la plus totale régnait encore, en 1930, en ce qui concerne la composition, la localisation et la distribution des acides nucléiques : on croyait, bien à tort, que l'acide « thymo-

nucléique » (notre ADN) ne se rencontre que dans les cellules animales, tandis que l'acide « zymonucléique » (l'ARN d'aujourd'hui) serait caractéristique des tissus végétaux. Il était impossible d'émettre la moindre hypothèse au sujet de leur rôle : des chercheurs qui avaient consacré leur vie entière à l'étude des acides nucléiques et apporté des contributions importantes à nos connaissances sur leurs propriétés chimiques et physiques en arrivaient à conclure que leur rôle serait celui d'un tampon, destiné à maintenir constant le pH du noyau cellulaire (cf. Levene, 1931).

C'est le développement de la *cytochimie,* c'est-à-dire celui des méthodes destinées à identifier la composition chimique des divers constituants de la cellule, qui a permis de sortir de l'ornière et qui a conduit à l'efflorescence présente de travaux sur le rôle biologique des acides nucléiques. Un premier — et très important — pas en avant a été réalisé lorsque Feulgen et Rossenbeck, dès 1924, ont mis au point une élégante méthode de détection de l'ADN, basée sur les propriétés aldéhydiques de l'acide apurinique (produit de l'hydrolyse acide ménagée de l'ADN) : leurs recherches ont montré que l'ADN est un constituant spécifique du noyau cellulaire, plus précisément de la chromatine, dans la cellule au repos, et des chromosomes dans la cellule en voie de division. La localisation nucléaire de l'ADN est un fait constant, qu'il s'agisse de cellules végétales ou de cellules animales.

Il a fallu attendre plus de quinze ans pour que la question de la localisation et de la distribution de l'ARN soit, à son tour, résolue : des techniques cytochimiques basées sur l'absorption dans l'ultraviolet des acides nucléiques (Caspersson, 1941) et sur leur affinité pour les colorants basiques (affinité qui peut être éliminée spécifiquement par une digestion enzymatique préalable à la ribonucléase : Brachet, 1941) ont, enfin, permis de suivre la distribution de l'ARN. Ces observations ont conduit à des conclusions claires : tout comme l'ADN, l'ARN se rencontre tant chez les animaux que chez les plantes; mais, alors que l'ADN est essentiellement un constituant de la chromatine, c'est surtout dans le nucléole et dans le cytoplasme qu'on trouve de l'ARN. Comme Caspersson et nous-même l'avons montré simultanément et indépendamment en 1941, il existe une corrélation entre la teneur en ARN d'une cellule et son aptitude à synthétiser des protéines (voir ci-dessous).

Dès lors, deux conclusions importantes paraissent émerger : l'ADN, constituant spécifique des chromosomes qui sont le support des gènes mendéliens, interviendrait dans la transmission des caractères héréditaires; l'ARN jouerait un rôle dans la synthèse des protéines cellulaires. Ce sont ces deux aspects du problème du rôle biologique des acides nucléiques qui feront l'objet principal de ce rapport.

III. LE RÔLE DE L'ADN DANS LA TRANSMISSION DES CARACTÈRES HÉRÉDITAIRES

C'est probablement à Boivin et Vendrely (1948) qu'on doit d'avoir exprimé pour la première fois de façon claire l'idée qu'à chaque gène correspondrait une molécule spécifique d'ADN. Il découle nécessairement de cette hypothèse de base qu'il doit exister un très grand nombre d'espèces moléculaires distinctes d'ADN au sein d'un même noyau cellulaire (autant qu'il contient de gènes, c'est-à-dire des milliers). D'autre part, si on admet que les noyaux de toutes les cellules d'un même organisme contiennent les mêmes gènes, on doit en déduire que ces noyaux renferment tous la même quantité d'ADN. Il est évident aussi que, puisque l'un des attributs fondamentaux du gène est sa capacité de se reproduire identique à lui-même au cours des générations, la teneur en ADN du noyau doit très exactement doubler au moment de la division cellulaire.

A l'heure actuelle, il est devenu impossible de donner une définition simple du gène : on peut le considérer comme une particule chromosomiale, séparable par « crossing over » lorsque les filaments chromosomiaux se rompent et se reconstituent (le gène peut donc être considéré comme une unité de *recombinaison* génétique). On peut l'envisager, aussi, comme une « unité de *mutation* » : s'il n'y avait pas de mutations, c'est-à-dire si tous les gènes allèles étaient identiques, nous serions tous semblables les uns aux autres et... il n'y aurait pas de génétique ! Enfin, le gène est une unité *fonctionnelle* : c'est par les potentialités qu'il apporte que la synthèse de protéines spécifiques — d'enzymes, par exemple — qui pourront conditionner l'apparition de caractères morphologiques, sera ou non possible. Sans entrer ici dans le détail, disons que, dans de nombreuses expériences, le gène « unité de recom-

binaison », le gène « unité de mutation » et le gène « unité fonctionnelle » ne sont pas identiques : à l'heure présente, on renonce de plus en plus à l'idée du gène particulaire et on le considère plutôt comme un fragment d'une macromolécule géante, dont les différentes portions entrent en interaction les unes avec les autres.

Après ce trop sommaire examen de la notion même du gène — notion qui devient de plus en plus fluide — passons très rapidement en revue les nombreux faits qui indiquent que l'ADN joue un rôle primordial dans la constitution de cette unité héréditaire.

a) Les recherches de Chargaff (1957) sur la composition en bases des ADN extraits de nombreuses espèces animales, végétales et bactériennes ont clairement montré que les ADN présentent une *spécificité d'espèce* bien reconnaissable. Toutefois, les ADN extraits des organismes les plus divers présentent aussi des ressemblances (régularités) frappantes : la teneur en adénine est égale à celle en thymine et il y a autant de guanine que de cytosine. Ce sont ces régularités qui ont permis à Watson et à Crick (1953) de proposer un modèle extrêmement ingénieux de la structure de la molécule d'ADN, qui a le grand avantage d'expliquer le mécanisme de sa réduplication; l'hypothèse de Watson et Crick sera exposée en détails dans l'un des rapports suivants.

Ajoutons que les travaux de Bendich (1955) indiquent l'existence de plusieurs ADN distincts, dans un même organe. Certes, on ne peut prétendre que l'hypothèse initiale de Boivin (à chaque gène correspondrait un ADN spécifique) soit démontrée et que chaque noyau contienne autant de molécules d'ADN spécifiques que de gènes; il est plus probable, comme l'a souligné notamment Benzer (1957), que le gène n'est qu'une séquence de nucléotides dans une macromolécule géante d'ADN. Néanmoins, les données dont on dispose actuellement sur la spécificité et la structure des ADN sont en parfait accord avec les propriétés qu'on est en droit d'attendre du matériel génétique.

b) Les gènes mendéliens étant incontestablement localisés dans les chromosomes, la substance chimique qui s'identifie au matériel génétique doit avoir une localisation strictement chromosomiale : nous avons déjà vu plus haut que l'ADN est, effectivement, un

constituant spécifique de la chromatine et des chromosomes et qu'il remplit donc cette condition.

Au cours de ces dernières années, on a signalé à plusieurs reprises l'existence d'ADN « extra-nucléaire » : sans entrer ici dans une discussion de la question, qui reste controversée et que nous avons examinée en détails ailleurs (Brachet, 1957), disons qu'il est effectivement fort probable que les œufs vierges des insectes et des batraciens contiennent un excédent d'ADN dans leur cytoplasme. Mais rien n'indique que cet ADN cytoplasmique puisse jouer un rôle génétique quelconque et il est même douteux qu'il constitue un précurseur de l'ADN nucléaire au cours du développement embryonnaire.

c) Si toutes les cellules d'un même organisme contiennent les mêmes gènes (ce qui n'est d'ailleurs nullement démontré, car la génétique des cellules somatiques est encore dans l'enfance), il faut s'attendre à trouver une *teneur constante en ADN par noyau* diploïde au repos. C'est ce que de nombreux auteurs (Alfert, 1954; Vendrely, 1956, etc.) ont effectivement constaté; mais d'autres chercheurs, utilisant les mêmes méthodes, ont observé, au contraire, des variations notables de la teneur en ADN par noyau diploïde au repos, lorsque les conditions physiologiques changent (Fautrez, 1953; Pasteels et Lison, 1950; La Cour et collaborateurs, 1956, etc.). Il est malaisé de conclure : nous croyons qu'il serait dangereux d'accepter comme un dogme la constance *absolue* de l'ADN par noyau. Mais il est incontestable que l'ADN tend à demeurer remarquablement constant par série chromosomiale. Les variations observées ne sont pas surprenantes si on se souvient de ce que de grandes portions des chromosomes (les segments « hétérochromatiques ») ne présentent guère d'activité génétique. De jolies expériences autoradiographiques de Ficq et Pavan (1957) montrent que, dans les chromosomes géants des diptères, certains segments chromosomiaux incorporent fortement de la thymidine tritiée dans leur ADN, alors que d'autres demeurent presque inertes : la synthèse d'ADN, dans ces segments, est en rapport avec leur activité sécrétrice propre. Il est clair que, dans le cas étudié par Ficq et Pavan (1957), il ne peut y avoir une teneur absolument constante en ADN dans le noyau. Mais les variations sont faibles et, tout compte fait, sont à prévoir si on accepte les

conceptions plus dynamiques du gène que les généticiens modernes ont proposées.

d) Le gène se caractérise, nous l'avons vu, par sa capacité de *réduplication;* de nombreuses mesures cytophotométriques ont montré que la teneur en ADN du noyau double, en effet, très exactement avant la division cellulaire. Sans qu'il y ait de règle absolue à ce sujet, cette réduplication de l'ADN se fait généralement dans le noyau au repos et elle cesse quelques heures avant la division elle-même. Il est fort possible que la réduplication de l'ADN se fasse, au sein du chromosome, selon le schéma proposé par Watson et Crick (1953); il serait, toutefois, audacieux de l'affirmer parce que les données autoradiographiques dont nous disposons, à l'heure actuelle, sur l'incorporation de la thymidine radioactive dans l'ADN au cours de la division cellulaire demeurent contradictoires (Mazia et Plaut, 1955; Taylor et collaborateurs, 1957).

e) Une autre caractéristique importante du gène est sa *stabilité* vis-à-vis des variations du milieu externe et interne. Cette stabilité se retrouve dans le cas de l'ADN : en effet, les travaux soigneux de Kihara et collaborateurs (1956), Swick et collaborateurs (1956) et de Daoust et collaborateurs (1956) ont montré que l'ADN présente une exceptionnelle stabilité métabolique dans les cellules au repos du foie. Si leur ADN est le siège d'un « turnover », il est extrêmement faible et son existence même peut être mise en doute.

f) Si le gène présente, généralement, une très grande stabilité, il est cependant susceptible, comme nous l'avons rappelé plus haut, de subir des mutations. La fréquence des mutations peut être considérablement accrue par l'emploi d' « agents mutagènes » variés : radiations ionisantes ou ultraviolettes, substances chimiques comme les ypérites azotiques, etc. Il est frappant de constater que tous ces agents mutagènes touchent fortement la molécule d'ADN, qui finit par se dépolymériser. Le cas des rayons U. V. mérite une mention spéciale : dans de nombreux cas, le spectre d'action de ces radiations coïncide avec le spectre d'absorption des acides nucléiques; c'est-à-dire que, pour une même quantité d'énergie, l'effet mutagène est maximal aux longueurs d'onde où les acides nucléiques absorbent le plus (2.600 Å).

Donc, comme le gène, l'ADN présente une grande stabilité vis-à-vis des conditions extérieures, mais il est particulièrement sensible aux agents mutagènes.

g) Mais il existe un système biologique qui apporte des arguments autrement directs en faveur de l'idée d'une identité entre l'ADN et le matériel génétique : c'est celui des *bactériophages* et des bactéries. La complexité de ce système, au double point de vue biochimique et génétique, est telle que nous ne pouvons mentionner ici qu'une expérience, particulièrement frappante : c'est celle de la « seringue », due à Hershey et Chase (1952).

Les bactériophages sont des virus qui s'attaquent aux bactéries à l'intérieur desquelles ils se multiplient; ils ressemblent un peu à des têtards ultramicroscopiques, en ce sens qu'ils sont formés d'une tête et d'une queue. Celle-ci, tout comme la membrane qui recouvre la tête, est de nature protéique; c'est à l'intérieur de la tête que se trouve concentré l'ADN du phage.

L'expérience fondamentale de Hershey et Chase (1952) a consisté à marquer les protéines du phage au S^{35} et son ADN au P^{32}, puis à infecter les bactéries avec ces phages doublement marqués. L'étude des bactéries infectées a montré qu'il n'y pénètre que des quantités minimes de la protéine du phage; celui-ci enfonce sa queue, comme une aiguille, dans la bactérie et il y injecte son ADN. Cet ADN de phage, qui est chimiquement différent de celui de la bactérie, permet à lui seul la multiplication de nouvelles particules de phage, possédant toutes les particularités génétiques du phage infectant (il existe, en effet, de nombreuses lignées de phages, génétiquement distinctes).

L'expérience montre donc clairement que l'introduction, dans une bactérie, de l'ADN d'un phage permet la multiplication, au sein de cette bactérie, de particules spécifiques de phage et, par conséquent, la synthèse des protéines spécifiques de ce phage. Ajoutons que le mécanisme de cette réduplication de l'ADN est probablement fort complexe; il est établi que la formation de nouvelles particules de phage, après l'injection de son ADN, n'est possible que s'il se produit, dans la bactérie infectée, une synthèse initiale de protéines et d'ARN (Hershey, 1956; Burton, 1955).

h) La situation est beaucoup plus claire encore dans les expériences de *transformations bactériennes* (ou mutations dirigées). Le cas du phage vient de nous montrer que l'ADN constitue, selon toute apparence, le matériel génétique du virus. Mais, dans l'expérience de Hershey et Chase (1952), le phage injecte, outre son ADN, une faible quantité de protéines, auxquelles on pourrait attribuer aussi un rôle génétique. Cette objection tombe dans les expériences de transformations bactériennes, qui constituent la démonstration la plus directe que nous possédions du rôle génétique de l'ADN.

Voici un bref exposé de ces expériences, dont les premières ont été réalisées par Avery et ses collaborateurs (1944) : si on traite des bactéries (pneumocoques), qui sont génétiquement incapables d'élaborer une capsule protectrice, par de l'ADN purifié isolé à partir d'une autre souche, encapsulée celle-là, une proportion appréciable (10-20 %) des bactéries traitées forment une capsule. La capacité de synthétiser la capsule est, dorénavant, acquise définitivement (à moins d'une rare mutation en retour) et elle est donc transmise aux descendants, tout comme le serait un gène. En d'autres termes, l'ADN isolé à partir d'une souche encapsulée se comporte comme un authentique gène dès qu'il a pénétré dans les bactéries réceptrices et qu'il s'est intégré dans leur génome. On donne le nom de « facteur transformant » à l'ADN de la souche qui fournit le caractère (production d'une capsule dans l'exemple choisi); pour que le facteur transformant puisse exercer ses effets, il faut que les bactéries réceptrices soient dans un état physiologique particulier, qui permette la pénétration de l'ADN dans la bactérie et dans ses chromosomes : c'est l'état de « compétence », qui a été récemment analysé par R. Thomas (1955).

Hotchkiss (1949) a pris grand soin de purifier à l'extrême le facteur transformant capsulaire, qui demeure actif à de très grandes dilutions : ses préparations ne contenaient plus de protéines en quantités décelables (inférieures à 0.02 %); elles étaient très rapidement inactivées par une digestion à la désoxyribonucléase et elles résistaient, par contre, parfaitement aux enzymes protéolytiques et à la ribonucléase. L'identité du facteur transformant avec l'ADN fait d'autant moins de doute qu'il est inactivé par tous les agents mutagènes.

Les transformations bactériennes ont été retrouvées (toujours sous l'influence de l'ADN) chez d'autres bactéries que le pneumocoque; chez ce dernier, on connaît, à l'heure actuelle, plus de trente caractères biochimiques distincts qui ont été introduits dans les bactéries par des ADN purifiés et qui se sont, ensuite, maintenus de génération en génération (Hotchkiss, 1955).

En résumé, l'ADN se comporte donc, dans ces expériences, exactement comme un gène qui aurait été mis en solution : une fois introduit dans la bactérie compétente, il lui apporte la possibilité de réaliser des synthèses nouvelles et il se reproduit, identique à lui-même, à chaque génération. Le grand nombre de caractères qu'il est possible de transmettre par transformations bactériennes montre, en outre, qu'il doit exister, dans une même cellule bactérienne, un très grand nombre d'espèces moléculaires distinctes et spécifiques d'ADN; mais il se peut aussi, comme nous l'avons signalé plus haut, que le facteur transformant corresponde plutôt à une partie seulement de la macromolécule d'ADN, partie qui devrait son activité spécifique à la séquence des nucléotides qui le constituent.

Si les expériences faites sur les bactéries ne laissent aucun doute sur le rôle génétique de l'ADN, il faut cependant admettre que nous ne savons rien de ce qui se passe lorsque le facteur transformant s'intègre dans la génome (chromosome) bactérien; rien ne permet d'affirmer que d'autres constituants de ces chromosomes, les protéines par exemple, ne jouent pas aussi un rôle dans la réduplication et dans l'activité de l'ADN spécifique qui s'y est incorporé.

Récemment, Benoit et ses collaborateurs (1957, 1958) ont publié des expériences qui cherchent à démontrer l'existence de transformations comparables à celles des bactéries chez des organismes beaucoup plus élevés en organisation, les canards. Il ne fait aucun doute que les observations de Benoit et de ses collaborateurs (1957, 1958) ont de l'intérêt et qu'elles méritent d'être reprises et analysées; mais il est également hors de doute que, à l'heure actuelle, aucune conclusion sérieuse ne peut être tirée de ces expériences : trop de contrôles essentiels (effet éventuel de l'ADN homologue, disparition de l'activité par un traitement à la désoxyribonucléase, étude approfondie de la génétique des races

de canard utilisées, etc.) manquent pour qu'on puisse se hasarder à une conclusion quelconque.

IV. LE RÔLE GÉNÉTIQUE DE L'ARN

Nous avons vu plus haut qu'il y a de fortes raisons de penser que c'est l'ADN qui contient et transmet l' « information génétique » dans le cas des virus bactériens, les bactériophages. On possède des arguments tout aussi sérieux pour attribuer à l'ARN cette même information génétique dans le cas d'un virus végétal, celui de la mosaïque du tabac. Ce virus, comme le phage, est uniquement formé d'acide nucléique et de protéine; mais, dans les virus végétaux, c'est l'ARN qui prend la place de l'ADN.

Des expériences remarquables de Fraenkel-Conrat (1956) et de Gierer et Schramm (1956) ont montré qu'il est possible de séparer l'ARN de la protéine du virus de la mosaïque du tabac : si l'ARN est isolé par des méthodes suffisamment douces, il se révèle infectieux quand on le dépose sur une feuille de tabac; cela signifie que l'ARN du virus est capable, à lui seul, d'induire la synthèse de particules *complètes* de virus. Il n'y a pas seulement synthèse de l'ARN, mais aussi de la protéine du virus. Bien plus, si on combine, comme l'a fait Fraenkel-Conrat (1956), l'ARN d'une souche de virus de la mosaïque de tabac avec la protéine d'une autre souche et si on infecte des feuilles de tabac au moyen de ce virus « hybride », on constate que le virus récolté possède désormais la protéine qui correspond normalement à l'ARN qui a servi à l'infection. En d'autres termes, c'est l'ARN qui a déterminé la nature et la structure de la protéine qui s'est synthétisée et c'est donc lui qui possédait l'information génétique.

Il convient d'ajouter que c'est l'ARN *seul* qui possède cette information génétique et la capacité d'induire la synthèse de particules virulentes complètes : en effet, la fraction protéique du virus est totalement inactive. En outre, l'activité de l'ARN du virus est rapidement anéantie par une digestion à la ribonucléase, tandis qu'elle n'est pas affectée par les protéases et la désoxyribonucléase.

Ces expériences démontrent donc que c'est bien l'ARN qui est le déterminant génétique du virus de la mosaïque du tabac, que c'est lui qui transporte le « message génétique » et que c'est sous son influence que s'effectue la synthèse de la protéine spécifique du virus.

Voyons maintenant dans quelle mesure l'idée que l'ARN intervient directement dans la synthèse des protéines demeure valable lorsqu'on s'adresse à un système autrement complexe qu'un virus, la cellule vivante.

V. INTERVENTION DE L'ARN DANS LA SYNTHÈSE DES PROTÉINES

Comme nous l'avons fait pour le rôle génétique de l'ADN, nous passerons rapidement en revue les principales indications en faveur d'une intervention de l'ARN dans la synthèse des protéines, en terminant par les expériences les plus récentes et les plus directes.

a) *Observations cytochimiques.* C'est en 1941 que Caspersson et l'auteur de ce rapport ont conclu, indépendamment et simultanément, à une intervention de l'ARN dans la synthèse des protéines. Utilisant deux techniques extrêmement différentes pour la détection intracellulaire de l'ARN, ils ont constaté que seules les cellules qui synthétisent d'importantes quantités de protéines (cellules du pancréas exocrine, cellules sécrétrices de pepsine dans la muqueuse gastrique, cellules des glandes séricigènes des vers à soie, etc.) sont très riches en ARN. Par contre, des organes qui ont une activité physiologique très élevée, mais qui n'élaborent pas de protéines (le cœur, les muscles, les reins, par exemple) sont pauvres en ARN. Les microorganismes, qui se multiplient à un rythme rapide et qui sont, par conséquent, le siège de très importantes synthèses protéiques, sont très riches en ARN. De nombreux travaux ultérieurs ont confirmé l'hypothèse d'une relation entre la teneur en ARN d'une cellule et son aptitude à synthétiser des protéines : par exemple, la stimulation expérimentale des synthèses protéiques s'accompagne d'un accroissement de la teneur en ARN et *vice versa.*

Plus récemment, l'emploi de radioisotopes et de l'autoradiographie a apporté des arguments nouveaux en faveur de la même idée : ce sont les cellules les plus riches en ARN qui incorporent les acides aminés marqués dans leurs protéines le plus rapidement et le plus intensément (Ficq et Brachet, 1956; Niklas et Oehlert, 1956).

b) *Confirmations quantitatives.* Les techniques cytochimiques ne sont que grossièrement quantitatives; mais des dosages quantitatifs de la teneur en ARN des divers organes et tissus, effectués par des méthodes variées, ont entièrement confirmé les conclusions initiales d'une relation entre la teneur en ARN des cellules et leur capacité de synthèses protéiques (Brachet, 1941; Davidson, 1947; Leslie, 1955).

De nombreux travaux ont établi que, même chez les bactéries, il existe un excellent parallélisme entre la synthèse des protéines et celle de l'ARN; c'est seulement lorsque ces microorganismes sont traités par des antibiotiques (le chloramphénicol, notamment) que la synthèse de l'ARN se dissocie de celle des protéines : mais il est maintenant établi que l'ARN qui se synthétise dans les bactéries traitées au chloramphénicol est anormal à maints égards (Neidhardt et Gros, 1957; Gros et Gros, 1958).

Il existe d'ailleurs, dans le cas des microorganismes, un très grand nombre d'observations qui plaident fortement en faveur d'une intervention de l'ARN dans la synthèse des protéines : par exemple, en utilisant des mutants exigeant des dérivés, soit de l'ARN, soit de l'ADN pour leur croissance, H. et R. Jeener (1952) chez *Thermobacterium acidophilus,* Cohen et Barner (1954) chez *E. coli,* ont montré que l'arrêt de la synthèse de l'ADN n'empêche pas celle des protéines, ni même la synthèse induite d'enzymes; par contre, l'arrêt de la synthèse de l'ARN s'accompagne d'un arrêt rapide de la croissance et des synthèses protéiques. On peut obtenir des résultats analogues en irradiant des bactéries aux U. V. (Kelner, 1953; Kanazir et Errera, 1954) : de faibles doses arrêtent la synthèse de l'ADN, alors que celles de l'ARN et des protéines continuent et demeurent concomitantes. La synthèse induite d'enzymes est, elle aussi, beaucoup moins sensible aux U. V. que celle de l'ADN (Halvorson et Jackson,

1956); mais elle a la même sensibilité aux radiations que celle de l'ARN.

Ces résultats ont conduit les microbiologistes à une conclusion importante : la synthèse de nouvelles molécules d'ARN est nécessaire pour que la synthèse induite d'un enzyme, c'est-à-dire d'une protéine spécifique, soit possible (Pardee, 1954; Spiegelman et collaborateurs, 1955). Cette conclusion a été confirmée par Chantrenne (1956), qui a étudié l'incorporation de l'adénine dans l'ARN lors de la synthèse induite de la catalase dans la levure sous l'influence de l'oxygène : la synthèse de cette protéine spécifique paraît bien être étroitement associée à la synthèse d'un nouvel ARN.

c) *Siège des synthèses protéiques dans la cellule.* Nous avons déjà signalé plus haut que l'ARN se trouve principalement localisé dans le nucléole et le cytoplasme des cellules. S'il existe une corrélation entre l'ARN et la synthèse des protéines, on peut prédire que les protéines s'élaborent dans ces deux régions de la cellule.

En ce qui concerne le nucléole, les observations autoradiographiques de A. Ficq (1955) ont montré que, dans les œufs d'étoiles de mer, l'incorporation des acides aminés dans les protéines du nucléole est dix fois supérieure à celle observée dans le restant de la cellule. Des constatations similaires ont été faites dans de nombreux autres cas.

Passons au cytoplasme : les travaux de Claude (1943), ceux de Brachet et Jeener (1944) ont montré que l'ARN de la cellule est surtout lié aux *microsomes;* on entend par là des particules de petite taille, qu'il est possible d'isoler en centrifugeant à grande vitesse des cellules broyées. Nous savons maintenant que ces microsomes proviennent, en réalité, de la rupture artificielle de structures délicates, surtout développées dans les cellules qui sont le siège de fortes synthèses protéiques (ergastoplasme ou reticulum endoplasmique). Au microscope électronique, les microsomes sont formés de deux parties : des membranes et de très petits granules, les grains de Palade (Palade, 1955). Il est possible d'isoler les grains de Palade en lysant les microsomes au moyen de sels biliaires et en ultracentrifugeant ensuite, à très

grande vitesse. Alors que les microsomes contiennent 10-15 % d'ARN, les grains de Palade, isolés de la manière qui vient d'être esquissée, en renferment jusqu'à 45 % (Littlefield et collaborateurs, 1955).

D'après tout ce qui a été dit plus haut, on doit s'attendre, si l'hypothèse d'une intervention directe de l'ARN dans la synthèse des protéines est exacte, à trouver que les microsomes, et plus encore les grains de Palade, sont le siège d'une incorporation massive des acides aminés dans leurs protéines. C'est ce qu'ont effectivement montré de très nombreuses expériences, à commencer par celles de Borsook (1950) et de Hultin (1950). Mentionnons, notamment, les travaux de Allfrey et collaborateurs (1953), de Oota et Osawa (1954), de Martin et Morton (1955) qui conduisent tous à la conclusion que les microsomes, riches en ARN, jouent un rôle particulièrement important dans la synthèse des protéines, tant chez les animaux que les végétaux.

Il est plus frappant encore de constater que, comme l'ont montré Littlefield et collaborateurs (1955) et Zamecnik et collaborateurs (1956), les grains de Palade isolés sont de sept à huit fois plus actifs que la fraction des microsomes soluble dans les sels biliaires.

Enfin, argument décisif, un traitement des microsomes, qu'ils soient d'origine animale ou végétale, par la ribonucléase (enzyme qui digère l'ARN), inhibe fortement l'incorporation des acides aminés dans les protéines (Allfrey et collaborateurs, 1953; Zamecnik et Keller, 1954; Webster et Johnson, 1955). L'intégrité de l'ARN des microsomes est donc indispensable pour que l'anabolisme protéique puisse se produire.

Mais, si importants que ces résultats obtenus sur des broyats de cellules puissent être, le biologiste s'intéresse davantage à ce qui se passe dans la cellule intacte, *encore vivante :* existe-t-il des arguments directs en faveur d'une intervention de l'ARN dans la synthèse des protéines de la cellule vivante ? C'est ce que nous allons examiner maintenant.

d) *ARN et synthèse des protéines dans la cellule vivante.* Il convient de dire quelques mots, pour commencer, des importantes recherches effectuées par Gale et Folkes (1954, 1955) sur des

bactéries (staphylocoques) qui avaient été traitées par les ultrasons : ces bactéries ne sont plus vivantes et elles ne peuvent donc plus se multiplier; mais elles demeurent, néanmoins, capables de réaliser une synthèse *nette* de protéines si on les place dans des conditions de milieu favorables. Les expériences de Gale et Folkes (1954, 1955) ont montré clairement que la synthèse des protéines cesse, dans les staphylocoques brisés par les ultrasons, quand on leur enlève, par des moyens variés, leurs acides nucléiques; mais la synthèse d'enzymes et l'incorporation des acides aminés dans les protéines reprennent quand on leur ajoute, soit des acides nucléiques de staphylocoques, soit un produit de la digestion, par la ribonucléase, de l'ARN. Gale (1956) a concentré ses efforts, sans résultat vraiment concluant jusqu'à présent, sur l'identification du facteur qui, parmi les produits de la digestion par la ribonucléase, stimule l'incorporation de la glycine (GIF) dans les protéines; ce facteur, de faible poids moléculaire, peut efficacement remplacer l'ARN.

Des résultats comparables à ceux de Gale et Folkes (1954, 1955) ont été obtenus dans le cas des protoplastes, c'est-à-dire des bactéries dont la coque a été dissoute par un traitement au lysozyme : comme l'ont montré Landman et Spiegelman (1955) et Spiegelman (1956), la synthèse induite d'un enzyme (la β-galactosidase) est complètement inhibée par la ribonucléase, alors que la désoxyribonucléase demeure sans effets : il faut en conclure que l'intégrité de l'ARN est plus importante, pour la synthèse des protéines, que celle de l'ADN.

Ce sont les expériences de Gale et Folkes (1954, 1955) qui nous ont incité (1954, 1955, 1956) à étudier les effets de la ribonucléase sur des cellules vivantes (œufs d'amphibiens et d'étoile de mer, racines d'oignon, amibes). Un exposé, même sommaire, de ces expériences nous entraînerait trop loin et nous nous limiterons donc aux conclusions générales : dès que la ribonucléase pénètre dans une cellule, elle bloque de façon quasi-complète l'incorporation des acides aminés dans les protéines, la synthèse de ces dernières, la croissance et la division cellulaire. Si on ajoute aux cellules préalablement traitées par la ribonucléase un excès d'ARN, on assiste à une reprise de la division cellulaire, de la croissance et de l'incorporation des acides aminés dans les protéines; cette réversibilité est souvent totale si on ajoute l'ARN

à un moment où la ribonucléase a provoqué une inhibition de 50-60 % de la synthèse des protéines. Dans le cas des racines d'oignon, l'ARN homologue, isolé à partir des racines, est cinq fois plus actif que celui de la levure à cet égard.

En conclusion, on constate qu'il est possible de modifier presque à volonté l'incorporation des acides aminés dans les protéines de nombreuses cellules vivantes, la synthèse des protéines et la division cellulaire, en les soumettant à des traitements appropriés et successifs par la ribonucléase et l'ARN. L'ensemble des résultats ne permet guère d'échapper à la conclusion que, dans la cellule *vivante, l'intégrité de l'ARN est indispensable au maintien des synthèses protéiques.*

e) *Mécanismes biochimiques de la synthèse des protéines.* Peut-être le biologiste peut-il se satisfaire de la conclusion qui vient d'être présentée et se tourner vers d'autres problèmes, plus complexes encore (mécanisme de la morphogénèse, de la division cellulaire, de l'action des hormones, etc.). Mais le biochimiste désire comprendre le *mécanisme* par lequel l'ARN intervient dans la synthèse des protéines. C'est ce que nous commençons à faire, grâce surtout aux très beaux travaux de Hoagland (1955, 1957) et de son groupe.

On sait, depuis longtemps, que la synthèse des liaisons peptidiques exige de l'énergie et que cette énergie, dans les êtres vivants, provient de l'acide adénosinetriphosphorique (ATP) : c'est ce qui a conduit Lipmann (1949) et Chantrenne (1951) à supposer qu'un acide aminé activé par phosphorylation pourrait être un intermédiaire dans la synthèse des protéines. L'idée était intéressante et c'est elle qui a permis à Hoagland (1955) et à De Moss et Novelli (1955) de démontrer que le premier stade de la synthèse des protéines est effectivement une réaction entre un acide aminé et l'ATP : mais le produit de la réaction, qui est catalysée par des enzymes se trouvant en solution dans la cellule, est un produit du type amino-acyl-AMP. On peut écrire, schématiquement, la réaction de la manière suivante, où :

ATP = Adénosinetriphosphate;
AMP = Adénosinemonophosphate;
AA = Acide aminé;
PP = Pyrophosphate;

$$\text{ATP} + \text{AA} \longrightarrow \underset{\text{Acide aminé activé.}}{\text{AMP-AA}} + \text{PP}$$

Il existe un grand nombre de ces enzymes solubles, présentant une spécificité vis-à-vis d'un acide aminé défini. L'enzyme d'activation du tryptophane dans le pancréas a été cristallisé par Davie et collaborateurs (1956).

L'acide aminé activé est donc bien le résultat d'une réaction entre l'ATP et l'acide aminé, dont le groupe carboxylique se trouve désormais sous une forme activée.

C'est à l'étape suivante de la synthèse des protéines qu'on voit apparaître l'ARN : comme l'ont montré Hoagland et ses collaborateurs (1957), l'acide aminé activé (amino acyl-AMP) est incorporé dans un ARN soluble (c'est-à-dire la fraction qui n'est liée à aucune structure cellulaire). Ce second stade du processus, qui exige de façon absolue la présence d'un ARN spécifique, est extrêmement sensible à la ribonucléase. Enfin, dans un troisième et dernier stade, l'ARN auquel l'acide aminé demeure attaché est transféré aux microsomes; ce transfert exige la présence de guanosinetriphosphate, dont le rôle exact demeure obscur.

Nous ignorons tout d'ailleurs du mécanisme qui, au niveau des microsomes et sous l'influence de l'ARN qu'ils contiennent, conduit à l'assemblement des divers acides aminés en une protéine spécifique. La seule hypothèse valable dont nous disposions est celle du modèle (« template » des auteurs anglo-saxons) : l'ARN des microsomes, en raison de sa structure spécifique, provoquerait l'association des acides aminés dans un ordre défini et la formation de chaînes polypeptidiques spécifiques. Il n'existe à l'heure actuelle, aucun fait expérimental qui soit en contradiction ouverte avec cette hypothèse du modèle. Mais il faut bien reconnaître que nous ne disposons pas encore non plus d'une expérience cruciale en sa faveur. Ce sera la tâche de l'avenir d'affermir ou d'éliminer une hypothèse dont, à l'heure actuelle, nous ne pouvons pas nous passer.

VI. INTERVENTION DE L'ADN DANS LA SYNTHÈSE DES PROTÉINES

Nous avons vu plus haut qu'une synthèse de protéines est possible sans qu'il y ait synthèse concomitante d'ADN : c'est ce qui se produit, notamment, dans les mutants de *E. coli* qui exigent

de la thymine pour se multiplier (Cohen et Barner, 1954) et dans les bactéries irradiées aux U. V. de Kelner (1953) et de Kanazir et Errera (1954). Nous avons vu aussi que, dans les protoplastes bactériens, la synthèse induite des enzymes est inhibée par la RNase, mais non par la DNase (Landman et Spiegelman, 1955).

Ces constatations sont évidemment peu favorables à l'idée d'une intervention directe de l'ADN dans les synthèses de protéines. Il existe, cependant, deux observations qui suggèrent une telle intervention directe; elles ont, malheureusement, été faites sur des systèmes qui s'écartent fortement de la cellule vivante.

Tout d'abord, dans les expériences déjà signalées de Gale et Folkes (1954, 1955) sur des staphylocoques traités aux ultrasons et privés de leurs acides nucléiques, l'addition d'ADN restaure la synthèse de la β-galactosidase. Ensuite, Allfrey, Mirsky et Osawa (1955) ont montré que les noyaux *isolés* de cellules de thymus sont capables d'incorporer des acides aminés dans leurs protéines; cette incorporation est inhibée par la désoxyribonucléase, tandis que la ribonucléase est inactive. Mais, selon les observations les plus récentes de ces mêmes auteurs (1957), l'effet de l'ADN pourrait bien être indirect : sa destruction par la désoxyribonucléase provoquerait une inhibition des processus producteurs d'énergie (phosphorylations oxydatives) dans les noyaux isolés. Faisons remarquer à ce propos que la ribonucléase, dans le cas des cellules intactes, ne touche en rien ces réactions productrices d'énergie dans des conditions où elle inhibe puissamment les synthèses protéiques.

En conclusion, le rôle de l'ADN dans la synthèse des protéines demeure momentanément obscur; les trop rares indications dont on dispose actuellement indiquent qu'il s'agit plutôt d'un rôle indirect que d'une intervention directe.

VII. ESQUISSE DES RELATIONS EXISTANT ENTRE L'ADN, L'ARN ET LES PROTÉINES DANS LA CELLULE VIVANTE

Nous nous trouvons donc devant une contradiction apparente : la génétique nous apprend que la synthèse de nombreux enzymes et d'autres protéines (les hémoglobines, par exemple) est contrôlée par les gènes. Nous avons vu que le matériel génétique peut être

identifié à l'ADN. Mais nous venons de constater qu'il n'existe pas de démonstration satisfaisante que cet ADN contrôle *directement* la synthèse de protéines spécifiques; tout indique, au contraire, que c'est l'ARN qui exerce ce contrôle.

C'est pour résoudre cette contradiction qu'on a émis l'hypothèse, à de nombreuses reprises (voir Brachet, 1957, pour une discussion plus approfondie), que le contrôle exercé par l'ADN sur la synthèse des protéines serait complexe et indirect : des molécules spécifiques d'ADN, correspondant aux gènes, donneraient naissance à des molécules complémentaires d'ARN, suivant le principe du modèle; par le même mécanisme du modèle, les molécules spécifiques d'ARN induiraient la synthèse de protéines spécifiques. Il s'agirait, en somme, d'une relation entre les acides nucléiques et les protéines qu'on peut, très simplement, transcrire de la manière suivante : ADN $\longrightarrow$ ARN $\longrightarrow$ Protéines (c'est là l'un des « slogans » actuels de la génétique biochimique : « DNA makes RNA, and RNA makes protein »).

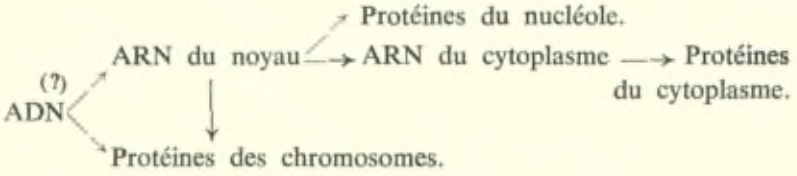
Il est difficile d'accepter cette relation entre les acides nucléiques et les protéines sous une forme aussi simpliste : il faut, tout d'abord, faire une distinction entre l'ARN du noyau et celui du cytoplasme. De nombreuses expériences, dont les plus convaincantes sont celles de Jeener et Szafarz (1950) et celles de Goldstein et Plaut (1955 ont montré que le noyau, et plus particulièrement le nucléole (Ficq, 1955), est le siège d'une synthèse d'ARN beaucoup plus importante et plus rapide que le cytoplasme. Il est vraisemblable — et même probable — qu'une partie de l'ARN cytoplasmique est d'origine nucléaire; il est, par contre, fort douteux que la *totalité* de l'ARN cytoplasmique provienne du noyau : en effet, les mécanismes de synthèse de l'ARN persistent dans les fragments *anucléés* de l'algue unicellulaire *Acetabularia* (Brachet et collaborateurs, 1955), qui sont toutefois incapables de réaliser une importante synthèse *nette* de cet acide nucléique (Richter, 1957).

Par ailleurs, il ne fait aucun doute que des fragments dépourvus de noyau de cette algue peuvent effectuer une synthèse nette de protéine (Brachet et collaborateurs, 1955; confirmé par Werz, 1957), y compris celle de protéines spécifiques, enzymatiquement actives (l'aldolase, par exemple : Baltus, 1956). En fait, l'ablation

du noyau *stimule* initialement, chez *Acetabularia,* la synthèse des protéines. On peut donc, dans le cas de cette algue, éliminer le noyau — c'est-à-dire l'ADN et le génome — et il se produit, néanmoins, une synthèse extrêmement importante de protéines. Cette synthèse se ralentit toutefois après une dizaine de jours et cesse presque complètement après trois semaines. Les expériences effectuées sur *Acetabularia* démontrent donc qu'il existe bien un contrôle du noyau (sans qu'on puisse affirmer qu'il soit dû à l'ADN) sur la synthèse des protéines cytoplasmiques; toutefois, le contrôle n'est pas immédiat, mais éloigné.

On a vu plus haut les nombreuses raisons qui nous font admettre une intervention de l'ARN dans la synthèse des protéines; par contre, nous n'avons, à l'heure présente, aucun argument sérieux en faveur de l'idée que cet ARN est synthétisé directement par l'ADN. Ce dernier, conformément aux observations de Allfrey et ses collaborateurs (1957), intervient certainement dans la synthèse des protéines du noyau cellulaire lui-même; encore, cette intervention est-elle probablement indirecte, comme nous venons de le voir.

On peut, en somme, résumer l'état actuel de nos connaissances dans le schéma suivant, que l'avenir se chargera certainement de compléter et de modifier :



VIII. CONCLUSIONS

Nous avons essayé, dans le présent rapport, de montrer l'intérêt que les acides nucléiques présentent pour le biologiste : le rôle génétique de l'ADN et celui de l'ARN dans la synthèse des protéines sont, désormais, plus que des hypothèses fructueuses. On voit qu'il reste beaucoup à faire, aux généticiens et aux biochimistes, pour préciser les relations existant entre l'ADN, l'ARN et les protéines, c'est-à-dire, en dernière analyse, les relations

existant entre le gène et la protéine dont il contrôle la synthèse. Mais la parole est désormais, avant tout, aux chimistes organiciens et aux physicochimistes : l'actuel Conseil de Chimie Solvay ne manquera sûrement pas d'apporter d'importantes contributions dans un domaine qui touche aux racines mêmes de la Vie.

BIBLIOGRAPHIE

Alfert, M. : *Internat. Rev. Cytology*, **3**, 131 (1954).

Allfrey, V.G., Daly, M.M. et Mirsky, A.E. : *J. gen. Physiol.*, **37**, 157 (1953).

Allfrey, V.G. et Mirsky, A.E. : *Proc. natl. Acad. Sci. U.S.*, **43**, 589 (1957).

Allfrey, V.G., Mirsky, A.E. et Osawa, S. : *Nature*, **176**, 1042 (1955).

Allfrey, V.G., Mirsky, A.E. et Osawa, S. : *J. gen. Physiol.*, **40**, 451 (1957).

Avery, O.T., Mc Leod, C.M. et Mc Carty, M. : *J. exptl. Medic.*, **79**, 137 (1944).

Baltus, E.: " Proc. 3rd intern. Congress Biochemistry ", commun., p. 76, Academic Press, New York (1956).

Bendich, A., Fresco, J.R., Rosenkrantz, H.S. et Beiser, S.M. : *J. amer. chem. Soc.*, **77**, 3671 (1955).

Benoit, J., Leroy, P., Vendrely, C. et Vendrely, R. : *C. R. Acad. Sc. Paris*, **244**, 2320 (1957).

Benoit, J., Leroy, P., Vendrely, C. et Vendrely, R. : *C. R. Acad. Sc. Paris*, **245**, 448 (1957).

Benoit, J., Leroy, P., Vendrely, C. et Vendrely, R. : *C. R. Acad. Sc. Paris*, **247**, 1049 (1958).

Benzer, S. : dans " The chemical basis of heredity ", p. 70. Edit. W.D. Mc Elroy et B. Glass, Johns Hopkins Univ. Press, Baltimore (1957).

Boivin, A., Vendrely, R. et Vendrely, C. : *C. R. Acad. Sc. Paris*, **226**, 1061 (1948).

Borsook, J.H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G. et Lowy, P.H. : *J. biol. Chem.*, **184**, 529 (1950).

Brachet, J. : *Enzymologia*, **10**, 87 (1941 *a*).

Brachet, J. : *Arch. Biol.*, **53**, 207 (1941 *b*).

Brachet, J. : *Nature*, **174**, 879 (1954).

Brachet, J. : *Pubbl. Staz. zool. Napoli*, **27**, 146 (1955).

Brachet, J. : *Exptl. Cell Res.*, **10**, 255 (1956).

Brachet, J. : " Biochemical Cytology ", Academic Press, New York (1957).

Brachet, J., Chantrenne, H. et Vanderhaeghe, F. : *Biochim. biophys. Acta*, **18**, 544 (1955).

Brachet, J. et Jeener, R. : *Enzymologia*, **11**, 196 (1944).

Burton, K. : *Biochem. J.*, **61**, 473 (1955).

Caspersson, T. : *Naturwiss.*, **28**, 33 (1941).

Chantrenne, H. : *Pubbl. Staz. zool. Napoli.* **23**, suppl., p. 70 (1951).

Chantrenne, H. : *Nature*, **177**, 579 (1956).

Chargaff, E. : dans " The chemical basis of heredity ", p. 521. Edit. W.D. Mc Elroy et B. Glass, The Johns Hopkins Univ. Press, Baltimore (1957).

Claude, A. : *Biolog. Sympos.*, **10**, 3 (1943).

Cohen, S.S. et Barner, H.D. : *Proc. natl. Acad. Sci. U.S.*, **40**, 885 (1954).

Daoust, R., Leblond, C.P., Nadler, N.J. et Enosco, M. : *J. biol. Chem.*, **221**, 727 (1956).

Davidson, J N. : *Cold Spring Hrbor Sympos.*, **12**, 50 (1947).

Davie, E.W., Koningsberger, V.V. et Lipmann, F. : *Arch. Biochem. Biophys.*, **65,** 21 (1956).

De Moss, J.A. et Novelli, G.D. : *Biochim. biophys. Acta*, **18,** 592 (1955).

Fautrez, J. et Fautrez-Firlefyn, N. : *C. R. Soc. Biol.*, **147,** 351 (1953).

Feulgen, R. et Rossenbeck, H. : *Z. physiol. Chem.*, **135,** 203 (1924).

Ficq, A. : *Arch. Biol.*, **66,** 509 (1955).

Ficq, A. et Brachet, J. : *Exptl. Cell Res.*, **11,** 146 (1956).

Ficq, A. et Pavan, C. : *Nature*, **180,** 983 (1957).

Fraenkel-Conrat, H. : *J. amer. chem. Soc.*, **78,** 882 (1956).

Gale, E.F. : " Ciba Foundation Symposium on ionizing radiations and cell metabolism ", p. 174, Churchill, London (1956).

Gale, E.F. et Folkes, J.P. : *Nature*, **173,** 1223 (1954).

Gale, E.F. et Folkes, J.P. : *Nature*, **175,** 592 (1955).

Gierer, A. et Schramm, G. : *Z. Naturforschg.*, **11b,** 138 (1956).

Goldstein, L. et Plaut, W. : *Proc. natl. Acad. Sci. U.S.*, **41,** 874 (1955).

Gros, F. et Gros, F. : *Exptl. Cell Res.*, **14,** 104 (1958).

Halvorson, H.O. et Jackson, L. : *J. gen. Microbiol.*, **14,** 26 (1956).

Hershey, A.D. : dans " Currents in biochemical research ". Edit. D.E. Green, Interscience, New York (1956).

Hershey, A.D. et Chase, M. : *J. gen. Physiol.*, **36,** 39 (1952).

Hoagland, M.B. : *Biochim. biophys. Acta*, **16,** 288 (1955).

Hoagland, M.B., Zamecnik, P.C. et Stephenson, M.L. : *Biochim. biophys. Acta*, **24,** 215 (1957).

Hotchkiss, R.D. : *Colloq. intern. C.N.R.S.*, **8,** 57 (1949).

Hotchkiss, R.D. : dans " Nucleic Acids ". Edit. E. Chargaff et J.N. Davidson vol. II, p. 435, *Academic Press*, New York (1955).

Hultin, T. : *Exptl. Cell Res.*, **1,** 376, 599 (1950).

Jeener, H. et Jeener, R. : *Exptl. Cell Res.*, **3,** 675 (1952).

Jeener, R. et Szafarz, D. : *Arch. Biochem.*, **26,** 54 (1950).

Kanazir, D. et Errera, M. : *Biochim. biophys. Acta*, **14,** 62 (1954).

Kelner, A. : *J. Bacteriol.*, **65,** 252 (1953).

Kihara, H.K., Amano, M. et Sibatani, A. : *Biochim. biophys. Acta*, **21,** 489 (1956).

La Cour, L.F., Deeley, E.M. et Chayen, J. : *Nature*, **177,** 272 (1956).

Landman, O.E. et Spiegelman, S. : *Proc. natl. Acad. Sci. U.S.*, **41,** 698 (1955).

Leslie, I. : dans " Nucleic Acids ". Edit. E. Chargaff et J.N. Davidson, vol. II, p. 1, Academic Press, New York (1955).

Levene, P.A. : " Nucleic Acids ", *The Chemical Catalog Co. Monograph Series*, n° 56, New York (1931).

Lipmann, F. : *Federation Proc.*, **8,** 597 (1949).

Littlefield, J.W., Keller, E.B., Gross, J. et Zamecnik, P.C. : *J. biol. Chem.*, **217,** 111 (1955).

Martin, E.M. et Morton, R.K. : *Biochem. J.*, **62,** 696 (1955).

Mazia, S. et Plaut, W.S. : *Biol. Bull.*, **100,** 335 (1955).

Neidhardt, F.C. et Gros, F. : *Biochim. biophys. Acta*, **25**, 513 (1957).

Niklas, A. et Oehlert, W. : *Beitr. pathol. Anat. u. allgem. Pathol.*, **116**, 92 (1956).

Oota, Y. et Osawa, S. : *Biochim. biophys. Acta*, **15**, 162 (1954).

Palade, G.E. : *J. biophys. biochem. Cytology*, **1**, 59, 567 (1955).

Pardee, A.B. : *Proc. natl. Acad. Sci. U.S.*, **40**, 263 (1954).

Pasteels, J. et Lison, L. : *C. R. Acad. Sc. Paris*, **230**, 780 (1950).

Richter, G. : *Naturwiss.*, **44**, 520 (1957).

Spiegelman, S. : " Proc. 3rd intern. Congress of Biochemistry ", p. 185, Academic Press, New York (1956).

Spiegelman, S., Halvorson, H.O. et Ben-Ishai, R. : dans " Amino acid metabolism ". Edit. W.D. Mc Elroy et B. Glass, p. 124, Johns Hopkins Univ. Press Baltimore (1955).

Swick, R.W., Koch, A.L. et Handa, D.T. : *Arch. Biochem. Biophys.*, **63**, 226 (1956).

Taylor, J.H., Woods, P.S. et Hughes, W.L. : *Proc. natl. Acad. Sci. U.S.*, **43**, 122 (1957).

Thomas, R. : *Biochim. biophys. Acta*, **18**, 467 (1955).

Vendrely, C. et Vendrely, R. : *Intern. Rev. Cytology*, **5**, 171 (1956).

Watson, J.D. et Crick, F.H.C. : *Nature*, **171**, 737 (1953).

Webster, G.C. et Johnson, M.P. : *J. biol. Chem.*, **217**, 641 (1955).

Werz, G.V. : *Experientia*, **13**, 79 (1957).

Zamecnik, P.C. et Keller, E.B. : *J. biol. Chem.*, **209**, 337 (1954).

Zamecnik, P.C., Keller, E.B., Littlefield, J.W., Hoagland, M.B. et Loftfield, R.B. : *J. cell. compar. Physiol.*, **47**, suppl.1, 81 (1956).

DISCUSSION SUR LE RAPPORT DE Mr. BRACHET

M. Watson. — Is there any information on the form of ribonucleic acid within the nucleus ? How much of it is present as microsomal (Palade) particles ?

M. Brachet. — We don't know much about the ultra-structure of the nucleus, which is a less favorable object than the cytoplasm for electron microscopy studies.

However, granules resembling Palade's particles have been described in nucleoli and in the loops of the lampbrush chromosomes. It has been speculated that these small particles might carry genetic information, be transferred from the nucleus into the cytoplasm and be integrated into the ergastoplasm.

M. Felix. — Professor Brachet, you did not say much about the proteins which are associated with the nucleic acids. As to the RNA which is synthesized in the nucleoli and transferred into the cytoplasm, is it free nucleic acid or a nucleoprotein ?

M. Brachet. — I can't give a definite answer to that question; it is highly probable that the RNA which is transferred from the nucleus to the cytoplasm is in the form of ribonucleoproteins, possibly similar to those present in Palade's small ergastoplasmic granules.

M. Butler. — In connection with Professor Brachet's discussion of the role of RNA in the nucleus I should like to mention the observation some years ago (1) that DNA's prepared from tumours were always contaminated with RNA, while those prepared from normal tissues were usually free. This might just be a reflection of the fact that the RNA content of tumour cells in exceptionally high.

We should of course like very much to find some intermediary between the DNA and the protein. One possible candidate for this

(1) J.A.V. Butler, E.W. Johns, J.A. Lucy and P. Simson, *Brit. J. Cancer*, **10**, 202, 1956.

function is the histone, but as a rule histone in non-dividing cells has been found to be metabolically inert, and it does not look as if histone molecules are used for transfering the pattern of DNA to RNA in protein. However, recently Busch (1) has found that in tumours histone is comparatively active and indeed he has obtained in them a very active constituent of a basic character.

M. Ochoa. — Is it your impression that synthesis of RNA occurs exclusively in the nucleus and, hence, that all cytoplasmic RNA is of nuclear origin ?

M. Brachet. — There is little doubt that part of cytoplasmic RNA is of nuclear origin; but it is certain that, in *Acetabularia*, incorporation of various precursors (orotic acid, adenine, CO^2) into RNA remains perfectly normal, in the absence of the nucleus, during several weeks.

Whether we are dealing with extensive turnover or exchange reactions in the absence of net synthesis of RNA is not known. What is certain is that enucleation has little effects on RNA metabolism in the case; in amoebae, removal of the nucleus quickly reduces the incorporation of precursors into RNA, but never suppresses it completely.

M. Ochoa. — In connection with my previous question, I now should like to ask whether DNA is synthesized only in the nucleus. Is there no evidence for cytoplasmic synthesis of DNA ?

M. Brachet. — If you are interested in the intracellular distribution of enzymes concerned in DNA synthesis, I think that Professor Davidson, who has studied the question, will give you a more precise answer than I could.

M. Davidson. — The question of the site of DNA synthesis is of considerable interest. It is known from the work of Kornberg that in bacterial systems DNA synthesis takes place from the deoxynucleoside triphosphates and a similar process occurs in mammalian systems — in regenerating rat liver (Bollum and Potter, *J. Biol.-Chem.*, **233**, 478, 1958; Mantsavinos and Canellakis, *Biochim.*

(1) H. Busch, J.R. Davis and D.C. Anderson, *Cancer Research*, **18**, 916, 1958.

Biophys. Acta, **27**, 661, 1958) and in Ehrlich ascites tumour cells (Smellie, Keir and Davidson, *Biochim. Biophys. Acta*, 1959, in the press).

We have found that the enzyme system involved, which can bring net synthesis of DNA *in vitro* in cell and particle free extracts, is apparently more active in extracts of cytoplasmic than of nuclear material. This suggestion that DNA might be synthesized in the cytoplasm is supported by the autoradiographic observations of our collegue Losan using ^{3}H-thymidine which is incorporated, apparently in bound form, into the cytoplasm of ascites cells. Dr. Plaut in Wisconsin has, I believe, also demonstrated the cytoplasmic incorporation of ^{3}H-thymidine.

All the evidence should be interpreted with great caution but it does suggest that it is not impossible for DNA synthesis to be a cytoplasmic process.

M. Brachet. — However, your enzyme system is found in the supernatant after ultracentrifugation and, in such cases, one is never sure that enzymes have not leached out of cellular organites, nuclei for instance.

M. Butler. — We have also examined the incorporation of thymidine by mammalian cytoplasmic fractions, in the presence of DNA, as was done first by van Potter.

I should like to make the point that the amount of incorporation observed is extremely small.

With tritiated thymidine it is possible to obtain counts of the order of 15 000 per min. per mg. DNA.

We were surprised to find, when we worked it out, that this corresponded to a labelling of DNA to the extent of only 1 particle of DNA in 1 000. This is very much smaller than the incorporation observed in bacterial extracts, and it is possible that the mammalian experiments give a misleading impression.

M. Kuhn. — Is it possible to give any information about the minimum of substances that must be present *besides* RNA and free aminoacids to make the synthesis of proteins possible ? Can any-

thing be said in particular about the additional substances which provide the free energy for the protein synthesis ?

M. Brachet. — A number of substances are required for protein synthesis : aminoacids of course, ATP (or a system generating ATP) as an energy source, soluble RNA and microsomal RNA. Recent work indicates that the aminoacids are first activated by combination with ATP (in order to form an amino acid — AMP complex) under the action of specific soluble enzymes. The activated aminoacids are then incorporated into specific (for each aminoacid) soluble RNA's; the latter are, in turn, transferred to the microsomes, where the big mystery occurs : a specific protein is formed, probably by a template mechanism.

M. Overbeek. — In relation to the question about the mechanism of protein synthesis, I want to signal that my collaborators Koningsberger, van der Grinten and Schuurs (*Biochim. Biophys. Acta*, **26**, 483, 1957) have isolated a number of carboxyl activated peptides from the soluble fraction of yeast cells and from yeast microsomes. The peptides of the soluble fraction have a molecular weight of 3-4 000 and their (terminal ?) carboxyl group is bound to AMP, very probably to one of the OH^- groups of the ribose ring. The identity of the nucleotide part of the peptides from the microsomes has not yet been established. These nucleotide-peptides contain *at least* 10 different amino acids, among them lysine, aspartic acid, alanine, proline, etc. but no histidine, arginine or tryptophane. In paper electrophoresis at pH = 3.9 or 7.0 they migrate slowly to the cathode.

These peptides may conceivably be intermediates in protein synthesis. More particularly they might be condensed under the influence of s-RNA, the AMP group attached to the peptides being the terminal group of s-RNA, from which the remainder has been broken away possibly by the action of RNAase.

The activated nature of the peptides points strongly to their intermediate character. Their molecular weight is such that they would use just about all the information present in the nucleotide sequence of s-RNA.

Apart from the obvious interest for protein synthesis the peptides might have a parallel in nucleic acid synthesis which according to previous discussions might start in the cytoplasm and be finished in the nucleus.

M. Butler. — Is it possible that the peptides are fragments of proteins produced by proteolytic enzymes ?

M. Overbeek. — I cannot conclusively rule out the possibility that the peptides have been formed by hydrolytic enzymes from larger molecules, but particularly in the case of the peptides isolated from microsomes this is improbable.

Yeast microsomes (" 80 S " particles) are isolated first, but they give up their activated nucleotide-peptides only after dialysis against citrate by which the microsomal structure is destroyed, so that the remaining material has sedimentation constants of 2-3 S. (The peptides themselves sediment still more slowly.)

The high energy bond to nucleotide would moreover not be in agreement with the formation from a bigger non activated peptide or protein.

M. Watson. — Is your small peptide basic ? There is now good evidence that the proteins associated with ribonucleic acid in RNA particles are basic.

M. Overbeek. — All our peptides travel to the cathode at pH 3.9 and 7.0. The isoelectric points have not yet been determined. They contain much lysine, but also much aspartic and some glutamic acid (or amide). They may be basic.

M. Ubbelohde. — Two quantitative questions inspired by physico-chemical concepts may be asked :

(i) If the sectioning of the algae is made progressively along the 5 cm of cell extension above the nucleus, is a threshold found beyond which no " umbrella » grows ?

(ii) How far can significant rate constants be established for two different processes :

(*a*) exchange or " turnover " reactions ?

(*b*) actual increase or decrease of a substance by positive or negative synthesis ?

To develop physico-chemical aspects of cytochemistry some idea of the ratio between such rate processes would be valuable.

M. Brachet. — Regarding the first question, experiments of Hämmerling have clearly shown, many years ago, that only " old " algae can form caps; furthermore, he has demonstrated that the morphogenetic substance responsible for cap formation is formed by the nucleus, but is accumulated at the tip of the stalk. The concentration of this substance thus decreases, according to a gradient, from the apical to the basal end of the stalk. Basal fragments devoid of nuclei can not form caps; but, as shown recently in Hämmerling's laboratory, they are capable of extensive protein synthesis. I recently obtained evidence that the frequency of cap formation can be modified at will by modifying the $— SH \rightleftharpoons SS$ equilibrium.

The second question is a more difficult one : it is a very difficult problem to distinguish between exchange or turnover reactions, especially in cases where nothing is known about enzymes concerned with nucleic acid metabolism (as in *Acetabularia* and *Amoeba*). The use of words such as synthesis, turnover and exchange is almost a matter of taste. There are people who talk of RNA *synthesis* in anucleate amoebae, although the RNA content actually drops, simply because incorporation of precursors into RNA has not ceased.

Mme Grunberg-Manago. — L'acide ribonucléique qui lève l'inhibition de la synthèse protéique par la RNAase, doit-il être spécifique et si non, peut-il être remplacé par les polynucléotides biosynthétiques ?

M. Brachet. — Il y a quelques années, le Professeur Ochoa m'a donné de petites quantités de ses polymères artificiels afin de les essayer sur des amibes traitées à la ribonucléase; malheureusement, les résultats ont été très variables, en raison même de la grande variabilité biologique du matériel. Il serait intéressant de reprendre ces expériences sur les racines d'oignon (à condition de pouvoir disposer de quantités plus importantes de polymères) : nous savons maintenant, que si on ne pousse pas trop loin l'action de l'enzyme,

de façon à ce qu'il n'inhibe les synthèses protéiques que de 50 % environ, il est possible de renverser entièrement l'inhibition par l'addition d'un excès d'ARN de levure. Dans une expérience unique (parce que le matériel est difficile à obtenir en quantité suffisante), nous avons constaté que l'ARN isolé à partir des racines d'oignon est cinq fois plus actif que l'ARN de levure pour obtenir cette restauration. Mais cette expérience doit évidemment être refaite à plusieurs reprises avant qu'on puisse tirer des conclusions.

M. Desreux. — Je désirerais signaler, en relation avec les multiples fonctions éventuelles du RNA, que l'antigène de la transplantation de la peau (greffe) semble être de nature ribonucléoprotéique.

Le fractionnement d'extrait de noyaux par chromatographie sur phosphate effectué par Casterman et Oth à Liège, donne plusieurs fractions dont l'activité antigénique évolue parallèlement avec la teneur en acide ribonucléique.

Les chimistes et physico-chimistes seraient très heureux si les biologistes pouvaient leur indiquer des sources d'ADN et d'ARN possédant des caractéristiques biologiques telles que la purification de ces molécules ou toute modification physique et chimique puisse être suivie par test biologique aisé et rapide.

M. Ochoa. — The supernatant cytoplasmic fluid contains RNA (soluble RNA) endowed with specific biological activity, namely that of binding certain aminoacids in a reaction catalyzed by specific (both for the aminoacid and the RNA) amino acid activating enzymes.

There would appear to be at least 20 different ribonucleic acids each specific for one amino acid. However, the fractionation and separation of these different ribonucleic acids has not as yet made much progress.

M. Sadron. — 1) Recherche d'un caractère spécifique du DNA commode pour les mesures physico-chimiques.

En injectant du DNA dans le sang d'un lapin (Dr. Zahn R.), on observe la formation d'une quantité considérable de DNAase. Nous avons observé ce phénomène nous-mêmes mais nous n'avons pas

pu encore mettre en évidence une spécificité de la DNAase relative au DNA. Quoique les expériences faites en ce sens semblent négatives, elles ne sont pas encore absolument sûres.

2) L'affaire des canards.

Sur trois séries d'injections de DNA, la première à provoqué une mutation génétique.

On ne peut pas nier la validité de cette observation, même si l'interprétation (rôle ou non rôle du DNA injecté) est encore à rechercher.

M. Felix. — Les expériences du Dr. R. Zahn on été faites dans mon institut. Il a injecté de l'ADN de hareng dans le sang d'un lapin et constaté que la DNAase a augmenté dans le sang, mais il ne peut pas dire si cette DNAase est spécifique de l'ADN injecté.

MOLECULAR STRUCTURE OF DEOXYRIBONUCLEOPROTEINS

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INTRODUCTION

Much has been learnt in recent years of the role of deoxyribonucleic acid (DNA) in heredity, and there is now some understanding of the function of DNA in terms of its molecular structure. In contrast, little is known of the role of the proteins to which DNA is attached *in vivo*, and knowledge of the structure of deoxyribonucleoprotein (DNP) is very incomplete (for review see Davidson, Conway and Butler, 1954).

When DNA combines with proteins it retains its characteristic molecular configuration. As a result, study of the molecular configuration of DNP is simplified. We may note that, in contrast, ribonucleic acid (RNA) shows little signs of having a definite configuration when isolated; its structure in nucleoproteins is probably determined by that of the protein, and in the case of some plant viruses at least, it is the protein that retains its configuration when isolated.

Sometimes DNA occurs *in vivo* not bound to protein, e.g. in bacteriophage and possibly in bacteria. In higher organisms DNA is bound to basic proteins by salt linkages to the phosphate groups. Little is known of other types of protein-DNA linkage which it has been suggested may exist (e.g. Kirby, 1958). Knowledge of the structure of the basic proteins is fundamental to understand-

ing the structure of DNP. The early studies of these proteins (reviewed by Kossel, 1928) are of great importance, but we shall be concerned here mainly with newer results obtained in our laboratory, by the use of physical techniques such as X-ray diffraction (with Dr. Zubay and Dr. Wilson) and infra-red absorption spectroscopy.

The early workers divided basic proteins into two groups — protamines and histones. The newer techniques show that the structures of nucleoprotamine and nucleohistone are very different. Whereas nucleoprotamine appears to be a structure found only in sperm of some species, nucleohistone is the major constituent of chromosomes in higher organisms. Probably the structure of nucleohistone forms the basis of chromosome ultrastructure, and, on the molecular level, there is little distinction between chromosome structure and nucleohistone structure.

THE STRUCTURE OF DNA

The structure of DNA has been elucidated mainly by chemical studies of the covalent bonds in the molecule and comparative studies of its chemical composition; by physico-chemical study of hydrogen bonding and molecular shape; and by X-ray diffraction and stereochemical studies of the molecular configuration (for a general review see Wilkins, 1956*a*). As a preliminary to discussing the structure of DNP we shall give briefly recent unpublished data on DNA structure.

X-ray diffraction studies of DNA are made on fibres. The double-helical configuration of the molecule may take various forms in these fibres. The most detailed information has been obtained for the *B* configuration of DNA which is the form found to occur in DNP *in vivo*. A recent X-ray diffraction photograph of the lithium salt of DNA is shown in Fig. 1. The observed X-ray diffraction intensities may be correlated with intensities calculated from a molecular model (Langridge *et al.*, 1957). The model is constructed using stereochemical data on the structure of the nitrogen base, deoxyribose, and phosphate parts of the molecule. The latest structure is shown in Fig. 2. It closely resembles that previously described (Langridge *et al.*, 1957) except that the phosphate group has been reoriented about

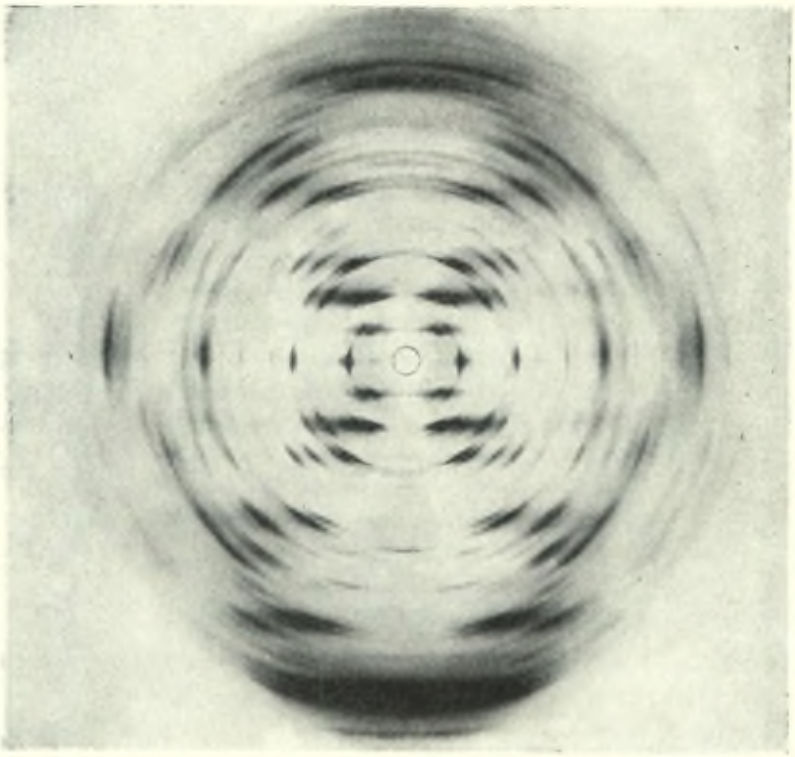


Fig. 1. — X-ray diffraction photograph of fibres of lithium salt of DNA. Material made by L. D. Hamilton and photograph by D. A. Marvin.

its centre. The basic groups of protein will be attached to the two charged oxygen atoms of the phosphate group. The reorientation of the phosphate groups has caused these atoms to move respectively about 1 Å and 1.5 Å. It is likely that the position of these atoms is not more than 0.5 Å in error. The measure of agreement between calculated and observed diffraction intensities is shown in Fig. 3. The new structure is preferred to the old because : (1) bond angles are less distorted; (2) the configuration of the sugar ring is puckered instead of planar; (3) the orientation of the phosphate group is in agreement with the observation that, with oriented material, infra-red absorption bands characteristic of the phosphate group show no dichroism. Calculation of the points of closest intermolecular approach in the microcrystals in the fibre confirms the correctness of the structure.

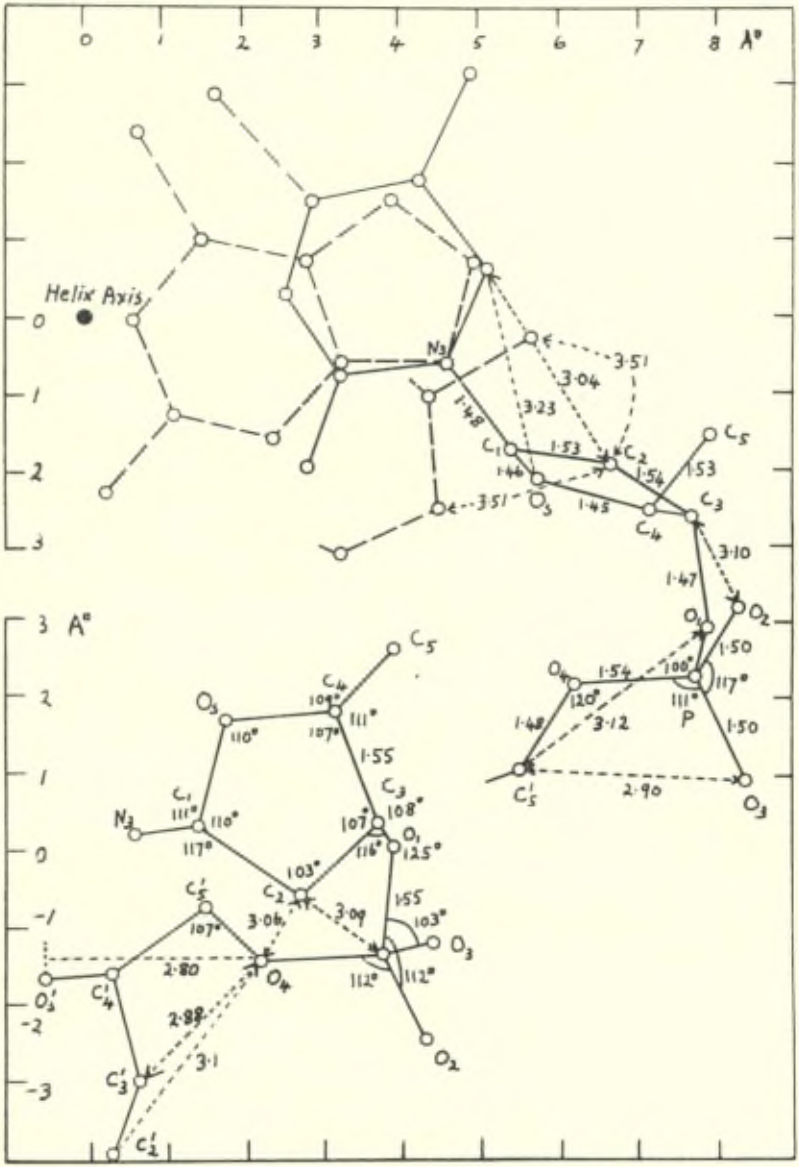


Fig. 2. — Structure of DNA. A nucleotide is shown projected on planes perpendicular to the helix axis and to a diad axis in the molecule. Bond lengths and short inter-atomic distances are given in Å and bond angles are shown.

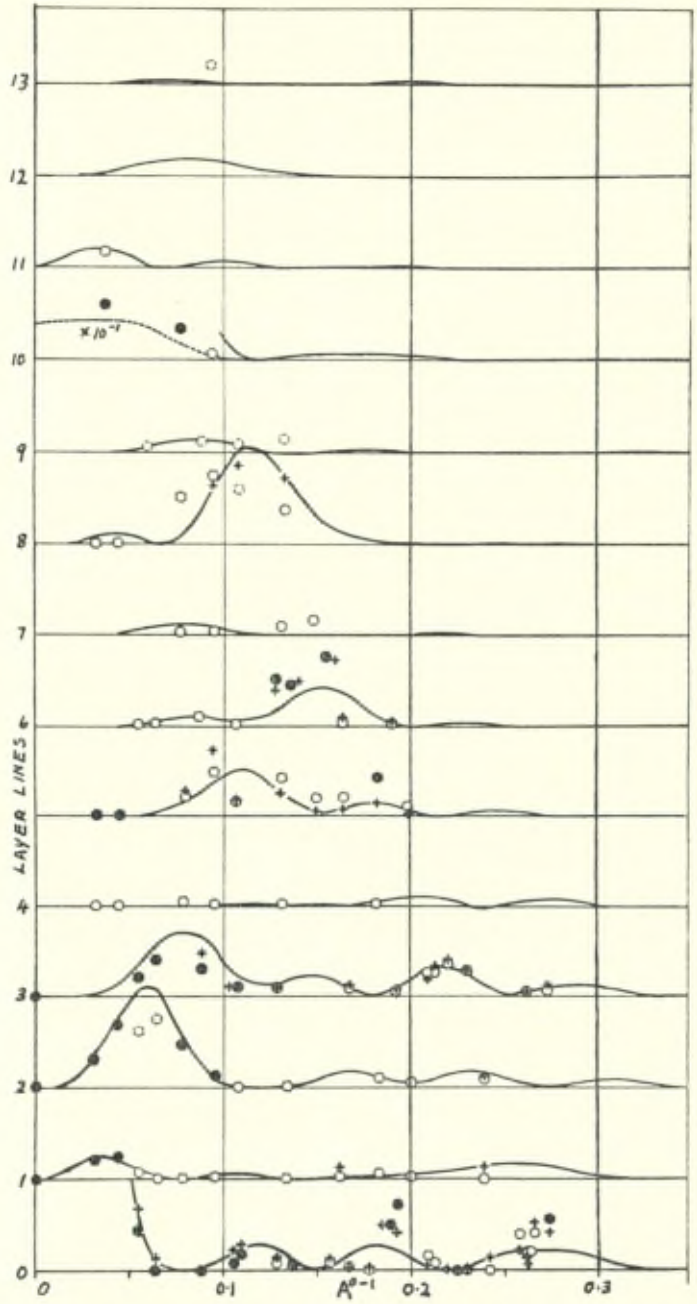


Fig. 3. — Comparison of the calculated diffraction from the DNA model with observed intensities of X-ray diffraction. The continuous line corresponds to the square of the Fourier transform (averaged by rotation about the helix axis). When values for individual reflections differ appreciably from the average, the value is shown +. Observed intensities are, in order of reliability, indicated by ●, O, O.

EVIDENCE THAT DNA HAS THE SAME CONFIGURATION WHEN FREE AND WHEN BOUND TO PROTEIN

Most evidence is obtained by comparing the X-ray diffraction patterns of oriented DNA and DNP. In both there is a strong 3.4 Å reflection corresponding to the spacing of nucleotides 3.4 Å apart along the helix axis, and a 34 Å layer-line spacing which shows that the pitch of the helix is 34 Å. Hence some of the dimensions of the molecule are the same. In the central region of the DNA and DNP patterns, diffracted intensity is concentrated in much the same way into a cross pattern. This shows the general helical form of both molecules is similar. There are intensity differences in this region and, in the case of nucleoprotamine, these indicate that the protein is wrapped around the DNA helix. In the outer part of the pattern, the 10th layer line (3.4 Å reflection) and the 5th and 8th layer lines may be distinguished; otherwise, on account of disorientation in the specimen and blurring of the pattern, the layer lines merge into a characteristic system of streaks. No significant difference is apparent in these features in DNA and DNP (Fig. 4). This confirms that the details of the structure of DNA, as well as its general form, are closely similar in free DNA and in DNP.

NUCLEOPROTAMINE

Protamines (Felix *et al.*, 1956) are polypeptides of average molecular weight about 5,000. Two thirds of the residue are arginine and the rest are small neutral amino acids. The main sequence in the polypeptide chain appears to be *AAAA* xy *AAAA*, where *A* is arginine and *x* and *y* are other amino acids. Proline is often an end group. Optical evidence suggests the polypeptide chain has an extended β form.

When solutions of protamine and DNA (sodium salt) are mixed, a precipitate is formed which has properties very similar to those of native nucleoprotamine in sperm heads. Like sperm heads, the precipitate contains few metal atoms (Feughelman *et al.*, 1955): these are displaced from the phosphate groups of the DNA by the basic groups of arginine.

Nucleoprotamine precipitates may be pulled into birefringent fibres which give oriented diffraction patterns. A somewhat sharper pattern of the same kind is obtained from elongated

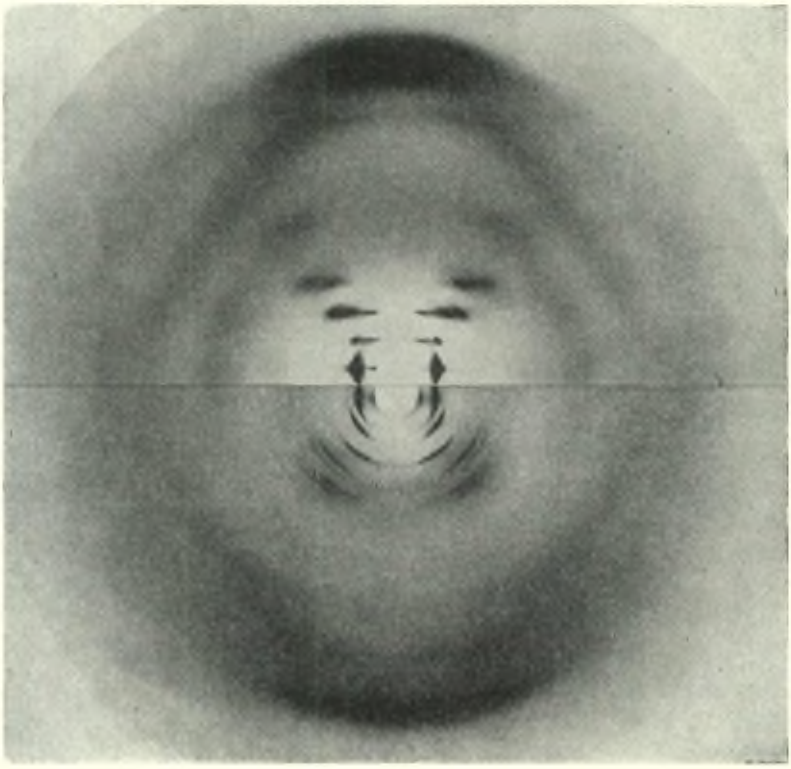


Fig. 4. — Similarity of the streaks in the outer part of the X-ray diffraction pattern of DNA (top) and of *Sepia* sperm heads (bottom).

sperm heads in which the DNA molecules lie along the length of the head. The central part of the pattern (Fig. 4) consists of well defined arcs, the positions of which show that the DNA molecules are packed together in a crystalline array. The outer part of the pattern consists of streaks similar to those obtained from DNA (Fig. 4). The fact that the well defined arcs characteristic of a crystalline structure are not present in the outer part of the pattern shows that the packing of the molecules in a crystalline structure is imperfect. The intensities of the arcs show that the first layer line of the DNA-like pattern is stronger than for DNA. This observation led to the suggestion that the protamine was wrapped around the DNA molecule and lay in the small groove on it (Feughelman *et al.*, 1955) and that in the *AAAA* regions the polypeptide chain had a β configuration.

The dimensions of the arginine groups are such that they form a regular arrangement attached to the phosphate groups of the DNA. Since the non-basic residues occur in pairs in the polypeptide chain, they can form loops in the chain and do not interfere with the arrangement of the arginine groups. As a result all phosphate groups can be neutralised by arginine. The complex of DNA and polylysine appears to have a similar structure to that of the *AAAA* part of nucleoprotamine (Wilkins, 1956*a*).

NUCLEOHISTONE

Histone consists of two distinct types of protein (e.g. Luck *et al.*, 1958). About 20 % is lysine-rich histone of molecular weight about 10,000, and consists largely of lysine and alanine and contains very little arginine and few acidic residues or residues of high molecular weight. We shall refer to the other fractions as arginine-rich. Their molecular weight is probably in the region of 20,000, and they contain arginine and lysine, tyrosine, and acidic and other residues. Optical evidence suggests that lysine-rich histone is of β form and this is confirmed by the observation that it combines with DNA to give a structure like nucleoprotamine (Wilkins, 1956*a*). Several lines of evidence indicate that in arginine-rich histone the polypeptide chain is folded, has internal hydrogen bonding, and is probably in a helical configuration. That histone can denature is indicated by the fact that it combines well with Kieselguhr and amino benzyl cellulose only after heating (Brown and Brown, 1958), and that the characteristic X-ray pattern of nucleohistone is obtained from reconstituted nucleohistone only if great care is taken to avoid denaturation. One of the most marked properties of histones is their tendency to form molecular aggregates in solution. This tendency is reduced by the presence of substances such as urea that break hydrogen bonds (Luck *et al.*, 1958).

Nucleohistone is soluble in water if the ionic strength is low, and is insoluble in salt solution of physiological molarity 0.14 and of molarity greater and less over a range. Nucleohistone, like nucleoprotamine, dissociates and dissolves in salt solutions of molarity about 0.7 M and above. When a solution in high salt concentration is diluted, a precipitate is formed. This reconstituted nucleohistone can be dispersed in water if the ionic

strength is low (Crampton, 1957), but the solution appears to contain aggregates of nucleohistone molecules. It has also been shown that reconstituted nucleohistone, when treated with barium chloride and acetic acid, liberates histone fractions in different proportion from those liberated from native nucleohistone (Crampton, 1957). However, reconstituted nucleohistone gives an X-ray diffraction pattern little different from that of intact nucleohistone, and it appears, therefore, that the differences between reconstituted and native material are not fundamental.

Light scattering and viscosity measurements on solutions of nucleohistone in water show that the particles in solution each contain a small number of DNA molecules. Although there is considerable tendency for nucleohistone particles to aggregate, it is possible to disperse the particles so that each contains only one DNA molecule. Light scattering measurements (Doty and Zubay, 1959) on such solutions show that the weight-average molecular weight of the particles is 18.5×10^6 and the radius of gyration is 3,000 Å. Uniform filaments of diameter about 30 Å are seen in electron miscroscope photographs of nucleohistone. These filaments are probably individual DNA molecules coated with histone. About 47 % of the weight of nucleohistone is DNA and hence the average weight of DNA per particle is equivalent to a molecular weight of 8.7×10^6. This is not much different from 8.0×10^6, the molecular weight of calf thymus DNA.

Nucleohistone contains sodium ions equivalent to about 20 % of the phosphate groups (Wilkins, 1956*b*). The arginine and lysine residues could neutralise about 76 % of the phosphate groups. Possibly some of the sodium is bound to glutamic and aspartic acid in the histone, but it seems more likely that it neutralises phosphate groups and, therefore, about 20 % of these are not attached to basic residues in the histone. The hydration of these phosphate groups will assist the nucleohistone to dissolve in water. Presumably aggregation of nucleohistone is connected with the ability of histone itself to aggregate. We can suppose that histone is attached to DNA molecules by salt linkage and that histone on one DNA molecule may bind to histone on another DNA molecule and thus form labile bridges. These bridges may break in water because the charged portions of neighbouring DNA molecules repel each other.

X-RAY DIFFRACTION PATTERNS OF NUCLEOHISTONE

The diffraction pattern of nucleohistone is more complicated than that of nucleoprotamine and is less well defined and more difficult to interpret. Unoriented nucleohistone gives a pattern consisting of rings. If the material is stretched into a fibre, the intensity in the rings orients and appears as spots drawn into arcs. When the relative humidity around the fibres is changed, the positions and intensities of the various spots change. These changes are largely reversible.

Nucleohistone *in vivo*

We have studied various nuclei under such conditions that we believe their structure is approximately the same as *in vivo*. Calf thymocytes, isolated in 0.14 M NaCl, show a diffraction ring at about 38 Å. Chicken erythrocyte nuclei in 0.14 M. NaCl show the same ring more clearly and traces of other rings. Diffuse scattering at 60 Å and above is also present. A similar pattern is given by wet gels of nucleohistone extracted from calf thymus and by sperm heads of some sea urchins. The basic proteins in these sperm heads contain much lysine and alanine and resemble histones more closely than protamine. When the specimens containing nucleohistone are dried somewhat by equilibrating in an atmosphere at 98 % relative humidity, or by shrinking in ethanol-water mixtures, the pattern becomes better defined, clearer in the centre, and a ring at 60 Å appears.

Nucleohistone at 98 % relative humidity

Unoriented material gives a pattern with strong rings at about 60, 36, 27, 12, 8, and 3.4 Å. The nature of the pattern becomes much clearer when the oriented pattern (Fig. 5) obtained from fibres is studied. This pattern consists of three main parts.

1. Part of the pattern resembles that produced by DNA. Near the centre there is an equatorial reflection of about 30 Å and the first, second and third layer lines may be seen. In the outer part of the pattern the usual 3.4 Å meridional arc shows clearly and there are diffuse streaks of the same form as those obtained from DNA. The whole pattern is poorly defined but spots may be seen on the second layer line and on the equator. The presence

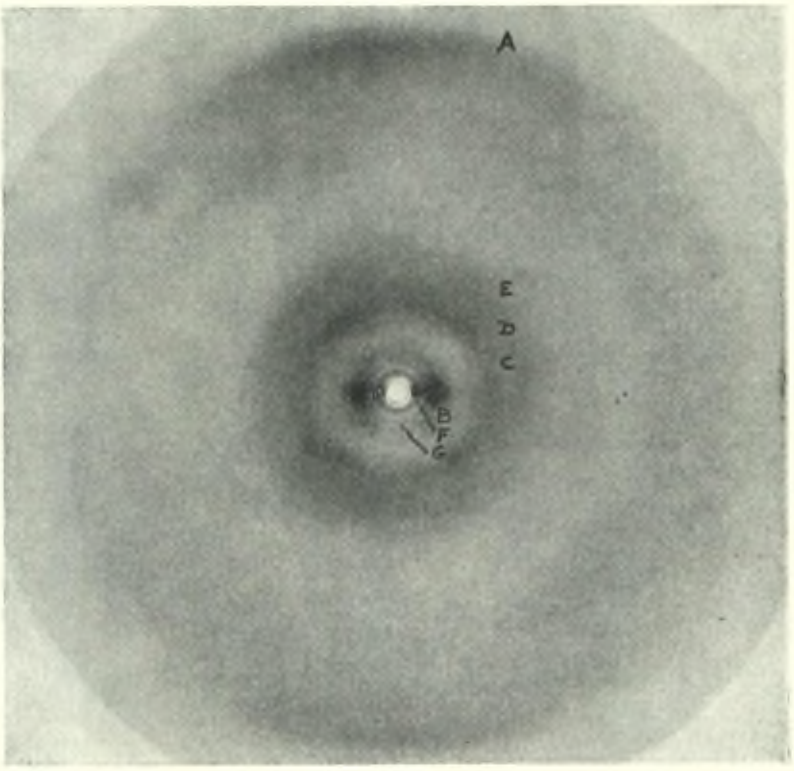


Fig. 5. — X-ray diffraction pattern of fibres of calf thymus nucleohistone at 98 per cent relative humidity. The fibre is vertical. *A* is the 3.4 Å arc, *B* is the 30 Å equatorial spot, *C*, *D*, *E*, are the first, second and third layer lines respectively, *F* is the 60 Å equatorial spot, *G* is the 36 Å meridional arc.

of these spots shows that the molecules are packed in a manner possessing some regularity in three dimensions. When extra-well oriented fibres are used, there are streaks on the layer lines. These streaks show that some of the oriented molecules are displaced irregularly along their length.

2. There is a poorly oriented arc on the meridian at about 36 Å. There are also signs of a similar arc at about 55 Å.

3. There is a strong well-oriented spot on the equator at about 60 Å. When sheet specimens are used and the sheets are parallel to the X-ray beam, this 60 Å spot is very strong and a faint 15 Å spot is also visible on the equator. When the sheets are perpendicular to the beam, the 60 Å spot is barely visible and no 15 Å spot is present. These observations show that the

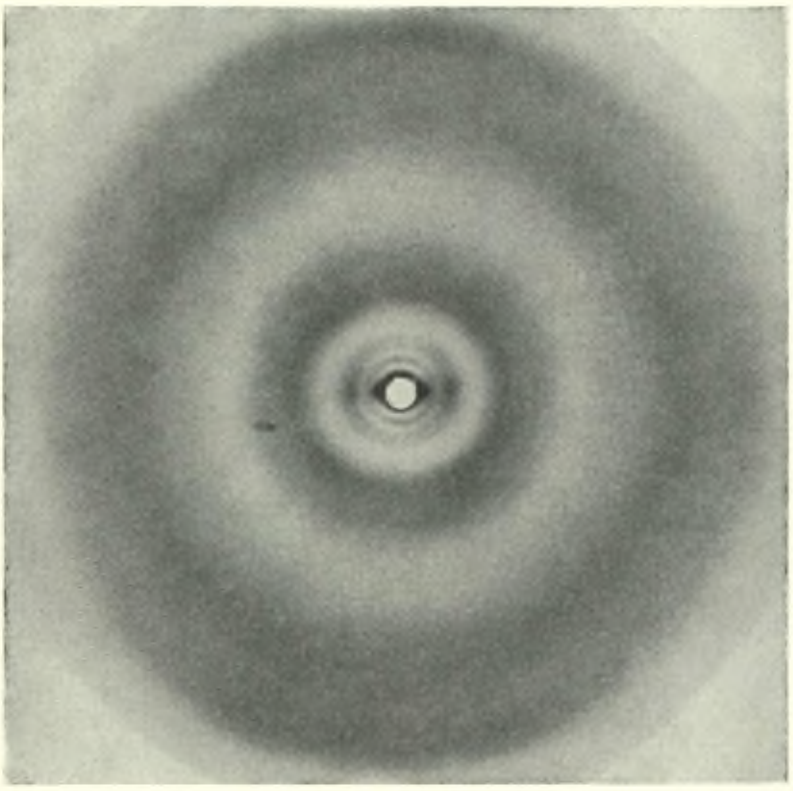


Fig. 6. — X-ray diffraction pattern of fibres of calf thymus nucleohistone at 75 per cent relative humidity. The main equatorial reflection is at about 23 Å. The 36 Å meridional arc shows clearly.

structure giving the 60 Å spot is a sheet structure. Specimens that have not been purified carefully show, in addition, a sharp meridional arc at 4.2 Å. Sphingomyelin is a sheet structure and gives similar 60, 15, and 4.2 Å diffraction, and, as we shall see later, we believe that the 60 Å spot in the nucleohistone pattern is entirely or largely due to lipid impurity.

The nucleohistone pattern at lower humidities

When the relative humidity around the fibre is lowered, there is a decrease in the spacings corresponding to the equatorial and second layer line spots of the DNA-like pattern. Also the DNA-like pattern becomes more diffuse, and as a whole becomes weaker — the equatorial spot especially so (Fig. 6). In contrast the 36 Å

meridional arc becomes somewhat stronger, and a strong diffuse and poorly-oriented meridional arc appears at about 75 Å. There is also strong diffuse scattering in the centre of the pattern. A sharp equatorial reflection is often observed between 35 and 40 Å (Fig. 7) and is believed to be due to lipid impurity. At 32 % humidity and below, the outer part of the pattern consists only of diffuse rings at about 4.7 and 9.7 Å.

THE PRESENCE OF LIPID IN NUCLEOHISTONE PREPARATIONS

X-ray diffraction data show in several ways that nucleohistone preparations contain lipid impurity. Sphingomyelin has a sheet structure and gives a very strong diffraction pattern consisting of a strong 60 Å and a weaker 15 Å spot on the equator, and a 4.2 Å meridional arc. These reflections are all observed in diffraction patterns obtained from nucleohistone if the material has not been carefully purified. Sometimes the 60 Å spot alone is observed if the nucleohistone has been repeatedly washed in an attempt to remove impurities. The experiments with nucleohistone sheets show that the 60 Å spot is produced by a sheet structure. Comparison of the intensity of this spot with that given by a exposure of oriented sphingomyelin, showed that 3 % of sphingomyelin in nucleohistone would be sufficient to give the observed intensity of the 60 Å reflection.

Adequate chemical estimation of the amount of lipid impurity in nucleohistone preparations has not been made (Chayen and Gahan, 1958), but there are many indications that a few per cent of lipid is present (e.g. Bakay *et al.*, 1955). Infra-red absorption data suggest that the amount of lipid is less than 5 %. Purification of the nucleohistone by dissolving it in water and precipitating with 0.14 M NaCl does not remove the 60 Å spot (Wilkins, 1956*a*). This suggests that lipid is attached to nucleohistone. A 60 Å ring is observed in the pattern given by some histone samples, but not in patterns from DNA. This indicates that the lipid in nucleohistone preparation is bound to the histone. Lipids resembling sphingomyelin contain phosphate groups and these could link to basic groups of histone.

It is clear that a large part of the 60 Å reflection from nucleohistone specimen is due to lipid impurity, but it is possible that

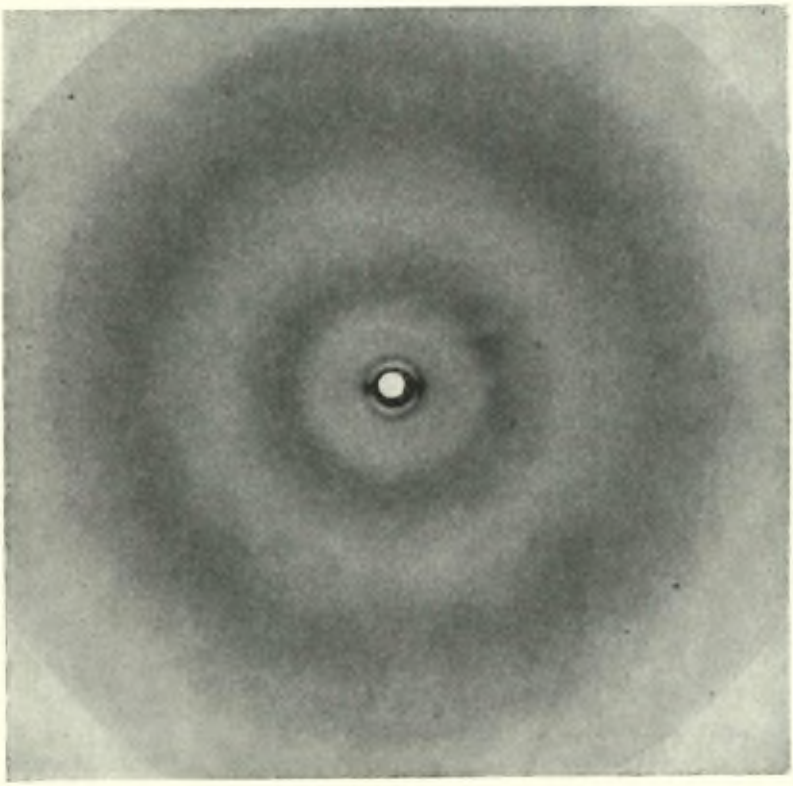


Fig. 7. — X-ray diffraction photograph of fibres of nucleohistone at 32 per cent relative humidity. The 36 Å meridional arc shows clearly. The sharp equatorial spot due to lipid is at about 40 Å.

the nucleohistone itself might also contain a sheet structure with a 60 Å periodicity. However, this seems rather unlikely because nucleohistone, reconstituted from DNA and histone free from lipid, gives a pattern with all the characteristics of the normal nucleohistone pattern except that the 60 Å equatorial spacing is absent.

Further X-ray evidence suggesting the presence of lipid is as follows. A characteristic of many lipids is that the sheet structure can shrink so that the 60 Å spacing moves to about 40 Å. This shrinkage is achieved by raising the temperature of the specimen to about 100° C (Elkes and Finean, 1953). We have not studied the diffraction pattern of hot nucleohistone but we have observed that a sharp equatorial reflection, in addition to that at 60 Å, often

appears when the water content of fibres of nucleohistone is reduced. This spacing moves to a limiting value of about 35 Å. Shrinkage of this kind is observed in many but not all specimens of nucleohistone, and has been seen in specimens purified by reprecipitation. Sometimes it seems that the 60 Å spot is replaced entirely by the smaller spacing, but this is not certain.

In early stages of our work we were reluctant to accept the idea that a small amount of lipid impurity might be responsible for the strong 60 Å reflection that appeared so characteristic of nucleohistone irrespective of the source from which it was obtained. We suggested that the reflection indicated that nucleohistone molecules might exist in the form of small bundles of parallel molecules (Wilkins, 1956*b*), e.g. in groups of four. We have now abandoned these ideas.

OPTICAL EVIDENCE CONCERNING THE STRUCTURE OF NUCLEOPROTEINS

Infra-red and ultra-violet data both show that DNA molecules tend to lie along the length of fibres and stretched sheets of nucleoprotamine and nucleohistone (Fig. 8). The phosphate absorption frequencies of nucleoproteins show a smaller change with humidity than is observed with salts of DNA. This suggests that the basic groups of the protein are, as one would expect, bound directly to the phosphate groups, whereas in salts of DNA the metal ion is surrounded by water and, as a result, the phosphate group is hydrated. There is no detectable dichroism of the absorption bands associated with the C = O and NH bonds in the proteins of nucleoprotein. This means that these bonds lie on the average at angle of $56° \pm 10°$ to the axis of the DNA molecules. This excludes the possibility that the polypeptide chains, in the α or β configuration, lie on the average parallel to the length of the DNA molecules.

The deuteration of nucleoproteins may be studied by measuring their infra-red absorption. Deuteration of NH groups is found to be fast in nucleoprotamine. This is consistent with the idea that the polypeptide chain has little internal hydrogen bonding and is largely in the extended β form. A considerable part of the deuteration of protein NH groups in nucleohistone takes place slowly at a rate similar to that observed in α proteins and

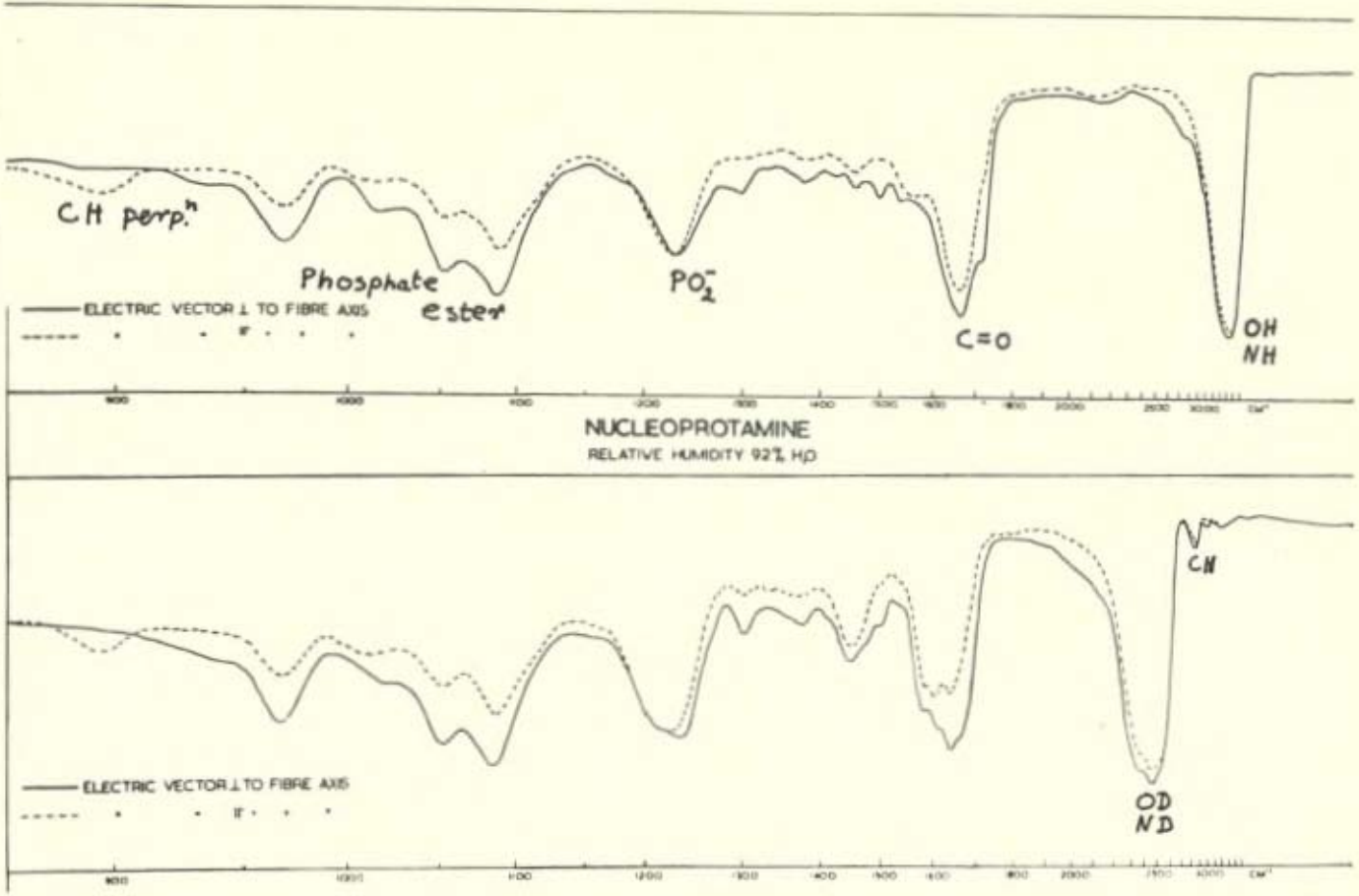


Fig. 8. — Absorption of polarised infra-red radiation by oriented sheets of nucleoprotamine. The lower pair of curves shows absorption when water in the specimen has been replaced by deuterium oxide.

collagen. We may expect, therefore, that there is a large amount of internal hydrogen bonding in histone polypeptide chains. The simplest assumption is that the configuration is the α-helix. The C = O vibration in nucleohistone occurs at about 1,660 cm^{-1} as is observed in α proteins; while this suggests the configuration is not β, it is in itself no proof that the configuration is α. Observation on the denaturation of histone, in experiments on reconstituting nucleohistone from DNA and histone, also suggest that histone has a specific molecular configuration stabilised by internal hydrogen bonds.

INTERPRETATION OF THE NUCLEOHISTONE DIFFRACTION PATTERN

The oriented DNA-like pattern shows that in nucleohistone fibres DNA molecules lie roughly parallel and are packed in a semi-regular manner in three dimensions. In unoriented material a similar structure is present because much the same spacings are observed. The relative intensities of the layer lines in the DNA-like pattern are not obviously different from those of DNA itself. This suggests that there may not be a very close association between histone and DNA. Previously Arndt and Riley (1953) had, on the basis of much less clear evidence, made a similar suggestion. The fact that the first layer line is not strong, as in nucleoprotamine, shows that histone is not concentrated in the small groove on the DNA molecule. Also the intensities of the layer lines do not correspond with the model of nucleohistone (Wilkins, 1956*a*) in which a polypeptide chain is wrapped around both the grooves of the DNA molecule. The spots in the pattern are less well defined than those given by nucleoprotamine and the arrangement of the molecules is, therefore, less regular.

The 36 Å semi-meridional arc is less well oriented than the DNA-like pattern. It is therefore likely that there are two phases present in nucleohistone fibres. This is also indicated by the fact that, with well oriented fibres, although the spots in the DNA-like pattern are well oriented, they are not so clearly defined as the less well oriented arcs. It appears that orientation of the DNA molecules tends to destroy some of the regularity of the structure.

The 36 Å spacing is oriented roughly along the fibre length and is about the same as the pitch length of the DNA molecules. This suggests that the spacing may correspond to the distance between protein bridges formed between adjacent DNA molecules. One would expect that bridges would occur at points of contact between one DNA molecule and its neighbour, or at other corresponding points along the line of junction between two molecules. The distance between the bridges is somewhat greater than the usual value for pitch length of DNA molecules. The distance would be greater than the pitch length if the DNA molecules were coiled in a right-handed direction around each other. A system of such coiled coils consists of coaxial cylindrical shells of coiled DNA molecules and has a structure resembling that of the metal core of an electric cable. The molecules are inclined to the axis of the cable, and lines through corresponding points on protein bridges are at right angles to the length of the molecules. As a result, diffraction from the protein bridges is poorly oriented with respect to the axis of the cable. In such a system intertwined pairs of DNA molecules are themselves twisted round other pairs. It has been suggested (e.g. Kaufmann and McDonald, 1956) that chromosomes consists of two helical structures twisted round each other and that each of these helical structures consists of two smaller helical structures twisted round each other and that this structurai relationship continues on smaller and smaller scales until the ultimate double helix of polynucleotide chains is reached.

When nucleohistone is stretched to form fibres, the three-dimensional system of nucleohistone might pull apart into individual nucleohistone molecules like those found in solution. We would expect these molecules to become well oriented and they would then give the well oriented DNA-like pattern. Since the protein bridges would be broken, no well oriented reflection from the bridges would appear.

Coiling of the DNA molecules is not essential to explain the 36 Å semi-meridional reflection. There is no direct evidence that the less well oriented DNA molecules in nucleohistone fibres do not have a pitch length somewhat greater than is usual for DNA molecules and equal to the semi-meridional spacing. If this were so, straight parallel DNA molecules could be joined by bridges and linked into groups. These groups might orient poorly on account of cross-linking and aggregation between groups,

or because their shape was not long and thin. As a result, diffraction from the system of protein bridges would be poorly oriented.

One might expect a strong reflection at right angles to the length of nucleohistone molecules, if the molecules, whether twisted or not, were arranged regularly in groups, and if the histone were closely attached to the DNA molecules and there was a large space filled with water between the DNA molecules. We believe that the 38 Å ring, observed with unoriented undried nucleohistone and in unfixed nuclei, corresponds to the semi-meridional reflection from fibres. Previously we believed we had evidence that this reflection could be oriented and was equatorial (Wilkins, 1956*a*). However, we have not been able to confirm this. If a ring corresponding to an equatorial reflection is absent, this could be explained if histone was distributed throughout the space between DNA molecules and, except for the basic ends of the side-chains of lysine and arginine, was not in close contact with the DNA molecules. Alternatively it may be that *in vivo* the DNA molecules are packed somewhat inclined to each other, and that an equatorial reflection appears only after drying has caused the molecules to condense into a parallel arrangement. If histone were loosely attached to DNA, the histone structure would collapse when the nucleohistone was dried, but the inter-bridge spacing would remain. If bridges tended to coalesce in pairs, the semi-meridional 75 Å spacing would be produced. In dry fibres of nucleohistone we believe the DNA molecules have collapsed and become irregular and contribute little to the diffraction pattern. The histone is then largely consolidated and diffraction from such histone would produce the diffuse 4.7 and 9.5 Å rings. Gaps between the bridges would give rise to the semi-meridional 36 Å reflection.

THE RELATIONSHIP OF DNA TO PROTEIN IN VARIOUS LIVING FORMS

We have shown that, in a wide variety of living things, DNA has the same structure (Wilkins, 1957). The only exception known is in Φ X-174 bacteriophage where DNA appears to occur as a single polynucleotide chain. In T_2 bacteriophage, DNA has its usual structure but is not bound to basic protein. The number of basic residues in the phage protein is much less than the

number of phosphate groups in the DNA, and the number of metal ions present is also small. Probably some of the phosphate groups are neutralised by poly-amines present in the phage head. In preliminary attempts we have failed to obtain a DNA-type X-ray diffraction pattern from insect virus. The DNA content of this virus is small and the protein is non-basic. Base ratios are unity in the DNA and this would suggest that the DNA has its usual structure and is not a single polynucleotide chain like RNA in tobacco mosaic virus.

Apart from somewhat incidental observations, bacterial DNP has been studied only in the case of *Escherichia coli*. Quantitative amino acid analysis shows that the protein present is not histone (Zubay and Watson, 1959); furthermore, the protein is not dissociated from DNA by concentrated NaCl. However, the qualitative results of Palmade *et al.* (1958) indicate that the protein attached to DNA is histone. X-ray diffraction study of the DNP preparations of Zubay and Watson shows that DNA is present largely free of protein (Wilkins and Zubay, 1959).

DISCUSSION AND CONCLUSION

We have mentioned some observations indicating that nucleoprotamine and nucleohistone have different structures. X-ray data show that nucleoprotamine consists of parallel DNA molecules fairly close together and bound to basic protein. This is confirmed by the observation that protamine-containing sperm heads are negatively birefringent, and the electron microscope shows that their structure before maturity consists of regularly arranged parallel fibrils. Nucleohistone, however, is different: parallel fibrils are not observed in nuclei containing nucleohistone; most chromosomes, unless they are stretched, have low birefringence and show little dischroism of the ultra-violet absorption band of DNA; sperm heads containing histone-like proteins are not birefringent. This suggests that in nucleohistone the DNA molecules are coiled and, when histone is replaced by protamine during sperm development, uncoiling takes place. This agrees with our deductions from the X-ray diffraction patterns and with the ideas of those who have, with the light microscope, observed coiled-coil structures in chromosomes. We believe that the com-

bination of histone with individual DNA molecules causes the DNA to coil, or that coiling is produced when histone on a DNA molecule links with that on another and the bridges between the molecules build the chromosome structure. We must, however, recognise that evidence for the existence in nucleohistone of protein bridges and of coiling of DNA molecules is indirect, and interpretation of the X-ray diffraction data in terms of bridges and coiled DNA molecules is not without ambiguity.

There is not much data to indicate the nature of the histone bridges. There is, however, good evidence that the polypeptide chain in arginine-rich histone is in a coiled form, probably the α-helix. An α-helix can fit into the large groove on the DNA molecule, and a well defined bridge between DNA molecules could be formed by an α-helix lying in the large grooves of two parallel DNA molecules. The role of lysine-rich histone may be to neutralise, on the DNA, the phosphate groups that have not been neutralised by the α-helix histone.

It may be noted that if histone is not closely in contact with DNA molecules, but is concentrated in a bridge structure, it is likely that the nitrogen bases of DNA molecules are not covered by protein. The bases would then be free for chemical reaction and genetical function. The hydration of nucleohistone *in vivo* is high and there is sufficient separation of DNA molecules for quite large molecules to pass into the three-dimensional nucleohistone structure (Wilkins, 1956*a*). These molecules may then interact with the bases of the DNA molecules.

The role of histone is probably to join together, in higher organisms, genetically equivalent DNA molecules into a polytenic chromosome, and to link the large number of molecules in each chromosome into a coherent group that is transportable during mitosis. It seems likely that, in bacteriophage, chromosomes are not polytenic and consist of single DNA molecules (possibly joined end to end) and that in bacteria the polyteny is low. Such small chromosomes may be manageable, during multiplication of DNA molecules or during nuclear division, without the complication of chromosome coiling present in higher organisms. We suggest that histone exists only for structural purposes and only in polytenic chromosomes. It may be that histone, in addition, takes part in gene action; but nothing is known of this at present.

ACKNOWLEDGEMENTS

The X-ray diffraction studies of nucleoprotein were made jointly with Dr. H. R. Wilson and Dr. G. Zubay; help was also given, and some X-ray photographs taken, by Dr. R. Langridge and Mr. C. W. Hooper. Dr. W. E. Seeds, Mr. W. Fuller and Dr. M. Spencer have helped by building molecular models and calculating diffraction patterns. New data on DNA structure were obtained with Mr. D. A. Marvin. Details of infra-red absorption data have been kindly supplied to me by Mr. G. R. Wilkinson. Professor J. T. Randall, F. R. S., has encouraged the work throughout. The work was aided by computer time made available by I. B. M. United Kingdom, Ltd., and I. B. M. World Trade Corporation. I am grateful to Dr. V. Luzzati for stimulating discussion of his unpublished X-ray data.

REFERENCES

Arndt, U.V. and Riley, D.P. : *Nature* [*London*], **172,** 294 (1953).

Bakay, B., Kolb, J.J. and Toennies, G. : *Arch. Biochem. Biophys.*, **58,** 144 (1955).

Brown, G.L. and Brown, A.V. : *Symp. Soc. Exp. Biol XII,* 6 (1958).

Chayen, J. and Gahan, P.B. : *Biochem. J.*, **69,** No. 4, 49 P (1958).

Crampton, C.F. : *J. Biol. Chem.*, **227,** 495 (1957).

Davidson, P.F., Conway, B.E. and Butler, J.A.V. : *Progress in Biophysics,* **4,** 148 (1954).

Doty, P. and Zubay, G. : *J. Mol. Biol.*, **1** (1959), in press.

Elkes, J. and Finean, J.B. : *Exp. Cell Res.*, **4,** 82 (1953).

Felix, K., Fischer, H. and Krekels, A. : *Progress in Biophysics,* 6,1 (1956).

Feughelman, M., Langridge, R., Seeds, W.E., Stokes, A.R., Wilson, H.R., Hooper, C.W., Wilkins, M.H.F., Barclay, R.K. and Hamilton, L.D. : *Nature* [*London*], **175,** 834 (1955).

Kirby, K.S. : *Biochem. J.*, **70,** 260 (1958).

Kossel, A. : "The Protamines and Histones", London, Longmans (1928).

Kaufmann, B.P. and McDonald, M.R., : *Cold Spring Harbor Symp. XXII,* 233 (1956).

Langridge, R., Seeds, W.E., Wilson, H.R., Hooper, C.W., Wilkins, M.H.F. and Hamilton, L.D. : *J. Biophys. Biochem. Cytol.*, **3,** 767 (1957).

Luck, J.M., Rasmussen, P.S., Satake, K. and Tsvetikov, A.N. : *J. Biol. Chem.*, **233,** 1407 (1958).

Palmade, C., Chevalier, M.R., Knobbloch, A. and Vendrely, R. : Compt. rend, **246,** 2679 (1958).

Wilkins, M.H.F. : *Cold Spring Harbor Symp. XXI,* 75 (1956a).

Wilkins, M.H.F. : *Biochem. Soc. Symp.*, No. 14, 13 (1956b).

Wilkins, M.H.F. : Special Publications. New York Academy of Sciences, **5,** 180 (1957).

Wilkins, M.H.F. and Zubay, G. : *J. Biophys. Biochem. Cytol.*, in press (1959).

Zubay, G. and Watson, M. : *J. Biophys. Biochem. Cytol.*, in press (1959).

DISCUSSION DU RAPPORT DE M. WILKINS

M. Watson. — What is the evidence for second order coiling of the fibrils ?

M. Wilkins. — The evidence is circumstantial :

1) Oriented fibres of nucleohistone disorient when wetted.

2) The 35 Å reflection that we believe is due to diffraction from histone bridges, can never be well oriented.

3) Birefringence and ultra-violet dichroism of chromosomes containing histone are low. In contrast DNA is well oriented in nucleoprotamine in sperm heads.

4) Electron microscopy of nucleohistone and nucleoprotamine supports the idea that nucleohistone consists of coiled fibres whereas nucleoprotamine is not coiled.

M. Felix. — May one conclude from your observations that only nuclei with loose structure can contain nucleohistone ? This would explain why the histones have to be replaced by protamines during sperm maturation. There the nuclear material is much more compact than it is in the somatic nuclei.

My second question regards the quantitative relative relationship between the basic amino acids in the histone and the phosphoric acid in the DNA. As far as I remember Dr. Butler (P.F. Davison and J.A.V. Butler, *Biochim. et Biophys. Acta*, **21**, 568, 1956) has found that there are not enough basic amino acid to neutralize the phosphoric acid of the DNA.

M. Wilkins. — It is true that nucleoprotamine in sperm heads is more compact and less hydrated than nucleohistone. However, some sperms, e.g. those of cod and sea urchin, contain a basic protein resembling histone.

Electron microscopy of sea urchin sperm heads shows that they have a structure resembling that of thymus nuclei. I do not know the hydration of these heads.

Dr. Crampton's measurements show that 90 % of the phosphate groups in nucleohistone are accounted for by basic amino acids. Dr. Zubay's measurements give about 75 %.

I think all workers agree that the figure is less than 100 %.

M. Butler. — We found indeed that the total amount of basic amino acids in thymus nucleohistone was only 15 % less than the total amount of phosphate groups.

It is difficult to understand this unless it has a stoichiometrical basis, i.e. the majority of the phosphates are neutralized by a basic group. This gives rise to considerable geometrical difficulties in Dr. Wilkins structure, e.g. it would be necessary to have ten DNA fibres round each DNA fibre.

There is another point. In histones it has been found that in general there are three non basic amino acids for one basic amino acid. This is true not only of all the samples of whole histone which we have examined; it is also true of all the fractions we have analysed.

It is difficult to see how a complete α-spiral can be obtained with only three residues, when the basics are arranged zig-zag from one DNA fibre to the next. It is true, as pointed out by Davison and myself (1), that you can get something approaching an α-spiral when the histone goes straight along one " vertical " row of phosphates.

M. Wilkins. — I agree with Prof. Butler that there is good evidence that a large proportion of the phosphate groups in nucleohistone are neutralized by basic groups of the histone and that a model of nucleohistone should satisfy this condition.

We think that one molecule of histone is attached to one DNA molecule, and that this molecule of histone is then attached, probably through hydrogen bonds, to another histone molecule on another DNA molecule. Because side chains of the basic residues in the histone are moderately long and flexible, the histone molecules do not need to be very closely attached to the DNA molecules.

M. Luzzati. — Je voudrais décrire très sommairement les résultats d'une étude effectuée à Strasbourg par M. Nicolaïeff et moi-même.

(1) *Biochem. J.*, **21**, 568, 1956.

Nous avons examiné la diffusion centrale des rayons X par des gels aqueux de ADN, nucléoprotamines (DNP) et nucléohistones (DNH) en fonction de la teneur en eau : nous avons étudié également des noyaux intacts contenant soit de la DNP soit de la DNH.

Dans le système ADN-eau, on trouve une seule phase, à toutes les concentrations : toute l'eau du système est logée entre les molécules d'ADN, et, à des concentrations supérieures à 20 % environ (en ADN), la structure s'ordonne selon un assemblage hexagonal.

Au contraire, dans les systèmes DNP-eau et DNH-eau, à chaque concentration on rencontre deux phases en équilibre : dans chacun de ces systèmes il existe plusieurs phases différentes. Les diagrammes de diffraction de certaines de ces phases sont identiques à ceux qu'on obtient avec des noyaux intacts : nous décrirons ici ces structures.

La phase " biologique " du système DNP-eau contient 30 % environ d'eau : sa structure est bien ordonnée, la maille est hexagonale, son paramètre est 31 Å environ, les raies sont très fines. La DNA au contraire forme un gel, dont les raies de diffraction sont peu nettes, indice d'une organisation médiocre; la concentration est de 35 % environ de DNH. On reconnaît dans les diagrammes de diffraction trois bandes, dont les espacements sont 110 Å et ses deux ordres supérieurs (55 Å et 37 Å).

Cette différence de structure entre DNH et DNP peut correspondre au rôle biologique différent de ces substances; l'ADN dans les DNP ne semble pas avoir une fonction métabolique importante, ce qui est compatible avec une structure compacte et peu hydratée, tandis que dans les DNH la structure est très ouverte (le taux d'hydratation atteint 65 %), ce qui peut permettre une circulation de matière, nécessaire pour que l'ADN puisse jouer son rôle métabolique.

Par ailleurs le type de diagramme de rayons X observé dans les DNH suggère une structure lamellaire, dont l'espacement serait 110 Å.

Cette étude a été effectuée avec des noyaux de spermatozoïdes de truite et d'érythrocytes de poulet, ainsi qu'avec les DNP et DNH extraites de ces noyaux : les résultats ont été généralisés à d'autres nucléoprotéines extraites de différents organes de plusieurs espèces

d'animaux. Au contraire les DNH obtenues en précipitant *in vitro* des solutions d'ADN mélangées avec des solutions d'histone ont souvent une structure différente.

Je désire remarquer, par ailleurs, qu'il ne me semble pas nécessaire que la neutralité des nucléoprotéines soit assurée par un rapport exact entre le nombre des phosphates de l'ADN et celui des groupements basiques des protamines ou des histones, car les ions de Na^+ ou Cl^- pourraient remplir ce rôle. Il me semble nécessaire au contraire, pour l'équilibre de systèmes colloïdaux de ce type que les deux espèces macromoléculaires soient polaires : or les histones sont riches en groupements basiques et *acides*.

M. Felix. — I should like to make a few remarks on the composition of nucleoprotamines. The nuclei of trout and herring sperm contain more amino acids than one finds in the corresponding protamines isolated by extraction with diluted HCl. The same is true for the reprecipitated nucleoprotamine fibres. Aspartic and glutamic acids are always among these " additional amino-acids ". If one separates the protamine part from the DNA part, these amino acids remain attached to the latter.

Since last year we are interested in the chemistry of the nuclei of trout eggs. We could not separate them from the yolk material. Then we investigated if a basic protein may be found. For this purpose we have removed the shell from the eggs and centrifuged the contents at high speed. Four layers were formed. The top layer was an orange red oil, the next one was a white yellow lipid material. The third and largest layer was a yellowish turbid fluid. At the bottom of the tube was a slight amount of insoluble material. The Feulgen reaction was positive only in the third layer. The extraction of this layer with diluted HCl yielded a basic protein which resembled a histone and contained cystine.

M. Ubbelohde. — Two questions arise about the water molecules retained in the structures under discussion :

1) If there are several layers of H_2O molecules separating the organic network when this is fully swollen, it can happen that the layer nearest the network is held by fairly strong hydrogen bonds and polarisation forces, whereas the H_2O molecules fully surrounded by others H_2O behave as in ice or water.

Properties such as the dielectric relaxation of such molecules can be quite different — with shorter relaxation times — in the strongly held layer, from those of the molecules in the intermediate layers with a near-ice bonding. Is there any evidence of this ?

2) If the fully swollen material can be quickly frozen by a deep-freeze technique, it may prove possible to remove the less strongly held H_2O by evaporation in high vacuo, leaving an open skeletal structure. On raising the temperature of such an open structure, it will of course shrink and collapse, since the rates of bond rearrangement leading to shrinkage when water is removed are obviously quite rapid at ordinary temperatures. But if the temperature of the rapidly frozen structure is never allowed to rise, the rates of bond rearrangement could become sufficiently slowed down to freeze in the non-equilibrium product and to permit a study of various interesting properties of the open skeletal structure, such as its internal surface in adsorption.

M. Wilkins. — Nuclear magnetic resonance data indicate that several molecular layers of water are bound in an ordered manner to DNA molecules. On the other hand, our X-ray diffraction data indicate that the water molecules do not occupy defined positions. Probably both sets of data may be reconciled by the idea that the structure of the bound water varies with time, so that, on the average, the electron density is fairly uniform.

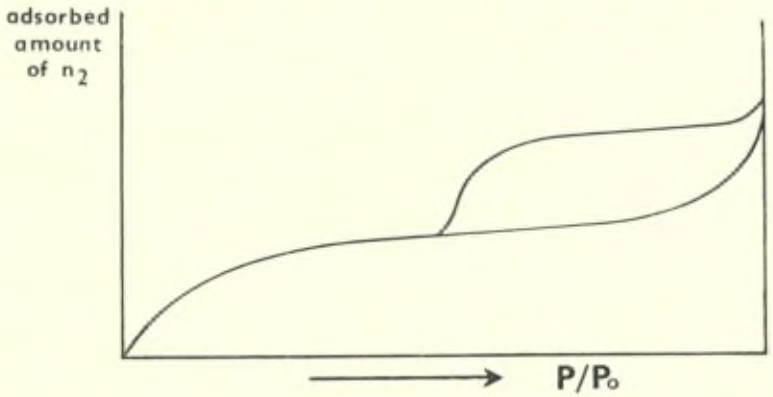
Concerning dehydration, it is a remarkable fact that DNA or nucleohistone may be dehydrated in vacuo at room temperature (and then heated to 100° C, to remove the remaining traces of water) and that the collapsed structure may then be rehydrated to give the original diffraction pattern. Apparently there is little irreversible denaturation on drying. Other techniques have, however been used to show that DNA undergoes some irreversible changes on drying.

M. de Boer. — 1) I should like to ask Dr. Wilkins a question about the swelling that nucleohistone shows with water, sometimes leading to complete dispersion. It reminds me of some work one of my pupils did (published: de Boer and van Doorn, *Proc. Kon. Ned. Akad. Wet.*, **B 61,** 242, 1958) with graphitic oxyde. This compound consists of laminae (not aromatic as graphite itself, but showing a puckered structure), completely hydrophilic due to oxygen-

containing groups. On swelling with water, the basic reflection of the sheets indicates an immense increase of distance and, at certain pH values a complete dispersion occurs. Does the dispersion which Dr. Wilkins observes, depend on pH ? One might expect a loosening of the bonds when, by ionic exchange, the hydrogen bonding is replaced by other cations, provided the ionic strength is not too high.

2) May I, secondly, make a remark with respect to the discussion whether the nucleoprotamines and nucleohistones are organised in fibrillic or laminar arragements ? Electron microscope observation of some laminar arrangements may, sometimes give the impression that one has to do with fibres, whereas a stereoscopic technique shows that the fibre-structure is simulated by the edges of very thin sheets. We had this experience with a certain form of aluminum hydroxide, which showed up as if it were cotton wool, in the normal microscope technique, but proved to consist of thin sheets in a stereoscopic electron microscope observation. Birefringence, of course, in itself, may be given by both structures.

3) It may, thirdly, perhaps be recommended to investigate the multimolecular adsorption of nitrogen, at liquid nitrogen temperature, with completely dried preparations. This technique is largely used in the field of adsorbents and of catalysts. By applying the Brunauer, Emmett and Teller (B.E.T.) equation, the specific surface area, exposed to other molecules, may then be found. Secondly, when a complete isotherm is measured (adsorption as well as desorption), a *laminar* arrangement *may* show up by a very special type of hysteresis curve, as shown in the accompanying figure.



Only pores caused by plan-parallel sheets give this type of hysteresis curve (*).

The drying of the preparations, prior to the low temperature adsorption of nitrogen, may, in case of collapsable structure, advantageously be done by the freeze drying technique, as already pointed out by Prof. Ubbelohde. Drying in liquid water, in many cases, leads to a serious shrinkage, because of the high surface tension of liquid water, drawing the structure together. Freeze drying may lead to an open structure, where the remaining capillaries may, in some cases, be investigated by adsorption methods.

M. Wilkins. — 1) As far as I remember, Zubay and Doty, in their study of the dispersal of nucleohistone in water, covered a range of pH from 6.5 to 8.0. Within this range there was no large effect of pH on dispersal.

2) I agree that it is possible, with the electron microscope, to mistake lamellar structures for fibrillar. This has indeed happened in some studies of immature sperm heads. There seems little doubt, however, that nucleohistone appears, in the electron microscope, to be fibrillar rather than lamellar. Ris has used stereoscopic technique in his electron microscope studies of nucleohistone.

In reply to Prof. Ubbelohde's point, I think it is true to say that freeze-drying techniques for preparing specimens for the electron microscope have not been so successful as one might have expected. Critical point drying has, however, been very useful and has been applied by Ris to nucleohistone.

M. Luzzati. — On ne peut pas retirer de l'eau de ces systèmes sans modifier leur structure, ce que montre l'étude des diagrammes des phases au moyen de la diffusion centrale des rayons X. Cette remarque pourrait expliquer pourquoi l'étude au microscope électronique a fourni jusqu'ici des renseignements si décevants dans l'étude de l'organisation interne des noyaux cellulaires; en effet nous avons constaté, au cours de quelques essais encore préliminaires, que l'organisation que mettent en évidence les diagrammes de rayons X disparaît au cours des manipulations inhérentes à la fixation de tissu.

(*) Cf. J.H. de Boer, „The Shapes of Capillaries", Proc. Tenth Symp. Colston Research Society 1958, X, 68-94.

M. Kuhn. — It would be interesting if filaments of microscopic size could be made with other experiments in view.

a) Optical birefringence and optical dichroism (besides the infrared dichroism mentioned) as well for histone, for DNA, for protamine separately, as for the combination of them.

If there exists in the combination of DNA and histone a superposition of two systems oriented at right angles to each other, it should be possible to predict the optical birefringence and dichroism of the combination from the data characterising the components.

b) Freezing point measurements. There exists a freezing point depression of a system consisting of a three-dimensional network relative to the freezing point of the embedding medium. The depression was of the order of 1° C for crosslinked polyvinylalcohol *swollen* in water, containing 96 % of water.

The freezing point depression depends on the size of undisturbed ice crystals which can be formed when the solvent is frozen in presence of the network. It thereby provides a means of determining the size of the three dimensional network formed by the filaments of gel (1).

c) Freeze-drying. In order to see the details of a structure in the electron microscope after freeze-drying, it is necessary to carry out not only the freeze-drying but also shadowing of the preparation at a low temperature, say at — 190° (without allowing the temperature of the preparation to rise to room temperature between the two operations. An apparatus allowing to achieve this is being constructed in our laboratory.

M. Champetier. — Des comparaisons ont-elles été effectuées entre les clichés de diffraction de rayons X des nucléoprotéines et des combinaisons éventuelles d'ADN et de polypeptides synthétiques, notamment en ce qui concerne les modifications structurelles dues à l'eau?

(1) W. Kuhn and H. Majer, *Z. physik. Chem.*, **8**, 330, 1955; *Angew. Chemie*, **68**, 345, 1956; *Kunststoffe-Plastics*, **3**, 129, 1956.
W. Kuhn, E. Peterli and H. Majer, *J. Polymer Sci.*, **13**, 21, 1954; *Z. Elektrochem.*, **62**, 296, 1958;
W. Kuhn, *Helv. Chim. Acta*, **34**, 1071, 1956.

M. Wilkins. — We have studied the X-ray diffraction pattern of DNA combined with polylysine. It resembles that of nucleoprotamine and varies with water content of the specimen in the same manner.

M. Brachet. — Je voudrais savoir de M. Wilkins :

1) Quels sont les arguments en faveur de l'idée que le noyau bactérien ne contiendrait que de l'ADN, non associé à des protéines ?

2) Quel est le rôle des protéines résiduelles dans la structure du chromosome proposée par le Dr. Wilkins?

M. Wilkins. — 1) The *E. coli* nucleoprotein extract prepared by Dr. Zubay gives, when partly dried, an X-ray diffraction pattern similar to that of a mixture of DNA and protein. It does not give a pattern like nucleohistone or nucleoprotein.

Kellenberger has compared, with the electron microscope, the appearance and fixation-behaviour of the phage DNA pool and of the bacterial nucleus in *E. coli*. He has concluded that the DNA in the nucleus is in a state similar to that in the phage DNA pool, i.e. not combined with protein.

2) For the present we are confining our attention to the nucleohistone and ignoring the residual protein because it does not form a large part of the chromosomes we are studying. We hope we are justified in doing this and that later we will be able to find how residual protein is connected with nucleohistone.

M. Watson. — Dr. Wilkins, is the protein fraction found by Zubay attached to *E. coli* DNA rich in the basic amino acids?

M. Wilkins. — It is not specially rich in basic amino acids and has a composition similar to that of the total protein of *E. coli*.

M. Sadron. — Quelle est la distance entre deux doubles hélices de DNA dans la fibrille ? Quelle est la longueur de l'hélice secondaire par rapport à la longueur de la double hélice constituée par une molécule de DNA?

M. Wilkins. — The DNA molecules are probably spaced about 40 or 50 Å apart. I think the pitch length of the secondary helix is probably in the region of 500 Å.

M. Duchesne. — Je crois utile de faire remarquer que nous avons mesuré par une méthode originale de radiofréquences (*C.R. Paris*, **241**, 749, 1955), les coefficients d'expansion thermique de DNA et de DNP. Nous avons trouvé le résultat remarquable que ce coefficient est très petit et de l'ordre de grandeur de celui du quartz. Il est à peu près le même dans les deux cas. Ceci semble en accord avec le fait signalé par le Dr. Wilkins que le DNA a la même configuration dans l'état libre ou lié à une protéine. Nos résultats indiquent, en outre, une conductivité thermique élevée. Il semble, en tout état de cause, qu'ils impliquent une structure rigide.

Il serait sans doute intéressant d'étudier plus à fond les relations de ces données avec la structure des fibres, et je voudrais spécialement savoir s'il existe quelque évidence en faveur d'une conductivité thermique élevée.

M. Ubbelohde. — When the change in vibrational free energy with volume is the only factor responsible for thermal expansion, as in solids whose units of structure are simple, a very small coefficient of thermal expansion does permit the inference that the bond structure is quite rigid. For example, in graphite parallel to the carbon hexagon networks the bond structure is quite rigid and thermal espansion *in this direction* is slightly negative. At right angles there is a large positive thermal expansion.

On the other had, in solids with complex polyatomic units of structure, various other factors can contribute to the thermal expansion, in addition to the vibrational effect. As one example, changes of configurational entropy with extension of a flexible molecule, as in rubber, can swamp ordinary vibrational contributions and can lead to negative terms. It would be important to know the thermal expansion in various directions of well oriented material, to help in elucidating the significance of the remarkably low thermal expansion coefficients reported by Prof. Duchesne.

M. Wilkins. — We have not measured the thermal expansion of DNA. When one does this I think it would be important to ensure that the water content of the specimen remains constant.

ON THE CONSTITUTION OF HISTONES

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INTRODUCTION

The study of the protein moiety of deoxyribonucleoproteins is characterized by some of the same types of problems encountered in the purification of the nucleic acids. The isolation of DNA is fraught with the difficulty of depolymerization by intracellular nucleases; in turn, the histones are unusually susceptible to the action of proteinases. An important part of any discussion of the preparation of a nucleic acid or a protein is the question of whether the isolated product is identical to the naturally occurring material or is perhaps a smaller molecule derived by cleavage of the parent polymer. The first part of the present discussion on histones will dwell on the primary problem of the adequateness of methods for their isolation. Two main steps are involved — the preparation of a total histone mixture and the subsequent examination of the mixture to determine the number of components that are present. Recent years have seen the application of both chromatographic and electrophoretic methods to the problem.

The development of more adequate procedures for the characterization of histones has made possible exploratory studies of the question of their cell specificity. Preliminary comparisons have been made of histones from the nucleohistone fractions of calf thymus, liver, and kidney. There is also the fundamental question of whether the combination of DNA and histone isolated by current methods represents an artifact (a non-specific union of polyacids and polybases) or is, at least in part, a specific combination characteristic of the nucleoproteins as they exist in the chro-

mosome. This problem has repeatedly received attention since the time of Miescher and of Kossel (Kossel [1], Mirsky and Ris [2], and Chargaff [3]). Recent evidence on this subject will be summarized.

The present report draws in the main upon a series of experiments carried out by Dr. Charles F. Crampton during his association with Dr. William H. Stein and myself. Dr. Crampton came to the Rockefeller Institute after two years as a postdoctoral fellow with Prof. Erwin Chargaff, and arrived with an interest in nucleic acids that had been stimulated by his sojourn at Columbia University. What we have tried to do is to facilitate the joining of his experience with ours to learn a little more about the protein moiety of nucleoproteins. All three of us recognize the result of this collaboration as a brief chapter in the study of a subject that has attracted much investigation over the years. The following discussion is not a review in the general sense, but is, rather, an attempt to summarize the results of a series of experiments carried out within the framework of the earlier as well as the contemporary studies that have contributed the main body of knowledge on the biochemistry of histones.

THE PROBLEM OF THE ALTERATION OF HISTONES DURING THEIR ISOLATION

Histones are basic proteins occurring in association with nucleic acid in the nuclei of somatic cells. The logical operational steps in their preparation are the separation of the nuclei from the cells, the isolation of the nucleoprotein from the nuclei, and finally, the dissociation of the histones from the DNA. Because of the chemical properties of histones, the danger that cellular proteinases will degrade the product sought during the isolation is greater than it is for many other tissue proteins. Proteins such as ribonuclease, for example, are completely resistant to a proteolytic enzyme such as trypsin. Some physically or chemically induced unfolding of the molecule is necessary before the protein is susceptible to tryptic hydrolysis. Histones, on the other hand, possess little or no such resistance. The isolated proteins are rapidly hydrolized by trypsin (Crampton *et al.* [4]). The fact that

they contain no cystine means that the structures are not stabilized by disulfide cross-linkages; viscosimetric data indicate that histones probably possess an open, relatively unfolded structure. To define further the susceptibility to enzyme action, a preparation of isolated histone was added to a dilute thymus homogenate ([4]). After 1 hour at 35°, chromatographic analysis of the mixture showed that the histone had been degraded with the formation of altered products.

The problem is also illustrated in a practical way by the results of varying the time required for extracting histones from homogenates of calf thymus ([4]). On the left in Fig. 1 are shown the results of chromatographic analyses of preparations obtained by relatively slow methods which required isolation of the nucleoprotein fraction, dissociation by 2 M sodium acetate and alcohol, and removal of salt by dialysis. On the right are shown chromatograms obtained by direct analysis of undialyzed barium acetate-alcohol extracts of nucleohistone that had been extracted ten times more rapidly. The conclusion drawn from these experiments is that the more basic Fraction B can be degraded to fragments which move through the column unretarded or overlap the position of Fraction A. The problem presented by catheptic digestion was noted in 1954 by J. A. V. Butler and his associates ([5]), and in the recent studies of Luck *et al.* ([6]) the importance of operating at temperatures of 3° to 4° to minimize degradation is emphasized.

The chromatographic system used to examine the preparations of histones referred to in Fig. 1 requires brief comment. The method illustrated in Fig. 1 is not as effective as we would like it to be, and the yield of histones is only about 70 % on a ninhydrin color basis. The procedure evolved from earlier studies on the chromatography of basic proteins on the carboxylic acid resin IRC-50 ([7]) by elution with sodium phosphate buffers. Histones proved to be held too tenaciously by the resin to be eluted by sodium ions but it was found that they could be eluted with increasing concentrations of divalent cations, such as those of calcium and barium ([8]). Although the system separates calf thymus histone into two main fractions (A and B) rather efficiently, it has limited resolving power. Evaluation of alternative methods of fractionation, however, is complicated by the question of the degradation of histones during their isolation. It has frequently

been difficult to decide whether a fractionation that shows many peaks or zones has resulted from better resolution of native histones or from the presence of a number of degraded histones in the particular preparation examined.

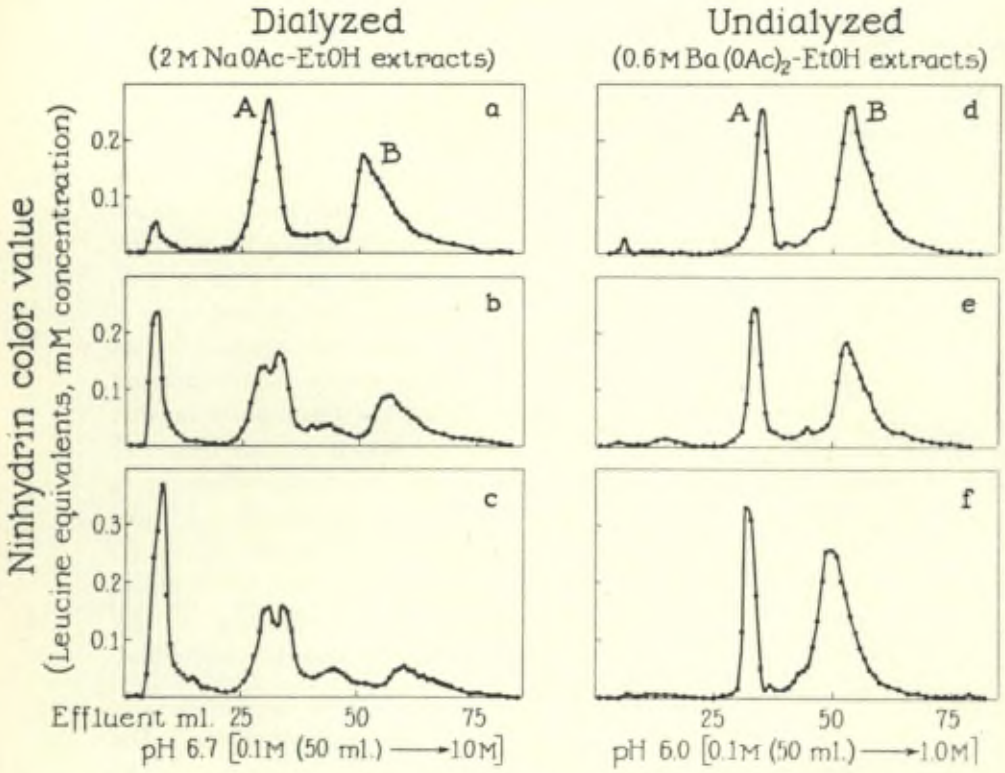

Fig. 1. — Chromatography of histone mixtures on columns of Ba IRC-50 (10 × 0.9 cm). The more complex patterns on the left (*a*, *b*, *c*) are to be compared with those obtained when a more rapid method of preparation was used for isolation of the histone fraction. The histone (ca 6 mg) from 0.21 — 0.25 gm of calf thymus was used for each chromatogram. The results are not dependent upon the *p*H of the eluent in the range from *p*H 6 to 7 (8). From Crampton *et al* (4).

Since the presently available methods of separating histones do not have inherently high resolving power, as chromatographic methods go, there is much room for new approaches. The experiments of Luck, Rasmussen, Satake, and Tsvetikov (5), who have used guanidinum chloride for the quantitative elution of histones from IRC-50, form one of the most recent studies on this subject.

The behaviour of histones in moving boundary and zone electrophoresis, particularly in conjunction with chromatography, will continue to provide important criteria for the homogeneity of different preparations.

THE LYSINE-RICH AND ARGININE-RICH HISTONE FRACTIONS

The methods of fractionation developed in several laboratories in recent years have led to the conclusion that, when degradation is minimized, the proteins of calf thymus nucleohistone can clearly be separated into two main fractions, one of which is characterized by a high lysine content and the other by a high arginine content. Many attempts are currently being made to sub-fractionate these materials. This research shares the difficulties inherent in the study of the purification and homogeneity of proteins in general, a topic that was reviewed in detail for an earlier Solvay Conference on Proteins (Desreux and Fredericq [3]). The best that we can do at the present writing is to describe how these main two histone fractions can be prepared reproducibly and to define some of their chemical properties. In the final analysis, the conclusion as to whether an isolated histone is homogeneous may have to rest on the determination of the amino acid sequence in the peptide chain.

Most of the work on histones has employed calf thymus as starting material, because of its relatively high content of nucleohistone (about 5 % of the wet weight of the tissue [3]). The nucleoprotein fraction is about half nucleic acid and half histone, and the histone, in turn, as will be pointed out, consists of about 9 to 1 on a weight basis of an arginine-rich component and a lysine-rich fraction.

The chromatographically purified lysine-rich Fraction A (Fig. 1, *d*) possesses the amino acid composition given in the second column of Table I. The analyses (4) were performed by ion exchange chromatography using automatic recording equipment (10). It will be noted that the basicity of histones results from the fact that they not only contain considerable amounts of

lysine and arginine, but also relatively small amounts of aspartic and glutamic acids.

Since the lysine-rich protein contains no methionine or histidine, both of which are present in Fraction B, the absence of these two amino acids (particularly histidine) has served as a sensitive indication of whether this histone fraction is contaminated with Fraction B or degradation products therefrom. For example, when material isolated from the Fraction A position in Fig. 1, *a* or *b*, was analysed, small amounts of histidine and methionine were found, confirming the conclusion that degradation of Fraction B into some more rapidly eluted components had occurred.

Crampton has been able to prepare the lysine-rich histone fraction without chromatography by direct selective extraction of calf thymus nucleohistone with aqueous 0.2 M $Ba(OAc)_2$ ([4]). After an alcohol precipitation step, a product is obtained which is indistinguishable from Fraction A in amino acid composition and chromatographic behaviour. Lysine-rich fractions very similar to A have been prepared previously by extraction and precipitation methods, and more recently by alternative chromatographic procedures. Studies of fractions differing in amino acid composition have included those of Stedman and Stedman ([11]), Davison and Butler ([12]), Daly and Mirsky ([13]), Bijvoet ([14]), Ui ([15]), Davison ([16]), Smillie *et al.* ([17]), Phillips ([18]), Cruft *et al.* ([19, 20]), and Luck *et al.* ([6]).

On the basis of chromatographic behavior and amino acid composition, it would seem that the Fraction A referred to in Table I is probably the least degraded material of this type obtained to date, but we hasten to add that, in all likelihood, it still is not a pure protein. For example, if it were homogeneous, it would have to possess a minimal molecular weight of about 20,600 in order to contain 1 residue of phenylalanine and 1 of tyrosine per molecule ([4]). Yet ultracentrifugal studies by Trautman and Crampton ([21]) of the molecular weights of the histones in urea solution indicate a molecular weight of about 10,000 for this preparation of Fraction A. End group determinations on purified samples will also be important; Phillips ([18]) has recently concluded that the lysine-rich fraction contains predominantly NH_2-terminal proline.

The amino acid composition of the arginine-rich Fraction B is given in the third column of Table I. In our experience, the

TABLE 1

Amino acid composition of histone fractions from calf thymus.

Amino acid	N as per cent of total recovered N		
	Total nucleohistone [a]	Lisine-rich Fraction A [b]	Arginine-rich Fraction B [c]
Aspartic acid	3.8	1.5	3.5
Glutamic acid	5.8	2.4	5.9
Glycine	6.0	5.0	6.2
Alanine	9.1	19.5	7.7
Valine	4.4	3.6	4.6
Leucine	5.6	3.1	5.8
Isoleucine	3.0	0.7	3.4
Serine	3.7	4.8	3.7
Threonine	3.9	4.3	4.0
Cystine	< 0.3	0	< 0.2
Methionine	0.8	0	0.7
Proline	3.5	7.0	2.7
Phenylalanine	1.3	0.35	1.3
Tyrosine	1.8	0.34	2.0
Histidine	4.1	0	4.6
Lysine	19.0	42.4	16.2
Arginine	24.8	5.1	27.7

[a] Average for preparation Nos. 24 and 25, Table IV, Crampton *et al.* (4). Tryptophan, less than 0.08 % (8). Amide-NH_3 nitrogen is not included in the calculations.

[b] Average for preparation Nos. 22, 22*d*, 24 and 25, Table II (4).

[c] Average for preparation Nos. 22, 22*d*, 24 and 24*d*, Table III (4).

best indication of whether degradation has taken place during the preparation of the Fraction B is the arginine content of the material in the B-peak when IRC-50 -$Ba(OAc)_2$ chromatography has been used for its isolation. Partially hydrolyzed samples which show additional components moving near or ahead of the A-peak usually have less than 20 % arginine-N in Fraction B, whereas preparations isolated more rapidly have 28 % arginine-N. This criterion applies only to the present methods of fractionation, and will require reconsideration if methods of higher resolving power are found.

The arginine-rich fraction has also been obtained by extraction without the use of chromatography. When nucleohistone from which the lysine-rich histone has been removed by extraction

with 0.2 M $Ba(OAc)_2$ is dissolved in water and treated with stronger $Ba(OAc)_2$ in the presence of ethanol, the arginine-rich histone fraction is obtained ([4]). The product prepared by extraction is very similar in amino acid composition to Fraction B and behaves similarly upon chromatography on IRC-50.

Trautman and Crampton ([21]) find the molecular weight of Fraction B in urea solution to be about 16,000, which may be compared with 19,700 calculated from the amino acid composition ([4]). The marked tendency of the arginine-rich fraction to form aggregates in aqueous solution has been noted and studied by several groups of investigators ([14, 15, 20, 22, 23, 24]). Phillips ([15]) reports that the arginine-rich fraction contains predominantly alanine in the NH_2-terminal position.

From the amino acid analyses of these two main fractions and that of the original mixture (Column 1, Table I), it can be calculated that a mixture of 1 part of lysine-rich histone and 9 parts of arginine-rich histone would have an amino acid composition very similar to that of the naturally occurring mixture ([4]). Since Fraction A has a higher ninhydrin color value than Fraction B and the yield of the latter from the chromatogram is a little lower, the presence of a 1 : 9 mixture is not incompatible with the chromatographic results. This calculation leaves room for the probable presence in the nucleohistone of small amounts of other proteins that differ from both the lysine-rich and the arginine-rich fractions.

Daly and Mirsky ([13]) have pointed out the importance of the demonstration that both lysine-rich and arginine-rich histones occur as well in nuclei prepared by the Behrens technique, using non-aqueous media for the extractions. The chances of obtaining artifacts as a result of interaction between DNA and cytoplasmic proteins are considered to be less than when aqueous media are used in the extraction. At the suggestion of Mirsky and Allfrey, the chromatographic analyses on IRC-50 ([5]) were extended to the demonstration that in thymus nuclei prepared by a modified Behrens technique histone Fractions A and B occur in about the same proportion as they do in nucleohistone isolated from the thymus.

STUDIES ON CELL SPECIFICITY

With methods available for isolating reproducible preparations of histones from calf thymus, it would be desirable to compare the results obtained when the same methods are applied to nucleoprotein preparations from cells of other tissues, with the object of learning, if possible, whether histones are cell specific. Since the studies which Dr. Crampton has carried out on this subject were performed contemporaneously with his investigation of the fundamentals of the isolation procedure, the histone preparation was made before he had learned how to keep degradative side reactions to a minimum. The results, so far as they go, speak for the similarity rather than the dissimilarity of histones from different tissues, but we wish to make it clear that the findings are of value only as a first step toward examination of the question of cell specificity. Any conclusions on similarity apply only within the limits of the resolving power of the methods used, and are open to revision as more sensitive criteria can be brought to bear on the subject.

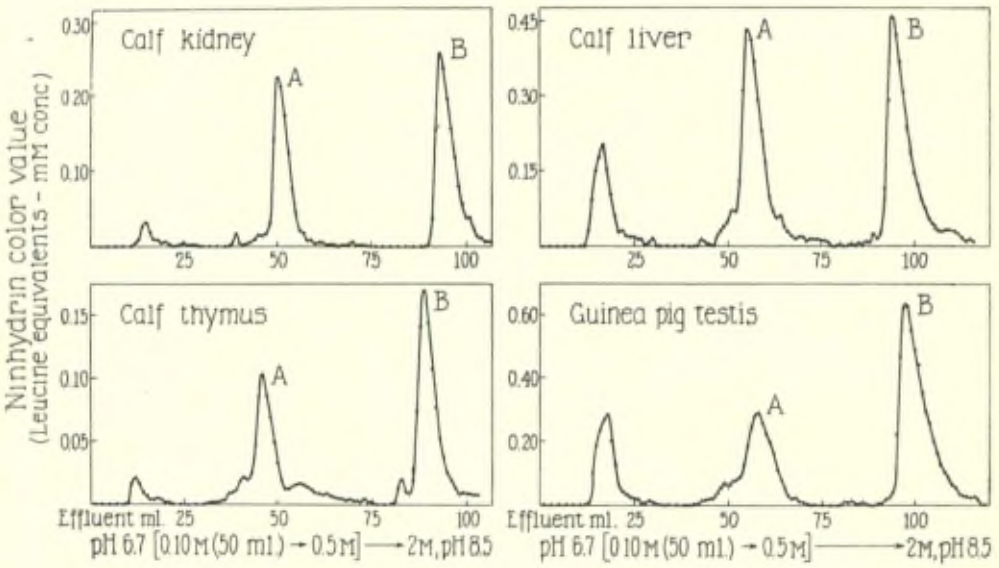

Fig. 2. — Chromatography of total histone mixtures from different organs on columns of Ba IRC-50 (30 × 0.9 cm). The samples of histones from calf kidney, calf liver, calf thymus, and guinea pig testis weighed 8, 20, 5 and 20 mg, respectively. The preparations were made before the preferable, more rapid method of isolation (cf. Fig. 1) was adopted. From Crampton *et al* (4).

In the studies under review, the nucleoprotein fractions from calf liver, kidney, and thymus and from guinea pig testis were prepared by procedures based upon the Mirsky-Pollister method for obtaining deoxyribonucleoproteins ([25, 26]). The chromatograms obtained with the total histone mixtures are shown in Fig. 2. All four tissues contain proteins emerging at the positions occupied by Fractions A and B. The amino acid compositions of the individual histone Fractions A (Fig. 3) and Fractions B (Fig. 4) were also similar. The corresponding histones from guinea pig testis seem to show significant differences, which would be expected on the basis of anticipated species specificity.

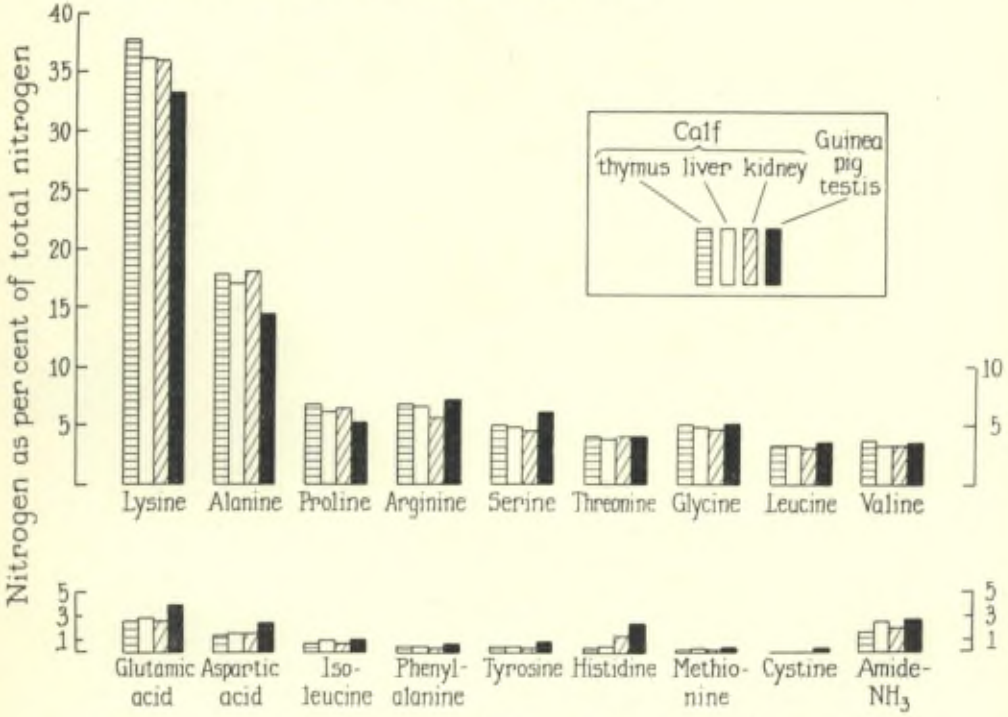

Fig. 3. — Amino acid composition of histone Fractions A from different tissues. The analyses were performed by ion exchange chromatography ([10]) with 22- and 70- hour acid hydrolysates. The analyses account for 96 to 98 % of the total nitrogen. The preparations for this series of comparisons were made before the methods of isolation were improved and the data show that the lysine content of Fraction A from calf thymus, for example, is lower than that of better preparations (Table 1). From Crampton *et al.* ([4]).

A further test was applied to each of the histone Fractions B. Proteins possessing the same amino acid composition can theoret-

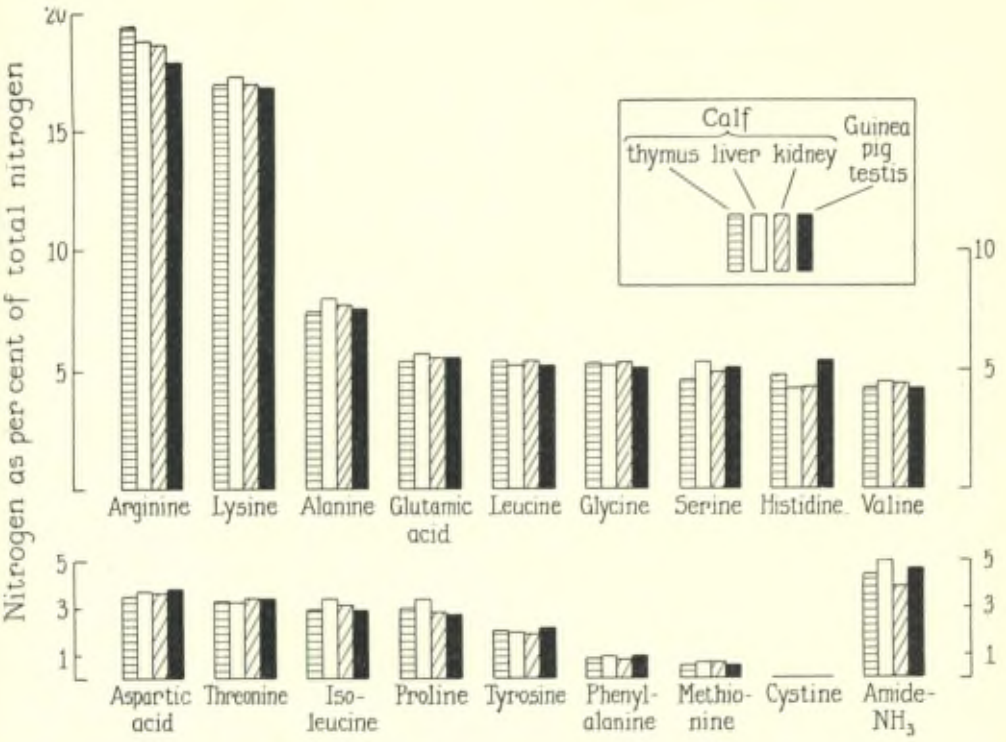

Fig. 4. — Amino acid composition of histone Fractions B from different tissues. (Cf. legend to Fig. 3). From Crampton *et al.* (4).

ically differ with respect to the order in which their constituent residues are arranged in the polypeptide chain. If, however, two proteins differ with respect to the sequences of their constituent amino acid residues, they would not be expected to yield similar mixtures of peptides when hydrolyzed by an enzyme. Therefore, tryptic hydrolysates of Fractions B from the different organs were prepared and chromatographed on columns of Dowex 50-X2 under conditions similar to those worked out during the structural investigation of ribonuclease by Hirs *et al.* (17).

The effluent curves (Fig. 5) yielded by the hydrolysates of the three protein fractions from calf tissues are remarkably similar. In each pattern there is evidence for about thirty-five peaks, some of which undoubtedly contain more than one peptide. The production by trypsin of numerous fragments from proteins so rich

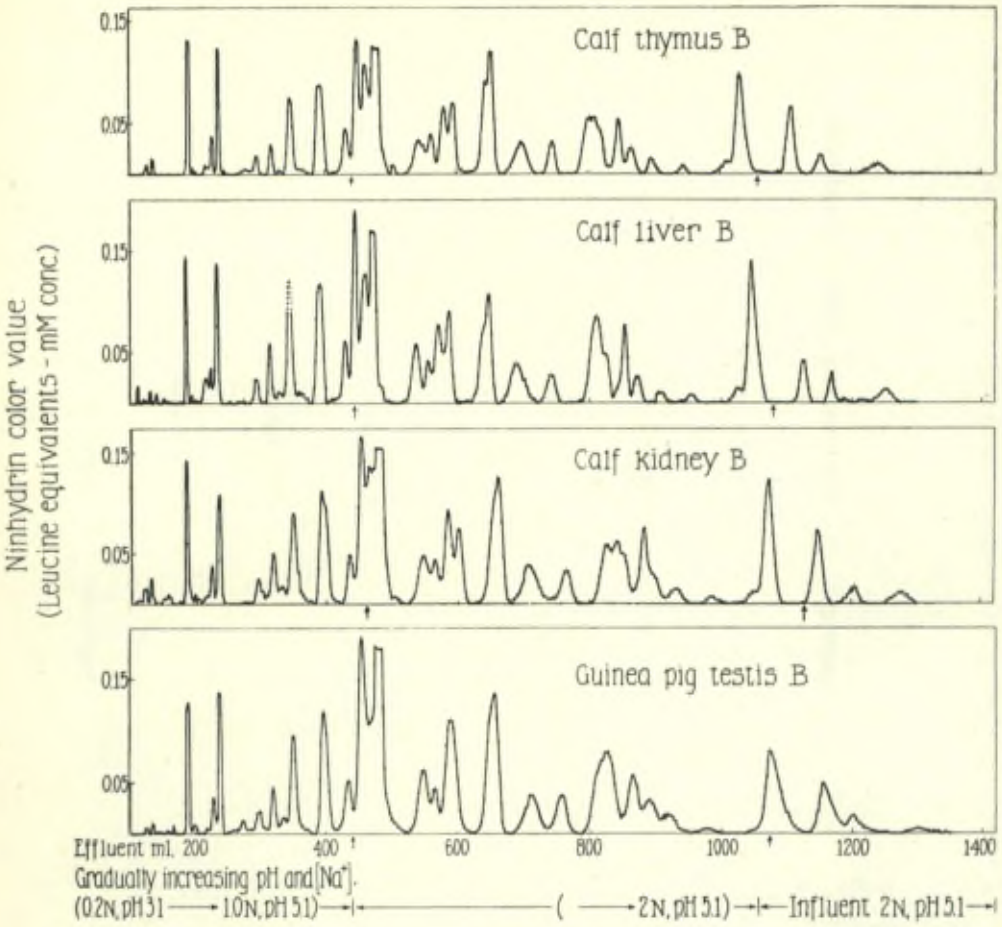

Fig. 5. — Chromatographic comparison of 21-hour tryptic hydrolysates of histone Fractions B from different organs. Columns of Dowex 50-X2 (150 × 0.9 cm, cf. (17)) were used for the chromatography and aliquots of hydrolysates corresponding to about 18 mg of Fraction B were used in each case. The histones were prepared before the adoption of methods designed to keep degradation during isolation to a minimum. From Crampton *et al.* (4).

in lysine and arginine is expected. The peaks at 400 ml and 590 ml may include free lysine and arginine, respectively, but the relatively small sizes of the peaks at these positions, coupled with the large number of peaks attributable to peptides, suggest that in the molecules composing histone Fractions B, the basic amino acids are, for the most part, not adjacent to one another. Although the amino acid compositions of the peptides responsible

for the different peaks have not been determined, the similarities among the patterns must reflect similarities in the way the amino acids are arranged in the original proteins. The Fraction B from guinea pig testis shows some clear differences in the number and the size of the peaks obtained, particularly at 450-500 and 590 effluent ml.

This type of experiment provides a means of comparing in considerable detail the structures of related proteins from different tissues and different species in a manner similar to the way in which paper chromatography and electrophoresis were used by Ingram ([28]) in his comparative studies of different hemoglobins. We hope to be able to facilitate comparisons of the type shown in Fig. 1 by using the automatic recording equipment for obtaining the curves, just as we now use this equipment for amino acid analyses ([16]).

From the experiments that have been summarized, we can only conclude that if cell specificity exists, it will have to be detected by techniques more refined than those which have been used to date. The present work has not dealt with the comparison of normal and malignant tissues, a subject which has recently been reviewed by Busch and Davis ([29]).

THE SPECIFICITY OF THE DNA-PROTEIN COMBINATION IN THE NUCLEUS

The question has frequently been raised as to whether nucleic acid and histone actually occur in combination in the nucleus or whether, perhaps, soluble histones react with DNA to form an insoluble salt of a polyacid and a polybase during the preparation and extraction of the nuclear material. In reviewing some experiments on this subject, I am summarizing research which Dr. Crampton started with Prof. Chargaff ([30]) and returned to in studies directed toward the protein half of the combination ([31]).

If a difference in physical or chemical properties could be demonstrated between nucleohistone isolated under non-dissociat-

ing conditions and the nucleohistone obtained by deliberately dissociating and recombining the two polyelectrolytes *in vitro*, it would be evidence for a uniqueness of the original mode of combination. Study of the purine and pyrimidine composition of the DNA liberated by fractional extraction of " native " and " reassociated " nucleohistone did not give evidence of differences ([30]), and Riley and Arndt ([32]) found that the x-ray scattering patterns of the two types of products were very closely similar. However, a difference was observed by Crampton ([31]) in a study of the relative ease of removal of lysine-rich and arginine-rich histones from the " native " and reassociated nucleohistones. The results are illustrated in Fig. 6. The term " native " is not used in the absolute sense, but to indicate samples which were prepared under conditions of low salt concentration (0.2 M NaCl) known to minimize dissociation of the component histones from DNA. The reassociated products were prepared by brief dissociation in 2 M NaCl before precipitation from 0.2 M NaCl.

The top pair of chromatograms shows that when the extracting solution is 0.05 M $Ba(OAc)_2$ to which 2 volumes of ethanol are added, the *more basic* histone Fraction B is eluted preferentially from the " native " nucleohistone, whereas the reconstituted product liberates the less basic lysine-rich histone more readily. This tendency is also evidenced by the second pair of chromatograms of extracts prepared using an initial $Ba(OAc)_2$ concentration of 0.2 M. The third set of chromatograms serves as a control to show that when the initial salt concentration is raised to 1.0 M, the two curves are the same, thus indicating that no fundamental alteration of the histones occurred during the preparation of the reassociated sample.

The differences observed by Crampton between the " native " and reassociated nucleohistones might depend upon a number of physical or chemical features of the nucleoproteins. The possible role of dilution effects must be considered, and the discussion in the original paper ([31]) can be consulted for a careful consideration of the interpretation of the experiments. Dr. Crampton concludes only that the findings suggest that at least a portion of the components of nucleohistones are originally combined *in vivo* in a manner which is not restored upon random reassociation.

If, as is likely, all of the histone in the nucleohistone of calf thymus is combined with DNA, the arrangement would require that more than 100 molecules of histone be associated with each molecule of DNA. Even apart from the consideration that there are as many nucleohistones in the nucleus as there are nucleic

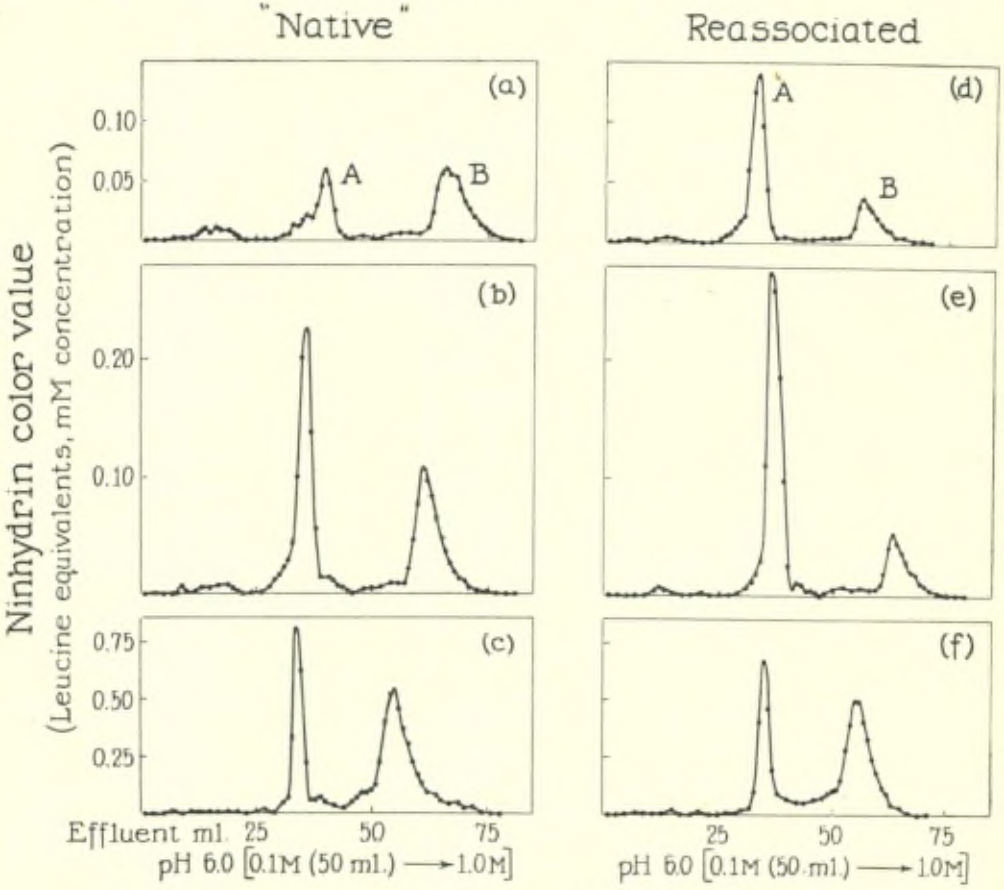

Fig. 6. — Comparison of the chromatography of histones obtained from "native" (*a*, *b*, *c*) and from reassociated (*d*, *e*, *f*) nucleohistones by addition of two volumes of ethanol to mixtures in 0.05 M Ba $(OAc)_2$ (*a* and *d*) 0.2 M Ba $(OAc)_2$ (*b* and *e*) and 1.0 M Ba $(OAc)_2$ (*c* and *f*). Each pattern has been scaled to represent the product from 0.4 gm of calf thymus. From Crampton [31].

acids, there is wide scope for structural variations. It may be significant in this connection [4] that the nucleohistone of calf thymus contains nearly the same number of residues of basic

amino acids as there are total residues of phosphoric acid in the DNA of the nucleohistone. The thoughts along this line parallel the considerations of Felix and coworkers ([33]) on the possible structural isomerism of nucleoprotamines and the approach of Wilkins and associated investigators ([34]) who have examined the molecular structure of the DNA-protamine combination with the aid of x-ray diffraction studies.

CONCLUSIONS AND SUMMARY

Research in a number of laboratories in the past few years has led to the development of improved methods for the isolation and characterization of histones. When degradative reactions are minimized (but not necessarily eliminated) during the isolation of the nucleohistones, two principal protein fractions can be obtained from calf thymus; a lysine-rich fraction containing 42 % of its total nitrogen as lysine-N, and an arginine-rich fraction containing 28 % arginine-N. The nuclei of calf liver and calf kidney yield lysine-rich and arginine-rich histones which cannot, by the techniques used to date, be distinguished from the corresponding fractions prepared from calf thymus of the same species. The question is left open as to whether more refined methods will lead to evidence for cell specificity.

Recent experiments on the mode of combination of nucleic acid and histone have given the first indications that the combination is a specific one *in vivo,* possessing a structure different from that of the salt that is obtained when the isolated DNA and histone are allowed to recombine under laboratory conditions. Some of the concepts of the properties of the nucleohistones of the chromosomes have previously rested on indirect evidence; the research of the past few years has added considerably to the chemical knowledge of this group of nucleoproteins and to the methods that can be used to study further the protein half of the combination.

REFERENCES

(1) A. Kossel : " The Protamines and Histones ", New York, Longmans Green and Co. (1928).

(2) A.E. Mirsky and H.J. Ris : *J. Gen. Physiol.*, **34**, 475 (1951).

(3) E. Chargaff : In " The Nucleic Acids ", edited by E. Chargaff and J.N. Davidson, New York, *Academic Press* (1955), Vol. I, p. 307.

(4) C.F. Crampton, W.H. Stein and S. Moore : *J. Biol. Chem.*, **225**, 363 (1957).

(5) J.A.V. Butler, P.F. Davison, D.W.F. James and K.V. Shooter : *Biochim. et biophys. acta*, **13**, 224 (1954).

(6) J.M. Luck, P.S. Rasmussen, K. Satake and A.N. Tsvetikov : *J. Biol. Chem.*, **233**, 1407 (1958).

(7) S. Moore and W.H. Stein : *Advances in Protein Chem.*, **11**, 191 (1956).

(8) C.F. Crampton, S. Moore and W.H. Stein : *J. Biol. Chem.*, **215**, 787 (1955).

(9) V. Desreux and E. Fredericq : *Neuvième Conseil de Chimie*, Institut de Chimie Solvay, " Les Protéines ", R. Stoops, Bruxelles (1953), p. 171.

(10) D.H. Spackman, W.H. Stein and S. Moore : *Anal. Chem.*, **30**, 1190 (1958).

(11) E. Stedman and E. Stedman : *Nature*, **166**, 780 (1950).

(12) P.F. Davison and J.A.V. Butler : *Biochim. et biophys. acta*, **15**, 439 (1954).

(13) M.M. Daly and A.E. Mirsky : *J. Gen. Physiol.*, **38**, 405 (1955).

(14) P. Bijvoet : *Biochim. et biophys. acta*, **25**, 502 (1957).

(15) N. Ui : *Biochim. et biophys. acta*, **25**, 493 (1957).

(16) P.F. Davison : *Biochem. J.*, **66**, 708 (1957).

(17) L.B. Smillie, G.C. Butler and D.B. Smith : *Can. J. Biochem. and Physiol.*, **36**, 1 (1958).

(18) D.M.P. Phillips : *Biochem. J.*, **68**, 35 (1958).

(19) H.J. Cruft, J. Hindley, C.M. Mauritzen and E. Stedman : *Nature*, **180**, 1107 (1957).

(20) H.J. Cruft, C.M. Mauritzen and E. Stedman : *Proc. Royal Soc. B.*, **149**, 21 (1958).

(21) R. Trautman and C.F. Crampton : *J. Am. Chem. Soc.*, in press.

(22) H.J. Cruft, C.M. Mauritzen and E. Stedman : *Nature*, **174**, 580 (1954).

(23) P.F. Davison D.W.F. James, K.V. Shooter and J.A.V. Butler : *Biochim. et biophys. acta*, **15**, 415 (1954).

(24) J.M. Luck, H.A. Cook, N.T. Eldredge, M.I. Haley, D.W. Kupke and P.S. Rasmussen : *Arch. Biochem. Biophys.*, **65**, 449 (1956).

(25) A.E. Mirsky and A.W. Pollister : *Proc. Nat. Acad. Sci.*, **28**, 344 (1942).

(26) A.E. Mirsky and A.W. Pollister : *J. Gen. Physiol.*, **30**, 117 (1946).

(27) C.H.W. Hirs, W.H. Stein and S. Moore : *J. Biol. Chem.*, **221**, 151 (1956).

(28) V.M. Ingram : *Nature*, **178**, 792 (1956).

(29) H. Busch and J.R. Davis : *Cancer Research*, **18**, 1241 (1958).

(30) C.F. Crampton, R. Lipshitz and E. Chargaff : *J. Biol. Chem.*, **206**, 499 (1954).

(31) C.F. Crampton : *J. Biol. Chem.*, **227**, 495 (1957).

(32) D.P. Riley and U.W. Arndt : *Nature*, **172**, 295 (1953).

(33) K. Felix, H. Fischer and A. Krekels : *Progress Biophysics and Biophys. Chem.*, **6**, 2 (1956).

(34) M. Feughelman, R. Langridge, W.E. Leeds, A.R. Stokes, H.R. Wilson, C.W. Hooper, M.H.F. Wilkins, R.K. Barclay and L.D. Hamilton : *Nature*, **175**, 834 (1955).

DISCUSSION DU RAPPORT DE M. S. MOORE.

M. Butler. — I am sure we have all enjoyed Dr. Stanford Moore's presentation. I am under the impression that in the experiments of Crampton only 50-60 % of the material added was recovered from the column. Dr. Moore will correct me if I am wrong.

M. Moore. — The recovery is 70-80 % from the IRC-50 columns when degradation of histones during their preparation is minimized.

M. Butler. — An alternative method using carboxymethyl cellulose was developed by Davison in my laboratory (1) and gave 100 % recovery in two principal and one subsidiary peaks. This procedure was improved by Phillips and Johns by the use of different solutions of hydrochloric acid as the eluting agent (2), which gives two main fractions, one relatively rich in lysine and the other relatively rich in arginine.

We found that we needed some criterion of homogeneity of these fractions and Phillips (3) therefore examined their N-terminal amino-acids. In the whole histone the principal N-terminals are alanine and proline, with smaller quantities of a number of others, such as glycine. It was found that the latter increased on keeping the histones under neutral conditions, which must be due to catheptic enzymes. This action could be reduced by keeping the solutions acid during the whole preparation or by adding an enzyme inhibitor (DFP = diisopropylphosphofluoridate). In good preparations the alanine and proline end groups together amount to more than 90 % of the whole.

All the histones examined from various sources (e.g. calf and rat tissues and also human placenta (Hirschbein) show predominantly proline and alanine end groups (see Table 1).

(1) *Biochem. J.*, **66**, 708, 1957.
(2) *Biochem. J.*, in the press.
(3) *Biochem. J.*, **68**, 35, 19.

TABLE 1

	Proline end	Alanine end
Calf thymus	52	37
Rat thymus	53	36
Rat liver.	42	22*
Human placenta (Hirschbein)	45	17*
* = no inhibitor present.		

When two principal fractions mentioned above were examined it was found that one peak was associated with the proline end group (the lysine rich component) and the other with the alanine end group.

It should however be mentioned that the " lysine rich " peak mentioned here is a larger fraction than the " very lysine rich " component of Crampton and Moore which amounted to about 10 % of the whole histone. The latter is contained in the former and can be separated from it in various ways. It follows that the main fractions obtained by carboxy-methyl cellulose chromatography are not homogeneous although they have for the most part a common N-terminal group and similar overall compositions. I might add that Phillips has been unable to detect any free N-terminal amino groups in the " very lysine rich " component. It is possible that these groups are acetylated, but this has not been demonstrated so far.

M. Wilkins. — The results Dr. Moore describes show that native and reassociated nucleohistone differ in structure. In our laboratory Dr. Zubay has been able to recombine histone and DNA so that the characteristic X-ray pattern of native nucleohistone is obtained. However, the discrepancy between these two results may not be so great as might appear. The X-ray observation shows only that the greater part of the histone recombines with DNA to give essentially the same structure as is present *in vivo*.

Differences might arise as a result of aggregation between histone molecules while these molecules are separated from DNA. If the arginine-rich fraction aggregates more than the lysine-rich, the lysine-rich might, compared with arginine-rich, be removed more readilly from reassociated material than from the native nucleohistone. Anyway, the lysine-rich fraction represents only 10-20 % of the total histone, and the X-ray diffraction might not be sensitive to the arrangement of this fraction. In the main, however, there is a characteristic combination of DNA and histone. If the histone is not very carefully prepared it apparently denatures and the X-ray pattern given by DNA combined with such histone does not show the special characteristics of the nucleohistone pattern but resembles a very diffuse nucleoprotamine pattern.

M. Felix. — Perhaps I could answer the question about the resistance of the arginyl-arginine bond against trypsin. Most of the arginine of clupein occurs in tetrapeptide sequences. After digestion with trypsin a relatively large amount of arginyl-arginine could be isolated besides free arginine. Thus some of the arginyl-arginine bonds are not split by trypsin (K. Felix, K. Inouye and K. Dirr, *Z. physiol. Chem.*, **211**, 187, 1932).

M. Butler. — With reference to the possible occurence of two or more basic amino acids adjacent to each other (Arg-Arg or Arg-Lys) I might mention some observations of Satake in Dr. Murray Luck's laboratory at Stanford University. I do not think he would mind these being mentioned here.

Satake has examined peptides in hydrolysis products of arginine-rich histones. In histone II (proline end group) not less than 17 % of the arginine was present as Arg-Arg or Arg-Lys groupings. Histone III had 19 % of arginine as Arg-Lys and 3 % as Arg-Arg.

Phillips in my laboratory has also obtained by tryptic digestion a histone product which has only one basic aminoacid in every seven residues.

It is therefore impossible, unless extensive re-arrangement occurs on hydrolysis, to maintain that the histone consists of repeated regular tetrapeptide units, with a basic group in each tetrapeptide.

At the same time it seems to me that the occurence on only two principal N-terminal groups in the histones must have a very definite

meaning. One possibility which could be put forward tentatively is that the two main classes of histones distinguish the two strands of nucleotides in the DNA fibre which lie on adjacent helices. It seems to be quite possible for a histone molecule to be combined with a single spiral of nucleotide (see fig. 1). However, there is

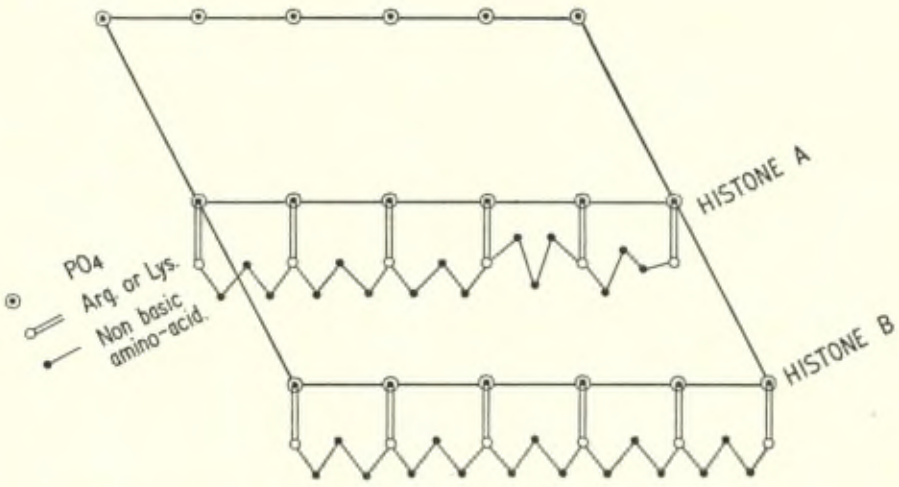

Fig. 1. — A possible arrangement of the two principal histones (proline ends and alanine ends) on adjacent nucleotide helices. The histones are represented as having a regular tetrapeptide structure.

one severe contra indication of this hypothesis, viz. that the amounts of the two classes of histones are not the same as they should be if the hypothesis were entirely correct. However it is possible that the alanine-ended histones have undergone proteolysis to some extent and we must remember the possibilities that some of the N-terminations are acylated.

M. Fraenkel-Conrat. — Attention is drawn to the fact that the nature of nucleoproteins, if they exist, may vary considerably. The association between histones and DNA, which was studied by Drs. Crampton and Moore, probably is close enough to justify the term nucleoprotein. On the other hand, in many viruses, this association between protein and RNA appears much looser.

M. Ubbelohde. — It seems important to have some kind of quantitative measure of the minimum differences of structure that would definitely become apparent in current X-ray methods. Clearly the resolving power of any method of differentiation is limited; but have any quantitative tests been made of the resolving power of X-rays for the types of structure under discussion, for example, by progressive small additions of samples of molecules definitely but only slightly different ?

M. Wilkins. — If we are studying a homogeneous crystalline substance, we can decide how small a difference of structure is detectable by the use of X-ray diffraction technique. Unfortunately, however, specimens of long-chain macromolecules are not homogeneous. In general, they consist of crystalline and amorphous regions which give, respectively, well defined and diffuse diffraction patterns. Small differences of structure may be detected in the crystalline regions but large differences may occur in amorphous and poorly-crystalline regions without causing much change in the diffuse diffraction pattern.

M. Luzzati. — Je voudrais rappeler que les renseignements qu'on peut obtenir des diagrammes de diffraction des rayons X des nucléohistones sont bien pauvres si on les compare à ceux que fournissent l'ADN ou même les nucléoprotamines. Ainsi, les différents modèles de structure qui ont été proposés jusqu'ici pour les nucléohistones, bien que compatibles avec les données cristallographiques, ne sont pas entièrement convaincants. Il semble donc difficile de définir un diagramme de rayons X de fibres, *aux grands angles*, qui soit caractéristique des nucléohistones.

Au contraire, il semble que l'organisation colloïdale des nucléohistones soit assez spécifique : c'est seulement dans les nucléohistones qu'on trouve une organisation à l'échelle de 100 Å environ, à la fois dans les noyaux intacts et dans le matériel extrait. Or les diagrammes de diffusion centrale des rayons X qu'on obtient avec les produits de la précipitation de solutions d'ADN et d'histone sont souvent différents de ceux qui sont caractéristiques des nucléohistones.

M. Wilkins. — Dr. Luzzati has suggested that nucleohistone may consist of lamellae spaced 100-120 Å apart.

I think it is true to say that the differences of interpretation of X-ray results made by Dr. Luzzati and myself arise largely because Dr. Luzzati has not studied diffraction from oriented material. The diffraction present of nucleohistone is not well defined and some of its most characteristic features are not clear unless orientation is present. Orientation greatly increases the amount of information obtained. Unfortunately, however, orientation cannot be obtained unless the material contains less wather than *in vivo*.

We have also studied the low-angle diffraction region referred to by Dr. Luzzati. We are fairly certain that the 60 Å reflection is due to phospholipid associated with the nucleohistone and we are inclined to the view that the reflection in the 100 Å region, which I think we have not obtained so clearly as Dr. Luzzati, is also due to lipid or lipoprotein. Since these reflections may be oriented at right angles to the 35-37 Å reflection, they cannot be various orders of the same fundamental reflection and due to diffraction from a lamellar system. It is not impossible, however, that the 35-37 Å reflection we obtain in our fibres does not correspond to the 37 Å observed *in vivo*. But, in any case, it is difficult to reconcile our observations with a lamellar nucleohistone structure.

M. Felix. — I should like to ask Dr. Moore two questions :

1) Have you analyzed the DNA, remaining after the extraction of the histone, for attached aminoacids ?

2) Is it possible that metal ions may play a role in the formation of larger complexes from the smaller fractions of histone ? In the last months we made some observations with clupein which may be of interest in this connection. We tried to confirm our previous findings that arginine is the C-terminal amino acid and used N-Bromosuccinimid for this purpose. We were very surprised to find that the amount of CO_2 liberated depended on the procedure we used for the preparation of clupein. If it is precipitated from a solution of nucleoprotein in 10 % NaCl by saturation with NaCl an oil results from which no CO_2 is liberated at all. But if one treats this oil with trichloracetic acid in order to separate the clupein from the salt, a different kind of preparation is obtained. It delivers the expected amount of CO_2 for each unit of about 4 000 molecular weight upon treatment with N-bromosuccininid. We assumed

that the presence of a metal ion could account for this phenomenon. In the different preparations we have found remarkable amounts of Cu in the salted-out clupein and none in that which had been treated with trichloracetic acid. If we removed the Cu from the salted-out clupein, by adding Na-diethyldithiocarbamate, CO_2 was produced by N-bromosuccinimid and if one added Cu again no reaction took place. Unfortunately we did not find a stoichiometric relationship between the CO_2 and the Cu.

M. Moore. — 1) Amino acid analyses of the proteins attached to DNA after the histones are largely extracted, have not been made in our studies. Results from other laboratories (cf. ref. (29) in our paper) indicate that the residual proteins may contain all of the amino acids common to tissue proteins.

2) The finding of an influence of metals upon end-group determinations is an interesting observation. I do not recall that the various studies on the aggregation of histones (refs. 14, 15, 20-24) have as yet implicated bivalent cations in the process; multivalent anions also might be of concern in this connection.

M. Watson. — I would ask Dr. Wilkins to expand on his statement that histones can be denatured. What exactly is your criterion for denaturation ?

M. Wilkins. — First, we find that unless histone is prepared very carefully it will not recombine with DNA to give the characteristic nucleohistone diffraction pattern. Second, Dr. G.L. Brown, in our laboratory, has found that histone will not fractionate DNA, nor will it bind so efficiently to *p*-amino benzyl cellulose unless it is heated (G.L. Brown and A.V. Brown, " The Biological Replication of Macromolecules ", p. 10, Cambridge University Press, 1958).

M. Luzzati. — Il y a lieu de rappeler que l'organisation des nucléohistones est très sommaire, si on la compare à celle du TMV. En effet, les diagrammes de diffraction des rayons X du TMV contiennent un grand nombre de taches, bien ordonnées sur les strates, correspondant à un excellent diagramme de fibre. Au contraire, les diagrammes relatifs aux nucléohistones ne contiennent

que quelques taches diffuses, à peine orientées dans les cas les plus favorables.

M. Bigwood. — Dr. Byvoet in Prof. Westenbrink's laboratory in Utrecht University has studied a method of fractionation of the histone components by ethanol precipitation. The arginine-rich component is precipitated between 13 and 17 % ethanol, whereas the lysine-rich component stays in solution.

Prof. Westenbrink and Dr. Monfoort, another of his co-workers, have asked us to determine the aminoacid content of this lysine-rich fraction obtained from calf's thymus by using Moore and Stein's chromatographic method on ion exchange columns. This was done in duplicate on Amberlite I.R.C. 120 × 8 columns, according to Moore, Spackman and Stein, 1958 (*Analytical Chemistry*, **30,** 1185-1190). Our duplicate determinations checked perfectly and gave us a yield close to 100 % of the total nitrogen as determined by Kjeldahl. The results obtained by R. Crokaert and Ch. Wodon in my laboratory, are expressend in aminoacid N in percentage of total nitrogen and compared in this way to the values given in Moore's report (see Table 2).

It appears from this comparison :

a) that Crampton, Stein and Moore's component is richer in lysine than Westenbrink's;

b) that it is poorer in arginine than the latter;

c) that it contains no histidine, whereas some of this amino acid is found in Westenbrink's preparation.

These three findings suggest :

— that the lysine-rich histone obtained by ethanol fractionation may still contain perhaps some of the arginine-rich component, or a degradation product therefrom, that failed to precipitate completely out of solution;

— that Moore's data for their respective contents in several other amino acids in histones are also in line with that suggestion;

— that the 9-1 ratio of the arginine-rich versus the lysine-rich components as observed by Crampton and Moore, in comparison to the 8-2 corresponding ratio obtained by Byvoet, points to the same interpretation.

TABLE 2

Histones from calf thymus.
(Lysine-rich fraction).

	N in % of total N	
	Fraction A (Moore's report, Table 1)	Fraction II (Westenbrink)
Aspartic acid	1.5	2.1
Glumatic acid	2.4	3.4
Glycine	5.0	5.5
Alanine	19.5	17.9
Valine	3.6	3.6
Leucine	3.1	3.4
Isoleucine	0.7	0.8
Serine	4.8	5.0
Threonine	4.3	4.1
Cystine	0	0
Methionine	0	0
Proline	7.0	6.7
Phenylalanine	0.35	0.4
Tyrosine	0.34	0.4
Histidine	0	1.1
Lysine	42.4	37.5
Arginine	5.1	8.3

Westenbrink agreed that I should communicate our findings in the course of the discussion of Dr. Moore's paper, both in his and in our name. He asked me however to stress on his behalf the following points based on his more recent attempts to obtain, if possible, a purer sample of the lysine-rich component that the one he has so far obtained by ethanol fractionation alone :

1) Byvoet noticed in 1958 that with 5 % trichloracetic acid it is possible to precipitate the arginine-rich component and to leave the lysine-rich one in solution.

2) that the electrophoretic diagram of his lysine-rich component obtained by ethanol fractionation indicated that it seemed to contain impurities migrating either faster or more slowly than the main component of the lysine-rich histone, in the electrophoretic field.

3) That his lysine-rich fraction yielded a very slight precipitate when treated with 5 % trichloracetic acid, but that after separation of the latter by centrifugation the supernatent fluid's electrophoretic behaviour was not substantially altered.

Prof. Westenbrink feels inclined therefore to believe that his lysine-rich component is not contaminated by some of the unaltered arginine-rich one which would not have been completely precipitated out of solution in the presence of 13-17 % alcohol.

The question could perhaps be solved by analysing chromatographically Westenbrink's preparation of his lysine-rich component obtained by ethanol fractionation alone or by this procedure completed by trichloracetic acid treatment, using Ba IRC-50 columns, such as those that have yielded figure 1 of Dr. Moore's report to this conference.

Dr. Westenbrink is also studying the question of how he could reduce the possibility of proteolysis during the preparation of histones. His thoughts on the importance of reducing proteolysis parallel the thoughts on that subject which Dr. Moore has mentioned in his talk.

Dr. Westenbrink has asked me to refer to the preliminary unpublished experiments he has made on the direct homogenation of thymus tissue with trichloracetic acid as the first step in the preparation of the lysine-rich histone.

LES PROPRIÉTÉS PHYSIQUES DE L'ACIDE DÉSOXYRIBONUCLÉIQUE EN SOLUTION

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INTRODUCTION

Il convient peut-être de commencer par définir dans quel esprit nous analyserons — avec sans doute de graves lacunes dont nous nous excusons à l'avance — l'ensemble des résultats établis au cours de l'étude des propriétés physiques des solutions étendues de DNA (acide désoxyribonucléique).

Tout naturellement en effet la plupart des expérimentateurs n'ont pas accompli leurs recherches dans le but louable mais vague, d'augmenter leurs connaissances générales soit en diversité soit en précision. Le plus souvent leurs travaux ont été orientés par des intentions assez bien définies. Il ne s'agit donc pas ici de ces expériences « pour voir », dont a parlé Claude Bernard, mais d'expériences d'une part inspirées par les nouvelles acquisitions de la biologie, et d'autre part combinées en fonction de développements récents que la physico-chimie macromoléculaire a connus notamment, dans le cas généralement plus simple où la matière étudiée n'a pas été élaborée au sein d'un organisme vivant.

De ce point de vue l'étude qui nous intéresse ici est l'un des chapitres de la physico-chimie biologique. Si l'on admet que l'un des problèmes centraux de cette science est d'expliquer — en termes de physique et de chimie — l'enchaînement des phénomènes organisés

dans un être vivant, il est évident que les bases de départ doivent être nécessairement l'étude de la structure élémentaire de ce dernier.

Au cours des années, et au fur et à mesure que se perfectionnaient les moyens d'observation, les structures mises en évidence ont atteint une finesse de détail de plus en plus délicate.

Mais si l'on dissèque encore plus avant la matière vivante, on arrive tout naturellement — comme pour la matière inerte — à rencontrer ses molécules constitutives. Celles-ci — tout au moins celles qui sont significatives du point de vue biologique — sont en général des macromolécules de structure très complexe comportant souvent des caractères très spéciaux.

Il est évident que leur description détaillée présente un intérêt fondamental et, de ce point de vue, l'étude physico-chimique des macromolécules d'origine biologique se présente-t-elle comme le prolongement de l'anatomie.

Il est intéressant de constater avec Fauré-Frémiet que notre époque voit se rencontrer dans le domaine macromoléculaire, les efforts d'analyse des biologistes tendant à mettre en évidence des structures de plus en plus fines et les efforts de synthèse des chimistes qui, venus des molécules « ordinaires » très simples, ont bâti et étudié des édifices de plus en plus considérables.

Cela posé, remarquons aussitôt que dans les êtres vivants les macromolécules sont le plus souvent arrangées d'une manière délicatement ordonnée au sein de l'organite qui les contient : c'est bien, par exemple, le cas des nucléoprotéines et des acides nucléiques dans la cellule. Un double problème se pose alors : le premier consiste à décrire cet arrangement « in vivo », le deuxième, qui touche plus directement la physico-chimie proprement dite, consiste à décrire les macromolécules elles-mêmes. Or, pour ce faire, il est nécessaire d'extraire celles-ci de l'édifice originel et presque toujours de les mettre dans un état (fibre, solution étendue, etc.) tel que les procédés habituels de la physico-chimie puissent être appliqués efficacement. On doit alors s'attendre à ce que les configurations présentées par les macromolécules dans le milieu d'étude « in vitro » soient en général différentes de celles qu'elles présentent « in vivo », bien qu'à l'occasion, ces dernières puissent réfléter un souvenir de l'organisation originelle. Par contre, restent intégralement conservés des caractères intrinsèques des macromolécules : tels que leur masse

et l'ordre dans lesquels se trouvent liés entre eux les groupes atomiques constitutifs.

C'est de ce problème limité dont nous nous occuperons ici dans le cas du DNA, et encore laisserons nous pratiquement de côté dans cet exposé l'étude — cependant fondamentale — des séquences pour nous attacher beaucoup plus à la détermination des masses.

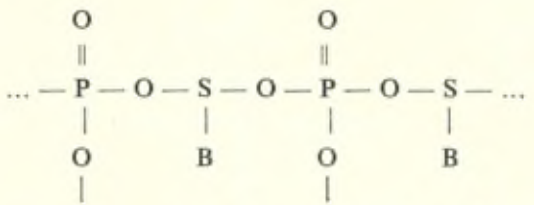
C'est justement et surtout dans ce but que les propriétés physiques des solutions étendues ont été explorées, et ainsi se trouve éclairée la position que nous entendons prendre ici.

* * *

En conclusion, nous préciserons ainsi les termes du problème dont la solution nous préoccupe.

Nous partons des connaissances obtenues sur la composition chimique du DNA ainsi que sur sa structure aux rayons X observée, par exemple, dans les fibres préparées à partir de sa solution à haute teneur d'humidité (forme B).

Nous admettrons donc que — dans cet état — le DNA se présente au moins en grande partie sous la forme de la double spirale maintenant familière, proposée par Crick et Watson (fig. 1). Celle-ci est constituée par l'assemblage de deux chaînes répondant chacune à la formule schématique :



où S représente un sucre, le désoxyribose, et B l'une des quatre bases adénine et guanine d'une part, cytosine et thymine d'autre part. Les deux chaînes sont enroulées l'une sur l'autre en hélices et soudées par des liaisons hydrogène entre adénine-thymine d'une part et cytosine-guanine d'autre part, comme le montre la figure 1.

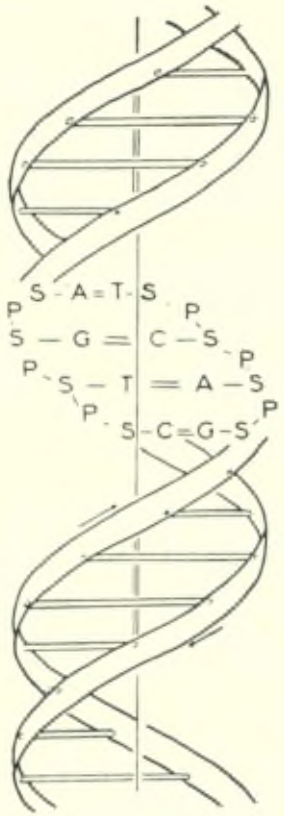


Fig. 1. — Schéma de la double hélice de Crick et Watson

Selon ce modèle, la distance entre les plateaux basiques est de 3,4 Å et la masse molaire correspondante est de 20.377 pour 100 Å de longueur.

Nous n'insisterons pas plus sur ces notions maintenant classiques bien qu'encore incomplètes, et qui nous serviront de base.

Cela posé, on extrait le DNA d'un milieu donné (thymus du veau, érythrocytes d'oiseaux, sperme de poissons, bactéries, etc.) au moyen d'un certain nombre d'opérations physico-chimiques. Celles-ci diffèrent selon les divers auteurs soit dans leur principe même (Hammarsten, Signer et Schwander, Sevag, Butler, etc.) soit dans des nuances de détail (Vendrely, Pouyet, etc.).

On obtient ainsi, à l'état final, une dispersion de la substance dans un milieux aqueux de pH et de force ionique déterminés.

Il s'agit alors de caractériser les particules de DNA dispersées, c'est-à-dire :

1. de déterminer la fonction de distribution de leurs masses, soit encore — faute de mieux — de mesurer des valeurs moyennes de celles-ci;

2. de déterminer leurs caractères morphologiques, c'est-à-dire leurs forme, dimensions ou statistique de configurations si — comme cela semble bien le cas — chaque particule peut présenter plusieurs de celles-ci.

C'est là un problème typique de physico-chimie macromoléculaire, tel qu'il se pose dans nombre de cas n'intéressant aucunement les substances d'origine biologique. C'est à son examen que nous nous bornerons ici. En particulier nous laisserons de côté la question — pourtant essentielle — de savoir si toutes les particules dispersées présentent ou non des compositions chimiques identiques ou si elles peuvent différer, à composition identique, par l'ordre dans lequel se succèdent les différentes bases B. Cette question n'est vraisemblablement pas justiciable des méthodes que nous allons employer, mais cependant il est possible que celles-ci puissent contribuer à sa solution complète.

* * *

PREMIÈRE PARTIE

RAPPEL DE NOTIONS GÉNÉRALES

Nous n'hésitons pas — pour la clarté de l'exposé — à rassembler brièvement dans ce chapitre quelques unes des bases sur lesquelles repose la détermination des masses et des paramètres morphologiques des macromolécules en solution. Nous nous appesantirons le moins possible sur les considérations théoriques qui n'auraient pas leur place ici, mais nous insisterons sur la nature de leurs conséquences de manière à permettre au lecteur non spécialisé de porter un

jugement motivé sur les résultats de leur application au cas, particulièrement délicat, des acides désoxyribonucléiques.

Il est bien entendu que ce court chapitre n'a aucunement la prétention de présenter de façon complète l'ensemble de cette importante question que l'on trouvera exposée dans des monographies plus importantes dont quelques unes sont signalées en bibliographie.

A. Mesures hydrodynamiques.

D'une manière très générale, on effectue sur la solution un certain nombre d'opérations, au moyen de techniques appropriées et qui fournissent la valeur de grandeurs telles que par exemple les constantes de diffusion brownienne, de translation A et de rotation B, la constante S de sédimentation, la viscosité intrinsèque $[\eta]$, etc. et qui, toutes, sont liées aux caractéristiques — masse et morphologie — des particules dispersées. On sait que A et B sont donnés par:

$$A = \frac{kT}{f} \quad (1) \qquad \text{et} \qquad B = \frac{kT}{C} \quad (2)$$

où k est la constante de Boltzmann, T la température de la solution et f et C les coefficients unitaires de frottement de la particule respectivement en translation et en rotation uniformes dans le solvant de viscosité η_o.

f et C sont proportionnels à η_o, les coefficients de proportionnalité dépendant de la forme et des dimensions des particules, et non de sa masse, par des lois qui sont soit établies théoriquement à partir des principes de l'hydrodynamique des fluides visqueux supposés valables encore à l'échelle submicroscopique (cas des sphères, des bâtonnets, des ellipsoïdes de révolution) ou à partir de relations semi-empiriques dans le cas des molécules en chaîne.

La vitesse uniforme de sédimentation des particules dans un champ de gravitation γ produit par exemple par ultracentrifugation est :

$$v = M(1 - V_{sp}\rho_o)\gamma/f \qquad (3)$$

où M est la masse de la particule, V_{sp} le volume spécifique partiel de cette dernière et ρ_o la densité du solvant.

L'expérience consiste à mesurer v, γ étant connu. On obtient ainsi la valeur de la constante de sédimentation $S = v/\gamma$, c'est-à-dire:

$$S = \frac{M\,(1 - V_{sp}\,\rho_o)}{f} \tag{4}$$

De (1) et (4) on tire :

$$S = M\,(1 - V_{sp}\,\rho_o)\,A/kT \tag{5}$$

de sorte que V_{sp} étant déterminé séparément par une mesure adéquate (et parfois critiquable) la mesure simultanée de S et de A fournit la valeur de M.

Si η est le coefficient de viscosité de la solution la théorie montre que $(\eta - \eta_o)/\eta_o$ est proportionnel au nombre de particules par centimètre cube, égal lui-même à c/M.

On a donc :

$$\frac{\eta - \eta_o}{\eta_o c} = F/M \tag{6}$$

Le coefficient F dépend encore uniquement de la forme et des dimensions de la particule. Tout comme f et C il peut s'exprimer dans les cas simples par les lois de l'hydrodynamique, et d'une façon semi-empirique dans le cas des molécules en chaîne.

On conçoit aisément que si l'on peut mesurer simultanément A, B, S et η l'on peut, en comparant les expressions théoriques de ces diverses grandeurs (données), obtenir M et certains paramètres morphologiques (inconnues) : volume et dimensions dans le cas d'un ellipsoïde de révolution compact, écart quadratique moyen entre les extrémités de la chaîne ou rayon de giration dans le cas des macromolécules caténaires.

On trouvera dans la bibliographie l'indication de quelques exposés généraux relatifs à cette analyse.

Remarquons cependant tout de suite que tout ce que nous venons de dire repose sur deux conditions essentielles.

a) Les valeurs de f, C, S, F ne sont caractéristiques de la particule qu'autant que les interactions de celle-ci avec ses voisines sont négligeables de manière à ce qu'elle puisse se comporter comme si

elle était seule dans le solvant indéfini. Il est donc nécessaire d'effectuer les mesures de A, B, S et η à des concentrations de plus en plus faibles et d'extrapoler les résultats obtenus à la concentration nulle. La limite à $c = 0$ de $(\eta - \eta_o)/\eta_o c$ est souvent appelée la viscosité intrinsèque de la solution, et c'est elle que nous désignons par $[\eta]$.

b) Toutes les particules sont identiques entre elles (solutions monodisperses). Sinon (solutions polydisperses) on obtient pour *f*, C, S et F des valeurs moyennes de natures diverses et d'expressions souvent compliquées. Dans ce cas la détermination de M et des caractères morphologiques par les méthodes dont nous venons de donner le principe ne peut être faite — et encore avec beaucoup de difficulté — que dans des cas très particuliers et avec l'aide d'hypothèses simplificatrices parfois arbitraires.

B. Méthodes optiques.

Nous insisterons avec plus de détail sur une méthode reposant sur l'analyse de la lumière diffusée par une solution macromoléculaire dont l'utilisation, relativement commode, a été largement employée dans le cas du DNA.

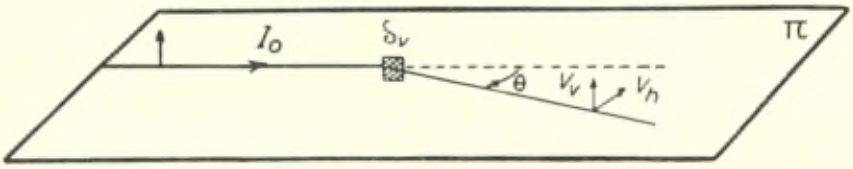


Fig. 2

On sait que la distribution de l'intensité lumineuse diffusée V_v dans une direction θ (fig. 2) est donnée — par exemple si le faisceau incident est polarisé verticalement — par la relation :

$$V_v = A\, c\, M\, P(\theta) \qquad (7)$$

où *A* est un facteur que la théorie de la diffusion de la lumière permet de calculer à partir des conditions de l'expérience, et $P(\theta)$ une fonction sans dimensions de θ, dont la valeur est au plus égale à l'unité quand $\theta = o$ et dont l'expression dépend seulement des

caractères dimensionnels et morphologiques des particules dispersées dans le solvant.

L'expression (7) peut être mise sous la forme :

$$\frac{c}{K} = \frac{1}{M\,P\,(\theta)} \tag{8}$$

où K représente une grandeur accessible à la mesure.

En réalité cette expression n'est valable que pour les concentrations infiniment petites et elle n'est que le premier terme de l'expression plus générale :

$$\frac{c}{K} = \frac{1}{M\,P\,(\theta)} + 2\,Bc \tag{9}$$

où B représente le deuxième coefficient du viriel de la solution.

De plus nous avons supposé que toute la lumière diffusée était — comme la lumière incidente — polarisée verticalement. Il peut arriver qu'il n'en soit pas ainsi et alors les résultats théoriques subissent une complication. Nous laisserons ce cas de côté puisque ce n'est pas celui que l'on rencontre pour les solutions de DNA.

Il est alors possible de calculer la fonction P (θ), dans le cas de particules de forme géométrique simple.

Posons :

$$h = (4\,\pi/\lambda)\,\sin\frac{\theta}{2} \tag{10}$$

où λ est la longueur d'onde de la lumière incidente dans la solution ($\lambda = \lambda'/n_o$ si λ' est la longueur d'onde dans le vide et n_o l'indice de réfraction du solvant).

Le calcul fournit les expressions suivantes :
Sphère de rayon L :

$$P\,(\theta) = \left[\frac{3}{h^3L^3}\left(\sin hL - hL \cdot \cos hL\right)\right]^2 \tag{11}$$

Bâtonnet de longueur 2 L :

$$P\,(\theta) = \frac{1}{hL}\quad \mathrm{Si}\left(2\,hL\right) - \left(\frac{\sin hL}{hL}\right)^2$$

$$\text{où}\quad \mathrm{Si}\,(2\,hL) = \int_o^{2\,hL} \frac{\sin x}{x}\,dx \tag{12}$$

Disque de rayon L :

$$P(\theta) = \frac{2}{h^2L^2}\left[1 - \frac{1}{hL} J_1\left(2\,hL\right)\right] \qquad (13)$$

où J_1 est la fonction de Bessel du premier ordre.

Chaîne gaussienne simple d'écart quadratique moyen L^2 :

$$P(\theta) = \frac{2}{(h^2L^2/6)^2}\left[e^{-h^2L^2/6} + \frac{h^2L^2}{6} - 1\right] \qquad (14)$$

On sait qu'ici :

$$L^2 = N\,b^2$$

où N représente le nombre de chaînons statistiques de la chaîne et b^2 le carré moyen de leur longueur.

On a représenté sur la figure 3 les variations de P (θ) en fonction de hL dans ces divers cas.

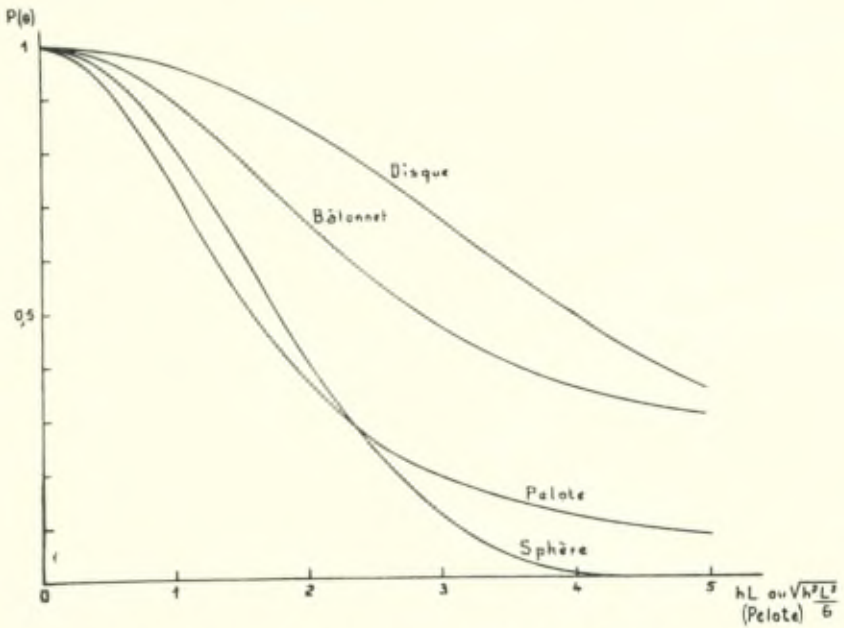


Fig. 3 — La fonction P (θ) pour des particules de forme simple.

a) *Cas limite des valeurs de* hL *infiniment petites :*

Lorsque hL tend vers zéro, la fonction P (θ) peut être représentée par un développement selon les puissances croissantes de h. Si l'on se limite aux deux premiers termes on a :

$$P(\theta) = 1 - h^2 \frac{R^2}{3} \tag{15}$$

où R^2 est le carré moyen du rayon de giration de la particule (*).

Il est très important de noter que cette expression est tout à fait générale et indépendante du modèle géométrique choisi par la particule. Elle est donc applicable même si l'on ne connaît pas celui-ci.

L'application de ces résultats à l'étude des particules dispersées se fait directement.

On voit en effet que, d'après (9) on peut écrire :

$$\left(\frac{c}{K}\right)_{\theta = o} = \frac{1}{M} + 2Bc \tag{16}$$

et :

$$\left(\frac{c}{K}\right)_{c = o} = \frac{1}{M} P^{-1}(\theta) \tag{17}$$

Ainsi donc, par deux extrapolations convenables de la grandeur mesurable $\frac{c}{K}$ il est possible d'obtenir expérimentalement d'une part la valeur de M et de B (mesure de $\frac{c}{K}$ à $\theta = o$ pour les valeurs décroissantes de c) et, d'autre part, la fonction P (θ) (mesure de $\frac{c}{K}$ à concentration nulle pour toutes les valeurs de θ). En particulier on pourra, d'après (15), mesurer R^2.

Ces opérations sont souvent effectuées par un procédé simple proposé par Zimm et qui consiste à porter en ordonnées les grandeurs mesurées de $\frac{c}{K}$ pour diverses valeurs de c et de θ, et en abscisses les valeurs de $\sin^2\left(\frac{\theta}{2}\right) + nc$ où n est un facteur numérique dont la grandeur est choisie pour des raisons de commodité.

(*) Le rayon de giration d'un ensemble de N points A_i de masse m et de centre de gravité G est défini par $R^2 = (\Sigma_i r_i^2)/N$ où r_i représente la distance de A_i à G.

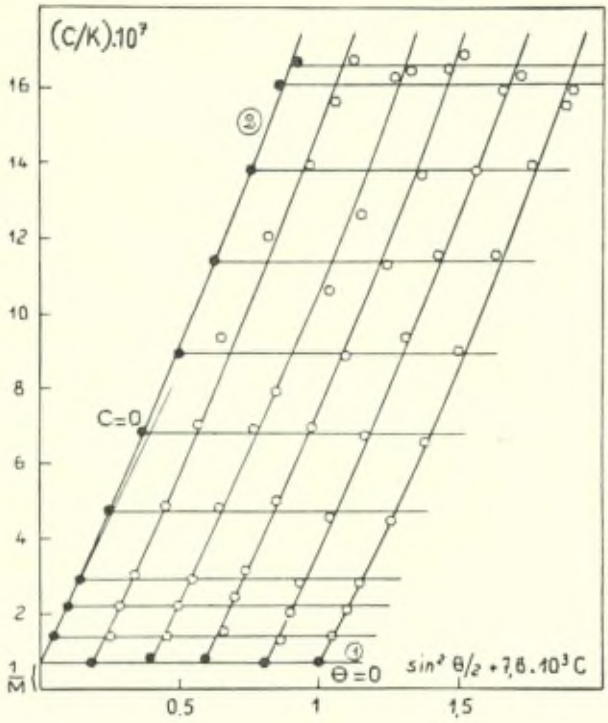


Fig. 4 — Utilisation des données des mesures de diffusion de la lumière avec le procédé de Zimm.

On obtient alors le réseau de la figure 4 où les deux courbes extérieures 1 et 2, correspondant aux valeurs extrapolées, ont la même ordonnée à l'origine donnant la valeur de 1/M et des pentes qui fournissent respectivement les valeurs de B et de R^2.

b) *Cas limite des grandes valeurs de* h*L :*

Dans ce cas, pratiquement réalisé lorsque θ tend vers π et L/λ vers l'infini, $P(\theta)$ tend asymptotiquement vers les expressions telles que :

sphère	$\frac{9}{2}/(hL)^4$	
bâtonnet	$\pi/2\, hL$	(18)
disque	$2/h^2L^2$	
chaîne gaussienne	$3/h^2L^2$	

Ces résultats montrent que de la manière dont varie l'extrémité des courbes P (θ) en fonction de sin θ/2 on peut déduire à quel modèle géométrique appartient la particule. Il y a cependant ambiguïté pour le disque et pour la chaîne gaussienne : dans les deux cas l'asymptote est en $1/h^2L^2$.

D'autres modèles pourraient être d'ailleurs envisagés. Par exemple la chaîne non gaussienne (1) et le ruban très plat (2).

Nous aurons d'ailleurs à revenir sur ce point lorsque nous examinerons le cas des particules de DNA.

A ce rapide rappel de notions nous ajouterons que si l'on fait le rapport entre les intensités diffusées par deux valeurs différentes de θ (par exemple θ = 45° et θ = 135°) on obtient, d'après (7) :

$$\frac{V_v(45^\circ)}{V_v(135^\circ)} = \frac{P(45^\circ)}{P(135^\circ)} = Z \qquad (19)$$

Fig. 5. — Le facteur de dissymétrie Z dans le cas de particules de formes simples.

ce qui a l'avantage d'éliminer le facteur K. Il est évidemment possible de calculer les valeurs de Z — appelé facteur de dissymétrie — si l'on connaît l'expression de P (θ), c'est-à-dire si l'on connaît la géométrie de la particule.

On sait que la valeur de Z, alors qu'elle croît indéfiniment en fonction des dimensions dans le cas général de la sphère, du disque ou d'une pelote, tend, pour le bâtonnet, vers une limite finie (fig. 5).

Celle-ci est de 2.47 si le bâtonnet est isotrope et de 3.36 s'il est anisotrope ([3]).

c) *Cas des rayons X :*

Nous avons admis que le champ électromagnétique était de longueurs d'ondes situées dans le domaine du visible. On conçoit qu'une semblable hypothèse ne soit pas restrictive et que les résultats que nous avons résumés aient une signification générale, quelle que soit la valeur de λ — à condition bien sûr que la transparence du milieu soit réalisée.

En particulier on peut utiliser des longueurs extrêmement petites correspondant au domaine des rayons X.

Dans ce cas le phénomène de diffusion (distinct du phénomène de Bragg donnant les diagrammes bien connus des cristallographes) est à nouveau observable.

L'intensité I diffusée dans la direction θ est à nouveau donnée par une équation analogue à (7) :

$$I(\theta) = B\,c\,M\,P(\theta)$$

Cependant des remarques importantes sont à présenter.

1) Les rayons X n'étant pas polarisés le facteur B est une fonction de θ :

$$B = B_o\,(1 + \cos^2\theta)$$

Le facteur $(1 + \cos^2\theta)$ apparaît également dans l'étude de la diffusion de la lumière incidente quand celle-ci n'est pas polarisée, mais les grandeurs physiques qui apparaissent dans B_o ne sont pas les mêmes que celles qui figurent dans A. Dans le premier cas ce sont en effet les électrons qui constituent les oscillations élémentaires,

tandis que, dans le deuxième cas, ce sont les hétérogénéités d'indice de réfraction qui sont les responsables du phénomène.

2) Cela posé, les fonctions P (θ) s'expriment de la même manière dans les deux cas. En particulier la variable qui s'introduit dans la fonction P est encore hL où, rappelons le, $h = \frac{4\pi}{\lambda} \sin \theta/2$.

Mais on voit aussitôt que λ étant, pour les rayons X, environ 4.000 fois plus petit que pour la lumière visible, les valeurs de h obtenues sont beaucoup plus grandes dans le premier cas que dans le second.

Il en résulte deux conséquences.

La première c'est que — la valeur de P (θ) tendant vers 0 quand h augmente indéfiniment — l'intensité du faisceau diffusé de rayons X ne sera mesurable que dans les directions très voisines de celle du faisceau incident : d'où le nom de diffusion centrale.

La seconde c'est que l'emploi des rayons X s'imposera tout naturellement chaque fois que l'on voudra déterminer la forme limite de P (θ) pour les grandes valeurs de hL, de façon à pouvoir utiliser les expressions asymptotiques dont nous avons énuméré ci-dessus quelques exemples.

Au contraire, c'est aux grandes longueurs d'onde qu'il faudra s'adresser lorsque l'on voudra extrapoler aux valeurs nulles de hL, c'est-à-dire déterminer la masse et le rayon de giration (équation 15).

C. Effet de la polydispersité.

Il est clair que, dans le cas où la solution renferme des particules d'espèces différentes, les simples conclusions que nous venons d'exposer ne sont plus valables.

Dans ce cas nous devrons considérer deux cas différents selon que la grandeur mesurée est donnée par l'observation de phénomènes permanents ou transitoires.

Dans le premier cas : viscosité, diffusion de la lumière ... l'analyse des grandeurs mesurées conduit à la détermination de certaines valeurs moyennes.

Dans le deuxième cas : diffusion brownienne, vitesse de sédimentation ... on obtient des courbes de répartition de valeurs dont l'examen est extrêmement précieux, ainsi que nous le verrons plus loin.

Nous nous bornerons ici à rappeler quelques définitions.

Considérons un paramètre p caractéristique de la particule (masse, dimension, ...) et supposons que la solution contienne un mélange de particules de p différents, tous les autres caractères étant identiques ou sans effet sur la grandeur mesurée.

Nous désignerons par $f(p)$ la loi de distribution en nombre des particules par rapport à p. L'expression $f(p)\,dp$ représente alors le nombre de particules par unité de volume pour lesquelles p est compris entre p et $p + dp$. On peut définir aussi leur loi de distribution en masse $g(p)$.

Si — ce qui n'est pas nécessairement toujours le cas — la masse M des particules est une fonction biunivoque de p on a :

$$g(p) = \mathrm{M}(p)\, f(p) \tag{20}$$

Si $\mathrm{Q}(p)$ représente une grandeur particulaire dépendant de p, on peut calculer la valeur moyenne de Q pour toutes les particules de la solution :

$$\mathrm{Q} = \frac{\int_0^\infty \mathrm{Q}(p) f(p)\, dp}{\int_0^\infty f(p)\, dp}$$

En explicitant l'expression de $\mathrm{Q}(p)$ on obtient des valeurs moyennes du paramètre p.

Celles-ci sont — avec la notation généralement admise — les suivantes :

— moyennes en nombre :

$$p_n = \int_0^\infty p\ f(p)\, dp \ / \ \int_0^\infty f(p)\, dp$$

— moyennes en poids :

$$p_w = \int_0^\infty p^2 f(p)\, dp \ / \ \int_0^\infty p\ f(p)\, dp \tag{21}$$

— moyenne « z » :

$$p_z = \int_0^\infty p^3 f(p)\, dp \ / \ \int_0^\infty p^2 f(p)\, dp]$$

On sait, par exemple, que si Q est la pression osmotique et M le paramètre p, la valeur moyenne mesurée est M_n.

Considérons tout particulièrement le cas de la diffusion de la lumière.

L'intensité diffusée dans une direction θ donnée est, d'après (7) :

$$V_v = \int_o^\infty M^2 P_M \ (\theta) f(M)\, dM \tag{22}$$

ce qui suppose évidemment qu'il n'y a dans la solution que des particules de même type morphologique (sphères, bâtonnets, pelotes, etc.) et pour lesquelles A est identique et $P(\theta)$ une fonction biunivoque de M.

On explicite sans difficulté particulière le deuxième membre de l'équation (22) dans les cas où $P(\theta)$ est connu.

Ainsi dans le cas d'un mélange de pelotes de Gauss (4) différant seulement par le nombre N d'éléments statistiques (b^2 identique pour toutes les chaînes) on a :

$$\frac{c}{K}_{\theta = o} = \frac{1}{M_w}\left[\ 1 \ + h^2 b^2 N_z/18\right] \tag{23}$$

et :

$$\frac{c}{K}_{hL \to \infty} = \frac{1}{2M_n}\left[1 + h^2 b^2 N_n/\ 6\right] \tag{24}$$

Si on porte $\frac{c}{K}$ en fonction de h^2 on voit que l'on obtient les résultats schématisés par les diagrammes de la figure 6.

On voit que si M_n, M_w et M_z sont entre elles comme les nombres 1, 2, 3, c/K varie linéairement en fonction de h^2.

Des calculs identiques ont été faits pour le cas d'un mélange de bâtonnets de longueur différente (5) ou d'un mélange de disques de diamètres différents (6).

Dans tous les cas l'ordonnée à l'origine de la courbe donnant c/K en fonction de h^2 fournit la masse moyenne M_w. La pente à l'origine fournit un rayon de giration moyen défini par :

$$R_z^2 = \frac{1}{M_w}\int_o^\infty R^2 M\, f(M)\, dM \tag{25}$$

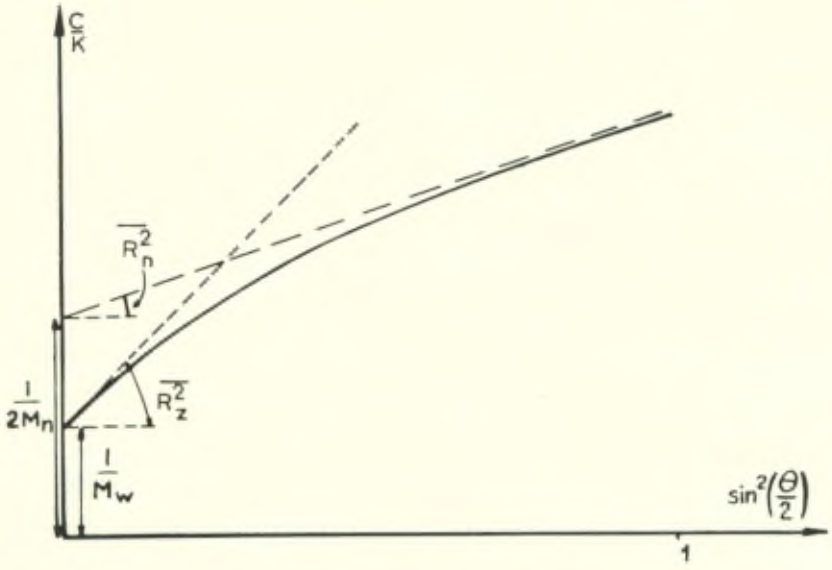


Fig. 6. — Intensité de la lumière diffusée dans le cas d'un mélange de pelotes de Gauss.

Enfin la variation de c/K en fonction de h^2 pour les grandes valeurs de h^2L tend, pour un mélange de bâtonnets de longueurs L différentes mais de même masse M/L par unité de longueur, vers l'asymptote d'expression :

$$\frac{c}{K}_{hL \to \infty} = \frac{2}{\pi^2 M_n} + \frac{h}{\pi} \cdot \frac{L}{M} \qquad (26)$$

ce qui permet de mesurer expérimentalement M/L.

Pour les disques, on obtient seulement la masse par unité de surface.

Pour terminer, insistons encore sur le fait que ces résultats ne sont valables que parce que le seul paramètre M suffit à caractériser la particule, même du point de vue de la diffusion de la lumière. C'est là une hypothèse simplificatrice dont, évidemment, rien ne permet a priori de dire qu'elle est de valeur générale. On peut parfaitement imaginer qu'un milieu contienne un mélange de particules de formes différentes. Dans ce cas l'analyse précédente ne s'applique généralement plus.

* * *

DEUXIÈME PARTIE

ÉTUDE DES SOLUTIONS DE DNA

Conformément aux intentions que nous avons annoncées en introduction, nous serons guidés ici par le souci de voir dans quelle mesure les propriétés physiques des solutions de DNA peuvent nous permettre d'acquérir des connaissances sur les particules dispersées dans la solution.

Nous ne cacherons pas que ce faisant nous insisterons surtout sur l'étude de la diffusion de la lumière, dont — à notre opinion du moins — l'emploi nous semble à l'heure actuelle le plus prometteur.

Mais auparavant, il est nécessaire de donner quelques précisions au sujet des conditions dans lesquelles doivent être effectuées les mesures, quel que soit le principe des méthodes employées.

I. CONDITIONS EXPÉRIMENTALES A RESPECTER

Les échantillons de DNA préparés par les méthodes habituelles sont solubles dans l'eau et dans les solutions chlorurées sodiques en toutes concentrations de NaCl. L'emploi de l'eau pure ou de la solution chlorurée n'est cependant pas indifférent.

En effet, nous avons indiqué ci-dessus que l'interprétation correcte des méthodes d'analyse physico-chimique supposait que les résultats de mesure fussent extrapolés à concentration nulle et cela afin d'éviter les difficultés qu'introduit l'existence, entre les particules, d'interactions dont l'effet peut être beaucoup plus grand que celui du phénomène que l'on peut interpréter théoriquement.

Or d'une part les particules de DNA, en solution, sont très encombrantes et d'autre part elles portent des charges électriques négatives qui proviennent de l'ionisation du groupe phosphorique salifié par le sodium.

Il en résulte, entre molécules voisines en solution aqueuse, des interactions intenses de type hydrodynamique et coulombien, qui se marquent par un taux considérable de variation, en fonction

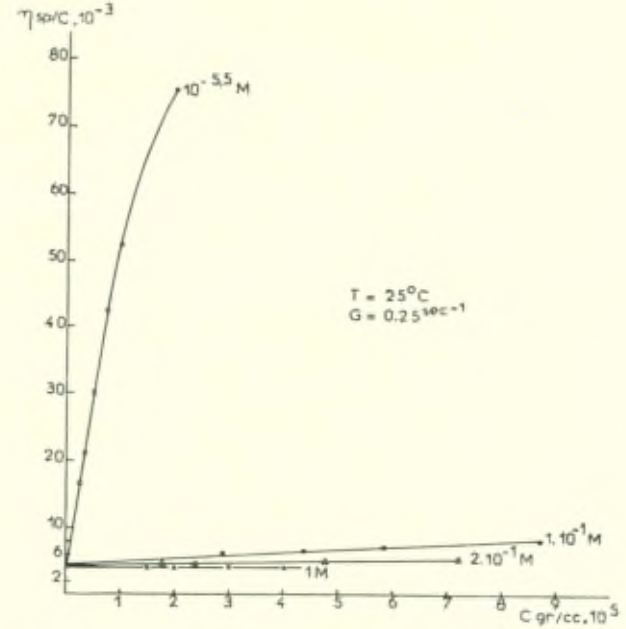


Fig. 7*a*. — Exemple de variation de $\eta_{sp}/c = (\eta - \eta_0)/\eta_0 c$ en fonction de c pour des solutions de diverses concentrations en NaCl (C V 9).

Mesures faites au viscosimètre de Couette avec un gradient hydrodynamique $G = 0{,}25$ sec.$^{-1}$.

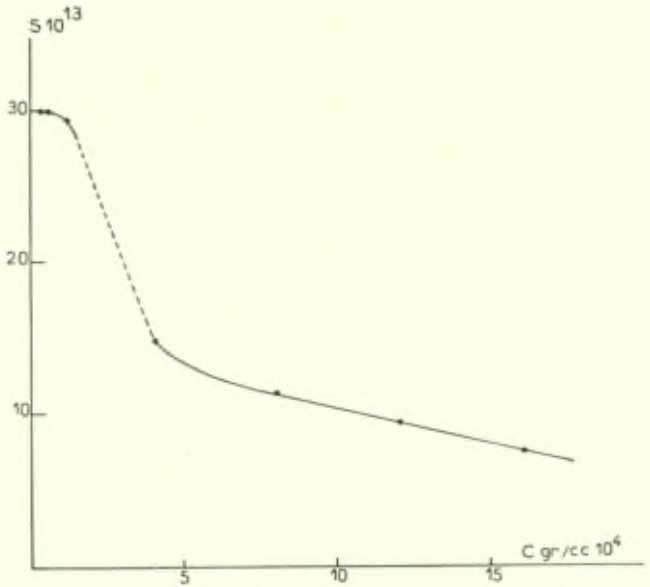


Fig. 7*b*. — Exemple de la variation de la constante de sédimentation en fonction de c (d'après J. Hermans [8]).

de la concentration, de grandeurs qui autrement auraient dû rester constantes.

Il est alors nécessaire d'employer de très hautes dilutions si l'on veut que les valeurs extrapolées à concentration nulle soient significatives, ce qui présente de réelles difficultés techniques.

La situation est considérablement améliorée si l'on opère en solution saline, chlorurée sodique par exemple. Alors les charges libres sont pratiquement saturées et les interactions coulombiennes disparaissent. Mais il est encore nécessaire d'opérer à de très hautes dilutions en DNA si l'on veut éviter les interactions hydrodynamiques.

On peut illustrer clairement ce fait très général en prenant comme grandeur la valeur de $(\eta - \eta_0)/\eta_0 c$. D'après (6) cette grandeur doit être constante si c est suffisamment petit. On constate, sur la figure 7*a*, qu'elle augmente beaucoup plus rapidement dans l'eau pure que dans les solutions chlorurées.

En conclusion il est donc nécessaire, dans le type de mesures que nous employons, d'opérer en solution saline. Il conviendra évidemment de se poser la question de savoir si la présence du sel introduit n'apporte pas de perturbations soit dans la masse de la particule, soit dans la validité des formules générales énoncées dans la Première Partie, soit enfin dans la morphologie de la particule, chose que nous examinerons plus loin.

Remarquons enfin, sur les exemples donnés, que les concentrations en DNA sont extrêmement faibles de l'ordre de quelques 1.10^{-5} g/cm^3. L'expérience montre d'une façon tout à fait générale (exemple de la fig. 7*b*) qu'il faut opérer dans ce domaine de dilution si l'on veut obtenir des résultats susceptibles d'être extrapolés avec quelque sûreté. Beaucoup de travaux, relativement anciens d'ailleurs, et pour lesquels cette règle n'a pas été observée ne peuvent être analysés utilement.

II. ÉTUDE DE LA DIFFUSION DE LA LUMIÈRE

Nous devons a priori, quitte à modifier plus tard cette façon de voir si l'expérience nous y oblige, nous placer dans l'hypothèse

générale qu'une préparation contient des particules de masses différentes.

Dans ce cas l'on a vu que les mesures de diffusion de la lumière peuvent permettre, au moyen des équations dont nous avons donné les principales, de tirer un certain nombre de conclusions sur les grandeurs moyennes relatives aux particules dispersées.

A. La diffusion aux petits angles.

Nous avons vu, dans la Première Partie, que la mesure de l'intensité lumineuse diffusée pour les valeurs de θ voisines de zéro permettait d'atteindre la valeur de la moyenne M_w des masses des particules ainsi que celle de leur rayon de giration.

1. *Critique des conditions d'extrapolation :*

Nous avons indiqué, ci-dessus, quelques unes des conditions que devait remplir l'expérience et notamment insisté sur le fait que la charge et la dimension des particules de DNA imposaient d'opérer avec des solutions d'extrêmes dilutions et en présence d'électrolytes tels que NaCl.

Supposons que les difficultés que soulèvent ces exigences aient été franchies de façon satisfaisante et portons notre attention seulement sur l'opération qui consiste, à concentration nulle, à extrapoler $\frac{c}{K}$ aux valeurs très petites de θ.

Pour simplifier l'exposé, admettons que la solution est monodisperse.

On a vu (équation 8) que, dans ce cas :

$$\left(\frac{K}{c}\right)_{c=o} = M\,P(\theta)$$

où la fonction P dépend de θ par le paramètre hL, et la détermination de M demande l'extrapolation à $h\mathrm{L} = 0$ de la valeur de K/c.

Or on sait bien que les angles θ auxquels on peut accéder expérimentalement ne descendent pas au-dessous d'une certaine

limite θ_l dépendant de la construction de l'appareil. Par exemple, pour l'appareil Wippler-Scheibling, utilisé au laboratoire, sin $\theta_l/2 = 0{,}25$. Par conséquent les valeurs mesurées du paramètre hL sont toutes supérieures à la valeur limite $\frac{4\pi L}{\lambda} \sin \frac{\theta_l}{2}$.

Or θ_l étant donné cette limite est d'autant plus élevée que L/λ est lui-même plus grand.

Donc, si les particules sont très grosses les points expérimentaux figurant les valeurs de K/c en fonction de sin $\theta/2$ risquent — même pour les petites valeurs de θ — de se trouver sur la partie asymptotique de la courbe.

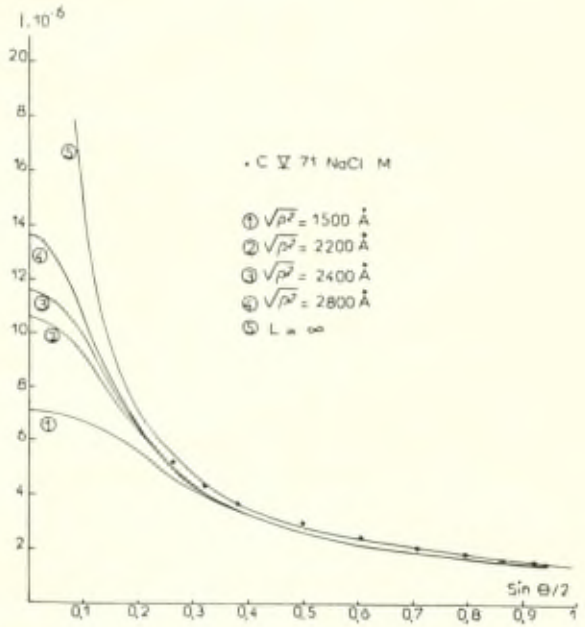


Fig. 8. — Les points représentent les valeurs mesurées de $I = k/c$ pour différentes valeurs de θ.

Les courbes représentent la variation de I, calculée théoriquement, pour des bâtonnets de rayon de giration $R = \sqrt{\rho^2}$ différents ($L^2 = 12\,\rho^2$) et de même masse par unité de longueur.

La courbe 1 correspond à la masse mesurée par le procédé classique de Zimm, soit $M_w = 7.10^6$.

On voit que ce n'est pas la courbe qui passe au mieux par les points expérimentaux.

Avec le mode de représentation directe, que nous employons ici, on s'aperçoit que l'extrapolation à $\theta = o$ est extrêmement incertaine.

Il semble bien que c'est dans cette situation que nous nous trouvons dans le cas des solutions de DNA. On s'en rend compte en consultant la figure 8 où les points expérimentaux (échantillon CV 71) se trouvent manifestement très loin de la région d'inflexion (les courbes représentent la fonction $P(\theta)$ pour des bâtonnets de diverses longueurs).

Dans ces conditions il sera très difficile, puisque l'on n'atteint même pas la région d'inflexion, d'effectuer correctement l'extrapolation vers les petites valeurs de θ.

D'ailleurs il faut remarquer que, à la limite, si les points expérimentaux sont relatifs à de suffisamment grandes valeurs de hL pour qu'ils se trouvent, aux erreurs d'expérience près, sur la courbe asymptote, il devient rigoureusement impossible d'extrapoler à $\theta = o$. En effet seul M/L est alors déterminé expérimentalement. Toute particule, de n'importe quelle masse M peut être considérée comme satisfaisante, pourvu que la valeur de M/L corresponde à la valeur expérimentale. Il existera donc une infinité de manières d'extrapoler vers $\theta = o$.

Il faut remarquer aussitôt que ce n'est pas en utilisant un procédé particulier, comme celui de Zimm, décrit plus haut, que l'on peut éviter une critique qui a une valeur de principe.

Au contraire : en portant en abscisses $\sin^2 \theta/2$ au lieu de $\sin \theta/2$ on resserre l'échelle des abscisses dans la région des petites valeurs de θ, où justement une grande précision serait importante, et en portant en ordonnées c/K au lieu de K/c on représente dans la même région, par des longueurs très petites les grandes valeurs de K/c, c'est-à-dire celles où la précision de la mesure est la plus grande.

Il est donc bien possible que, dans ce mode de représentation, on commette l'erreur de confondre la partie asymptotique de $P(\theta)$ avec sa partie initiale.

Ainsi nous trouvons nous amenés à une conclusion désagréable : c'est que, du point de vue de la stricte méthode, les valeurs de M_w tirées des mesures de diffusion de la lumière sont à ce point suspectes que nous ne savons pas très bien ce qu'elles signifient.

Pour sortir de cette incertitude il nous faudra :

— soit employer un nouveau dispositif expérimental permettant

d'effectuer des mesures correctes de l'intensité diffusée pour des valeurs de θ de quelques degrés seulement,

— soit employer un autre principe de méthode, ce que nous verrons plus loin.

Enfin nous soulignerons que les critiques que nous avons faites sur la validité de la mesure d'une ordonnée à l'origine (la masse M_w) sont encore plus inquiétantes en ce qui concerne la mesure d'une pente initiale (rayon de giration moyen R).

2. *Etude de la reproductibilité des dispersions de DNA :*

Cependant, avant de continuer la discussion de ce que peuvent donner les mesures de diffusion de la lumière, notamment aux grands angles, nous allons examiner une importante question : celle de la reproductibilité des préparations de DNA.

TABLEAU I.

Echantillon	Organe	Auteur *	Méthode	$M_w.10^{-6}$	Z	$[\eta]$
JJA	Thymus de veau	C.R.M.	NaCl	11	5.2	6.000
CV 91b	Thymus de veau	C.R.M.	NaCl	7.9	5.8	2.980
R H	Thymus de veau	$Leyde_2$	NaCl	5	3.4	3.900
HB 1	Thymus de veau	$Leyde_1$	Chymo-trypsine	7	4.3	4.490
AH 1a	Thymus de veau	$Leyde_1$	Chymo-trypsine	18	4.6	3.600
AH 1b	Thymus de veau	$Leyde_1$	Chymo-trypsine	36	4.9	3.170
B 1	Thymus de veau	$Leyde_1$	Chymo-trypsine	6.3	4	3.420
DH 1	Thymus de veau	$Leyde_2$	Détergent	24	3.7	1.900
DH 2	Thymus de veau	$Leyde_2$	Détergent	8	3.6	—
Doty	Thymus de veau	$Leyde_2$	Détergent	8	—	6.000
DH 3	Thymus de veau	$Leyde_2$	Sevag	14	4.4	1.200
JC 1	Thymus de veau	$Leyde_1$	Chymo-trypsine	11.6	4.2	2.700
JC 2	Thymus de veau	$Leyde_1$	Chymo-trypsine	8.8	3.8	3.650
JCK 1	Thymus de veau	$Leyde_1$	Phénol	16	4.55	4.000
JCK 2	Thymus de veau	$Leyde_1$	Phénol	13	4.9	5.600
J 40	Thymus de veau	Londres	Détergent	5.9	4.4	6.800
J 35	Thymus de veau	Londres	Détergent	8.8	4.1	3.300
T 1	Thymus de veau	Londres	Détergent	9.2	4.2	6.800
J 31	Thymus de veau	Londres	Sevag	8.2	4.8	5.000
B B	Thymus de veau	Londres	Détergent	11.5	3.93	2.300

* C.R.M. Centre de Recherches sur les Macromolécules, Strasbourg.
$Leyde_1$ Laboratoire de Biochimie.
$Leyde_2$ Laboratoire de Chimie Physique.
Londres Chester Beatty Research Institute.

TABLEAU II.

Echantillon	Organe	Auteur	Méthode	$M_w.10^{-6}$	Z	$[\eta]$
B.G.	Thymus de veau	B. Bunce	Gulland	3.5	3	3.070
SX	Thymus de veau	R. Bütler	Signer	3.8		
CV 42	Thymus de veau	Vendrely	Signer	4.0	3.55	1.360
CV 49	Thymus de veau	Vendrely	Signer	4.5	3.35	4.300
Simmons	Thymus de veau	Doty	—	5.8		5.340
SV II	Thymus de veau	Schwander	Signer	5.9		5.100
—	Thymus de veau	G. Brown	—	6.0		
SV	Thymus de veau	Schwander	Signer	6.0	3.85	1.500
I 69	Thymus de veau	Vendrely	Signer	6.0	3.4	1.500
CV 74a	Sperme de truite	Vendrely	Mirsky	6.0	4.2	3.400
CV 69	Thymus de veau	Vendrely	Signer	6.0	2.1	4.600
CV 71	Thymus de veau	Vendrely	Signer	6.0	2.7	4.800
SVIII	Thymus de veau	Schwander	Signer	6.0	3.7	5.700
CV 74b	Sperme de truite	Vendrely	Mirsky	6.0	3.3	5.900
Varin	Thymus de veau	Varin	—	6.87		4.800
—	E. Coli	Rowen	Kay	7.0		
Varin	Thymus de veau	Varin	Signer	7.7		
Doty-Rice	Thymus de veau		—	7.7		
Brown	E. Coli	Brown	—	9.1		
SXII	Thymus de veau	R. Bütler	Signer	11.6		
CV 51	Erythrocyte de poulet	Vendrely	Signer	11.6	4.5	4.000
CV 62	Thymus de veau	Vendrely	Signer	13.0	4.8	3.450
CV 64	Thymus de veau	Vendrely	Signer	13.0	4.65	5.900
I 62	Thymus de veau	Vendrely	Signer	14.0	4.6	2.600
CV 9	Thymus de veau	Vendrely	Kay	15.0	4.6	4.000
CV 78	Erythrocyte de poulet	Vendrely	Signer	15.0	5.1	3.400
CV 5	Thymus de veau	Vendrely	Signer	16.5	4.3	3.600
Brown	Bacille tuberc. aviaire	Brown	—	14.0		
				13.5		

Nous constatons en effet que les nombres M_w — résultats de l'extrapolation que nous venons de critiquer — même s'ils ne peuvent être interprétés comme des valeurs vraisemblables de la masse moyenne des particules, représentent chacun cependant une grandeur caractéristique d'une solution de DNA donnée. Deux solutions de particules identiques doivent évidemment fournir la même valeur de M_w.

Aussi récapitulerons nous tout un ensemble de données numériques, que nous avons rassemblées dans le tableau I, déjà utilisé par nous ([9]) et dans le tableau II, tiré de la thèse de J. Hermans ([8]).

L'on est immédiatement frappé par la dispersion des résultats obtenus.

On ne peut soutenir vraisemblablement que les différences observées — qui dépassent de beaucoup les écarts dus à la précision des mesures — peuvent provenir de la nature du matériel d'où l'échantillon a été extrait puisque l'on trouve (tableau I) que les valeurs de M_w peuvent être différentes pour des matériels identiques et identiques pour des matériels aussi différents que peuvent l'être le thymus du veau ou une bactérie.

On est donc amené à se poser la question de savoir si, pour un matériel donné, la dispersion obtenue ne dépend pas de la méthode de préparation.

C'est ce que nous allons maintenant examiner.

Admettons, comme hypothèse de travail, que les valeurs de M_w représentent, sinon un ordre de grandeur de la masse moyenne des particules, mais tout au moins un nombre qui caractérise celle-ci avec plus ou moins de certitude.

L'impression qui se dégage de la dispersion des résultats est alors que, dans certains cas, l'on peut au cours de la préparation provoquer accidentellement la formation d'agrégats.

A ce sujet remarquons aussitôt que les grandeurs numériques ne semblent pas réparties au hasard. L'examen du tableau I montre que plus de 40 % des valeurs de M_w se trouvent comprises entre 6.10^6 et 8.10^6. Le tableau II donne les mêmes pourcentages pour des valeurs comparables, comprises entre 6.10^6 et 10.10^6.

De plus, 30 % des valeurs se trouvent comprises entre 10.10^6 et 16.10^6.

Enfin, très peu de valeurs se trouvent inférieures à 6.10^6 ou supérieures à 16.10^6.

Il serait donc possible — toujours selon l'hypothèse de travail avancée plus haut — de suggérer, comme nous l'avons déjà fait ([10]) que les préparations donnent des particules dont la masse la plus probable M_o correspondrait à une valeur M_w comprise entre 6.10^6 et 8.10^6 ou bien des agrégats constitués par deux de ces particules élémentaires, donc de masse $2\,M_o$.

Recherchons quels pourraient être les facteurs influant sur la formation de ces agrégats.

a) *Effet des précipitations en cours de préparation :*

Une première idée qui se présente naturellement est que, en cours de préparation, le nombre des précipitations par l'alcool et destinées soit à séparer le DNA du complexe protéique, soit à le purifier, peut jouer un rôle déterminant.

Ce point de vue semble justifié par certaines observations de R. Vendrely qui avait remarqué que certains échantillons pour lesquels des précautions spéciales avaient été prises (emploi d'organes frais traités immédiatement, purification du mélange DNA-protéine par précipitations successives du DNA à l'alcool) présentaient de préférence la masse limite $2\,M_o$.

Nous avons dès lors suspecté un possible effet de la précipitation par l'alcool sur l'état d'agrégation du DNA et nous avons cherché à élucider ce point.

A cet effet, J. Pouyet a procédé à partir des érythrocytes du coq, à une extraction du DNA effectuée de telle manière qu'à aucun moment cette substance ait été précipitée (échantillon AMJ 1, rapport azote phosphore N/P = 1.7).

A partir de la solution de DNP obtenue classiquement on sépare la protéine par un procédé au chloroforme dérivé de la méthode de Sevag. Le DNA reste toujours en solution.

Le résultat des opérations est une dispersion en solution aqueuse saturée en NaCl. La masse des particules, *en cet état*, est trouvée égale à 14.10^6.

Sur cette solution on a ensuite procédé aux opérations suivantes :

1. Le DNA est précipité par l'alcool à partir de la solution saturée en NaCl.

Une première partie A du précipité est redissoute dans l'eau pure, puis amenée à la concentration NaCl M. La masse mesurée par diffusion de la lumière est trouvée égale à $11{,}5.10^6$.

Une deuxième partie B du précipité est dissoute dans l'eau pure et amenée à la concentration NaCl 0,15 M, puis reprécipitée par l'alcool et enfin ramenée à la concentration NaCl M. On trouve $M_w = 8{,}3.10^6$.

2. La solution initiale de DNA en NaCl saturé jamais précipitée, est dialysée contre l'eau pure pendant une dizaine de jours à la

chambre froide. On obtient ainsi une solution dans l'eau pure. La masse trouvée est $M_w = 10.10^6$.

A partir de cette solution on prépare des solutions à diverses forces ioniques et, dans chaque cas, on mesure la masse par diffusion de la lumière. Le résultat obtenu est représenté par la courbe I de la figure 9.

Une autre partie de la solution initiale en NaCl saturé est dialysée contre une solution de NaCl 0,15 M. Le DNA, précipité par l'alcool à cette force ionique, est remis en solution et dialysé contre de l'eau pendant trois jours. La masse obtenue par diffusion de la lumière sur cette solution est de 30.10^6.

On augmente alors progressivement la force ionique par des dialyses successives, et pour chaque solution de force ionique μ on effectue une précipitation par l'alcool, puis on remet le DNA en solution à la même force ionique. Les masses obtenues sont représentées par la courbe II de la figure 9.

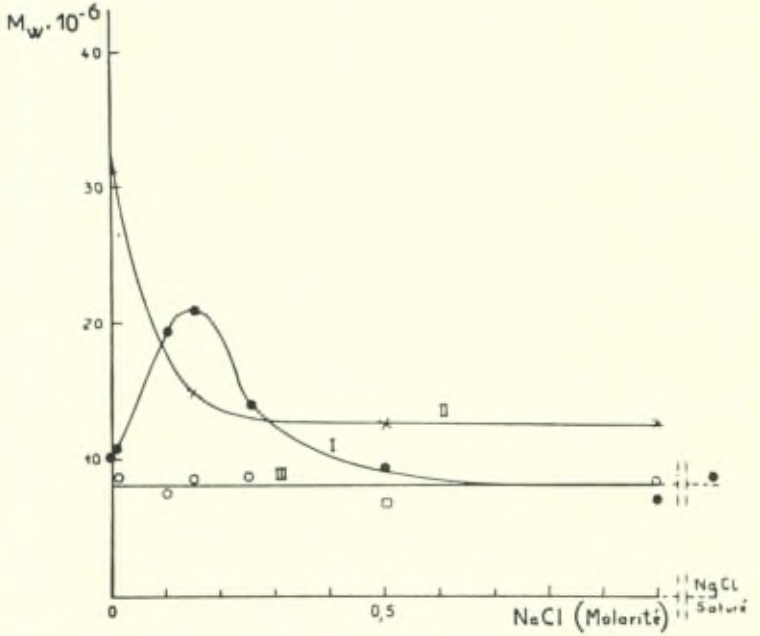


Fig. 9 — Les courbes I et II montrent les valeurs de M_w trouvées pour l'échantillon AMJ_1 après différents traitements.
La courbe III montre que la valeur de M_w est indépendante de la concentration en NaCl quand l'échantillon a été traité à la chymotrypsine.

Ces expériences montrent que les valeurs de M_w mesurées dépendent de l'histoire antérieure de l'échantillon. L'état d'agrégation des particules dépend donc de ce qu'il y ait eu ou non précipitation, et l'effet de celle-ci dépend de la force ionique de la solution à partir de laquelle elle a été provoquée.

b) *Causes encore inconnues :*

Si cette conclusion semble difficilement discutable, il n'en reste pas moins que la précipitation n'est pas la seule responsable d'irreproductibilité.

Nous avions soupçonné depuis longtemps que d'autres causes indéterminées devaient entrer en ligne de compte, et nous en avons obtenu une preuve frappante dans l'observation suivante.

Après avoir préparé l'échantillon AMJ_1 ($M_w = 14.10^6$) dans les conditions que l'on sait, J. Pouyet, quelques semaines après, ayant besoin de nouvelles quantités de DNA, a procédé avec le même matériel (érythrocyte de coq) et par la même méthode (sans précipitation) à la préparation d'un échantillon de DNA catalogué MAV et dont l'étude à la diffusion de la lumière a fourni $M_w = 7.10^6$, c'est-à-dire une valeur moitié moindre que celle observée sur l'échantillon AMJ_1. Rien, dans le protocole des expériences relatives aux deux extractions, ne nous a permis jusqu'ici de discerner quel était le facteur responsable de la différence observée pour les deux échantillons.

c) *Recherche d'une dispersion totale :*

Si l'on admet que la solution de DNA contient des agrégats, il est tout naturel de rechercher les conditions dans lesquelles ceux-ci peuvent être détruits.

A notre connaissance tout au moins, cela a été fait selon deux méthodes très différentes.

1) La première consiste à utiliser l'effet de la chaleur.

On chauffe la solution à une température T pendant des temps θ progressivement croissants, et on détermine les caractères physico-chimiques principaux (diffusion de la lumière, viscosité, constante de sédimentation, etc.) en fonction du temps de chauffe pour chaque température T.

On trouvera ailleurs ([8]) ([11]) les résultats détaillés de ces opérations et nous en extrairons seulement les conclusions suivantes, illustrées par la figure 10.

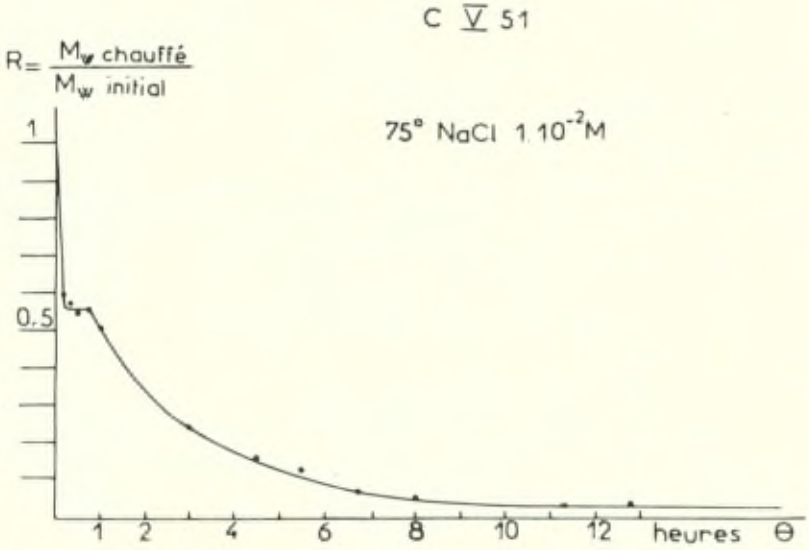


Fig. 10. — La « masse » M_w de l'échantillon CV 51 tombe de 12.10^6 à $6.5.10^6$ après 30 minutes de chauffage à 75° C (destruction de l'agrégat).
Elle recommence à décroître après 1 heure de chauffe (dégradation normale).

On voit que M_w, pour l'échantillon CV 51 chauffé à $T = 75°$ dans une solution à NaCl 10^{-2} M, décroît immédiatement de 12.10^6 à $6,5.10^6$. Quand les durées de chauffe dépassent 50 minutes M_w décroît à nouveau et atteint, pour θ de l'ordre de 12 heures, une valeur constante voisine de 350.000.

Un échantillon CV 71 de masse égale à 7.10^6, chauffé dans les mêmes conditions, ne voit pas son poids moléculaire changer jusqu'à $\theta = 20$ minutes. Pour les durées de chauffe plus grandes M commence à décroître et atteint, au bout de plusieurs heures, la même valeur limite de 350.000 que plus haut.

Il est permis de conclure de cette expérience que, dans les conditions où l'on s'est placé, l'agrégat de masse $2\,M_o$ a été scindé très rapidement en deux constituants de masse M_o voisine de 6.10^6 qui présente ainsi une stabilité particulière.

Ce n'est que pour les durées de chauffe plus grandes qu'on assiste à un deuxième phénomène, la dégradation thermique de la particule de masse M_o.

2) La deuxième méthode est peut-être plus convaincante encore. Depuis longtemps on avait soupçonné que les particules de DNA en solution pouvaient être souillées par des résidus protéiques susceptibles, au cours de la préparation, de souder ces particules entre elles par une sorte de pontage et ainsi de provoquer la formation d'agrégats.

Ce point de vue a été surtout développé par Butler et son école ([12]), et ces auteurs ont été logiquement amenés à étudier l'influence d'un enzyme protéolytique, la chymotrypsine, sur les solutions de DNA. La méthode employée a été ici non pas l'étude de la diffusion de la lumière, mais celle de la sédimentation sur laquelle nous reviendrons plus loin. Elle montre que les agrégats les plus lourds sont détruits par l'enzyme.

Ces expériences ont été répétées par divers auteurs. J. Hermans (1958) étudie la diffusion de la lumière dans des solutions de DNA avant et après traitement par la chymotrypsine et il obtient les résultats rassemblés dans le tableau III.

TABLEAU III.

Nom	Original M_w 10^{-6}	Traité M_w 10^{-6}	Z
CV 69	15	6.3	4.2
JJA	11	6.0	3.7
		4.3	3.5
JC 1	11.6	6.5	3.8
JC 2	8.8	6.7	3.8
JCK 1	16	5.2	3.7
		5.7	4.1
		5.3	4.2
JCK 2	13	6.8	3.7
		5.8	4.5
		5.4	3.7
J 40	5.9	7.7	4.0
J 35	8.8	6.9	3.8
T 1	9.2	6.1	3.8
J 31	8.2	6.2	4.1
AMJ 1	12.5	8.0	5.0
MAV	8.0	6.0	4.0
BB	11.5	2.9	3.65
AH 1a	18	14	4.1
AH 1b	36	20	—

Ils montrent d'une façon parfaitement nette que les agrégats ont été détruits par l'enzyme pour restituer des particules de masses moléculaires comprises entre 6.10^6 et 8.10^6 qui correspondent justement à la masse M_o.

Il reste cependant à remarquer que certains agrégats : BB, AH 1*a* et AH 1*b* de masses respectives $11{,}5.10^6$, 18.10^6 et 36.10^6 pour J. Hermans, se comportent différemment.

Enfin, J. Pouyet et A.M. Freund ont également étudié l'effet de la chymotrypsine sur l'échantillon AMJ_1 dont nous avons déjà parlé plus haut. On part de la solution aqueuse obtenue par dialyse et on ajoute la chymotrypsine en tampon phosphate 0,01 M à pH = 7.38.

La solution ainsi obtenue est ramenée à différentes forces ioniques pour chacune desquelles on mesure M_w. Les résultats, figurés sur la courbe III de la figure 9, montrent que les anomalies ont disparu. M_w reste constant et égal à 8.10^6 quelle que soit la concentration en NaCl.

Conclusion.

De cet ensemble de travaux il apparaît que les méthodes d'extraction du DNA fournissent des solutions contenant, sans qu'on puisse exactement diriger le phénomène, soit des particules de masse M_o caractérisées par des valeurs M_w comprises entre 6.10^6 et 8.10^6, soit des agrégats de masse généralement égale à $2\,M_o$.

Ces agrégats sont décomposés en particules de masse M_o par l'action ménagée de la chaleur ou par celle d'un enzyme protéolytique comme la chymotrypsine. Toutes les anomalies disparaissent alors.

Peut-être, comme il a déjà été suggéré, sont-ils constitués par deux (ou plus de deux) particules M_o liées entre elles par un pont protéique, ou par un amino-acide. Du fait de la très haute valeur de la masse de la particule de DNA, la masse du pont peut constituer une impureté indosable et même peut-être indécelable.

Enfin, bien que, comme nous l'avons vu, le nombre et les conditions des précipitations effectuées en cours de préparation influent sur l'état d'agrégation, la non reproductibilité est encore observée même si a aucun moment le DNA n'a été précipité.

On obtient ainsi au hasard soit des particules « simples » de masse M_o, soit des particules « doubles » de masse $2\,M_o$.

L'emploi du mot hasard signifie pour nous que nous ignorons quel est le détail expérimental, certainement très délicat, dont un changement conduit à une telle conséquence.

Celui-ci semblerait néanmoins lié à la présence d'un résidu protéique puisque (courbe 1, figure 9) on observe pour AMJ_1 un maximum apparent de M_w à la concentration NaCl 0,15 M à laquelle, on le sait, correspond un minimum de solubilité des complexes nucléoprotéiques.

B. La diffusion aux grands angles.

Ainsi donc, nous avons montré que les données de diffusion de la lumière, dans le cas des grosses particules comme celles de DNA, correspondaient à des valeurs de hL si grandes que l'extrapolation à $\theta = o$ devenait difficile, sinon illusoire.

Il nous reste donc à utiliser les mêmes données expérimentales pour tenter l'extrapolation vers les valeurs infinies de hL, ce qui nous permettrait de tirer d'intéressantes conclusions, comme nous l'avons souligné dans la Première Partie de cet exposé.

Tout d'abord les mêmes observations que celles que nous avons faites plus haut relativement à l'irreproductibilité des résultats aussi bien qu'à l'effet de l'histoire de l'échantillon subsistent. Seules, en toute logique, resteront à reprendre certaines interprétations que nous avons données et qui reposaient sur l'hypothèse que les nombres M_w étaient liés simplement à la masse moyenne des particules, comme par exemple l'existence d'agrégats de masse double de celle d'une particule élémentaire.

Au contraire de ce que nous avons fait dans la section A, nous ne reprendrons pas l'examen statistique de l'ensemble des données expérimentales qui ont permis de dresser les tableaux I et II et nous nous intéresserons à des cas peu nombreux et aussi simples que possible.

C'est pourquoi les échantillons AMJ_1 et MAV retiendront notre attention et dans les figures ci-dessous nous représenterons les résultats de mesure en portant c/K en fonction de $\sin \theta/2$.

1°) *Echantillons traités à la chymotrypsine :*

Dans ce cas, le plus simple, nous avons déjà observé que les données de diffusion de la lumière étaient pratiquement indépendantes de la concentration ionique du milieu. Nous avons, sur les figures 11*a* et 11*b* reporté ces données en prenant les valeurs de c/K comme ordonnées et celles de $\sin \theta/2$ comme abscisses.

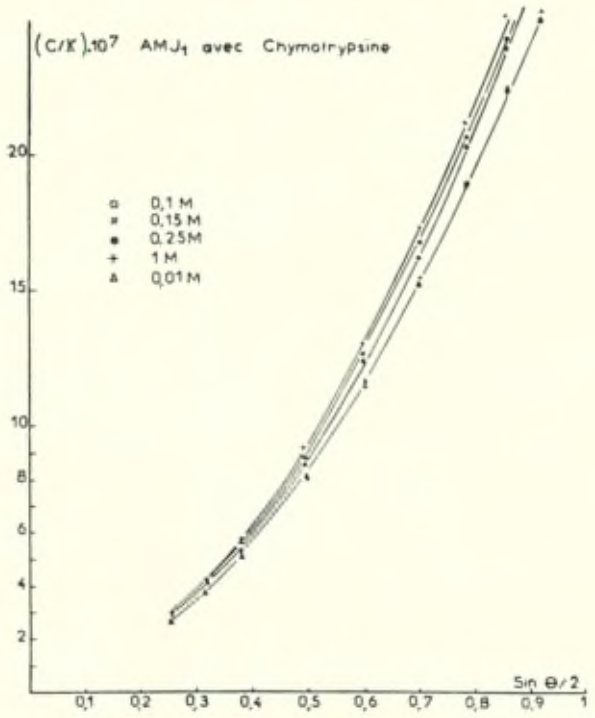


Fig. 11*a*.

Nous constatons à nouveau le peu d'importance de la concentration en NaCl pour l'échantillon MAV. Des différences plus sensibles apparaissent pour l'échantillon AMJ_1. Peut-être ces nuances de comportement tiennent-elles à ce que, dans les deux cas, le mode opératoire a été différent.

Dans le premier cas on a traité directement à la chymotrypsine la solution originelle (NaCl saturé), puis on a réalisé les solutions à différentes forces ioniques en dialysant contre des solutions de NaCl à concentration convenable.

Dans le deuxième cas la solution originelle (NaCl saturé) a été transformée en solution aqueuse par dialyse. Cette solution a ensuite été traitée par la chymotrypsine en tampon phosphate 0,01 M

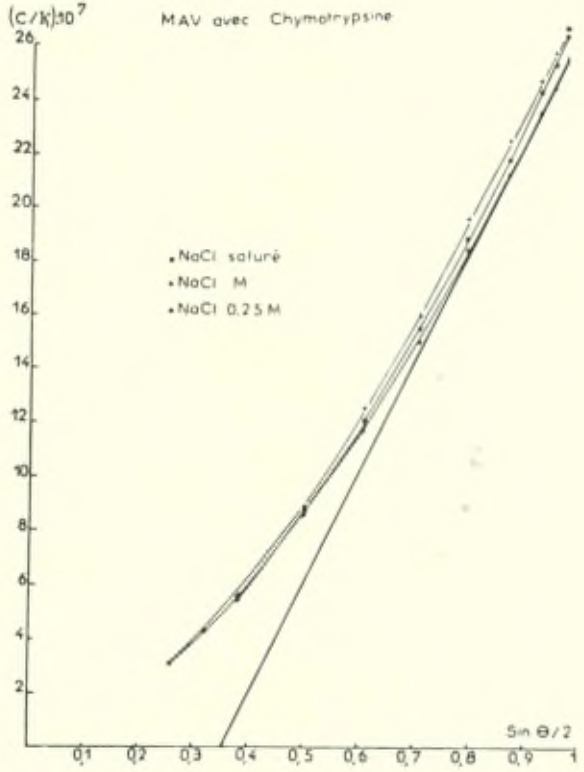


Fig. 11*b*.

pendant le temps convenable (une dizaine d'heures à 37 °C). On a ensuite préparé des solutions de différentes forces ioniques par dialyse contre la solution de NaCl à concentration convenable.

Néanmoins, et sans nous dissimuler que la dispersion des résultats introduit un élément sérieux d'incertitude, il semble raisonnable de considérer que toutes les courbes des figures 11*a* et 11*b* se comportent, pour les grandes valeurs de sin $\theta/2$, de façon linéaire.

S'il en est bien ainsi, on tire immédiatement (équation 26) la conclusion que la particule se conduit comme le ferait un bâtonnet.

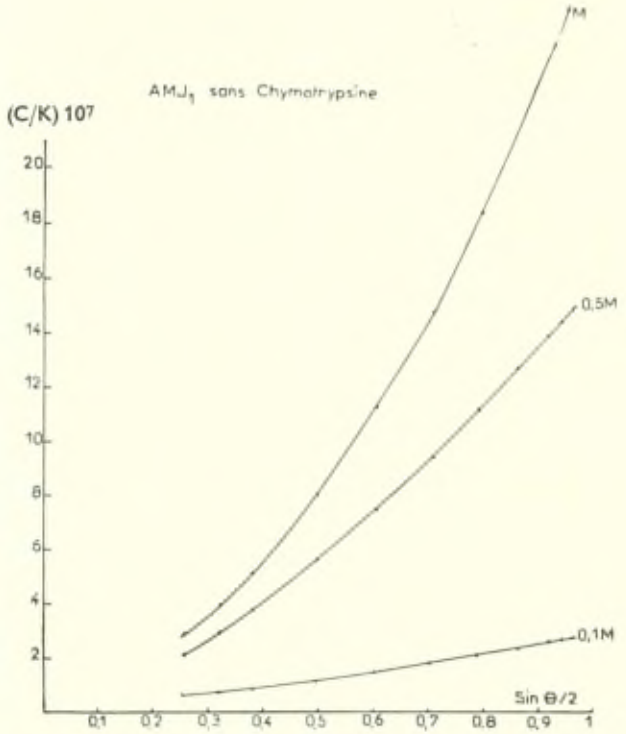


Fig. 12*a*.

On peut dès lors, en mesurant la pente de cette droite asymptote, déterminer la valeur correspondante de M/L que nous exprimerons en masse molaire par 100 Å.

On trouve :

— pour l'échantillon AMJ_1, M/L compris entre 20.000 et 22.000,

— pour l'échantillon MAV, M/L compris entre 24.000 et 26.000.

Il est alors très intéressant de souligner que, eu égard à la précision des mesures, ces valeurs sont compatibles avec la valeur théorique calculée d'après le schéma de la double hélice de Crick et Watson et qui est, rappelons le, de 20.377 pour 100 Å.

2°) *Echantillons non traités par l'enzyme :*

Cette fois, nous le savons, l'influence de la concentration ionique est considérable.

C'est ce que nous constatons à nouveau avec la représentation de c/K en fonction de sin $\theta/2$ employée dans les figures 12*a* et 12*b*.

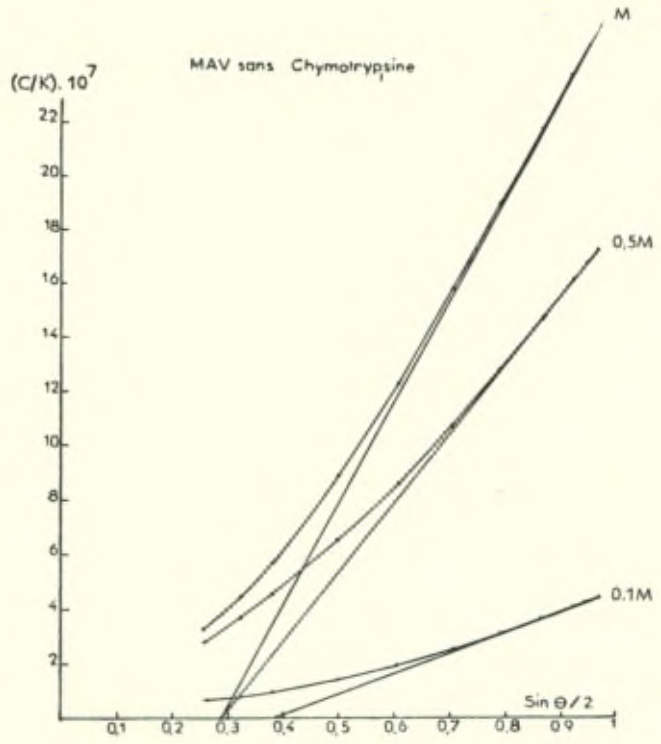


Fig. 12*b*.

Bien que, notamment pour les petites valeurs de la force ionique, les conclusions soient peu nettes, il semble bien qu'encore une fois l'asymptote de c/K soit linéaire et que, encore une fois par conséquent, la particule ait un comportement de bâtonnet.

Mais visiblement la pente des asymptotes, donc la valeur de M/L, dépend de la concentration ionique. Nous avons représenté sur les courbes de la figure 13 les diverses valeurs mesurées pour M/L en fonction de la concentration de la solution en NaCl.

Pour les deux échantillons, M/L présente un maximum pour la concentration 0,15 M de NaCl, c'est-à-dire pour la région de

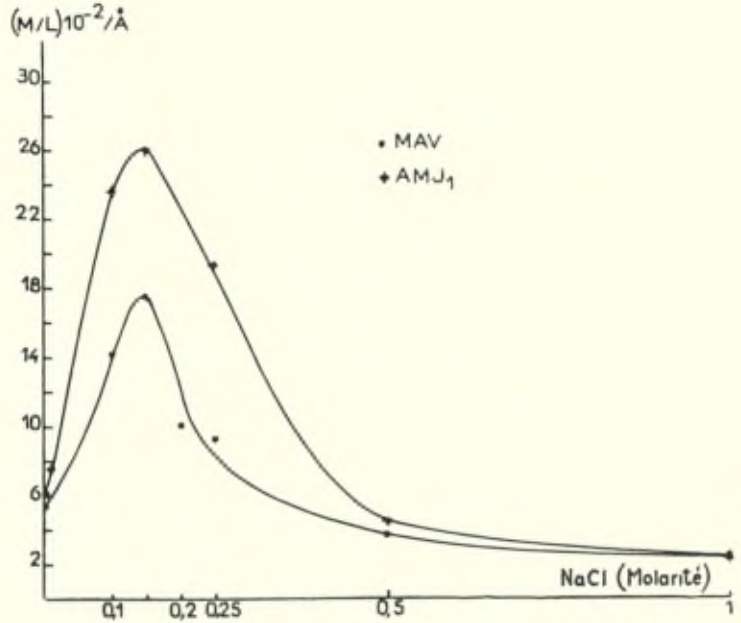


Fig. 13. — La valeur de la masse par unité de longueur déterminée par diffusion de la lumière dépend de la concentration de la solution en NaCl pour les échantillons non traités à la chymotrypsine.

La concentration NaCl 0.15 M est celle du minimum de solubilité des complexes nucléoprotéiques.

solubilité minima du complexe nucléoprotéique. C'est là un phénomène déjà constaté pour M_w (courbe I, figure 9).

De plus M/L est toujours plus élevé pour l'échantillon AMJ_1 pour lequel M_w a la valeur double de celle correspondant à l'échantillon MAV, sauf pour les forces ioniques extrêmes où M/L est le même dans les deux cas avec une valeur de 25.000 pour 100 Å, égale à celle que l'on observe pour les échantillons traités à la chymotrypsine.

3°) *Essai d'interprétation des données expérimentales :*

De ce qui précède découle une conclusion simple : c'est que — sauf lorsque les conditions sont telles que la présence de résidus protéiques puisse se manifester — les particules de DNA ont, à la diffusion de la lumière, le même comportement que des dispersions de particules en forme de bâtonnet présentant une masse par unité de longueur

pratiquement égale à celle que l'on calcule pour la double spirale de Crick et Watson.

Doit on en déduire que ces particules sont réellement des fragments rectilignes en double spirale?

Cette question se divise plus précisément en deux propositions :

Est-ce que la particule a ou non une structure de double spirale?

Est-ce que la particule se présente comme un bâtonnet rectiligne?

a) A la première d'entre elles nous répondrons que les données de diffusion de la lumière ne permettent pas de juger. En effet, les mesures fournissent une valeur de M/L compatible avec celle que l'on calcule pour la double spirale mais ne donnent aucune indication sur le diamètre du bâtonnet.

Si, par exemple, on suppose que la double spirale subisse un effort de torsion, dans un sens ou dans l'autre, lui imposant une déformation qui respecte néanmoins la distance de 3,4 Å existant entre les plateaux puriques et pyrimidiques, alors M/L reste le même et les données de mesures de diffusion de la lumière ne sont pas affectées.

Par exemple — d'accord avec V. Luzzati — nous ne pouvons pas savoir si la colonne nucléique ne peut présenter — en tout ou en partie — non plus l'allure d'une double spirale de Crick et Watson, mais celle d'une échelle plate.

V. Luzzati pense que les mesures qu'il poursuit en ce moment à Strasbourg sur la diffusion centrale des rayons X lui permettront d'obtenir — outre une bien meilleure détermination de l'asymptote de P (θ) — des données précises permettant d'éclaircir ce point très important.

b) Pour répondre à la deuxième question, considérons les courbes des figures 11 donnant c/K en fonction de $\sin \frac{\theta}{2}$.

Nous constatons que si la pente de l'asymptote est bien — comme nous l'avons vu — celle d'un bâtonnet, il se trouve que son ordonnée à l'origine est, sans ambiguïté, négative. Cette observation est en opposition formelle avec l'équation (26) qui montre que pour un mélange de bâtonnets de différentes longueurs l'ordonnée à l'origine ne peut être que positive.

Cette contradiction peut être expliquée à la lumière de récents travaux théoriques de H. Benoît [13] qui s'est attaché à calculer la

fonction P (θ) dans le cas où l'on a affaire avec des configurations constituées par des éléments rectilignes liés les uns aux autres, chaque élément étant — et c'est là la chose nouvelle — d'une dimension supérieure à λ/20.*

Considérons par exemple une ligne brisée — formant une pelote de Gauss si l'on veut — dont les segments ont la longueur L. Si L est plus petit que λ/20 chaque segment se conduit comme un oscillateur unique et l'on est ramené au calcul classique de P (θ) pour une pelote de Gauss (voir Première Partie). Si L est supérieur à λ/20, cette simplification n'est plus acceptable : il faut tenir compte des interférences entre les ondelettes diffusées par oscillateurs portés par un même segment. Dans ces conditions H. Benoît démontre que la fonction P (θ) est donnée par l'équation :

$$P(\theta) = \frac{1}{N} P_L(\theta) + \left[\frac{1}{hL} \mathrm{Si}(hL)\right]^2 P_G(\theta) \qquad (27)$$

Dans cette expression N représente le nombre de segments rectilignes, $P_L(\theta)$ la fonction P (θ) relative au bâtonnet qui constitue chacun de ceux-ci et $P_G(\theta)$ la fonction P (θ) relative à l'ensemble des oscillateurs situés par exemple aux points de jonction entre ces bâtonnets.

Il est clair que si N est infiniment grand le deuxième membre de (27) donne la fonction $P_G(\theta)$ relative à la pelote classique.

Mais, si N est petit, on trouve que l'asymptote prend la forme linéaire :

$$\frac{c}{K}_{hL \to \infty} = \frac{hL}{M\pi} + P$$

$$\text{avec } P = \frac{N}{\pi^2 M}\left[2 - \frac{\pi^2}{2} \cdot \frac{N-1}{N}\right]$$

Sa pente est bien la même que pour un bâtonnet unique.

Mais il y a plus : le terme P est toujours négatif sauf pour N = 1.

Dans ces conditions, la forme des fonctions $\frac{c}{K}_{hL \to \infty}$ détermi-

* Dans un récent article, J. of Phys. Chem., **62** 1543 (1958), J. Hermans Jr. et J. J. Hermans ont traité de la même question et calculé P (θ) pour les chaînes en zig-zag. (Note ajoutée en cours de correction)

nées expérimentalement pour le DNA nous incite à admettre que la configuration des particules est celle de lignes brisées constituées par un tout petit nombre de segments rectilignes de longueur supérieure à $\lambda/20$, soit environ 250 Å.

Cependant, il reste une ambiguïté. H. Benoît a en effet considéré le cas des configurations cruciformes de grands bâtonnets. Il a calculé, par exemple, la fonction P (θ) pour une croix formée par deux segments rectilignes se coupant en leur milieu, chaque bâtonnet ayant par rapport à l'autre et à probabilité égale toutes les orientations possibles.

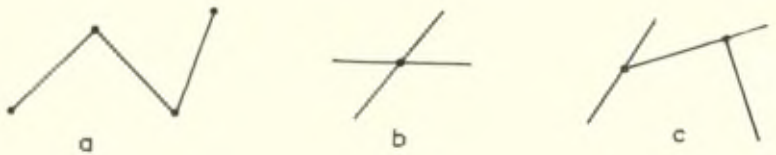
Il trouve alors que l'asymptote est donnée par :

$$\frac{c}{K} = \frac{1}{M}\left[\frac{hL}{\pi} + \frac{4 - 2\pi^2}{\pi}\right]$$

La pente est encore celle qui correspond à un bâtonnet unique, mais l'ordonnée à l'origine est nettement négative.

De ce point de vue, une configuration fourchue, chacune des trois branches étant longue d'au moins 200 Å, donnerait un résultat compatible avec l'expérience.

En conclusion, des configurations telles que celles de la figure 14, peuvent être également adoptées sans s'exclure l'une l'autre.



configurations compatibles avec la forme experimentale de l'asymtote $\left(\frac{C}{K}\right)_{hl \to \infty}$

Fig. 14.

III. EMPLOI DE DIVERSES AUTRES MÉTHODES

Conformément aux principes qui nous ont guidés dans cet exposé et qui nous ont conduit à analyser avec quelque détail les conséquences de l'étude de la diffusion de la lumière, nous ne considérerons les autres propriétés physiques des solutions que dans la mesure où l'on peut, actuellement, en tirer des conclusions nous permettant de préciser — ou de compléter — la descriptions des particules de DNA telle qu'elle résulte du chapitre précédent.

La viscosité.

a) Rigidité des configurations :

Quand une molécule en chaîne contient des groupements fortement ionisables elle porte, en solution aqueuse, une succession de charges d'un même signe — négatives par exemple — entre lesquelles s'exercent des forces coulombiennes de répulsion.

Si la molécule est une longue chaîne flexible, l'effet de ces forces est de dilater les configurations en pelote. L'introduction d'un électrolyte, tel que NaCl, en provoquant la diminution et même la disparition de ces forces, amène une contraction de la pelote.

L'encombrement de celle-ci diminue donc en fonction de la concentration en NaCl, sans évidemment que la masse moléculaire ait varié.

Il s'ensuit que le facteur F de l'équation (6) diminue ainsi que, par suite, la viscosité intrinsèque de la solution.

La figure 15 montre bien cet effet dans le cas d'une chaîne souple de bromure de polyvinylbutylpyridinium. Elle montre aussi l'existence d'un maximum de $(\eta_i-\eta_o)/\eta_o c$ pour certaines concentrations du polyélectrolyte, phénomène tout à fait général pour ce genre de molécules.

Il est clair que le comportement des filaments de DNA en solution (fig. 7) est tout à fait différent. On voit que — au moins à la précision des mesures — $[\eta]$ est indépendant de la concentration en NaCl. Des travaux effectués par la suite par d'autres auteurs ([15]), ([16]), ([17]), confirment ces conclusions.

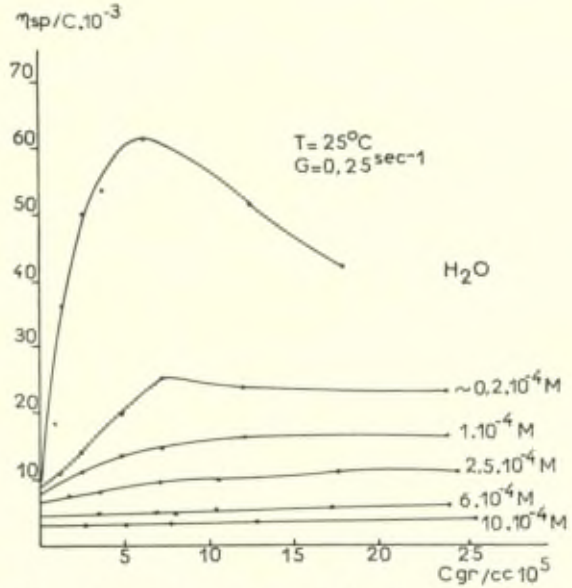


Fig. 15. — Comportement typique de η_{sp}/c dans le cas d'une solution aqueuse d'un polyélectrolyte en chaîne souple, pour diverses concentrations en NaCl. La viscosité intrinsèque $[\eta]$ décroît rapidement en fonction de la teneur en NaCl.

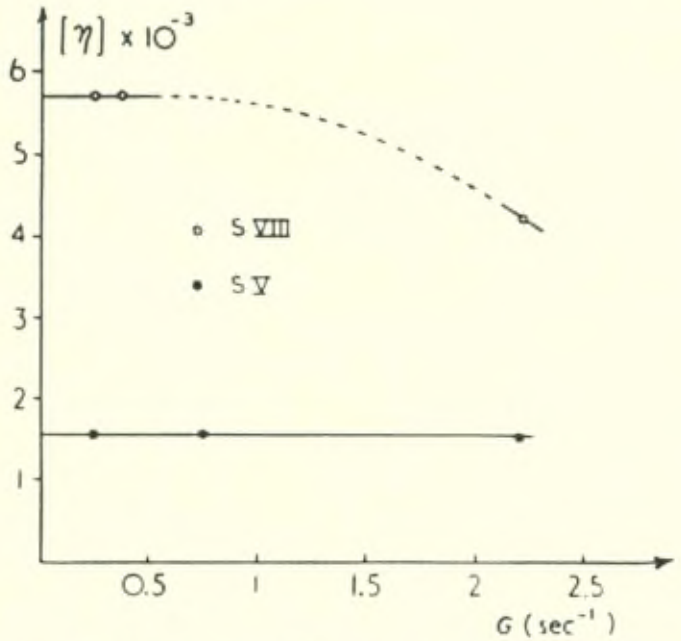


Fig. 16. — Effet du gradient d'écoulement sur la valeur de $[\eta]$. Les particules de l'échantillon S. VIII subissent un effet d'orientation.

Ainsi les particules de DNA présentent elles des configurations rigides.

b) Anisodiamétrie des configurations :

Quelle que soit la force ionique de la solution étudiée, on constate — par exemple en employant un viscosimètre de Couette — que la viscosité de la solution dépend du gradient de vitesse réalisé dans l'appareil (fig. 16) ([18]).

Cela ne peut être interprété que de deux manières : ou bien les configurations macromoléculaires sont déformées par le champ de frottement hydrodynamique, ou bien elles sont orientées par celui-ci.

La première hypothèse ne peut être raisonnablement retenue puisque nous avons trouvé que les configurations ne changeaient pas dans le champ électrique, c'est-à-dire que la particule est rigide.

La deuxième impose alors que les configurations n'aient pas la symétrie sphérique (ou cubique). L'ensemble des bâtonnets doit donc être tel qu'on puisse grossièrement l'inscrire dans un ellipsoïde allongé ou aplati, et non dans une sphère ou un cube.

Il ne peut s'agir d'un bâtonnet unique. Cela serait d'abord en contradiction avec certaines données de la diffusion de la lumière (ordonnée à l'origine négative pour l'asymptote).

De plus la valeur de $[\eta]$ calculée dans ce cas au moyen de l'équation (6) avec les valeurs de $F(p)$, où p est l'allongement du bâtonnet, et données par l'hydrodynamique des fluides visqueux, se trouverait trop élevée (voir fig. 17) ([9]).

c) Polydispersité des configurations :

Enfin l'étude de la viscosité nous montre encore (fig. 17) qu'il n'y a pas de corrélation entre les valeurs de $[\eta]$ et celles de M_w. En particulier, à une valeur M_w donnée (7.10^6 par exemple), on constate qu'il peut correspondre des valeurs de $[\eta]$ variant dans des proportions aussi élevées que 1 à 5.

Dans la mesure où les nombres M_w sont — même grossièrement — liés à la masse moyenne des particules, cela indique que des particules de masse approximativement identique peuvent présenter des configurations extrêmement diverses.

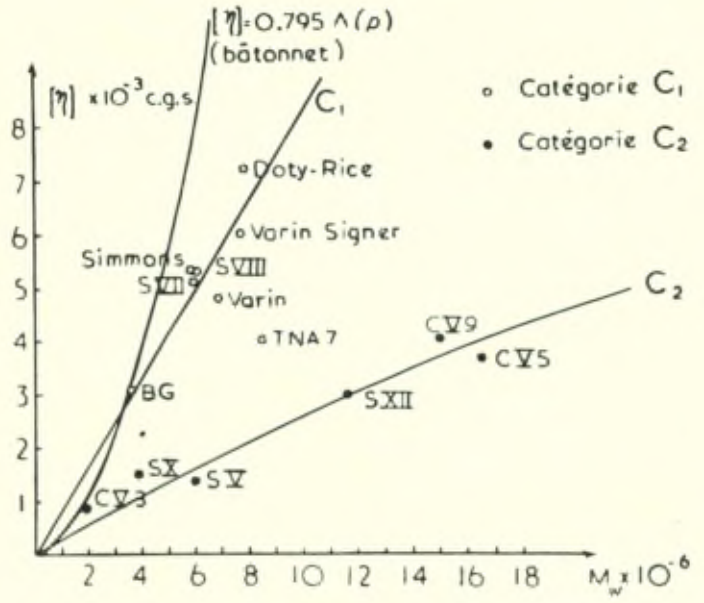


Fig. 17. — Représentation de $[\eta]$ et de M_W pour quelques échantillons. La courbe parabolique, à gauche, a été calculée pour les bâtonnets de masses différentes et de structure donnée par la double hélice. On voit qu'il y correspond des viscosités trop élevées.

La représentation montre de plus qu'il n'y a pas de corrélation nette entre $[\eta]$ et M_W, bien que les points se groupent de préférence sur deux droites extrêmes C_1 et C_2.

Ainsi donc on devrait admettre l'existence d'une double polydispersité dans les solutions de DNA :

— une polydispersité en masse, que nous n'avons pas encore mise en évidence au cours de notre exposé et dont l'existence doit être logiquement supposée a priori, et

— une polydispersité de forme : les configurations des particules sont diverses, même pour une masse donnée.

La sédimentation.

Ce n'est que récemment ([20]) que l'on est parvenu à mesurer les vitesses de sédimentation des particules de DNA. La migration de celles-ci n'a pu en effet être observée, aux très basses concentrations

nécessaires, que par la technique de visualisation par absorption de l'ultraviolet dans la bande de 2.600 Å.

Si on désigne par $f(s)\,ds$ le pourcentage de particules dont la constante de sédimentation est comprise entre s et $s + ds$, le pourcentage de particules dont la constante de sédimentation est comprise entre 0 et s est :

$$F(s) = \int_0^s f(s)\,ds$$

C'est la courbe F (s) que fournit la technique de mesure par visualisation à l'*u.v.*

De l'ensemble des observations effectuées, et qui reflètent évidemment la même dispersion que nous avons signalée dans les autres cas, on tire cependant que les courbes F (s) ont l'allure générale suivante fig. 18) même si l'échantillon a été traité à la chymotrypsine ([12]).

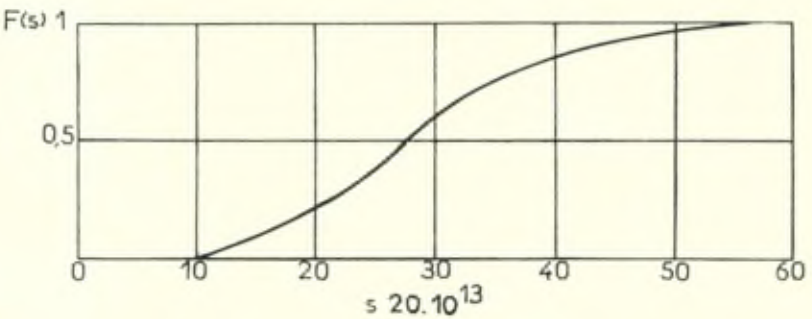


Fig. 18. — Courbe de distribution intégrale de s, comme elle apparaît en général pour les solutions de DNA.

Elles montrent sans ambiguïté que la solution contient des particules présentant des valeurs de s différentes.

Mais là s'arrêtent les conclusions précises que nous pouvons tirer de ce genre de mesure. En effet, le nombre s (voir équation 4) dépend à la fois de la masse M et du coefficient de frottement f, c'est-à-dire de la forme des configurations.

L'expérience de sédimentation seule ne peut nous dire si la polydispersité en s provient seulement d'une polydispersité de masse, ou de forme, ou des deux.

En tout cas l'existence d'une telle polydispersité nous empêche de déterminer M par la simple application de l'équation (5) à partir de la mesure de s et de A.

D'ailleurs, jusqu'ici néanmoins, les mesures du coefficient de diffusion brownienne de translation n'ont pas été systématiquement accomplies. Nous pensons combler prochainement cette lacune et tenter alors la détermination de la courbe de distribution en M à l'aide de procédés qui ont été spécialement étudiés par M. Daune ([19]).

Enfin certains auteurs ont cherché d'ingénieuses combinaisons entre s et d'autres grandeurs, comme $[\eta]$, pour en tirer des données sur la masse des particules ([20]). Par exemple certains ont trouvé, par ce moyen, que des échantillons caractérisés par la valeur $M_w = 7.10^6$ à la diffusion de la lumière avaient, avec ce système, une masse pratiquement double et égale à 14.10^6.

Sans nier l'intérêt de ces recherches nous pouvons cependant dire que toutes ces tentatives se heurtent, on le comprend bien, à une même difficulté inhérente : c'est qu'il ne semble pas y avoir de corrélation entre la configuration et la masse. Dans ces conditions nous avons plus d'inconnues que de données et l'on doit, pour aboutir, faire appel à trop d'hypothèses souvent arbitraires. De plus les nombres trouvés correspondent à des moyennes mal définies.

Nous ne pensons donc pas qu'une analyse plus détaillée de ces travaux ait sa place ici. Aussi utiles puissent-ils être pour l'élaboration des idées et des plans de travail, leurs résultats sont encore trop incertains pour que nous risquions, en les rapportant, d'alourdir un exposé déjà long et touffu

Microscopie électronique.

La description des particules de DNA, telle que nous l'avons esquissée à partir de l'analyse de certaines propriétés des solutions, est trop vague et trop incomplète pour que nous résistions au désir de la compléter par l'examen des particules au microscope électro-

nique, même si l'on peut hésiter à admettre que ce domaine entre vraiment dans le cadre de notre exposé.

En effet, le microscope électronique donne l'image de particules précipitées sur un support, tandis que les méthodes « en solution » que nous avons exposées donnent la description des particules baignées dans leur solvant.

Mais si, de ce fait, les caractères morphologiques présentés par les particules, dans ces deux états, peuvent être différents, on peut espérer qu'une étude de la photographie puisse permettre d'évaluer un ordre de grandeur de leurs masses.

1. De nombreux auteurs ont obtenu des photographies qui montrent que les particules de DNA se présentent sous la forme de longs filaments, de diamètre approximativement égal à 20 Å, ce qui correspond bien à la structure de Crick et Watson.

Ces filaments ont une forme quasi rectiligne et en tout cas très allongée.

Sur beaucoup d'entre eux on observe la présence de fourches, sans qu'il soit possible d'avancer avec certitude qu'il s'agit là de véritables ramifications et non l'effet de simple enchevêtrement de particules individuelles (fig. 19).

Cela rend fort difficile une étude statistique des longueurs des filaments, qui conduirait à une loi de distribution de leur masse.

D'après une étude approdondie faite par C. E. Hall ([21]), il semble qu'on puisse constater l'existence de filaments entiers de plusieurs microns de longueur, ayant par conséquent des masses de l'ordre de plusieurs millions.

2. Il est peut-être plus facile de tenter une étude statistique des masses particulaires en étudiant les images sphérulaires — et non plus filamenteuses — des particules de DNA.

Ces images — déjà signalées par divers auteurs ([22]), ([23]), ([24]) — ont été étudiées récemment d'une façon plus approfondie ([25]) et conduisent aux observations suivantes.

On part simplement de solutions de DNA privées au mieux de leurs ions. On peut, par exemple, dialyser complètement une solution

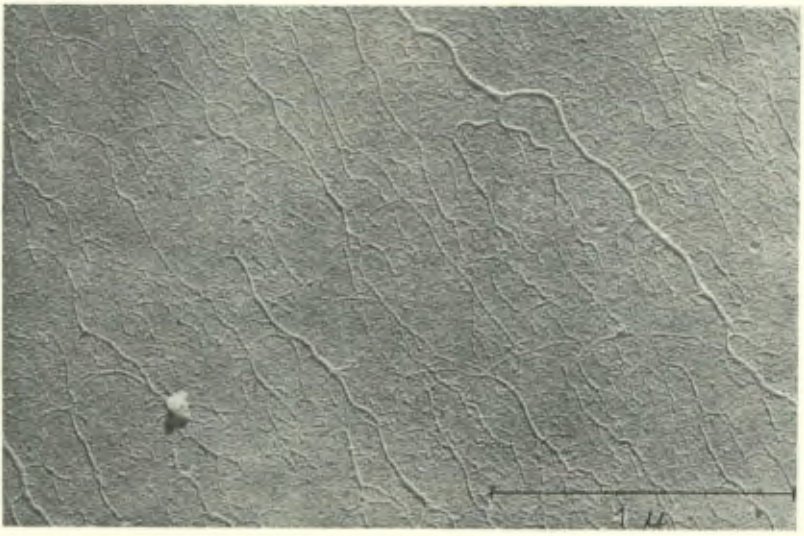


Fig. 19. — Forme filamenteuse des particules de DNA.

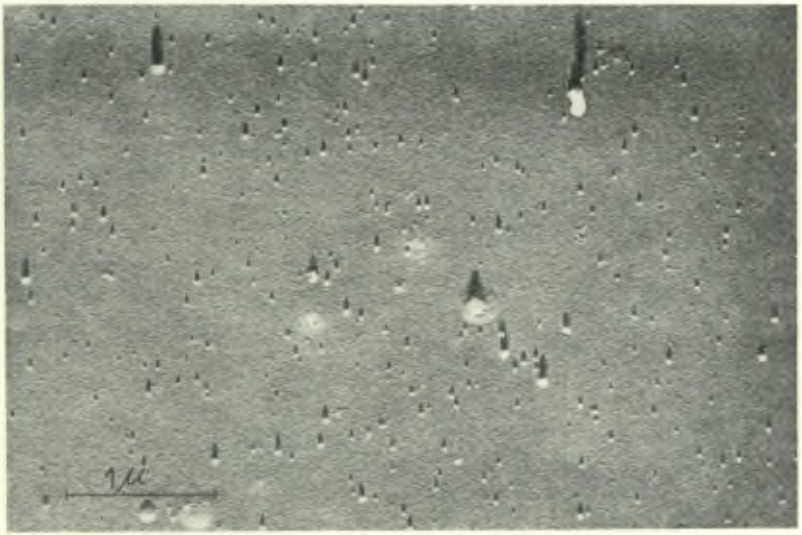


Fig. 20. — Forme globulaire des particules de DNA.

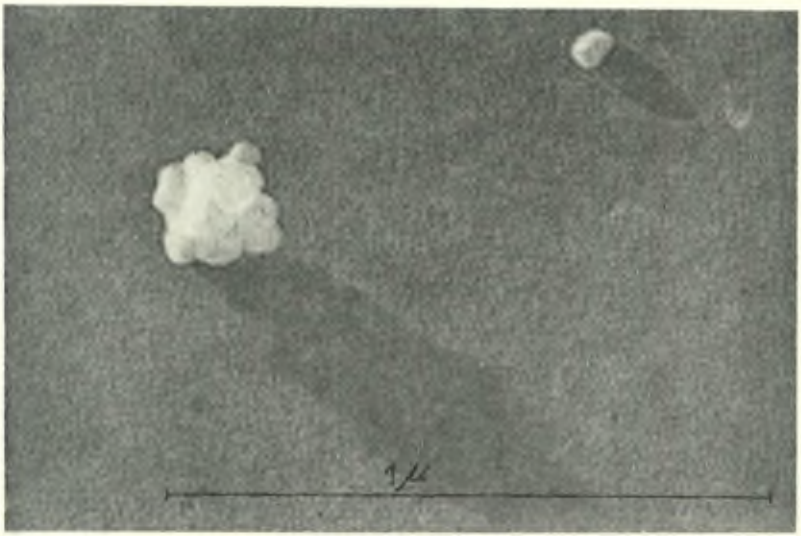


Fig. 21. — Agglomérats de particules de DNA dispersées à sec.

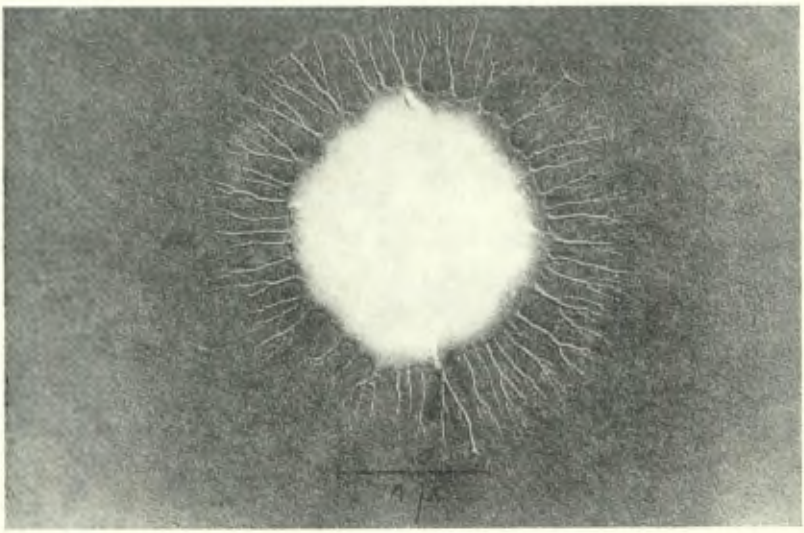


Fig. 22. — « Germination » d'un amas de DNA sous l'influence de l'humidité en présence d'ions alcalins.

de DNA, à la concentration de quelques mg par litre, de façon à extraire tout résidu de sel. Une gouttelette de cette solution déposée sur le porte objet et évaporée dans le vide donne, après traitement à la vapeur de chrome, une dispersion globulaire comme celle qui est représentée par la figure 20.

Visiblement, les sphérules obtenues sont de diamètres différents. Une mesure approximative montre que près de 30 % ont un diamètre d'environ 125 Å et 65 % un diamètre d'environ 250 Å. Des diamètres supérieurs sont exceptionnels.

Si l'on admet que la densité de la matière qui forme les particules est de l'ordre de l'unité, on calcule aisément que les masses molaires des divers glomérules sont respectivement de 6.10^5 et 5.10^6.

Bien que l'on retrouve ici pour les masses des ordres de grandeurs familiers il n'en reste pas moins que l'expérience conduit à conclure à une polydispersité de masses, si vraiment les filaments sont enroulés avec la même densité dans tous les glomérules.

Si l'on admet que les différences de volume proviennent d'une différence dans la compacité, il n'en reste pas moins qu'il existe des particules dont la masse minima est à peu près 10 fois plus petite que M_w.

Est-ce à dire que ces conclusions sont indiscutables? Peut-être pas : il n'est en effet pas absolument hors de vraisemblance que le passage du DNA en solution aqueuse étendue soit accompagné d'une dénaturation comme le montre l'étude de l'hyperchromie ([26]).

3. Il peut être intéressant de montrer comment s'effectue le passage de la forme globulaire à la forme filamenteuse.

Pour cela, les auteurs cités ([25]) partent d'une solution en eau distillée et, après plusieurs précipitations par l'alcool, obtiennent un matériau soluble dans l'alcool isopropylique à 75 %. L'addition d'une petite quantité de NaCl à la solution provoque la formation d'un fin précipité qui est recueilli et séché au dioxane.

La poudre est dispersée sur le porte-objet et traitée à la vapeur de chrome. La figure 21 montre que le DNA est rassemblé en agglomérats de sphérules. Celles-ci — à la différence de celles qui sont représentées sur la figure 20 — contiennent du chlorure de sodium.

Si on laisse alors séjourner pendant quelques minutes les glomérules — avant ombrage — dans une atmosphère humide à 25°C, on constate, après ombrage, que des filaments s'échappent du magma central comme on le voit sur la figure 22.

Ce phénomène ne se produit pas en l'absence de NaCl.

CONCLUSIONS

Puisque nous avons volontairement, dans cet exposé, examiné quelques propriétés physiques des solutions de DNA dans le but d'approfondir nos connaissances sur les particules dispersées, quels sont, en conclusion générale, les résultats qu'une étude aussi longue et parfois aussi délicate peut nous permettre de proposer?

Pour répondre il nous suffit de rassembler, avec quelque recul et quelques commentaires, les conclusions partielles éparses dans les diverses sections de notre texte.

Les masses.

D'abord une constatation peu encourageante. Si nous savons, sans risque de grossière erreur, que la masse des particules de DNA atteint des valeurs de plusieurs millions, nous n'en connaissons pas exactement la grandeur. Nous ne savons même pas encore si ces particules ont ou non une masse uniforme. Certes, les données du microscope électronique nous laissent à penser qu'il n'en est pas ainsi, mais rien n'est sûr, nous l'avons vu, une dégradation partielle pouvant se produire au cours des opérations.

Si l'on admet cependant que les données de la diffusion de la lumière conduisent, par chance, à une valeur correcte de M_w égale à 6 à 8 millions quand on a détruit les agrégats provoqués par la présence de résidus protéiques, on est frappé par le fait que les particules de DNA extraites d'organismes aussi divers qu'une bactérie ou un mammifère apparaissent identiques de ce point de vue.

Il serait tentant, alors, de supposer que tous les organismes fabriquent des particules de même masse. Sinon il faudrait admettre qu'un artefact de préparation conduise à un mélange final caractérisé

par la même valeur de M_w. Il serait alors presque obligatoire de supposer que ce mélange final est composé d'agrégats de particules de masses plus petites, mais alors de grandeur uniforme pour toutes les espèces. C'est sans doute aller un peu loin.

Il est frappant aussi de trouver que — sans l'action de la chymotrypsine — les préparations donnent au hasard, sans qu'on sache clairement pourquoi, des masses simples ou doubles. Peut-être ce fait a-t-il une signification intéressante.

Mais il est, à notre avis, imprudent de nous aventurer dans ces voies tant que des mesures indiscutablement correctes n'auront pas été faites. Par exemple, il faut construire des dispositifs permettant de mesurer les intensités de lumière diffusée à des angles θ limite beaucoup plus faibles. Des tentatives sont en cours; attendons, avant de pousser la discussion, d'en connaître les résultats. Ceux-ci montreront peut-être que tout ce que nous venons de dire n'est qu'illusion.

Les configurations.

Assez curieusement, c'est dans un domaine particulier, et en général difficile à explorer, que quelques résultats clairs ont été obtenus.

Tout d'abord, dans les solutions étendues, les configurations des particules de DNA sont des lignes construites avec une sorte de fil de fer dont la masse par unité de longueur correspond bien au schéma de la double hélice de Crick et Watson, sans qu'on puisse encore être parfaitement sûr que, par régions, cette hélice ne soit pas « détordue » en prenant l'allure d'une échelle.

Nous avons bien dit « fil-de-fer » et non pas « corde », voulant illustrer ainsi le fait que ces configurations semblent rigides. Mais toutes les particules n'ont pas la même configuration.

On pourrait comparer l'ensemble des particules de DNA en solution à une collection de morceaux de fil de fer, ayant ou non tous la même longueur, tous coudés de façon variée mais de façon que chacun dessine une ligne brisée dont les segments rectilignes auraient des dimensions bien supérieures à quelques centaines d'angströms, et dont l'allure générale serait allongée. Peut-être, parfois, certaines de ces particules comporteraient elles des ramifications.

Il serait tentant d'admettre que le long filament nucléique, dont la longueur peut atteindre plusieurs microns, serait fortement replié

sur lui même dans la nucléoprotéine dont il a été extrait, par exemple en une sorte d'accordéon. Quand on arrache la protéine l'accordéon serait distordu, ses plis plus ou moins ouverts, et prendrait les configurations variées que nous avons décrites.

Dès lors se pose la question de savoir — comme nous l'avons fait déjà remarquer ([9]) en examinant le schéma proposé par Dekker et Schachman ([27]) — si la forte pliure n'impose pas qu'à l'endroit du filament où elle se produit la double spirale soit détruite, ce qu'on peut illustrer par les schémas des figures 23 et 24.

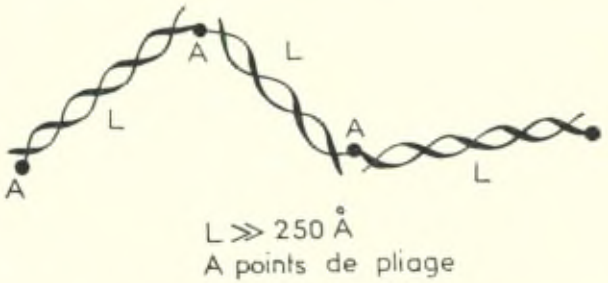


Fig. 23. — Modèle de configuration d'un filament de DNA respectant la double spirale, avec points de moindre résistance mécanique ou chimique (points A). L'échelle n'est pas respectée : le diamètre de l'hélice est égal à 20 Å et L supérieur à plusieurs centaines d'Å.

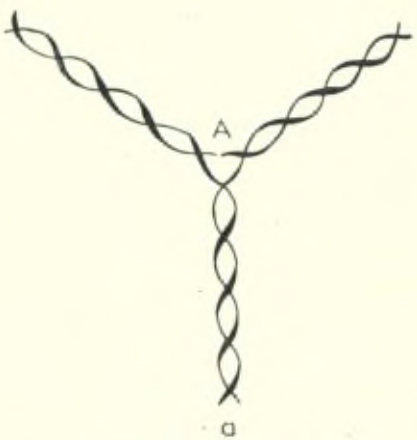


Fig. 24. — Bien que l'interprétation des mesures d'intensité lumineuse diffusée soit compatible avec le schéma de la figure précédente, elle laisse cependant supposer l'existence de ramifications. Celles-ci pourraient correspondre au schéma ci-dessus déjà proposé par Crick et Watson pour d'autres raisons.

Enfin, dans un tel schéma, les liaisons hydrogènes n'existeraient pas aux points de pliage qui se trouveraient être de ce fait des points de fragilité maxima. Peut-être serait-ce en ces sites que se produirait sélectivement la dégradation ménagée, découpant la chaîne nucléique en segments de longueur L. Ainsi pourraient s'interpréter aisément les effets de la dégradation thermique ([11]) ou sonique ([28]) qui scindent la particule en tronçons de masse molaire 300.000. Dans cette hypothèse L serait de l'ordre de 1.500 Å ce qui est compatible avec les données de la diffusion de la lumière.

Nous retrouvons donc, peut-être avec quelques précisions supplémentaires, des configurations déjà proposées par nous-même ou par d'autres auteurs tels que J. A. V. Butler par exemple.

Il est satisfaisant de voir qu'une étude plus poussée des données de la diffusion de la lumière, telle que nous l'avons résumée ci-dessus, tend à confirmer les conclusions déjà acquises en laissant espérer dans le proche avenir quelques progrès appréciables de nos connaissances.

*
* *

Peut-être sera-t-on déçu de la pauvreté de l'apport des méthodes que nous avons analysées eu égard à l'importance de problèmes tels que, par exemple, celui de l'ordre des séquences le long de la particule nucléique.

Nous pensons qu'une telle impression serait trop pessimiste.

La connaissance de la masse des particules et de leur éventuelle distribution autour d'une valeur moyenne nous semble, en elle même, fondamentale pour le biologiste. Lorsque nous saurons — ce qui ne saurait tarder — déterminer ces caractères, ils pourront — avec les caractères morphologiques essentiellement — servir à identifier les différences éventuelles et significatives qui peuvent exister entre les particules de DNA provenant des diverses espèces d'être vivants, suivre la formation du DNA dans la vie cellulaire, et chercher à voir dans quelle mesure des modifications du DNA accompagnent ou non certains états pathologiques. Ils pourront aussi nous permettre de mieux saisir l'effet que certains agents physiques, chimiques ou biologiques exercent sur la particule de DNA, qu'il s'agisse de déna-

turation, de dégradation ou d'association. Enfin, de toute manière, ils sont nécessaires à la parfaite connaissance des structures in vivo au sein desquelles le DNA se trouve engagé.

Nous pensons donc qu'en dépit de son aridité, que la lourdeur de notre exposé rend encore plus manifeste, ce sujet de biologie macromoléculaire mérite qu'on s'y attache avec fermeté et obstination.

Je tiens à remercier mes collaborateurs du Centre de Recherches sur les Macromolécules, tels que MM. H. Benoît, V. Luzzati, J. Pouyet et R. Vendrely, de l'aide précieuse qu'ils ont bien voulu m'apporter dans la rédaction de ce travail.

BIBLIOGRAPHIE

On aura intérêt à consulter les ouvrages ou articles généraux suivants :

Diffusion de la lumière	Light Scattering in Physical Chemistry by K. A. Stacey, Butterworths Scientific Publ. London, 1956.
Ultracentrifugation	J. W. Williams, K. E. van Holde, R. L. Baldwin, H. Fujita, *Chem. Rev.* **58**, p. 716 (1958).
Méthodes générales de détermination des formes et des dimensions des macromolécules en solution.	Ch. Sadron, *Progress in Biophysics*, **3**, p. 237 (1953).
Etude des particules de DNA en solution	Ch. Sadron, J. Pouyet, Proc. of the IVth International Congress of Biochem, Vienna (1958). P. Doty, Proc. of the IIIrd Intern. Congress of Biochemistry, Brussels (1955). K. V. Shooter, Progress in Biophys. **8**, p. 310 (1957). J. Hermans, Thèse, Leyde (1958).

(1) H. Benoît : *C. R. Ac. Sc. Paris*, **245**, p. 2244 (1957).
(2) A. R. Stokes :*Proc. Phys. Soc.*, **70 B**, p. 379 (1957).
(3) P. Horn, H. Benoît : *J. Pol. Sc.*, **10**, p. 29 (1953).
(4) H. Benoit : *J. Pol. Sc.*, **11**, p. 507 (1953).
(5) A. Holtzer : *J. Pol. Sc.*, **17**, p. 432 (1955).
(6) O. Kratky, G. Porod : *J. Coll. Sc.*, **4**, p. 35 (1949).
(7) J. Pouyet : non publié.
(8) J. Hermans : Thèse, Leyde (1958).
(9) C. Sadron : Proc. of the Third International Congress of Biochemistry, Brussels (1955), p. 125.
(10) C. Sadron, J. Pouyet, R. Vendrely : *Nature*, **179**, p. 263 (1957)
(11) A. M. Freund, J. Pouyet, C. Sadron : *C. R. Ac. Sc. Paris*, **246**, p. 1306 (1957).
(12) J. A. V. Butler, D. H. Philipps, K. V. Shooter : *Arch. Biochem. Biophys.*, **71**, p. 423 (1957).
(13) H. Benoit, non publié.
(14) H. Eisenberg, J. Pouyet : *J. Pol. Sc.*, **13**, p. 85 (1954).
(15) J. Pouyet : *C. R. Ac. Sc. Paris*, **234**, p. 152 (1952).
(16) B. E. Conway, J. A. V. Bulter : *J. Pol. Sc.*, **12**, p. 199 (1954).
(17) H. Eisenberg : *J. Pol. Sc.*, **25**, p. 257 (1957).
(18) J. Pouyet : *J. Chim. Phys.*, **48**, p. 1 (1951).
(19) M. Daune, Thèse, Strasbourg (1958).
(20) K.V. Schooter and J.A.V. Butler, *Trans. Far. Soc.* **52**, p. 734 (1956). J. A. V. Butler : Symposium No. IX, IVth Intern. Cong. of Biochem. Vienna (1958). Voir aussi : J.A.V. Butler, D. J.R. Laurence, A.B. Robins et K.V. Shooter, *Proc. Roy. Soc.* A 250, p. 1 (1959).
(21) Voir par ex. C. E. Hall : Symposium IX, IVth Intern. Congr. of Biochemistry, Vienna (1958).
(22) S. T. Bayley : *Nature*, **168**, p. 470 (1951).
(23) J. Liquier - Milward : *Bioch. Biophys. Acta*, **10**, p. 5 (1953).
(24) C. E. Hall : *J. Biophys. Biochem. Cytol.*, **2**, p. 625 (1955).
(25) R. Vendrely, C. Vendrely, C. Sadron : *Exp. Cell. Res.*, **15**, p. 222 (1958).
(26) R. Thomas : *Bioch. Biophys. Acta*, **14**, p. 231 (1954).
(27) C. A. Dekker et H. K. Schachman : *Proc. Nat. Ac. Sc. Washington*, **40**, p. 894 (1954).
(28) P. Doty, B. Bunce, S. A. Rice : *Proc. of the Nat. Ac. Sc. Washington*, **44**, p. 432 (1958).

DISCUSSION DU RAPPORT DE M. SADRON

M. Kuhn. — Three remarks are made, not with the intention to criticise but with the intention to emphasise some points.

a) The first remark concerns a question of terminology : we should carefully distinguish between *rigidity* and *degree of coiling*. The degree of coiling can be expressed by the ratio of the total length L of the stretched filament and the actual distance of the two ends of the filament in the statistical coil. By the degree of coiling nothing is said about the rigidity of the coil. Strong coiling of the filament (small distance of the two ends) does not necessarily mean that the coil is made of a soft material; also an iron wire can be strongly coiled. In the case of chain molecules the degree of coiling is an affair of the mean statistical equilibrium shape. The rigidity on the other hand is to be measured as the force the molecule opposes if by some means a swift change of the shape of the molecule is attempted.

b) By the second remark we wish to recommend, besides light scattering, the use of hydrodynamic observations, in particular of viscosity and, in addition to it, the value of the birefringence and the orientation angle of the birefringence of flow. If the results of the relevant experiments are expressed in terms of the quantities :

$$\left[\eta\right] = \left[\frac{\eta_i - \eta_{i0}}{\eta_{i0}\, c}\right]_{\substack{q=0 \\ c=0}}$$

$$\left[n\right] = \left[\frac{n_1 - n_2}{q\eta_{i0}\, c}\right]_{\substack{q=0 \\ c=0}}$$

$$\left[\omega\right] = \left[\frac{\omega}{q\eta_{i0}}\right]_{q=0}$$

where $n_1 - n_2$ is the observed value of the birefringence, q the velocity gradient in the solution, ω the angle of orientation of the birefringence (see fig. 1) we would, for a suspension of straight cylindrical rods of length S, radius r and density ρ, i.e. of mass : $M = \pi r^2 S \cdot \rho$; approximately have :

$$\left[\eta\right] \simeq \frac{1}{48\rho}\frac{S^2}{r^2} = \frac{\pi\, S^3}{48M} \qquad (1)$$

W. Kuhn, Intervention 2, 6 (1959) (Sadron).

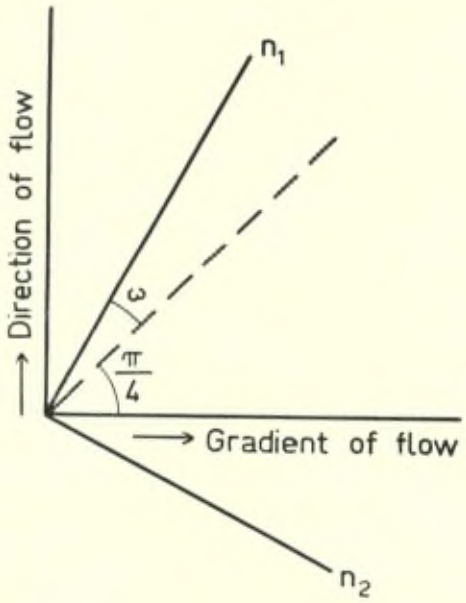


Fig. 1. — Definition of the orientation angle of the birefringence of flow. The vectors n_1 and n_2 indicate the direction in which the main refractive indices n_1 and n_2 are observed.

$$[n] = \frac{(n_0^2+2)^2}{6n_0}\,\frac{4\pi}{3}\left(\gamma_1-\gamma_2\right)\frac{1}{kT}\,\frac{1}{120\rho}\,\frac{S^2}{r^2}$$

$$= \frac{(n_0^2+2)^2}{6n_0}\,\frac{4\pi^2}{3}\,\frac{\gamma_1-\gamma_2}{120kT}\,\frac{S^3}{M} \qquad (2)$$

$$[\omega] = \frac{\pi S^3}{96kT} \qquad (3)$$

n_0 is the index of refraction of the embedding medium and $\gamma_1 - \gamma_2$ the difference of the polarisabilities of the rod parallel and perpendicular to the axis.

Corresponding equations can be given for suspensions containing ellipsoids or statistically coiled chain molecules (1). It will for

(1) For a review and references see W. Kuhn, H. Kuhn and P. Buchner, *Ergebn. exakt. Naturwiss.*, **25**, 1-108 (1951).

example be noticed that the orientation number according to equation (3) depends on the length of the rod only. The inspection of the corresponding equation valid for ellipsoids and statistically coiled chain molecules would show that $[\omega]$ indeed practically exclusively depends on the value of the long axis of the ellipsoid or statistical coil, the exact expression being rather insensitive with respect to the thickness of the particle. Equation (3) (or the corresponding more exact formulae) give therefore a possibility to determine the length S of the particle, if it is approximated by a rod or an ellipsoid in a simple and sensitive way and in a way which is not much dependent on assumptions concerning other parameters characterizing the shape.

It will furthermore be noticed that forming the quotient of equation (1) and (3) an expression is obtained for the mass M of the particle again free (and with the more exact expressions nearly free) of further parameters.

A good knowledge of the long axis of the particle is valuable for many considerations.

Dividing equation (2) by équation (1) gives $\gamma_1 - \gamma_2$ in the case of the rod-like particle. In the case of the statistical coil $\alpha_1 - \alpha_2$ (the difference of the polarisabilities of the statistical chain element parallel and perpendicular to the axis of the chain element) is practically given by the same quotient (2). From the value of the quotient of equation (2) and (1) therefore information will be obtained in many cases concerning the coiling or relative straightness of the particle, especially when the birefringence of the pure 100 % oriented substance is known.

c) Information about the rigidity of the particle can be obtained if the magnitudes listed in formulae (1), (2) and (3) are measured in the case of higher values of the velocity gradient q. For example if $[n]$ (see équation 2) is plotted as a function of q, an increase which is more than proportional to q is obtained for soft particles (fig. 2, curve 1) and an increase which is less than proportional to q for rigid particles.

The intermediate case of moderately rigid particles has recently been solved by W. Kuhn and P. Buchner (3). The difference between

(2) See reference 1), especially pp. 73 and 75.
(3) P. Buchner, Diss. Univ. of Basel (1958).

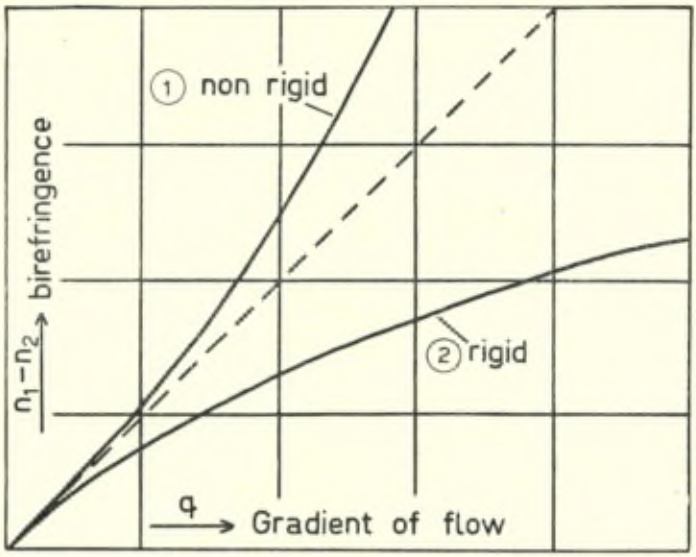


Fig. 2. — Value of the birefringence of flow as a function of the velocity gradient, q, for dilute solutions: (1) for non-rigid statistically coiled chain molecules and (2) for rigid geometrically anisotropic particles. For more details see W. Kuhn, H. Kuhn and P. Buchner, *Ergebn. exakt. Naturwiss.*, **25**, 1-108 (1951).

curve 1 and 2 of figures 2 is due to the circumstance that soft particles are, at high values of the velocity gradients, partly uncoiled and thereby more birefringent.

M. Champetier. — Quelle est l'influence de la nature du milieu de dispersion sur la rigidité des macromolécules d'ADN? Il y aurait sans doute intérêt à substituer aux solutions de NaCl des solutions salines ayant un effet désassociant sur les liaisons hydrogène (par exemple solution de LiBr ou de $(CNS)_2Ca$). Peut-être observerait-on une séparation des deux hélices formant la macromolécule d'ADN et le passage d'une forme macromoléculaire rigide à une forme plus souple?

En tous cas, la formation des agrégats doit dépendre de l'influence plus ou moins désassociante du sel sur les interactions hydrogène si celles-ci jouent un rôle dans ce phénomène.

M. Sadron. — Je n'ai présentement pas à l'esprit les travaux qui ont pu être faits dans l'ordre d'idées proposé par M. Champetier.

Il me semble me souvenir que l'action des sels de lithium, et notamment du LiBr a été examinée et a conduit à des conclusions intéressantes concernant la dénaturation du DNA. Mais je ne puis, sans consulter mes références, donner plus de précisions.

M. Fraenkel-Conrat. — The significance of the smallest molecular units seen on electron-micrograms was discussed. Since it is assumed that exposure to low ionic strength does not cause the breakage of primary bonds, while possibly being able to cause a dissociation of two hydrogen-bonded strands, the smallest observed unit must actually mean that the molecular weight is maximally double that observed. Thus particles of a mass or 600 000 would signify that the molecular weight of DNA may be as low as 0,6 to 1,2 $\times$ 10^6. This would not necessarily be so, if one assumes breaks in the polynucleotide chain, as suggested by Schachman and Dekker.

M. Sadron. — I think that Dr. Fraenkel-Conrat's remark is very interesting, being granted that it is perfectly established that only secondary bonds are broken at low ionic strengths.

M. Kuhn. — It is comparatively easy to tell the effect of different possible ways of molecular association on : $(\eta - \eta_0)/\eta_0 c = [\eta]$.

a) if two filaments of rod-like shape are connected so as to form a straight rod of twice the length of each of the original rods (end-to-end-connection), $[\eta]$ will be increased by a factor 4 ;

b) if two filaments each forming a statistical coil are similarly connected to form a filament of twice the length of each original filament, the latter having again the shape of a statistical coil, $[\eta]$ will be increased by a factor 2 approximately (1) ;

c) if two filaments of rodlike or of statistical shape are associating by arranging themselves parallel to each other over the whole of their lenght, $[\eta]$ will decrease to half original value.

M. Sadron. — I quite agree with the consideration given by Prof. W. Kuhn, except perhaps for the value of the change of vis-

(1) (Communiqué après la réunion.) The factor would be exactly equal to 2 for perfectly draining and to $\sqrt{2}$ for non-draining statistical coils. I perfectly agree therefore with the remark made by Prof. Sadron in response to my statement. In the case of DNA, the coils, if they exist, will be extremely loose and therefore completely draining, causing a factor 2, or for uncomplete coiling greater than 2.

cosity of the end-to-end association of two coiled particles; ($[\eta]$ should be increased by a factor probably slightly less than 2). However it has to be reminded that other experiments could be usefully made. Specially, it has been shown in my paper that light scattering investigations were giving the value of the particle weight per unit length. It has been found that, for chymotrypsin treated samples, M/L is of the order of 220 to 240 (molar mass/Å), that is to say compatible with the Watson and Crick double helix. For untreated samples M/L depends on the NaCl concentration (fig. 13). It is maximum for a solution 0.15 M NaCl that is to say at the minimum of solubility of nucleoprotein, and decreases to the theoretical value for solutions NaCl M. This quite general behaviour strongly suggests that there is a side to side association of double helices, at least along a certain length — if not the total length — of these helices. We have several times observed — in certain untreated samples — that this association could be much more important and could concern up to ten double-helices. The action of chymotrypsin is to cut the bridges which make the double helices stick together, and one than observes the regular dispersion of single double-helices with the right value of 220 to 240 for M/L.

M. Thomas. — La question de M. Champetier et les allusions, qui viennent d'être faites, à l'existence d'agrégats de molécules d'ADN, m'incitent à rappeler quelques résultats concernant l'action de l'urée sur les ADN.

1) L'action, même prolongée, de l'urée 6M à la température ordinaire ne suffit pas à induire la dénaturation des ADN (Thomas, 1954; critère de la dénaturation : accroissement irréversible de l'absorption ultraviolette), et n'altère pas l'activité génétique de l'ADN transformant de Pneumocoque;

2) Ces conditions dissocient les agrégats de molécules d'ADN, comme le montrent les observations suivantes (Ephrussi-Taylor, Latarjet et Thomas). Certaines préparations d'ADN transformant de Pneumocoque sont inactivées par les rayons X selon une courbe à plusieurs coups, par suite, de la présence d'agrégats; ces mêmes préparations fournissent une courbe d'inactivation à un coup après traitement à l'urée.

M. Champetier. — Ce sont justement les intéressantes observations faites avec l'urée qui m'ont incité à poser ma question.

L'emploi de solutions salines diverses comme milieu de dispersion de l'ADN permettrait sans doute de vérifier ce point important sur la solidité de l'édifice à double hélice et sur le rôle des interactions hydrogène dans la formation des agrégats.

D'autre part, l'hydratation des macromolécules hydrophiles dépend de la nature de la solution saline employée comme milieu de dispersion ainsi que l'a montré l'un de mes collaborateurs, M. Neel, dans le cas de composés macromoléculaires synthétiques ou naturels (alcool polyvinylique, protéines). L'emploi de solutions salines diverses pourrait donner des renseignements intéressants sur la configuration et la rigidité des macromolécules d'ADN en fonction de leur degré d'hydratation.

M. Desreux. — Les mesures physiques relatives aux solutions d'ADN doivent être extrapolées à concentration zéro pour être significatives; d'autre part on se base sur la viscosité de ces solutions à force ionique très faible pour adopter ou rejeter le modèle du polyélectrolyte. Les faits suivants montrent que des mesures à concentration très faible en ADN et à force ionique faible sont difficiles à interpréter à des pH inférieurs à 5 (A.Oth). L'analyse à l'ultracentrifugation de solutions d'ADN à des concentrations égales ou inférieures à 0,05 g/100 ml et de force ionique 0,005-0,0005, révèle entre pH 5 et 4 la présence de deux constituants. La quantité du constituant lent augmente lorsqu'on abaisse le pH, la force ionique ou la concentration en ADN. A pH 5 on n'observe qu'un constituant A et à pH 4 le constituant B. D'autre part la viscosité à gradient zéro diminue considérablement lorsque le pH passe de 5 à 4 dans ces conditions. Il y a donc transformation d'ADN "natif" A en ADN "dénaturé" B. Les mesures de S et de $[\eta]$ semblent en effet montrer que le P.M. ne change pas mais que cette transformation consiste en une modification de configuration. La présence des deux constituants peut encore être mise en évidence par chromatographie sur cellulose; aux pH 3 et 5 on observe un constituant mais deux constituants apparaissent entre ces limites de pH. D'un point de vue pratique, il est donc dangereux au cours de préparation ou de manipulation de dissoudre l'ADN dans des solutions de faible force ionique ou de pH inférieurs à 5 à moins que la concentration en ADN soit assez élevée.

Il faut d'autre part soigneusement vérifier le pH des solutions diluées d'ADN à faible force ionique, les échantillons étant souvent

incomplètement neutralisés et abaissant de ce fait le pH en dessous de 5 lors de la dissolution.

M. Sadron. — Je crois qu'il faut distinguer deux actions : celle de la force ionique, celle du pH.

En ce qui concerne la force ionique il est bien connu que pour les concentrations salines très basses signalées par M. Desreux, l'ADN subit une modification qui se manifeste par une diminution de l'hypochromie. C'est pourquoi l'on doit sans doute suspecter l'extrapolation de la courbe $10^{-5,5}$ M du graphique de la figure 7*a*. Mais cela ne change rien aux conclusions qui ont été par la suite confirmées par Butler et Conway ainsi que par Eisenberg.

En ce qui concerne le pH on sait, depuis Gulland, que l'ADN subit une modification — probablement par rupture des liaisons hydrogène — à pH inférieurs à 5 ou supérieurs à 11. Par contre, l'observation faite par Desreux de l'existence de deux constituants chromatographiques dans l'ADN traité à pH < 5 est très intéressante. Elle mérite à notre opinion d'être étudiée de près.

M. Ubbelohde. — It seems important to point out that various contributors to the discussion are not using terms such as "denaturation", " degradation ", " dissociation " with quite the same meaning for any one of these terms. Ideally, it would be desirable for the various scientific disciplines to use the same terms. For example, dissociation might be used to imply reversible splitting up, and degradation irreversible splitting up of a macromolecule, whereas denaturation could be used to imply permanent changes of structure not involving any split up or aggregation. I do not suggest that these are the actual usages of any one of the scientists present at this conference, but wish to point out that different scientists appear to have different usages of the same term. Such confusion makes discussion of an already complex subject even more difficult. As an interim measure, even if the different scientists cannot at once conveniently agree on a truly common terminology, they should specify what they mean by any term which may involve some doubt concerning their usage of it.

M. Desreux. — La transformation observée entre pH 5 et 4 est interprétée sur la base d'une modification de configuration et non

par un changement de poids moléculaire. La transformation est partiellement réversible en ce sens que la viscosité d'une solution à pH 4 ramenée à pH 7 augmente rapidement jusqu'à 50 % de la valeur initiale; elle augmente ensuite plus lentement pendant plusieurs jours jusqu'à atteindre 75 % de cette valeur initiale après trois semaines.

L'ADN ainsi reformé ne semble cependant pas être strictement identique au produit de départ. Par dénaturation nous entendons donc en ce cas, une transformation impliquant une modification partiellement réversible de la structure secondaire des molécules, le poids moléculaire étant supposé inchangé.

Sir Alexander Todd. — The term " denaturation " is used much too loosely. To say it is a change in which only secondary linkages are broken without change in molecular weight and which may be reversible or irreversible seems peculiarly dangerous. We don't know what secondary linkages are involved and we don't know exactly what is meant by molecular weight in this connection. Particle weight and true molecular weight in the ordinary chemical sense seem to be frequently confused.

M. Fraenkel-Conrat. — It is suggested that the term " denaturation " be reserved for changes in secondary and/or tertiary structure, not accompanied by any change in covalents bonds.

M. Rich. — One must consider the use of the word " denaturation " in working with the hemoglobin protein. It has been taken to describe the process whereby the molecule splits into two-halves, without breaking any covalent bonds. In this case, the reaction is reversible and it is described as " a reversible denaturation ". However, the particle weight has changed.

M. Wilkins. — I think most people would agree that denaturation could be defined as change in chain (e.g. polynucleotide or plypeptide) configuration associated with breakage or alteration of secondary linkages e.g. hydrogen bonds. If a macromolecule is composed of sub-units joined to each other by secondary bonds, denaturation might also give rise to reduction in molecular or particle weight.

M. Butler. — I should like to offer a definition : that denaturation is an important change of the molecule involving a destruction of the secondary structure.

M. Duchesne. — Il me paraît que, méthodologiquement, il ne convient pas d'espérer actuellement une définition très précise de ce concept. Il est en effet, impossible de lui donner un contenu qui dépasse notre connaissance de la structure et de la nature des liaisons chimiques des acides nucléiques. Celle-ci, bien que déjà très poussée, si l'on tient compte de la complexité des systèmes, est encore dans ses premiers débuts, par comparaison avec les systèmes simples. Je songe, par exemple, à deux questions fondamentales :

1) comment se remanie la structure électronique de la molécule lors d'une scission d'un nombre de liaisons " hydrogène " intermoléculaires ?

2) quelle est la nature des liaisons intermoléculaires dans lesquelles les molécules d'eau semblent intervenir ? S'agit-il également de liaisons " hydrogène " ?

Il est capital de pouvoir répondre à ces questions si l'on veut pouvoir comprendre, structuralement parlant, l'évolution de ces systèmes et, par conséquent, concrétiser davantage le sens du mot " dénaturation ", selon la définition proposée par le Prof. Butler.

M. Overbeek. — It has been pointed out that the use of high ionic strength diminishes the interaction between particles. This is quite correct and is caused by a decrease of the size of the ionic atmospheres. However, at high ionic strength the interaction from the individual particles with the small ions is by no means abolished. In light scattering measurements for instance, the adsorption (positive or negative) of salt molecules to the macromolecule, if not taken into account, may falsify the molecular weight, even after extrapolation to zero concentration of macromolecules. If the effect is purely electrostatic (repulsion of negative ions by the polymeric ion) it is of the order of 10 % for NaCl and more for more highly refractive electrolytes, like bromides or thiocyanates. The size of the correction can be accurately determined from the salt distribution in the corresponding Donnan equilibrium (Overbeek and Vrij, to be published).

A similar correction should be made to the molecular weight as determined from sedimentation and diffusion.

M. Sadron. — I have had the great advantage to be informed previously by Dr. Overbeek and his collaborators about the effect of heterogeneity in the distribution of small ions near a large polyelectrolyte particles on the turbidity. This effect is somewhat comparable to the observed effect in measuring molecular weights of organic high polymers in a mixture of two solvents, where one of them can be selectively adsorbed on the polymer. In this case we do not obtain the right molecular weight by light scattering. However in the case of DNA, I have to stress that the precision of our determination of M is still too poor to make us able to see if the measurements give results depending or not from the NaCl concentration in the solution. In fact, I have shown in my report that such a dependence is existing but that it disappears when the DNA samples have been treated by chymotrypsin. After such a treatment, the light scattering results do not depend on a NaCl concentration (fig. 9, curve III). However not clear conclusions can be given before we have complete trust in the determination of M.

M. Wilkins. — Peacocke showed that evidence for the existence of secondary phosphoryl groups in DNA, based on electrotitrimetric or dye-binding studies, was not valid. Also, as Prof. Butler states in his paper, the evidence given by Dekker and Schachman in support of their idea for the structure of the DNA molecule has been showed to be unsound. I think, therefore, it is true to say that there is no evidence of breaks in the phosphate-ester chains in DNA molecules. I understand that a study has been made in Prof. Doty's laboratory of the effect of enzymatically-produced breaks on the thermal denaturation of DNA. The conclusion was that the number of breaks in native DNA molecules was very small and much less than is indicated in figures 23 and 24 of Prof. Sadron's paper.

M. Sadron. — I agree with Dr. Wilkins that there seems not to be any evidence of the existence of breaks in the phosphate-ester chain in DNA molecules as Dekker and Schachman have suggested it.

Our argument was that these breaks could explain the ability of the DNA double strand to be very strongly curved at different points, as it is suggested by light scattering properties of the solutions.

However this does not prove, of course, that these breaks are

actually taking place. Other types of local dislocations could be assumed (see Discussion Générale, p. 3).

Now I want to stress that — as I have indicated in the legend — the proportions are not respected in figures 23 and 24 of my paper. It is therefore impossible — as Dr. Wilkins does — to derive any conclusions from these schemes as to the number of breaks per molecule, which can be of a few units only.

General commentary on the question of " denaturation ".

The question raised by Prof. Ubbelohde is of a wide interest as it appears from the number of interventions.

(Ubbelohde, Desreux, Todd, Fraenkel-Conrat, Rich, Wilkins, Duchesne, Butler).

It seems that the opinions could be divided into two categories :

Category 1 : denaturation means a change in the particle without a change of its weight.

Category 2 : denaturation means a change brought by the disruption or alteration of secondary bonds, with or without a change in the particle weight.

These two definitions are flatly in opposition since, in the case of haemoglobin for example, we have no denaturation in the first class of definitions, and denaturation in the second one. Then a choice has to be decided between the two definitions. In concordance with Sir Alexander's opinion, it could be thought that the second definition is a dangerous one. In many cases, for instance, we do not know the structure of the particle with enough accuracy to be able to decide if an observed decrease of weight is due to the disruption of only secondary — and not primary — bonds. In these cases, however, it could be useful to describe the experimental situation by using one word : degradation or denaturation. From this point of view the first type of definition could be used, and not the second. Then, if we have later on a better notion of the particle structure, we could indicate more precisely the mechanism of denaturation or degradation by saying for instance :
we observe a degradation with rupture of covalent (or, of secondary) bonds (or both types of bonds) and the same explanation for denaturation.

As a summary the first type of definition seems better to me because it describes clearly an experimental fact without attempting, in the same time, an interpretation which could be sometimes premature.

M. Thomas (communiqué après la réunion). — Etant responsable dans une large mesure de l'extension aux ADN de la notion de dénaturation (voir principalement *Biochim. Biophys. Acta*, **14**, 231, 1954), je voudrais préciser quelque peu la nature du phénomène. Divers traitements doux — dont on sait qu'ils sont sans effet sur la liaison fondamentale (phosphodiester) des chaînes polynucléotidiques de l'ADN — entraînent l'altération simultanée d'un ensemble de propriétés physiques, chimiques et biologiques de l'ADN (effet hyperchrome, modification de l'affinité pour les colorants basiques, hystérésis de la courbe d'électrotitration, chute de la viscosité, disparition de l'activité transformante...). Pour éviter un néologisme inutile, j'ai appliqué à cette altération de la structure des particules d'ADN le terme de dénaturation, utilisé depuis longtemps dans le cas des protéines. L'étude de l'effet hyperchrome a montré de manière particulièrement claire que la dénaturation des ADN consiste en l'écroulement d'une configuration (" structure secondaire ") maintenue par des interactions entre les bases azotées. Depuis, le modèle de Watson et Crick a permis de visualiser la dénaturation comme la destruction de la structure régulière en double hélice, due à la rupture des ponts d'hydrogène entre les bases complémentaires.

Les définitions de la dénaturation faisant intervenir les notions de constance de la masse des particules, d'irréversibilité ou d'attaque de liaisons secondaires seulement, viennent d'être critiquées. Il me semble possible d'échapper à beaucoup d'objections en appliquant le terme de dénaturation aux modifications de la configuration d'une macromolécule, dues à *la rupture d'un ensemble de liaisons autres que la liaison covalente fondamentale* (phosphodiester pour l'ADN, peptidique pour les protéines) *qui assure la continuité des chaînes polynucléotidiques* (ADN) *ou polypeptidiques* (protéines). Ainsi définie, l'altération :

— peut entraîner ou non une chute de la masse des particules (cf. le cas de l'hémoglobine, soulevé par le Dr. Rich);

— peut être irréversible (ADN) ou réversible (homopolynucléotides associés, certaines protéines);

— concerne en général des forces de valence secondaires, mais pas nécessairement (rupture de ponts disulfures des protéines).

AVIS AU LECTEUR. — Les propriétés physico-chimiques des solutions de nucléoprotéines ont fait l'objet d'importantes discussions complémentaires que le lecteur trouvera au chapitre de la " Discussion générale ".

HETEROGENEITY OF NUCLEIC ACIDS AND EFFECTS OF CHEMICAL AND PHYSICAL AGENTS

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1. HETEROGENEITY OF DNA

Since it will be necessary eventually to determine the order of the bases along DNA fibres, in the first instance we must have a means of ascertaining whether all the DNA particles in a preparation are of the same kind. No doubt some preparations, e.g., from bacteriophage, may be expected to be much simpler than DNA from animal cells, because the information to be carried by them is much less extensive. When many genes are present, the DNA must be expected to be correspondingly complicated and the problem of distinguishing individual kinds from each other will be formidable. Since the DNA particles are, so far as has been observed, apparently very similar in general structure, being long double stranded fibres, the distinction of types differing in base composition at different points along the fibre becomes correspondingly difficult. However, a number of processes have been found which permit of some degree of fractionation.

A. Physical fractionation procedures.

When DNA is examined at low concentrations in the ultracentrifuge with ultra-violet optics, a wide range of sedimentation coefficients is observed (1). For one solution the curve showing

the distribution of sedimentation coefficients is quite reproducible (fig. 1) and as a rule very similar curves are obtained from one preparation made up in different ways. It has been shown that the distribution of sedimentation coefficients is a real property of different

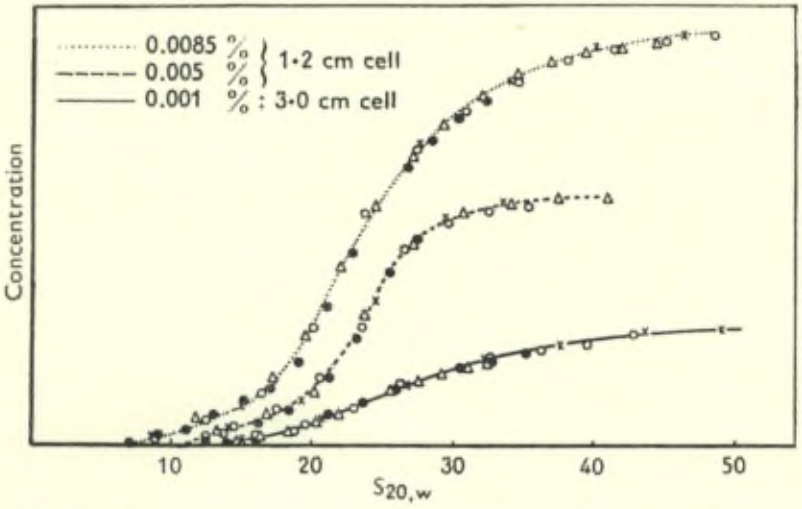

Fig. 1. — Integral sedimentation coefficient curves of calf thymus DNA at different concentrations. (The different symbols refer to different times of sedimentation.) (From Shooter and Butler, *Trans. Faraday Soc.*)

particles, by sedimenting that part of the preparation having higher sedimentation coefficients (2). When this fraction is redissolved and examined, it is found that the mean sedimentation coefficients observed are higher than those of the original solution (fig. 2). The sedimentation coefficient is also related to the physical dimensions of the particle. It has been found that over a large number of preparations of DNA from different sources (fig. 3) there is a fairly good correlation between the mean value of S and the intrinsic vsicosity at low shear rates ($S \propto [\eta]^{1/3}$) (3). For a range of sonically degraded DNA's, it was found by Doty that a relationship between the molecular weight and S exists, viz. $M \propto S^{0.37}$ (4). When the relation $S \propto [\eta]^{1/3}$ is inserted into the Flory and Mandelkern relation (5)

$$\frac{K S_0 [\eta]^{1/3}}{M^{2/3}} = \beta$$

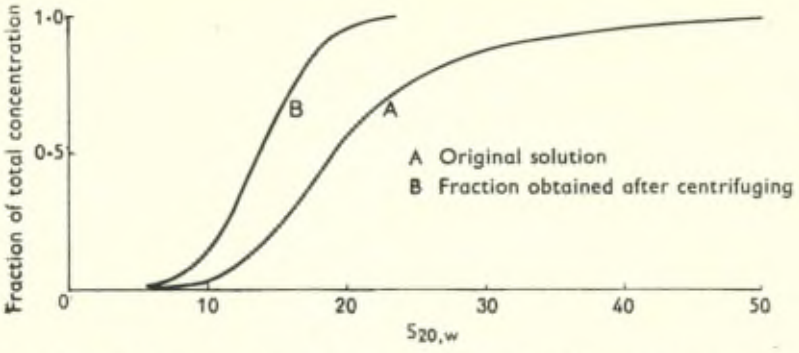

Fig. 2. — Fractionation of calf thymus DNA by centrifuging. (From Butler and Shooter, *Chemical Basis of Heredity*, 1957.)

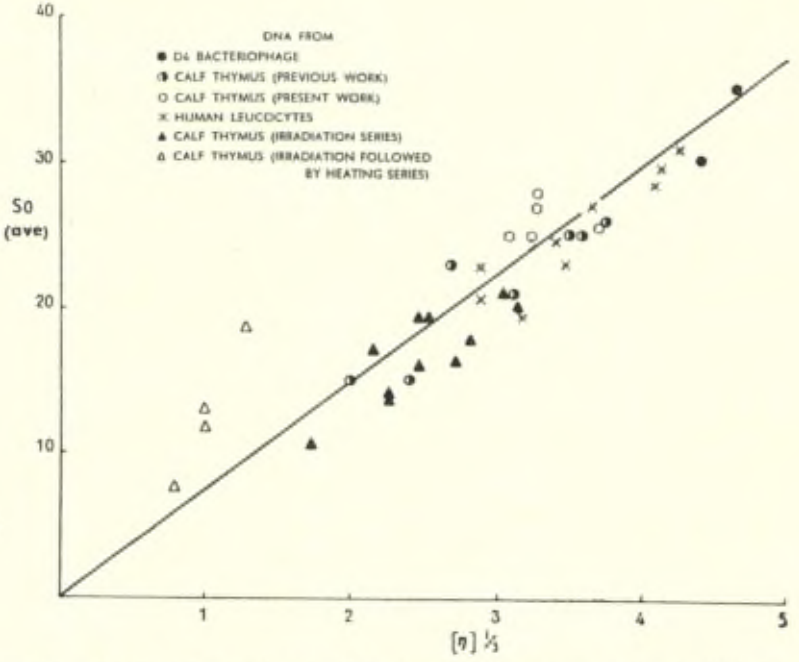

Fig. 3. — Mean sedimentation coefficients and intrinsic viscosities of DNA in aqueous solutions. (From Butler, Laurence, Robins and Shooter, *Proc. Royal Society*, 1959.)

where β is a constant for a particular shape or configuration we obtain :

$$[\eta] \propto M \text{ and } S \propto M^{1/3} .$$

However molecular weights determined in this way do not agree as a rule with those derived from light scattering observations on undegraded DNA, although reasonable agreement is often obtained with degraded samples of lower molecular weight.

From this it was concluded ([3]) that the different specimens of DNA have a similar configuration which is compatible with the relation $S \propto [\eta]^{1/3}$, and that the variable factor determining both the mean properties and also the distribution of sedimentation coefficients is the molecular weight of the particles.

The variation of molecular weight observed in a single preparation is quite considerable. Figure 4 shows some typical distribution

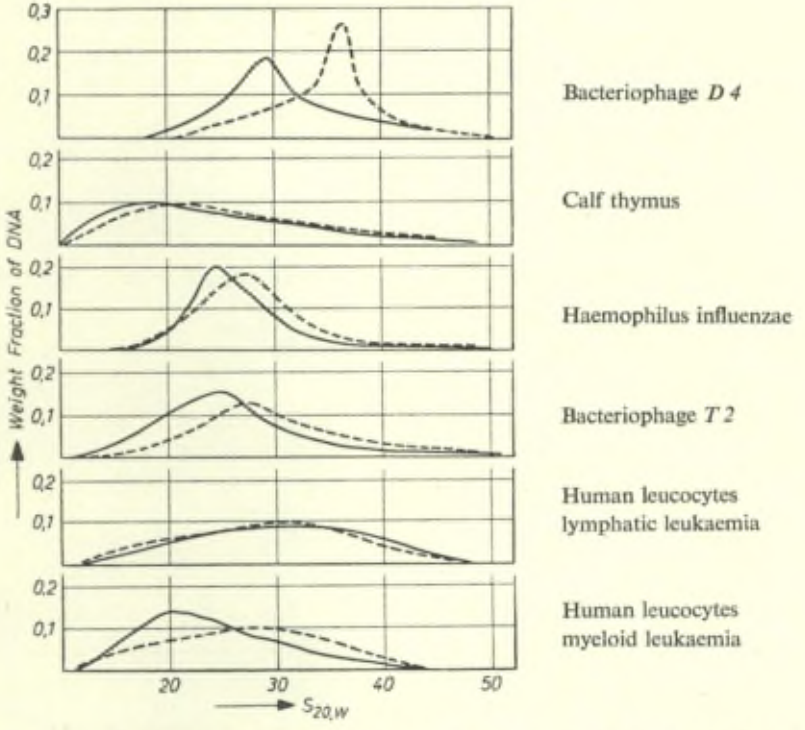

Fig. 4. — Distribution curves of various DNA's. (From Butler and Shooter, *Z. physik. Chem.*, 1958.)

curves. S_0 may vary from 10 to 40 (with the greater part between 15 and 30), which corresponds to a molecular weight variation, if the above theory is valid, of no less than 1 : 64. It is also found that preparations even from the same tissue differ considerably, e.g., mean molecular weights of DNA from calf thymus have been reported ranging from 3 to 18 $\times$ 10^6. Possible causes of this variability include :

(1) *Aggregation*, such as might be caused by the cross-linking of DNA particles by residual amounts of protein.

All DNA preparations contain small quantities of proteins, which are not histone in character (6). The amount of such impurity can be reduced by treating the DNA with chymotrypsin in salt solution and in other ways and after such treatments more reproducible values of the sedimentation coefficients are obtained. But there is usually a small amount of residual protein which cannot be removed in this way.

(2) *Degradation*, e.g. that produced by the action of DNAase during the preparation.

That this occurs has been shown by the fact that when DNP is kept at room temperature in its gel-like form, the DNA contained in it is found when separated from the protein by strong salt, to be slowly degraded (7). Although various substances (e.g. citrates) have been suggested as DNAase inhibitors, no really good inhibitor has been found and the effect of such actions is difficult to assess.

However the degree of heterogeneity observed in good preparations in which the protein content has been reduced as much as possible, is fairly reproducible and some characteristic differences of DNA's from different sources have been observed (8). In the case of some organs (e.g. rat spleen) it has been found difficult to obtain reproducible characteristics.

B. Extraction procedures.

It was observed by Chargaff, Crampton and Lipschitz (9) that DNA fractions having different base ratios, were obtained by extraction of a nucleoprotein gel (which had been denatured by shaking the solution with chloroform) with solutions of sodium chloride of increasing concentration. The fraction extracted by the higher salt concentrations were relatively rich in adenine and thymine.

Lucy and Butler ([10]) showed that it was unnecessary to vary the concentration of the salt solution as similar effects were observed on successive extractions with one salt solution. They found that the fractionation of the DNA was accompanied by the extraction of histones of different composition, lysine-rich histones being extracted first. However they did not think that this necessarily meant that the cytosine-guanine rich nucleic acids were associated with lysine rich histones, although this may be the case, as the two different fractionation processes may have been acting independently. Significant fractionations of complexes of DNA with polylysine and with polysulphates (e.g. heparin and dextran sulphate) have been observed ([11]). Fractional precipitation procedures, e.g. by ethanol or by histone, have also been tried, without much success.

C. **Chromatographic procedures.**

It would appear to be necessary to use a basic column which does not bind nucleic acids too firmly. From some ion exchange resins elution is very difficult. Brown and Watson ([12]) used a basic material by heating Kieselguhr with histone, and reported fractionations especially of transforming principle DNA, in which the activity was concentrated. They also obtained fractions from T_{22} bacteriophage one of which is identified with the " large piece " of Levinthal and Thomas ([15]). Bendich et al. ([13]) employed the slightly basic " ecteola cellulose " of Peterson and Sober. The DNA is eluted first with salt solutions of increasing concentrations and with various buffers and finally with alkali. A large number of fractions were obtained, giving a "chromatographic profile", which the authors claim is characteristic of the preparation. The fractionation involves differences of size of the particles to some extent, as the earlier fractions have been found by sedimentation and viscosity to be of lower mol. weight.

Some related observations with RNA might be mentioned here. Bradley and Rich ([16]) experimented with ecteola resin and found that concentrated sodium hydroxide was required to remove part of the RNA, and further more the fractions obtained did not appear in the same place on rechromatographing. Butler and Johns ([17]) have found it possible to obtain two distinct bands by the elution of RNA with calcium chloride or with sodium hydrogen phosphate, from ecteola resin. Smith, Rebhun and Kaplan ([17a]) have made

use of a cationic starch known as Cato-8, which permits complete elution of RNA and DNA with salt and pH gradients within 1 or 2 days.

D. **Counter-current distribution between two solvents.**

No suitable solvent has been found for DNA, but the distribution of RNA between 2-butoxy-ethanol and aqueous potassium acetate has been studied by Kirby ([18]) who obtained some evidence of fractionation.

2. ACTION OF PHYSICAL AND CHEMICAL AGENTS ON DNA

A. **Effects of heat.**

Heating DNA solutions causes a collapse of the hydrogen bonded structure, as shown by the great decrease in viscosity. This itself indicates that the resulting particles are much less extended. There has been some controversy as to whether the molecular weight is at the same time diminished. Dekker and Schachman ([19]) observed at the same time a diminution of the sedimentation constant which could only be attributed to a decrease of molecular weight. They therefore proposed that there were imperfections (single breaks) in the original nucleotide threads which resulted in a dissociation into smaller units when the hydrogen bonded structure was broken down. Doty and Rice ([20]) however observed no change on molecular weight on heating and concluded that the nucleotide threads were continuous. Shooter, Pain and Butler ([21]) observed, on the basis of sedimentation and viscosity, changes of molecular weight much less extensive than those of Schachman and Dekker. The question however is a complex one; since the hydrogen-bonded structure is more stable in salt solutions than in water, and especially in the latter on continued heating hydrolysis of the nucleotide chain undoubtedly takes place ([22]). There are other complications in that in moderately concentrated solutions aggregation processes also occur ([21]) owing to hydrogen bonding between exposed parts of two fibres so that what is actually observed is a balance between dissociation and aggregation.

The denaturation of DNA by heat is a typical "co-operative phenomenon", since the high temperature coefficient of the denatura-

tion process shows that a number of adjacent hydrogen bonds must be broken simultaneously in the process (23). Hydrogen bond breaking agents like urea assist the denaturation, as shown by the fact that the denaturation temperature is lowered. There has been discussion as to whether urea causes twin threads to separate from each other giving rise to two particles, each having half the original molecular weight. This was asserted in some preparations on the basis of light scattering measurements (25); although Doty and Rice found no change in the optical absorption density which accompanies denaturation by heating (23). It is of course possible that urea dissociates loosely aggregated DNA particles from each other without breaking up the main hydrogen bonded structure.

Another technique has been used by Meselson and Stahl (26). The substance is centrifuged in a gradient of caesium chloride and ultimately reaches equilibrium at a point where the density of this particle is the same as that of the medium. The width of the band a particle of any density occupies is determined by its molecular weight through the diffusion rate. On heating DNA at 100^0 for 30 minutes in the caesium chloride changes indicating a decrease of molecular weight by one half were observed. Moreover, when a bacterial DNA containing nitrogen, half of which was N^{15} and half N^{14}, gave a band intermediate in position between that of the N^{14}-DNA and the N^{15}-DNA; and on heating this split into two bands in positions expected for denatured DNA containing N^{14} and N^{15} respectively.

B. Effects of acids.

The earlier study of the titration curves showed the well known hysteresis phenomenon, i.e., the titration curves towards and away from an acid or alkaline pH are not identical (27). This is due to the fact that many of the titratable groups are involved in hydrogen bonds which are not broken until pH's substantially different to those at which they titrate when isolated. It has recently been shown by Peacocke (24, 28) that when the titrations are carried out at 0^0 C, the titration curves become reversible, i.e., the hydrogen bonding is not easily broken at the lower temperature. There has been some disagreement as to whether breaking of the hydrogen bonds in acid pH results in separation of the two nucleotide fibres. It is likely that small quantities of protein may influence the result.

C. **Effects of irradiation by X-rays.**

A large number of investigations have been carried out, and a complete summary is impossible. The more important effects observed are :

a) *Chemical effects on bases*, particularly on purines, e.g., deamination, dehydroxylation, fission of purine ring ([29]). Hems has recently observed ([30]) that the fission of the imidazole ring of the purine occurs on the irradiation of guanylic acid, the product being 2,4-diamino-5-formamido-6-hydroxypyrimidine (fig. 5). A similar degrada-

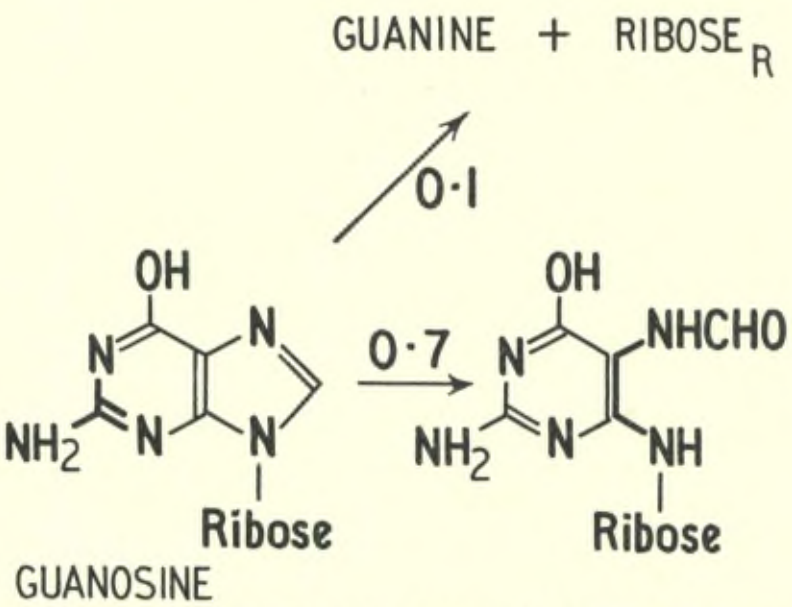

Fig. 5. — Effect of X-rays on guanosine (Hems). The numbers represent the G values of the two processes.

tion of the purine occurs in DNA ([31]) and will result in a disruption of hydrogen bonding, and probably also in an instability of the nucleotide chain. It is also found that adenine is liberated in appreciable amounts.

b) *Breakage of nucleotide chains.* This can occur either by the direct fission of the phosphate-sugar bond, or as a consequence of the oxidation of the sugar moiety, followed by hydrolysis of the

keto-ester, as suggested by Weiss and Scholes [32] or as a consequence of effects mentioned in (a). By the action of radiations, free $—PO_4$ is liberated, but since the breakage of two adjacent phosphate-ester links is required to form free $—PO_4$, it would be expected and is found that the amount formed increases with the square of the dose [33].

The breakage of a single nucleotide chain does not necessarily cause a fission of the whole double stranded thread. In order to divide the DNA particle into two halves, it is necessary for both threads to be broken at points separated by, at most, a few bases from each other. For this reason the diminution of molecular weight is not closely related to the actual number of fissions in the individual nucleotide threads. The way in which this quantity is related to the molecular weight change has been discussed by Alexander and Stacey [34], by Doty [35] and by Peacocke and Preston [36]. Cox, Overend, Peacocke and Wilson find that the number of breaks of the particle (expressed as M^{-1}) is proportional to the square of the dose of radiation [37]. This is as would be expected if two scissions of the threads were required to break the particle. From this relation it is possible, assuming that the primary nucleotide breaks are random, to deduce how close the breaks in the two chains must be in order to cause scission of the particle.

c) *Breakage of hydrogen bonding.* A study of the titration curves and of optical absorption spectra of DNA gives evidence of extensive breakage of hydrogen bonding as the result of irradiation [37]. It was estimated that for each break in a nucleotide chain, 16.4 hydrogen bonds were broken, which may be due to the ordered hydrogen bonding being disrupted on each side of a break over a region of about eight base pairs. If this is the case the effect of irradiation will parallel to a considerable extent that of heat. It has in fact been found [38] that after irradiation the critical temperature for heat denaturation is reduced. Extensive breaking of hydrogen bonds should produce a marked change in the flexibility of the particle. However, experiments on light scattering, as well as sedimentation and viscosity, show little evidence of a change of flexibility [3]. For this reason it is possible that the breakage of hydrogen bonds is more potential than actual, as it would be if a breakage of the nucleotide thread provides a point at which the threads can become unwound in the course of titration.

d) *Effects of oxygen.* Early in these studies it was found that after irradiation the viscosity of DNA solutions continued to decrease for a considerable time after the completion of the irradiation ([39]). It was shown by Conway and Butler ([40]) that this effect did not happen in the absence of oxygen, and they suggested that it was due to the slow decomposition of an oxygenated product, formed by a reaction in which oxygen is involved. More recently it has been found ([41]) that when thymine is irradiated in the presence of oxygen a hydroperoxide is formed, and the same occurs with DNA itself. Scholes and Weiss ([42]) also suggested that the after effect was due to the slow hydrolysis of keto-sugars formed during the irradiation, presumably only in the presence of oxygen.

D. **Action of radiations on DNA in vivo.**

The situation of DNA in the living cell is so different from that in purified solutions that it must be regarded as uncertain how far the effects observed in solutions are relevant. The main differences are 1) existence of protective substances in solution which will undoubtedly diminish the effect of free radicals, but are unlikely to eliminate their effects completely; 2) the fact that DNA is combined with histone in the chromosome. An attempt was made by Limperos and Mosher ([43]) to study the effect of irradiation *in vivo* of DNA in rat thymus. They found that DNA prepared from animals killed 24 hours after irradiation with 1000 *r* was much depolymerised; however, this is not conclusive as it is known that many of the thymus cells are killed by this treatment and the observed changes may be due to necrotic degradation. Butler and Johns ([44]) attempted to repeat this and found that although the amount of DNA obtainable from each organ was much diminished by irradiation, that which was isolated did not differ appreciably from the normal. A further study by Butler, Johns and Laurence ([45]) has shown that even with the radio-insensitive organ, the liver, the amount of DNA was sometimes much diminished by prior irradiation of the animal. However, further experiments have shown that this does not always occur and is a function of the time the irradiated liver homogenate is left to stand during the preparation. It appeared, therefore, that DNA in the homogenate of the liver of the irradiated rat was undergoing a fairly rapid degradation, which must be ascribed to deoxyribonuclease present in the irradiated homogenate, but not in homo-

genate from normal rat livers. It has been observed by Goutier-Pirotte and Thonnard (46) that deoxyribonuclease is released from mitochondria (lysosomes) by irradiation.

E. **Actions of radiomimetic chemicals.**

Since substances of the " mustard „ class produced lesions very similar to those produced by X-rays, attempts to replace therapeutic X-rays by these substances were made during or shortly after World War II. It was also observed that these substances produce chromosome aberrations very similar to those of X-rays and at a later stage it was demonstrated that they can act both as mutagens and carcinogens. For these reasons much work has been done on the effects of substances of this kind on biological substrates, particularly nucleic acids. Early investigations were mainly with proteins [for review, see Golumbic *et al.* (47)]; and it was shown that esterification reactions occurred with the $-NH_2$, $-OH$ and $-CO_2H$ groups. It was also found (48) that nucleic acids exhibited a high degree of reactivity. The following chemical changes have been shown to occur with DNA : 1) esterification of phosphate groups; 2) reac-

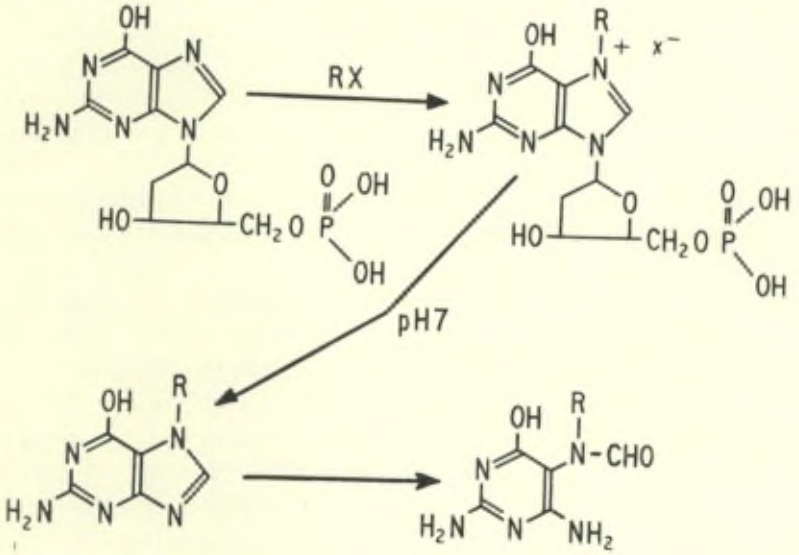

Fig. 6. — Action of alkylating agents such as „ nitrogen mustards " on guanylic acid and subsequent fission (from P.D. Lawley, *loc.cit.*).

tion with NH_2 of guanine; 3) reactions with ring nitrogens particularly of guanines (49). Lawley (50) has recently shown more precisely by optical measurements and chromatography that the guanine moiety is alkylated in the 7-position (fig. 6). This leads to hydrolytic fission of the sugar-base linkage, with the liberation of the 7-alkyl guanine. The imidazole ring of the purine is also rendered rather unstable and undergoes fission in alkaline solution. Alkylation of the adenine occurs to a smaller extent in the 1- and 3-positions and also to some extent cytosine in the 3-position (50).

The macromolecular effects of these changes on the DNA particle have also been extensively studied. Following the observation of Chanutin and Gjessing (51) of some limited changes of viscosity of the DNA solutions when treated with certain nitrogen mustards, Butler and Smith (52) found that an almost complete loss of the viscosity occurred in 2 - 3 days. A study of the molecular weight changes occurring showed that there was an initial decrease of viscosity with little change of molecular weight, followed by a definite degradation into smaller units (53). The former is ascribed to the loss of flexibility and folding up of the particle owing mainly to breakage of the hydrogen bonding; the latter may arise from instability of the nucleotide chain 1) at positions at which the phosphate has become tri-esterified or 2) arising from the alkylation and subsequent breakdown of the guanine as described above.

It is a general experience (54) that the " bifunctional " mustards with two or more chloroethyl groups are more active in tumour inhibition than similar monofunctional compounds. It has been suggested (53) that the reason for this is their ability to effect cross-linkage of two substrate particles, or two distinct groups of the same substrate molecule. The difference, however, is not an absolute one, as monofunctional compounds usually have similar effects, although frequently a concentration 30 - 50 times higher is necessary. The monofunctional mustard $(CH_3)_2N . CH_2 . CH_2 . Cl$ is capable of degrading DNA in a similar way to the bifunctional $CH_3 N . (CH_2CH_2Cl)_2$ except that there is a considerable lag period before any loss of viscosity occurs (55).

The precise way in which the bifunctional compounds bring about their biological actions is still somewhat uncertain. They are undoubtedly capable of cross-linking proteins and therefore may cross-link different parts of the chromosome fibres which are physic-

ally adjacent. It is also possible that they can cross-link the two separate nucleotide chains present in a single DNA fibre and so prevent their separation in the replication process. They could certainly do this at points where two guanine groups are close together in the two strands. However, as pointed out above, this can also lead to fission of the purine base and possibly also to the disruption of the nucleotide chains at this point. A possible reason for the greater effectiveness of the bifunctional component would then be seen to be in its ability to cause the rupture of both strands at points close to each other. The monofunctional mustard will also be able to do this, but less efficiently because the probability of two fissions being opposite each other will be much smaller. If we ask why a double fission is necessary, the answer may be that in a single fission, the continuity of the " message " may still be carried by the undamaged chain. Other consequences of cross-linking can no doubt occur, e.g., preventing the separation of the two strands which must necessarily occur in replication.

In the cell the DNA is usually combined with basic proteins as nucleoprotein in which nearly all the phosphate groups are neutralized by basic groups of the protein (mainly lysine and arginine). It might be asked if reactive compounds are able to react with the DNA in this combination. Experiments have been made by Crathorn and Butler ([56]) with the radioactive nitrogen mustards, p-di-(2-chlorethyl)amino-DL-phenylalanine (PAM) and also 2-di-(2-chlorethyl) aminophenyl butyric acid (PBM). Deoxyribonucleoprotein from a number of sources was used and it was found that generally appreciably more of the radioactive compound had combined with the DNA than with the protein; and in the case of the DNA, the radioactivity was not easily removable by treating the product with KOH.

It has been suggested by Stacey ([57]) that crosslinking of nucleoprotein by di-functional mustards occurs more readily than that of the DNA, as shown by the inhibition of swelling of nucleoprotein gels. Crosslinking of the protein may occur, but the swelling of nucleoprotein is easily modified by slight changes of polyelectrolyte character ([58]) and is also easily brought about by monofunctional mustards.

F. Reactions of radiomimetic chemicals in vivo.

The situation *in vivo* is different from that discussed above in that there are many competing substances present and the compound

often has to travel a considerable distance and penetrate cell membranes before reaching DNA or DNP in the cell nucleus. It might well happen that only a small proportion of the compound introduced reaches these substances in a reactive form. *In vivo* experiments with a radioactive nitrogen mustard PAM (see above) were made by Cohn ([59]). After administering the compound interperitoneally to rats, the protein, RNA and DNA fractions were obtained from various organs. The levels of radioactivity of the fractions are given in the following table.

It can be seen that even with non-lethal doses of the nitrogen mustard, appreciable combination of the drug with DNA occurs, but there is no *a priori* reason for believing that one of these reactions is more effective than the others.

Greater doses can be applied to bacteria and here again DNA and RNA can be isolated which is appreciably radioactive. Crathorn and Hunter ([60]) found, using *S. aureus*, that with some nitrogen mustards a marked inhibition of the ability of the growing micro-

TABEL 1

In vivo combination of a C^{14}-labelled nitrogen mustard (PAM) with proteins and nucleic acids.

	Dose : how administered	Specific radioactivity of		
		Protein	RNA	DNA
Spleen	10 mg/kilo : rats killed after 1 or 2 days.	0.017	0.004	0.010
Thymus		0.021	0.007	0.010
Liver mitochondria .	10 mg/kg twice at interval of 2 days : rat killed on fourth day.	0.083	0.037	—
Liver microsomes. .		0.073	0.037	—
Kidney supernatant		0.37	0.20	—

organism to incorporate amino acids into its protein (exchange conditions) occurred, and the extent of inhibition was roughly parallel with the inhibition of growth of the Walker tumour by the same compounds.

REFERENCES

(1) K.V. Shooter and J.A.V. Butler, *Trans. Faraday Soc.*, **52,** 734 (1956).
(2) K.V. Shooter and J.A.V. Butler, *Chemical Basis of Heredity*, p. 540 (Johns Hopkins Press, 1957).
(3) J.A.V. Butler, D.J.R. Laurence, A.B. Robins and K.V. Shooter, *Proc. Royal Soc.* (A), **250,** 1 (1959).
(4) P. Doty, B.B. McGill and A. Rice, *Proc. Nat. Acad. Sci.*, U.S.A., **44,** 432 (1958).
(5) P.J. Flory and L. Mandelkern, *J. Chem. Phys.*, **20,** 212 (1952).
(6) J.A.V. Butler, D.M.P. Phillips and K.V. Shooter, *Arch. Biochem. Biophys.*, **71,** 423 (1957).
(7) K.V. Shooter and J.A.V. Butler, *J. Polymer Sci.*, **23,** 705 (1957).
(8) J.A.V. Butler and K.V. Shooter, *Z. physikal Chem.*, **15,** 6 (1958).
E.E. Polli and K.V. Shooter, *Biochem. J.*, **69,** 398 (1958).
(9) E. Chargaff, C.F. Crampton and R. Lipschitz, *Nature*, Lond., **172,** 289 (1953).
(10) J.J. Lucy and J.A.V. Butler, *Nature*, Lond., **174,** 32 (1954); *Biochim. Biophys. Acta*, **16,** 431 (1955); *Bull. Soc. Chim. Belges*, **65,** 133 (1956).
(11) P.W. Kent, M. Hichens and P.R.V. Ward, *Biochem. J.*, **68,** 568 (1958).
P. Spitnik, R. Lipschitz, E. Chargaff and S.M. Benger, *J. Amer. Chem. Soc.*, **77,** 376 (1955); *J. Biol. Chem.*, **215,** 765 (1955).
(12) G.L. Brown and M. Watson, *Nature*, Lond., **172,** 971 (1955); G.L. Brown and A.V. Martin, *Nature*, Lond., **176,** 971 (1955).
(13) A. Bendich, *et al. J. Amer. Chem. Soc.*, **77,** 3671 (1955); *Nature*, Lond., **180,** 1356 (1957).
(14) C. Levinthal and C.A. Thomas, *Biochim. Biophys. Acta*, **23,** 465 (1957).
C. Levinthal and C.A. Thomas Jr., *Chemical Basis of Heredity*, p. 737 (Johns Hopkins Press, 1957).
(15) D.F. Bradley and A. Rich, *J. Amer. Chem. Soc.*, **78,** 5898 (1956).
(16) J.A.V. Butler and E.W. Johns, Reports IV Int. Congress Biochem. Vienna (1958), Symposium No. 9.
(17) In course of publication; see also K.C. Smith and H.S. Kaplan, *Fed. Proc.*, **17,** 1799 (1958).
(18) K.S. Kirby, *Trans. Faraday Soc.*, **55,** 490 (1959).
(19) C.A. Dekker and H.K. Schachman, *Proc. Nat. Acad. Sci.*, **40,** 894 (1954).
(20) P. Doty and S.A. Rice, *Biochim. Biophys. Acta*, **16,** 446 (1955).
(21) K.V. Shooter, R.H. Pain and J.A.V. Butler, *Biochim. Biophys. Acta*, **20,** 497 (1956).
(22) C. Sadron, Proc. Third Int. Congress Biochem., Brussels (1955), 210 (1956).
(23) P. Doty and S.A. Rice, *Biochim. Biophys. Acta*, **16,** 446 (1955).
(24) R.A. Cox and A.R. Peacocke, *J. Polymer Sci.*, **23,** 765 (1957); *J. Chem. Soc.*, 2499, 2646 (1956).
(25) P. Alexander and K. Stacey, *Biochim. J.*, **60,** 194 (1955); *Trans. Faraday Soc.*, **53,** 251 (1957).
(26) M. Meselson, F.W. Stahl and J. Vinograd, *J. Proc. Nat. Acad. Sci.*, U.S.A., **43,** 581 (1957).
(27) J.M. Gulland, D.O. Jordan and H.F.W. Taylor, *J. Chem. Soc.*, 1131 (1947).
(28) A.R. Peacocke, in the press.
(29) G. Scholes and J. Weiss, *Exptl. Cell Research.*, Supp. 2, 219 (1952).
(30) G. Hems, *Nature*, Lond., **181,** 1761 (1958).
(31) G. Hems (personal communication).
(32) G. Scholes and J. Weiss, *Nature*, Lond., **171,** 920 (1953); *Nature*, Lond., **178,** 157 (1956).
(33) J.A.V. Butler and B.E. Conway, *Proc. Royal Soc.*, B **141,** 56 (1953).
(34) P. Alexander and K.A. Stacey, in *Progress in Radiobiology*, p. 105 (Oliver and Boyd, Edinburgh, 1956).
(35) P. Doty, Proc. Third Int. Congress Biochem., Brussels (1955), 135 (1956).

(36) R.A. Cox, W.G. Overend, A.R. Peacocke and S. Wilson, *Nature*, Lond., **176,** 919 (1955).
A.R. Peacocke and B.N. Preston, *J. Polymer Sci.*, **31,** 1 (1958).
(37) R.A. Cox, W.G. Overend, A.R. Peacocke and S. Wilson, *Proc. Roy. Soc.*, **149 B,** 511 (1958).
(38) J.A.V. Butler, CIBA Found. Symp. on Ionising Radiations, **59,** (1957).
(39) B. Taylor, S.P. Greenstein and A. Hollaender, *Arch. Biochem.*, **16,** 19 (1948).
(40) J.A.V. Butler and B.E. Conway, *J. Chem. Soc.*, 3418 (1950).
(41) J. Weiss, *Les peroxydes organiques en radiobiologie*, Ed. R. Latarjet, p. 44 (Masson et Cie, Paris, 1958).
(42) G. Scholes and J. Weiss, *Nature*, Lond., **177,** 1033 (1956).
(43) G. Limperos and W.A. Mosher, *Am. J. Roentgenology*, **63,** 681 (1950).
(44) J.A.V. Butler and E.W. Johns, see *Radiation Research.*, Suppl. 1, 403 (1959).
(45) J.A.V. Butler, E.W. Johns and D.J.R. Laurence, unpublished work.
(46) M. Goutier-Pirotte anp A. Thonnard, *Biochim. Biophys. Acta*, **22,** 396 (1956).
(47) C. Golumbic, J.S. Fruton and M. Bergman, *J. Org. Chem.*, **2,** 518 (1946).
(48) R.M. Herriott, *J. Gen. Physiol.*, **32,** 221 (1948); *J. Gen. Physiol.*, **34,** 761 (1951).
(49) E.M. Press and J.A.V. Butler, *J. Chem. Soc.*, 626 (1952).
(50) P.D. Lawley, *Biochim. Biophys. Acta*, **26,** 450 (1957).
P.D. Lawley and C.A. Wallick, *Chem. and Ind.*, London, 633 (1957).
(51) A. Chanutin and E.C. Gjessing, *Cancer Res.*, **6,** 593 (1946).
(52) J.A.V. Butler and K.A. Smith, *J. Chem. Soc.*, 3411 (1950).
(53) J.A.V. Butler, L. Gilbert and D.W.F. James, *J. Chem. Soc.*, 3268 (1952).
(54) R.J. Goldacre, A. Loveless and W.C.J. Ross, *Nature*, Lond., **163,** 667 (1949).
(55) J.A.V. Butler, *et al. B.E.C.C. Ann. Report* (1952), p. 30.
(56) A.R. Crathorn and J.A.V. Butler, unpublished results.
(57) K.A. Stacey, in press.
(58) E.J. Ambrose and J.A.V. Butler, *Faraday Soc. Discussion*, No. 13, 261 (1953).
(59) P. Cohn, *Brit. J. Cancer*, **11,** 258 (1957).
(60) A.R. Crathorn and G.D. Hunter, *Biochim. J.*, **67,** 37 (1958).

DISCUSSION DU RAPPORT DE M. J.A.V. BUTLER.

M. Kuhn. — By the action of a divalent reagent on a macromolecule, an increase as well as a decrease of the viscosity of the solution may be expected and has been observed, depending on the circumstances. An illustrative example is the action of terephtalaldehyde on polyvinylalcohol (1). If the concentration of polyvinylalcohol is high (1 % or more) the action of the bivalent reagent will connect two originally independent molecules of polyvinylalcohol; i.e., the molecular weight and thereby the viscosity of the solution will increase; at sufficiently high concentrations a gel will be obtained. If the bivalent reagent on the other hand acts on a very dilute solution of polyvinylalcohol, the first reactive group will attach the reagent to one given polyvinylalcohol molecule; the second reactive group will subsequently react with another group of the same macromolecule; a ring formation and a certain contraction of the macromolecule is thus produced and thereby a decrease of the viscosity of the solution. Both an increase and a decrease of the viscosity have thus been observed, depending on the concentration of the macromolecular substance. This shows that one has to be very careful when interpreting the effect of chemical agents or also of associations occurring between parts of different or eventually parts of the same macromolecule.

M. Butler. — These difunctional reagents may of course give rise to cross-links between adjacent molecules, which will increase the viscosity. This may be the predominant effect with polymers of the type mentioned by Dr. Kuhn, because degradation does not occur. With DNA the predominant effect is degradative. As I mentioned in the paper, reaction with the two strands of the DNA is to be expected, and this will give rise on hydrolysis to a complete breakage of the DNA fibre.

(1) W. Kuhn and G. Balmer, unpublisched. For experiments of partly similar character produced through the action of X-rays or a nitrogen mustard see e.g. P. Alexander and M. Fox, *Nature*, **169**, 572, 1952; K.A. Stacey, M. Cobb, S.F. Consens and P. Alexander, *Ann. New York Acad. Sci.*, **68**, 682, 1958. For the theory of internal crosslinking through bivalent agents see W. Kuhn and H. Majer, *Makromol. Chem.*, **18**, 239,1956.

M. Julia. — A propos de l'action des moutardes azotées sur les acides nucléiques, a-t-on observé des actions analogues avec les agents alkylants utilisés en chimiothérapie du cancer, comme les éthylène-imines ou les alkylidènediméthane sulfonates ? Si oui, a-t-on observé la même dépendance entre l'action et la distance séparant les fonctions alkylantes que dans l'effet sur les tumeurs ?

M. Butler. — Ethylene-imines produce effects similar to the nitrogen mustards; but the alkyl dimethane sulphonates have very little effect under *in vitro* conditions. It has been found by experiments with a radioactive compound (" myleran ") that reaction with DNA does occur to a small extent, about one tenth of that of nitrogen mustard under similar conditions. If degradation of the DNA occurs after " myleran " treatment, it is a very slow reaction.

M. Fraenkel-Conrat. — Dr. Butler was asked what his criteria for the efficacy towards DNA of an alkylating agent were. With TMV-RNA, inactivation and mutation is evoked by extremely small amounts of e.g. dimethylsulfate. Possibly single-chain molecules are more highly susceptible towards such agents than the double-stranded helix.

M. Schramm. — 1) In our hands, alkylation of RNA of TMV caused only inactivation but no mutation.

2) Dr. Butler observed, as Dr. Sadron did, that the homogeneity of DNA is increased and its molecular weight is reduced by treatment with chymotrypsin. Could this be due to a contamination with DNAase ? In this case the molecular weight should not remain constant after longer treatment.

M. Butler. — If the chymotrypsin contained DNAase, a slow degradation of the DNA would be observed. Usually this does not occur.

M. Sadron. — Dans nos expériences nous n'avons pas observé que le DNA ait changé au bout de 12 heures de mise en contact avec la chymotrypsine, sauf dans le cas des " masses " de l'ordre de 14.10^6 où la diminution à 7.10^6 était rapide.

M. Watson. — Recent work in our laboratory suggests that T_4 DNA shows a more homogenous behaviour in the ultracentrifuge than the various DNA samples shown in Dr. Butler's report. We suspect, in fact, that we may be dealing with a homogenous population of very similar if not the same molecular weight.

M. Butler. — We also examined DNA from bacteriophages (T_2 and D_4) and found a sharper distribution than with mammalian DNA's (see fig. 4 in my paper). However, these were probably not very good preparations and were not as sharp as we thought they should be.

M. Schramm. — What is the size of the DNA in the T_2 or T_4 bacteriophages; are there several DNA molecules in one phage ?

M. Watson. — Our data suggest a molecular weight of about 12×10^6. There are thus 8-10 DNA molecules in the T_4 particle.

M. Overbeek. — Is there any information as to whether these 8 or 10 molecules are all identical, or could they be dissimilar in some respects ?

M. Watson. — The answer is not yet known.

M. Butler. — Our observations with T_2 (see fig. 4) are not compatible with the suggestion (see C. Levinthal and C.A. Thomas, Jr., " Physical Basis of Heredity ", p. 737, 1957), that the DNA is present as one large piece of molecular weight 45×10^6. The molecular weights most frequently represented in our preparations, as derived from the sedimentation coefficients, were between $5\text{-}12 \times 10^6$, but the spread of the S curve showed a spread of low values, probably derived from degraded particles.

M. Ubbelohde. — Dr. Kuhn has pointed out how different macromolecules undergo very different deformations when subject to mechanical shearing stresses. For sufficiently high stresses, they may also be expected to exhibit very different rupture limits. Is there any evidence of this kind, for example in experiments on ultrasonic rupture ?

M. Butler. — We have studied the effect of shearing stresses produced by high speed flow through a capillary tube. No change of the intrinsic viscosity was observed up to 40,000 sec^{-1}, although the *interaction* of the particles at higher concentrations was affected. The effect is a kind of combing out of the fibres. I agree that this is not quite the same as the stress produced by ultrasonics.

M. Fraenkel-Conrat. — To the chairman's question concerning susceptibility to ultrasonication of nucleic acids of different structure, recent experiments with TMV-RNA might be briefly summarised. It has been observed that this material can reversibly be transformed from a random coil to a more structured form through the addition of divalent metal ions (Ca^{++}, Mg^{++}) in amounts equivalent to the phosphorus present. When both forms are subjected to ultrasonication, the more organized structure is found considerably more resistant than the random coil form, as judged by the rate of inactivation, and probably also by the rate of decrease in chain length.

M. Wilkins. — Because liquids are very little compressible, it is difficult to see how ultrasonic waves, which are compression waves, can break chain polymers. Presumably cavitation is necessary.

M. Kuhn. — Among the influences which may physically produce a degradation of macromolecules, we will have to consider in the first place the *degradation through the action of a high gradient of flow* in a streaming liquid (1) and the *degradation through ultrasonics*. The mechanical action to which the chain molecule is exposed is in both cases the same, namely a mechanical force pulling the ends of the molecule in opposite directions. In the case of the ultrasonic mechanism a chemical mode of action through the formation of cavitations, and thereby through the formation of OH-radicals and so on, is assumed or stated by some authors (2) while a mechanical degradation independent of the appearance of cavitation or heating is observed by other investigators (3).

(1) H. Staudinger, *Ber. d. Chem. Ges.*, **67**, 1159, 1934.

(2) H.H.G. Jellineck, *J. Polymer Sci.*, **6**, 745, 1951; **13**, 403, 1954; R.O. Prudhomme, *Journ. de Chim. Phys.*, **46**, 667, 1949; **47**, 795, 1950; H.W.W. Brett and H.H.G. Jellineck, *J. Polymer Sci.*, **13**, 441, 1954.

(3) G. Schmid und O. Rommel, *Z. phys. Chem.*, **185 A**, 97, 1939.

M. Sadron. — As far as I know, the ultrasonic degradation of high polymers in solution is concomitant with a local cavitation. If this relation between cavitation and degradation is true in all cases, the degradation is a chemical phenomenon and not a mechanical one (breaking of a covalent bond by mechanical tension).

NUCLEIC ACIDS OF MICROORGANISMS

by. A N. BELOZERSKY

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The problem of nucleic acids of microorganisms is a large and complicated one. In my communication I shall dwell only on some aspects of this problem, namely on quantitative changes of nucleic acids occurring during the process of ontogenic development of microorganisms, and on the composition specificity of this group of compounds. These are the problems our laboratory has been studying during recent years.

It is well known at present that nucleic acids undergo quite definite and regular changes during the life cycle of microorganisms whereby it is mainly RNA which undergoes these quantitative changes.

The character of these changes may be illustrated by the data obtained in our laboratory by Zaitseva (1) for the culture of Azotobacter agile. In Fig. 1 there is presented a curve of growth and biomass accumulation for both nitrogen-fixating culture and a culture grown on ammonium-nitrogen, i.e. under the conditions of depressed nitrogen fixation. Fig. 2 shows graphically the analytic data on the content of total nitrogen, RNA, DNA and protein in these two cultures during their life cycle.

Comparison of the above two figures, makes one draw the conclusion on the change of above mentioned compositions both in the developmental cycle of Az. agile and in dependence on the form of nitrogen assimilated.

Fig. 1 shows the culture grown on ammonium nitrogen to have a somewhat shorter latent phase (about 10-12 hours) than that of nitrogen-fixating culture (16-17 hours). Then, nitrogen-fixating

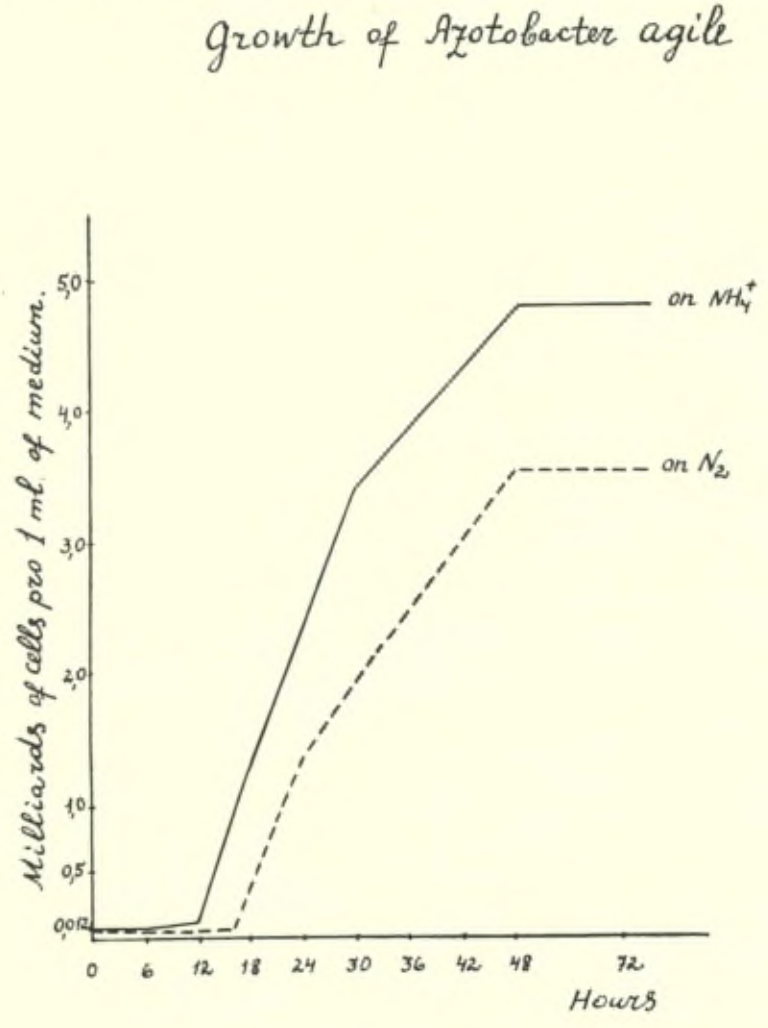


Fig 1.

culture is characterized by a somewhat delayed development when compared to the culture grown on ammonium nitrogen. This conclusion arises from the fact of a significantly greater and quicker biomass accumulation at depressed nitrogen-fixation. In

accordance with this fact, some difference in the time of going through developmental phases was observed in the above cultures. So, the logarithmic phase of nitrogen-fixating culture took from 18 to 43-45 hours of development, whereas in the culture with the depressed nitrogen-fixation it took from 12 to 40-45 hours. Thus, logarithmic phase was somewhat more prolonged in the presence of ammonium nitrogen. To be able to carry out comparative analytic investigation we thought it advisable to take under analysis such cultures the age of which differed with respect to the hours of their development but which were physiologically equivalent, when judging by the growth curve.

Hence, to investigate nitrogen-fixating culture, bacterial mass of 18, 24, 36 and 48 hours of development was taken, whereas that of 12, 18, 30 and 48 hours, respectively, was taken to investigate the culture grown on ammonium nitrogen. The above times of sampling corresponded to the onset, middle and end of the logarithmic phase, and to the early period of the stationary phase. Besides, material was taken in the latent developmental phase of 6, 12 and 16 hours, for the nitrogen-fixating culture and that of 4, 8 and 12 hours — to study the culture on ammonium nitrogen. Analysis of the initial culture which served as inoculum was carried out simultaneously.

When considering analytical data presented in Fig. 2, attention is first of all drawn to the intensive RNA synthesis proceeding in Azotobacter cells during the period of latent phase. During this period RNA-amount increases 2.5 times in nitrogen-fixating culture, whereas it increases even more in the culture with the depressed nitrogen-fixation. Thus, the most typical change occurring during the period of latent phase is the intensive RNA-synthesis, which in a given species of cells and under given conditions of existence attains a definite and a rather significant value; this seems to be one of the factors determining the further development of the culture. As it results from other experimental observations carried out in our laboratory, as well as from the data available in literature, the above mentioned regularity of chemical changes occurring in the protoplasm of microorganisms during the latent phase, seems to be typical of all the microorganisms.

Quantitative RNA level in the cell attaining the value typical of a given species of cells in accordance with their conditions intensive growth and reproduction of microorganism cells becomes possible. When examining Fig. 2, the attention is drawn to greater RNA accumulation in a cell, when the culture is grown on ammonium nitrogen. One should think that this higher RNA level in a given culture results in an increased pace and intensity of development accompanied by the accumulation of larger

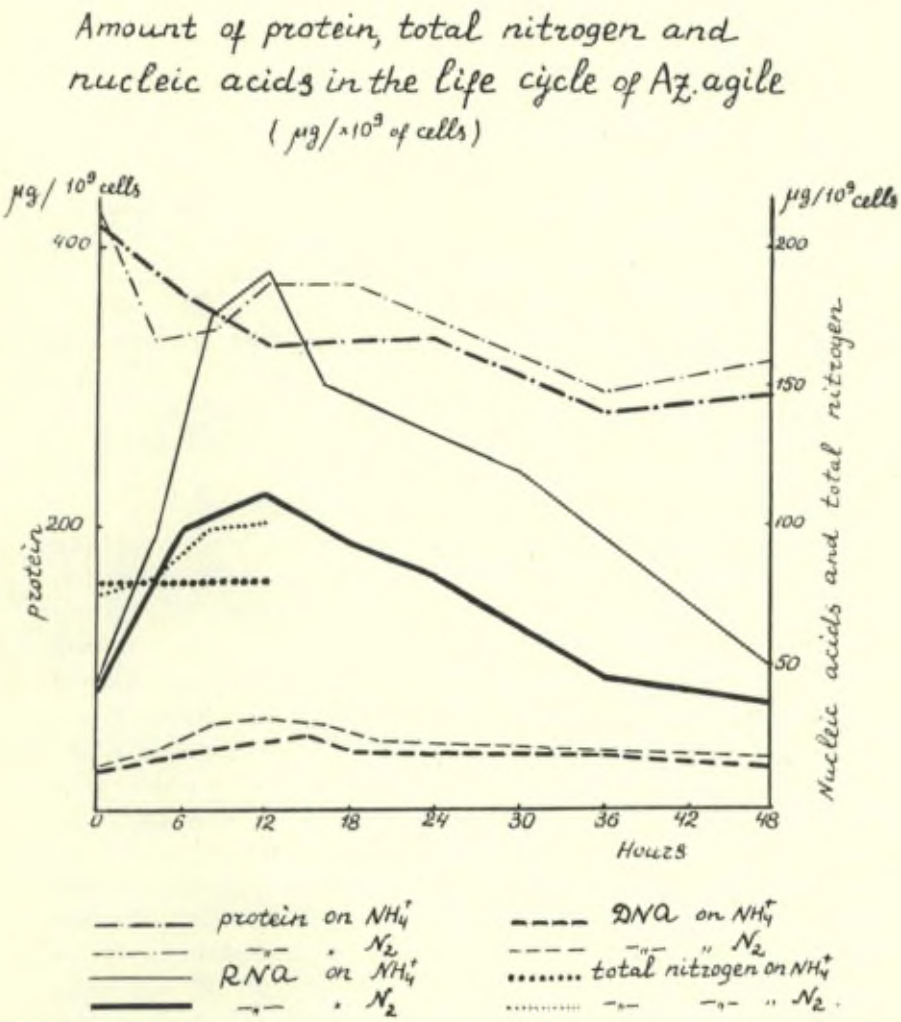


Fig. 2

amount of biomass than is the case with nitrogen-fixating culture (cf. Fig. 1).

RNA content in a cell decreases during the logarithmic developmental phase, whereas it attains initial relatively low level typical of the culture applied as inoculum during the stationary phase.

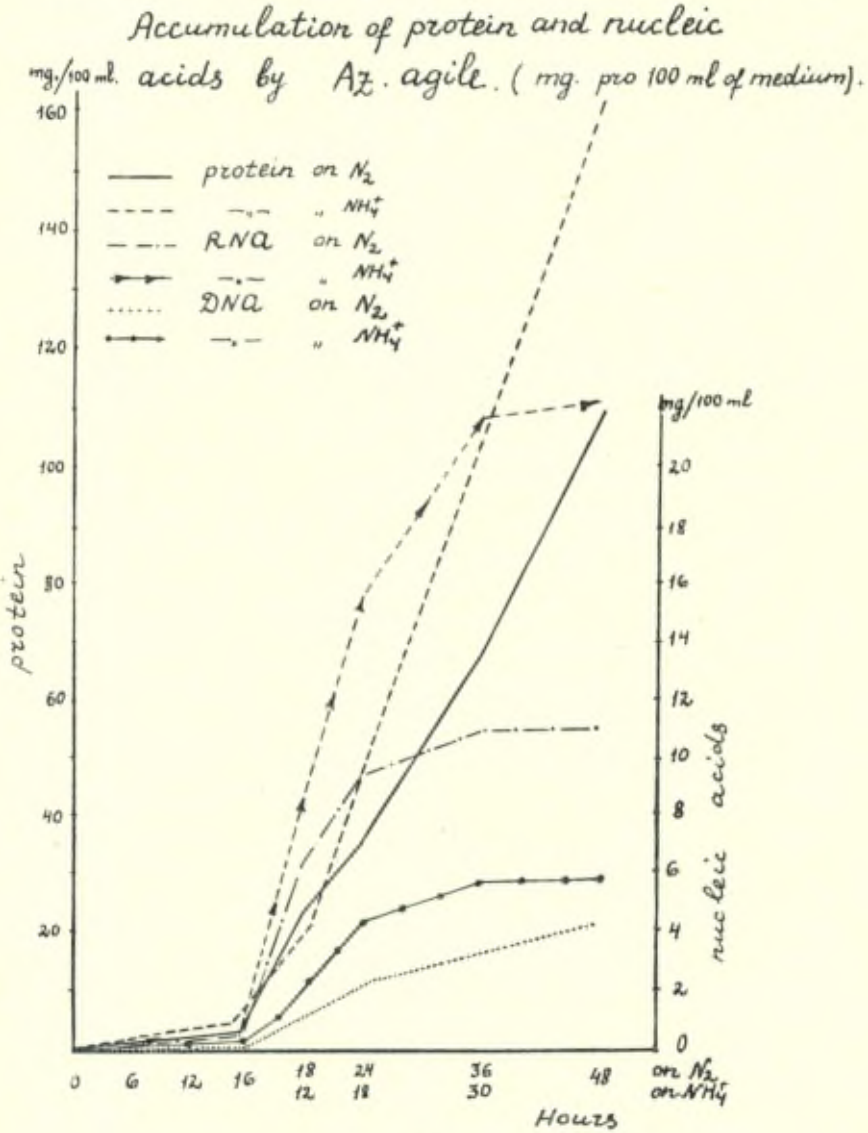


Fig. 3

Comparison of Figs. 1, 2 and 3 shows the intensity of growth and multiplication of cells to correlate with their RNA content. A decrease of RNA content in the cells is observed during the logarithmic phase compared to latent one. A larger surplus of RNA is apparently needed for the « starting » period preceeding that of intensive growth and development, than for the period of activity itself. Continuous intensive RNA biosynthesis during the logarithmic phase is proved by the data presented in Fig. 3 which shows accumulation of nucleic acids and protein in the biomass of Az. agile collected from 100 ml of medium and grown both under the conditions of nitrogen fixation and on ammonium nitrogen. This form of computation is of significant interest as an intensity index of biosynthesis of these or those cellular compounds at different substages of its development.

The level of nitrogen content in a cell remains constant during the latent developmental phase in the nitrogen fixating Azotobacter culture (Fig. 2). The lack of nitrogen fixation during this period and simultaneous intensive neoformation of RNA suggests nitrogen of protein to be consumed for nucleic acid synthesis in the latent phase. Investigations carried in our laboratory by Zaitseva on the amino acid composition ([2]) and electrophoretic study of protein components ([3]) of Azotobacter at different substages of its development showed that these correlations appear to exist. Thereby, the data obtained by us make one suppose that only a definite protein fraction can be utilized during this period. These fractions, at their turn, rise during the stationary developmental phase, i.e. during the period when the intensity of RNA biosynthesis is minimized.

As to DNA, its content in Azotobacter somewhat increases to the end of the latent phase and then undergoes no significant changes during the whole developmental cycle. Spirin ([4]) in our laboratory showed that the DNA content in E. coli cells does not significantly differ both when the culture passes from the logarithmic phase to the stationary one, and during the stationary developmental phase, whereas a considerable decrease of RNA content is observed in the cells during this period. In earlier works by Caldwell and Hinshelwood ([5]), where the count of nucleic acids per cell was also carried out, the constancy of DNA con-

tent in a cell at most various changes of physiological conditions of cultures was also noticed.

Thus, there are grounds to consider the quantitative DNA amount in a microorganism cell, unlike that of RNA, to be rather a constant one. There may be, however, particular cases when quantitative DNA amount in microorganism cells drastically decreases. So, in 1954 in our laboratory Demianovskaya ([6]) applying routine determination technique (e.g., Dische method) failed to detect DNA at all in Actinomyces globisporus streptomycini under certain developmental conditions of this organism. Guberniyev and coll. ([7]) also failed to determine DNA in Act. aureofaciens under the same developmental conditions, namely during the first 6-8 hours of its rearing after transferring the inoculum into large fermentyers. Trivial mycelium of the above Actinomycetes contains about 2-2.5 % of DNA. Spirin and Shugayeva studying DNA nucleotide composition of Actinomycetes in our laboratory showed chromatographically that in the cases when no DNA could be found out, it was actually present but its amount did not exceed 0.05-0.2 %. Thereby, it should be pointed out that no differences in nucleotide composition of this DNA were found out when compared to DNA of the mycelium in which normal amount of DNA was present. Venner ([9]) has also observed a drastic decrease of DNA content, down to 0.005 %, at a certain moment of the development of the fungus Polystictus versicolor.

These facts show that the theory of DNA constancy does not hold universally for the whole organic world and for all the conditions of existence of organisms.

Polyphosphates were recently found to play an ever important role in the activity of many microorganisms (Wiame; Ebel; Hoffmann-Ostenhof; Yoshida; Winder; Krishnan; Lohmann; Langen and Liss; Belozersky, Korchagin, Kulayev, Buchovich and Zaitseva; and others). These compounds were for a long time held for the derivates of metaphosphoric acid and were ascribed the cyclic structure of hexametaphosphate. However, as a result of recent works (Ebel, Yoshida, Thilo and others), the so called " metaphosphate " of yeasts and other lower organisms were shown to be linear polymers of ortophosphoric acid of high molecular weight.

Polyphosphates were shown to be macroergic compounds ([10]), comparable in this respect to such an energy accumulator as ATP. One succeeded to isolate enzymes from yeasts which carry out the transfer of phosphate residues from polyphosphates to ATP and back. These facts in themselves show the role of paramount importance which can be played by polyphosphates in the activity of organisms possessing them. Polyphosphates are undoubtedly most closely connected with the metabolism of nucleic acids and other phosphorus compounds of microorganisms. It is very likely that with the study of polyphosphate metabolism some amendments will have to be made with respect to phosphorus metabolism of those organisms to which polyphosphates are peculiar.

Up to now polyphosphates are found only in a number of lower organisms, such as bacteria, fungi and algae, as well as in the tissues of some insects. The attempts carried out in our laboratory in order to find polyphosphates in the tissues of higher plants and animals, have not yet given positive results.

Polyphosphates may exist in the cell in two forms: on the one hand, in the form of relatively low molecular fragments present in the cell in the free state, without any connection with organic compounds (acid-soluble fraction); on the other hand, in the form of high polymeres connected with some organic components of the cell (acid-insoluble fraction). Wiame ([12]) has found at his time that these are bound polyphosphates of the acid-insoluble fraction which are physiologically active. This important observation was later on proved in a number of laboratories, including ours ([13, 14, 15]).

In this connection one of the burning issues is to elucidate the problem, just with which cellular components may active polyphosphates be bound and at which organic basis their activation takes place.

According to many authors (Wiame, Ebel, Hoffmann-Ostenhof, Lohmann), polyphosphates may be bound to proteins. In 1955 experimental data were presented by us ([13]) which suggested that there is the possibility that a connection between polyphosphates and RNA may exist in yeasts. It should be noted that

the possibility that polyphosphate-RNA complexes may exist in yeasts was simultaneously pointed out by Chayen [11]. Later on in our laboratory [14, 15], and by Krishnan *et al.* [16] data were obtained showing the possibility that such complexes exist in mould fungi.

Further study of this problem by means of electrophoresis carried out in our laboratory by Kulayev [17] showed again that the possibility that polyphosphate-RNA complexes exist should not be overlooked. It should be pointed out that at purification of polyphosphate-RNA complexes by means of electrophoresis we readily succeed in the isolation of the above complex from the protein.

In 1958, Ebel [18] who was sceptic about the existence of polyphosphate-RNA complexes submitted to the Biochemical Congress in Wienna a number of experimental data which made him draw the conclusion that the reality of these complexes should not be overlooked. In his opinion, if they really do exist, the bounds between their components are labile. Thus, though the problem, to which cellular components physiologically active polyphosphates are bound, is not completely settled at present, the elucidation of this problem seems extremely important. Of peculiar principal interest is the problem on the possibility of RNA connection with polyphosphates. The elucidation of this problem seems extremely important and could contribute to the precision of our ideas on the physiological role of this nucleic acid. The problem of the existence of phosphorylated RNA periodically arises in literature again and again.

For the first time phosphorylated RNA was isolated by us as early as 1941 [20] from the cells of Spirillum volutans. On the basis of quantitative change of this RNA fraction occurring during ontogenesis we supposed that the basis of the volutine of this organism is the so-called volutine-nucleic acid, i.e. RNA enriched in phosphorus. Subsequent works of Bressler, Brachet, Hakim and others also do not overlook the possibility that RNA may undergo phosphorylation.

The connection of polyphosphates with some other cellular components, polysaccharides in particular, had not to be over-

looked either. This can be proved by a number of observations carried out in our laboratory.

Physiological role of polyphosphates was studied in our laboratory on a number of objects by means of both analytical technique and by the application of P^{32}. It was found that during the spore germination of mould fungi (Asp. niger and Pen. chrysogenum) ([14, 15]) acid-soluble polyphosphates present as a reserve in the form of low polymers transit to the acid-insoluble fraction associating there, apparently, with RNA. Acid-insoluble polyphosphates activated in this way are utilized for the intensive growth of the micelium. In particular, their consumption was shown by experimental data to be connected in the first place with the synthesis of protein and nucleic acids which may be clearly seen from the data presented in Fig. 4. Possibility of polyphosphates utilization in nucleic acid synthesis is clearly shown by our experiments with the application of P^{32} ([15]). It is important to point out that acid-soluble polyphosphates are almost entirely absent during the period of intensive synthetic activity of the growing mycelium. The processes of active growth slowing down or stopping, transition of polyphosphates from acid-insoluble fraction to acid-soluble one takes place.

Under the conditions of the surface growth of mould fungi during the sporogenesis, the degradation of polyphosphates proceeds through the stage of low polymers, in the form of which polyphosphates are transported into spores and stored there as the reserve. It should be noticed that, according to our data it is pyrophosphate which can serve as the form to be transported. In the absence of sporogenesis under the conditions of a submerged culture, transition of the polyphosphates to the acid-soluble fraction proceeds in the form of higher polymers.

Further degradation of acid-soluble high polymer polyphosphates under these conditions eludes the stage of low polymers and follows the way of direct orthophosphate formation.

Experimental data obtained in our laboratory with the application of both the analytical technique and of P^{32} have clearly shown that the synthesis of polyphosphates both in the mould fungi ([15]) and in yeast ([19]) proceeds in the acid-insoluble fraction, pre-

Fig. 4. Changes of acid-insoluble Polyphosphates, NA and Protein N during the Development of Penicillium Chrysogenum (per 1 g. of dry weight).

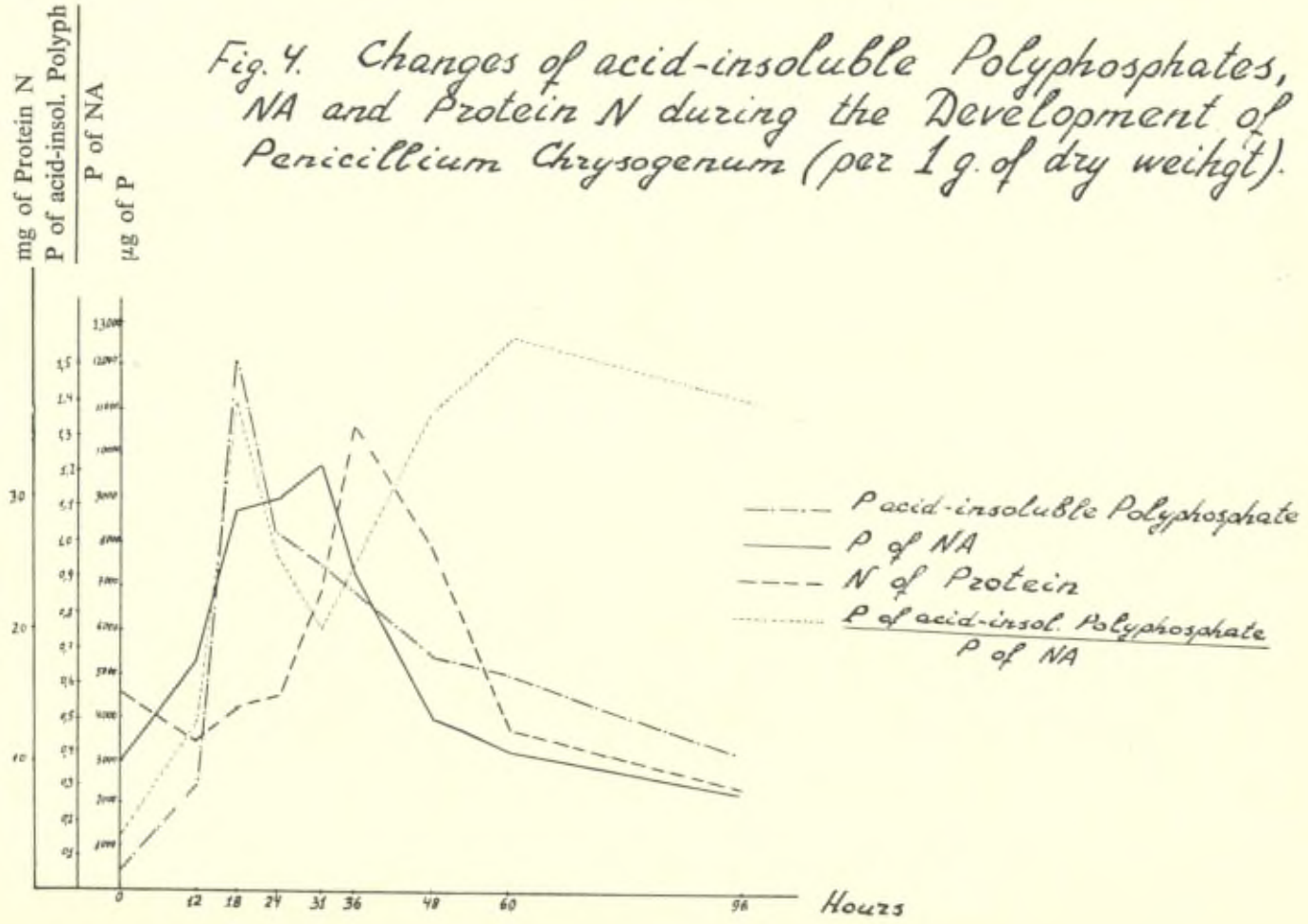


Fig. 4

sumably, on the basis of RNA. This synthesis needs oxidative phosphorylation, as the inhibition of this latter, for example by 2,4-dinitrophenol, leads to the complete cessation of polyphosphate synthesis ([19]). Our experiments showed some stable forms of phosphorus of the acid-soluble fraction to participate in the synthesis of polyphosphates. Thereby, oxidative phosphorylation plays a significant role in the reactions of phosphorus transfer from these stable phosphorus compounds to the polyphosphates of the acid-insoluble fraction. There are experimental grounds to assume that ATP participates in this synthesis.

We think that perhaps polyphosphates may be regarded not only as a peculiar accumulator of energy and phosphate groups but as a mechanism of ADP regeneration as well. The content of this latter is not very high in a number of microorganisms, and a rapid regeneration of this compound is required, in aerobic conditions in particular, i.e. when most intensive polyphosphate accumulation actually takes place.

Under cultivation of mould fungi on various nutritive media, certain shifts in phosphorus metabolism are observed; thereby principal regularities of polyphosphate metabolism are preserved. On the other hand, a number of observations shows that under certain conditions of cultivation stable phosphorus compounds can be accumulated among other phosphorus compounds in some fungi and Actynomycetes. In some cases this may be phytin, in others — the presence of polysaccharides containing phosphate groups in their molecule ([21]) must not be overlooked.

In our laboratory the formation and metabolism of polyphosphates were studied on yeasts ([13, 18]), mould fungi ([14, 15]) and Azotobacter, thereby these different microorganisms show a number of features of polyphosphate metabolism in common.

In Fig. 5 those possible ways of polyphosphate metabolism are schematically shown which may be outlined on the basis of experimental data obtained in our laboratory.

A question arises, how can the fact be explained that polyphosphates are typical of lower organisms only. On the one hand, it may be connected with the extreme intensity of life tempo of microorganisms. On the other hand, the presence of poly-

phosphates may be restricted, in the whole, to microorganisms because this is one of the form of evolution of energy-donor systems. One may suppose, that inorganic polyphosphates, i.e. compounds which, due to their structure and properties acted as energy accumulators, were present in nature during the period

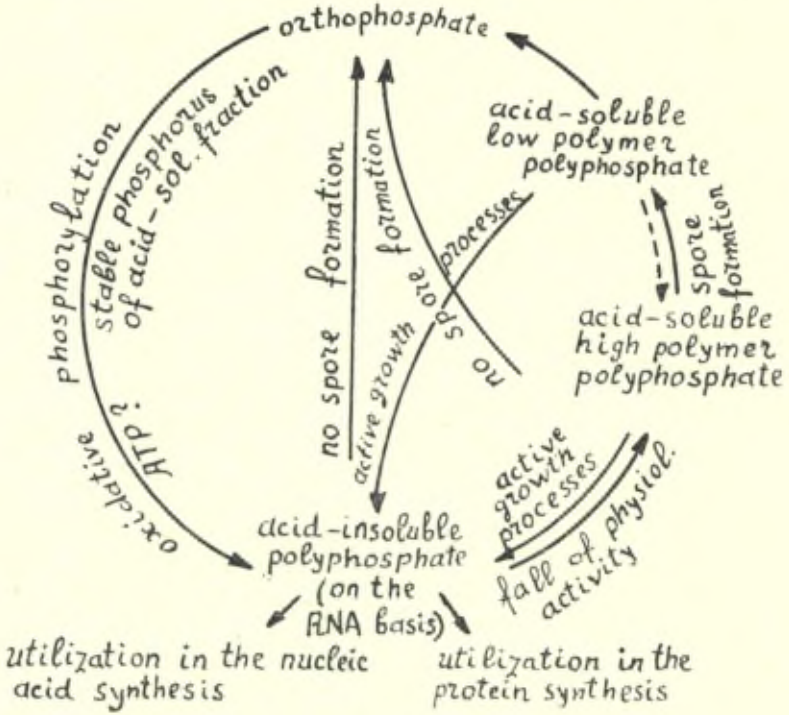


Fig. 5

of the formation of living matter. As a result of polyphosphate association with these or those organic compounds, precursors of the forthcoming energy donor systems could have risen which were being perfected by means of selection during the course of further development. That is why the fact that energy donor systems occur throughout the whole organic kingdom in the form of phosphoric acid derivatives or, more precisely, of polyphosphate derivatives, may be not fortuitous. Such a manner of energy accumulation may have been introduced in its time into organic kingdom from inorganic nature in the form of mineral polyphosphates.

The next problem I should like to dwell upon in my report concerns the specificity of nucleic acids of microorganisms. The works in this direction were carried out in our laboratory during recent years.

The problem of nucleic acid specificity was widely discussed in literature during the recent decade in most various aspects.

In our opinion, however, it is the question of the species specificity of this group of compounds which is the main task in the problem of nucleic acid specificity.

Species specificity of the DNA composition was found out by Chargaff on the basis of studying nucleotide composition in a relatively small number of objects very distant in their systematic position. Taking into consideration the paramount importance of the problem, a systematic investigation in this direction would be of interest in order to clarify, how far this specificity extends. On the other hand, it would be of interest to examine, how much the nucleotide composition of nucleic acids changes under the effect of hereditary variability of organisms. In our investigations we did not restrict ourselves to the study of nucleotide composition of the DNA only, but investigated that of the RNA in the same organisms as well. We thought it the more important and interesting as at that time only scarce data on the nucleotide composition of the RNA were available in literature so that no definite conclusions could be drawn on the RNA behaviour with regard to the species specificity of its nucleotide composition. Besides the parallel study of nucleotide composition of the DNA and RNA in the same species was of interest from the viewpoint of revealing correlation between the specific DNA and RNA composition both in the aspect of a species and under experimental variability.

At present the most suitable technique to determine qualitative changes of, or differences in, nucleic acids is the determination of the ratios of their purine and pyrimidine bases. Hence, we choose a comparative study of nucleic acid composition and the objects under study by means of the widely applied technique of quantitative paper chromatography in combination with ultraviolet spectrophotometry ([24, 25, 26]).

The study of the DNA and RNA composition was carried out in our laboratory without preliminary isolation of preparations, directly on the material under study. Such an approach to the study of the nucleotide composition of nucleic acid is necessary, due to the possible secondary changes of nucleic acid composition and, in particular, of the RNA composition during the isolation process ([27, 28]).

The data for Azotobacter were obtained in our laboratory by Zaitseva ([28]) and for all other bacterial cultures — by Spirin ([24, 25, 26]).

Before studying the nucleotide composition of the DNA and RNA in different systematic groups of bacteria, age variability of the DNA and RNA composition of E. coli and Azotobacter was studied. If the composition of these nucleic acids underwent a change during the development, investigation in systematic aspect would encounter difficulties, as it would be necessary to study cultures at just the same developmental stage. The data obtained in our laboratory in this respect ([25, 28]), as well as those appearing in literature, suggest the absence of age specificity of nucleic acids for a wide range of organisms, at any rate, with respect to the specificity of their composition.

The results obtained by us may also be connected with the problem of intracellular heterogeneity of the RNA with respect to its composition. RNA content is well known to undergo great quantitative changes during ontogenesis. In particular, in bacteria at the latent and logarithmic stages it is 1,5-3 times that at the stationary state. The constancy of composition of the total RNA obtained by us under considerable changes of its quantitative content may be observed, provided all the RNA molecules in the cell have about the similar ratio of nucleotides, i.e. if an expressed intracellular heterogeneity of the RNA molecules with respect to their composition is lacking.

Another supposition that there is a number of RNA fractions of different composition in the cell but that an identical equal decrease or increase of the RNA molecules takes place with age alterations in each of these fractions seems to us less likely as functionally different fractions must behave in a different manner.

The DNA and RNA nucleotide composition was studied in our laboratory in species respect on 21 bacteria species belonging to 9 different families by Bergey's classification ([26]) and on 6 Actinomycetes species ([8]).

The results of these analyses are set up in Tables I and II.

TABLE I

DNA composition in different bacteria.

SPECIES	Content of bases in molar per cent				Pur. / Pyr.	G + T / A + C	G + C / A + T
	G	A	C	T			
Clostridium perfringens	15,8	34,1	15,1	35,0	1,00	1,03	0,45
Staph. pyogenes aureus	17,3	32,3	17,4	33,0	0,98	1,01	0,53
Pasteurella tularensis	17,6	32,4	17,1	32,9	1,00	1,02	0,53
Proteus vulgaris	19,8	30,1	20,7	29,4	1,00	0,97	0,68
Escherichia coli	26,0	23,9	26,2	23,9	1,00	1,00	1,09
Proteus morganii	26,3	23,7	26,7	23,3	1,00	0,98	1,13
Shigella dysenteriae	26,7	23,5	26,7	23,1	1,01	0,99	1,15
Salmonella typhosa	26,7	23,5	26,4	23,4	1,01	1,00	1,14
Salmonella typhi-murium	27,1	22,9	27,0	23,0	1,00	1,00	1,18
Ervinia carotovora	27,1	23,3	26,9	22,7	1,02	0,99	1,17
Corynebact. diphtheriae	27,2	22,5	27,3	23,0	0,99	1,01	1,20
Azotobacter agile	28,3	21,4	26,5	23,8	0,99	1,08	1,21
Azotobacter vinelandii	27,4	22,1	28,9	21,7	0,98	0,97	1,28
Azotobacter chroococcum	28,7	20,5	28,5	22,2	0,97	1,04	1,34
Aerobacter aerogenes	28,8	21,3	28,0	21,9	1,00	1,03	1,31
Mycobacterium vadosum	29,2	20,7	28,5	21,6	1,00	1,03	1,37
Brucella abortus	29,0	21,0	28,9	21,1	1,00	1,00	1,37
Alcaligenes faecalis	33,9	16,5	32,8	16,8	0,98	1,03	2,00
Pseudomonas aeruginosa	33,0	16,8	34,0	16,2	0,99	0,97	2,03
Mycobacter. tuberculosis BCG	34,2	16,5	33,3	16,0	1,03	1,01	2,08
Sarcina lutea	36,4	13,6	35,6	14,4	1,00	1,03	2,57
Actinomyces globisporus streptomycini	36,1	13,4	37,1	13,4	0,98	0,98	2,73
Actinomyces globisporus flaveolus	36,3	13,8	37,2	12,7	1,04	0,96	2,77
Actinomyces griseus	35,8	13,8	37,4	13,0	0,97	0,96	2,73
Actinomyces viridochromogenes	36,6	13,3	37,2	12,9	0,99	0,98	2,80
Proactinomyces citreus	35,3	14,2	36,7	13,8	0,98	0,96	2,57
Micromonospora coerulea	36,2	14,3	35,6	13,9	1,02	1,04	2 53

Abbreviations : G = guanine; A = adenine; C = cytosine; T = thymine; Pur. = purine bases; Pyr. = pyrimidine bases.

Summing up the data on the DNA nucleotide composition in different bacteria species suggests, in the first place, that the DNA possesses an exhibited composition specificity; at the second

TABLE II

RNA composition in different bacteria.

SPECIES	Nucleotide content in molar per cent				Pur. / Pyr.	G + U / A + C	G + C / A + U
	G	A	C	U			
Clostridium perfringens . . .	29,5	28,1	22,0	20,4	1,36	1,00	1,06
Staphyl. pyogenes aureus . .	28,7	26,9	22,4	22,0	1,25	1,03	1,05
Pasteurella tularensis	29,8	27,3	21,0	21,9	1,33	1,07	1,03
Proteus vulgaris	31,0	26,3	24,0	18,7	1,34	0,99	1,22
Escherichia coli	30,7	26,0	24,1	19,2	1,31	1,00	1,21
Proteus morganii	31,1	26,0	23,7	19,2	1,31	1,01	1,21
Shigella dysenteriae	30,4	25,9	24,4	19,9	1,29	0,99	1,21
Salmonella typhosa	30,8	26,1	24,0	19,1	1,32	1,00	1,21
Salmonella typhi-murium . .	31,0	26,1	23,8	19,1	1,33	1,00	1,21
Ervinia carotovora	29,5	26,5	23,7	20,3	1,27	0,99	1,14
Corynebact. diphtheriae . . .	31,6	23,1	23,8	21,5	1,21	1,13	1,24
Azotobacter agile	31,0	24,2	26,0	18,7	1,23	0,99	1,33
Azotobacter vinelandii . . .	30,3	23,9	25,5	20,2	1,19	1,02	1,27
Azotobacter chroococcum . .	30,4	24,7	24,7	20,1	1,18	1,02	1,23
Aerobacter aerogenes	30,3	26,0	24,1	19,6	1,29	1,00	1,19
Mycobacterium vadosum . .	31,7	23,8	23,5	21,0	1,25	1,12	1,23
Brucella abortus	30,2	25,4	24,9	19,5	1,26	0,99	1,23
Alcaligenes faecalis	30,9	25,7	24,1	19,3	1,31	1,01	1,22
Pseudomonas aeruginosa . .	31,6	25,1	23,8	19,5	1,31	1,05	1,24
Mycobacter. tuberculosis BCG	33,0	22,6	26,1	18,3	1,25	1,05	1,45
Sarcina lutea	32,7	23,2	24,2	19,9	1,27	1,11	1,32
Actinomyces globisporus streptomycini	31,1	23,8	25,2	19,9	1,22	1,04	1,29

Abbreviations : G = guanylic acid; A = adenylic acid; C = cytidylic acid; U = uridylic acid; Pur. = purine nucleotides; Pyr. = pyrimidine nucleotides.

place, that the differences in the DNA composition in closely related species are usually significantly smaller than in those distant in systematic aspect; at the third place, the DNA nucleotide composition of bacteria drastically varies from species to species revealing all the diversity of types, from the extreme AT-type (e.g. Clostridium perfringens) up to the highest of the known GC-type (e.g. Actinomyces) with all the intermediate ratios.

In some cases the existence of though small, but appreciable differences in the DNA nucleotide composition may be shown in closely related species, sometimes even in species belonging to the same genus. It is very likely that in closely related species the differences in the nucleotide composition exist only among

some DNA molecules (if we admit the idea on the DNA heterogeneity in the same object to be true). In consequence of this the total DNA composition shows only small fluctuations which it is often difficult to reveal.

On the other hand, there is a possibility that the DNA molecules in closely related species, even with an identical nucleotide composition (e.g. S. typhosa and Shigella dysenteriae) may differ to this or other degree in the sequence of nucleotides.

Simultaneously with us, DNA nucleotide composition in a large group of bacteria was studied by Ki Yong Lee, Wahl and Barbu at Institut Pasteur in Paris, and the results and conclusions of both works closely agree ([29]).

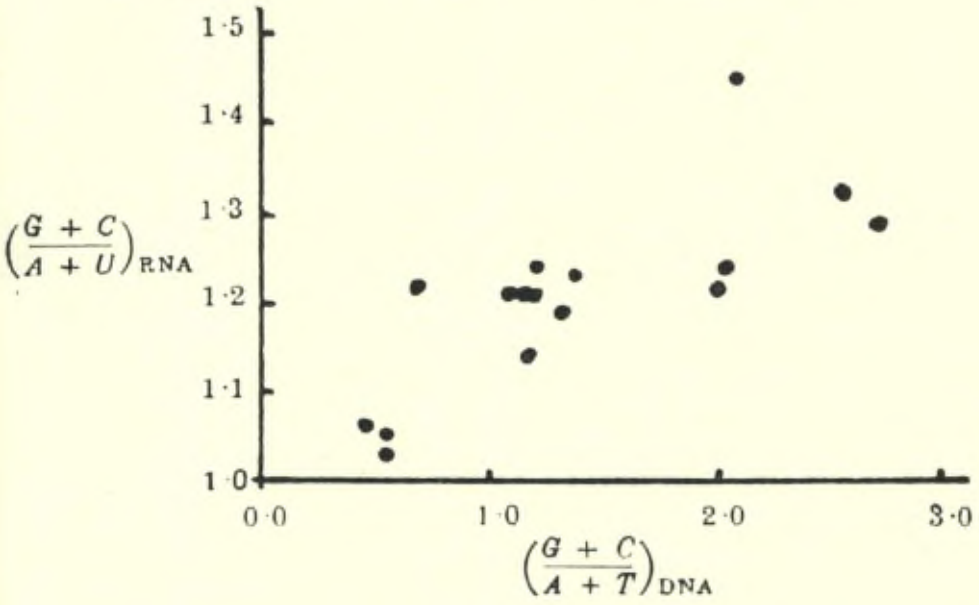
The data of the above authors, as well as our own data, suggest that DNA nucleotide composition due, on the one hand, to its specificity and, on the other hand — to a certain propinquity in closely related species may be used for the classification. A number of examples referred in our papers published earlier ([8, 26]) may actually show the taxonomic significance of the DNA nucleotide composition; there are grounds to hold this character for a very useful one for the establishing and precision of phylogenetic relations in bacteria.

Whereas the DNA nucleotide composition in bacteria varies widely, that of the RNA, as it follows from our data set up in Table II, is very similar in very distant species, and variations present are small ([26, 30]). All the bacterial RNA studied may be referred to GC-type irrespective of the type (GC- or AT-type) to which these or those bacteria belong by their DNA.

Thus, whereas with respect to DNA we may speak about a rather great specificity even when studying its total composition, with respect to RNA we may speak about the differences only in distant species and even then to a much smaller degree. However, in spite of the identity of the DNA composition in closely related species the possibility of the RNA specificity must not be overlooked, as the specificity may consist in a different nucleotide sequence. Nevertheless, propinquity of the nucleotide composition in different RNA favours a relatively smaller specificity of this nucleic acid when compared with the DNA. It is obvious however that definite, though small differences in the RNA com-

position do exist in distant species, i.e. that the RNA may to a certain degree also show the species specificity with regard to the composition. Species specificity of the RNA composition is restricted, however, only to species distant in systematic aspect.

Comparison of the DNA nucleotide composition with that of the RNA in species studied (cf. Tables I and II) shows the lack of an exhibited correlation in the nucleotide composition of these two nucleic acids. However, a mere examination of Table II suggest that the value $\left(\frac{G + C}{A + U}\right)$ still shows a general tendency towards an increase, if passing from the top of the Table downwards.



Fig, 6

This may be illustrated by the graph (cf. Fig. 6) were the values $\left(\frac{G + C}{A + T}\right)_{DNA}$ are plotted against those of $\left(\frac{G + C}{A + U}\right)_{RNA}$, each point corresponding to a species having characteristic values of the two ratios.

It should be noted that three species of Azotobacter were not included when analytical data were statistically treated, Nucleotide

composition of these three species was analysed by means of a somewhat modified technique.

A simple examination of this graph shows that all the species with lower ratio $\left(\frac{G + C}{A + U}\right)_{RNA}$ are grouped in the left part of the graph, i.e. they show also a lower ratio of $\left(\frac{G + C}{A + T}\right)_{DNA}$. The species with a higher ratio of $\left(\frac{G + C}{A + U}\right)_{RNA}$ are, on the contrary, grouped in the right part of the graph, i.e. they show also a high ratio of $\left(\frac{G + C}{A + T}\right)_{DNA}$. Thus, there exists a clearly seen positive shift of $\left(\frac{G + C}{A + U}\right)_{RNA}$ value on $\left(\frac{G + C}{A + T}\right)_{DNA}$ value, i.e. these two values exhibit positive correlation. The correlation coefficient of these two values is 0,72 with a mean error of 0,17; that is, $r = + 0{,}72 \pm 0{,}17$. The correlation coefficient is thus 4,3 times as large as its mean error which proves the reliability of the correlation found ([26, 31]).

Thus, with the increase of $\left(\frac{G + C}{A + T}\right)$ ratio in the DNA the value of $\left(\frac{G + C}{A + U}\right)$ ratio in the RNA somewhat increases. However, the $\left(\frac{G + C}{A + U}\right)_{RNA}$ value shifts thereby only for a very small interval with a significant change of $\left(\frac{G + C}{A + T}\right)_{DNA}$ value, i.e. the value of regression of $\left(\frac{G + C}{A + U}\right)_{RNA}$ on $\left(\frac{G + C}{A + T}\right)_{DNA}$ is very small in this correlation. That fact explains why one encounters difficulties in studying the differences in composition of the RNA in species differing drastically in the composition of their DNA. It should be noted that the positive correlation has been established on a relatively small number of different species of bacteria : further investigations are therefore necessary to show that this correlation is not fortuitous.

Nevertheless, our result suggests that part at least of RNA of the cell, and it seems to be a very small part which results from the very small value of the regression $\left(\frac{G + C}{A + U}\right)_{RNA}$ on $\left(\frac{G + C}{A + T}\right)_{DNA}$, may correlate by its composition with the DNA composition. On the other hand, the greatest part of the RNA of the cell may be thought to show no correlation with the DNA, and its composition is similar in different species.

In this connection the question arises whether we may unreservedly hold the opinion on the direct genetic connection of the DNA and RNA in the cell. Certainly, it is hard to think that with such a great difference in the DNA composition this latter will determine the synthesis of the RNA very identical by their composition. A possibility still exists thereby that some small part of the RNA, may be the functional analogue of the nuclear RNA, depends on the DNA, is determined by its structure or is directly transformed into it and vice versa. Still with respect to the main mass of the RNA in the cell we hold a possibility of direct transformations in the DNA and vice versa for a very unlikely one. The idea on the possibility of direct inter-transformations of these two nucleic acids during the development was widely accepted after Brachet's works carried out in the thirties, but for the main mass of the RNA in the cell the possibility of transformation does not directly correspond with the results obtained in our laboratory, at any rate, for the bacteria; perhaps, for a small specialized RNA fraction the above possibility should not be overlooked.

On the other hand, one may think that the part of the RNA correlating with the DNA may be the connecting link in the sense of the transmission of hereditary information from the DNA to some other, perhaps, protein substrates of the cell. The specific synthesis of the greater part of the RNA is independent of the DNA, and if a direct control of the DNA does exist it is realized indirectly, through some other links and substrates of the metabolism.

More strongly exhibited specificity of the DNA compared to that of the RNA may be well illustrated by the data obtained in our laboratory by Spirin when studying the DNA and RNA composition in some intestinal bacteria during the process of their experimental variability ([32]). In this work material obtained by Kudlai ([33]) was utilized. The above material represented hereditary stable, so called " saccharolytically inert " forms, which may be obtained from intestinal bacteria under unfavourable affects of different kinds. They may be divided in two groups absolutely differing in their properties. One group is represented by the so called " neutral forms " showing neutral reaction on peptone medium with carbohydrates; they appear to be identical with

TABLE III

The DNA composition of intestinal bacteria and forms experimentally obtained from them.

The form of bacteria	Content of bases in molar per cent				Pur. / Pyr.	G + T / A + C	G + C / A + T
	G	A	C	T			
Escherichia coli I							
(Initial)	26,0	23,9	26,2	23,9	1,00	1,00	1,09
Neutral form.	21,0	29,2	20,6	29,2	1,01	1,01	0,71
Alkali-producing form . .	33,3	16,7	33,8	16,2	1,00	0,98	2,04
Salmonella typhosa TY-2							
(Initial)	26,7	23,5	26,4	23,4	1,01	1,00	1,13
Neutral form.	21,9	28,2	21,7	28,2	1,00	1,00	0,77
Alkali-producing form . .	32,2	17,3	32,4	18,1	0,98	1,01	1,83
Shigella dysenteriae 913							
(Initial)	26,7	23,5	26,7	23,1	1,01	0,99	1,15
Alkali-producing form . .	32,8	17,0	33,2	17,0	0,99	0,99	1,94
Alkali-producing form . .	32,8	16,7	33,9	16,6	0,98	0,98	2,00

G-forms described in literature. The second group is represented by " alkali-producing forms ", which show alkali reaction on the same media. It is shown that under the formation of the above forms a drastic deviation of a whole number of properties takes place when compared to the initial culture : morphological, cultural, serological, biochemical, resistance to antibiotics and others. On the other hand, the forms belonging to one type are extremely similar by their properties, from whichever species of the intestinal bacteria they originate ([32]).

When comparing these data with the DNA composition presented in Table III, one may find a whole number of regular correlations.

Thus, all the initial intestinal bacteria are close by a number of their biological properties, with respect to resistance to antibiotics, they all possess one group antigene in common as one of

the main ones in the cell. This biological propinquity of them comes to a good agreement with the similarity of their DNA composition (feebly expressed GC-type, $\left(\frac{G + C}{A + T}\right) = 1{,}2$). The propinquity of alkali producing forms with respect to their properties is accompanied by the similarity of the DNA composition in alkali producing forms obtained by most different effects from different initial cultures (strongly expressed GC-type, $\left(\frac{G + C}{A + T}\right) = 2$). The same holds true also for the studied " neutral (G) form " (AT-type of DNA, $\left(\frac{G + C}{A + T}\right) = 0{,}7 - 0{,}8$).

On the other hand, radical changes of properties under the transformation of an intestinal bacteria, for example, in an alkali producing form, are accompanied by significant changes of the DNA composition. Thereby, changes of biological, serological and other properties being of one type, they are accompanied by changes of the DNA composition of the same kind. These data make once more draw the conclusion on the close connection between the DNA and specific hereditary characters of the cell.

From the data for the RNA presented in Table IV one may see that changes of the RNA nucleotide composition under the transformation of intestinal bacteria into " saccharolytically inert " forms are, on the contrary, small.

Nevertheless, the RNA composition of " neutral " (G) forms does still differ from that of initial and alkali producing forms. Hence, the composition of the total RNA may also change to a certain degree under certain hereditary changes, though much less than the DNA composition.

It is typical that under the transition to neutral form the RNA content changes towards the decrease of the ratio value $\left(\frac{G + C}{A + U}\right)$, i.e. in the same direction in which the DNA composition changes when transiting from the feeble GC-type to the AT-type. This is one more fact — now in the line of experimental variability — which prove the above shown positive correlation of the RNA composition with that of the DNA. This case, as well as the investigation of different species of bacteria, suggests that only a small fraction of the cellular RNA correlates with the DNA composition which results only in a small shift in the nucleotide composition of the total RNA under considerable changes in DNA.

Having found out that the DNA nucleotide composition in bacteria is characterized by an extremely high variability, we wanted to know how great are the shifts in the DNA nucleotide composition in the organisms belonging to other systematic groups.

TABLE IV

Nucleotide composition of the RNA in initial bacteria and in experimentally obtained forms.

The form of bacteria	Content of nucleotides in molar per cent				Pur. / Pyr.	G + U / A + C	G + C / A + U
	G	A	C	U			
Escherichia coli I							
(Initial)	30,7	26,0	24,1	19,2	1,31	1,00	1,21
Neutral form.	28,9	27,1	23,2	20,8	1,27	0,99	1,09
Alkali-producing form . .	30,4	25,8	24,1	19,7	1,28	1,00	1,20
Salmonella typhosa Ty-2							
(Initial)	30,8	26,1	24,0	19,1	1,32	1,00	1,21
Neutral form.	29,8	26,4	22,6	21,2	1,28	1,04	1,10
Alkali-producing form . .	30,7	25,7	24,3	19,3	1,29	1,00	1,22
Shigella disenteriae 913							
(Initial)	30,4	25,9	24,4	19,3	1,29	0,99	1,21
Alkali-producing form . .	30,5	25,8	23,8	19,9	1,29	1,02	1,19
Alkali-producing form . .	29,9	26,9	23,8	19,4	1,31	0,97	1,16

In this connection Serenkov ([24]) in our laboratory carried out a study of the DNA nucleotide composition in different algae. The results of this work are set up in Table V.

Analytical data presented in Table V show the DNA in the group of algae, as well as that in the bacteria to be characterized by a significant variability of its nucleotide composition. The representatives with strongly pronounced AT-type of the DNA and those the DNA of which belongs to the GC-type may be found here at the same time.

In this report I should like to refer only some data on the DNA nucleotide composition in higher plants obtained in our

TABLE V

DNA composition in different algae.

ALGAE	Content of bases in molar per cent				Pur./Pyr.	G + T / A + C	G + C / A + T
	G	A	C	T			
Diatomic							
Rhabdonema adriaticum .	18,6	31,4	18,3	31,7	1,00	1,01	0,58
Chaetoceras decipiens . . .	19,9	30,8	19,2	30,1	1,02	1,00	0,64
Thalassiosira Nordenscheldii	20,1	29,8	20,2	29,9	0,99	1,00	0,67
Green							
Hydrodictyon reticulatum .	27,3	23,1	26,2	23,4	1,01	1,02	1,15
Ankistrodesmus sp.	29,7	21,8	29,0	19,5	1,06	0,97	1,42
Scenedesmus quadricauda .	30,8	20,2	30,2	18,7	1,06	0,98	1,57
Scenedesmus accuminatus .	32,9	18,7	30,9	17,5	1,06	1,01	1,76
Brown							
Cystosira barbata.	29,5	20,8	29,3	20,4	1,01	0,99	1,42

laboratory by Uryson and Serenkov. Perhaps, it is not directly connected with the topic of my communication but I think, it can be of some interest from the view point of comparative biochemistry.

The ratios of bases in the DNA of various higher plants are presented in Table VI.

Various representatives of Mono- and Dicotyledones are set up in this Table. Thus, if our materials can not be regarded as complete in the sense of the characteristics of the higher plants kingdom, they give some idea on the fluctuation amplitude of the DNA nucleotide composition in this group of plants.

Examination of analytical data presented in Table VI made us draw the conclusion on the unusually small variability of the DNA nucleotide composition in higher plants. Indeed, whereas in different bacteria (cf. Table I) the specificity index, the ratio $\left(\frac{G + C}{A + T}\right)$, varies, according to our data, within the range from

0,45 to 2,80, and in algae (cf. Table V), from 0,58 to 1,76, in higher plants it varies from 0,58 to 0,94.

TABLE VI

DNA composition in various higher plants.

PLANT SPECIES	Content of bases in molar per cent					Pur./Pyr.	$\frac{G+T}{A+C+MC}$	$\frac{G+C+MC}{A+T}$
	G	A	C	MC	T			
Triticum vulgare . . .	23,8	25,6	18,2	6,4	26,0	0,98	0,99	0,93
Allium cepa	18,4	31,8	12,8	5,4	31,3	1,01	0,99	0,58
Pinus sibirica	20,8	29,2	14,6	4,9	30,5	1,00	1,05	0,67
Phaseolus vulgaris. . .	20,6	29,7	14,9	5,2	29,6	1,01	1,03	0,68
Arachis hypogaea . . .	20,3	29,3	14,4	6,1	29,8	0,99	1,05	0,69
Cucurbita pepo	20,8	30,2	16,1	3,8	29,0	1,04	0,99	0,69
Papaver somniferum. .	20,6	29,6	14,8	5,3	29,8	1,01	1,01	0,68
Corylus avelana. . . .	22,5	27,6	22,1	—	27,8	1,00	1,01	0,80
Salix caprea	20,3	29,8	19,9	—	29,9	1,09	1,00	0,68
Salix acubifolia	21,9	27,6	21,0	—	29,4	0,99	1,04	0,75
Alnus barbata	24,6	25,8	23,5	—	26,7	0,99	1,03	0,94

It should be noted that, according to data available in literature, variations in the DNA nucleotide composition in higher animals keep within the same range as in higher plants. Besides, higher plants and higher animals have the following features in common: their DNA belongs to AT-type and is characterized by the presence of the fifth nucleotide which contains 5-methylcytosine as the base. In animals the amount of this nucleotide is small and seems not to exceed 1,5-2 % of all the nucleotides, whereas its amount in higher plants makes up to 6-7 %.

What is the reason of such a small variability of the DNA nucleotide composition in higher forms when compared to micro-organisms, to bacteria in particular, remains an enigma up to the present time. At any rate, the DNA specificity seems to exist in higher organisms too, being due, perhaps, to the nucleotide sequence in the chain of the molecule.

This phenomenon may be connected with the monocellular structure and significant differentiation of species in bacteria in functional aspect, whereas in higher forms this is somewhat smoothed away.

It was mentioned above that the RNA in different bacteria is characterized by the extremely small variability of its nucleotide composition. In this connection we were interested in the problem, what variations of the RNA nucleotide composition may be expected in plant of other systematic groups.

In our laboratory a corresponding study of the RNA nucleotide composition in algae and higher plants was carried out by Vanyushin, Serenkov and Uryson. The results of these analyses are presented in Table VII.

TABLE VII

RNA composition in algae and higher plants.

ORGANISM	Content of nucleotides in molar per cent				Pur. / Pyr.	G + U / A + C	G + C / A + U
	G	A	C	U			
Algae							
Rhabdonema adriaticum .	28,6	24,0	26,2	21,2	1,11	0,99	1,21
Chaetocerus decipiens . . .	27,7	25,7	24,4	22,2	1,15	0,99	1,09
Thalossiosira Nordenscheldii	31,3	24,1	25,2	19,4	1,24	1,03	1,30
Hydrodictyon reticulatum .	30,1	23,2	26,3	20,4	1,14	1,02	1,29
Ankistrodesmus sp.	30,5	22,8	25,1	21,6	1,14	1,09	1,25
Scenedesmus quadricauda .	30,1	23,6	26,0	20,3	1,16	1,02	1,28
Scenedesmus accuminatus .	30,1	23'2	25,1	21,6	1,14	1,07	1,23
Higher plants							
Equisetum sp. (spores) . .	29,1	26,4	23,8	20,7	1,25	0,99	1,12
Triticum vulgare (germs) .	29,6	24,5	25,8	20,1	1,18	0,99	1,24
Zea mays (germs)	29,2	24,7	25,6	20,5	1,17	0,99	1,21
Eucommia ulmoides (pollen)	29,0	24,9	24,9	21,2	1,17	1,01	1,17
Hamamelis japonica (seeds)	29,6	26,2	24,5	19,7	1,26	0,97	1,18
Brassica oleifera (seeds) . .	29,4	25,3	24,8	20,5	1,21	1,00	1,18
Phaseolus vulgaris (sprout).	31,4	24,9	24,1	19,6	1,26	1,04	1,25
Arachis hypogaea (sprout).	30,9	25,3	24,6	19,2	1,28	1,01	1,25

The data presented in Table VII suggest that the RNA both in algae and in higher plants is very close by its nucleotide composition; if some variations are present, they are very small and lacking any regular character in connection with the systematic position of these or those groups of organisms. More than that,

comparison of these data with the results of the RNA analyses in bacteria (cf. Table II) shows the RNA of the whole plant kingdom to belong to the GC-type and to be very close by its nucleotide composition.

In this connection it should be admitted that the study of the nucleotide sequences in the chain of the RNA molecule is one of very important questions. Up to the solution of this problem our judgements with respect to the physiological role of this nucleic acid will be restricted to hypotheses and speculations.

I should like to note as one of the conclusions of our work that a relatively large experimental material obtained in our laboratory on the nucleotide composition of the DNA and RNA completely corroborates those principal regularities of nucleotides interrelations which were found by Chargaff for these two nucleic acids.

When analysing experimental data obtained in our laboratory on the nucleotide composition of the DNA and RNA and when comparing them with other facts described in literature, a whole number of considerations of a general character does rise.

Proceeding from a relatively constant nucleotide composition of the RNA and significant changes in the DNA nucleotide composition in connection with the differentiation and isolation of these or those groups of bacteria, a suggestion was put forward by us at the Symposium on the Origin of life on the Earth, held in Moscow in 1957 that the rise of the ribonucleotides and of the RNA was prior with respect to the DNA. DNA arose much later and parallel with the complication of functions and ever greater differentiation of the protoplasm ([25]). Besides, a number of arguments was presented in favour of the RNA priority which suggest that the RNA is connected with more general activity manifestations, whereas the rise of the DNA is connected with the formation of narrower and phylogenetically later properties of organisms. In compliance with this the RNA seems to be connected with those mechanisms of heredity which provide the transmission of general links of metabolism, whereas the DNA is connected with the mechanisms providing the transmission of narrower and more specialized properties of the organism which

rise in connection with the differentiation and isolation of species. Thus, we are convinced that the sum of hereditary properties is connected with the cell as a whole.

This conception results not only from a number of various data obtained in the field of biochemistry but it finds a support in extremely interesting works of nice performance carried out in Danielli's laboratory on the nucleus transportation from one amoeba species into the cytoplasm of another amoeba species.

If nucleic acids are important and powerful material factors of heredity, this function of them is realized, in our opinion, in the interaction with the protein substrates of the cell.

LITERATURE CITED

(1) A.N. Belozersky, G.N. Zaitseva, L.P. Gavrilova and L.V. Minayeva : *Mikrobiologia*, **26**, 409 (1957).

(2) G.N. Zaitseva and A.N. Belozersky : *Mikrobiologia*, **26**, 722 (1957).

(3) G.N. Zaitseva and A.N. Belozersky : *Biokhimia*, **24**, 1 (1959).

(4) A.S. Spirin, A.G. Skavronskaya and A. Pretel-Martines : *Mikrobiologia*, **27**, 273 (1958).

(5) P.C. Caldwell and C.J. Hinshelwood : *J. chem. Soc.*, 1415 (1950); *J. chem. Soc.*, 3151 (1950).

(6) N.S. Demyanovskaya and A.N. Belozersky : *Biokhimia*, **19**, 688 (1954).

(7) M.A. Guberniyev, N.A. Ugoleva and L.I. Torbochkina : *Antibiotiki*, No. 3, (1956); No. 6 (1956).

(8) A.N. Belozersky, N.V. Shugayeva and A.S. Spirin : *Dokl. Akad. Nauk SSSR*, **119**, 330 (1958).

(9) H. Venner : IV International Congress of Biochemistry, Vienna (1958).

(10) A. Yoshida : *J. Biochem.*, **42**, 381 (1955).

(11) R. Chayen, S. Chayen and E.R. Roberts : *Biochim. biophys. acta*, **16**, 117 (1955).

(12) J.M. Wiame : *J. Biol. chem.*, **178**, 919 (1949).

(13) A.N. Belozersky : " On metaphosphate-nucleic complexes of yeasts and on the chemical nature of volutine ". Report read at the 3-rd Inter. Congr. of Biochem. in Brussels (1955).

(14) A.N. Belozersky and I.S. Kulayev : *Biokhimia*, **22**, 29 (1957).

(15) I.S. Kulayev and A.N. Belozersky : *Biokhimia*, **22**, 587 (1957).

(16) P. Krishnan, S. Damle and V. Bajaj : *Arch. of Bioch. Bioph.*, **67**, 1 (1957).

(17) I.S. Kulayev and A.N. Belozersky : *Dokl. Akad. Nauk SSSR*, **120**, 1080 (1958).

(18) J.P. Ebel : IV Internat. Congress of Biochemistry, Wien (1958).

(19) E. Bukhovich and A.N. Belozersky : *Biokhimia*, **23**, 254 (1958).

(20) A.N. Belozersky : *Mikrobiologia*, **10**, 185 (1941).

(21) A.N. Belozersky and I.B. Naumova : *Dokl. Akad. Nauk SSSR*, **115**, 957 (1957).

(22) G.N. Zaitseva and A.N. Belozersky : *Mikrobiologia*, **27**, 308 (1958).

(23) A.N. Belozersky : Report read at the IVth Intern. Congr. of Biochem. in Vienna, Moscow (1958).

(24) A.S. Spirin and A.N. Belozersky : *Biokhimia*, **21**, 768 (1956).

(25) A.S. Spirin, A.N. Belozersky and A. Pretel-Martines : *Dokl. Akad. Nauk SSSR*, **111**, 1297 (1956).

(26) A.S. Spirin, A.N. Belozersky, N.V. Shugayeva and B.F. Vanyushin : *Biokhimia*, **22**, 744 (1957).

(27) D. Elson, T. Gustafson and E. Chargaff : *J. Biol. chem.*, **209**, 285 (1954); D. Elson and E. Chargaff : *Bioch. et Biophys. acta*, **17**, 367 (1955).

(28) G.N. Zaitseva and A.N. Belozersky : *Mikrobiologia*, **26**, 722 (1957).

(29) Ki Yong Lee, R. Wahl and E. Barbu : *Ann. Inst. Pasteur*, **91**, 212 (1956).

(30) A.S. Spirin and A.N. Belozersky : *Dokl. Akad. Nauk SSSR*, **113**, 650 (1957).

(31) A.N. Belozersky and A.S. Spirin : *Nature*, **182**, 111 (1958).

(32) A.S. Spirin, A.N. Belozersky, D.G. Kudlai, A.G. Skavronskaya and V.G. Mitereva : *Biokhimia*, **23**, 154 (1958).

(33) D.G. Kudlai : *Variability of microorganisms*, 2, 54, 133, 146, 186, 192, 202 and 211, Moscow (1958).

(34) G.P. Serenkov : *Vestnik MGU*, No. 3 (1957).

(35) A.N. Belozersky : Report read at the Symposium on the Origin of Life on the Earth, Moscow (1957).

DISCUSSION DU RAPPORT DE M. BELOZERSKY

M. Belozersky n'ayant pu, pour raison de santé, participer à la réunion, MM. Watson et Brachet ont bien voulu introduire son rapport. Le texte de celui-ci ayant été préalablement communiqué aux participants, la discussion a pu en être faite. Après la réunion, les textes des interventions ont été communiquées à M. Belozersky pour lui donner l'occasion d'y répondre par écrit. Sa réponse est reproduite à la fin de la discussion.

M. Watson. — I shall introduce Prof. Belozersky's paper commenting on several very striking facts reported by Belozersky and Spirin. Firstly there is the fact that the RNA base ratios are remarkably similar in all cells. This suggests to me that perhaps it serves a common fonction which may in large part be the synthesis of proteins, common to all cells. In this categories I include the proteins associated with cytoplasmic ribonucleic acid particles, cellular lipoprotein membranes, etc. It does not seem inconceivable to me that such proteins account for 30-50 % of all cellular proteins. I would also deduce from the constancy of base ratios that the " code " relating RNA nucleotide sequence to amino acid sequence is common in all cells. There is much greater difficulty in understanding how the base ratios in DNA can vary so greatly, a variation which Doty and collaborators has shown to extend to the level of almost all DNA molecules within bacterial cells. We would guess that many of the bacterial enzymes must be common and might contain roughly similar amino acid sequences. It is thus surprising to find that the DNA base sequence within homologous " genes " may be very different. This opens the unexpected possibility that the translation of " information " from DNA to RNA may not be either simple or universal.

M. Brachet. — I wish to make a few points about Prof. Belozersky's paper.

1) There is a marked RNA synthesis during the lag phase in Azotobacter, as already shown in Casperson's laboratory long ago.

This leads to the following questions : is there concomitant RNA and protein synthesis only in dividing cells? It is possible that the two processes are independent in non-dividing cells (resting bacteria, liver, pancreas).

2) In the cases where Prof. Belozersky found a big drop in the DNA content during the life cycle, one would like to know whether this is true for the DNA per cell. In eggs, for instance, the DNA content per cell is normal; but this DNA is diluted by the large amount of cytoplasm; if the estimations are expressed in percent there is almost no DNA in these eggs.

3) I hope that the interesting problem of the association of polyphosphate with RNA will be discussed by the audience. We observed often, with cytochemical methods, an increase in polyphosphates in injured cells. This might be due to reduced utilization of the polyphosphates for synthetic purposes or perhaps to an insufficient ADP content in theses cells : they would accumulate polyphosphates instead of marking ATP.

4) Regarding my old RNA → DNA conversion hypothesis, I agree with Prof. Belozersky's conclusion : there is no massive conversion, but we cannot exclude the possibility that it occurs on a lesser scale.

Two of my coworkers, Miss Tencer and M. Bielavsky, recently found that dissociated cells of amphibian eggs incorporate labelled cytidine exclusively into DNA during cleavage RNA's as well.

5) Concerning the idea that RNA is phylogenetically older than DNA, I expressed it at the same time as Prof. Belozersky. But I must frankly say that I don't know!

6) The idea that the cell as a whole is important in heredity is identical with the old suggestion, made by Albert Brachet, that one should distinguish, especially in the case of embryogenesis, between a general (cellular) and a special (chromosomal, mendelian) heredity.

M. Ochoa. — It is possible that, in spite of the similar base composition of RNA in different bacterial cells, the nucleotide sequences vary. It is known that RNA's from different strains of Tobacco mosaic virus (TMV) have the same base composition and their different specificity must depend on different nucleotide sequences.

The recent work of Reddi, at the Berkeley virus Laboratory, does in fact suggest that the nucleotide sequences vary in RNA's from different strains of TMV.

M. Moore. — In discussing the mechanism of protein synthesis in bacteria, Dr. Watson has referred to the probability that the proteins in different species of bacteria are the " same ", whereas, in the present state of our knowledge, he probably means that they are " similar ".

M. Watson. — Il should qualify my remarks to suggest that a large fraction of the proteins are similar, that is, their amino acid composition are about the same and so it may not be surprising that Belozersky has noted that the RNA contents of all cells are remarkably alike.

Sir Alexander Todd. — I would point out that the word " same " in Watson's RNA-protein picture is not to be taken too seriously. It only means that in general the gross compositions are alike — it does not mean they are the same in detail. Sequence of residues could be and of course, probably are quite different. Surely all that is emphasised is that the DNA's differ in gross composition and the RNA's are apparently very similar.

M. Schramm. — The small variation in the composition of RNA may be due to the fact, that a mixture of nuclear and cytoplasmic RNA is analysed. There are some data published by Chargaff suggesting that the base compositions of DNA and nuclear RNA are related.

Further this correlation may be obscured by the fact that we have a constant number of genes and therefore DNA molecules in each cell, whereas the amounts of different RNA's vary considerably depending on the metabolic state of the cell.

Volkin showed a correlation between the base composition of phage DNA and the incorporation of the corresponding bases in the specific RNA formed in the infected cell.

M. Davidson. — In the case of bacteria it is of course usually impossible to distinguish between nuclear and cytoplasmic RNA or even to know whether nuclear RNA exists. But in mammalian

cells the nuclear RNA can be subdivided into at least two fractions differing in rates of incorporation of labelled precursors and probably also in base ratios. There may be many nuclear RNA's. The important point is not to refer to nuclear RNA as if it were a single entity.

Sir Alexander Todd. — Is it possible that a small amount of the RNA — the nuclear RNA (or what in a bacterium corresponds to the nuclear RNA of higher organisms) is produced on the DNA templates but that some other mechanism than direct replication on the DNA or nuclear RNA template is involved in the main cytoplasmic RNA synthesis? What is the mechanism of cytoplasmic RNA synthesis? In general such ideas would not necessarily conflict with Belozersky's data.

M. Rich. — Belozersky's work shows that the RNA composition weakly reflects the change in composition of DNA. This weak dependence would arise if the DNA produced directly about 15-20 % of the cytoplasmic RNA, and the rest was produced in a non-specific manner as by polynucleotide phosphorylase. In this regard, it is worth wondering whether it is possible to vary the RNA composition by varying the environment of the cells.

Mme De Deken-Grenson. — In relation to the question of Dr. Rich, there is a paper by Brawerman and Chargaff which shows that environmental changes can bring about important changes in the composition of the RNA : when the green flagellate Euglena is cultivated in the dark instead of in the light the bases ratio of the RNA is altered in the same manner as it is in a white mutant.

M. Butler. — With reference to Prof. Davidson's point that in bacteria it is not possible to distinguish between nuclear and cytoplasmic RNA, I might mention the work of my colleagues Drs. Hunter and Crathorn, who have been investigating *Bacillus Megatherium* (*Biochem. J.*, **69**, 544, 1956). This organism consists of a cell wall, which is easily removed by lysozyme digestion, leaving a protoplast, which can be divided into a " membrane fraction " and cytoplasm. Both membrane and cytoplasm countain RNA; the former about one third and the latter two thirds. The membrane fraction is metabolically active in protein synthesis and is the initial receptor of added amino acids. It would be interesting to be able to report

their nucleotide compositions, but unfortunately I do not have that of the smaller membrane constituent. I might add that Mrs. Sutherhand has found that the cytoplasm is the active initial site of nucleic acid (RNA and DNA) synthesis.

M. Davidson. — Bacteria which readily yield protoplasts, of course, form a special case. In relation to mammalian cells there is a school of thought which maintains that at least some cytoplasmic RNA is synthesized in the nucleus and is secreted, as it were, into the cytoplasm. Prof. Brachet is well qualified to express an opinion on this point.

M. Brachet. — It is now generally thought that part of the cytoplasmic RNA is of nuclear origin. On the other hand, there is no doubt that anucleate fragments of *Acetabularia* are the site of intensive RNA turnover. We have some indications for the view that in this organism, net RNA synthesis occurs, in the absence of the nucleus, in the chloroplasts and that this RNA synthesis is compensated by a drop of the RNA present in the other fractions. If so, the RNA present in these fractions would be more dependent upon the nucleus than chloroplastic RNA. In any event, net synthesis of proteins, including enzymes, occurs in anucleate pieces of *Acetabularia*.

M. De Duve. — There is a basic difference between DNA on one hand and RNA and protein on the other. It seems possible that cells contains approximately the same number of each species of DNA molecules, corresponding to the various genes. Therefore, the average composition of DNA may be considered as a true average. This is certainly not the case for the proteins, of which some are represented by a much greater number of molecules than others. The average composition of the proteins is therefore weighted in favour of the more abundant ones. The same is probably true of the RNA and correlation between the average compositions of DNA and RNA should therefore not necessarily be expected.

M. Markham. — I do not think that Belozersky's figure 6 shows that there is only a poor relationship between his ratios for DNA and RNA components. The fit, though not perfect, is quite good.

M. Fraenkel-Conrat. — There seems to me no valid reason to assume that the occurence of identical enzymatic function suggests the occurence of identical proteins in different species. Therefore, one might not need to expect the occurence of detectable amounts of identical « genes », i.e. ultrasonic DNA fragments, as studied by density gradient centrifugation.

Sir Alexander Todd. — What is the dilemma? (1) Surely it exists only if one sticks to the simple template idea of DNA → RNA → protein. If one is prepared to accept nuclear RNA being made on the DNA template and the cytoplasmic RNA being made by another mechanism merely under control of the nuclear RNA or protein e.g. via production of enzymes rather like polynucleotide phosphorylase, then the « dilemma » disappears. We must be careful not to get too wedded to simple theories of replication or coding.

M. Watson. — I completely agree and should also like to emphasize the shaky basis for most of the coding arguments.

Mme Grunberg-Manago. — I would like to add some information to the observation presented by Dr. Watson in which he indicated that the soluble RNA is different from the particulate RNA especially with respect to its high content of odd bases. With the exception of soluble RNA, all the RNA's studied by us, including RNA of microsomes, yeast RNA and TMV-RNA, are phosphorolysed 100 % by polynucleotide phosphorylase. Soluble RNA is only phosphorolysed to the extent of about 40 %, which may perhaps be due to its high content of odd bases.

I would also like to report on some results obtained by Dr. Ebel which Dr. Brachet mentioned in his discussion. These results relate to the existence of polyphosphate-RNA complexes.

When yeast is grown on a medium containing no inorganic phosphate, there is no synthesis of polyphosphates and the RNA has a ratio of P to nucleotide of 1. If the yeast is initially grown on a

(1) Allusion à la difficulté soulevée par M. Watson dans son introduction. Dans l'hypothèse d'une traduction simple et universelle de l'« information » de l'ADN vers l'ARN, comment concilier le fait que les rapports des bases des ADN varient dans de si larges limites avec la constance relative des rapports des bases des ARN et avec la similitude (supposée) de la composition des protéines remplissant les mêmes fonctions dans des organismes différents. Voir à ce sujet : N. Sueoka, J. Marmur et P. Doty, *Nature*, **183**, 1429-1431 (1959).

medium containing no inorganic PO_4 and then transferred to a medium containing glucose and inorganic PO_4 but no N_2 source, there is an active synthesis of polyphosphates and the RNA extracted from this yeast contains a high excess of PO_4, in a ratio of 6 PO_4 to 1 nucleotide, the excess PO_4 being present as bound polyphosphates.

When synthetic polyphosphates are subsequently added to yeast previously grown on a medium containing limited PO_4, the RNA extracted has a ratio of P to nucleotide of about 1.3, very close to the one observed in the first case, suggesting that the RNA-polyphosphate complex formation is not due to simple adsorption. The linkage of the polyphosphate-RNA complex seems to be very labile. For example, chromatography on paper or electrophoresis at pH 2, 4.5 or 10, cause a separation of the polyphosphates from the RNA. Dr. Belozersky reports that this does not occur in his case. However, Dr. Ebel feels that the labile complex of polyphosphates and RNA may be due to hydrogen bonding. Polyphosphates are strong inhibitors of hexokinase. The inhibition by synthetic polyphosphates is decreased in the presence of RNA; the RNA-polyphosphate complex inhibit to a still lesser extent.

Sir Alexander Todd. — Is the linear nature of the polyphosphates discussed by Dr. Grunberg-Manago and by inference those of Dr. Belozersky really established? I am not entirely happy about the structures usually assigned to inorganic polyphosphates.

M. Wiame. — La difficulté réside en partie en ce que la chromatographie des polyphosphates longs est restée non résolue jusqu'à présent.

Mme Grunberg-Manago. — Dr. Ebel has developed a solvent system (Isopropanol, 60 ml; water, 40 ml; TCA, 5 g) which separates RNA from polyphosphates; RNA remains at the origin, while long chain polyphosphates migrate.

M. Fraenkel-Conrat. — Mrs. Grunberg-Manago was asked whether the « natural » binding of polyphosphate by yeast RNA might be through divalent metals, and might then be abolished by versene or other chelating agents. Attention is drawn to the finding that inorganic phosphate, presumably monomeric, is bound by

calcium or magnesium ion-treated TMV-RNA, and removable only together with the divalent ion.

M. Markham. — (Référence au point 4 de l'exposé de M. Brachet.) In Hurwitz's experiments the (ribo) cytidine residues are incorporated into the polynucleotide adjacent to nucleoside residues containing deoxyribose. They have then been isolated in the form of dinucleotides having both ribose and deoxyribose in their structure.

M. Brachet. — I quite agree with Dr. Markham that, in the experiments of Tencer and Bieliavsky, it has not been proved that the cytidine which has been incorporated into DNA has been transformed into a deoxy compound. It will be difficult to prove it, since this work is autoradiographic so far (and there are many snags in this method) and since the material (amphibian eggs) is unfavorable for chemical analysis of DNA. We hope to be able to extend this work to see urchin eggs, in which it could be easier to isolate DNA and to analyse it in details.

M. Rich. — Nature seems to be able to differentiate between the two chains of DNA, as we can see in the small bacteriophage Φ X 176. May be the plant viruses, such as TMV, are analogous in that they are single stranded and may be the products of a system of complementary replication, presumably just as the Φ X 176 arises from complementary replication of DNA.

Sir Alexander Todd. — On the simple replication theory the production of the virus RNA's which are single stranded molecules is really a difficulty — much greater in my opinion than one posed by Dr. Watson. For unless one has a very unusual base sequence in the viral RNA chain there must always be two different chains in production of which only one — so the virus experts would have us believe — is actually combined with the protein in the complete virus.

M. Watson. — Dr. Schramm now has data showing that there is a large accumulation of infectious TMV-RNA before a significant appearence of infectious virus particles. It thus seems unlikely to me that the virus protein preferentially choses one of the complementary RNA chains.

M. Ochoa. — It is unfortunate that the cellular localisation of bacterial polynucleotide phosphorylase cannot be ascertained, since it is of considerable importance to know whether the enzyme is localized in the nucleus or the cytoplasm. Hilmoe and Heppel, however, have found a polynucleotide phosphorylase — like enzyme — in liver nuclei. It is possible, therefore, that this kind of enzyme, the only one known so far to bring about a net synthesis of RNA, is localized in the nucleus. This would be in line with the fact that the cell nucleus plays an important role in RNA synthesis.

Sir Alexander Todd. — With reference to Dr. Watson's comment on the three types of RNA found in cells, I believe it is alleged by Lipmann and others that the « soluble RNA » is concerned with what is called « amino-acid activation ». The actual protein synthesis is on this view presumably done by either or both of the other types of RNA i.e. those with higher molecular weight (i.e. sedimentation constants). The difficulty of similar composition of these RNA's from different organisms therefore remains unchanged.

M. Ochoa. — I should like to put emphasis on Sir Alexander Todd's suggestion that the nature of cytoplasmic RNA may be controlled by RNA from the nucleus whose nature may have been determined by DNA. Present evidence suggests that RNA (or an oligoribonucleotide which is firmly bound to the enzyme) may have some controlling influence on the nature of the RNA synthetized by polynucleotide phosphorylase. DNA, on the other hand, has so far not been found to be a primer for this enzyme.

M. Belozersky (text sent after the meeting). — First of all I would like to express my gratitude to Professors Brachet and Watson who undertook to do the work of acquainting the participants of the Symposium with my paper. I am also much obliged to all those having taken part in the interesting and useful discussion of it.

In addition to the data on the small variability of nucleic acid composition of RNA which were presented in my paper, I would like to note that recently Dr. Vanyushin of my laboratory has undertaken to study nucleic acid composition of RNA in 29 species of higher plants which belonged to 5 types, 6 classes, 23 orders and 25 families. The result obtained was the same : $\frac{G+C}{A+U}$ ratio varied

only within the range from 1.06 to 1.31 (Doklady Akademii Nauk SSR, **127**, No. 2, 1959).

Some of the comments to my paper touched in this or other form upon the subject of intracellular heterogeneity of RNA. I briefly dwelled on this subject in my paper where on the basis of both data available in literature and my own investigations I draw the conclusion that the existence of a great RNA heterogeneity with respect to its composition is hardly to be expected. However, it does not rule out that there may exist in one cell the RNA molecules with different nucleotide sequences and perhaps with other structural differences. Prof. Ochoa's reference to the work of Dr. Reddi gives grounds to believe that the different sequences are actually possible. The virus RNA's, however, may have some peculiar structural features, for example, regularities of nucleic acid ratios found for RNA of cellular organisms can not be observed here. At any rate, the problem of the nucleotide sequences in RNA chain is one of the most actual problems.

Different metabolic activity of several RNA fractions does not imply in itself that these fractions are characterized by differences in their RNA. This may be due to some other reasons, first of all to different state of RNA itself in the cell. In particular, RNA metabolic activity may be undoubtedly considerably affected by its complex formation with various proteins.

As to the effect of the conditions of existence of organisms upon RNA composition, the analysis of corresponding data available in literature shows uniformly that RNA composition does not depend on the age of culture as well as on the conditions of culturing and functional state of microbial cells. The sole exception to this makes the recent paper of Brawerman and Chargaff. The difference of RNA composition found by these authors in green and etiolated cells of Euglena gracilis may represent a special case which is presumably associated with the formation of specific RNA molecules during the development of chloroplasts which are connected with the photosynthesis.

As to polyphosphate-RNA complexes, Drs. Spirin and Antonov in our laboratory recently obtained some indications on the possibility of artificial formation of polyphosphate complexes with RNA. This formation of complexes seems to take place even at the simple

mixing of the solutions of polymer polyphosphate and RNA at a low ionic strength and non-acid pH, i.e. under the conditions of extended configurations of both polymers. Following complex formation, both polymers twine very closely. No principal differences of these complexes are found from those obtained by extraction of cells of lower organisms. A number of data gives evidence both against hydrogen bonds between RNA and polyphosphate, and against decisive participation of bivalent or heavy metals.

There are some grounds to believe that the bonds in complexes may be not purely chemical, i.e. they are formed not between several chemical groups of polyphosphate and RNA chains, but they are rather a result of the interaction of both polymers at macromolecular level. This may be exemplified by the following supposition : a polyphosphate molecule having considerable affinity to metal cations, univalent ones including, it will accept cations forming ionic spheres of RNA molecules at low ionic strength of the solution, thus getting a positive charge. As a result of the arising electrostatic attraction between the polyphosphate and RNA particles, their molecules come into a close contact, and, due to the mutual shading of charges (in particular in the case of phosphate group shading by RNA bases), coiling of chains does take place. The resulting molecular coil can prove to be rather stable on the account of twining of chains. These but preliminary data indicate that the problem of the presence of polyphosphate-RNA complexes in a living cell is a very complicated one and still far from being solved. Its solution seems to require the development of new methods and new approaches of direct finding out the presence of complexes in the cell itself.

BIOSYNTHESIS OF RIBONUCLEIC ACID

by **SEVERO OCHOA**

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The mechanism of biosynthesis of the nucleic acids remained obscure until 1955 when the announcement was made (Grunberg-Manago and Ochoa, 1955) that an enzyme isolated from bacterial sources catalyzed the synthesis of ribopolynucleotides from nucleoside 5′-diphosphates. The reaction was shown to be reversible and to lead to a phosphorolytic cleavage of ribopolynucleotides with formation of nucleoside 5′-diphosphates. It is, therefore, analogous to the reversible synthesis and breakdown of polysaccharides catalyzed by phosphorylase and, for this reason, the enzyme was named polynucleotide phosphorylase.

Polynucleotide phosphorylase catalyzes the synthesis of ribopolynucleotides of high molecular weight; these can contain either one or several kinds of mononucleotide units. From a mixture of the 5′-diphosphates of adenosine, guanosine, uridine, and cytidine, the enzyme catalyzes the synthesis of polynucleotides containing all four nucleotide units present in natural RNA (*). Chemical and enzymatic degradation of the biosynthetic polynucleotides, carried out

(*) Abbreviations : ribonucleic acid, RNA; deoxyribonucleic acid, DNA; synthetic polynucleotides of adenylic, guanylic, uridylic, or cytidylic acid, poly A, poly G, poly U, or poly C; copolymer of adenylic and uridylic acids, poly AU; synthetic ribonucleic acid, poly AGUC; 5′-monophosphates of adenosine, guanosine, uridine, or cytidine, AMP, GMP, UMP, or CMP; 5′-disphosphates (pyrophosphates) of the above nucleosides; ADP, GDP, UDP, or CDP; adenine containing di-, tri-, or tetranucleotides bearing monoesterified phosphate at C-5′, pApA, pApApA, or pApApApA; trinucleotide containing two adenosine residues and a terminal uridine residue, with monoesterified phosphates at the C-3′ of the uridine unit and the C-5′ of the initial adenosine, pApApUp.

in collaboration with Dr. L. A. Heppel, of the National Institutes of Health, has shown that they are made up of 5′-nucleoside monophosphate units linked to one another through 3′-phosphoribose ester bonds as in RNA. Present information on structure size, X-ray diffraction patterns, and behavior toward different enzymes, indicates that the polynucleotides synthesized by polynucleotide phosphorylase are closely related to RNA. Indeed, the polynucleotide containing adenylic, guanylic, uridylic, and cytidylic acid residues is indistinguishable from RNA.

The isolation and properties of polynucleotide phosphorylase from *Azotobacter vinelandii* and the preparation, structure and properties of biosynthetic polynucleotides have been described in several publications (Grunberg-Manago *et al.*, 1955, 1956; Ochoa, 1956; Ochoa and Heppel, 1957; Heppel, Ortiz and Ochoa, 1957). Therefore, while reviewing briefly the general aspects of the problem, this paper will deal in more detail with such topics as the distribution of polynucleotide phosphorylase, the relation of the biosynthetic polynucleotides to RNA, the interaction between polynucleotides, and the mechanism of polynucleotide synthesis.

POLYNUCLEOTIDE PHOSPHORYLASE

Polynucleotide phosphorylase partially purified from *A. vinelandii* (Grunberg-Manago *et al.*, 1956) and other bacterial sources (Beers, 1956; Littauer and Kornberg, 1957; Brummond *et al.*, 1957) catalyzes Reaction 1,

$$(1)\quad n\ \text{X-R-P-P} \rightleftharpoons (\text{X-R-P})_n + n\ \text{P}$$

where R stands for ribose, PP for pyrophosphate, P for orthophosphate, and X for one or more of the following bases : adenine, hypoxanthine, guanine, uracil, or cytosine. The reaction requires the presence of magnesium ions. Due to its reversibility, the reaction brings about the incorporation of radioactive phosphate into the terminal phosphate group of nucleoside 5′-diphosphates. This incorporation or " exchange " has been used in our laboratory as an assay for the purification of the enzyme. The rate of liberation of orthophosphate from nucleoside diphosphates (Reaction 1, left to right) or the rate of phosphorolysis of polynucleotides (Reac-

tion 1, right to left), for example, that of synthetic polyadenylic acid, can also be used for assay.

Stoichiometry and Equilibrium. — Table I illustrates the stoichiometry of the synthesis of poly I from IDP, and of the phosphorolytic cleavage of the polynucleotide. The results are in accordance with Reaction 1.

The equilibrium constant of the reaction catalyzed by polynucleotide phosphorylase would be given by the expression

$$Keq \frac{[(X\text{-}R\text{-}P)_n]\ [P]}{[X\text{-}R\text{-}P\text{-}P]}$$

However, due to the high molecular weight of the reacting polymer, the equilibrium position is mainly determined by the ratio of the molar concentration of orthophosphate to that of nucleoside diphos-

TABLE I.

Stoichiometry of Reversible Reaction Catalyzed by Polynucleotide Phosphorylase *

Experiment No.		IDP	Orthophosphate	Poly I
la	Initial	9.8	1.0	0.0
	∇ 90 min.	— 5.5	+ 6.0	+ 5.0
lb	Initial	0.0	14.0	3.2
	∇ 60 min.	+ 2.3	— 2.2	— 2.0
2	Initial	0.7	8.1	7.5
	∇ 90 min.	+ 1.3	— 1.4	— 1.3

In experiment *la*, IDP was incubated at 30° with *Azotobacter* enzyme (0.5 mg. of protein) in the presence of $MgCl^2$ (4.8 μ moles) and tris (hydroxymethyl) aminomethane (Tris) buffer, pH 8.1, (36 μ moles). The reaction was stopped by heating 1 minute at 100°. The IDP remaining in an aliquot of the supernatant solution was removed, by hydrolysis to IMP and orthophosphate, with an excess of ox liver inosinediphosphatase (Plaut, 1955) for 40 minutes and this enzyme then destroyed by heating 1 minute at 100°. In experiment *lb*, an aliquot of the inosinediphosphatase supernatant solution from experiment *la* received some additional orthophosphate plus *Azotobacter* enzyme as above, and was incubated, at pH 7.4, as indicated. In experiment 2, poly I was incubated with *Azotobacter* enzyme (0.47 mg. of protein) in the presence of $MgCl^2$ (5 μ moles), Tris buffer, pH 8.1, (57 μ moles), and orthophosphate. Values are given in μ moles (for poly I, as μ moles of mononucleotide) per ml. of reaction mixture. Partially purified enzyme was used in these experiments.

(*) (Grunberg-Manago and Ochoa, 1955).

phate as is the case with the reaction catalyzed by (polysaccharide) phosphorylase. Data on the position of the equilibrium of the polynucleotide phosphorylase reaction are still scarce. With ADP or IDP as substrate the reaction comes to a standstill when the molar ratio [P] / [X-R-P-P] is from 1.5 to 2.0. Under these conditions, the reaction favors polynucleotide synthesis as would be expected from the fact that the pyrophosphate bonds of nucleoside diphosphates are converted to the phosphodiester bonds of the polynucleotide. However, the equilibrium may be displaced further in the direction of polynucleotide synthesis when polynucleotides containing AMP and UMP, or all four mononucleotides present in RNA, are synthesized. This is due to formation of multistranded chains which are not easily phosphorolyzed (Ochoa, 1957).

Distribution. — As reported elsewhere (Brummond and Ochoa, 1956; Brummond *et al.*, 1957) polynucleotide phosphorylase is widely distributed in bacteria and, following our initial report, the enzyme was isolated and purified from microorganisms other than *A. vinelandii*. Table II gives data on the bacterial distribution of the enzyme.

TABLE II.

Polynucleotide Phosphorylase in Bacteria*

Organism	Relative Activity
Azotobacter vinelandii	100
Mycobacterium phlei	50
Micrococcus lysodeikticus	35
Escherichia coli (Crookes)	25
» » (4157)	40
Micrococcus pyogenes var. *aureus* (Duncan)	70
Streptococcus hemolyticus	15
» *faecalis*	2
» *lactis R.*	15
Pneumococcus (type III)	7
Bacillus cereus	25
Clostridium kluyveri	2
Corynebacterium diptheriae	5
Alcaligenes faecalis	90
Hydrogenomonas facilis	20

Approximate relative specific activity of cell-free extracts (*Azotobacter* = 100) based on phosphate « exchange » assays (Grunberg-Manago *et al.*, 1956).

(*) From Brummond *et al.*, 1957.

Indications for the occurrence of the enzyme in plant tissues (Brummond *et al.*, 1957) and yeast (Grunberg-Manago and Wisniewski, 1957) have been obtained and recent work of Hilmoe and Heppel (1957) has shown its presence in animal tissues. The wide distribution of polynucleotide phosphorylase suggests that the enzyme may be generally concerned with the intracellular synthesis of RNA.

BIOSYNTHETIC POLYNUCLEOTIDES

Polynucleotides containing AMP, UMP, CMP, or IMP as the only basic unit have been obtained by incubating the *A. vinelandii* enzyme with the corresponding 5′-nucleoside diphosphates. Other polynucleotides prepared with the *A. vinelandii* enzyme include: one prepared from approximately equimolar mixtures of ADP and UDP, one from mixtures of ADP, GDP, UDP, and CDP in approximately molar proportions 1 : 0. 5 : 1 : 1, and another in which the four diphosphates were used in equimolar amounts. Recently Griffin, Todd and Rich (1958) have described the preparation of a polyribothymidylic acid with synthetic ribothymidine 5′-diphosphate as substrate.

While the above polynucleotides can be prepared in any amounts, it has only been possible to prepare traces of guanylic acid polynucleotide or polynucleotides containing both GMP and UMP. In each of these cases (*) there is slow liberation of inorganic phosphate on incubation with the nucleoside diphosphates, but it stops when only about ten per cent of the easily hydrolyzable phosphate has been split off as orthophosphate. On isolation by precipitation with ethanol, small amounts of alcohol-insoluble material are obtained but much of the ultraviolet absorbing material in the precipitate is lost on dialysis against distilled water. Of the amount of polynucleotide formed, judging from the observed liberation of orthophosphate, only about 20 % is non-dialyzable. It is possible that dialyzable oligonucleotides are formed in these cases to some extent. Perchloric acid or alkaline hydrolysis of the non-dialyzable material indicates the presence of the expected bases or mononucleotides. The reason why a

(*) Mii, S., unpublished results.

GMP polynucleotide, or polynucleotides containing GMP and other nucleotides, are not readily synthesized is not clear. In any case, when all four nucleoside diphosphates are present, GMP is readily incorporated into the resulting polynucleotide. Similar observations have been made by Littauer and Kornberg (1957).

Structure. — As already mentioned, the polynucleotides synthesized by polynucleotide phosphorylase consists of 5′-nucleoside monophosphate units linked to one another through 3′-phosphoribose ester bonds. The chains are terminated by a phosphate group esterified at carbon 5′ of the nucleoside moiety (Fig. 1). This is indicated by end-group assays (Ochoa, 1956) by the method of Markham *et al.* (1954) and is in agreement with the fact that the biosynthetic polynucleotides are polymers of nucleoside 5′-monophosphate units resulting from the splitting of orthophosphate from nucleoside 5′-diphosphates. The products yielded by hydrolysis of biosynthetic polynucleotides with alkali or with specific phosphodiesterases, such as snake venom phosphodiesterase, spleen phosphodiesterase, or pancreatic ribonuclease, are as expected from what is known of the action of these agents on RNA.

The preparation of synthetic RNA in which one of the nucleotide species is labelled with P^{32}, makes possible studies on the distribution of the labelled nucleotide in the polynucleotide chain (Ortiz and Ochoa, 1959). Fig. 1 presents the structure of a polynucleotide in which labelled AMP is randomly distributed. Hydrolysis of such a polymer with snake venom phosphodiesterase (Fig. 1 A) will yield a mixture of nucleoside 5′-monophosphates of which only AMP will be labelled and its specific radioactivity will be the same as that of the AMP incorporated. On the other hand, hydrolysis with spleen phosphodiesterase (Fig. 1 B) will yield nucleoside 3′-monophosphates all of which will be labelled and, as non-labelled adenosine 3′-monophosphate is also released, its specific radioactivity will be lower than that of the AMP incorporated. Hydrolysis with alkali will give the same result except that the nucleotides will consist of the isomeric mixtures of nucleoside 2′- and 3′-monophosphates. If the polymer contains P^{32}-labelled UMP, or other labelled nucleotide, what has been said of adenylic acid will apply to this nucleotide.

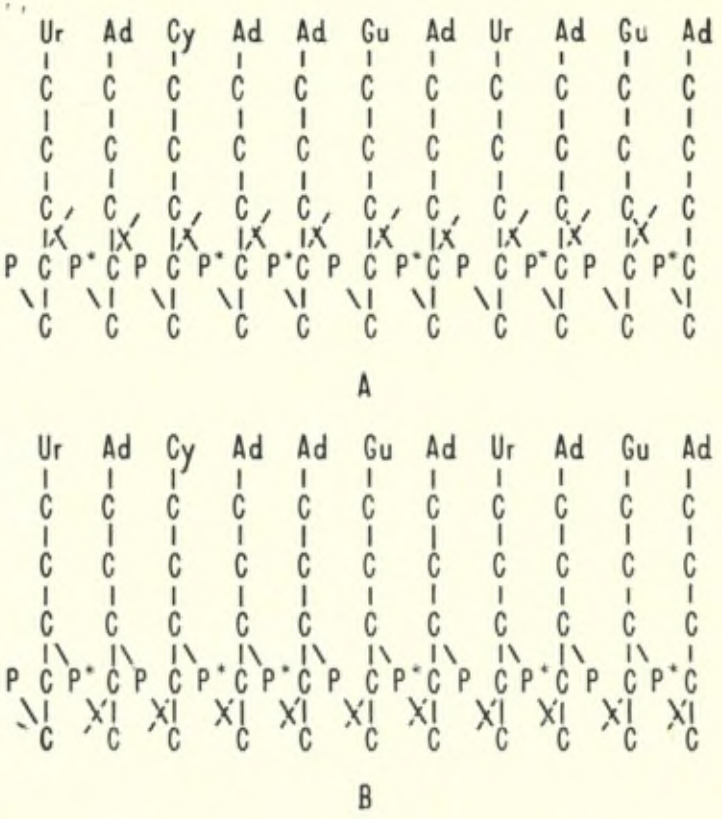

Fig. 1. — Scheme of hydrolysis of poly A*GUC by snake venom (A) or spleen (B) phosphodiesterase. The bonds hydrolyzed are indicated by dashed lines. The asterisk denotes P^{32} labelling. (From Ortiz and Ochoa, 1959).

Two kinds of labelled RNA have been used. One (poly A*GUC) was prepared with approximately equimolar amounts of P^{32}-labelled ADP (adenosine-P^{32}-P^{32}) and non-labelled GDP, UDP, and CDP. The other (poly AGU*C) was prepared in the same way but with P^{32}-labelled UDP (uridine-P^{32}-P) and non-labelled ADP, GDP, and CDP. After hydrolysis of poly A*GUC or poly AGU*C with venom diesterase the only labelled nucleotides released are AMP or UMP, respectively, whereas following hydrolysis with spleen enzyme (or alkali) each of the four nucleoside 3′- (or 3′[2′]) monophosphates is labelled.

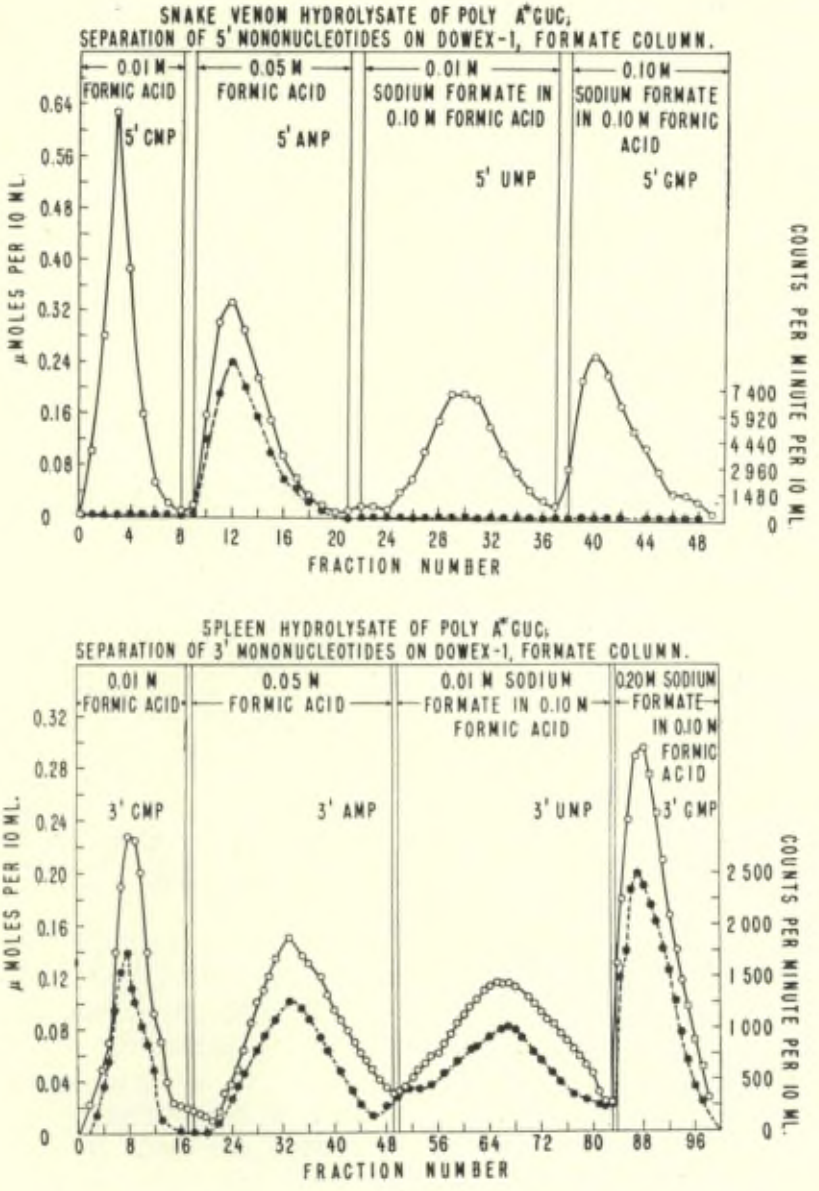

Fig. 2. — Hydrolysis of poly A*GUC with snake venom (A) or spleen (b) phosphodiesterase with separation of nucleotides by ion-exchange chromatography. Plot of nucleotide concentration (solid lines -o-o-) and radioactivity (dashed lines -----.) against effluent fraction number. (From data of Ortiz and Ochoa, 1959).

These results are illustrated in Fig. 3 for poly A*GUC, Table III shows that after hydrolysis of the radioactive polymers

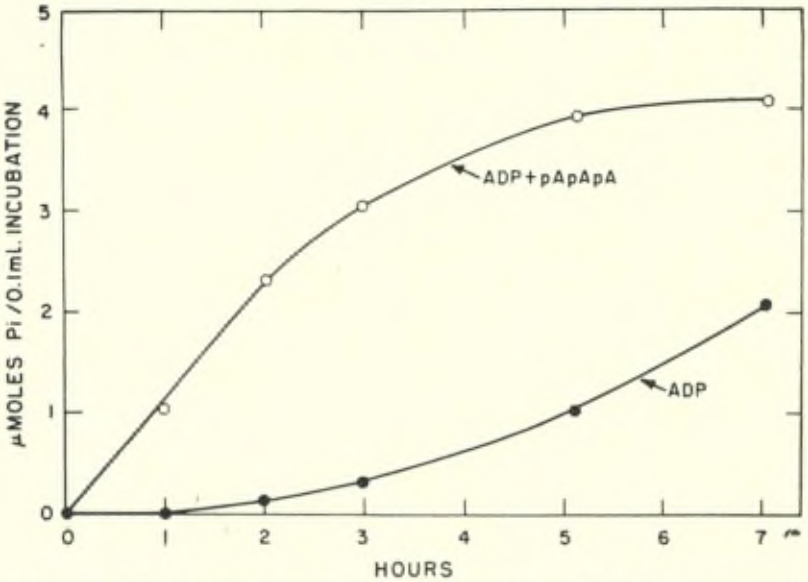

Fig. 3. — Priming of poly A synthesis by triadenylic acid (*p*A*p*A*p*A). Synthesis of poly A from ADP followed by the liberation of orthophosphate (P*i*). The reaction mixtures (0.1 ml. volume) contained 15 μmoles of tris(hydroxymethyl) aminomethane buffer, pH 8.2; 1.0 μmole of $MgCl_2$; 0.04 μmole of ethylene diamine tetraacetate; 6 μmoles of ADP and highly purified *Azotobacter* polynucleotide phosphorylase with 1.7 μg. of protein. In the experiment of the upper curve the sample contained, in addition 0.14 μmole of triadenylic acid. Incubation at 30°. (From data of Singer *et al.*, 1957).

TABLE III.

Specific Radioactivity of Nucleotides Released by Hydrolysis of P^{32}-Labelled RNA with Snake Venom Phosphodiesterase, Spleen Phosphodiesterase, or Alkali*.

Nucleotide	Poly A*GUC (8390)		Poly AGU*C (7000)		
	Venom	Spleen	Venom	Spleen	Alkali
Adenylic acid	26 500	7 580	0	5 579	7 655
Guanylic acid	0	7 627	0	6 144	7 488
Uridylic acid	0	7 592	39 800	6 522	7 011
Cytidylic acid	0	5 677	0	7 013	7 311

The values give the specific radioactivity in counts per minute per micromole of nucleotide. Separation of the nucleotides was carried out by ion exchange chromatography following hydrolysis with venom or spleen diesterase and by paper electrophoresis following hydrolysis with alkali. For comparison, the specific radioactivity of the unhydrolyzed RNA is shown in parentheses at the top of the column corresponding to each polymer.

(*) From Ortiz and Ochoa, 1959.

with spleen diesterase or alkali the specific radioactivity of the various nucleotides from each polymer is about the same and, as expected, of the same order of magnitude as that of the intact polynucleotide. These results show that the different nucleotide species of synthetic RNA are extensively linked to each other.

Relation to RNA

Many different lines of evidence indicate that the biosynthetic polynucleotides are closely related to RNA. This is, of course, particularly the case with poly AGUC.

Molecular Weight. — The molecular weight of biosynthetic polynucleotides varies from about 30,000 to 2,000,000. These values are in the range of molecular weights determined for RNA from different sources by various investigators. It has further been found (*) that RNA isolated from *A. vinelandii* cells had the same sedimentation constant as poly AGUC. Comparative data on molecular weights of biosynthetic polynucleotides and various ribonucleic acids have been previously presented (Ochoa, 1956; Ochoa and Heppel, 1957).

X-ray Diffraction. — X-ray diffraction patterns of fibers prepared from some of the biosynthetic polynucleotides have been obtained by Rich (1957). He has found that polynucleotides such as poly A, containing but a single nucleotide unit, give X-ray diffraction patterns which although very similar are not identical to the fiber pattern of RNA. On the other hand, polynucleotides containing both purine and pyrimidine bases, give virtually the same patterns as RNA. This is the case with poly AU and poly AGUC.

Interaction with Tobacco Mosaic Virus Protein. — The finding of Hart and Smith (1956) that biosynthetic ribopolynucleotides, as well as non-viral RNA, interact with tobacco mosaic virus protein to form virus-like rods, is another indication that the macromolecular structure of biosynthetic polynucleotides is very similar to that of RNA, as a close structural fit must be involved in the recombination. The observed non-infectivity of

(*) Warner, R. C., unpublished results.

such artificial " viruses " would be expected from the fact that the RNA is the carrier of biological specificity (Stanley, 1956).

Nucleotide Composition. — Studies on the nucleotide composition of synthetic ribopolynucleotides, carried out by Dr. R. M. S. Smellie in our laboratory (*), have shown that it is dependent on the relative concentrations of the nucleoside diphosphates used for preparations of the polynucleotide. With equimolar concentrations of diphosphates the nucleotide composition of poly AGUC synthesized by *A. vinelandii* enzyme is very similar to that of *A. vinelandii* RNA (Table IV). It should be emphasized that

TABLE IV.
Nucleotide Composition of Ribonucleic Acids
Relative values; adenylic acid taken as 10)

Source	Adenylic acid	Guanylic acid	Uridylic acid	Cytidylic acid	$\frac{A+C}{G+U}$	Reference
A. vinelandii	10	13.0	7.5	9.0	0.93	Smellie, unpublished.
A. vinelandii	10	13.0	7.3	9.0	0.94;	Ortiz and Ochoa, 1959.
A. vinelandii	10	12.5	8.0	10.5	1.00	Lombard and Chargaff, 1956
Poly AGUC+	10	14.5	8.5	12.0	0.95	Smellie, unpublished.
Poly AGUC+	10	11.6	6.6	7.2	0.94	Ortiz and Ochoa, 1959.
Poly AGUC+	10	12.5	6.9	7.3	0.89	Ortiz and Ochoa, 1959.
Staph. aureus	10	10.5	7.0	8.5	1.05	Smellie, unpublished.
A. faecalis	10	11.5	5.5	9.0	1.12	Smellie, unpublished.
Mycobact. phlei	10	15.0	7.0	8.0	0.82	Smellie, unpublished.
*E. coli**	10	10.0	8.5	8.5	1.00	Elson and Chargaff, 1954.
Rabbit liver	10	17.0	10.0	15.5	0.94	Volkin and Carter, 1951; Davidson and Smellie, 1952
Tobacco mosaic virus	10	8.5	9.0	6.0	0.91	Knight, 1952.
Turnip yellow mosaic virus	10	7.5	10.0	17.0	1.54	Markham and Smith, 1951.

(*) Analysis without prior isolation of RNA.

\+ Prepared from equimolar amounts of ADP, GDP, UDP and CDP with *A. vinelandii* enzyme (three different preparations).

although the polynucleotide was prepared with equimolar concentrations of nucleoside diphosphates, the proportions of nucleotides in the synthetic polymer were not equimolar just as they are not equimolar in ribonucleic acids in general. It should further

(*) Smellie, R. M. S., unpublished results.

be noticed in Table IV that the rabbit liver and plant virus ribonucleic acids differ very significantly in nucleotide composition from poly AGUC and bacterial RNA. It appears justified to conclude that starting with equimolar concentrations of nucleoside diphosphates and using partially purified enzyme, the polynucleotide phosphorylase of *A. vinelandii* catalyzes the synthesis of RNA of a characteristic composition which resembles that of other bacterial ribonucleic acids and, in particular, that of *Azotobacter* RNA.

Biological Activity. — If poly AGUC is indeed RNA, one would expect the synthetic compound to exhibit any biological activity characteristic of the latter. It has been shown by Okamoto (1939) and confirmed by Bernheimer (1949) that the formation of streptolysin *S* by hemolytic streptococci is markedly stimulated by the ribonuclease resistant fraction of RNA from various sources.

The effect of synthetic polyribonucleotides on the formation of streptolysin *S* by growing and resting cells of *Streptococcus pyogenes* was investigated by Tanaka *et al.* (1957); some of their results are summarized in Table V. It will be seen that poly

TABLE V.

Effect of Polyribonucleotides and their Ribonuclease Digestion Products on Streptolysin *S* Formation by Resting Cells of Streptococcus Pyogenes *

Polynucleotide	Concentration in assay (μ g./ml.)	Ribonuclease digestion + (hours)	Streptolysin (units/ml.)
Yeast RNA	400	0	10
	40	2	140
AGUC (1:1:1:1)	400	0	226
	40	2	447
AGUC (1:0. 5:1:1)	400	0	0
	40	2	33
AU	400	0	0
	100	4	0
AC	400	0	0
	100	4	0

(*) Data from Tanaka *et al.* (1957). + At 37° with 50 μ g. of crystalline ribonuclease per ml.

AGUC synthesized from equimolar concentrations of ADP, GDP, UDP, and CDP, was highly active whereas that synthesized with equimolar concentrations of ADP, UDP, and CDP, and half the molar concentration of GDP (a polynucleotide which contains about half the amount of guanylic acid present in the other) was almost inactive. Polynucleotides containing no guanylic acid were inactive. These experiments show not only that synthetic RNA has a biological activity exhibited by natural RNA, but they also throw some light on the nature of the oligonucleotides, produced by ribonuclease digestion of RNA, which are effective in stimulating streptolysin *S* formation. In the light of the above results and further experiments of Egami and his collaborators (*), only oligonucleotides rich in guanylic acid are active.

Interaction of Polynucleotides

The fact that polynucleotide phosphorylase can catalyze the synthesis of ribopolynucleotides containing but one single nucleotide species has provided unexpected means to gain a deeper insight into the structure, the chemical and physical properties, and possibly the biological properties of nucleic acids. Study of these polynucleotides has already provided strong confirmatory evidence of current ideas on the specificity and mechanism of action of the nucleic acid-hydrolyzing enzymes (Heppel *et al.*, 1957).

An important result of the investigations on biosynthetic ribopolynucleotides has been the demonstration (Warner, 1957), that polynucleotides containing a single mononucleotide species interact in aqueous solution to form stable complexes. This is the case with poly A and poly U. When solutions of these polymers are mixed there occurs a rapid and marked increase in viscosity. Occurrence of interaction is also indicated by a large decrease in ultraviolet absorption at the wavelength of maximum absorption together with a small shift of the peak towards shorter wavelengths. The formation of a complex is further indicated by the following observations: (*a*) electrophoresis of the mixture of poly A and poly U (poly A + U) yields a single component

(*) Egami, F., personal communication.

which migrates at a rate intermediate between those of the individual A and U polynucleotides and, (*b*) ultracentrifugation yields a single component with a higher sedimentation constant than that of the parent compounds.

Rich and Davies (1956) have found that fibers drawn from solutions of poly A + U produce well-oriented X-ray diffraction patterns very similar to those given by DNA. They have interpreted their results as indicating that the poly A + U aggregate is a two-stranded helix containing one strand of poly A and one of poly U, and consider that the bases adenine and uracil make two hydrogen bonds with each other in the same manner postulated for adenine and thymine in DNA (Watson and Crick, 1953). Thus, the RNA backbone may assume a configuration not unlike that found in DNA using the same complementarity in the base pairs.

More recently Rich and collaborators (Felsenfeld and Rich, 1957; Felsenfeld *et al.*, 1957; Davies and Rich, 1958; Rich, 1958) have reported on the formation of double-stranded helical structures by other polyribonucleotide pairs and on that of triple-stranded structures involving poly A, poly U, and other synthetic polynucleotides. This work has provided not only the first experimental proof of Watson and Crick's hypothesis on the structure of DNA but it has stimulated interesting speculation on the possible mode of action of DNA in RNA replication which might be visualized as the laying out of the growing RNA chain on the helical groove formed by the two DNA strands (*) (see also Zubay, 1958).

MECHANISM OF POLYNUCLEOTIDE SYNTHESIS

While the preparations of *A. vinelandii* polynucleotide phosphorylase available until recently were sufficiently pure for the synthesis of a number of polyribonucleotides, they were not suitable for studies of reaction mechanism. In particular, the question whether, in analogy with polysaccharide phosphorylase, a primer was required for polyribonucleotide synthesis had remained unans-

(*) Rich, A., personal communication.

wered. Further purification of the enzyme (Mii and Ochoa, 1957; Ochoa *et al.*, 1957) has yielded preparations with which a requirement for priming has been established (Mii and Ochoa, 1957; Singer *et al.*, 1957). Ultracentrifuge and other studies suggest a purity of 60-70 % for the best preparations and indicate that polynucleotide phosphorylase is a single enzyme capable of reaction with individual nucleoside diphosphates or with mixtures thereof (*).

With highly purified preparations of the enzyme the formation of polynucleotides from nucleoside diphosphates occurs only after an initial lag period which can be completely eliminated by the addition of small amounts of polynucleotides or of certain oligonucleotides.

Priming by Oligonucleotides. — Heppel and collaborators (Singer *et al.*, 1957, 1958) have found that small polynucleotides of adenylic acid, such as di-, tri-, and tetraadenylic acid, obtained from poly A by partial hydrolysis with an enzyme from guinea pig liver nuclei (Heppel, Ortiz and Ochoa, 1956), prime the synthesis of either poly A or poly U by highly purified polynucleotide phosphorylase. Priming of poly A synthesis by triadenylic acid is shown in Fig. 3. The structure of this oligonucleotide is shown in Fig. 4 A. The maximal rate of polynucleotide synthesis which can be obtained by priming with the above oligonucleotides

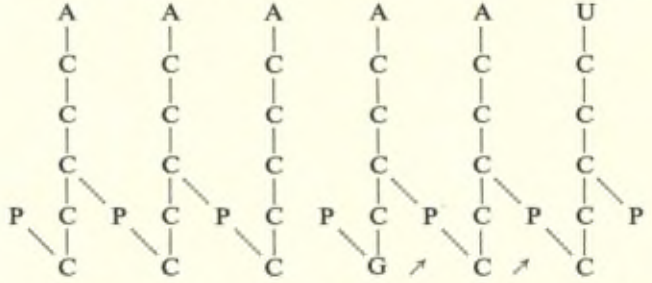

Fig. 4. — A. Structure of triadenylic acid (*p*A*p*A*p*A). B. Structure of the trinucleotide (*p*A*p*A*p*U*p*) obtained by ribonuclease digestion of the polynucleotide synthesized from UDP with diadenylic acid (*p*A*p*A) as primer, the arrows indicate the points of cleavage of this trinucleotide by alkali to give, from left to right, adenosine-5', 3'-diphosphate, adenosine 3'-monophosphate, and uridine 3'-monophosphate.

(*) Work in progress (Mii and Ochoa) at time of writing resulted in the preparation of chromatographically homogeneous *Azotobacter* polynucleotide phosphorylase. It is hoped to report at the meeting on the results of work with this enzyme.

is about the same with each of them although the concentration required to give that rate decreases with increasing chain length of the primer molecule.

In elegant experiments, Heppel and his associates have shown that the primer oligonucleotide forms a nucleus for starting the chain and becomes incorporated into the newly made polynucleotide. Thus, incubation of P^{32}-labelled oligonucleotide with phosphorylase and nucleoside diphosphate results in incorporation of radioactivity in the isolated polynucleotide. On incubation of diadenylic acid with UDP, they have demonstrated the formation of a polynucleotide consisting of two adenylic acid residues followed by a number of uridylic acid residues. Digestion of this polynucleotide with pancreatic ribonuclease, yielded uridine, uridine 3′-monophosphate, and the trinucleotide illustrated in Fig. 4 B. The synthesis and ribonuclease cleavage of the above polynucleotide can be represented as follows :

$$\text{pApA} + \text{n UDP} \xrightarrow{\text{phosphorylase}} \text{pApApUpUpU} \text{ --- pUpU}$$

$$\text{pApApUpUpU} \text{ --- pUpU} \xrightarrow{\text{ribonuclease}} \text{pApApUp} + \text{(n-2)}$$
$$\text{3′-UMP} + \text{uridine.}$$

The above experiments show that polynucleotide phosphorylase catalyzes very slowly, if at all, the initial condensation of two mononucleotide units, whereas it readily catalyzes the elongation of oligonucleotide chains by stepwise addition of nucleotide residues. Such a mechanism is depicted in Fig. 5.

Priming by Polynucleotides. — The synthesis of polyribonucleotides by polynucleotide phosphorylase is also primed by polyribonucleotides (Mii and Ochoa, 1957; Singer *et al.*, 1957; Ochoa *et al.*, 1957). Fig. 6 A shows " self " priming of the synthesis of poly A, poly U, and poly C, while Fig. 6 B shows the priming of RNA synthesis by several ribonucleic acids. However, contrary to priming by oligonucleotides of adenylic acid which is nonspecific (i.e., they prime the synthesis of poly A as well as that of poly U), priming by polynucleotides shows a certain degree of specificity. For example, poly A primes its own synthesis but not that of poly U or *vice versa.* Neither of these polynucleotides primes the synthesis of RNA. On the other hand, RNA and

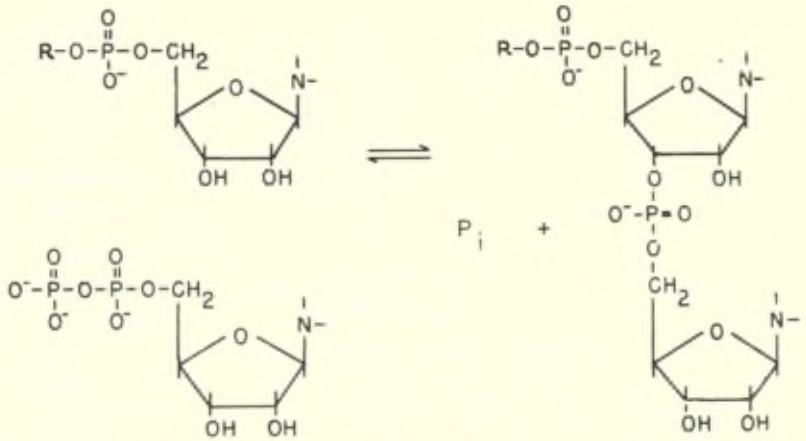

Fig. 5. — Scheme of lengthening of the chain of a primer oligonucleotide through addition of mononucleotide units to the nucleoside and by polynucleotide phosphorylase. R represents two or more mononucleotide units adjacent to the terminal nucleotide of a primer oligonucleotide.

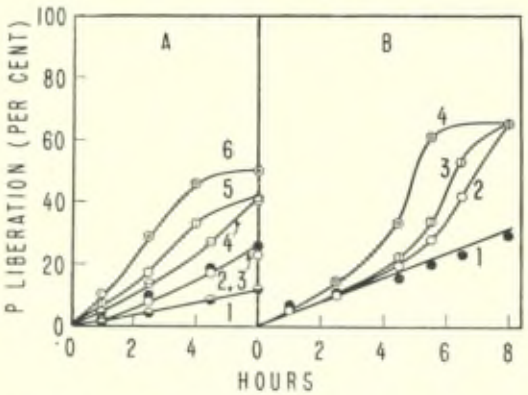

Fig. 6. — Effect of primers on the enzymatic synthesis of polynucleotides. Reaction followed by the liberation of orthophosphate from nucleoside 5′-diphosphates. The reaction mixtures (0.1 ml. volume) contained tris (hydroxymethyl) aminomethane buffer, $MgCl^2$, ethylene diamine tetraacetate, and highly purified polynucleotide phosphorylase as in fig. 3, with other additions as indicated below. A. Synthesis of « single » polymers. Curves 1 and 4, ADP, 6.0 μ moles and enzyme with 0.2 μ g. of protein, without and with 100 μ g. of poly A, respectively; enzyme with 0.4 μ g. of protein used for the remaining curves; curves 2 and 5, UDP, 6.0 μ moles, without and with 100 μ g. of poly U respectively; curves 3 and 6, CDP, 6.0 μ moles, without and with 25 μ g. of poly C, respectively. B. Synthesis of RNA (poly AGUC). 1.25 μ moles of each ADP, GDP, UDP, and CDP, and enzyme with 20 μ g. of protein. Curve 1, no primer; curve 2, with 50 μ g. of yeast RNA; curve 3, with 50 μ g. of poly AGUC; curve 4, with 32 μ g. of *Azotobacter* RNA. Incubation at 30°. (From Mii and Ochoa, 1957).

poly AU, which contain the bases present in poly A and poly U, prime the synthesis of these two polynucleotides. The behavior of poly C is quite exceptional in that it primes the synthesis of every polynucleotide thus far tried, including RNA, whereas its own synthesis is primed only by poly C and not primed or inhibited by all other polynucleotides. The synthesis of RNA is also primed by RNA natural or synthetic. These results are summarized in Table VI.

TABLE VI

Effect of Polyribonucleotides on the Enzymatic Synthesis of Poly A, Poly U, Poly C, Poly I, Poly AU and Poly AGUC*

Substrates for polynucleotide synthesis	Effect of					
	Poly A	Poly U	Poly C	Poly I	Poly AU	RNA or AGUC
ADP	+	—	+	O	+	+
UDP	—	+	+	O	+	+
CDP	—	—	+			—
IDP	—	O	+	+		
ADP + UDP					+	
ADP + GDP + UDP + CDP	O	O	+			+

The signs +, O, and —, denote priming, no effect, or inhibition, respectively.

(*) From data of Mii and Ochoa (1957), Singer *et al.* (1957), and unpublished results.

Considerations on the Role of Primers. — The need for a primer suggests that polynucleotide phosphorylase is unable to catalyze the synthesis of polynucleotides starting from nucleoside diphosphates as the only reactants. The sluggish reaction obtained under these conditions, i.e., in the absence of added primer, might be due to the presence of priming oligonucleotide in the best preparations of the enzyme so far available which contain up to 3 % nucleotide. Further purification may answer the question whether this nucleotide material is present as a contaminant or represents a " built in " primer or firmly bound

prosthetic group. At present all that can be said is that treatment with activated charcoal or Dowex-1 resin has failed to reduce the nucleotide content of the enzyme or affect its activity.

As regards the mode of action of oligonucleotide primers there is no doubt that, like muscle (polysaccharide) phosphorylase, polynucleotide phosphorylase adds nucleotide units to a pre-existing primer chain. It is not possible to decide at present whether the priming of polynucleotide synthesis by fully grown polynucleotides operates through the same mechanism but this appears doubtful as it is unlikely that long polynucleotide chains can undergo further elongation. One is led to consider that, in this case, priming may be due to a laying out of the growing polynucleotide chain alongside the chain of the primer. The specificity of the priming by polynucleotides further suggests that it may reflect the operation of a template mechanism of replication controlling not only polynucleotide synthesis but also the nature of the polynucleotide synthesized. Preliminary experiments of Dr. Sanae Mii in our laboratory indicate that the nature of the RNA used as primer has a marked influence on the nucleotide composition of the resulting RNA and that the presence of RNA in partially purified *Azotobacter* polynucleotide phosphorylase may be responsible for the similar nucleotide composition and other properties of *Azotobacter* RNA and the RNA synthesized by such preparations.

OTHER SYSTEMS OF POLYNUCLEOTIDE SYNTHESIS

Kornberg and his collaborators (Kornberg, 1957; Lehman *et al.*, 1958; Bessman *et al.*, 1958; Adler *et al.*, 1958; Schachman *et al.*, 1958) have isolated in highly purified form from *E. coli* an enzyme which catalyzes the synthesis of DNA from deoxyribonucleoside triphosphates with liberation of pyrophosphate. From the point of view of mechanism the reaction is similar to that catalyzed by polynucleotide phosphorylase since in both cases a pyrophosphate linkage is exchanged for the phosphodiester bond of the polynucleotide, but each enzyme is strictly specific for either the nucleoside di- or triphosphates. The DNA enzyme is much more active if all four triphosphates of deoxyadenosine,

deoxyguanosine, thymidine, and deoxycytidine, are present than if any one of them is absent and the reaction is but moderately reversible. The system, which requires priming by DNA, appears to yield DNA of the same molecular weight and nucleotide composition as that of the primer, suggesting a method of replication like the one discussed above for RNA. There is evidence for the occurrence of a similar DNA synthesizing system in animal tissues (Bollum, 1958; Mantsavinos and Canellakis, 1958).

Recently, work from several laboratories (Canellakis, 1957; Edmonds and Abrams, 1957; Hecht *et al.*, 1958; Goldthwait, 1958) has disclosed a mechanism for the addition of a few nucleotide residues to a pre-existing RNA chain. This involves the reversible interaction of the RNA with some ribonucleoside triphosphates such as ATP or CTP, with liberation of pyrophosphate. There is some degree of specificity of this reaction in that (*a*) only a certain kind of RNA can accept the nucleotide residues, and (*b*) these appear to be added in a preferred sequence. Thus, ATP or CTP alone cause the addition of a terminal AMP or CMP residue to the RNA chain, but both together result in the addition of one CMP followed by that of a terminal AMP unit. Crude enzyme preparations catalyzing the above reaction have been obtained from supernatant solutions derived from liver homogenates and homogenates of other animal tissues. There is no evidence so far that the operation of such systems results in a net synthesis of RNA. Reactions of this kind, in conjunction with nucleases and phosphoesterases, may serve to change the nature, and possibly the biological specificity, of RNA molecules in the cell.

Another way in which alterations of the nature of RNA chains can occur is suggested by recent work of Heppel and his associates (*). On incubation of triadenylic acid with preparations of polynucleotide phosphorylase, they have observed the formation of oligonucleotides of adenylic acid larger than the trinucleotide. This occurs at the expense of the trinucleotide which is simultaneously converted to the dinucleotide. The reaction, which occurs in the absence of orthophosphate and ADP, can

(*) Heppel, L. A., personal communication.

be considered to be a transnucleotidation and its first step can be formulated thus :

$$2\ \text{pApApA} \rightleftharpoons \text{pApA} + \text{pApApApA}.$$

Although this reaction has been observed with highly purified preparations of *Azotobacter* polynucleotide phosphorylase, there remains the possibility that it may be catalyzed by a separate enzyme contaminating the phosphorylase preparation. Heppel *et al.* (1955) have previously described the catalysis of similar nucleotide transfer reactions by ribonuclease.

A further possibility for changing sequences in nucleic acids could be the exchange of bases either between two polynucleotide chains or between a polynucleotide and a nucleoside, nucleotide or nucleoside polyphosphate. This reaction has been postulated (Brown, 1956) but there is not evidence thus far for its occurrence.

CONCLUDING COMMENTS

Biosynthesis of polynucleotides occurs through the stepwise polymerization of nucleoside 5′-monophosphate units from nucleoside 5′-diphosphates or triphosphates. Orthophosphate is released in the former and pyrophosphate in the latter case. In both cases the internucleotidic phosphoester linkages are made at the expense of the pyrophosphate bonds of nucleoside polyphosphates. This reaction pattern is essentially the same as that operating in the synthesis of polysaccharides, another biologically important class of high polymers, from glucose 1-phosphate. In the case of nucleic acids a new problem arises since these compounds are copolymers of at least four different monomeric units and may differ from one another, other than in size, in the sequence of nucleotide residues in the chain. Hence, the question of how a given sequence is produced or, in other words, how a given nucleic acid is replicated, is of considerable biological importance. At the present time it appears that the nucleic acid primer, required for nucleic acid synthesis, may imprint its sequential pattern on the newly formed polynucleotide. This would mean that nucleic acid would act as a template for its own replication. However, despite the suggestive results of the specificity of polyribonucleotide primers in our experiments with polynucleotide phosphorylase,

and the more decisive results of Kornberg and collaborators with the DNA-synthesizing enzyme of *E. coli*, such a suggestion should be taken only as a possibility which requires further exploration.

ACKNOWLEDGMENTS

The experimental work reported in this paper was aided by the grants from the Institute of Arthritis and Metabolic Diseases, and from the National Cancer Institute, United States Public Health Service, Bethesda, Maryland, the American Cancer Society, Inc., New York, N. Y., the Rockefeller Foundation, New York, N. Y., and the Office of Naval Research, Washington, D. C.

The author is indebted to Miss Priscilla J. Ortiz, Mr. Morton C. Schneider, and Mr. Horace Lozina for assistance in some of the experimental work reported in this paper, and wishes to acknowledge the collaboration of Dr. Sanae Mii in much of this work.

REFERENCES

Adler, J., Bessman, M. J., Lehman, I. R., Schachman, H. K., Simms, E. S. and Kornberg, A. : *Federation Proc.*, **17,** 178 (1958).

Adler, J., Lehman, I. R., Bessman, M. J., Simms, E. S. and Kornberg, A. : *Proc. Nat. Acad. Sci.*, **44,** 641 (1958).

Beers, R. F., JR. : *Nature*, **177,** 790 (1956).

Bernheimer, A. W. : *J. Exp. Med.*, **90,** 373 (1949).

Bessman, M. J., Lehman, I. R., Adler, J., Zimmerman, S. B., Simms, E. S. and Kornberg, A. : *Proc. Nat. Acad. Sci.*, **44,** 633 (1958).

Bessman, M. J., Lehman, I. R., Simms, E. S. and Kornberg, A. : *J. Biol. Chem.*, **233,** 171 (1958).

Bollum, F. J. : *J. Am. Chem. Soc.*, **80,** 1766 (1958).

Brown, G. B. : *Federation Proc.*, **15,** 823 (1956).

Brummond, D. O. and Ochoa, S. : *Federation Proc.*, **15,** 225 (1956).

Brummond, D. O., Staehelin, M. and Ochoa, S. : *J. Biol. Chem.*, **225,** 835 (1957).

Canellakis, E. S. : *Biochim. et Biophys. Acta*, **25,** 217 (1957).

Davidson, J. N. and Smellie, R. M. S. : *Biochem. J.*, **52,** 594 (1952).

Davies, D. R. and Rich, A. : *J. Am. Chem. Soc.*, **80,** 1003 (1958).

Edmonds, M. and Abrams, R. : *Biochim. et Biophys. Acta*, **26,** 226 (1957).

Elson, D. and Chargaff, E. : *Nature* **173,** 1037 (1954).

Felsenfeld, G., Davies, D. R. and Rich, A. : *J. Am. Chem. Soc.*, **79,** 2023 (1957).

Felsenfeld, G. and Rich, A. : *Biochim.* et *Biophys. Acta*, **26,** 457 (1957).

Goldthwait, D. A. : *Biochim.* et *Biophys. Acta*, **30,** 643 (1958).

Griffin, B. E., Todd, A., and Rich, A. : *Proc. Nat. Acad. Sci.*, **44,** 1123 (1958).

Grunberg-Manago, M. and Ochoa, S. : *J. Am. Chem. Soc.*, **77,** 3165 (1955).

Grunberg-Manago, M., Ortiz, P. J. and Ochoa, S. : *Science*, 122, 307 (1955).

Grunberg-Manago, M., Ortiz, P. J. and Ochoa, S. : *Biochim. et Biophys. Acta*, **20,** 269 (1956).

Grunberg-Manago, M. and Wisniewski, M. J. : *Compt. rend. Acad. Sci.*, **245,** 750 (1957).

Hart, R. G. and Smith, J. D. : *Nature*, **178,** 739 (1956).

Hecht, L. I., Zamecnik, P. C., Stephenson, M. L. and Scott, J. F. : *J. Biol. Chem.*, **233,** 954 (1958).

Heppel, L. A., Whitfeld, P. R. and Markham, R. : *Biochem. J.*, **60,** 8 (1955).

Heppel, L. A., Ortiz, P. J. and Ochoa, S. : *Science*, **123,** 415 (1956).

Heppel, L. A., Ortiz, P. J. and Ochoa, S. : *J. Biol. Chem.*, **229,** 679 (1957).

Heppel, L. A., Ortiz, P. J. and Ochoa, S. : *J. Biol. Chem.*, **229,** 695 (1957).

Hilmoe, R. J. and Heppel, L. A. : *J. Am. Chem. Soc.*, **79,** 4810 (1957).

Lehman, I. R., Bessman, M. J., Simms, E. S. and Kornberg, A. : *J. Biol. Chem.*, **233,** 163 (1958).

Littauer, U. Z. and Kornberg, A. : *J. Biol. Chem.*, **226,** 1077 (1957).

Lombard, A. and Chargaff, E.: *Biochim. et Biophys. Acta*, **20,** 585 (1956).

Knight, C. A. : *J. Biol. Chem.*, **197,** 241 (1952).

Kornberg, A. : " Proceedings of International Symposium on Enzyme Chemistry, Tokyo and Kyoto " 47 (1957).

Mantsavinos, R. and Canellakis, E. S. : *Biochim. et Biophys. Acta*, **27**, 661 (1958).

Markham, R., Matthews, R. E. F. and Smith, J. D. : *Nature*, **173**, 537 (1954).

Markham, R. and Smith, J. D. : *Biochem. J.*, **49**, 401 (1951).

Mii, S. and Ochoa, S. : *Biochim. et Biophys. Acta*, **26**, 445 (1957).

Ochoa, S. : *Federation Proc.*, **15**, 832 (1956).

Ochoa, S. : *Arch. Biochem. Biophys.*, **69**, 119 (1957).

Ochoa, S. and Heppel, L. A. : " In Chemical Basis of Heredity ", McElroy, W. D. and Glass, B., ed., Johns Hopkins Press, Baltimore, 615 (1957).

Ochoa, S., Mii, S. and Schneider, M. C. : " Proceedings of International Symposium on Enzyme Chemistry ", Tokyo and Kyoto, 44 (1957).

Okamoto, H. : *Japan J. Med. Sci., IV Pharmacol.*, **12**, 167. (1939)

Ortiz, P. J. and Ochoa, S. *J. Biol. Chem., in press* (1959).

Plaut, G. W. E. : *J. Biol. Chem.*, **217**, 235 (1955).

Rich, A. : " In Chemical Basis of Heredity ", McElroy, W. D. and Glass, B., ed., Johns Hopkins Press, Baltimore, 557 (1957).

Rich, A. : *Nature*, **181**, 521 (1958).

Rich, A. and Davies, D. R. : *J. Am. Chem. Soc.*, **78**, 3548 (1956).

Schachman, H. K., Lehman, I. R., Bessman, M. J., Adler, J., Simms, E. S. and Kornberg, A. : *Federation Proc.*, **17**, 304 (1958).

Singer, M. F., Heppel, L. A., and Hilmoe, R. J. : *Biochim. et Biophys. Acta*, **26**, 447 (1957).

Singer, M. F., Hilmoe, R. J. and Heppel, L. A. : *Federation Proc.*, **17**, 312 (1958).

Stanley, W. M. : *Federation Proc.*, **15**, 812 (1956).

Tanaka, K., Egami, F., Hayashi, T., Winter, J. E., Bernheimer, A. W., Mii, S., Ortiz, P. J. and Ochoa, S. : *Biochim. et Biophys. Acta*, **25**, 663 (1957).

Volkin, E. and Carter, C. E. : *J. Am. Chem. Soc.*, **73**, 1516 (1951).

Warner, R. C. : *J. Biol. Chem.*, **229**, 711 (1957).

Watson, J. D. and Crick, F. H. C. : *Nature*, **171**, 737 (1953).

Zubay, G. : *Nature*, **182**, 388, 1290 (1958).

DISCUSSION DU RAPPORT DE M. OCHOA

Mme Grunberg-Manago. — I would like to summarize briefly two points produced by our studies, in relation to the topic discussed by Dr. Ochoa.

The first point concerns the phosphorolysis of ribopolynucleotides and RNA and their molecular configuration. Dr. Ochoa has told us that the mixture of poly A and poly U in a ratio of 1/1 (conditions where a two-stranded helical structure is formed) is phosphorolysed more slowly than either poly A or poly U separately. The mixture containing an excess of poly U to poly A, under conditions where a triple-stranded helix is formed, is highly resistant to phosphorolysis. The same observation is illustrated in studies on the effect of salt concentration on the phosphorolysis of poly I. This polymer exists as a single chain in dilute salt and is phosphorolysed as readily as is poly A. However, in the presence of high salt concentrations, it forms a triple-stranded helix which is phosphorolysed very poorly. On the other hand, the rate of phosphorolysis of poly A and poly U is not decreased in the presence of high salt concentrations, their molecular structure not being affected to a large degree under these conditions. There is some direct evidence that one of the factors responsible for the rate of phosphorolysis of natural RNA is the extent of inter- or intramolecular hydrogen bonding. An increase of temperature produces O.D. changes of the RNA preparations which can be partially attributed to the cleavage of the internal hydrogen bonds. As a result of studies on these optical density changes, Doty and his collaborators have suggested that at a given temperature, different RNA samples exhibit different degrees of hydrogen bonding the presence of which interfers with phosphorolysis. Rates of phosphorolysis were measured at 40° and 25° for a number of different samples. The increase in optical density at the two temperatures was also measured and the results indicate that the relative increase in the rate of phosphorolysis of polyribonucleotides is linearly related to the increase in the optical density. It is therefore possible to conclude from these studies that the phosphorolysis of RNA and ribopolynucleotides depends not only on the relative concentrations of the substrates, but also on the

degree of intra- or intermolecular hydrogen bonding in the polymer chains. It is conceivable therefore that in the intact cell RNA may be protected against phosphorolysis, particularly if it is bound to a protein, even in the presence of high amounts of orthophosphate.

The second point I would like to discuss is the relation of phosphorolysis and polymer formation to the exchange reaction between inorganic P and the nucleoside diphosphates. All three reactions are catalysed by the purified Azotobacter enzyme. We have purified an enzyme from yeast about 200 fold, which catalyses the exchange of P^{32} labelled orthophosphate specifically with the terminal PO_4 of all the diphosphates. There is no reaction with the mono- or triphosphates. This enzyme has a 280/260 ration of about 1.75, indicating, in contrast to the Azotobacter enzyme, that it does not contain bound oligonucleotides. The yeast enzyme which appears to be free from contamination by nucleases, does not catalyse any detectable polymer formation, even in the presence of Polynucleotide phosphorylase primers (including oligonucleotides, poly A, AGUC, or RNA). It also does not phosphorolyse any of the biosynthetic polymers or oligonucleotides in spite of its ability to catalyse the exchange reaction.

M. Ochoa. — With regard to your first point, I was pleased to hear that your phosphorolysis studies reinforce and extend my conclusion that the phosphorolytic cleavage of polynucleotides is markedly dependent on the degree of intra- or intermolecular hydrogen bonding. Since most RNA's are phosphorolyzed with difficulty, it would appear, as you have emphasized, that in RNA synthesis by polynucleotide phosphorylase, the formation of hydrogen bonds would markedly favor the progress of the reaction in the direction of polymerization.

As regards your second point, catalysis of the exchange of P^{32} with nucleoside diphosphates but not of polymer synthesis, or phosphorolysis, by your purified yeast enzyme, it is indeed a baffling observation. It would be interesting if this were due to the absence of bound oligoribonucleotide from the preparations of the yeast enzyme. Another possibility might be damage of the enzyme, possibly by catheptic enzymes during extraction and isolation. Partial degradation might lead to loss of synthetic and phosphorolytic activity with retention of the P^{32} exchange activity. I must say, however, that this does not appear to me very likely, as I am

inclined to believe that the exchange reaction is a reflection of the occurrence of the overall reaction catalysed by the enzyme.

M. Rich. — In your table of primers, it is striking that each polymer chain is a primer for making more of itself. It is difficult to understand this, unless it suggests that there is an activation energy barrier for making an internucleotide bond between two different purines or pyrimidines, which is absent when the same base is used. However, I can see no good reason for this phenomenon.

M. Ochoa. — I am afraid that there is no satisfactory explanation for the experimental results you have pointed out. All one can say at present is that they are intriguing. However, the rule of priming by the same polynucleotide that is being synthesized has a striking exception in the case of poly C which primes, not only its own synthesis, but also that of other polyribonucleotides.

Mme. Grunberg-Manago. — I want to ask Dr. Ochoa if he thinks it is possible that the reason poly U does not act as a primer for poly A formation may be ultimately due to the interaction of poly U with poly A. It is possible for example that a small amount of poly A may be formed, but upon reaching a specific size reacts with poly U, the resulting complex might inhibit polynucleotide phosphorylase, thereby prevent further synthesis and this would explain the observation that poly U inhibits the synthesis of poly A which occur after a lag of time in the absence of poly A primer.

M. Ochoa. — I doubt whether this explanation is correct. The synthesis of poly G is primed, not inhibited, by poly C. Yet these two polymers would be expected to interact readily to form a double-stranded helix. As I previously pointed out, poly C is a universal primer; it primes the synthesis of all the polyribonucleotides so far studied, including that of poly G and poly AGUC.

M. Todd. — It is curious that we should have a polynucleotide synthesizing system in the ribonucleotide series based on nucleoside pyrophosphates whereas the DNA-synthesizing system of Kornberg is based on nucleoside triphosphates. It would be surprising if the synthetic methods used by nature for DNA and RNA should differ in this way and this makes me wonder whether there may be an

enzyme similar to that of Kornberg, still to be discovered, which is normally involved in RNA synthesis. From a purely chemical standpoint one might well imagine that the triphosphates would be better starting materials for polynucleotide synthesis than the pyrophosphates. Perhaps Dr. Ochoa's enzyme is of special importance in nature for its degradative action.

M. Ochoa. — No enzyme, or enzyme system, other than polynucleotide phosphorylase, has as yet been found capable of catalysing a net synthesis of RNA. A system utilising triphosphates has been detected in the cytoplasmic supernatant of certain animal cells but this system appears to be capable of adding only a couple of nucleotide units to a preexisting RNA chain.

M. Fraenkel-Conrat. — I wonder to what one could attribute the different phosphorolysis rates for TMV-RNA, as compared to other natural RNA preparations. It seems that all are similarly dependent upon the ionic medium and other factors in regard to the extent to which they have a structure, as indicated by hypochromicity, optical rotation, and other criteria.

M. Ochoa. — The work of Doty and that of Warner in our laboratory, has shown the existence of intramolecular hydrogen bonds, under appropriate conditions of ionic environment, in ribonucleic acids and synthetic polynucleotides such as poly AU. This hydrogen bonding appears to interfere with the phosphorolytic cleavage of RNA to a greater or lesser extent. If, under similar conditions, the nucleotides in tobacco mosaic virus RNA are less extensively hydrogen-bonded than those in other RNA's, tobacco mosaic virus RNA would be more readily phosphorolyzed.

M. Fraenkel-Conrat. — The possibility that the different behaviour might be due to different chain end groups is un turn suggested. Have synthetic polymers ever been prepared carrying a 3'-terminal phosphate, by the use of a nucleoside-3'-phosphate-5'-pyrophosphate as a primer, with the 5'-diphosphate as substrate ?

Mme. Grunberg-Manago. — The structure proposed by Doty and his coll. for the various RNA's is a single chain with internal hydrogen bonds. But at any given temperature different RNA

samples exhibit different degrees of hydrogen bonding. The hydrogen bonds are more labile in the case of TMV-RNA than in the other RNA's. May be Dr. Watson would like to discuss the structure proposed by Doty and coll. for the various RNA's.

M. Watson. — Doty and his collaborators have emphasized that while RNA is obviously not a double stranded complementary molecule, it, under certain conditions, possesses attributes suggesting extensive hydrogen bonding. Among these are its optical rotation and its marked hyperchromicity. Both of these attributes disappear at high temperature or in low ionic environment. We must be careful to compare RNA samples suspended in identical environments.

M. Rich. — It is important to point out that the thermal denaturation of RNA is quite different from what is observed with DNA. As we heat RNA, hydrogen bonds are broken and the optical density of the molecule rises. However, on cooling the RNA solution reverses itself completely, presumably forming all of its hydrogen bonds again. The DNA molecule shows only a small amount of reversibility. This contrast clearly demonstrates the specificity of the hydrogen bonding in DNA in contrast to what is observed with RNA. The synthetic polynucleotides show this same reversibility in their thermal denaturation curves.

M. Markham. — In the case of turnip yellow mosaic virus ribonucleic acid solutions, the increase of optical density with temperature is not completely reversible. I do not regard this as necessarily showing that this indicates the breaking of hydrogen bonds.

M. Schramm. — The RNA of TMV is stable until 50°. At 60° the infectivity is slowly reduced; that means that hydrolytic cleavages occur.

M. Ochoa. — In connection with utilization of nucleoside di- or triphosphates for polynucleotide synthesis it may be pointed out that, in biological activation reactions, ATP can be broken down either to AMP and pyrophosphate or to ADP and orthophosphate.

M. Todd. — The fact that ATP can at times transfer phosphate and at times adenylic acid residues is readily understood if it be

assumed that the effect of the protein component of the enzymes involved is to localise protons on the polyphosphate residue. This will give lability at one end of the polyphosphate group or the other according to the charge distribution. This is a subject which I have discussed in various lectures and publications and I shall not go into it in detail now. I shall be mentioning some matters relevant to it in my talk to-morrow and will gladly discuss it in more detail privately with Dr. Ochoa. It would take up too much of the meeting's time this afternoon to go into the subject now.

M. Sadron. — 1) Have measures of $[\eta]$ been made as a function of temperature ? If $[\eta]$ increases there should be hydrogen-bonding. If it decreases, it is an indication that no hydrogen-bonding exists.

2) In connection with Belozersky's and Ebel's work, have you incorporated polyphosphates in the solution during the RNA synthesis ?

M. Ochoa. — In answer to your first question, the intrinsic viscosity of solutions of poly AU has been found to increase with temperature in experiments of Warner and Breslow in our laboratory. As regards your second question, the effect of polyphosphates on the enzymatic synthesis of polyribonucleotides has not been studied, but it would certainly be interesting to do so.

M. Schramm. — Gierer found that the viscosity of RNA of TMV is increased at higher temperature.

M. Rich. — If the RNA molecule in solution were in the form of an elongated molecule with a moderately large number of intramolecular bonds, one would expect an initial rize in viscosity as the temperature of the solution is raised. The thermal motion could break the intramolecular hydrogen-bonding leading to a more extended molecule. However, this result may occur with either a randomly organized or regularly wound helical molecule, as long as they both unfolded under the thermal stimulus.

M. Markham. — I should like to ask for more details of the action of the Kornberg's enzyme in making poly AT. As I understand it, the enzyme makes this compound quite rapidly, but it has a rather long time lag. Does this not suggest that the enzyme is not really replicating its primer ?

M. Ochoa. — Recent experiments in Kornberg's laboratory have shown that, when poly AT is used as a primer, the enzyme replicates poly AT even in the presence of all four deoxyribonucleotide triphosphates.

M. Davidson. — Apparently that is the case as far as the nucleotide composition of the DNA, made with phage DNA as a primer, is concerned.

M. Rich. — As you know, Kornberg and his associates need a DNA primer for their enzyme. However, it now seems probable that a *single* strand of DNA rather than a double stranded molecule may be the primer *in vivo*. They find that heat denatured DNA is a better primer than the native material; in this form the chains have been partially separated. However, the best primer by far is the single stranded DNA which is found in the small virus Φ X-176. The product which is produced with this primer appears to be the usual two stranded form of the molecule, which supports the concept of using the complementarity of the two chains as the mode of reproduction.

THE FORMATION OF HELICAL POLYNUCLEOTIDE COMPLEXES

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INTRODUCTION

There is a great deal of interest today in the metabolism, structure and function of the nucleic acids. This interest is generated in large part by a growing picture which we have of the role of the nucleic acids as one of the major information bearing macromolecules in biochemical systems. It is widely held that the deoxyribosenucleic acid (DNA) is itself capable of acting as a carrier of genetic information. This interpretation rises out of the work which has been done on the transforming factor as well as from the observations that the infectivity of the bacteriophage viruses is carried by their DNA alone (Hershey and Chase, 1952).

We find in nature that the functions of the nucleic acids are divided into two groups for reasons that are unclear to us at the present time. The other class of polynucleotides are the ribosenucleic acids (RNA) which appear to have a different function in the cell than the DNA. Most of the cellular RNA is found in the microsomal particles where it constitutes over half the mass of the particle (Littlefield, et al, 1955). These units are believed to be the sites of protein synthesis and accordingly it is widely held that the ribosenucleic acid molecule plays an important role in the synthesis of proteins. It may be that

the sequence of nucleotide bases somehow influences the sequence of amino acids in the manufacture of protein. At the present time this is considered a reasonable hypothesis; however, it is important to realize that it is only a hypothesis rather than an established fact.

The functional differentiation between DNA and RNA is perhaps obscured somewhat by the observation that the purified RNA from the tobacco mosaic virus as well as from other viruses is able to act as an infectious agent alone (Gierer and Schramm, 1956; Fraenkel-Conrat, 1956; Colter, Harris and Brown, 1957). This demonstrates that the RNA polynucleotide chain is able to act as a carrier of genetic information in a way which is similar to that ascribed to the DNA molecule in the bacteriophage infection.

If we compare the information available today concerning the molecular structure of the two types of nucleic acids, we note a considerable disparity. A great deal is known about the details of the molecular configuration of DNA as will be discussed by Wilkins in an accompanying paper at this conference. But much less is known about the configuration of RNA. Attempts have been made to study the structure of isolated RNA molecules utilizing X-ray diffraction techniques, however, these studies were unsuccessful (Rich and Watson, 1954). The isolated molecules were too short to orient properly for X-ray diffraction analysis and it is quite likely that the RNA as normally isolated is heterogeneous. It was not until the enzyme polynucleotide phosphorylase was discovered that it became possible to study the configurational potentialities inherent in the ribosephosphate backbone of RNA.

THE SYNTHETIC POLYRIBONUCLEOTIDES

In 1955 Grunberg-Manago and Ochoa isolated polynucleotide phosphorylase, which has the unusual property of converting nucleotide diphosphates into polyribonucleotides. The enzyme cleaves off the terminal phosphate groups of the diphosphate and polymerizes the nucleotide residue. The resulting polymers resemble naturally occuring ribosenucleic acid in that they have the same covalent ribophosphate backbone as RNA and they have been shown to undergo similar enzymatic hydrolysis (Heppel, Ortiz and Ochoa, 1956). Polymers can be made with this enzyme which contain all the natur-

ally occurring purines and pyrimidines plus some which do not normally occur in RNA. Thus polymers can be made containing inosinic acid in which the purine hypoxanthine is found. The enzyme will also polymerize material which contains thymine, the pyrimidine which is a normal constituent of DNA rather than RNA (Griffin, Todd and Rich, 1958). Polyribonucleotide molecules can be made as homo-polymers containing only one base or as mixed polymers which have two or more purines or pyrimidines. This paper will discuss some of the reactions which are characteristic of the homo-polyribonucleotides.

When the synthetic polyribonucleotides were first synthesized it was not at all apparent that they were very interesting chemically. However, it was soon discovered that an unusual complex occured when polyadenylic acid was mixed with polyuridylic acid. The formation of this complex was associated with a drop in the ultraviolet spectra of these two substances as well as with an increase in the sedimentation properties as measured in the ultracentrifuge (Warner, 1956). By X-ray diffraction analysis it was found that these two molecules wrap around each other in solution to form a two stranded helical molecule which is very similar to naturally occurring DNA (Rich and Davies, 1956). The discovery of this remarkable interaction has been followed by a variety of similar discoveries among the other polynucleotides (Rich, 1958; Rich, 1958a; Davies and Rich, 1958). At the present time, we know of the existence of several of these elongated macromolecules which form two and three stranded helical complexes. Some of these will be discussed here and various features of their interaction will be described.

THE REACTIVITY OF POLYADENYLIC ACID

In an aqueous solution at neutral pH, polyadenylic acid exists as a more or less random coil. If, however, polyuridylic acid, polyinosinic acid or polyribothymidylic acid are added to polyadenylic acid in the presence of a small amount of salt, an interaction can be seen in which many of the solution properties are altered. Initially there is an increase in the viscosity of the solution. At the same time the optical density of the solution decreases. Accompanying this, there is a substantial rise in the molecular weight of the solution.

An X-ray diffraction analysis of fibers made from a 1:1 mixture of these polynucleotides shows a typical helical distribution in the scattering intensity and an overall diffraction pattern which is somewhat similar to that produced by the B form of DNA. The layer line spacing on the 1:1 mixture of polyadenylic acid (poly A) and polyuridylic acid (poly U) is 34 Å at 78 % relative humidity, i.e., just equal to that which is found in DNA (Rich, 1957). This represents the helical pitch of the molecules. From the strong meridional reflections in the region of 3 to 4 Å it can be shown that there are ten residues per turn of the helix. In both DNA and the poly (A + U) molecules the birefringence of the fiber is strongly negative which is associated with the fact that the planar purine and pyrimidine residues are oriented at right angles to the fiber axis. The poly (A + U) molecules are organized in a hexagonal lattice with a distance between the molecules of 28.8 Å which is somewhat larger than that which is observed for the DNA hexagonal packing.

With the exception of the diameter of the molecule, the two diffraction patterns are similar enough to suggest that they arise from a common structure. The uracil residue of adenine is quite similar to the thymine residue of DNA and it is identical with respect to its hydrogen bond forming properties. The diffraction pattern has been interpreted as indicating the formation of a two stranded helical molecule with a pair of hydrogen bonds between the uracil residue and the adenine residue which are the same as those between adenine and thymine in DNA. Because there is an additional hydroxyl group in the backbone of the polyribonucleotides the diameter of the molecule is increased slightly. This accounts for differences which are observed in the hexagonal spacing mentioned above and small differences which occur in the distribution of scattering intensity on the lower layer lines.

Similar studies have been carried out with the interaction of polyadenylic acid with polyribothymidylic acid, which results in the formation of a similar two stranded helical complex (Rich, to be published). An analogous reaction can also be seen in the interaction of polyadenylic acid with polyinosinic acid (Rich, 1958). The unusual feature of the polyinosinic acid reaction is the fact that both polynucleotide chains now contain purine bases instead of the purine-pyrimidine combination found in DNA. This interaction is accompanied by similar optical density and viscosity changes. When a

fiber of a 1:1 mixture of polyadenylic acid plus polyinosinic acid (poly I) is examined by X-ray diffraction analysis the characteristic helical distribution of scattering intensity is also observed. This diffraction pattern is also similar to the B form of DNA. The equator of the diffraction pattern can be indexed in the hexagonal system with a = 24.4 Å. The layer line spacing in the helical diffraction pattern indicates that the pitch of the helix is 38.8 Å. There is a meridional reflection near 3.4 Å due to the packing of the planar purine-purine pairs at right angles to the helix axis. Thus the overall configuration of the poly (A + I) molecule is very similar to that seen in DNA or in poly (A + U). The two polynucleotide chains interact by forming systematic hydrogen bonds as shown in figure 1. This shows that the adenine and hypoxanthine residues are joined together by hydrogen bonds which are similar to those which hold together the adenine and thymine residues in DNA.

The poly (A + I) helical complex is of considerable interest because the hydrogen bonding shown in figure 1 could also be formed if the hypoxanthine base were replaced by guanine, since the additional amino group attached to C_2 of the purine ring would not introduce any steric interference. This interaction also demonstrates that many of the features which give rise to a stable molecule of the DNA type with purine-pyrimidine hydrogen bonding are also present in a molecule with only purine-purine hydrogen bonding.

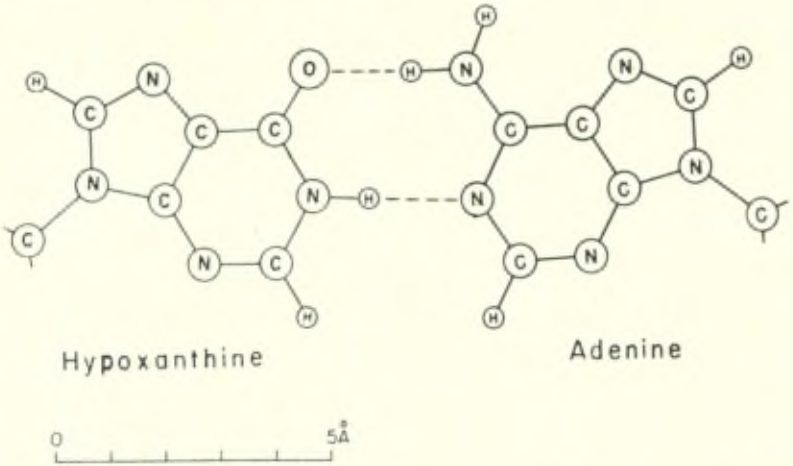

Fig. 1. — The hydrogen bond system which is found in the polyadenylic acid plus polyinosinic acid complex. One carbon atom is shown from the ribose ring attached to each base.

THREE STRANDED POLYNUCLEOTIDES

Three types of two stranded helical complexes have been described above, each of which contain polyadenylic acid. It has been possible to show that each of these two stranded complexes will take on an additional polynucleotide strand to form a three stranded helical molecule. Thus poly (A + U) will combine with an additional polyuridylic acid to make a molecule poly (A + 2U). Similarly, poly (A + I) combines with another polyinosinic acid strand to form poly (A + 2I), and poly (A + T) combines with polyribothymidylic acid to form poly (A + 2T). The formation of three stranded complexes was first observed by studying the optical density change which occurs when the helical complex is formed (Felsenfeld, Davies and Rich, 1957). In the case of polyadenylic acid plus polyuridylic acid, a minimum of optical density was first seen in solutions containing an equal number of adenine and thymine residues. However, under slightly different conditions a new minimum appeared at a

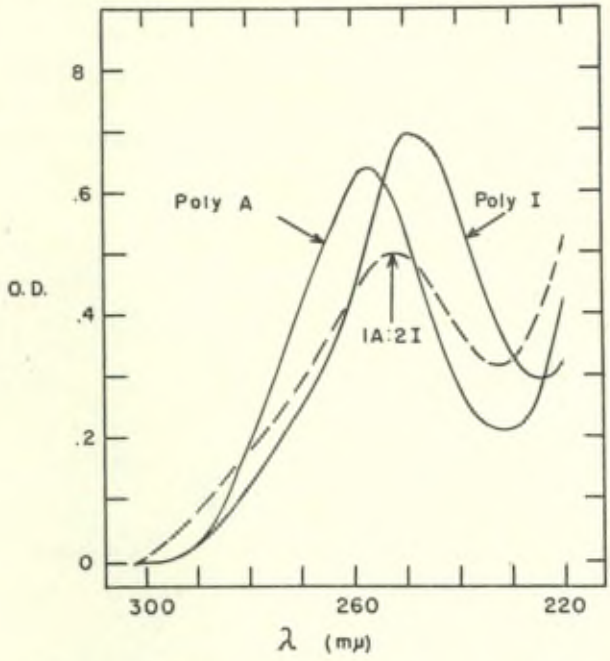

Fig. 2. — The ultraviolet spectrum of equal concentrations of polyadenylic acid (poly A), polyinosinic acid (poly I) and a 1:2 mixture of both solutions. The solutions are in 0.05M NaCl at neutral pH, T = 23° C.

mole ratio of two uracils to one adenine. Accompanying this additional change in optical density was an additional change in the sedimentation constant of the complex. Fibers can be drawn from a 2:1 molar mixture of polyuridylic acid and polyadenylic acid and studied by X-ray diffraction analysis. These produce a modified diffraction pattern relative to the 1:1 complex and this pattern has been interpreted in terms of the formation of a three stranded complex in which the third strand of polyuridylic acid is located in the deep helical groove between the polyadenylic acid and polyuridylic acid strands (Davies and Rich, unpublished observations).

Similar effects can be seen with the formation of the three stranded molecule involving two polyinosinic acid and one polyadenylic acid. During the formation of this three stranded complex there is a significant change in the optical density of the mixture as shown in figure 2. Here the initial curve of polyadenylic acid alone and polyinosinic acid alone intersect at a wave length of 254 mμ. However, when they are mixed together in 0.05 M sodium chloride at neutral pH there is a gradual decrease in the optical density which ultimately produces the spectrum as shown by the dotted line which has a new maximum at 254 mμ. The drop in optical density is larger for the formation of the three stranded complex than for the formation of the two stranded complex.

KINETICS OF POLYNUCLEOTIDE HELIX FORMATION

The speed with which polynucleotide strands wrap around each other is strongly dependent upon the ionic strength of the medium. However, at a given ionic strength the rate of this reaction can be measured by carrying out a kinetic analysis of the drop in optical density. In such a study we assume that the drop in optical density is proportional to the number of hydrogen bonds formed between the bases. A study of this type is shown in figure 3 for the combination of polyadenylic acid with polyinosinic acid where the optical density at 254 mμ is plotted as a function of time for various mole ratios of polyadenylic acid and polyinosinic acid. In these reactions the total number of moles of nucleotide is constant for all points in the figure even though the mole ratio varies. These reactions were carried out in 0.05 M sodium chloride solutions at neutral pH and at 23° C.

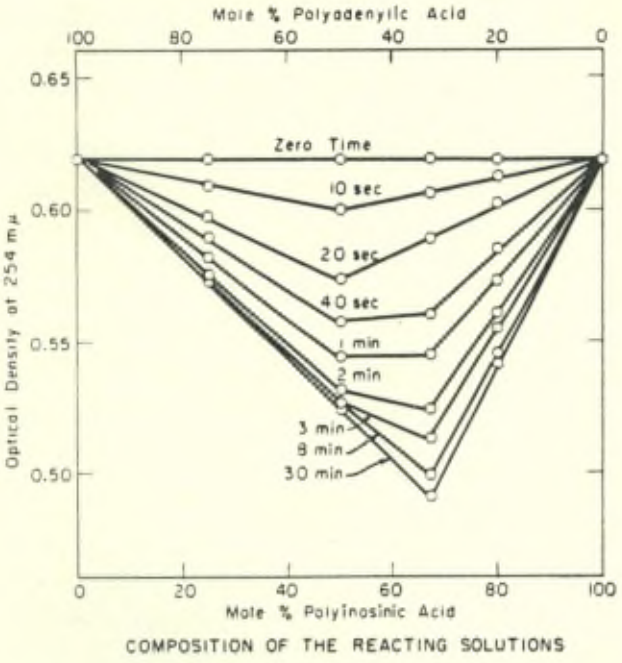

Fig. 3. — The optical density at 254mμ of various mixtures of polyadenylic acid and polyinosinic acid as a function of time. All points have the same total concentration of nucleotides. The solution are in 0.05M NaCl at neutral pH, T = 23° C.

It can be seen that at zero time no reaction has occurred and the various mixtures of polyadenylic acid with polyinosinic acid all have the same optical density. However, the optical density drops very quickly and at 10 seconds a maximum drop is seen at the 1:1 mole ratio position. The drop in optical density at the 1:1 position is still maximal at 20 seconds and only at 40 seconds is there evidence for the formation of a substantial amount of the 2:1 complex. This is shown by the break in the curve at 67 % polyinosinic acid. At 2 minutes the drop in optical density at the 2:1 position is greater than it is at the 1:1 position and at equilibrium (30 minutes), the drop in optical density is much greater at mole ratio 2:1 than 1:1. This kinetic profile shows the consecutive formation of an initial 1:1 complex followed by a 2:1 complex.

The final equilibrium curve shows that the drop in optical density is linear with increasing amounts of polyinosinic acid on the left side

of the minimum. Thus, complexing by the second strand of polyinosinic acid lowers the optical density in a manner similar to that produced by the addition of the first strand. By analyzing ultracentrifugal patterns, it can be shown that the 1:1 equilibrium mixture consists of two stranded polynucleotides rather than a mixture of three stranded and single stranded molecules.

We can learn something about the 1:1 combination of polyadenylic acid with polyinosinic acid by studying the rate of formation of the complex. In figure 4(a) is plotted the percent of poly (A + I) formed as a function of time. In figure 4(b) it is shown that the logarithm of the concentration of polyadenylic acid or polyinosinic acid varies in a linear fashion with time. Thus, the reaction as carried out under

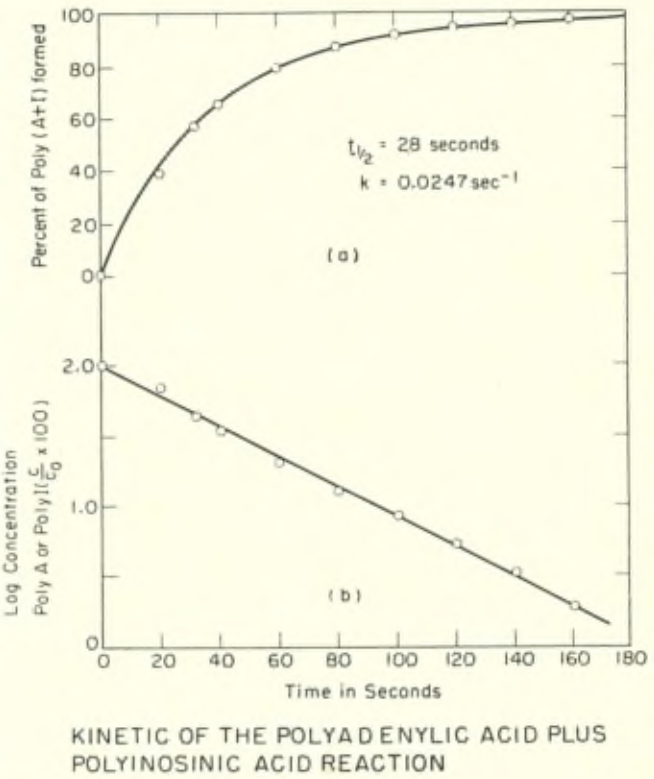

Fig. 4. — Kinetics of the reaction between polyadenylic acid (poly A) and polyinosinic acid (poly I) leading to the two stranded complex of poly (A + I), (a) Rate of formation of poly (A + I) as a function of time (b) Plot of the logarithm of either reactant as a function of time.

these special conditions of equal initial concentration follows first order kinetics with a half time of 28 seconds. The reaction has a « quasi » first order rate constant of $k = 0.0247$ sec^{-1}. The term « quasi » is used to indicate that this is the result of an initial exploratory study of the kinetics, and no final conclusions can be drawn as yet.

However, it is rather surprising that the reaction seems to proceed in such a simple fashion. Of the several possible interpretations, two will be discussed here.

One mechanism is shown in figure 5(a). The two polymer chains meet in solution and rapidly form an unorganized complex, such that most of the molecules are initially in this intermediate form, with very few free polynucleotide chains. In this complex, only a small number of hydrogen bonds are formed randomly, and there is no significant drop in optical density. Following this there is a much

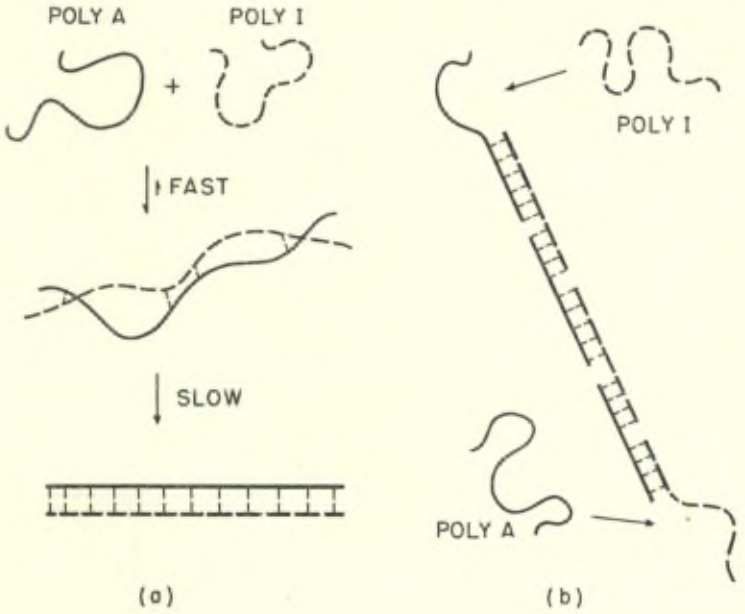

Fig. 5. — Possible mechanisms leading to the formation of two stranded complexes. (a) A random intermediate complex forms rapidly, and then slowly organizes into a regular two stranded helix (shown flattened out for simplicity). (b) The complex forms by continually adding polymer chains at the ends.

slower reaction in which these initial hydrogen bonds are broken and new hydrogen bonds are systematically formed leading to the production of the two stranded helix. Since we are only observing the last step in this sequence of reactions, it would follow quasi first order kinetics. However, the rate constant should change on varying the initial concentrations of poly I and poly A in such a mechanism. Alternatively, changes in the molecular length of the individual polymer molecules need not have a great effect in this mechanism if the rate limiting step is the formation of the hydrogen bonds between each individual pair of bases.

Another interpretation of these results is based upon a rapid formation of the hydrogen bonds through a cooperative process in which there is a « zippering » together of the polynucleotide strands once they have made an initial attachment. In this mechanism, the kinetic unit is the entire polynucleotide chain rather than the individual nucleotide. This could give rise to first order kinetics due to the formation of a very elongated complex with overlapping of alternative poly A and poly I molecules (figure 5(b)). As mentioned above, formation of the two stranded complex is accompanied by a large increase in viscosity and an increase in molecular weight which is greater than the sum of the individual poly A and poly I molecules. We can imagine a growing complex (figure 5(b)) which is able to add new polymer molecules only at the ends. However, the number of reactive ends remains constant since the complex is linear. This mechanism could produce first order kinetics. In such a case, the rate limiting step might be the attachment of a given random polymer chain to the organized complex. After a critical number of hydrogen bonds are formed, the remaining ones may form very rapidly by a cooperative process. Such a mechanism might also entail a preferential attachment of the oncoming polymer chain by one end so that there are no large gaps with unbonded adenines between the successive molecules of polyinosinic acid. This might account for the fact that there are very few bases which are not hydrogen bonded in the final equilibrium state (Felsenfeld, 1958).

If a mechanism of this second type is occurring, we would expect an alteration in the molecular length of the reactive units to have a great effect on the kinetic constant. Preliminary experiments with a variety of polymer lengths suggest this may be the case.

In this second mechanism, the reaction should not follow first order kinetics at the very onset of complex formation when the nuclei are forming. However, this process may be rapid and reversible, so that it is not reflected in the rate limiting process.

These experiments are preliminary and we can only draw tentative conclusions from this work. It is, however, of considerable interest to pursue this study in order to understand these mechanisms since it is quite likely that processes occur in the cell which involve the coiling and uncoiling of polynucleotide helices in the formation and replication of DNA as well as RNA.

EFFECT OF CATIONS ON POLYNUCLEOTIDE HELIX FORMATION

In the kinetic experiments described above, both polynucleotide solutions were in 0.05 M sodium chloride. At this low ionic strength the combination of the two polynucleotide strands occurred very slowly with a half time of 28 seconds. However, when the ionic strength is raised the speed of the reaction increases considerably so that special recording techniques are required to record the reaction.

This clearly indicates that the ionic strength of the environment has a significant influence on polynucleotide interactions. Further, it can be shown that there is a considerable difference in the effect of divalent and monovalent cations on the extent of these combinations (Felsenfeld and Rich, 1957).

Solutions of polynucleotides can be prepared from ion-free distilled water and then dialyzed against ethylenediamine tetraacetic acid to insure the complete removal of any excess ions. When these ion-free polynucleotide preparations are mixed together no reaction occurs. Since both of the polymers are negatively charged it is not unreasonable that the electrostatic forces should be strong enough to keep the polyelectrolytes from combining.

The effect of adding various amounts of monovalent and divalent cations is illustrated in figure 6. Solutions were made which had equal molar amounts of polyadenylic acid and polyuridylic acid. To these were added various amounts of sodium chloride or manganese chloride and the optical density was measured. As can be seen, the

addition of very small amounts of the divalent cation produces a striking effect. At a concentration of about 10^{-4}M Mn^{++} the two polynucleotide chains react as shown by the drop in optical density. There is an observable hypochromic effect when the molar ratio of manganese to phosphate is 2:1 and the reaction is completed at a mole ratio near 3:1. Similar results are obtained if magnesium chloride is used.

The effect of the sodium ion on the combination of polyadenylic acid and polyuridylic acid is similar to that of the divalent cation except that about one hundred times the concentration of sodium ion is necessary to produce the interaction. Thus in figure 6 at 10^{-2}M sodium ion the two polyelectrolyte molecules interact and a drop in optical density is produced.

The reversibility of this combination can be demonstrated. The addition of ethylenediamine tetraacetic acid makes the optical density rise if it had been lowered by the presence of manganese ion.

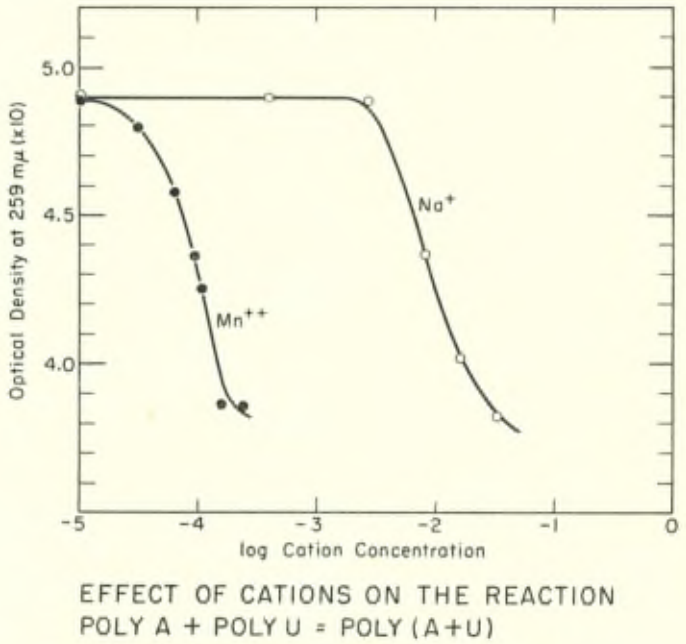

Fig. 6. — The effect of different salts on the formation of a two stranded complex between polyadenylic acid and polyuridylic acid.

Similar studies can be carried out on the effect of cations on the formation of the three stranded polynucleotide complexes. In figure 7, the effect of monovalent and divalent cations is shown for the formation of the three stranded complex from a mixture of the two stranded complex of poly (A + U) and a single additional strand of polyuridylic acid. In these experiments a 1:1 mixture of polyadenylic acid and polyuridylic acid was prepared in 0.1 M sodium chloride. To this complex was added another mole of polyuridylic acid and the additional lowering of the optical density was measured as a function of added salt. As shown in figure 7 there is a drop in optical density upon the addition of divalent cation which occurs over a wider range of concentration than in the formation of the two stranded complex, but with a midpoint near 10^{-3}M manganese or magnesium ion. This is in contrast to the 10^{-4}M manganese ion shown in figure 6.

The addition of sodium ion brings about the formation of the three stranded complex only when it is present at concentrations

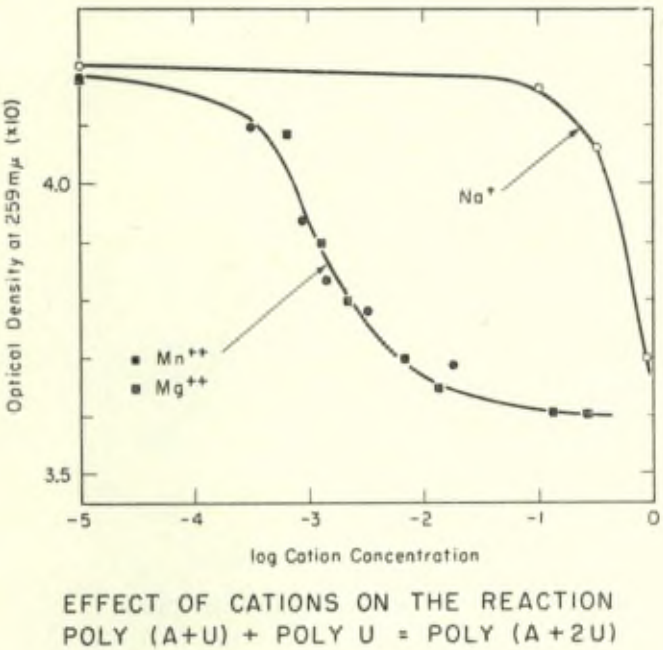

Fig. 7. — The effect of different salts on the formation of a three stranded complex from a two stranded complex and a single polynucleotide strand.

approaching 1 molar. Thus there is again a factor of 10^2 in the effectiveness of monovalent versus divalent cations in the formation of the three stranded complex. Similar experiments have been carried out with zinc ions and calcium ions, both of which have an activity similar to that shown for manganese and magnesium ions.

It is possible to analyze the data for the interaction of manganese or magnesium ion with the three stranded polynucleotide complex and show that the reaction is approximately second order in the divalent cation. Thus there are two cations for each group of three nucleotides, one adenine and two uracils.

In a solution of any given ionic composition, the addition of the first strand of polyuridylic acid to polyadenylic acid occurs much more readily than the addition of the second strand. This is of course to be expected from electrostatic considerations. The role of the cations in this complex is to neutralize the negatively charged phosphate groups so that the chains can approach each other. This can be accomplished in two ways. There may be a Debye-Huckel electrostatic screening, a measure of which is the ionic strength of the medium. However, the difference in the effectiveness of divalent and monovalent cations (10^2) is greater than can be expected due to the change in ionic strength. Consequently, it is likely that there is also a more specific interaction between the positively charged divalent cations and the negatively charged groups on the polymer. It is well known that magnesium and manganese ions complex with phosphate groups and it is highly likely that they play an important role in stabilizing the negatively charged polymer chains by this type of specific binding.

CONCLUDING REMARKS

These studies with synthetic polyribonucleotides make it possible for us to determine some of the important physical and chemical parameters in the specificity of polynucleotide chain interaction. These experiments have given rise to a variety of suggestions concerning the mode of action of the nucleic acids in vivo. In particular, the existence of three stranded polynucleotide complexes has led to the suggestion that the formation of RNA may occur via a process in which a two stranded DNA molecule acts as a template for a single

stranded RNA. However, the existence of a two stranded complementary DNA-like molecule containing the RNA backbone also reinforces the concept that RNA production may occur through the formation of a two stranded complementary helix, one strand of which is DNA and the other RNA. Only future work will enable us to discover whether either of these mechanisms actually occur in nature.

ACKNOWLEDGMENTS

Some of the experimental work reported in this paper was aided by a grant from the National Cancer Institute, National Institute of Health, Bethesda, Maryland.

REFERENCES

Colter, J.S., Harris, H.B. and Brown, R.A. *Nature*, **179**, 859 (1957).
Davies, D.R. and Rich, A. *J. Am. Chem. Soc.*, **80**, 1003 (1958).
Felsenfeld, G., *Biochim. Biophys., Acta*, **29**, 133 (1958).
Felsenfeld, G., Davies, D.R. and Rich, A., *J. Am. Chem. Soc.*, **79**, 2023 (1957).
Felsenfeld, G. and Rich, A., *Biochim. Biophys. Acta*, **26**, 457 (1957).
Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, **78**, 882 (1956).
Gierer, A. and Schramm, G., *Nature*, **177**, 702 (1956).
Griffin, B., Todd, A. and Rich, A., *Proc. Nat. Acad. Sci.* (Washington) **44**, 1123 (1958).
Grunberg-Manago, M. and Ochoa, S., *J. Am. Chem. Soc.*, **77**, 3165 (1955).
Heppel, L.A., Ortiz, P.J. and Ochoa, S., *Science*, **123**, 415 (1956).
Hershey, A.D. and Chase, M., *J. Gen. Physiol.*, **36**, 39 (1952).
Littlefield, J.W., Keller, E.B., Gross, J. and Zamecnik, P.C., *J. Biol. Chem.*, **217**, 111 (1955).
Rich, A. in *The Chemical Basis of Heredity*, McElroy, W.D. and Glass, B., ed., Johns Hopkins Press, Baltimore, 557 (1957).
Rich, A., *Nature*, **181**, 521 (1958).
Rich, A., *Biochim. Biophys. Acta*, **29**, 502 (1958, a).
Rich, A. and Davies, D.R., *J. Am. Chem. Soc.*, **78**, 3548 (1956).
Rich, A. and Watson, J.D., *Proc. Nat. Acad. Sci.* (Washington) **38**, 139 (1954).
Warner, R.C., *Fed. Proc.*, **15**, 379 (1956).

DISCUSSION DU RAPPORT DE M. RICH

M. Fraenkel-Conrat. — The remarkable parallelism in changes of U.V. absorbance under the influence of various salts of natural RNA and the base-pairing or base-tripling synthetic polymers is pointed out. Is it true that the X-ray diffraction diagrams do not support the conclusion that the underlying structure is also the same for the synthetic helices and the natural RNA in presence of divalent ions.

M. Rich. — As you know there are many effects which produce changes in the U.V. absorption of nucleic acids. It is hazardous to infer that a lowering of the optical density upon the addition of cations means the formation of hydrogen bonds in a helical complex. However, in the cases mentioned, the helical nature of the complexes is shown by the X-ray diffraction investigation.

Fibres of natural RNA produce an X-ray diffraction pattern which is distinctly helical in character. However, the diffraction pattern is not sufficiently resolved to yield a unique structural interpretation. The diffraction patterns produced by the synthetic polynucleotides are of many different types which reflect the fact that they form a variety of different helical structures. Only in the case of the complex of polyinosinic acid plus polycytidylic acid is the diffraction pattern similar to that seen in RNA.

M. Felix. — Has one ever investigated how these synthetic nucleic acids react with polyamines, protamines and histones ?

M. Rich. — Dr. Gary Felsenfeld has been investigating the reactivity of the synthetic polynucleotides in the presence of polyamides. He has found that they act on the polyadenylic plus polyuridylic acid system in a manner which is very similar to that described for metallic cations. Thus the divalent polyamine is about as effective as a divalent metal cation in bringing about the formation of the two-stranded complex.

I do not know of any studies with protamines or histones. It is conceivable that they might inhibit or delay the formation of the two-stranded helical complex if they combined firmly with the individual polynucleotides.

M. Wilkins. — It seems to me fairly clear that the X-ray diffraction patterns obtained by Drs Rich and Watson in 1954, although not well defined, indicate fairly clearly that RNA has a helical structure consisting of two (or possibly three) polynucleotide chains. I understand that recent work in Prof. Doty's laboratory lends support to this type of structure for RNA in solution.

M. Rich. — I agree that our earlier X-ray diffraction studies on RNA fibres strongly support a helical configuration for RNA. In view of the similarities between that diffraction pattern and 1 : 1 complex of polyinosinic plus polycytidylic acids, it is reasonable to believe that the RNA molecule is in the form of a two-stranded helix. However, it is very important to differentiate a structure of this type from the kind of structure found in DNA. Doty has clearly indicated the reversible nature of the heat denaturation curves found in RNA in marked contrast to the behaviour of DNA. Furthermore, the same reversibility is found with the synthetic polynucleotides containing all four of the RNA bases (poly A G U C). Poly A G U C produces the same diffraction pattern as found in naturally occuring RNA. Hence, the helical structure in the molecule cannot be dependent upon the specificity of nucleotide interactions over very long sequences. It seems quite clear that this is not a " definite " structure in the same sense as is found in DNA, where the integrity of the structure is dependent upon nucleotide sequence.

It may be that a sequence-dependent structure may be found for RNA in the microsomal particle.

M. Luzzati. — Il n'est peut-être pas toujours possible d'extrapoler à des solutions des renseignements structuraux obtenus avec des fibres assez peu hydratées. Ainsi, dans certains cas (RNA, peut-être), on pourrait avoir une structure bien organisée en solution, bien que l'organisation des fibres soit médiocre.

M. Wilkins. — In reply to Dr. Luzzati I may mention that Dr. Fresco (*J. Mol. Biol.*, vol. 1, 1959), has obtained a surprisingly sharp X-ray diffraction pattern from the acid-stable form of polyadenylic acid in solution. The data show that poly A has the same structure in solution (1-10 % concentration) as in oriented fibres.

M. Duchesne. — Il me paraît intéressant d'attirer l'attention sur un travail récent de Sutherland (1955). Cet auteur a mis en évidence la présence d'un dichroïsme, variable avec le degré d'humidité, de certaines bandes infrarouges de sels sodiques de DNA sous forme de films. Ce fait semble impliquer que le plan des nucléotides, plus ou moins perpendiculaire à l'axe moléculaire, est susceptible de subir une torsion sous l'action de l'humidité. L'incidence remarquable de ce facteur pourrait bien se rattacher à des effets de diffusion des molécules d'eau régis par les " défauts de réseau " de l'eau liée. C'est pourquoi l'hypothèse du Dr. Rich, selon laquelle des " défauts de structure " pourraient se produire au sein des molécules de DNA elles-mêmes, m'a spécialement réjoui. Il me semble que ces deux modalités d'un même concept devront être de plus en plus prises en considération pour comprendre certains aspects du comportement de ces systèmes, où de petites causes peuvent entraîner des effets importants. Une étude systématique de la conductivité électrique serait sans doute, à cet égard, utile. En tout état de cause, je serais heureux de connaître l'opinion du Dr. Rich sur ces points de vue.

M. Rich. — The results of the infra-red studies of Sutherland complement the X-ray diffraction results of Wilkins in that they both show that the DNA helix will form another type of structure when they are deprived of water molecules due to a lowering of the relative humidity. In this altered structure the base pairs are tilted so that they are no longer in a plane perpendicular to the helix axis. However, in aqueous solutions, all evidence points to a configuration in which the base pairs are oriented at right angles to the helix axis. Most of the studies of the polynucleotide interactions are carried out in aqueous media and it seems likely that there is only one stable configuration in this environment.

However, the fact that the DNA molecule is capable of transforming into another configuration is of great interest and tells us

something about the flexibility inherent in the sugar-phosphate backbone. This is, of course, related to the presence of possible defect sites in the polynucleotide molecules.

M. de Boer. — The striking results which Dr. Rich reports on the lowering of the optical density are measured at one wave-length. If the optical change is directly caused by and connected with the formation of hydrogen bonds, one might expect a shift of the absorption maximum and a general lowering of the absorption spectrum. By choosing a suitable wave-length one could then perhaps also obtain cases where the optical density (at that particular wave-length) would be increased. Is there a shift in the absorption maximum ?

M. Rich. — During the formation of the complexes between polyadenylic acid and polyuridylic acid there is no shift in the absorption maximum. Both of these polymers have an absorption maximum near 259 mμ. The helical complex also has a maximum at the same wave-length.

M. de Boer. — If there is no shift of absorption maximum one might perhaps conclude that the formation of hydrogen bonds (or the change of the nature of already existing hydrogen bonds) only indirectly influences the optical density, e.g. by altering number of hydrogen bonds per unit volume (locally). This might then lower the absorption probability without altering the energy of absorption.

M. Rich. — The fundamental nature of the hypochromic effect in the nucleic acids is still open to debate. There are several effects which could produce a change in the absorption spectrum. Among these are :

(1) An alteration in the number or type of hydrogen bonds formed by the polynucleotide molecule. This could produce a change since it may be associated with the stabilization of one or another tautomeric form.

(2) An alteration of the stacking of nucleotides might bring about a distortion of the π-electron systems of the purines and pyrimidines which could be reflected in the absorption spectrum.

(3) Finally, there is the possibility of a local deviation from Beers Law. Thus, in concentrating a large number of strong chromophores in a small region of space, some of the nucleotides may shield others from the ultraviolet light. However, when the nucleotides are depolymerized, they would all absorb the light and this would result in an increase in the optical density.

It is difficult to make a choice between these various effects. However, it should be born in mind that even a simple dinucleotide such as adenylic-guanylic acid has a considerable hypochromic effect relative to the free nucleotides.

M. Overbeek. — M. Rich has suggested that the big difference in concentration between monovalent ions and bivalent ions in producing two-stranded strains points to specific effects. On the other hand apparently there is little or no specificity within the groups of monovalent ions or bivalent ions. Similar large differences in flocculating power of ions of different valences are well known in colloid science. There they have been explained as purely electrostatic effects. The influence of bi- and multivalent ions is much higher than which corresponds with the ionic strength principle. This is due to the fact that the attraction between the local accumulation of negative phosphate charges and the positive ions increases exponentially with the valence. Particularly, if the chains attract each other at relatively large distances by Van der Waals forces and repel each other electrostatically one expects the concentration, at which the attraction can overcome the repulsion to vary inversely as the sixth power of the valence of the counter ion, that is as 1/64 for divalent and monovalent ions.

This suggest that the electrolyte effect observed by Dr. Rich may be purely electrostatic and not specific.

M. Rich. — I agree that the electrostatic effects may account for a large part of the difference in effectiveness between monovalent and divalent cations. However, Dr. Felsenfeld has carried out experiments such as are illustrated in figure 6 in which he decreased the concentration of the reacting polynucleotides. The resulting curves show not only a lowering of the inflection, but also a shift in the salt concentration at which the polynucleotides are half combined. A shift of this type implies that there is some specificity in the interaction in addition to the electrostatic effects.

M. Julia. — On sait que des ions métalliques peuvent favoriser énormément une réaction en établissant une chelation. Je demande alors si la distance entre les deux anions phosphoriques des restes à joindre permet l'établissement d'une chelation qui faciliterait le rapprochement des noyaux entre lesquels doivent s'établir les liaisons hydrogène ?

M. Rich. — At one time I wondered whether it was possible for a hydrated magnesium ion to be bound to two phosphate groups — one on each chain of a two-stranded helix. The distance is too great, however, so that this is unlikely. However, it is not unreasonable to believe that such a configuration may be an intermediate in the formation of the two-stranded helical complex from the two random-coil molecules.

M. Fraenkel-Conrat. — In considering the mode of binding of Mg^{++}, one must keep in mind that the binding of divalent ion enables the macromolecule to also bind inorganic phosphate in a manner which can be reversed only concomitant with release of the Mg^{++}, e.g. by versene. Thus at least one ionic valence of the RNA-bound metal seems to be free.

M. Rich. — In the combination of polyadenylic acid with two polyuridylic acid molecules to form a three-stranded complex, two magnesium ions seem to be used for each group of three nucleotides. This would also suggest that at least one positive charge in the magnesium ion is free to complex with other anions.

Sir Alexander Todd. — Is there any difference in the stability of the double helical structures prepared from poly A and poly U and the DNA double helices ? I ask this because I was just wondering whether interaction between the 2'-OH and the adjacent internucleotidic linkage might perhaps so distort the polyribonucleotide chains that they would not form the double-stranded structure easily unless some method (e.g. complexing with Mg^{++}) were first applied to remove this interaction. This is, of course, sheer speculation and I have not thought about the problem in detail. But it would be nice to have some explanation for the fact that natural RNA seems not to occur in the double helical structure when the

only difference from DNA lies in the presence of a hydroxyl at C_2, in the sugar residues.

M. Wilkins. — Molecular model building shows that it is not possible to build ribonucleic acid in the B configuration of DNA. There are prohibitively close distances between the 2'-OH of the ribose and other groups.

M. Rich. — There is some evidence concerning the effect of ions on DNA. In distilled water or in salt solutions of less than 10^{-5} M, the DNA molecule denatures — that is, the chains pull apart and there is a rise in optical density. This process can be partially reversed by adding salt to the DNA solutions which results in a partial lowering of the optical density. The effectiveness of various mono- and divalent cations in this process is almost exactly what is observed in the synthetic polyribonucleotides accompanying the helix formation. Thus, the two-stranded DNA molecule and the two-stranded poly A plus poly U molecules appear to respond to cations in the same way. I think this makes an interaction with the 2'-hydroxyl groups less likely, although it does not rule it out.

It is possible that natural RNA does occur in the form of a two-stranded complementary structure of the DNA type in some phase of its metabolic cycle in the cell. The indications for this are slight, but perhaps not negligible.

Sir Alexander Todd. — In discussing the effects of ions on the formation of double helical structures analogous to DNA in these polyribonucleotides no one seems to have considered the possible significance of the 2'-hydroxyl group adjacent to the phosphate in ribonucleotides. This is the only chemical difference between the ribo- and deoxyribosides. Does this perhaps mean that interaction between the 2'-OH and the phosphates in Dr. Rich's polyribonucleotides (and in RNA) has an adverse effect on the double helix formation or indeed on any helical structure ? Mg ions could by complexing with the phosphate and hydroxyl make the situation normal so that the helical structure forms.

M. Rich. — I believe that the 2'-hydroxyl group does not interfere with the formation of helical complexes and that it only has a small effect on the configuration of the ribose-phosphate backbone when

it is compared with the backbone of DNA. If it were possible to make large synthetic homopolynucleotides of the DNA type, I believe they would show the same type of reactivity relative to each other — that is, they would form two- and three-stranded helical complexes and would require roughly the same concentration of divalent cations to overcome the electrostatic barrier to the formation of the helix.

M. Ubbelohde. — Two questions arise in regard to different parts of Dr. Rich's paper :

(i) when he describes the closely equivalent behaviour of divalent ions such as Mg^{++} and Mn^{++}, both of which are rather small and can form complexes with phosphate and OH groups, how far has he extended this comparison :

a) to much larger divalent ions which would not complex as easily. For example, Ba^{++}, or even better, large divalent complex ions of metals such as cobalt;

b) to trivalent ions with very different tendencies towards complex formation, such as the rare earths, or Fe^{+++}. A fairly careful quantitative comparison could give useful clues about the role of positive ions generally.

(ii) It is a real puzzle to those who are familiar with the statistical thermodynamics of defect formation in solids and in macromolecules, if assemblies with molecular weight of the order of 10^6 or more are completely free from random defect sites, especially when these involve energies of formation of only 2-3 kT.

How well is this absence of defects established by the experimental methods at present available? They could have important functions in controlling rate processes for these macro-molecules.

M. Rich. — We have not done a great deal of work with other cations as yet. Preliminary work has shown that Fe^{+++} as well as Ba^{++} will bring about the formation of the helical complexes, but these have not been compared quantitatively. I agree that the result of such a study would be quite useful.

It is quite likely that defects occur in the formation of two- and three-stranded helical complexes. Indeed it is surprising that we do not have evidence for many more defects. In the combination

of poly A with poly U, we have reasonably reliable evidence that over 95 % of the purines and pyrimidines are hydrogen bonded to each other. This rules out the possibility of having large stretches of poly A or poly U which are unpaired when the two polymers are together at equimolar concentrations. The evidence for this is in the lowering of optical density which appears to be completely linear right up to the equivalence point. However, it is possible that the energy of forming a defect is much greater than a few kT. Thus, for example, if water molecules were prevented from gaining access to the nucleotides in the defect site, the energy loss would be considerable.

M. Watson. — Wat is the evidence that the free proton in poly I is located on N_1 as postulated in your three-stranded helical structure ?

M. Rich. — Infra-red studies have been carried out by workers in Australia on a variety of hypoxanthine derivatives. From this they concluded that the oxygen in the six position is in the keto form rather than enol. Thus it is likely that the proton is attached to N_1 of the purine ring.

M. Wilkins. — As Dr. Rich has said, the 3-chain polynucleotide structures have interesting biological implications. Of the various structures he has studied, the AUU polymer is probably the most interesting. There are at present two proposals for the structure of AUU, the one proposed by Dr. Rich originally, and the other by Dr. Zubay. Dr. Rich has mentioned that he has built molecular models of these two structures and has calculated the diffraction from them and that he has obtained a new and improved X-ray photograph of AUU. He said that this new data was in agreement with his structure but not with that of Dr. Zubay. In view of the interest in this matter could he give us further information ?

M. Rich. — Dr. Davies and I have recently obtained an improved diffraction photograph of the helical complex containing polyadenylic acid plus two polyuridylic acid chains. We have compared this data with the predicted Fourier transform of our model and we find there is quite good agreement. Dr. Zubay's proposed model has

a drastically different type of hydrogen bonding and, what is of more importance, the three ribose-phosphate chains are arranged about the helical axis at angles which are substantially different from those in our model. The different arrangement has a significant effect on the Fourier transform, and accordingly we can make a reasonably clear distinction between the two proposals. We hope to have this work finished reasonably soon so that we can submit it for publication.

THE STRUCTURE OF RNA CONTAINING VIRUSES

by **GERHARD SCHRAMM**
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Nucleoproteins isolated from normal cells are in general mixtures of substances with a multitude of different functions. In contrast, viruses are a class of nucleoproteins particularly suitable for structural investigations, since their biological activity can be determined quantitatively. Genetically homogeneous strains can be isolated from many viruses. Spontaneous and induced mutants can be obtained and thus chemical alterations corresponding to genetic changes can be studied. Further methodological progress may allow a correlation between virus nucleic acid structure and the structure of protein produced under the control of these nucleic acids.

Virus nucleic acid can be of deoxypentose or ribose type. All viruses also contain protein and some more complicated viruses contain lipids and carbohydrates. Important groups of DNA containing viruses are summarized in Table I and groups of RNA containing viruses in Table II ([1, 2]). Only some typical representatives are mentioned.

In this paper only RNA containing viruses will be discussed. It could be demonstrated in some cases that RNA *per se* is infectious and responsible for the process of multiplication. The first evidence for this important fact was achieved with tobacco mosaic virus (TMV) ([3, 4]). Gierer and Schramm ([3]) extracted the protein from a solution of TMV with phenol, using a method developed by Schuster, Schramm, and Zillig ([5]). The aqueous phase contains free RNA, which proved to be infectious. By chemical

TABLE I
DNA containing viruses.

Group and typical representative	Molecular weight	% DNA	DNA/virus
Papilloma virus	$45 \cdot 10^6$	8.7	$3.9 \cdot 10^6$
Adeno-viruses		?	
Pox-viruses			
Vaccinia	$3{,}200 \cdot 10^6$	5.6	$200 \cdot 10^6$
Bacteriophages			
T^2	$200 \cdot 10^6$	44	$90 \cdot 10^6$
Polyhedral viruses			
Bombyx mori	$300 \cdot 10^6$	13	$40 \cdot 10^6$

TABLE 2.
RNA containing viruses

Group and typical representative	Molecular weight	% RNA	RNA/ virus
Spherical plant viruses			
Tomato bushy stunt virus	$10.65 \cdot 10^6$	16.5	$1.8 \cdot 10^6$
Rod-like plant viruses			
Tobacco masaic virus	$40 \cdot 10^6$	5.6	$2.2 \cdot 10^6$
Entero viruses (Polio, Coxsackie, ECHO)			
Poliomyelitis-virus	$6.8 \cdot 10^6$	22-30	$1.8 \cdot 10^6$
Anthropod-borne encephalitis virus			
Western equine encephalitis virus	$24 \cdot 10^6$	4.4	$1 \cdot 10^6$
Foot and mouth disease virus	$7 \cdot 10^6$	—	—
Influenza-group			
Influenza-virus	$200 \cdot 10^6$	1	$2.2 \cdot 10^6$
Fowl plague virus	$150 \cdot 10^6$	1.8	$2.7 \cdot 10^6$
Multiform viruses			
Newcastle disease virus	$800 \cdot 10^6$	4	$32 \cdot 10^6$

analysis it was shown that the protein content was less than 0.4 %. Virus protein could not be detected serologically, meaning that the contamination is less than 0.02 %. Evidence was obtained that the infectivity of the RNA was not due to contamination with intact virus or virus protein ([3]). The infectivity is not diminished by repeated extraction with phenol. TMV antiserum does not neutralise the infectivity of RNA preparations whereas a virus solution of the same activity is inactivated. The activity of RNA is rapidly destroyed by ribonuclease (RNase) whereas TMV is not affected by this enzyme. In RNA preparations the infective particles have a sedimentation constant of about 20 S whereas TMV has a constant of 190 S under the same conditions. Fraenkel-Conrat ([4]) prepared RNA by treating the virus with sodium dodecylsulfate and came to the same conclusions.

The size and shape of infectious RNA was studied in some detail by Gierer ([6]). The RNA preparations were not homogeneous with regard to molecular weight. The main component has a sedimentation constant of $s_{20} = 31$ S ($c \longrightarrow 0$) in 0.02 M phosphate buffer, but there is also some polydisperse RNA material which sediments slower. Only the high molecular component proved to be infectious. It has a molecular weight of about $2.1.10^6$, determined by sedimentation and viscosity measurements. The shape of the molecule depends on the ionic strength of the medium. When the ionic concentration is extremely low the sedimentation constant decreases to a value of 6-7 S, but the viscosity increases so that the molecular weight remains unchanged. The RNA molecule is uncoiled and stretches in water as can be expected for such a polyelectrolyte. These changes of shape are reversible and have no influence on the infectivity which obviously does not depend on the configuration or superstructure of the molecule. The viscosity of RNA is about 100 times less than that of DNA of the same molecular weight, i.e. RNA is much more flexible than DNA. The reason for this flexibility is that RNA from TMV consists of a single chain of nucleotides in contrast to the double stranded DNA. The evidence for a single chain was obtained by Gierer by studying the kinetics of degradation with RNase. By incubation with RNase the viscosity and optical rotation decreased and the UV absorption increased; simultaneously acidic groups were liberated. The simplest explanation

for these effects is that RNA is coiled in salt solutions. The mutual influence of adjacent nucleotides depresses the UV extinction and increases the optical rotation. By splitting phosphate bonds between nucleotides, values characteristic for isolated nucleotides are reached. From the decrease of viscosity and activity during incubation with RNase, Gierer calculated that each cleavage of a phosphate bond reduces the length of the molecule and destroys the activity. If RNA were polystranded, a reduction of the length could be expected only when several cleavages were accumulated in different strands. This was actually observed in double stranded DNA (7). In contrast, the degradation of RNA strictly follows a one hit curve and has no latent period. X-ray data on TMV are further evidence for the single strand (see below). From these experiments it follows that the intact strand is necessary for the multiplication. It can be calculated that one RNA molecule contains 6,000 nucleotides. This corresponds to the total amount of nucleotides in the virus particle. Therefore we have only one nucleotide chain in each particle which runs through the whole molecule.

FUNCTION OF RNA DURING THE MULTIPLICATION OF TMV

The infectivity of RNA assayed on Nicotiana glutinosa is about 0.3 % of that of an equivalent amount of RNA within the complete virus. Probably the lower infectivity is due to the fact that the RNA is no longer protected by a protein coat. Fraenkel-Conrat and Singer (8) were able to reconstitute the complete virus by mixing RNA with virus protein. The reconstituted particles have an activity of about 30-60 % of the original virus. This is good evidence that the lower infectivity of free RNA is only due to the loss of the protein shell.

The separation of protein from RNA and the recombination take place during the multiplication of the virus in the host. Siegel *et al.* (9) and Schramm and Engler (10) demonstrated that the synthesis of the virus in cells starts earlier after infection with free RNA than after infection with intact virus. The latent period is shortened from 30 to 20 hours in tobacco plants at

23° C. The difference probably corresponds to the time for stripping the protein coat. Studying the amount of free RNA and of complete virus immediately after infection, Schramm and Engler [11] found first a production of free RNA followed later on by a synthesis of protein. With the commencement of protein synthesis, the amount of free RNA decreases, due to its combination with protein to form a complete virus. Zech [12] employed ultraspectrophotometrical methods to show that the RNA content of TMV infected hair cells rises at first in the nucleus. Schramm and Röttger [13], using fluorescent antibodies, demonstrated that the formation of virus protein occurs only in the cytoplasma. Thus the multiplication of TMV can be described by the following steps: (1) stripping of the protein coat from RNA; (2) multiplication of virus RNA in the nucleus; (3) formation of virus protein in the cytoplasma under the control of RNA, and (4) combination of protein and RNA to form complete virus.

ISOLATION OF INFECTIOUS RNA FROM OTHER VIRUSES.

Infectious RNA could also be extracted with phenol from other viruses. The isolation of infectious RNA from cells infected with Mengo, West Nile, and Type II Poliomyelitis (MEF_1 strain) virus has been described by Colter and co-workers [14, 15]. Mengo and West Nile viruses were grown in cells of the Ehrlich ascites carcinoma, while polio virus was propagated in the central nervous system of suckling hamsters. The infectivity of these RNA preparations was about 2.5-3 log units less than that of the starting material. The conclusion that the infection in each case was due to a RNA component was based on evidence obtained from experiments similar to that with TMV-RNA. The infectivity was abolished by brief treatment with RNase. The infectious principle of virus and RNA preparations could be differentiated on the basis of their sedimentation velocities and further by their different behaviour in 1M NaCl, where the virus activity was reduced to 0.1 % whereas the RNA remained stable. Centrifugation experiments on these infectious RNA preparations suggested that the infectious principle has a sedimentation constant of about

32 S whereas that of the virus is 160 S [16]. Alexander and co-workers [17, 18] have reported the isolation of infectious RNA from partially purified type I polio virus concentrates. The cytopathogenic action of the RNA preparations has been completely inhibited by RNase but not by the same concentration of DNase, papain, or chymotrypsin. The infectivity of this RNA was inhibited by type I antiserum, but also by normal monkey serum. The isolation of infectious RNA from purified virus and from tissue extracts after long storage suggests that the infectious RNA was derived from virus particles themselves and not from free RNA or other precursors. From the sedimentation constant of infectious RNA and from the RNA content of the whole virus particle, it can be concluded that the molecular weight of this RNA is of the same order of magnitude as that of TMV. The phenol method was also useful with other entero-viruses. Franklin, Wecker, and Henry [19] isolated an infectious RNA from brains of mice infected with mouse encephalomyelitis virus. The results suggest that the infectious principle is RNA and is derived from the virus. The dose response curve shows that one RNA unit is sufficient to cause infection. An inhibitory effect of DNA was observed.

Slightly different results were obtained by Huppert and Sanders [20] by extraction of mouse ascites cells infected with murine encephalomyocarditis virus. The amount of infectivity extractable by phenol was not directly related to the virus content of the cells. It is suggested that the infected cells contain, in addition to virus, a second component related in some way to virus synthesis. This material may be a form of viral RNA which has failed to be assembled into complete virus.

Wecker and Schäfer [21] and Wecker [22, 23] reported the extraction of infectious RNA with cold phenol from cells infected with eastern equine encephalitis (EEE) or western equine encephalitis (WEE). The active RNA could only be obtained when the phenol was added to the tissue before homogenization. The treatment of purified WEE with 80 % phenol at low temperature (about 4° C) failed to yield any viral RNA. This suggests that the infective RNA is not derived from intact virus but from some precursor. Later Wecker [23] found that infectious RNA could also be extracted from purified WEE virus by treatment with

phenol at 40-50° C. The infectivity of such RNA preparations was low compared to the infectivity of the original virus preparation (about 10^{-7}). The infectivity is sensitive to RNase. It can be precipitated by ethanol without any loss of infectivity, in contrast to the virus. It sediments in the ultracentrifuge with a speed similar to the RNA from TMV.

The phenol extraction was also successfully applied to Semliki forest virus by Cheng [24] and to the virus of foot and mouth disease by Brown *et al.* [25] and by Mussgay and Strohmaier [26]. Further it should be mentioned that Latarjet *et al.* [27, 28] were able to prepare nucleic acid from leukemic cells of mice. In this case the method of Simmons [29] was employed, using xylene sulfonate. The nucleic acid preparation produced leucemia in other mice, but it was not decided whether the active agent was RNA or DNA.

It has not been possible to extract an infectious RNA from myxoviruses such as fowl plague virus or Newcastle disease virus. It is not clear whether this failure is due to methodological difficulties or whether multiplication of these viruses cannot be initiated by a single RNA molecule. Further, perhaps other substances besides RNA are necessary for the virus synthesis.

In general we should remember that the purification of animal viruses is much more difficult than that of TMV, and therefore the evidence that nucleic acid alone is the infectious principle is not so conclusive as with TMV.

For comparison high molecular weight RNA was also isolated with phenol from various normal tissues. Gierer [30] studied the high molecular weight RNA from microsomes of animal tissues and from unfractionated plant tissue. In both cases two main components were observed in the sedimentation diagram. Their molecular weight is calculated on the basis of sedimentation and viscosity measurements to be around $1.8.10^6$ and $0.6.10^6$, respectively. The kinetics of degradation by RNase suggests that the RNA molecule is composed of a single strand of nucleotides. Its structural features are similar to those of viral RNA from TMV. The molecular weight of the larger component corresponds approximately to the RNA content of cytoplasmatic ribonucleoprotein particles as well as to that of small viruses. This

suggests a fundamental relation of that component of cellular RNA to viral RNA. Timasheff *et al.* [31] have studied isolated RNA from Ehrlich ascites cells by means of light scattering. The mean molecular weight of this material was estimated to be $1.2.10^6$, compatible with the results of Gierer. Comparing the RNA content per virus in Table II we can assume that the amount of RNA per virus particle is of the same order for all RNA containing viruses except Newcastle disease virus. This fact was first stated by Frisch-Niggemeyer [32]. It is interesting that this amount is similar to the RNA content of cytoplasmatic ribonucleoprotein particles. It seems that a certain molecular weight of the RNA is suitable for the synthesis of protein.

ARRANGEMENT OF RNA WITHIN VIRUSES

Detailed data on the arrangement of RNA in virus particles are only available for TMV. Franklin [33] developed the following model for TMV, mainly based on her X-ray data and on chemical investigations carried out by other laboratories [1]. TMV is a hollow cylinder constructed of peptide subunits having a molecular weight of about 17,000 arranged in a helix. The nucleic acid is embedded in the hollow cylinder. The phosphate groups are located some 40 Å from the center. The molecular weight of the peptide subunits calculated from X-ray data fits well to the molecular weight determined by amino acid and endgroup analysis [1]. Recently Anderer [34] determined the size of the peptide units by sedimentation and diffusion measurements. In agreement with the other data, a molecular weight of 18,800 ± 10 % was found. The TMV rod consists of 2,300 of these subunits, resulting in a total length of 3,000 Å. The RNA strand follows the pitch of the protein helix. Assuming a single strand of 6,000 nucleotides the length of the chain would be just sufficient to run through the whole cylinder. A double molecule would be too short.

A protein cylinder of the same pitch can be obtained by reaggregation of the peptide subunits alone [35]. These rods are not infectious. The diameter and internal structure are the same as in the virus, but the length is variable. From these results it can be concluded that the internal structure of nucleoproteins is determined

mainly by the properties of the peptide subunits, but the length of the aggregate is determined by the RNA.

The spherical plant and animal viruses are not so precisely analysed as is TMV. The stability of these viruses against RNase suggests that the nucleic acid is arranged inside the particle and is protected by a protein shell. These general features are also supported by the electrophoretical studies of turnip yellow mosaic virus by Markham and Smith ([36]). X-ray data and physical investigations show that the protein shell of the spherical plant viruses consists of numerous subunits ([37]).

INACTIVATION AND MUTATION BY CHEMICAL MEANS

The RNA of TMV is the carrier of genetic information. Therefore it is interesting to elucidate whether chemical alteration of the nucleotides leads to inactivation or mutation. Schuster and Schramm ([38]) studied the reaction of HNO_2 with the amino groups of the purines and pyrimidines in TMV-RNA. According to the reaction scheme $R—NH_2 + ONOH \longrightarrow R—OH + H_2O + N_2$ the amino groups are transformed to hydroxy groups. Adenine is transformed to hypoxanthine, guanine to xanthine, and cytosine to uracil, whereas uracil remains unchanged. It was possible to deaminate infectious RNA without splitting the nucleotide chain. By hydrolysis of treated RNA, the reaction rate of adenine to hypoxanthine, of guanine to xanthine, and of cytosine to uracil was determined. Simultaneously the inactivation of RNA was measured by bioassay. The deamination of one nucleotide out of 3,300 inactivates the whole molecule. Since the RNA of TMV contains 6,000 nucleotides, more than half of all nucleotides are necessary for infectivity.

It was believed that the alteration of the other 3,000 nucleotides leads to a mutation rather than to an inactivation. This was proved by Gierer and Mundry ([39, 40]). Treating RNA or virus particles with HNO_2 under different conditions they observed a high mutation rate. Using a certain marker, namely the production

of local lesions on Nicotiana tabacum (Java), the mutation rate was determined quantitatively. The absolute maximum of mutations was reached when an average of one deamination has taken place per molecule, i.e. the infectivity decreased to 1/e of the original value. Continuing the treatment with HNO_2 a state can be reached where probably all surviving particles are mutated. By isolating the treated virus particles a very broad spectrum of mutations was observed. Other authors have attempted to induce mutations in isolated virus particles *in vitro* by irradiation or chemical agents, but no convincing results were obtained. It was only possible to inactivate the particles. The reaction with HNO_2 seems to be the first case of mutations induced by chemical alteration of isolated virus particles. The inactivation and mutation of TMV by this reaction follows a one-hit curve, i.e. each nucleotide of 6,000 is relevant for the multiplication.

Since every hit (deamination) has a 50 % probability to inactivate the virus, very few hits are sufficient to completely inactivate the RNA. Since this inactivation is very rapid under mild conditions this method may also be used for the preparation of vaccines from killed viruses ([11]). For this purpose it is necessary to completely inhibit the multiplication of a virus, without destruction of the antigenic properties. Considering the new results on the dominant rôle of the nucleic acid we have to look for reactions, which change the nucleic acid with a minimum of alterations in the protein part. Careful studies on TMV and several animal viruses showed that deamination by HNO_2 fulfils these demands. The viral protein coat seems to be permeable to nitrite. Thus the alteration of the RNA inside intact virus occurs with a fast and constant rate until total inactivation has been achieved.

REFERENCES

(1) References see : G. Schramm : „ Biochemistry of Viruses ", *Annual Review of Biochemistry*, **27**, 101 (1958).
(2) References see : W. Schäfer in Viruses, Academic Press, New York in press.
(3) A. Gierer and G. Schramm : *Z. f. Naturforschung*, **11 b**, 138 (1956); *Nature*, **177**, 702 (1956).
(4) H. Fraenkel-Conrat, B. Singer and R. C. Williams : *Biochim. Biophys. Acta*, **25**, 87 (1957).
(5) H. Schuster, G. Schramm and W. Zillig : *Z. f. Naturforschung*, **11 b**, 339 (1956).
(6) A. Gierer : *Nature*, **179**, 1297 (1957); *Z. f. Naturforschung*, **13 b**, 477 and 485 (1958).
(7) C. A. Dekker and H. K. Schachman; *Proc. Nat. Acad. Sci.*, **40**, 894 (1954).
(8) H. Fraenkel-Conrat and B. Singer : International biochemical congress, Vienna, 1958.
(9) A. Siegel, W. Ginoza and S. G. Wildman : *Virology*, **3**, 554 (1957).
(10) G. Schramm and R. Engler : *Nature*, **181**, 916 (1958).
(11) R. Engler and G. Schramm, *Nature*, **183**, 1277, 1959.
(12) H. Zech and L. Vogt-Köhne : *Naturwissenschaften*, **42**, 337 (1955).
(13) G. Schramm and B. Röttger : *Z. f. Naturforschung*, in press.
(14) J. S. Colter, H. H. Bird and R. A. Brown : *Nature*, **179**, 859 (1957).
(15) J. S. Colter, H. H. Bird, A. W. Moyer and R. A. Brown : *Virology*, **4**, 522 (1957).
(16) C. E. Schwerdt and F. L. Schaffer : *Virology*, **2**, 665 (1956).
(17) H. E. Alexander, G. Koch, I. M. Mountain, K. Sprunt and O. v. Damme : *Virology*, **5**, 172 (1958).
(18) H. E. Alexander, G. Koch, I. M. Mountain and O. v. Damme : *J. Expt. Med.*, **108**, 493 (1958).
(19) R. M. Franklin, E. Wecker and C. Henry : *Virology*, **7**, 220, 1959.
(20) J. Huppert and F. K. Sanders : *Nature*, **182**, 515 (1958).
(21) W. Wecker and W. Schäfer : *Z. f. Naturforschung*, **12 b**, 415 (1957).
(22) E. Wecker : *Z. f. Naturforschung* in press.
(23) E. Wecker : *Virology*, **7**, 241, 1959.
(24) P. Y. Cheng : *Nature*, **181**, 1800 (1958).
(25) F. Brown, R. F. Sellers and D.L. Stewart : *Nature*, **182**, 535 (1958).
(26) M. Mussgay and K. Strohmaier : *Ztbl. f. Bakteriologie, Parasitenkunde u. Infektionskrankheiten* **173**, 163 (1958).
(27) R. Latarjet and M. de Jaco : *Comptes rendus*, **246**, 499 (1958).
(28) R. Latarjet, N. Rebeyrotte and E. Moustacchi : *Comptes rendus*, **246**, 853 (1958).
(29) N. S. Simmons, S. Chavos and H. K. Obrach : *Fed. Proc.*, **11**, 390 (1952).
(30) A. Gierer : *Z. f. Naturforschung*, **13 b**, 768 (1958).
(31) S. N. Timasheff, R. A. Brown, J. S. Colter and M. Davies : *Biochim. Biophys. Acta*, **27**, 662 (1958).
(32) W. Frisch-Niggemeyer : *Nature*, **178**, 307 (1956).
(33) R. E. Franklin, A. Klug and K. C. Holmes : „ Ciba Foundation Symp. on the Nature of Viruses", London 1957.
(34) F. A. Anderer : *Z. f. Naturforschung* **14 b**, 24 (1959).
(35) G. Schramm and W. Zillig : *Z. f. Naturforschung*, **10 b**, 493 (1955).
(36) R. Markham and K. M. Smith : *Parasitology*, **39**, 330 (1949).
(37) D.L.D. Caspar : *Nature*, **177**, 475 (1956).
(38) H. Schuster and G. Schramm : *Z. f. Naturforschung*, **13 b**, 697 (1958).
(39) A. Gierer and K. W. Mundry : *Nature*, **182**, 1457 (1958).
(40) K. W. Mundry and A. Gierer : *Z. f. Vererbungsforschung*, **89**, 614 (1958).
(41) W. Schäfer, Th. Zimmermann and H. Schuster : *Z. f. Naturforschung*, in press.

DISCUSSION DU RAPPORT DE M. SCHRAMM

M. Sadron :

a) How did you measure the RNA molecular weights ?

b) Is it possible to obtain, from your experiments, data on the rate of replication of infectious RNA ?

M. Schramm ;

a) The molecular weight of TMV-RNA was measured by A. Gierer, using sedimentation and viscosity determinations (*Z. Naturforsch.*, **13b**, 477, 1958).

b) Yes.

M. Markham. — I believe that Prof. Sadron is under the impression that the figures in Table 2 of Prof. Schramm's paper refer to the measured molecular weights of the virus ribonucleic acids. They are theoretical values based upon the weights of the virus particles and the percentage of nucleic acid in the virus. They thus represent the maximum value the molecular weights could have.

M. Brachet. — Regarding the cytochemical part of Dr. Schramm's work, I think that the present observations do not allow us to decide whether the increased content in RNA in the nuclei is due to the virus itself and not to a synthetic reaction of the infected cell (as in the case of phage). It is anyway very difficult to measure accurately the RNA content of nuclei, which are so rich in DNA. Would it be possible to prepare ^{14}C- or tritium- labelled TMV-RNA and follow its intracellular localization by autoradiography ?

M. Schramm. — We have measured the increase of infectious RNA, thus we are sure that we have an increase of specific virus RNA before the complete virus appears.

It would not appear feasible to use labelled virus since only a few molecules can be introduced in the cell.

M. Overbeek. — Is virus RNA degraded by other agents than ribonuclease ? For instance, by proteolytic enzymes or by substances like EDTA, citrate or pyrophosphate which are known to complex bi- and multivalent cations.

M. Schramm. — The virus RNA is stable against proteolytic enzymes and chelating agents.

M. Watson. — Is there any data yet available on the proportion of nitrous acid induced mutations in TMV which affect the coat protein ?

M. Schramm. — In the protein of the mutants we could expect an alteration of the amino acid composition or sequence. Investigations are going on in several laboratories.

M. Rich. — Have you noticed any decrease in the molecular weight (or sedimentation constant) of the TMV-RNA with this prolonged treatment of the RNA with nitrous acid ? I believe you exposed it for about 100 hours near pH 4.

Furthermore, it seems to me that your data on the reactivity of native DNA suggest that the guanine-cytosine base pair is less stable to attack by nitrous acid than the adenine-thymine pair. This is contrary to the evidence on thermal denaturation.

M. Schramm. — The molecular weight is not altered by treatment with nitrous acid for several hours. In this time the activity decreases more than 1 000 times.

Schuster found that after treatment of native DNA with nitrous acid adenine and thymine do not react at all, cytosine reacts very slowly, whereas the amino group of guanine reacts with the same velocity as in RNA. After heat denaturation with DNA all amino groups react with a rate of the same order as in RNA. It seems that the adenine-thymine bond is completely stable in weak acid solutions, whereas guanine-cytosine bond splits slowly.

M. Hamers ;

a) Do you find any mutants in the descendants of tobacco mosaic virus which contains base analogs ? In that case you should expect

a more specific mutagenic effect than that of nitrous acid; unless the mutagenic effect of nitrous acid is due to the modification of only one of the four bases.

b) In the case of the mutagenic action of nitrous acid, you mentioned you had some idea of the components whose deamination brought about a mutation. Would you care to specify this idea ?

M. Schramm :

a) As far as I know, no mutants of TMV by base analogs have been observed.

b) Probably the alteration of cytosine to uracil leads to a mutation, but we do not know that exactly.

M. Fraenkel-Conrat. — I would like to describe some experimental attempts (Gordon, Singer and Fraenkel-Conrat, in press) to determine the end groups of TMV-RNA. One approach was by means of phosphomonoesterase which was found to split off about one inorganic phosphate per 3 000-5 000 nucleotides in 30-60 minutes and not appreciably more after 3 hours. In view of the occurence of broken chains in RNA preparations, which would increase the number of ends over the theoretical, the observed values are regarded as indicative of one terminal phosphate per intact 6 000-nucleotide chain, although the possibility that this terminal phosphate was produced in the course of isolation of the RNA cannot be excluded. These analyses were possible through the use of highly P^{32} labelled viral RNA.

Another approach to the problem was to search for terminal glycol groups by means of periodate oxidation followed by treatment with S^{35} labelled thiosemicarbazide. In agreement with J.D. Smith and R. Dulbecco, who had developed this method, few if any glycol groups were found prior to enzymatic dephosphorylation of the chain ends. After this enzyme treatment, higher values were obtained, but these still corresponded to less than one glycol end per chain (about 0.5 over the background binding of reagent by the RNA).

Thus it seems that end group analyses support the belief that TMV-RNA is a single long chain, not an aggregate of shorter chains. Attention is also drawn to the electronmicrographs of extensively degraded TMV obtained by R. Hart (*Bioch. Biophys.*

Acta, **28**, 457, 1958). These have shown continuous RNA threads of lengths approaching but never exceeding 30,000 Å, the length of an extended 6 000-nucleotide chain.

Finally, a few words concerning the chemical modification of TMV-RNA. The production of mutants by nitrous acid was confirmed in our laboratory, although with much lesser frequency than observed by Mundry and Gierer. By the same technique, mutants were also occasionally found after treatment of the RNA with N-bromo succinimid or dimethylsulfate, but not with other reagents.

Sir Alexander Todd. — Dr. Fraenkel-Conrat has given us a lot of very interesting information. I do not think he need worry too much about his periodate results. That his results are lower than he hoped is probably due merely to the reaction not being truly quantitative. There is, however, one unfortunate feature of all determinations showing 3'-phosphate end groups; one must remember that any degradation of an RNA during isolation, either by chemical or by ribonuclease action, would necessarily give a product with a 3'-terminal phosphate. In other words, one cannot unfortunately, at this stage draw any conclusions from the discovery of 3'-terminal phophate groups, since they could merely indicate that some degradation had occured during isolation.

M. Markham. — I should like to suggest that the problem of finding the nature of the terminal residues of tobacco mosaic virus ribonucleic acid might be resolved by using Lipkin's method of methanolysis in anhydrous formamide. Using ^{32}P as a label, and an internal dehydrating agent such as aluminium methoxide, it should be possible to isolate the terminal nucleotide from the nucleotide methylesters formed from the middle of the chain and estimate its amount. Naturally if a 5'-phosphate is present at the other end of the chain this would be recognized as a monomethyl-ester of a nucleoside diphosphate. I should perhaps emphasize that such an experiment would require experimental technique of an exceptional order.

M. Watson (to Dr. Fraenkel-Conrat). — Does your phospho-monoesterase destroy the infectivity of TMV-RNA ?

M. Fraenkel-Conrat. — Only at so slow a rate, that the tentative conclusion was drawn that the loss of infectivity was not due to the terminal dephosphorylation, but to incipient diesterase action.

M. Schramm. — Schuster found that phosphomonoesterase does not destroy the infectivity of TMV-RNA. Treatment with periodate leads to inactivation, but the mechanism is not yet clear.

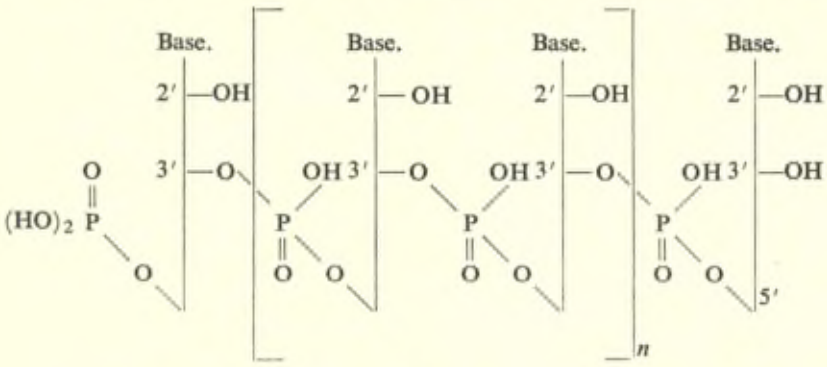
CHEMICAL SYNTHESIS OF POLYNUCLEOTIDES

by **SIR ALEXANDER TODD**
University of Cambridge.

LECTURE FOR SOLVAY CONGRESS, 1959

With the establishment of the detailed structure of the nucleosides and simple nucleotides obtained by hydrolysis of nucleic acids and the recognition that the ribonucleic acids (abbreviated structure I) and deoxyribonucleic acids (abbreviated structure II) are 3′:5′-linked polynucleotides ([1]) the foundation was laid for a closer study of the relation between structure and function of the nucleic acids by degradative and synthetic methods. Interest in this general field was further stimulated by the discovery of the double helical structure of the deoxyribonucleic acid (DNA) macromolecules through X-ray analysis, and the ingenious concept of specific base-pairing (adenine/thymine and guanine/cytosine) in the DNA double helix due to Watson and Crick ([2]). This concept, which provided for the first time a possible explanation for the genetic role of DNA, has undoubtedly led to the very large upsurge both in chemical and biological work in the nucleotide field which has been evident in the last four or five years. In this paper I propose to discuss only one aspect of the work which has been going on, namely, the synthesis of polynucleotides. True, synthesis in the polynucleotide field has not yet gone very far, but it may be of interest to consider it, even at this early stage, since it has certain features which may indicate the likely lines of further development.

From the chemical viewpoint, polynucleotide synthesis from



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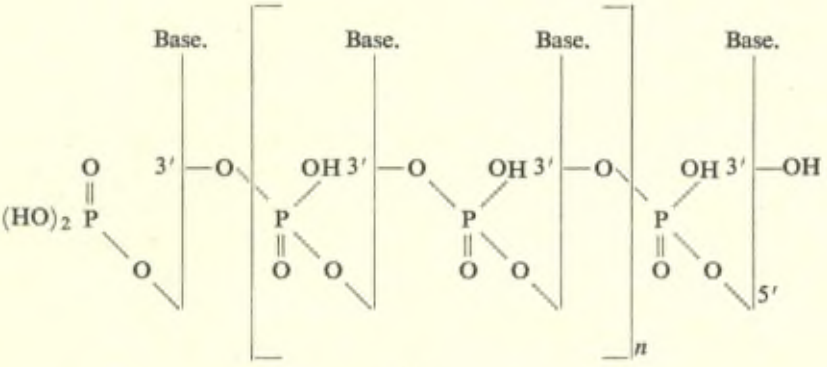


II

nucleotides has a close analogy to polypeptide synthesis from amino-acids, which has made very great progress in recent years. There are, however, certain complicating factors which must be recognised. Firstly, the nucleic acids are polydiesters of phosphoric acid, i.e., they are themselves strongly acidic, and esterification by an inorganic acid such as phosphoric acid is generally more difficult than the amide formation involved in polypeptide synthesis. Moreover, whereas in the amino-acids there is, in most cases, only one amino-group which is available for reaction with the acid to form the peptide bond, the simple nucleotides each contain several groups capable of reacting with a phosphorylating agent. This is particularly so in the case of the ribonucleotides, where there are two hydroxyl groups (at C_2· and C_3·) of very

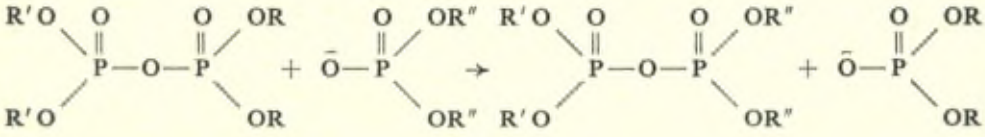
similar reactivity in the sugar residue, which can react indiscriminately in polynucleotide synthesis making exclusive formation of the desired 3′:5′-internucleotidic linkage a matter of considerable difficulty; this particular difficulty is not encountered in the deoxyribonucleotides which lack the hydroxyl at C_2· and hence synthesis in the DNA series, despite the lesser stability of the nucleotides, is a somewhat simpler task. On the other hand, the danger of racemisation which complicates polypeptide synthesis is negligible in the nucleotide field.

Polynucleotide synthesis can be regarded as having two main objects: (1) the synthesis of small polynucleotides (oligonucleotides) of moderate molecular weight but with a definite and predetermined sequence of residues, so that problems of reactivity, enzyme specificity and methods of sequence determination can be studied, and (2) the synthesis of large polynucleotides by polycondensation methods so as to produce analogues of the natural nucleic acids for physical and biological study. These two objects demand related, but nevertheless rather different, methods of approach and will be treated separately, but since both entail ester formation from alcohols and phosphoric acid it is necessary first to consider the basic methods which are available for reactions of this type.

The phosphorylation of organic compounds has been studied during the past fifteen years in Cambridge as an important facet of nucleotide chemistry, and it is now possible to make some general observations about the methods developed [3]. So far, it would appear that four general types of phosphorylation procedure are known; the differences between them are significant and easily recognisable in practice, although they cannot be regarded as fundamental.

The first and hitherto the most widely used type of method is that which employs fully esterified pyrophosphates or mixed anhydrides of diesters of phosphoric acid with other, stronger, acids. This, the classical phosphorylation procedure, is illustrated by the behaviour of the mixed pyrophosphate (III) derived from two different diesters of phosphoric acid, of which one, $(RO)_2P(O)OH$, is a stronger acid (or, better put, has a more stable anion) than the other $(R'O)_2P(O)OH$. This pyrophosphate

will react with a nucleophile X, usually in presence of a base, to yield primarily $(R'O)_2P(O)X$, i.e., it will yield with an alcohol an ester or with an amine an amide derived from the weaker acid; with the anion of a diester of phosphoric acid weaker than $(RO)_2P(O)OH$ the mixed pyrophosphate (IV) will be produced ([4]).

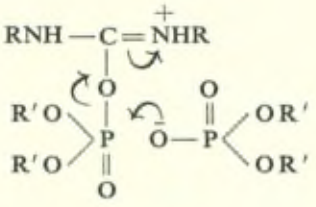


III IV

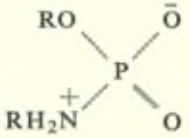
The reason for this is that attack by a nucleophile on phosphorus does not proceed by addition to the P = O grouping, but entails simultaneous expulsion of another group; the driving force can therefore be regarded as the tendency to expel the more stable anion so that phosphorylation by the weaker acid occurs. It should be noted that ionisation of the anhydride before reaction would lead to the same result, since the positive phosphorylium ion (the phosphorylating entity) would derive from the weaker acid. No accurate physicochemical studies have as yet been made of these reactions, so that the precise mechanism remains in doubt; but it would seem likely that when pyrophosphates are used the intact anhydride molecule reacts, whereas ionisation may occur when the reagent is a dialkyl phosphorochloridate or a mixed anhydride of a dialkyl phosphate with, e.g., benzenesulphonic acid or trifluoroacetic acid. The anhydride method of phosphorylation was the one employed in the synthesis of the simple nucleotides ([1]) and, in principle at least, is should be equally applicable to polynucleotide synthesis. As will be seen later, it was used successfully in the first recorded synthesis of a dinucleotide containing a 3′:5′-internucleotide linkage ([5]).

The second type of phosphorylation procedure to be noted involves the use of what may be described generally as protonated imidoyl phosphates as the actual phosphorylating reagents. Examples of such reagents are the protonated forms of simple imidoyl phosphates prepared from imidochlorides ([6]) or *via* the Beckmann rearrangement of oxime sulphonates in presence of phosphoris acid derivatives ([6, 7]), the O-phosphoryl-

ψ-ureas (e.g., V) formed as intermediates in the reaction of phosphoric acids with carbodi-imides ([8]), and similar intermediates formed by addition of phosphates to cyanamide ([9]) and ketenimide ([10]) derivatives. In such cases, where diesters of phosphoric acid are used to prepare the phosphorylating reagent, electronic displacements indicated in (V) render attack by a phosphate anion with expulsion of, in the case of (V), a disubstituted urea, a very ready reaction, so that these reagents are extremely effective for the formation of pyrophosphates. The reaction of carbodi-imides with diesters of phosphoric acid in, e.g., pyridine, to give symmetrical tetraesters of pyrophosphoric acid in almost quantitative yield is a well known example. As might be expected, this type of reaction, where diesters of phosphoric acid are used as starting materials, is not effective in phosphorylating alcohols, reaction of the intermediate with an anion being always preferred. Phosphorylation using monoesters of phosphoramidic acids in protonated form (VI) as reagents is of a similar type; used in the manner so far recorded in the literature they are only of practical importance for preparing pyrophosphates ([11]).



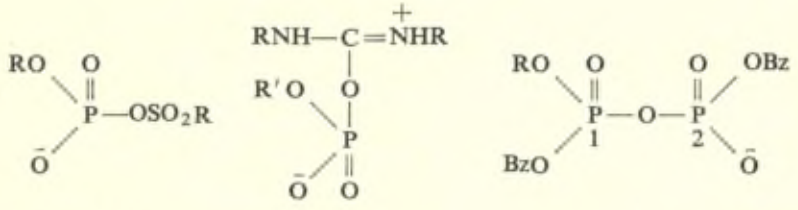
V



VI

The third type of phosphorylation procedure to which attention should be drawn is one of considerable interest and importance in polynucleotide synthesis, since the actual phosphorylating reagent derives from a mono-ester of phosphoric acid (to which class the ordinary nucleotides belong so that they can be used directly without any complications). It embraces methods using mixed anhydrides of mono-esters of phosphoric acid with much stronger acids (e.g., sulphonic acids) (VII) — analogous to those of the phosphodiesters mentioned above under Type I — and protonated imidoyl phosphates analogous to all those discussed

under Type II but derived from phosphomonoesters (e.g., the carbodi-imide adduct VIII).

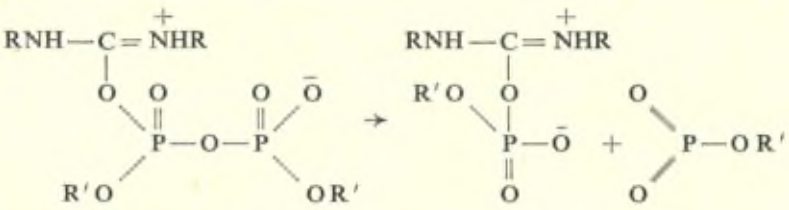


VII VIII IX

A priori one can assume that the attack of a nucleophile on the phosphorus atom in (VII) would be hindered, if not altogether prevented, by the formal negative charge. Indeed, there is some, admittedly qualitative, evidence from studies on the synthesis of nucleoside polyphosphates that in compounds as (IX; R = nucleoside residue) no attack by benzyl phosphate anions occurs at P_2 in presence of base ([12]). Yet compounds of type (VII), which are undoubtedly formed when sulphonyl halides react with phosphomonoesters in presence of pyridine, are effective phosphorylating agents ([13]). I suggest that the explanation of this fact is the ionisation of the anhydride (VII) to the stable sulphonate ion on the one hand and alkyl metaphosphate on the other; it is this "nascent" metaphosphate which is the phosphorylating agent. In the case of trialkyl pyrophosphates the disparity in strength of the two acids involved is not great enough to induce ionisation, so that they are of little value as phosphorylating agents.

The situation with regard to the phosphorylating action of (VIII) I suggest is similar, the compound generating alkyl metaphosphate in presence of base and so being an effective phosphorylating agent for alcohols as well as for anions. It is for this reason that phosphomonoesters react with dicyclohexyl-carbodiimide in presence of a large excess of an alcohol to produce phosphodiesters ([14]), and that nucleoside-2′ and -3′ phosphates are converted to cyclic 2′:3′-phosphates by action of carbodiimide ([15]). It is of considerable interest to note that in anhydrous pyridine stoichiometric amounts of a phosphomonoester and an alcohol react with dicyclohexyl-carbodi-imide to give good yields of phosphodiesters ([16]) although under these conditions the primary

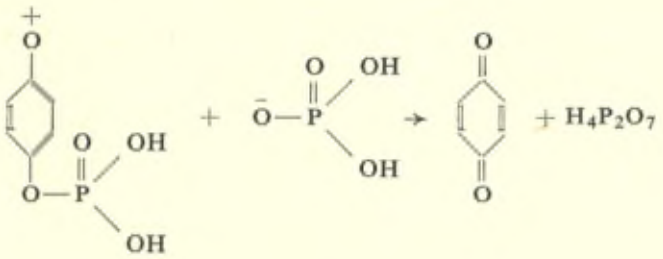
reaction is undoubtedly formation of a P_1:P_2-dialkyl pyrophosphate. It seems likely that the phosphorylating agent in this case, too, is alkyl metaphosphate. The initially formed pyrophosphate will react additively with the carbodi-imide to yield the intermediate (IX) (shown in protonated form). Substance IX is now analogous to VII in that it is in effect the anhydride of a monoester of phosphoric acid with a much stronger acid; it therefore yields metaphosphate and compound (VIII), itself a generator of metaphosphate. Since the only effective nucleophile present is the alcohol, the overall reaction corresponds to simple esterification of the latter. The fact that tri-*n*-butylamine inhibits this reaction ([16]) is in agreement with the mechanism here proposed, since this amine, presumably on account of its basic strength, prevents the addition of di- but not of mono-esters to dicyclohexyl-carbodi-imide; P_1:P_2-dialkyl pyrophosphates will clearly be analogous to phosphodiesters in this respect. This concept of metaphosphate production as the driving force of reactions involving monoesters of phosphoric acid provides a rational explanation of the known facts, although further quantitative studies are necessary to establish its correctness.



IX VIII

The fourth type of phosphorylation to be considered is oxidative phosphorylation ([17]). In this method a phosphorylating agent is generated by electron withdrawal. Examples of this are to be found in the quinol phosphates ([17]) and the enol-phosphates ([18]). It has been established that quinol phosphates react with phosphoric acid extremely readily in presence of an oxidising agent to yield quinones and pyrophosphates. As can be seen from the simple example shown below, this type of reaction is really analogous to the imidoyl phosphate methods, the only difference

being that the phosphorylating entity (e.g., X) is formed by removal of an electron rather than by addition of a proton.

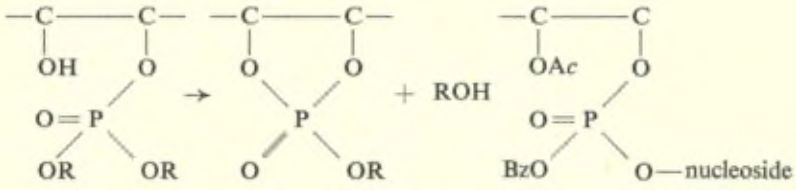


X

Oxidative phosphorylation of this nature has not yet been exhaustively studied from a preparative standpoint, but its general scope and limitations should be like those of the imidoyl phosphates. It should be noted, too, that while simple enol phosphates such as XI may normally phosphorylate only under oxidising conditions it is possible, given appropriate structural features giving a very strong electron withdrawal from the P-O linkage, that enol phosphates might phosphorylate directly; such appears to be the case with enol phosphates derived from malonic ester derivatives ([19]).

Having considered these four main types of phosphorylation procedures, it is possible to make some observations on their probable value in polynucleotide synthesis, taking as the object the rational synthesis of oligo- and poly-nucleotides in which the sequence of residues is predetermined. In principle, of course, all of them can be used, but one might expect that the use of oxidising agents in Type IV reactions would often be undesirable because of possible side effects. Reactions of Type II are unlikely to be of value since in most of the practical methods belonging to this type the actual phosphorylating agent is not isolated as such, but is produced transiently by allowing a phosphodiester to react with some unsaturated compound, e.g., a carbodi-imide, so that pyrophosphate formation is favoured as against esterification. Type I and Type III methods are clearly those most likely to be effective in practice.

Reactions of Type I have a disadvantage in that they yield initially triesters of phosphoric acid. This is particularly serious in work on polyribonucleotides, since in each ribonucleoside or 5′-ribonucleotide residue there are *cis* vicinal hydroxyls on $C_{2'}$ and $C_{3'}$. It has been shown that phosphotriesters bearing a *cis* vicinal hydroxyl in one of the esterifying groups are extremely unstable and decompose, even at neutral pH, into (initially) a cyclic phosphate and an alcohol:



XI

It follows, then, that if, e.g., a ribonucleoside-5′ benzyl phosphorochloridate were brought into reaction with a 5′-substituted ribonucleoside, the initial product would almost certainly break up to give a cyclic phosphate of the latter. This difficulty is, of course, avoided if, say, the 2′-hydroxyl in the 5′-substituted ribonucleoside is protected by acylation. The initial reaction product is then of Type XI, but it is not always easy to ensure that the acyl group on 2′ remains firmly in position until the benzyl group has been removed; retention of the acyl group until then is essential for success. Difficulties of this nature vitiated the earliest experiments on polyribonucleotide synthesis ([20]), although the phosphorochloridate route using protected nucleoside derivatives was later used to synthesise adenosine-2′ uridine-5′ phosphate ([21]) by reacting 2′:3′-diacetyluridine-5′ benzyl phosphorochloridate with 3′:5′-diacetyl-adenosine and subsequent removal of protecting groups; yields were, however, very low and extension of the method to dinucleotides in the ribo-series has not yet been reported.

The simpler situation encountered in the deoxyribonucleosides which contain hydroxyl groups only at $C_{3'}$ and $C_{5'}$ in the sugar residue allow Type I phosphorylation methods to be more readily applied. Using the phosphorochloridate route with suitably

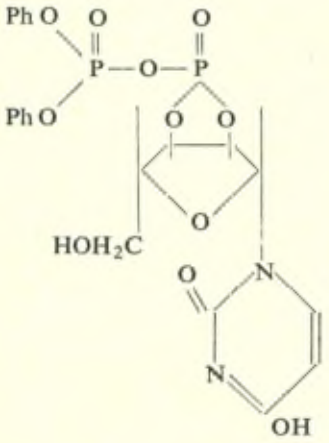
protected starting materials, the first successful synthesis of a 3′:5′-linked dinucleotide — thymidylyl-(5′→3′)-thymidylic-5′ acid — was achieved, as well as that of dithymidine-3′:5′ phosphate ([5]). Here again, however, the yield was rather low, the method giving various by-products including substantial amounts of pyrophosphates, although it could doubtless be improved by modification of the reaction conditions.

An ingenious application of a Type I method to polyribonucleotide synthesis has been described by Michelson ([22]). In this, polynucleotides are formed from ribonucleoside-2′:3′ cyclic phosphates by treatment with, e.g., tetraphenyl pyrophosphate. This method, which gives a mixture of 2′:5′- and 3′:5′-linked products, is not readily applicable to the preparation of polynucleotides with a predetermined nucleotide sequence, and it will therefore be discussed under polycondensation methods.

It is, however, methods of Type III which have so far shown most promise in the polynucleotide field. Not surprisingly, their use has been restricted in the present initial phase of polynucleotide synthesis to deoxyribonucleotides in which the complication of 2′:5′- and 3′:5′-linkages does not arise. Thus it is known that reaction of 5′-deoxyribonucleotides with arylsulphonyl halides ([13]), trifluoroacetic anhydride ([23]), oxalyl chloride ([23]) or thionyl chloride ([23]) in presence of pyridine yields di — and some higher — polynucleotides. A similar result can be obtained using dicyclohexyl carbodi-imide ([16]). This latter reagent has been used successfully by Khorana to prepare several dinucleoside phosphates and dinucleotides in the deoxyribo-series. Treatment of equimolar quantities of, e.g., 3′-acetylthymidylic-5′ acid and thymidine-5′ dibenzyl phosphate in dry pyridine with dicyclohexyl-carbodiimide followed by removal of protecting groups gives a moderate yield of the dinucleotide thymidylyl-(5′→3′)-thymidylic-5′ acid ([16]). This type of synthesis, which almost certainly proceeds by the mechanism suggested above rather than that proposed by Khorana, seems to offer considerable promise for stepwise synthesis of oligonucleotides and it is probable that other methods of the same general type will be developed for use in this important field. With further development one can expect a range of synthetic oligonucleotides to become available.

A number of attempts have been made to develop polycondensation procedures which would yield polynucleotides of considerable chain length in one operation. Although some success has been achieved, published chemical methods still fall considerably short of the enzymic synthesis of polynucleotides from ribonucleoside-5′ pyrophosphates in efficiency, and further work in this direction is required.

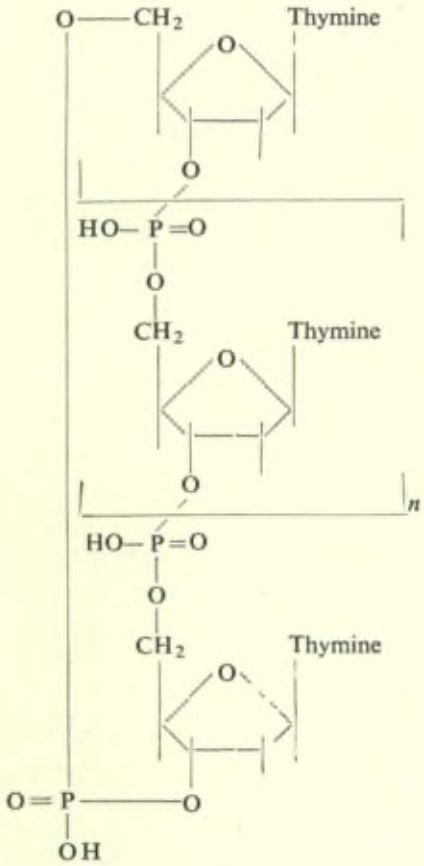
Perhaps the most successful method as regards size of polynucleotide produced is that of Michelson ([22]) already mentioned. True, it applies only in the ribonucleotide field and it always yields mixtures of 2′:5′- and 3′:5′-linked polynucleotides, but it appears to give products of chain length up to 8-10 units in appreciable amount. Moreover, by using a mixture of different nucleoside-2′:3′ cyclic phosphates it is possible to obtain polynucleotides containing several different nucleotide residues although not, of course, in any prescribed order. As to mechanism, presumably the initial reaction of tetraphenyl pyrophosphate with, say, uridine-2′:3′ phosphate is to form an anhydride (XII) which will then react with the free C_5· hydroxyl in an adjacent molecule with subsequent opening of the cyclic phosphate to give a 2′:5′- or 3′:5′-linked product. Termination of the chain by phosphorylation by the tetraphenyl pyrophosphate used occurs apparently at a fairly early stage, so that for the most part relatively short chains are produced.



XII

The synthetic products obtained by Michelson, or at least the 3′:5′-linked materials present in them, show chemical and enzymatic behaviour very similar to yeast ribonucleic acid (which may well consist largely of relatively small polynucleotides), and he has been able to carry out a number of interesting comparisons with them ([22, 24]).

In the deoxynucleotide field, Khorana has reported the synthesis of polynucleotides whose maximum chain-length probably does not exceed that attained by Michelson in the ribo-series and may be somewhat lower. The method employed is treatment of a 5′-deoxynucleotide such as thymidylic acid in pyridine with a



XIII

sulphonyl halide or preferably with excess of dicyclohexyl carbodi-imide ([25]); the probable mechanism of this reaction I have discussed in general terms above (Type III). As might be expected, the yields of polynucleotides, apart from dinucleotide, are very small, the formation of cyclic oligonucleotides (XIII; $n = 0, 1, 2, 3$) is a prominent side reaction, and some nucleoside-3′:5′ cyclic phosphate is also produced. Thus, in the thymidine series the following yields of linear 3′:5′-polynucleotides were obtained (as fractions from cellulose-type ion-exchange columns) — di, 5 %; tri, 7 %; tetra, 6 %; penta, 3 %; simultaneously, the following amounts of cyclic oligonucleotides were obtained — di, 15-20 %; tri, 5-6 %; tetra, 3 %; penta, 0.5 %.

Somewhat similar results have been obtained by reaction of 5′-deoxynucleotides with oxalyl and thionyl chlorides ([25]) and polynucleotides are also formed by a Type I procedure, when thymidine-5′ benzyl phosphorochloridate is generated from the corresponding phosphite in presence of pyridine ([26]).

These results are of considerable interest and importance, but the results so far obtained underline the defects in the methods and indicate the directions in which further work is needed. The production of cyclic oligonucleotides is a defect inherent not only in the carbodi-imide procedure described above but will be found in any similar Type III phosphorylation procedure applied in this field, unless some method of blocking the free hydroxyl on the terminal residue is found. True, cyclic oligonucleotide formation is not likely to be serious in larger polynucleotides, but its ready occurrence with the lower members is likely to cause a large wastage of material in polycondensations of this type. Secondly, the fact that dinucleotide is the major product in both the Type I and Type III procedures so far used is to be expected from the nature of the reactions employed. In every case a bifunctional molecule is used containing a phosphorylating group and a hydroxyl group capable of phosphorylation, and condensation is brought about by adding a condensing agent; it is surely inevitable that the main initial reaction in these circumstances will be formation of dinucleotide rather than stepwise chain growth. It will be recalled that in the polypeptide field the production of long chain molecules from anhydrocarboxyamino acids has been achieved ([27]). Success in that case is due to the fact that reaction is initiated

by a little free amino-acid and that each molecule of anhydride as it reacts with a second molecule liberates the amino group necessary for further reaction — in other words, we are dealing with a true chain-forming reaction. The development of such a logical chain-forming procedure in the polynucleotide field is a difficult problem, but it is probable that only when it is solved will the synthesis of really long-chain polynucleotides by polycondensation become practicable.

REFERENCES

(1) For general reviews see, for example : " Nucleic Acids ", Vol. I, Chapter 4 (Baddiley), Chapter 12 (Brown and Todd), New York (1955); " Nobel Lecture " (Todd, 1957), published in *Chem. Ind.*, 170 (1958).

(2) Watson and Crick : *Nature*, **171**, 737, 964 (1953).

(3) *Cf.* Todd : *Chem. Technik*, **10**, 677 (1958).

(4) Corby, Kenner and Todd : *J. Chem. Soc.*, 1234 (1952).

(5) Michelson and Todd : *ibid.*, 2632 (1955).

(6) Atherton, Morrison, Cremlyn, Kenner, Todd and Webb : *Chem. Ind.* 1183 (1955).

(7) Kenner, Todd and Webb : *J. Chem. Soc.*, 1231 (1956).

(8) Khorana and Todd : *ibid.*, 2257 (1953).

(9) Kenner, Reese and Todd : *ibid.*, 546 (1958).

(10) Unpublished experiments by Dr. R.J.W. Cremlyn.

(11) Clark, Kirby and Todd : *J. Chem. Soc.*, 1497 (1957); Chambers and Khorana : *Chem. Ind.*, 1022 (1956).

(12) Griffin and Todd : *J. Chem. Soc.*, 1389 (1958).

(13) Khorana, Tener, Moffatt and Pol : *Chem. Ind.*, 1523 (1956).

(14) Smith, Moffatt and Khorana : *J. Amer. Chem. Soc.*, **80**, 6204 (1958).

(15) Dekker and Khorana, *ibid*, **76**, 3522 (1954).

(16) Gilham and Khorana : *ibid.*, **80**, 6212 (1958).

(17) Clark, Kirby and Todd : *Nature*, **181**, 1650 (1958); Wieland : *Angew. Chemie*, **70**, 313 (1958).

(18) Spencer, Todd and Webb : *J. Chem. Soc.*, 2968 (1958).

(19) Cramer and Gärtner : *Chem. Ber.*, **91**, 704 (1958).

(20) Unpublished experiments by Dr. R.H. Hall; *cf.* also Hall, Todd and Webb, *J. Chem. Soc.* 3291 (1957).

(21) Michelson, Szabo and Todd : *ibid.*, 1546 (1956).

(22) Michelson : *Nature*, **181**, 303 (1958).

(23) Unpublished experiments by Drs. R.F. Webb and J.A. Schofield.

(24) Michelson : *Nature*, **182**, 1502 (1958).

(25) Tener, Khorana, Markham and Pol : *J. Amer. Chem. Soc.*, **80**, 6223 (1958).

(26) Unpublished experiments of Dr. B.E. Griffin.

(27) Schramm and Woodward : *J. Amer. Chem. Soc.*, **69**, 1551 (1947).

DISCUSSION DU RAPPORT DE SIR ALEXANDER TODD

M. Rich. — Although it would be very inefficient, do you think longer polynucleotides would be obtained by fractionating the reaction mixture to separate the longer chains (8-12 residues) and then using them to seed the next reaction mixture. In this way, by using a cyclic process, it might be possible to get very small amounts of much longer polynucleotides.

Sir Alexander Todd. — This is certainly a possibility, but as far as I know it has not yet been explored.

M. Markham. — I should like to ask Sir Alexander if he has any views about suitable solvent systems for use as media for making polynucleotides. As I understand it, it would be advantageous to use as high a concentration of reagents as possible (for several reasons) and it is not practicable to use water-containing solvents. The quantity of nucleotide which can be used is therefore limited rather severely.

Sir Alexander Todd. — The problem of finding suitable solvent systems is a difficult one. A considerable variety of non-aqueous solvents have been tried, but so far no really satisfactory answer has been found.

M. Markham. — Has 3′ : 5′-cyclic phosphate been used as a starting material for polynucleotide synthesis ?

Sir Alexander Todd. — The nucleoside 3′ : 5′-cyclic phosphates cannot be used for polynucleotide synthesis in the way that Michelson has used the 2′ : 3′-phosphates as the ring system is too stable. They have recently been used, however, by Khorana for the stepwise synthesis of 3′ : 5′-linked dinucleotides in the ribo-series; the method rests essentially on blocking the 2′-position with a pyranyl group and then opening the cyclic phosphate with alkali to give the 2′-pyranyl-3′-phosphate and the 2′-pyranyl-5′-phosphate, which then serve as starting materials for synthesis.

M. Julia. — Est-ce que l'éthoxyacéthylène peut être employé à la place de la dicyclohexyl-carbodiimide pour préparer des esters phosphoriques ?

Peut-on obtenir un phosphate d'α-glycol par action d'acide phosphorique, par exemple, sur un oxyde d'éthylène ?

Sir Alexander Todd. — Quite a number of substances, e.g., ketenimides, cyanamide derivatives and ethoxyacetylene, can be used as alternatives to dicyclohexyl carbodiimide, but with the possible exception of dimethylcyanamide they are less convenient.

The production of glycol by action of phosphoric acids on ethylene oxides has been described in the literature.

M. Ubbelohde. — Have calculations of free energies been made, to test whether the synthetic difficulties described by Sir Alexander arise from real thermodynamic trends or merely from kinetic obstacles ? Do the syntheses go uphill thermodynamically ?

Sir Alexander Todd. — There is a certain amount of information, and I think you may take it that the syntheses I have described are not going uphill thermodynamically.

M. Ochoa. — In the reaction catalyzed by polynucleotide phosphorylase, the pyrophosphate bonds of nucleotide diphosphates are converted to the phosphadiester bonds of the polynucleotide chains. From rough determinations of the equilibrium constant, the polymerisation reaction appears to proceed with a small decrease of free energy (about 1000 cal. per mole of nucleotide).

Sir Alexander Todd. — Dr. Ochoa's point supplements my answer to Prof. Ubbelohde.

M. Ubbelohde. — Consideration of the fact that some of the difficult synthetic reactions in solution, described by Sir Alexander Todd, readily occur when appropriate enzymes are added, suggests that " in vitro " syntheses of nucleotides should be studied in the presence of suitable heterogeneous catalysts. These could act by absorbing the monomer at weakly basic sites on the catalyst, thus facilitating approach to the phosphorus atom. This gives some

clue as to the kind of heterogeneous catalyst that seems most promising.

Sir Alexander Todd. — We have given some thought to this point and have made some preliminary experiments on these lines, but we have not gone far enough yet to say anything definite.

M. Ubbelohde. — Further to the suggestion to study the synthesis of nucleotides in the presence of heterogeneous catalysts with weakly basic sites, the possibility of using ion exchange resins of weakly basic character may be considered. These could be used in bulk or in a column, and should help to " cover up " the hydroxyl groups whose repulsion effects have been discussed by Sir Alexander Todd.

Sir Alexander Todd. — The idea of using ion exchange resins is one which has been considered.

M. De Boer. — I should like to make a few remarks with respect to the chairman's suggestion that polymerisation of nucleotides might be a problem which should be solved along the lines of heterogeneous catalysis. It is, indeed, along these lines that a physico-chemist would think.

Polymerisation of ethylenic bonds may take place via a radical mechanism or via positive or negative ions. I should like to draw the attention to the last mechanism. Long linear polymers may be formed by a heterogeneously catalyzed reaction, using negative ions, as e.g. carbanions. NATTA's isotactic polymerisations are excellent examples of this kind. A suitable, negatively charged, X^--ion is adsorbed on a surface. It may, on this catalytic surface, be added to an ethylenic bond of a molecule :

$$R\text{-}CH = CH_2 \qquad (A)$$

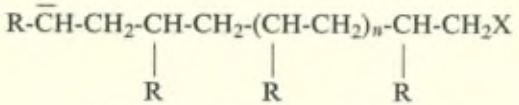
which is also adsorbed on the surface. The result is an adsorbed carbanion :

$$R\text{-}\overline{CH}\text{-}CH_2X \qquad (B)$$

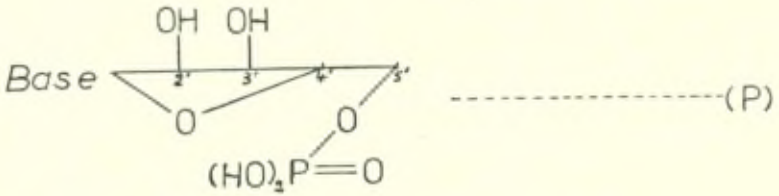
(B) in its turn may be added to another molecule (A) :

$$R\text{-}\overline{CH}\text{-}CH_2\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}CH_2X \qquad (C)$$

More molecules (A) may in succession react similarly, leading to a polymer :

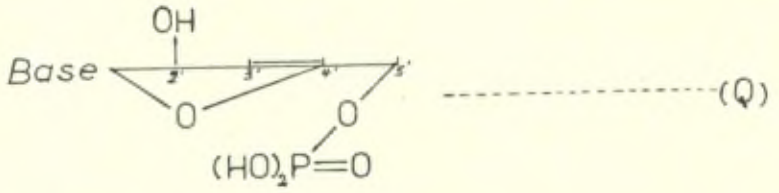


If, following the schematic formulae of Sir Alexander Todd's paper, we symbolise a nucleotide with :



(P)

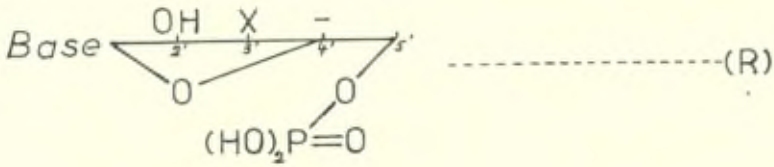
one might imagine it to be possible to synthesize a similar monomeric compound with a double bond between the third and fourth carbon atom :



(Q)

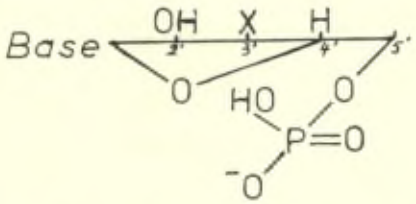
which would be molecule (P) minus one H_2O molecule.

If then a suitable catalyst could be found as in the case of the polymerisation of ethylenes, having ions X^- on its surface (which may be phosphoric acid ions in this case), a similar addition reaction may be imagined, leading to :



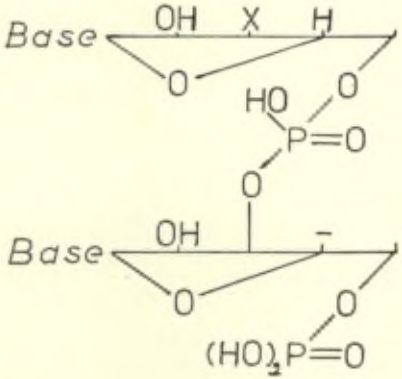
(R)

One might then imagine a hydrogen ion of one of the OH-groups of the phosphoric acid residue to neutralize the negative charge on carbon atom 4', leading to :



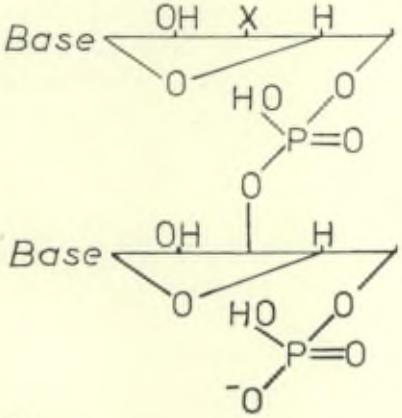
----------(S)

Such a transfer of a hydrogen ion should, sterically, be an easy one. Molecule (S), adsorbed on the surface, might then be visualized to react with another molecule (Q) to :



----------(T)

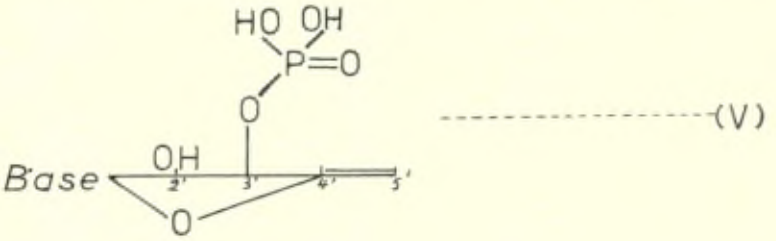
Again a hydrogen ion transfer would lead to :



----------(U)

and the reaction could go on.

A similar scheme can be drawn up, starting with a molecule :



instead of (Q).

These purely imaginary schemes may not work at all; they are only meant as an indication of a trend of thought which might, ultimately, lead to a possible way of a heterogeneously catalysed polymerisation reaction in this field.

Sir Alexander Todd. — The general idea is certainly interesting, although the scheme actually used to illustrate it by Prof. de Boer has a number of snags in it from the organic chemical standpoint.

M. Butler. — Do you have any views on Bendich's report that he added small nucleotides to an ion exchange resin and was able to elute polynucleotides ?

Sir Alexander Todd. — I have only seen a brief report by Dr. Bendich and have therefore no comment to make at present.

DISCUSSION GÉNÉRALE

M. Ubbelohde. — We have been discussing various physical parameters such as " molecular weight ", " molecular size ", " aggregate size ", " particle size " without getting it really clear which of these parameters can be used in a precise metrical sense. It would be useful to examine these physical concepts more closely before going much further with the discussion. By way of a start, I am going to ask Dr. Sadron to review critically some of the methods of determining " molecular weight " and to specify the limits of significance of the figures given by each of the available methods.

M. Sadron (Détermination des masses moléculaires). — Il y a trois principales méthodes à examiner :

1. *Diffusion de la lumière.*

Pour une solution monodispersée dans un solvant unique on obtient par extrapolations graphiques convenables, la masse de la particule. A condition que certaines conditions d'indice de réfraction soient remplies, il n'y a aucun doute sur la validité de la théorie. Dans le cas du DNA l'erreur provient, comme je l'ai montré, d'une insuffisance technique (angle limite θ_l trop grand quand les particules sont très grandes).

2. *Méthodes hydrodynamiques, en particulier emploi de* $[\eta]$ *et de* S.

Ici pas de notable difficulté technique, mais au contraire difficulté très grave du point de vue du principe.

a) On choisit, parmi toutes les valeurs de S une valeur S_0 qui est la plus probable, puis on néglige l'effet des molécules pour lesquelles S est différent de S_0.

b) On admet a priori qu'il y a une loi de correspondance entre S et M d'une part, $[\eta]$ et M d'autre part. Ceci est une hypothèse

dont on ne peut, dans l'état actuel des choses, connaître la valeur. Il se pourrait par exemple que pour une valeur donnée de M, il y ait plusieurs configurations de la particule, donc plusieurs valeurs de S et de $[\eta]$. Dans ce cas on n'a absolument aucune idée de l'approximation faite et, par suite, on ne connaît pas l'ordre de grandeur de l'erreur commise en déterminant M par ce genre de méthode. Seules des méthodes utilisant la distribution de S et de A (constante de diffusion) permettront d'échapper à ces critiques.

3. *Méthode de Meselson.*

Extrêmement intéressante, cette méthode — (équilibre de sédimentation dans un gradient de densité) — donne, en théorie, la valeur de M, sans qu'aucun doute soit permis. Si toutes les molécules de DNA étaient identiques, l'analyse de la densité optique dans la bande d'absorption dans la cellule d'ultracentrifugation donnerait M sans ambiguité. La difficulté reste d'ordre technique : celle de la micrographie dans une bande étroite, mais cela n'est pas très important. Mais si, entre les différentes molécules de DNA, il existe un léger écart de densité, alors on ne peut plus connaître M sans hypothèses supplémentaires qu'il faudra justifier par ailleurs. Même observation pour le coefficient d'absorption u.v. Donc ici encore, incertitude fondamentale.

N'ayant pas encore pu — pour des raisons non scientifiques — utiliser cette méthode nous-mêmes, je regrette de ne pouvoir donner une opinion plus précise.

En résumé, pour les différentes raisons énumérées (principe, technique) nous ne pouvons dire dans quelle mesure les nombres M_W (diffusion de la lumière) ou M_{η^g}, ou M_M (Meselson), représentent les masses moléculaires.

Le fait que pour un échantillon donné, on n'observe pas de différences supérieures à 30 ou 40 % est très encourageant.

Il ne faut pas oublier en effet que si les molécules de DNA n'ont pas une masse uniforme la mesure parfaite de M par les trois diverses méthodes donne trois types de moyenne différents. Donc les nombres trouvés doivent être différents, toute erreur étant mise à part.

Au contraire, si on trouvait que ces trois nombres expérimentaux sont pratiquement égaux, on pourrait en conclure qu'il y a une quasi certitude pour que la masse des particules de DNA soit uniforme.

* * *

Je désire par la même occasion ajouter un court commentaire sur la forme des configurations prises par le filament nucléique.

C'est une conclusion rigoureuse de la théorie de la diffusion de la lumière que l'ordonnée négative de l'asymptote à la courbe c/K indique l'existence de configurations du type *a*, *b* ou *c* (fig. 14), ou de *a*, *b* et *c* à la fois, pour le filament nucléique.

Si l'on admet que la particule de DNA est constituée par un fil unique, sans ramifications (comme en *c*) ni points de croisement (comme en *b*), il reste que ce fil doit nécessairement subir des coudes brusques de façon à avoir la forme du zig-zag.

Ces coudes ne sont pas nécessairement en angle aigu, mais le rayon de courbure de la chaine doit être très petit. En tout cas — toujours à cause de la diffusion de la lumière — ils ne peuvent exister qu'en des points de la chaîne éloignés d'au moins $\lambda/20$.

Dans ce cas, il serait normal de conclure qu'en ces points le filament n'a pas la structure en double hélice. Il y aurait, pour employer l'expression du Prof. Ubbelohde, une dislocation.

J'ai donné comme exemple (fig. 23) une dislocation d'un type déjà proposé, sans cependant affirmer que ce schéma est absolument nécessaire, ce que la diffusion de la lumière ne permet pas de dire.

D'autres types de dislocations sont faciles à imaginer. J'ai cité la possibilité d'une structure en " échelle " comme l'avait suggéré Ambrose. Mais bien d'autres schémas sont possibles.

Enfin il convient de dire que si ces dislocations existent, il n'est pas impossible qu'elles aient une importance significative sur les propriétés spécifiques du DNA.

M. Rich. — The density gradient floatation method is very sensitive to density heterogeneity which can arise from differences in base composition of the DNA. Doty has been able to obtain a measure of this by looking at the temperature of denaturation of DNA as a function of composition. Phage DNA denatures sharply at a given temperature, as judged by the rise in optical density in the U.V.; in contrast to mammalian DNA which denatures over a wider range of temperature. This may imply that the phage DNA is more homogeneous in terms of composition (and hence denaturation temperature) than mammalian DNA. Thus, the errors of

measuring molecular weights in mammalian DNA will be much greater than with phage DNA.

M. Wilkins. — Has the Meselson method of measuring molecular weight been tested with particles of known and uniform weight?

M. Sadron. — Experiments of this type should be very necessary indeed. I do not know that any of them have been performed.

M. Markham. — I should like to point out that the width of the band obtained in Meselson's technique may be increased merely by using slower centrifuge speeds (with the disadvantage that the experiment takes much longer), and that it is not imperative to use ultraviolet absorption methods for detecting the band.

M. De Boer. — All particles of DNA, RNA and proteins are very hydrophilic, hence they bind a great number of water molecules. Some of the methods for the determination of molecular (or particle) weight, mentioned by Prof. Sadron, in his very clear summary, will be influenced by this hydratation, others not.

In the method of the density measurements in a CsCl solution in a centrifugal field, the particles are also influenced by the strong ionic atmosphere of that solution, so that more or less Cs^+ ions can be " bound ".

Suppose we have particles of exactly the same all over chemical composition, but having a difference in the sequence of the constituents, the hydration may be different and also the influentiation of the double layer by the CsCl solution may be different. The various methods might deviate then noticeably in giving different figures for the molecular (particle) weight, though the water-free molecules (particles) have the same weight.

M. Sadron. — I of course agree with Prof. de Boer's remark. In general, methods of determining the particle weight by means of parameters depending on shape or dimension such as brownian diffusion constants and sedimentation or viscosity will be very sensitive to the adsorption on the particle.

Light scattering is much less sensitive, except perhaps when the solvent contains ions (Overbeek).

Equilibrium of sedimentation is not sensitive, except if there is a buoyancy change due to the adsorption. I have discussed these effects in *Progress in Biophysics* (1953).

M. Markham. — In the method of Messelson the "molecular weight" calculated is independent of the degree of solvation, provided that the CsCl is able to dissolve in this solvation water as freely as it can in liquid water. This may not be so.

M. Sadron. — The eventuality suggested by Prof. Markham is certainly to be contemplated. A difference in the ionic surrounding of DNA particles, due to difference of sequences, would affect not only the hydrodynamical parameters, but the optical data too. However these differences will be measured, and eventually used as a means of analysis, only when the different methods that we have reviewed will be much more accurate than they are at the present time. That is a reason why these methods have to be improved.

M. Overbeek. — In a centrifugation equilibrium the distribution of particles is completely determined by the weight of the *dry* particles and by the buoyancy as determined from the partial specific volume. In a two component system it is not necessary to take hydration into account separately. However, in a multicomponent system as is used in gradient centrifugation one has to take into account that one of the solvent components (CsCl or H_2O) may be preferentially adsorbed to the particles. This is not sufficiently reflected in the partial specific volume of the particles. The necessary additional information may be derived from the CsCl distribution in a DONNAN (membrane) equilibrium (cf. Hooijman, *Physica*, 1957, Mijnlieff, thesis, Utrecht, 1958).

M. Butler. — The molecular weight determination of Meselson is essentially a measure of diffusion. In the case of heterogeneous systems such as DNA, we all know how difficult it is to derive diffusion coefficients of individual species from the Gaussian curves and for this reason I think the measurements must be very approximate and that the contour of the curve which is measured must depend on the distribution of diffusion coefficients.

M. Markham. — When DNA is "denatured" does not the position of the band in the ultracentrifuge cell and hence the density of the particles, change?

If this is so then the quantity of CsCl bound by the nucleic acid must be a variable. I suggest that this may indicate that the CsCl solution is not in equilibrium with the water of solvation of the particles.

M. Luzzati. — Il semble difficile d'expliquer la densité du RNA qui apparaît comme très élevée dans la sédimentation dans un gradient de CsCl, sans admettre qu'il y a association préférentielle du CsCl et du RNA.

M. Rich. — In the density gradient method, RNA bands in caesium formate at a density of 2.04. This is much higher than DNA, and may reflect a binding of caesium ions. However, this phenomenon is far from clear, since the synthetic polynucleotides band at a density of 1.85 if they are combined together into a two stranded helical complex. This change (2.04 to 1.85) is much greater than what is observed in the denaturation of DNA (1.71 native goes to 1.73, denaturated).

M. Ubbelohde. — Water molecules of hydration bound to the organic macromolecule will vary according to the experimental conditions, and this should affect the rival methods of determining molecular weight in different ways. From the comments of de Boer, of Overbeek, and of Sadron, it seems clear that in a buoyancy method, water of swelling will only affect the result if its partial molal volume within the macromolecule differs from that outside. But surely its effects on molecular weight determined by migration will be very marked? And in light scattering, a lowering of the average optical density of the scattering molecule by swelling it with H_2O dipoles may have an effect which is not quite evident at first sight. Has M. Sadron made any calculations about this?

M. Sadron. — If it is assumed that a particle is swelling by adsorption of a monocomponent solvant, it is clear that some hydrodynamical data such as sedimentation constant, intrinsic viscosity, brownian constants of diffusion, will change since they are depending on the size of the mobile complex (particle plus absorbed solvant).

However it does not seem that buoyancy or optical polarization are significantly modified, specially in water. If we have a multicomponent solvant (mixture of organic solvents for organic polymers, electrolyte aqueous solutions for DNA) things appear differently. In that case a selective absorption of one of the components brings a modification of the buoyancy and of the optical properties which play a role in light scattering.

M. Defay. — Il me semble résulter clairement de la discussion précédente que la masse par particule mesurée par la diffusion de la lumière pourra différer de celle mesurée par l'équilibre de sédimentation. En effet, la première, mesurée dans l'eau, est indépendante de l'eau d'association qui se fixe sur la particule tandis que la seconde en dépend parce que l'eau absorbée a à peu près la même densité que l'eau libre mais non pas la même densité que le solvant mixte. Je voudrais savoir si M. Sadron est bien d'accord avec cette conclusion.

M. Sadron. — Deux cas sont à considérer : pour l'équilibre de sédimentation la valeur de la masse apparente $M(1 - V_{sp}\rho_0)$ joue un rôle. Qu'il s'agisse de solvant unique ou d'un mélange de solvants, on mesure V_{sp} par pycnométrie et l'on obtient M. Naturellement une grave difficulté se présente si toutes les molécules n'absorbent pas également l'un des solvants. Alors on n'a plus une simple répartition exponentielle des particules à l'équilibre. Pour la diffusion de la lumière, ainsi que je viens de le dire, la valeur M est correcte s'il s'agit d'un solvant unique, mais elle n'est pas correcte dans le cas du solvant mixte avec absorbtion préférentielle. Dans ce cas les valeurs de M obtenues par équilibre de sédimentation et par diffusion de la lumière sont différentes, la première étant la plus correcte.

M. Kuhn. — *Curvature through thermal agitation of a straight cylinder of length L, radius* r, *made of a homogeneous material possessing an elastic modulus E.* The viscosity and streaming birefringence data are highly dependent on the shape of the particles. It is therefore, especially with the measurements on DNA in view, intersting to form an idea about the conditions under which a straight cylindrical particle of length L and radius r will be bent through thermal agitation (fig. 1).

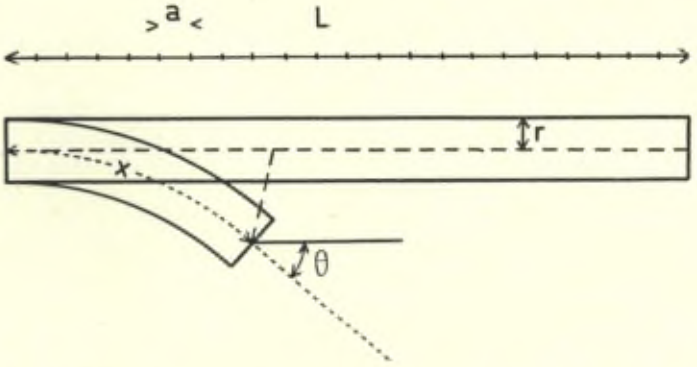


Fig. 1. — Bending of a straight elastic cylindrical rod by thermal agitation. The cylinder of length L is throught to be composed of $\nu = L/a$ sections of length a each. The angle θ between the horizontal and the axis of the rod at a distance x from the beginning of the rod will be a function of x.

A difficulty arises at first sight from the fact that the curvature produced by thermal agitation in subsequent parts of the cylinder (along the X-axis) will change in magnitude and in sign. In order to describe the phenomenon let us divide the length L of the cylinder into :

$$\nu = \frac{L}{a} \tag{1}$$

sections of length a each. The angle θ which the axis at a distance X from the beginning of the cylinder makes with the X-axis will vary continually when we pass from $X = O$ to $X = L$. The rate of the angle θ with X in a given section of length a will be described by :

$$\frac{d\theta}{dX} = \frac{1}{R} \tag{2}$$

where R is the radius of curvature in the segment considered. Let further E be the elastic modulus of the substance the cylinder is made of. The energy U which is necessary to produce the deformation (2) in the segment of length a will then be :

$$U = \frac{\Pi}{8} E \left(\frac{d\theta}{dX}\right)^2 a \cdot r^4 \tag{3}$$

The change of θ occuring along the segment considered is then :

$$(\Delta\theta)_a = \left(\frac{d\theta}{dX}\right) \cdot a \tag{4}$$

We have therefore :

$$U = \frac{\Pi}{8} E (\Delta\theta)_a^2 \cdot \frac{r^4}{a} \tag{5}$$

The energy U is thus a quadratic function of the deviation $(\Delta\theta)_a$ occuring along the segment of length a. The mean energy corresponding to a variable of which the energy is a quadratic function is, however, at a temperature T, equal to $kT/2$ where k is the Boltzmann constant. We have therefore according to (5) at the temperature T for the mean square deviation $\overline{(\Delta\theta)_a^2}$

$$\overline{(\Delta\theta)_a^2} = \frac{4}{\Pi} \cdot \frac{kT}{E} \cdot \frac{a}{r^4} \tag{6}$$

When passing along the cylinder (fig. 1) from one end to the other, the mean square deviation of the total cylinder $\overline{(\Delta\theta)_L^2}$ will be the sum of the mean square deviations contributed by the ν sections into which the length L has been divided. We therefore have, taking equation (1) into account :

$$\overline{(\Delta\theta)_L^2} = \overline{(\Delta\theta)_a^2} \cdot \frac{L}{a} = \frac{4}{\Pi} \cdot \frac{kT}{E} \cdot \frac{L}{r^4} \tag{7}$$

From this equation the length a of the segments into which the cylinder had been divided, has cancelled out. We can therefore be confident that (7) gives a true estimate of the mean square deviation of θ that will be found when passing along the total length L of the cylinder. Besides $\overline{(\Delta\theta)_L^2}$, we will be interested in the distance A through which we have to pass along the axis of the cylinder in order to obtain a deviation : $\Delta\theta = \frac{\Pi}{2}$
(i.e. a deviation of 90°. From (7) we obtain immediately :

$$A = \frac{\Pi^3}{16} \frac{Er^4}{kT} \tag{8}$$

A will be approximately equal to the statistical chain element of the coil which the originally straight cylinder will form due to its flexibility under the influence of thermal agitation. For a filament of DNA we put tentatively $r = 10^{-7}$, and $E = 10^9$dyn cm^{-2}.

The evaluation of (8) gives for T = 300°K :

$$A = \frac{\Pi^3}{16} \cdot \frac{10^9 \cdot 10^{-28}}{1.37 \cdot 10^{-16} \cdot 3 \cdot 10^2} \simeq 4.7 \cdot 10^{-6} \text{ cm.} \tag{9}$$

In the case of DNA the actual value of A may be somewhat smaller than the value given in equation (9) due to the fact that the local cross-section of the filament is elliptical instead of circular. Furthermore the value $E = 10^9$ dyn cm^{-2} is an estimate. The actual value can be determined by measurements on macroscopic filaments. Even if E is measured, corrections may be necessary taking account of the circumstance that the retractive forces observed when stretching a single DNA filament may be unequally distributed over the filament's cross-section.

The evaluation of (8) may be, therefore, subject to various amendments. It is however interesting that a first estimate gives a value for A which seems to be in a good agreement with general observations concerning the behaviour of DNA solutions.

M. Ubbelohde. — In the analysis of crumpling of the macromolecules by Dr. Kuhn, presumably differences of hydrogen bonding within such a molecule will affect the modulus E, and possibly the molecular thickness to which this model refers. This could lead to apparent "denaturation" by which I mean increased crumpling, whose explanation would be primarily physical since they could be attributed to changes of E and ν. This is in contrast to crumpling to be attributed to strong cross linkages in the crumpled form of the macro-molecule, which could be much more effective in preventing changes of shape with time due to molecular fluctuations.

M. Wilkins. — The cross section of the DNA molecule is approximately an ellipse the major axis of which is 20 Å. Because the minor axis is considerably less than 20 Å, the bending of the molecule will be greater than calculated by Prof. Kuhn.

M. Sadron. — La remarque de M. Kuhn est très intéressante. C'est un exemple de l'essai qui est souvent tenté de la transposition des observations macroscopiques à l'échelle microscopique. En particulier, la notion de module d'Young à l'échelle d'une molécule en chaîne est très peu précise. En tout cas, supposé qu'on puisse le définir, l'ordre de sa grandeur ne peut pas être tiré sans précaution de celui que l'on observe dans des assemblées de molécules en chaîne entre lesquelles des interactions jouent — dans l'échantillon solide — un rôle fondamental.

M. Desreux. — Quelle serait, dans la méthode proposée par M. Kuhn, l'influence du pourcentage de cristallinité de la fibre utilisée dans la mesure du module de Young?

M. Kuhn (answer communicated after the council). — The influence of cristallinity on the elastic modulus of a fibre in which the molecules are oriented in the direction of the fibre axis will be probably small. The deformation each molecule is undergoing when the fibre is stretched will be approximately independent on the degree of order which is realized in the system in a direction perpendicular to the molecular of fibre axis.

M. Todd (texte non communiqué). — Question concerning the distinction between aggregates and " true " molecules of ADN.

M. Sadron. — In DNA solutions we have particles which can be aggregates or true molecules. If we had aggregates of real molecules, one could expect that the weight of the aggregates should be distributed at random, except if some kind of specific phenomenons would occur. If it turns out that the weight of the particles is homogeneous, it could be reasonably assumed that these particles are real molecules.

M. Ubbelohde. — Some guide to the range of " aggregate " size due merely to general intermolecular forces can be obtained from statistical considerations, and from experiments on the range of micelle size in colloids. It seems likely that a narrow band of " aggregate size " implies quite specific forces of interaction. General dispersion and dipole forces acting more or less at random between different parts of a macromolecule probably lead to a much broader spectrum of aggregate sizes, but I would like to ask if any relevant concrete figures can be quoted from colloid chemistry?

M. Butler. — The effect of urea is a controversial one, in that some investigators claim to have shown dissociation and others the reverse. The question of aggregates is a practical one — whether desaggregation can be achieved by mild procedures. Broadly speaking as judged in this way, RNA is often highly aggregated but DNA is not.

Fraenkel-Conrat. — As will be discussed tomorrow, it seems now established on chemical ground, i.e. by end group analyses, that the molecular weight of RNA is really of the order of 1-2 million. No similar evidence for DNA seems to exist to chemically support the concept that a particle weight of 10 million represents actually two polynucleotide chains of 5 million molecular weight, but in view of the results with RNA this does not seem improbable.

M. Rich. — The only clearly defined molecular species of DNA known at present is the DNA extracted from the small phage, S-13 or ΦX-174. In this case the molecular weight of the extracted DNA is just equal to the DNA content of a single phage particle. However, as Sinsheimer has shown, this DNA is unusual in that it is a single stranded molecule rather than the usual two stranded helical form.

M. Butler. — Prof. Sadron has discussed the two kinds of molecular weight determination of large molecules like DNA i.e. light scattering and that based on hydrodynamic quantities (sedimentation coefficient and viscosity) and has given some criticisms of the latter. The light scattering method has technical difficulties of at least two kinds and these are extremely difficult to overcome. First there is the great difficulty of completely removing extraneous dust. I believe that many of the determinations recorded are too high and would have been reduced by more efficient clarification. Very long centrifuging (i.e. up to 20 hours at high speed) is required before fairly constant turbidities are reached. Since some fractionation may occur in this process it is necessary when comparing the values obtained by several methods to use the identical solutions which have been clarified in this way also for the sedimentation and viscosity measurements.

The second difficulty is the extrapolation to zero angle which is a large one and even if the lines look straight a slight difference of slope makes a big difference. My colleague D.J.R. Laurence has shown that even when a good straight line is obtained from 30° upwards, in certain circumstances the line of c/τ against $\sin^2 \theta/2$ may dip downwards at smaller angles. Finally I might mention a theoretical difficulty. These fibres are longer than the wave length of the light and I am not convinced that even at zero angle under these circumstances the Debye calculation is true.

Now with regard to the hydrodynamic methods, Prof. Sadron has criticized these on the ground that while two quantities are measured there are three unknowns viz. molecular weight and two other parameters such as volume and axial ratio, and he suggests that it is impossible to obtain all these quantities from the observations. However the great value of the Mandelkern-Sheraga equation :

$$\frac{K S_0 [\eta]^{1/3}}{M^{2/3}} = \beta$$

lies in the fact that it separates almost completely the molecular weight variable from the volume and shape terms, so that it is reasonable to use it for the determination of M if we do not try to get the other parameters as well. This can be seen if we write :

$$S_0 \propto M^{2/3}(M/V)^{-1/3} t(p)$$

$$[\eta] \propto (M/V)\, \Lambda\,(p)$$

which give :

$$\frac{S_0 [\eta]^{1/3}}{M^{2/3}} \propto t(p)\, \Lambda^{2/3}\,(p)$$

This product is almost independent of the axial ratio. Laurence has calculated β in a number of cases. It is true that for very long rigid rods it can reach a value twice that given for flexible coils. But the rigid rod model would require S_0 independent of M and the dissymetry observed is also incorrect. Laurence (*Proc. Roy. Soc.*, A 250, 18, 1959), tried various degrees of flexibility put in by introducing a variable persistence length i.e. distance over which the fibre is incapable of bending. Even when the persistence length is 2 000 Å (i.e. 10 to 20 flexible kinks per fibre) the configuration does not differ very much from a flexible coil and the β value is nearly the same.

M. Sadron. — I think that there is a discrepancy between Prof. Butler's findings and our own in the field of light scattering. We observe that for large values of θ the reverse turbidity curve has a linear asymptote in sin $\theta/2$ and not, as he finds, in $\sin^2(\theta/2)$. Our results are consistent with the results of central diffusion of X-rays and we do not think that they could be seriously questionned. On a second part, it is very comforting to see that, from the use of hydrodynamical methods which are so much criticisable in our view he concludes to a structure of the DNA particle very much alike to ours.

M. Kuhn. — When determining molecular weights by hydrodynamic methods it must be stated that the reliability is different depending on the combination of data chosen. When M is determined from *diffusion* and *velocity of sedimentation* the accuracy is undisputed because in both phenomena the translatory resistance of the particle is acting in essentially the same way : In diffusion and in sedimentation the main phenomenon is a simple translation of the particle. However complicated the relation between the hydrodynamic translatory resistance and the molecular weight and shape of the particle may be, we will allways have the relation :

Driving force = translatory friction constant × translatory velocity :

$$\breve{R}_{trans} = R_{trans} \times u_{trans} \qquad (1)$$

The inaccurracy of our knowledge about R_{trans} does not make itself felt when sedimentation and diffusion are combined. R_{trans} can (strictly) be said to be determined by the *diffusion* experiment, the acting force $\breve{R}_{trans}$ (in equation 1) corresponding in this case to the free energy of the transfer of the diffusing molecule from a solution of high to a solution of low concentration. If R_{trans} thus determined (from the diffusion experiment) is consequently introduced into équation (1) for evaluating a sedimentation experiment [where $\breve{R}_{trans}$ is now proportional to the molecular weight M of the particle] an *accurate* determination of the particles M is obtained. This satisfactory situation is *not* realised when a determination of M is attempted e.g. by a combination of the sedimentation constant S with the intrinsic viscosity $[\eta]$.

Here again the combination of the data (S and $[\eta]$) can be said to express R_{trans} in equation (1) by the aid of some other data, in this case by the aid of $[\eta]$. It is true indeed that $[\eta]$ is resulting from some additional friction due to the presence of dissolved particles when a gradient of flow (q) is maintained in the solution. This additional friction is however connected rather to *a rotatory not to a translatory motion* of the dissolved particles. The rotatory friction constant R_{rot} is however in a different way dependent on the magnitude and shape of the particle than the translatory friction constant R_{trans}. An elimination of R_{trans} in equation (1) with the help of $[\eta]$ is therefore not possible in a simple and general way.

The formula :

$$\beta = \frac{KS_0\,[\eta]^{1/3}}{M^{2/3}}$$

mentioned in Dr. Butler's remark amounts to the assumption that the relation of the rotatory to the translatory friction constant will always be the one which is valid for *a non-draining statistical coil* (about double as long as thick) i.e. for a standard shape of the dissolved particle. In many cases, e.g. in the case of DNA the assumption of a non-draining coil will probably not be true; in those cases inaccuracies in the M-determination from S and $[\eta]$-determinations must be expected.

Inaccuracies of this kind again disappear if combinations of $[\eta]$ are made with the values of $[\omega]$ and $[n]$ characterising the orientation and the value of the birefringence of flow (1). By combination of these quantities which all are connected to the *rotatory diffusion constant* of the particle, the latter can be eliminated, yielding relations from which the long axis of the particle, the molecular weight and other characteristic parameters can be obtained with the help of the accessible experimental data (2). It has been mentioned in a previous remark (concerning the paper of Prof. Sadron) that observations of these phenomena at higher values of the gradient of flow are fundamental for the determination of the rigidity or non-rigidity of the particle. An extensive use of observations of this kind can therefore be expected to give further information on essential properties of the particles.

M. Sadron. — Prof. Cerf in my laboratory has made an extensive theoretical study of streaming double refraction and intrinsic viscosity of coils. He has shown that one could obtain, among other things, the weight of the particle from a convenient comparison of the two types of data. Experiments have been done by Leray on several DNA samples. They show that the molecular weight measured in this way is very much in accordance with those determined by light-scattering work. This comes in complete agreement with your remark.

M. Wilkins. — Are the light scattering data on DNA obtained by Prof. Sadron similar to those of Prof. Doty and do Prof. Sadron's criticism apply to the work of all laboratories?

(1) For the definition of $[\omega]$, $[n]$ and $[\eta]$ see contribution by W. Kuhn to the paper of Prof. Sadron.

(2) For details and further references see a review article by W. Kuhn, H. Kuhn and P. Buchner, *Ergebn. exakt. Naturwiss.*, **25**, 1-108 (1951) (in particular, pp. 69-82).

M. Sadron. — I think that, as I have tried to show it, the extrapolation to $\theta = 0$ in the case of large particles such as DNA particles is highly criticisable. This applies — if I may say so — to results obtained in this way in all laboratories using scattering devices where the θ-values cannot come under the usual limiting value of 20° to 30°.

M. Butler. — Prof. Sadron mentioned the use of light scattering at higher angles in which you get a straight line if you plot HC/τ against $\sin \theta/2$. This only gives the linear density of the rod M/L and is not a method of determining molecular weight. With regard to Dr. Kuhn's remarks, I agree of course that it would be better to use diffusion coefficients or birefringence coefficients instead of viscosities for the unambiguous determination of molecular weights, but the experimental problems are much greater for these quantities, at very low concentrations. However the situation with viscosity is, I think, not quite so bad as he suggests. For a given model, which may either be a draining or non-draining coil or some kind of ellipsoid, or a zig-zag rod, it is possible to derive both sedimentation coefficient and intrinsic viscosity from molecular dimensions, and both types of information can be combined to give molecular weights. The validity of the result will depend on the model being reasonably correct. The correlation we have found between viscosity and sedimentation coefficient suggests that all the preparations, within limits, could be treated as examples of the same model.

M. Luzzati. — Malgré les limitations soulignées par M. Sadron, l'examen des données expérimentales de diffusion de la lumière combinées à celles de diffusion centrale des rayons X (mesurée à l'échelle absolue), par des solutions d'ADN, permettent de dégager certaines conclusions.

1. La totalité de l'ADN (dans les limites de l'erreur expérimentale) est organisée en particules filiformes, dont la masse par unité de longueur est en excellent accord avec le modèle de Crick et Watson.

2. La solution contient un ensemble de segments droits d'ADN, liés entre eux par des points d'articulations ou de croisement, sans qu'on puisse affirmer que la configuration soit linéaire, au sens topologique du terme.

3. Le modèle d'un seul filament long et flexible, sans points singuliers, semble devoir être rejeté.

M. Ubbelohde. — Dr. Butler has mentioned the difficulty of obtaining completely reproducible specimens for physical measurements in any one laboratory. Are there any standard preparations that could be used in different laboratories, or means of exchange of specimens between laboratories to test calibrations?

M. Butler (communiqué après la réunion). — Samples have been exchanged and on the whole the observations made in different laboratories with one sample agree fairly well. Different preparation vary very greatly owing to (1) the possibility of enzymic degradations during the preparation; (2) the effects of traces of protein. There are also disagreements in the interpretation of the data; but so far as I know our own measurements are the only ones in which light scattering, viscosity and sedimentation were all measured on identical solutions.

M. Hamers (à M. Sadron). — Vous nous dites que la molécule de DNA pourrait avoir la configuration d'une ligne brisée composée d'une série de bâtonnets rectilignes (l'ordonnée à l'origine de la fonction C/K est négative). Ne pourrait-on pas vérifier cette hypothèse en incorporant du ^{32}P en petite quantité dans la molécule de DNA? Après désintégration du ^{32}P on obtiendrait une valeur différente pour la fonction C/K qui dépendrait du nombre de ruptures additionnelles occasionnées dans chacune des chaînes de la double hélice de DNA.

M. Sadron. — Si on a une chaîne en zig-zag contenant N segments de longueur b ($b > 300$ Å pour les longueurs d'onde utilisées en diffusion de la lumière), H. Benoit a montré que l'asymptote de la courbe donnant C/K (K proportionnel à l'intensité diffusée dans la direction θ) est :

$$\frac{C}{K} = \frac{N}{\Pi^2 M}\left[2 - \frac{\Pi^2}{2}\,\frac{N-1}{N}\right] + \frac{h}{\Pi}\,\frac{Nb}{M}$$

$$\left(h = \frac{4\Pi n_0}{\Lambda}\sin\theta/2\right)$$

La pente de la courbe donne $\frac{N\varrho}{M}$, c'est-à-dire la masse spécifique par unité de longueur. L'ordonnée à l'origine qui est négative si $N > 1$ donne, si l'on connaît M, la valeur de N. Nous n'avons pas cherché encore à interpréter quantitativement les résultats expéri-

mentaux obtenus chez nous à cause de l'incertitude sur M et, en général, sur les extrapolations diverses qui sont nécessaires et délicates.

M. Brachet. — I would like to ask two questions, which have great biological importance and which can perhaps be answered by the X-ray crystallographers present here. 1) Is there some evidence for the existence of DNA-RNA complexes, comparable to those obtained by Dr. Rich with synthetic polynucleotides? This is of course important for the vexing question whether DNA makes RNA or not. Can one propose a theoretical model in which a two-stranded DNA would stick together with a single stranded, complementary, RNA? Has one attempted to isolate such a complex from isolated nuclei? 2) What is the size of the gene, if defined as a mutation unit? Can we accept the idea that a change in a single pair of bases in the DNA helix necessarily leads to a gene mutation?

M. Rich. — I do not know of any experimental evidence at the present for naturally occuring complexes of DNA or RNA. Hurwitz has recently isolated parts of an enzyme system which apparently has the ability to polymerize ribonucleotides and desoxyribonucleotides in the same covalent chain, but it is unlikely that this represents a reaction which is carried out normally in vivo. A great deal of theoretical effort has been devoted to the stereochemical problems involved in making complexes of DNA and RNA. At the present time it is reasonable to say that no convincing models have been put forward yet for a three stranded DNA-RNA complex. There are, of course, four different sites along the DNA molecule which could in principle serve as recipients for four ribonucleotide bases, i.e. the informational content of the DNA molecule is suitable. However, almost all configurations suffer from one or another stereochemical difficulty. The only scheme which does not suffer from stereochemical difficulty is that in which a single stranded DNA chain is hydrogen bonded to a single stranded RNA chain using the same system of hydrogen bonds which is found in DNA itself. However, at the present time there is no experimental evidence supporting such a mechanism.

Sir Alexander Todd. — Such complexes, if they exist, should be looked for in cell nuclei.

M. Watson. — Three-stranded structures are sterically difficult to conceive.

M. Ubbelohde. — One could expect the concentration of three-stranded complexes to be extremely low.

M. Wilkins (to a question by M. Rich). — Il would expect that, if the two-strand DNA molecules that had an RNA strand attached to them (thus forming a three-strand complex) did not comprise more than 10 or 20 % of the total DNA, the presence of the three-strand complex might not be detected by means of X-ray diffraction.

M. Markham. — A point which may be relevant to this discussion concerns the " unusual " bases in nucleic acids. One of these, which has been isolated by Dunn in our laboratory both from RNA and from DNA, is 6-methylaminopurine. This purine was isolated from nucleic acid treated with N-NaOH. Recently Dunn has reexamined this point to see whether the compound may not have originated as 1-methyl adenine and have been converted to 6-methylaminopurine by molecular rearrangement by the treatment with alkali. In fact this has been found to be so in RNA, but in DNA, where a methyl group on N_1 would prevent the hydrogen bonding, no such group is found, the methylamino group being at C_6 in the intact nucleic acid.

M. Felix. — I would be very happy if a few minutes could be spent to discuss the protein part of the nucleoproteins. I have been very much impressed by what Dr. Moore reported on the residual protein, which is found together with the nucleic acid after the extraction of the histone. I am interested in this problem because I have the feeling that this part of the nucleoprotamines which cannot be extracted with hydrochloric acid could be something similar to the residual protein. The other day I reported about this not extractable part of the nucleoprotamines and called it " additional amino acids ", because it includes such amino acids which are not constituents of the isolated protamines. Apart from these amino acids we have found all the usual constituents of the protamines attached to the nucleic acid. Among them is arginine, roughly estimated to about one fourth of the total arginine of nucleoprotamine. This is the situation in the nucleus of the mature fish

testis. At the time of the first appearence of the nucleoprotamine things are different. The nucleoprotamine can be extracted from the nuclei for the first time about 40 days before spawning. In the earlier stages we have found nucleohistone only. It is also present during the later stages besides the nucleoprotamine. When the nucleoprotamine appears the content of N and P in nuclei rises quite suddenly, and at this time the nucleoprotamine has the same composition as in the mature sperm but the protein part can be extracted completely, no amino acids remain attached to the nucleic acid. Thus the so called " residual protein " of the fish sperm nucleus refers only to the final phase in maturation. Perhaps there exist also situations in the thymus cell nucleus at which the residual protein can be extracted and removed from the nucleic acid.

M. Brachet. — It is generally accepted that there is a relationship between the amount of residual proteins present in a nucleus and the intensity of protein synthesis in the cell. Since mature sperm cells are inert from the viewpoint of protein synthesis, it is not surprising that they contain very little or no residual proteins. One should not forget that nuclei contain a number of enzymes and, therefore, must contain a number of different, distinct proteins.

M. Schramm. — I would like to suggest tissue culture as a cleaner way to characterize and correlate metabolic activity.

M. Felix. — In this connection I would like to make an assumption. Il could be that the peptides and amino acids of the " residual protein " are more firmly bound to the DNA in order to prepare the protein synthesis after fertilization. But according to the present view of protein synthesis the amino acids are bound to one OH-group of ribose in RNA. There is no OH-group left in DNA. Hence the amino acids must be bound in other way.

M. Brachet. — During egg cleavage, 90 % at least of the nucleoprotein synthesis occurs in the nucleus. It is only later in development that RNA and protein synthesis become detectable in the cytoplasm. Unfortunately, we do not know anything about the nature of the proteins (histones, residual proteins, etc.) associated to chromatin and chromosomes during egg cleavage.

M. Butler. — Dr. Busch has found that in tumour cells which are actively dividing there is a metabolically active protein present in the nucleus, which is associated with the histone, but is not apparently a regular histone. I do not think this is a residual protein as it is extracted by acid.

M. Schramm. — Question concerning RNA leaving the nucleus (texte non communiqué).

M. Brachet. — This question is linked to the still open question of the existence of pores in the nuclear membrane; the interpretation of the electron micrographs remains discussed among the experts. It is usually supposed that nucleolar small particles, similar to Palade's granules, come out of the nucleus through these hypothetical pores and become incorporated in ergastoplasmic lamellae. There is also some evidence for the view that, especially in growing oöcytes, the nuclear membrane can undergo a process of delamination : fragments of this membrane could be expulsed in the cytoplasm and become part of the ergastoplasm.

Sir Alexander Todd. — Binding of protein aminoacids to nucleic acids could very well occur through the phosphorus OH-group.

M. Felix. — Since the ratio of P to arginine is almost 1:1 we have concluded that all phosphoric acids are occupied by arginine. We are just investigating how those amino acids are bound. Perhaps we shall have to revise our opinion on the role of arginine in neutralizing the phosphoric acid residues.

M. Wilkins. — The early work on the non-basic or residual protein in chromosomes led to the suggestion that this protein was the major structural component of chromosomes and formed the backbone of the chromosome thread. The more recent experiments of Callan on lampbrush chromosomes show that it is DNA, and not protein, which forms the basic chromosome thread. Furthermore, recent study of liver nuclei shows that the non-basic protein can be washed out of chromosomes, and a nucleohistone thus prepared from liver has about the same composition as that from thymus. It appears a reasonable hypothesis that nucleohistone is the basic

structural substance of chromosomes and that non-basic protein is associated with metabolic activity. However, chromosomes from different tissues in higher organisms show a wide variety of forms, e.g. lampbrush chromosomes, salivary gland chromosomes with bands, meiotic chromosomes which show special structure when studied in the electron microscope, and the various types of chromosomes observed in immature sperm heads. Until the proteins in these widely-differing chromosomes have been studied, we will not know whether nucleohistone is the basic structural component in all chromosomes in higher organisms.

M. Overbeek. — At the request of the Chairman, I like to tell something about electrostatic interactions, with particular emphasis on non specific effects. Three aspects will be distinguished.

1. *Interaction of individual particles with the surrounding electrolyte.*

As electroneutrality has to be conserved, the charge of a particle will be compensated by adsorption of ions of opposite charge (counterions) or by pushing away ions of similar charge (co-ions). In the neighbourhood of a negatively charged particle the electrical potential, Ψ, is negative (far from the particle $\Psi = 0$) and the ions are distributed according to a Boltzmann equilibrium as shown in figure 1. The double layer formed in this way has a thickness of

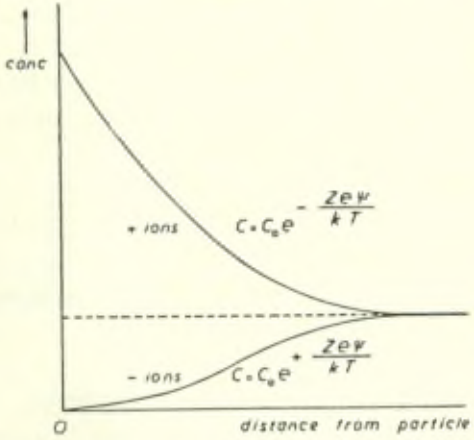


Fig. 1. — Distribution of ions near a negative particle. C = local concentration, C_0 = concentration far away from the particle, Z = valency of the ions, e = elementary charge, k = Boltzmann's constant, T = absolute temperature, Ψ = local potential.

the order $1/\varkappa$, the characteristic length of the Debye-Hückel theory. According to this theory $\varkappa$ is proportional to $Z\sqrt{C_0}$ where Z is the valency of the ions and C_0 their concentration. When $C_0 = 0{,}1$ molar and $Z = 1$, $1/\varkappa$ is 10 Å. For $C_0 = 0.001$ molar $1/\varkappa$ is 100 Å.

If C_0 is large, the number of ions needed to compensate the charge of the particle is *relatively* small, the potentials stay low, the exponent in the Boltzmann equation is small and the curves for cations and anions become symmetrical around $C = C_0$. Consequently the particle charge is compensated halfway by adsorption of counterions and halfway by negative adsorption of co-ions.

Or in other words, an amount of salt equivalent to half the particle charge is pushed away from the particle. If, however, C_0 is small, potentials become high, the positive exponential increases much faster than the negative one decreases and the electrolyte pushed away is equivalent to less than one half of the charge. Now in light scattering or in sedimentation it is not the particle as such but the particle with its surroundings, which is the scattering or sedimenting unit and for highly charged particles such as nucleic acids the correction may be easily 10 or 20 %. The precise amount of the electrolyte pushed away from the particles can be conveniently determined in a (Donnan) membrane equilibrium. (See J.Th. G. Overbeek, The Donnan Equilibrium, *Progress in Biophysics and Biophysical Chemistry*, **6**, 57 [1956]).

2. *Interaction between particles of similar charge.*

Particles of similar charge repel each other. As the particle charge is compensated at a distance of the order $1/\varkappa$, the range of the repulsion is also of the order of $1/\varkappa$. The value of the repulsion can be calculated from the theory of the electrical double layer. It depends on size, shape and charge of the particles, on their distance, on concentration and valence of the electrolyte, on temperature and dielectric constant (ε) of the solution. For flat particles of high charge a very simple expression can be given for the energy of repulsion V_R which is the energy necessary to bring the particles from infinity to a distance H. For spherical or cylindrical particles the mathematics are more complicated but the physical effects are very similar to those for flat plates. For flat plates of high charge :

$$V_R = A \frac{\varkappa}{Z^2} \cdot e^{-\varkappa H}$$

where A is a constant given by the theory ($A = 2k^2T^2\varepsilon/\pi e^2$). The repulsion as discussed will prevent particle contacts.

Its range can be decreased by the addition of electrolytes but this will not bring the repulsion to zero. If it is observed that particles nevertheless make contact, as is the case in the flocculation of hydrophobic colloids or in the pair formation of nucleic acids, this points to the existence of an attractive force of comparatively long range. In hydrophobic colloids this attractive force is very probably the London-Van der Waals force, which, though of short range if considered for individual atoms or molecules, has a long range between agglomerations of molecules. It is suggested that long range London-Van der Waals forces are also acting in bringing nucleic acid molecules together. After contact has been made the attraction is increased over and above the London-Van der Waals force by the formation of short range hydrogen bonds. For flat plates the London-Van der Waals attraction energy has the form :

$$V_A = -\frac{B}{H^2}$$

where B can be calculated in a simple way. The combination of repulsion and attraction leads to a curve for the total energy of interaction with minima for $H = 0$ and for large H and possibly, if the repulsion is large enough a maximum in between.

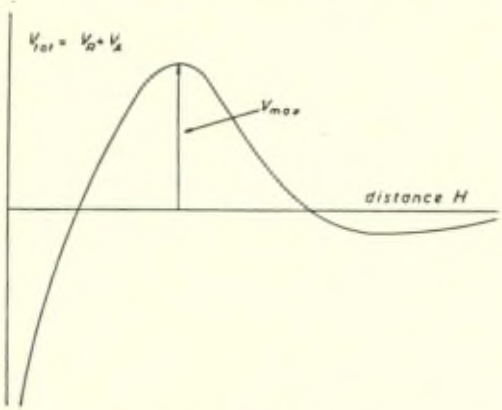


Fig. 2. — Energy of interaction composed of electrostatic repulsion and London-Van der Waals attraction.

Particles will be able to make contact if V_{max} is small compared to kT, the energy of Brownian motion. A very simple relation is

found from condition that V_{max} should just be zero. From the simultaneous conditions $V = 0$ and $dV/dH = 0$ one derive esasily that:

$$C = \frac{\text{const.}}{Z^6}$$

This inverse sixth power which has already been mentioned in an earlier remark has been very amply confirmed in hydrophobic colloids of great variety and might very well be applicable in nucleic acids too. The rather extreme influence of the valency of the ions, particularly of the counterions, is directly connected with the steep increase of the positive exponential in the Boltzmann equation. For further details, see E.J.W. Verwey and J.Th.G. Overbeek, *Theory of the Stability of lyophobic Colloids*, Elsevier, Amsterdam, 1948.

3. *Interaction amongst particles of opposite charge.*

Particles of opposite charge attract each other and if the charges are sufficiently high they form a complex which separates from the solutions. This complex usually entrains solvent and small ions and has a liquid nature. This phenomenon has been called (complex) coacervation by Kruyt and Bungeberg de Jong, who with their collaborators have studied it extensively. In a coacervate the particles of opposite charge neutralize each other almost exactly even when it separates from a solution in which the amounts of the two components differ greatly. This " neutralization " does not necessarily point to the formation of a specific stoichiometric compound, but is already explained by the difference in entropy needed to bring one large poly ion or an equivalent amount of small ions in the coacervate. Electrostatically the energy gain will be similar but the entropy loss will be much worse for the many small ions than for the single large ion. Applying these ideas to the formation of nucleoproteins one expects indeed a fairly exact compensation of negative phosphate with positive lysine or arginine, for electrostatic reasons only, no specific compounds being formed. It may be of course that in a later stage of the process a more localized and specific " snapping in " would follow. For general information on complex coacervation, see H.G. Bungenberg de Jong, several chapters in H.R. Kruyt, *Colloid Science*, Vol. II, Elsevier, Amsterdam, i1949. Further: J.Th.G. Overbeek and M.J. Voorn, Phase separation in polyelectrolyte solutions; theory of complex coacervation, *J. Cellular and Comparative Physiology*, **49**, Suppl., 1, p. 7 (1957).

M. Thomas. — La participation des ions métalliques à la configuration des particules d'ADN, à laquelle M. Overbeek vient de faire allusion, est illustrée par l'accroissement, partiellement irréversible, de l'absorption ultraviolette des ADN à faible concentration saline. Le seuil de concentration saline sous lequel l'ADN est dénaturé dépend essentiellement — à pH et température donnés — de la valence de l'ion métallique utilisé : de l'ordre de 10^{-3} M pour Na^{+}, et de 10^{-5} M seulement pour divers ions bivalents (Mg^{++}, Ba^{++}, Fe^{++}). A force ionique égale les cations bivalents empêchent donc beaucoup plus efficacement la dénaturation des ADN; il est probable qu'ils forment avec les ADN, des combinaisons peu dissociées. Ces résultats sont, à beaucoup d'égards, semblables à ceux que Rich a obtenus, plus récemment, sur les " homopolynucléotides " de synthèse. La principale différence réside dans le caractère irréversible de la dénaturation des ADN, qui s'explique par le fait que seul le rétablissement rigoureusement exact — hautement improbable — de la configuration originale, après la fin du traitement dénaturant, pourrait permettre à toutes les bases azotées de se trouver à nouveau associées par des ponts d'hydrogène.

M. Ubbelohde. — Are coacervates reversible?

M. Overbeek. — Coacervation is completely reversible, at least if no specific effects like denaturing occur. Kinetically the reversibility is the easier the more solvent the coacervate contains and this again is obtained at relatively high salt concentrations. There is an upper limit of salt concentration for coacervations, roughly connected with the fact that at high salt concentration the range of attraction is short and the gain in electrical energy insufficient to counterbalance the loss in entropy. The fact that nucleoproteins swell and finally dissolve in salt solutions is indicative for their coacervate character.

M. Markham. — Dr. Overbeek's discussion is very relevant to the structure of the small spherical viruses such as the small plant viruses, which consist of a spherical shell of protein enclosing a mass of ribonucleic acid some 170 Å in diameter. The number of basic groups in the protein part is nothing like sufficient to neutralize the primary phosphate groups of the nucleic acid, and, of course, the length of the arginine side chains is not sufficient to penetrate

more than a short distance into the nucleic acid. Dr. Overbeek has pointed out that it would be unsound in principle to neutralize these groups by small univalent cations, and indeed in practice we have found that, as has been found in bacteriophages by Ames, the plant virus nucleic acid is neutralized to a large extent by polyvalent organic cations. The substance present is apparently a triamine (but is not spermidine) and has been found in a number of plant viruses.

M. Rich. — Dr. Felsenfeld has recently carried out experiments comparing the effectiveness of diamines relative to divalent cations, such as magnesium or manganese, in the formation of two or three stranded helical polynucleotide complexes. He finds that the diamines and the divalent cations are equally effective. Hence it is likely that the most important effect is the overall electrostatic charge.

M. Wilkins. — The infra-red absorption bands associated with the phosphate group in DNA shift in frequency when the water content of the specimen is varied. The corresponding shifts for nucleoprotamine and nucleohistone are smaller. These observations, made by Wilkinson and Bradbury in our laboratory, suggest that there is a degree of direct binding between phosphate and the basic groups of protein in nucleoproteins. In DNA one expects, at high hydration, that water molecules occur between the metal ions and the phosphate group; there appears to be less hydration of the phosphate groups in nucleoprotein.

M. Luzzati. — Nous avons effectué quelques mesures sur l'action du sel (ClNa) sur la structure des gels de nucleoprotéines, et les résultats semblent confirmer les remarques de M. Overbeek. En effet, en absence de sel, les gels de nucléoprotamines contiennent une phase paracristalline, dont la teneur en eau est de 35 % environ; toute l'eau en excès forme une phase séparée. L'ADN, au contraire, forme avec l'eau des gels bien organisés, en absorbant toute l'eau du système. Or, si l'on ajoute assez de sel à la nucléoprotamine les gels cessent d'être formés de deux phases : à toute concentration l'eau est absorbée en totalité entre les molécules d'ADN et protéine. Cette observation semble indiquer que les nucléoprotéines sont une association colloïdale bien plus qu'une espèce moléculaire, comme l'affirme le Dr. Wilkins. En partageant l'avis de M. Overbeek, je

ne vois aucune nécessité pour que les charges libres des phosphates de l'ADN soient neutralisées exactement par les groupements basiques des protamines (ou des histones).

M. Overbeek. — Indeed the 50 : 50 balance of charges of particles in a coacervate is only exact if the supernatant also contains them in the same ratio. But if e.g. positive and negative particles are mixed in the ratio 10 : 90 or 20 : 80 the coacervate formed will have a ratio 40 : 60 or 45 : 55. This has been demonstrated experimentally by Bungenberg de Jong and is in agreement with the theoretical work by Voorn.