BIOCHEMICAL SOCIETY SYMPOSIA
NO. 14

THE STRUCTURE OF NUCLEIC ACIDS AND THEIR ROLE IN PROTEIN SYNTHESIS

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equally natural for others to let the assumption pass; often without realising that it was an assumption. Hopkins' prejudices favoured small molecules but the outlook was changing and he encouraged a sceptical and non-conformist attitude in his laboratory. As a student in this wholesome atmosphere I was accustomed to hearing the tetranucleotide hypothesis derided and the quantitative inadequacy of its foundations exposed. When therefore, in 1936, the nucleic acid from tobacco mosaic virus appeared to be much bigger than a tetranucleotide the observation did not have the barrier to overcome that would have been present in a student less fortunate in his environment. It did not take subsequent analytical evidence to overthrow the hypothesis; it seemed to us that the original evidence was sufficient for this.

General acceptance of the overthrow was easy because the phase of thinking that molecules are small until they have been shown to be large has passed and an assumption such as Levene's would probably not now be made. It may be that the pendulum has swung too far and there is a tendency to think that particles in the cell are very large until they get broken up in the course of extraction. Undoubtedly this often happens but aggregation as well as dissociation takes place on rough handling and evidence is needed before we accept as axiomatic the conclusion that, if two preparations of, for example, nucleic acid, differ in average particle size, the one with the larger particles is more nearly in the native state.

Many other types of dangerous assumption are commonly made; two may be discussed because they are particularly prevalent in current nucleic acid literature. It is obvious that the invariable presence of a substance in a system carrying out a certain process does not necessarily mean that that substance is one of the effective agents. Thus we have N_2 in our lungs but we do not assign much of a role to it. There is ribonucleic acid in the mitochrondria and microsomes from many tissues but they also contain 60% or more of other material. The observed correlation is between the concentration of microsomes, of which nucleic acid is an index, and protein synthesis or other forms of metabolism. It may well be that the effective component is indeed the nucleic acid and experiments in which the addition of RNA or its fragments stimulates protein synthesis strongly suggest this. But the conclusion does not follow of necessity from RNA determinations on tissues of varying metabolic capacity.

The other dangerous assumption may be called " aesthetic special pleading ". The prettier or tidier of two structures is not necessarily the correct one. It would have been tidier if the seven metals and the sun, moon and five planets had, as the alchemists thought, influenced one another; or if the demonstration that one amino acid or nucleotide accounted for a quarter of the residues in a protein or nucleic acid had meant that it appeared in every fourth position along a chain; or if proteins had had the cyclol structure. Similarly it would be tidy if

nucleic acids were all and always cross-bonded double helices. They may be; but biologists who do not understand the nature of the evidence on which the conclusion is based, would be wise not to build too much theoretical superstructure on what is for them a pure assumption. Spirals are fashionable at the moment but fashions change.

So much for destructive criticism. I will finish by asking two constructive questions. There is some evidence for nucleic acids with enhanced phosphorus content. Are we keeping a sufficiently sharp lookout for parts at any rate of the nucleic acid molecule playing a metabolic role similar to that of ADP and ATP? Do we not need a new word to avoid the circumlocution of "purines and/or pyrimidines"? The old word "nuclein" is not now being used. Would it be useful to reintroduce it, or to coin another word, to cover the nitrogen-containing heterocycles that have been, or will be, found in nucleic acids?

Recent Views on the Chemical Structure of the Polynucleotides

By R. MARKHAM

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The nucleic acids were discovered by Miescher (1871) as constituents of animal cell nuclei and their presence in plant cells was demonstrated some eighteen years later by Altmann (1889). These substances attracted a great deal of interest because of their composition, being phosphorus-containing and being related in some way to nitrogen-metabolism, while their evident connexion with the staining reactions of cell nuclei also provided a stimulus to their investigation. In consequence the nucleic acids were studied by a number of very famous workers with the result that, by 1931 when the classic monograph of Levene & Bass was published, it appeared that only a few minor structural points remained to be clarified. That this was not so, and, indeed, was very far from correct, only became apparent very gradually, and it is only in the past decade that the complexity of the problem and its probable importance in biology has been fully recognised.

The views on the general structure of the nucleic acids which were held until the more recent work, and which are to be found in most text books, represent a considerable oversimplification, but many of the essential points, particularly with regard to the nature of the sub-units, the nucleotides, and their constituents, were recognised. There appeared to be two kinds of nucleic acid, one containing ribofuranose (ribonucleic acid, RNA), the other containing 2-deoxyribofuranose (deoxyribonucleic acid, DNA). Both contained phosphoric acid and both had four nitrogen-containing heterocyclic compounds, namely the purines adenine and guanine, and the pyrimidines cytosine and uracil in RNA, while in DNA uracil was replaced by 5-methyluracil (thymine). These substances were present as *N*-glycosides or nucleosides, and the nucleotides, the fundamental subunits, were derived from these by phosphorylation, in the case of RNA at C3′. The position of the phosphate residues in deoxyribonucleotides was not known with certainty, but pyrimidine nucleoside diphosphates containing phosphate residues both at C3′ and C5′ were isolated from acid digests of DNA a long time ago (Levene & Jacobs, 1912). As it was possible to isolate four nucleosides from DNA, the general opinion was that nucleic acids were composed of four mononucleotides. It was also recognised that the linkage between the individual mononucleotides was a phosphate ester bridge. The problems outstanding were (*a*) the order in which the four nucleotides were arranged and (*b*) the exact nature of the internucleotide link.

Although a certain amount of uneasiness about the tetranucleotide hypothesis of nucleic acid structure was felt by some workers, principally because of the difficulty of explaining some of the physicochemical behaviour of nucleic acids, it was not until chromatographic methods of analysis were applied to these substances that it was realised how much the earlier workers had been mistaken in their interpretations. Vischer & Chargaff (1948) and Hotchkiss (1948) first began this type of investigation, and in a few years a number of accurate analytical methods were evolved, which, when they were applied to nucleic acids from a number of sources, showed that not only were the nucleic acids not composed of equimolar proportions of the four mononucleotides, but that some actually contained five nucleotides. The most striking example was the DNA from wheat germ, which Wyatt (1950) showed to contain a high proportion of 5-methylcytosine, a pyrimidine which occurs in a number of other DNA's. Since this observation other new constituents which have been recognised are 6-methylaminopurine in DNA from some bacteria (Dunn & Smith, 1955) and 5-hydroxymethylcytosine (Wyatt & Cohen, 1952) which occurs in T_2, T_4 and T_6 bacteriophage DNA in place of cytosine, and in which it is present largely as the *O*-glucoside.

RNA's from the smaller plant viruses have proved of considerable interest, because they can be analysed either directly without preliminary isolation of the RNA, or after its isolation under conditions in which minimal degradation has occurred. These nucleic acids exhibit a great variation in the proportions of the constituent nucleotides from virus to virus, although they are characteristic of the individual viruses (Markham, 1953). It is, of course, from sources such as these that one might expect to obtain nucleic acids having the greatest variation in composition. When they are obtained from sources such as animal tissues where, as we now know, they are present as mixtures, the great variety of molecules present will tend to obscure variations in the composition of the individual molecules. In the small viruses, on the other hand, the number of types of molecule which can exist is more restricted, so that differences become more apparent.

THE NATURE OF THE INTERNUCLEOTIDE LINK

Although the chromatographic analysis of nucleic acids exploded the tetranucleotide hypothesis more or less instantly, the problem of the nature of the internucleotide linkage or linkages remained. In DNA the number of possibilities was limited to 5′ : 5′, 3′ : 3′ and 3′ : 5′ phosphodiester links. In RNA's, on the other hand, the presence of a hydroxyl group at C2′ doubled the number of possibilities. Whether C5′ was involved was, however, regarded as doubtful, because it was known, from a study of the isomeric adenylic acids obtained from muscle (adenosine 5′-phosphate) and from yeast RNA by hydrolysis (adenosine 3′-phosphate), that phosphate in this position was more

stable to hydrolysis than it was at C3′. Consequently the internucleotide link in RNA was generally thought to involve C2′ and C3′ and the former position was regarded as having unusual lability, because nucleoside 2′-phosphates were not to be found on hydrolysis of RNA which occurs extremely readily in acid or alkali.

However, by using chromatographic techniques, Carter (1950) and Cohn (1950) demonstrated that the so-called nucleoside 3′-phosphates were in fact mixtures of isomeric pairs having characteristic chemical and physical properties. The nucleosides obtained from these pairs of nucleotides were identical, so that isomerism was evidently in the position of the phosphate, and as neither was the 5′-phosphate which is very easily characterised, it seemed that the new nucleotides were the nucleoside 2′-phosphate and the nucleoside 3′-phosphate. This has since been confirmed and the allocation of the phosphate group has been decided by a number of methods including degradative techniques, synthesis and X-ray crystallography (Khym *et al.*, 1953 ; Brown *et al.*, 1953). On the face of these observations, it might be thought that here was conclusive evidence for the linkage between C2′ and C3′, but it did not explain the remarkable instability of the RNA towards hydrolysis by alkali.

The interpretation of these observations was finally given by Brown & Todd (1952) who pointed out that it was known that phosphate diesters of compounds having a 1 : 2 glycol structure were unusually unstable. This is because transesterification can take place on to the adjacent hydroxyl group with simultaneous splitting off of the other alcohol. The ' cyclic ' diester formed is then degraded in its turn leaving monoesterified phosphate on one or other of the hydroxyl groups. In a sugar phosphate ester a similar situation can occur if the hydroxyl groups are not only adjacent but in the *cis* relationship, as they are in ribofuranose at C2′ and C3′. They therefore proposed that the instability of RNA was due very largely to the presence of this structural feature, and this theory is now well established because the nucleoside ' cyclic ' 2′ : 3′-phosphates have all been isolated as intermediates in the alkaline hydrolysis and the enzymic degradation of RNA (Markham & Smith, 1952). The theory, however, did not throw any further light on the nature of the internucleotide link except to show that C2′ or C3′ was definitely involved, and that C5′ was certainly not excluded. In fact any of the hydroxyl groups could be involved. The final solution of the problem came very largely from the study of the small polynucleotides arising from the partial degradation of RNA, and from the use of specific enzymic methods of degradation.

In 1951 Cohn & Volkin confirmed the earlier work of Gulland & Jackson (1938) who had obtained indirect evidence for the existence of nucleoside 5′-phosphates in snake venom digests of RNA ; and the former actually isolated the nucleoside 5′-phosphates from enzyme digests, thus, incidentally, establishing a direct relationship between the

nucleotide coenzymes and RNA. In this year too, Markham & Smith (1951, 1953*b*) reported the isolation of a number of dinucleotides and other fragments from digests of RNA made with the use of pancreatic ribonuclease. Some of these dinucleotides had one phosphate residue present as a 2′ : 3′ cyclic phosphate and in addition the two nucleotide residues were linked together (Fig. 1). Hence, by inference, C′5 was

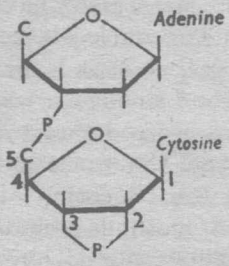

Fig. 1. Schematic formula of the ' cyclic ' dinucleotide of adenine and cytosine obtained from ribonucleic acid by the action of pancreatic ribonuclease.

involved in the internucleotide linkage. Other methods of degradation gave similar information. Thus for example a ' cyclic ' dinucleotide of the type mentioned is split by the phosphodiesterase of snake venom to give a nucleoside and a nucleoside 3′, 5′-diphosphate. Ordinary dinucleotides not having a 2′ : 3′ phosphate residue may be dephosphorylated by phosphomonoesterase and they are then split by the snake venom phosphodiesterase to give a nucleoside plus a nucleoside 5′-phosphate (Fig. 2).

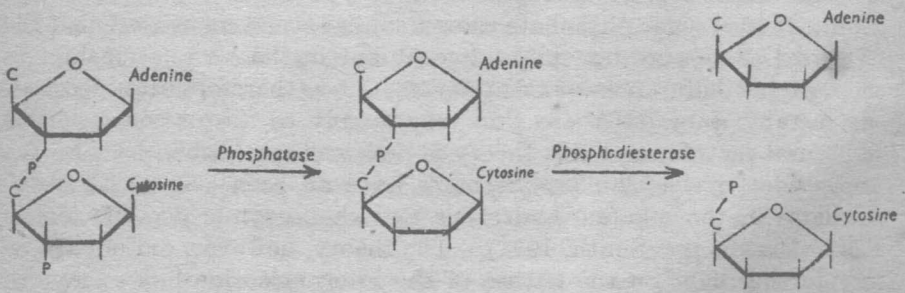

Fig. 2. Enzymic degradation of the dinucleotide of adenine and cytosine to show structure.

The position of the other end of the internucleotide link was determined in a number of ways. As normal chemical hydrolysis was inevitably associated with phosphate migration, such a degradative method could not be used to determine the position of the phosphate residues in intact RNA. The enzyme pancreatic ribonuclease, however, was used to show that in pyrimidine nucleotides, at least, the phosphate was at C′3. This enzyme has been found to be a specific phosphodiesterase attacking esters of pyrimidine nucleoside 3′-phosphates (Markham &

Smith, 1952 *a*, *b*; Brown & Todd, 1953). In this enzyme action 'cyclic' nucleotides are formed as intermediates, but it has been shown by the use of synthetic substrates of known structure that phosphate migration does not occur. In the case of the purine nucleotide residues this method cannot, of course, be used, but in these, too, the phosphate group was shown to be on C'3, by two different methods. The first involved the use of a phosphodiesterase from spleen, which splits internucleotide linkages without producing a cyclic intermediate (Heppel, Markham & Hilmoe, 1953), and the second method, which is of some theoretical interest, involves the observation that a polynucleotide chain having a free 2', 3' glycol exposed by means of phosphomonoesterase treatment, can then be degraded by oxidation of the glycol by $NaIO_4$ followed by exposure to a pH of 10. Under these conditions the remains of the terminal nucleotide split off and leave the penultimate nucleotide residue with its phosphate group on C'3 (Whitfeld & Markham, 1953). This reaction, which does not involve a hydrolytic splitting and which does not cause degradation of ordinary internucleotide links, is theoretically capable of use for a step-by-step degradation of a polynucleotide chain, and in fact has been used for the analysis of a number of small polynucleotides (Whitfeld, 1954). Its potential uses are not as great as might be anticipated, because it seems probable that polynucleotides greater than a few residues in length will always occur as rather complex mixtures which are themselves difficult to resolve. It can, however, be used to determine the nature of the terminal nucleotides of nucleic acids, but a simpler method is to do the dephosphorylation and follow it by alkaline degradation. Terminal residues are then released as nucleosides. This type of method is, of course, rather difficult to apply to large polynucleotides because the presence of a small amount of phosphodiesterase could cause spuriously large values for the number of terminations. A much better method which may be used to detect some only of the ends is the use of ribonuclease. When digestion by this enzyme is complete any free purine mononucleotides liberated must have originated from chain ends where they were linked to a pyrimidine nucleoside residue in the rest of the chain. In several RNA's many such ends have been found to be 2' : 3' 'cyclic' nucleotides (Markham & Smith, 1952 *c*; Matthews, personal communication) which would be difficult to detect by other methods. A method which is potentially of great use for the determination of nucleoside 2' or 3' phosphate terminations is by methanolysis in an anhydrous medium (Lipkin, Talbert & Cohn 1954). Such treatment splits internucleotide links to give rise to mononucleotide methyl esters which may be distinguished quite readily from the terminal residues, which are liberated as free mononucleotides. The success of such a method is dependent upon the elimination of all traces of water, and, of course, if the polynucleotides are terminated by cyclic nucleotide residues, no ends will be observed. Some polynucleotides and possibly most

RNA's have phosphate in the 5′ position in a terminal nucleotide at the other end of the chain. Such ends are easily recognised because they appear as nucleoside diphosphates (2′, 5′ and 3′, 5′) on alkaline degradation (Markham, Matthews & Smith, 1954).

The application of the degradative procedures mentioned above has led to a number of conclusions about RNA structure, one of the most important of which is probably that it is very difficult indeed to prepare RNA in an undegraded state. Many specimens of RNA, especially those obtained from animal tissues, are sufficiently contaminated with nucleases to break down when simply dissolved in water. Again most of the early preparations of RNA were badly degraded by chemical treatment during their isolation. The number of end-groups which are found by the various methods which have been discussed, varies from preparation to preparation, but it seems clear the RNA's are not as large as DNA's and that they consist of rather complex mixtures. A certain amount of speculation has arisen around possible branching structures, which could be formed by triply esterified phosphate groups on the one hand, and by subsidiary chains arising from C2′ in some of the nucleoside residues. The former is improbable because model substances of this type are unstable (Brown, Magrath & Todd, 1955). The latter is possible, but appears unlikely because several ribonuclease catalysed transesterification reactions involving the —OH group at C2′ are now known (Heppel, Whitfeld & Markham, 1955, and unpublished) and this mechanism is probably important in normal RNA metabolism. An obstruction of C2′ by a branch point would interfere with this type of reaction and would waste much of the potentiality of the structure. It should also be noted that no really good evidence for the existence of such branches in RNA has yet been put forward and all the experimental observations made to date may be explained on the assumption that RNA is a linear structure.

THE STRUCTURE OF DNA

In the case of DNA the locating of the internucleotide link is not quite as complex because the absence of a hydroxyl group on C2′ restricts the number of possible internucleotide phosphate ester links. This very simplicity, however, has greatly impeded the study of DNA structure, for the very reason that this nucleic acid is not nearly so amenable to selective method of degradation. In consequence, most of our information has been obtained by the use of enzymes, of which one, namely pancreatic deoxyribonuclease, has been used with some success. Deoxyribonucleic acid treated with this enzyme yields a number of small polynucleotides terminating with phosphate at C5′ and having 3′ : 5′ internucleotide linkages (Smith & Markham, 1952; Sinsheimer & Koerner, 1952). From the analysis of such polynucleotides two interesting facts have emerged which may have a bearing on DNA structure. The first is that the isomeric pairs of dinucleotides are not released in equal amounts, and in some cases, for example,

the dinucleotide containing cytosine and adenine, only one of the two possible isomers is found. The other, which is of interest with regard to the macromolecular structures which have been proposed by Watson & Crick (1953), is that 5-methylcytosine is not, as would be expected, distributed in the same way as cytosine, but has a definite tendency to occur in the nucleotide adjacent to deoxyguanylic acid. This may, of course, be explained by supposing that cytosine and 5-methylcytosine do not occur in the same nucleotide chain, and it might be of interest to try to isolate the dinucleotide containing both bases.

A certain amount of chemical degradation may be carried out on DNA by virtue of the fact that the purine glycosidic links are very unstable to acid, and may be removed by a selective hydrolysis to give what has been termed " apurinic acid " (Tamm, Hodes & Chargaff, 1952). As deoxyribose is highly aldehydic the straight chain sugar residues thus exposed are then sensitive to alkaline degradation, the reaction being thought to be analogous to that in periodate oxidised ribonucleoside residues (Brown & Todd, 1955), although hydrolysis through a 3 : 4 cyclic phosphate might also occur. Experiments such as this have, however, yielded little information so far except to indicate that pyrimidine nucleotide residues occur together on occasion, a fact which had been evident from the enzymic degradations referred to previously.

One of the most remarkable facts which have so far been noted in the DNA's is the numerical relationship first pointed out by Chargaff (1950) and since confirmed by a number of workers. This relationship, which appears to hold for most of the DNA specimens analysed so far, is that there is an equivalence (*a*) of purines to pyrimidines, and (*b*) of adenine to thymine, and guanine to cytosine (or cytosine+methyl cytosine if the latter is present). This relationship is, of course, the basis of the Watson-Crick structure which will be referred to by later speakers and which involves the assumption of specific base pairing. This theory is particularly attractive in that it would appear to offer a means whereby DNA could duplicate itself, but a very serious objection is the fact that mechanisms of this type would not appear to allow the incorporation of the various methylated cytosines and 6-methylaminopurine in other than a random manner, nor of course, does it explain why uracil could not be used in place of thymine. It is possible that such discrepancies can be resolved by experiment. The methods which would have to be employed are not evident, but progress has been so rapid in the past few years that there is good reason to hope that we shall know a great deal more about DNA structure in the near future.

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DISCUSSION

Dr F. H. C. Crick (*Cambridge University*). I should like to make a suggestion about the incorporation into DNA of certain unusual bases such as 5-methylcytosine and methyladenine. The immediate precursors of DNA are unknown, but for the purposes of explanation let us call them nucleotides. It is often tacitly assumed that these precursors (in this terminology) are mononucleotides. If, on the other hand, they were di-nucleotides, or larger combinations, certain experimental facts might fall into place. Thus it has been shown that 5-methylcytosine appears to occur next to guanine. The way in which methyladenine is incorporated could be explained if it were carried into the structure by other bases. A natural hypothesis is that it neighbours thymine. This could be tested experimentally by examining all the di-nucleotides containing methyladenine in a partial hydrolisate. If, as I suspect, there are restrictions on the possible sequence of bases (though of a subtle kind) such experiments might also help towards discovering them.

Molecular Structure of Deoxyribose Nucleic Acid and Nucleoprotein and Possible Implications in Protein Synthesis

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THE THREE-DIMENSIONAL CONFIGURATION OF DEOXYRIBOSE NUCLEIC ACID MOLECULES

BEFORE we consider the implications of nucleic acid structure in the synthesis of proteins we will review our knowledge of the structure. This knowledge has increased greatly in the last few years : chemical study showed that the nucleosides were linked by 3′, 5′-phosphodiester groupings in both DNA and RNA ; titration experiments indicated a large amount of intra-molecular hydrogen-bonding between bases in DNA ; analysis of DNA showed that, although the ratio of adenine to guanine could vary considerably with species, there were always equal proportions of adenine and thymine and of guanine and cytosine. Physical studies demonstrated that nucleic acid molecules were long thread-like molecules with the planes of the bases tending to lie perpendicular to the length of the molecule. Fibres of DNA gave excellent X-ray diffraction photographs and these, together with the chemical data, have enabled the structure of DNA, i.e., its three-dimensional configuration, to be established with reasonable certainty. RNA gives inferior X-ray data and its structure has not yet been elucidated.

The DNA X-ray experiments strongly suggested a helical structure and provided the main dimensions of the helix. About the same time an important advance was made by Watson & Crick who built a molecular model of DNA. The factors which guided the building of this model were as follows. First, the X-ray data provided in a general way the dimensions of the helix and showed that the molecule consisted of 2 or more polynucleotide chains; second, the base analysis data suggested that the bases occurred in pairs ; third, the titration studies suggested the polynucleotide chains in a molecule were joined by hydrogen bonding between the bases. Watson & Crick made the important assumption that the number of polynucleotide chains in the molecule was two, and the ingenious manner of their base-pairing resulted in both pairs being equivalent and symmetrical with regard to the joining of the two phosphate-ester chains. This scheme was most attractive because it explained how DNA, consisting of 4 bases of different dimensions, could exist as molecules of great regularity which crystallised with considerable perfection irrespective of the ratio of