THE CHEMISTRY OF NUCLEIC ACIDS

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PREFACE

Our present knowledge of the structure and function of the nucleic acids has accumulated from studies involving a wide variety of different disciplines, ranging from physics through chemistry and biochemistry to biology and genetics. A comprehensive work covering all aspects of the structure and function of nucleic acids would require several volumes and would be beyond the compass of any one author. I have therefore concentrated, in this book, on the chemistry and structure of the nucleic acids. The word structure is used here in its widest sense, thus in addition to the detailed architecture of the nucleic acid molecule giving the relative positions of the atoms, the nature of the bonds, and the size and shape of the macromolecule, the heterogeneity of nucleic acid preparations brought about by different sequences of nucleotides, by different nucleotide composition, and different molecular size is also discussed. Such an interpretation is clearly necessary, since if the nucleic acids carry the genetic code, as is currently believed, and also possess other biological functions, the structure must be all important in determining these functions.

My interest in the chemistry of the nucleic acids was first aroused by the late Professor J. M. Gulland with whom I was privileged to work for several years. I wish to record my appreciation of his guidance and encouragement when our ideas of the macromolecular structure of nucleic acids were first taking shape.

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CONTENTS

1

INTRODUCTION

THE combination of chemical, cytological and genetical studies has led to the realization that nucleic acids are of fundamental importance in controlling the metabolism, reproduction and growth of living systems. Almost all metabolic processes function through enzymes whose synthesis is controlled by the genes, the units of heredity. The gene must be capable of replication during cell division and it must also be able to control the synthesis of a specific protein molecule. It has been known for some time that the chromosomes, which are the cellular structures incorporating the genes, consist of nucleic acids and proteins, but convincing evidence has only recently been given that the genetically significant part of the gene is composed of nucleic acid and not protein.

The idea that it is the nucleic acid which carries the genetic information stems from the discovery by Avery, Macleod and McCarty[1] that the 'transforming principle,' first described by Griffith[2], which under appropriate conditions will induce the transformation of unencapsulated R variants of pneumococcus type II into fully encapsulated S cells of type III, is a highly polymerized deoxypentose nucleic acid[3]. This work did not immediately receive the widespread recognition that it deserved, largely because ideas concerning the structure of nucleic acids were still prejudiced, at that time, in favour of the erroneous tetranucleotide structure. However, with the appreciation of the macromolecular character of nucleic acids and in particular through the work of Hershey and Chase[4] concerning the nature and action of phages, the implications of the work of Avery and his collaborators have become widely accepted. Hershey and Chase[4] were able to demonstrate that when coliphage is added to *Escherichia coli* cells in a suitable environment, the nucleoprotein constituting the phage dissociates, the deoxypentose nucleic acid and a small amount of soluble protein enter the cell while most of the protein component remains outside. In the subsequent stages of phage reproduction occurring in the host cell, specific protein and deoxypentose nucleic acid are synthesized which are different from any of the components of the non-infected host. Such species-specific materials, it is

generally assumed, are produced under the influence of specific genetic determinants, in this case either the deoxypentose nucleic acid or the soluble protein entering the cell or both. Hershey[5] has shown that the material which enters the cell, what he calls the 'germinal substance,' is 97 per cent deoxypentose nucleic acid and 3 per cent protein and that a decisive assignment of the phage genetic functions to one component in preference to the other cannot be made at present. However, Herriott[6] considers that in view of the nature of the bacterial transforming factors and from structural considerations, the deoxypentose nucleic acid appears the more attractive choice. This view is substantiated by the study of the rescue of genetic markers from ultra-violet-inactivated phage by Doermann, Chase and Stahl[7] and the isotopic tracer studies of Stent[8]. Further evidence for the unique part played by nucleic acids in biological processes comes from the observation, obtained independently by Fraenkel-Conrat[9] and by Gierer and Schramm[10], that the pentose nucleic acid isolated from tobacco mosaic virus possesses virus activity which can account for almost all the activity of either the native virus or the reconstituted virus formed by the recombination of the dissociated nucleic acid and protein.

From studies such as these, it has proved evident that the once held view that the primary coding of genetic information resided in the protein portion of the chromosomes must be abandoned, and it is now generally accepted that the primary genetic material is deoxypentose nucleic acid, except in the plant viruses, where pentose nucleic acid takes its place[11].

In order that the nucleic acids may convey genetic information, a pattern or code must be incorporated in their chemical structure. The nucleic acids are co-polymers in which the monomers, known as nucleotides, consist of a purine or pyrimidine linked to a sugar phosphate. In any one nucleic acid there is only one kind of sugar, but generally four different purines or pyrimidines. Although there is no direct information concerning the sequence of purines and pyrimidines, the fact that they are the only variable constituents suggests that they are arranged in a definite sequence and that this sequence constitutes the genetic code. The length of the nucleic acid molecule varies and is dependent not only on the particular type of tissue extracted, but preparations from a given tissue are invariably heterogeneous, as shown by a wide distribution in the sedimentation coefficient[12,13]. This heterogeneity may, in part, be produced artificially during extraction, but in view of the observation that the fractionation of nucleic acids by anion exchangers[14,15] shows a correlation between the composition of the eluting solution

and the sedimentation coefficient, and that the sedimentation coefficient distribution within the fractions is much narrower, the possibility arises that the nucleic acids of different genes are of different lengths. Thus in addition to the genetic code being associated with the sequence of purines and pyrimidines, it may also be determined by the length of the nucleic acid molecules.

The study of nucleic acids and in particular the study of their biological function, has been considerably stimulated in recent years by the proposal in 1953 by Watson and Crick[16] of a structural model for deoxypentose nucleic acid which, with one or two minor modifications, is now widely accepted as the basic structure for this type of nucleic acid. This structure, which is fully discussed in later chapters, consists of two polynucleotide chains, which run in opposite directions, are coiled round each other to form a double helix, and are bound to each other by specific hydrogen bonds between the purines and pyrimidines. The complementary arrangement of the purines and pyrimidines of the two polynucleotide chains produced in this way suggested to Watson and Crick[17] that the nucleic acid molecule replicates itself directly by having each chain serve as a template for the formation of its complement. This stimulating suggestion presents many problems involving the separation of the intertwined polynucleotide chains, and several modifications of the replication procedure have been suggested which have been fully discussed by Delbrück and Stent[18]. Although modifications have been made to both the original structure and replication mechanism suggested by Watson and Crick, their contribution, based, as it was, on the accumulated chemical and physical evidence from several laboratories, represents an important landmark in the development of our knowledge of nucleic acids.

The discovery of the nucleic acids was the result of the work of Miescher, who, working in the laboratory of Hoppe-Seyler in Tübingen in 1868–69, isolated from the nuclei of pus cells a substance which he called ' nuclein.' This substance, which was acidic in nature, warranted special interest as it contained a relatively high proportion of phosphorus and at that time lecithin was the only known organic compound in tissue which contained phosphorus. Miescher submitted an account of his work to Hoppe-Seyler who found it so interesting and surprising that he repeated, personally, the experiments of Miescher on pus cells and engaged two of his students, Plósz and Lübavin to test the methods of Miescher on materials other than pus (*viz.* egg yolk, yeast, casein and the red cells of birds and reptiles). Having confirmed the discovery of ' nuclein ' the work of Miescher was published along with that

of Hoppe-Seyler and his pupils in Hoppe-Seyler's *Medicinisch-chemische Untersuchungen* in 1871[19]. A convenient and general method of preparation of 'nuclein' was later described by Altmann[20], to whom also must be credited the introduction of the term 'nucleic acid.'

The work of Miescher, which amply demonstrated the highly polymeric character of nucleic acid, was unfortunately rather overlooked by later workers, who, for investigations using the degradative methods of organic chemistry, made use of material which had been extracted from tissue by methods involving heat treatment and the use of acid and alkali. Such methods of extraction yielded a product which, while no doubt suitable for the degradative studies and the examination of hydrolysis products, bore little resemblance to the native nucleic acid.

Many assumptions based on investigations making use of degraded material led to erroneous conclusions, some of which may have seriously retarded the development of ideas on the structure and function of nucleic acids. For example, the conception that the nucleic acid molecule was a tetranucleotide, which was accepted for some ten to fifteen years, grew up through the discovery of four nucleotides in approximately equimolecular proportions in the hydrolysate of yeast nucleic acid and a determination of molecular weight[21], later shown to be erroneous[22] which indicated a particle size corresponding to a molecule composed of four nucleotides. In spite of this criticism, however, the degradative studies by the classical methods of organic chemistry were of fundamental importance in determining the elementary molecules which together constitute the nucleic acids.

From this work there emerged the idea of the existence of two distinct types of nucleic acids. One, the nucleic acid from yeast, on hydrolysis yields adenine, guanine, cytosine, uracil, phosphoric acid and a sugar recognized by Hammarsten as a pentose and identified by Levene and Jacobs[23] as ribose. The other, the nucleic acid from thymus gland, yields adenine, guanine, cytosine, thymine (5-methyl uracil), phosphoric acid and a sugar shown by Levene and London[24] to be a deoxypentose and identified by Levene, Mikeska and Mori[25] as deoxyribose. These two nucleic acids thus came to be called ribonucleic acid and deoxyribonucleic acid, respectively, and since most nucleic acids of animal origin appeared to resemble that from thymus while those of plant origin were similar to that from yeast, the idea became prevalent that the pentose nucleic acids were characteristic of plants while the deoxypentose nucleic acids were characteristic of animal tissues.

This classification, however, was never free from objection and from its inception exceptions were continually being discovered. Conclusive evidence of its invalidity came from the histochemical studies of Brachet[26], the ultra-violet spectrophotometric examination of tissues introduced by Caspersson[27] and the chemical analysis of cells by Davidson and Waymouth[28]. More precise analysis of the chemical constituents of cells and tissues became possible with the introduction of the methods of paper[29–31] and ion-exchange[32] chromatography. From studies such as these, it is generally concluded that the cytoplasmic components contain pentose nucleic acid, while the nuclei contain deoxypentose nucleic acid and small amounts of pentose nucleic acid. It has thus been demonstrated that both types of nucleic acid are present in all types of cell, whether of plant or animal origin and that the main biological distinction between pentose nucleic acid and deoxypentose nucleic acid is that the former is mainly found in the cytoplasm, while the latter is exclusively, or almost exclusively, found in the nucleus.

The identification of the sugar in the nucleic acids has only been rigorously determined in a few instances. The only sugars isolated from nucleic acids so far are D-ribose and 2-deoxy-D-ribose. The pentose sugar has been identified as D-ribose in the pentose nucleic acids of yeast[23] and liver[33], but has been shown to be chromatographically identical with D-ribose in pentose nucleic acids from a large number of sources[31]. The deoxypentose sugar in the deoxypentose nucleic acid of thymus has been identified as 2-deoxy-D-ribose[25], but again the sugars in deoxypentose nucleic acids from other sources have been shown to be identical with it by chromatographic methods[30]. Throughout this book the generic names 'pentose nucleic acid' and 'deoxypentose nucleic acid' will in general be used, except where reference is made to a specific nucleic acid of known composition.

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2

THE ISOLATION OF NUCLEIC ACIDS

INTRODUCTION

THE aim of research in nucleic acids, in common with many biochemical studies, is to relate the chemical structure and reactivity of the isolated nucleic acids to their function in living organisms. The isolation of cell constituents of complex structure and high molecular weight must always raise the question as to whether the isolated preparation may be regarded as in any way equivalent to the original substance in the cell. Ideally, isolation procedures should be such as will produce a nucleic acid preparation which retains, in every detail, the structure existing in the original cell. However, nucleic acid has always been found associated with protein in the cell. Whether all of the nucleic acid in cells occurs as a nucleoprotein cannot at present be stated, but there can be little doubt that most, if not all, of the nucleic acid is present in the cell as a biologically active nucleoprotein. It is possible that nucleoproteins may be isolated more or less unchanged, but it is very difficult to distinguish between native nucleoprotein and artifacts such as protein nucleates, produced by the simultaneous isolation of protein and nucleic acid from cells. To separate the nucleic acid from the protein various procedures have been adopted, but whether these be mild or strong, the fact remains that if the nucleic acid occurs in the cell combined with protein, groups originally joined to protein will become free and the danger of secondary reactions may be considerable.

The isolation of nucleic acids is further complicated by our lack of knowledge of their exact functions in the cell. Unlike many of the proteins, such as enzymes and antibodies, nucleic acids possess no easily measurable biological activity. The criteria for the satisfactory isolation of a specimen of nucleic acid are therefore much more difficult to define than for the analogous case of the proteins and many arbitrary standards have from time to time been employed; these have included chemical analysis, molecular weight, ultra-violet absorption spectra, viscosity and acid–base titration. These criteria can strictly only be used for comparing

preparations and make use of such not necessarily valid assumptions as, for example, the higher the molecular weight the better the preparation. It is to be emphasized, therefore, that at present there is no absolute criterion of a satisfactory preparation of nucleic acid such as exists for some proteins and viruses.

The first preparations of nucleic acids described by Miescher[1], Altmann[2], and Kossel and Neumann[3] were devised on the assumption that nucleic acids were labile substances and that reactive reagents and heat would produce fundamental changes in the nucleic acid. This assumption is now known to have been correct and all modern methods of preparation employ the mildest possible conditions. However, in 1899, Neumann[4] described a modification of the original Altmann procedure which involved the heating of the minced organs in a 3 per cent solution of sodium hydroxide, and which was to set the pattern for preparations for the next thirty years[5]. Thus Levene and Bass[6] state 'the old traditional fear of using energetic methods for the separation of the nucleic acid from the protein was abandoned'.

This development probably led to an acceleration of the study of the products of the hydrolysis of nucleic acids by the classical methods of organic chemistry, but nevertheless was also responsible for the acceptance of a very erroneous value for the molecular weight of the nucleic acids. Thus while it appears very probable that both Miescher and Kossel realized the complex nature and macromolecular structure of nucleic acids, the later workers considered nucleic acids to be much smaller. This view was not discredited and the true macromolecular character of nucleic acids again realized until a return was made to the mild methods of preparation.

Nucleic acids occur in the cell in close association with protein and since the most difficult step in the preparation of nucleic acids is the separation of the nucleic acid from the protein, the isolation is now generally carried out in two stages: firstly, the isolation of the nucleoprotein, which may or may not be isolated in the solid state, and secondly the separation of the nucleic acid from the protein. In this procedure the separation of the pentose from the deoxypentose nucleic acid is carried out while both acids are combined with protein by making use of the marked difference in the solubility of pentose and deoxypentose nucleoproteins in electrolyte solutions, described by Mirsky and Pollister[7]. Procedures which have been described for the separation of pentose and deoxypentose nucleic acids[8] after separation from the protein, and which employ strong reagents, are not satisfactory.

THE ISOLATION OF DEOXYPENTOSE NUCLEOPROTEIN

Excluding those methods which involve the use of acid or alkali, two main methods are used for the isolation of the nucleoprotamines and nucleohistones. The difference between these methods lies in the ionic strength of the solution used for the extraction of the nucleoprotein from cell tissue; the first method employs solutions of water or of low ionic strength[9–28]; the second method uses solutions of high ionic strength[7, 29–39]. For the purpose of isolating a sample of deoxypentose nucleic acid, there is probably little to choose between the two procedures, but for a preparation of nucleoprotein the former method is to be preferred since there exists considerable evidence that in strong solutions (1M) of sodium chloride a dissociation of the nucleic acid and protein takes place[7, 12, 26, 36, 37, 40–42] with, on precipitation, the formation of a protein nucleate which is unlikely to resemble in specific detail the original nucleoprotein.

The remarkable variation of solubility of deoxypentose nucleoprotein in solutions of electrolytes[7] enables a complete separation of this nucleoprotein from pentose nucleoprotein to be carried out. The solubility of deoxypentose nucleoprotein in solutions of sodium chloride of different concentration has been fully investigated by Frick[36] who showed that the solubility in 0·14M sodium chloride solution (physiological saline) was one hundredth of the solubility in pure water, while the solubility in 1M sodium chloride was at least twice that in water. The solubility of deoxypentose nucleoprotein thus shows a minimum in solutions of 0·14M sodium chloride. On the other hand, the pentose nucleoproteins are more generally soluble and in particular are soluble in 0·14M sodium chloride. Thus by washing minced tissue with 0·14M sodium chloride solution a separation of the deoxypentose from the pentose nucleoprotein may be effected.

During the isolation of the nucleoprotein from the cell, enzymic degradation must be prevented, or, if this is not possible, kept at a negligible level. Specific depolymerizing enzymes for nucleic acids appear to be present in most cells and during the isolation procedure the enzyme and the nucleoprotein are free in the same solution and the possibility of reaction exists. To reduce or prevent enzymic action a variety of precautions have been recommended. All preparations are made at 0°C or lower to reduce the rate of enzymic action and generally enzyme poisons such as arsenate[18], citrate[34], fluoride[33] or a chelating agent such as sodium ethylenediamine tetra-acetate are added to further suppress enzymic action.

Emphasis has already been laid on the necessity, during the isolation of nucleoproteins and nucleic acids, of avoiding the use of heat and of strong chemical reagents which will alter the pH of the solutions significantly from neutrality. Also, as first pointed out by Chargaff[43], the use of high speed mincers is to be avoided. There is now little doubt that the excessive use of such mincers (blendors or homogenizers) during preparation leads to degradation of the macromolecules. This is probably brought about either by local heating or ultrasonic cavitation effects. However, the use of such a mincer appears to be necessary in order to disrupt the cells, but it is essential that it should be used for as short a period as possible.

Two typical preparations, the first using solutions of low ionic strength and the second solutions of high ionic strength are given below.

Preparation of calf thymus nucleohistone using solutions of low ionic strength[26]

Calf thymus gland from freshly slaughtered animals is trimmed to remove fat, chilled immediately and processed without delay. All subsequent operations should be performed in a cold room at 4–6°C. Fifty-gramme portions of gland are triturated for 30 seconds in a high speed mixer equipped with cutting blades, with 50 ml. of an ice-cold mixture of 0·1M sodium chloride and 0·05M sodium citrate previously adjusted to pH 7. The supernatant fluid resulting from centrifugation at 2000*g* for 30 minutes is discarded and the sediment resuspended in 100 ml. of the sodium chloride–citrate solution and again centrifuged. This washing procedure may be repeated.

The sediment is then washed three times by thorough resuspension and centrifugation in order to remove electrolytes, using each time 50 ml. of distilled water, previously adjusted to pH 7 by the addition of sodium bicarbonate solution. During the final washing the sediment should swell, but not appreciably dissolve in the supernatant fluid. The gelatinous sediment is then blended for 15 seconds in a high speed mixer with 250 ml. of distilled water (adjusted to pH 7) and shaken overnight. A very viscous solution should result which after briefly stirring in the high speed mixer, is centrifuged for 30 minutes at 2000*g*. The nucleoprotein is precipitated by making the aqueous solution 0·15M with respect to sodium chloride by adding the calculated amount of a solution (approximately 0·2M) of sodium chloride. The precipitate is collected after 30 minutes by centrifugation and washed in the centrifuge with 0·15M sodium chloride and then, very briefly, with a small amount of distilled water.

Preparation of calf thymus nucleohistone using solutions of high ionic strength[7, 37]

Calf thymus gland is obtained as described above and all subsequent operations should be performed in a cold room at 4–6°C.