NUCLEOTIDES AND COENZYMES

D. W. Hutchinson

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General Editors' Foreword

Methuens' Biochemical Monographs are similar in form and aim to the series of Methuen's Monographs on other subjects. The volumes can be regarded as chapters of a large work which records progress in biochemistry in general. Each volume in the series aims to provide an authoritative survey of the present position in a particular field of biochemistry by an expert in the subject, written in such a way that the book can provide an introduction both for the student who is reaching the end of his undergraduate studies and for the research worker who wishes to have an account of a subject cognate with his own. Although these accounts of the subject are intended for those who wish to study biochemistry, in practice the books have proved to be attractive to a much wider group.

The books are intended to be handy and to be rather more than a review but less than a detailed monograph. No attempt has been made to include a complete bibliography, but references are given which should provide a key to the essential relevant literature.

As biochemistry widens its interests so the diversity of the subjects included in this series will grow. They naturally range from some which are primarily chemical in their emphasis to those which are essentially biological in their outlook.

The present book by Dr D. W. Hutchinson is primarily chemical in its range. It is a companion book to Professor Davidson's *The Biochemistry of the Nucleic Acids*, which has now run through several editions. The enormous extension of the literature in the field of Nucleic acids due to the activity in research, has made it essential to discuss the more chemical side in a separate volume. This Dr Hutchinson's monograph succinctly does, always with a view to the biochemical importance of the substances under discussion.

Preface

This book is intended as a companion to the Methuen Monograph *The Biochemistry of the Nucleic Acids* by Professor J. N. Davidson and in it the chemistry and biochemistry of nucleosides, mono- and polynucleotides, nucleotide coenzymes, and the two 'non-nucleotidic' coenzymes, pyridoxal phosphate and thiamine pyrophosphate, will be described.

It is obvious that an exhaustive and detailed review of a subject of this nature would be beyond the scope of a Methuen Monograph (and probably beyond the scope of the author). If a more detailed treatment is required of certain aspects of the subjects which are dealt with, I would refer the reader to one of the following excellent, and more extensive, works: *The Nucleic Acids* edited by Chargaff and Davidson, *The Enzymes* edited by Boyer, Lardy, and Myrbäck, and *The Chemistry of the Nucleosides and Nucleotides* by Michelson.

The field is reviewed up to the end of September 1963, but one of the necessary evils of producing a book of this kind is that some of the statements made in it may be out of date by the time of publication. This is particularly true of the subject of protein biosynthesis which is discussed at the end of Chapter 5, as this field is expanding rapidly at the present time.

Throughout, I have attempted to use the nomenclature for coenzymes and enzymes which is approved by the International Union of Biochemistry. Thus, I have used nicotinamide-adenine dinucleotide (NAD^+ and its reduced form NADH) instead of diphosphopyridine nucleotide or Coenzyme I. I have, however, symbolized inorganic orthophosphate and inorganic pyrophosphate as P_i and PP_i respectively in descriptions of biochemical reactions for which the ionic forms of the ortho- and pyrophosphate are not defined.

In addition, a shorthand nomenclature for oligo- and polynucleotides has been adopted. The nucleosides are represented by their initial capital letters, e.g. A for adenosine and T for thymidine. Assuming a 3′→5′ linkage by a phosphate residue between the nucleosides, the phosphate residue may be represented by the suffix p, and the polynucleotide may be built up from the 5′ hydroxyl end in the following manner. Adenylyl (3′→5′) adenosine is represented by ApA, adenylyl (3′→5′) adenosine-3′ phosphate by ApAp, and the 5′ phosphate of adenylyl (3′→5′) adenosine by pApA. Deoxynucleosides are represented by the prefix d, and oligodeoxynucleotides are represented as follows, deoxycytidylyl (3′→5′) deoxycytidine is d-CpC.

I have used abbreviations for the Journals which are approved by the Chemical Society of London, except that the following shortened forms have been employed:

Journal of the Chemical Society	*JCS*
Journal of the American Chemical Society	*JACS*
Journal of Biological Chemistry	*JBC*
Biochemical Journal	*BJ*
Biochimica et Biophysica Acta	*BBA*
Proceedings of the National Academy of Sciences (Washington)	*PNAS*

The series *The Enzymes* Volumes I to VIII edited by Boyer, Lardy and Myrbäck which is published by the Academic Press is referred to in the following manner, *The Enzymes* (1962), **6**, 301.

I would like to thank Messrs Macmillan for permission to use the diagram of the Watson–Crick helical form of DNA on p. 122.

I should like to thank Professor Lord Todd for his interest in this book and Drs V. M. Clark, J. F. P. Richter, and S. G. Warren for their kindness in helping me to prepare this volume. Not least I should like to thank Sir Rudolph Peters and Professor F. G. Young without whose encouragement this book would never have been written. The award of an Imperial Chemical Industries Research Fellowship is gratefully acknowledged.

D. W. HUTCHINSON

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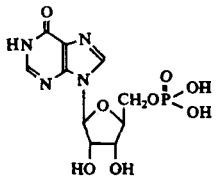
CHAPTER ONE

Historical Introduction

The discovery of the first nucleotide, inosinic acid, by Liebig in 1847 (1), preceded the discovery of nucleic acids by Miescher (2) by some twenty years.



Liebig isolated inosinic acid from extracts of meat along with creatine and sarcosine, but unfortunately he did not detect the presence of phosphorus in inosinic acid. The correct structure was not established until 1911 when Levene and Jacobs (3) proved that it was the 5′ phosphate ester of hypoxanthine riboside.

Miescher (2) obtained a material with a high phosphorus content from the nuclei of pus cells which he had treated with pepsin and dilute hydrochloric acid. On the addition of ether to the digests of the pus cells, a material which Miescher called 'nuclein' was precipitated from the reaction mixture. Nuclein was insoluble in organic solvents and acids but was readily soluble in alkali. A most remarkable feature about nuclein was its high phosphorus content as lecithin was the only naturally occurring organo-phosphorus compound which had been discovered at that time. Hoppe-Seyler, to whom Miescher presented his results for publication, was sceptical about the nature of nuclein and refused to publish until he and two of his students had repeated Miescher's experiments (4).

Altmann, who continued Miescher's work after the latter's death, was the first to introduce the term 'nucleic acid' (5). Altmann

obtained protein-free nucleic acid from yeast as well as animal tissues. Shortly afterwards, Kossel and Neumann (6) discovered a nucleic acid in thymus glands.

The second stage in the development of the chemistry of nucleic acids, the elucidation of the components of the nucleic acids, was begun by Piccard who isolated guanine and hypoxanthine from sperm nuclei. The determination of the constituents of nucleic acids was continued mainly by Kossel and Levene, and by 1914 all the main purine and pyrimidine bases together with D-ribose had been identified. The existence of two distinct types of nucleic acid was apparent when the nucleic acid from yeast on hydrolysis yielded adenine, guanine, cytosine, and uracil together with orthophosphate and D-ribose, whereas the nucleic acid from thymus glands gave adenine, guanine, cytosine, and thymine together with orthophosphate and a pentose. This pentose was later identified as 2-deoxy D-ribose. These nucleic acids came to be called ribonucleic and deoxyribonucleic acids and it was assumed that ribonucleic acid was the component of plant tissues and deoxyribonucleic acid was the component of animal tissues. This sweeping assumption was finally disproved by the histochemical studies of Brachet (7), and by the analytical studies on cells of Davidson and Waymouth (8).

Although the structures of most of the nucleosides and nucleotides had been determined by 1940, the study of nucleosides and nucleotides did not progress rapidly until the introduction of paper chromatography (9), paper electrophoresis (10), and ion exchange chromatography (11). These techniques enable small quantities of nucleosides and nucleotides to be separated and identified unequivocally as a routine operation. This is an enormous improvement on 'classical' separation techniques involving precipitation and crystallization, as it is almost impossible to separate chemical compounds with very similar properties by such methods.

The first nucleotide coenzyme was reported by Harden and Young in 1906 (12) as the heat stable cofactor of alcoholic fermentation; this coenzyme is now known as nicotinamide-adenine dinucleotide (NAD^+). Some years then elapsed until the discovery of other coenzymes such as adenosine triphosphate (13) and nicotinamide-adenine dinucleotide phosphate (14); here again the numbers and

diversity of nucleotide coenzymes were not realized until the introduction of paper and ion exchange chromatography. This was largely due to the extreme lability of the coenzymes under the conditions of 'classical' isolation techniques.

The chemical synthesis of nucleosides and nucleotides, which was begun by Levene, has been developed by Todd, who together with his co-workers, has been responsible for the rigorous structural proof and synthesis of many nucleosides, nucleotides, and coenzymes. The synthetic approach to nucleotide chemistry has been continued by Khorana who has synthesized polynucleotides and such complex coenzymes as Coenzyme A (15).

After the determination of the ratios of the base pairs adenine + thymine and guanine + cytosine in DNA by Chargaff (16), and the demonstration by Brown and Todd that the 3′- and 5′-hydroxyls of successive nucleosides in RNA and DNA were joined by a phosphodiester group, the way was open for Watson, Crick, and Wilkins (18) to demonstrate the helical coil structure of DNA.

The biological role of nucleic acids and the nucleotide coenzymes has been demonstrated at a much later date than the elucidation of their structure. In 1944, Avery, MacLeod, and McCarty (19) showed that the 'transforming principle', which under suitable conditions could transform one type of pneumonococcus bacterium into another, was DNA and hence DNA must have some effect on the genetic structure of cells. This discovery was followed by others on the mode of action of bacteriophages. These consist in the main of nucleoprotein, and when the bacterial cell becomes infected by the phage, the DNA separates from the nucleoprotein and enters the cell (20) to direct the synthesis of DNA and proteins which are alien to the host cell. Additional evidence concerning the genetic role of nucleic acids was provided by Fraenkel–Conrat (21) and Schramm (22) from a study of the tobacco mosaic virus (TMV). The genetic information in TMV, which is a ribonucleoprotein, lies exclusively in the RNA component of the virus.

Another biological function of RNA is the direction of the biosynthesis of protein; this subject is discussed in detail in the final section of Chapter 5 of this volume. Once RNA had been implicated in the biosynthesis of protein, the question arose as to how the RNA

could 'code' amino acids into specific positions in the protein. This led to the development of the theory of the triplet code for the transference of genetic information (23). In this theory, three successive nucleosides in the RNA code for one specific amino acid. There has recently been a great burst of activity throughout the world in an effort to crack the triplet code as to which three nucleosides (or more particularly which three bases) code for which amino acid.

To summarize, the discovery of the nucleic acids and coenzymes was followed by the determination of their structure, and structural determination methods were simplified by the introduction of such modern analytical techniques as paper and ion exchange chromatography, together with ultraviolet and infrared spectroscopy. This development in analytical procedures has been followed by considerable effort to try to discover the exact biological function of nucleic acids and nucleotide coenzymes. Thus the arbitrary boundaries between organic and biochemistry have been broken down and the study of nucleotides and nucleic acids could perhaps best be described as a 'molecular science' rather than any particular branch of chemistry.

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CHAPTER TWO

Nucleosides and Nucleotides

2.1 Introduction

Nucleosides can be defined as compounds in which a purine or pyrimidine base is linked glycosidically to a carbohydrate. Phosphate esters of nucleosides are called nucleotides. Nucleotides, the sub-units of nucleic acids, are also the constituents of various important coenzymes.

2.2 Occurrence

The most important source of nucleosides and nucleotides are the nucleic acids which can be degraded to nucleosides and nucleotides by either chemical or enzymic methods.

Nucleic acids normally occur in association with proteins, such aggregates being known as nucleoproteins. Very mild isolation techniques are necessary to separate the nucleoprotein from cell tissue and to prevent denaturation of the nucleic acid portion of the nucleoprotein. For example, denaturation of the nucleic acid takes place if the nucleoprotein is subjected to heat, acid, alkali or even to the excessive use of high-speed mincers during isolation. A common technique for the isolation of nucleic acids is the extraction of the nucleoprotein and cell tissue with solutions of salts at low ionic strength or phenol, followed by separation of the nucleic acid from the nucleoprotein by means of anionic detergent (1, 2). This procedure gives nucleic acids of differing molecular weight and these have been fractionated by chromatography on either ion-exchange celluloses (3), or ion-exchange resins (4); or by counter-current distribution (5).

Chromatography on columns of cross-linked polysaccharide (Sephadex) (6) appears to be a very satisfactory method of isolating nucleic acids. The Sephadex column removes low molecular weight

electrolytes from solutions of the nucleic acid, enabling the nucleic acid to be separated into homogeneous fractions. Using Sephadex, bacterial RNA has been separated into ribosomal RNA and transfer-RNA (7); and aminoacyl transfer-RNA's have been separated from ATP and the amino acid.

Even using these mild isolation methods there is no rigid proof that the nucleic acid has not decomposed on isolation as normal chemical criteria of purity cannot easily be applied to *in vivo* systems.

Acid hydrolysis of nucleic acids liberates the purine bases since purine-sugar bonds are much more labile in acid than are pyrimidine-sugar bonds. Hydrolysis can be effected with alcoholic or aqueous acid, and Levene has shown that the purine-sugar bond is split at pH 2 before an appreciable amount of inorganic phosphate is produced (8, 9). Pyrimidines are liberated only after vigorous treatment with acid and some decomposition of the bases may take place at the same time.

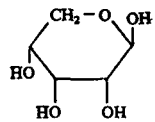
Since the purine-sugar bond is comparatively stable in the presence of strong alkali, RNA can be hydrolysed under these conditions to orthophosphate, and purine and pyrimidine nucleosides (10); nucleotides can be isolated if milder conditions are employed (11). Chemical methods of hydrolysis are unsatisfactory for DNA, as the purine nucleotides are removed in acid to leave 'apurinic' acid. Further degradation of 'apurinic' acid with acid does, however, liberate pyrimidine deoxynucleotides. Under the conditions required for alkaline hydrolysis, complete deamination of the purines and cytosine occurs (12). Enzymic hydrolysis has been used for the preparation of deoxyribonucleotides, and digestion of DNA from calf pancreas with deoxyribonuclease gives a good yield of the nucleoside-5′ phosphates (13, 14). Hydrolysis of RNA with ribonuclease gives ribonucleoside-2′,3′ cyclic phosphates and nucleotides which terminate in pyrimidine ribonucleoside-3′ or -2,3′ cyclic phosphates (15, 16).

When ribonucleoside-5′ phosphates are obtained from RNA by the action of snake venom phosphodiesterase, pyrimidine nucleoside 2′,5′- and 3′,5′-diphosphates and purine nucleosides are also produced (17). The enzymic hydrolysis of polynucleotides is described in more detail in Chapter 5.

The bases, nucleosides, and nucleotides produced by the hydrolysis of nucleic acids may most conveniently be identified, separated, and isolated by column chromatography. Both ion-exchange resins (18) and ion-exchange celluloses (19) have been used.

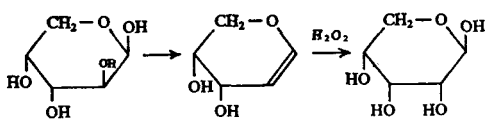
2.3 D-Ribose

Hydrolysis of RNA with acid gave D-ribose (I) (20, 21), which was identified from differences between physical constants of the sugar and other known pentoses, as D-ribose was unknown at the time of isolation.



(I)

D-Ribose was first synthesized by the epimerization of D-arabonic acid to D-ribonic acid followed by the reduction of the lactone of D-ribonic acid to give D-ribose. The original synthesis from D-arabinose has been improved and, for example, D-arabinose (II) can be converted into D-ribose by the action of hydrogen peroxide on the intermediate D-arabinal (III) (24).



(II) (III) (I)

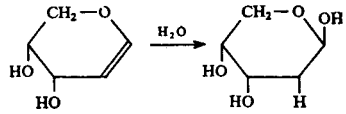
Ribonucleic acids from many different sources have been subjected to hydrolysis; but, so far, only D-ribose has been isolated as the sugar component. It is, therefore, generally assumed that this is the only sugar present in RNA.

2.4 Deoxy-D-ribose

The carbohydrate component of thymonucleic acid was first isolated by Levene and London (24) who degraded thymus DNA enzymically

to the purine nucleosides and prepared the deoxy sugar, 2-deoxy-D-ribose (IV), from these purine nucleosides (25). Levene and London were unable to isolate a sugar from the pyrimidine nucleosides, as the drastic conditions needed to cleave the pyrimidine-sugar bond caused the sugar to decompose to levulinic acid. The isolation of deoxynucleosides has been improved by the introduction of ion-exchange chromatography and 2-deoxy-D-ribose has been prepared by the acidic hydrolysis of purine nucleosides (26). Several syntheses of the sugar have been reported (27–30), for example, D-arabinal (III) has been converted into 2-deoxy-D-ribose by the addition of water to the carbon-carbon double bond with dilute sulphuric acid at low temperature (28).

2-Deoxy-D-ribose (31) like D-ribose (32) is in the pyranose form in the solid state.



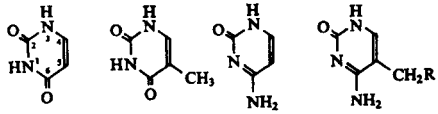
(III) (IV)

2-Deoxy sugars are much more reactive than their oxygenated counterparts, and are readily converted to O- and N-glycosides. Deoxy sugars are sensitive to acid and, for example, treatment with acid will degrade 2-deoxy-D-ribose to levulinic acid.

From paper chromatographic examination of hydrolysates of DNA, obtained from many different sources, Chargaff (33) has concluded that 2-deoxy-D-ribose is the only sugar present in DNA.

2.5 Pyrimidine bases

The three main pyrimidine bases which occur in nucleic acids are uracil (V), thymine (VI), and cytosine (VII). Several pyrimidine bases

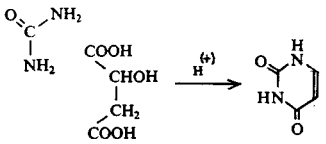


(V) (VI) (VII) (VIII)

are minor components of DNA and RNA. For example, 5-methyl cytosine (VIII, R = H) is a minor component of both DNA and RNA (34, 35) and 5-hydroxymethyl cytosine (VIII, R = OH) replaces cytosine completely in the DNA of 'T even' phages (36, 37).

The isolation, proof of structure, and synthesis of uracil, thymine and cytosine are reviewed by Levene and Bass (38) and will not be considered here.

Synthesis. In general, the pyrimidine ring system may be synthesized from a derivative of urea and an activated three-carbon fragment. For example urea and malic acid in the presence of fuming sulphuric acid condense to give uracil (39).



Thymine may be prepared in an analogous manner from urea and 3-methyl malic acid.

Properties. The pyrimidine ring shows some similarities to pyridine in its chemical behaviour (40, 41). Thus the 2-, 4-, and 6-positions are electron deficient, and the 5-position exhibits 'normal' aromatic reactivity. However, the only property which will be considered here is the keto-enol tautomerism of the hydroxy and amino groups. This has an important influence on the hydrogen bonding between bases which is required by the Watson and Crick double helical structure of DNA (42).

From a study of ultraviolet spectra of 2- and 6- substituted pyrimidines, Marshall and Walker concluded that the hydroxy group exists in the keto form, but could not reach a conclusion in the case of amino substituents (43). Brown, Hoerger, and Mason, from a study of basic strengths, and ultraviolet and infrared spectra, concluded that 2- and 6-amino pyrimidines exist largely in the amino, as opposed to the imino, form (44). Kenner, Reese, and Todd have also shown that