Nucleic Acids.

A LECTURE DELIVERED BEFORE THE CHEMICAL SOCIETY ON MAY 5TH, 1938.*

By J. MASSON GULLAND.

THE nucleus of a cell consists invariably and largely of nucleoproteins, compounds of nucleic acids and proteins. Miescher and Schmiedeberg, for example, found that the heads of salmon spermatozoa consisted of 60·5% of nucleic acid and 30·6% of a protamine, but the proportion of nucleic acid is in general lower. In this connection, it may be noted that the chromosomes contain nucleic acids (Caspersson, *Naturwiss.*, 1935, **23**, 527; 1936, **24**, 108; Caspersson, Hammarsten, and Hammarsten, *Trans. Faraday Soc.*, 1935, **31**, 367; Signer, *ibid.*, 1936, **32**, 296).

Glandular tissues, such as thymus and pancreas, are particularly rich in nucleoprotein, and these, together with yeast, are at the present time the chief sources of nucleic acids, which are liberated from nucleoproteins by enzymic, or mild chemical hydrolyses. Steudel and Takahata (*Z. physiol. Chem.*, 1924, **133**, 165; see also Clarke and Schryver, *Biochem. J.*, 1917, **11**, 319) state, however, that in yeast the nucleic acid is present in a free state and not in combination with protein. The linkages between nucleic acids and proteins in nucleoproteins are probably partly electrovalent and partly covalent, but further discussion is not warranted here.

The nucleic acids are colourless, amorphous powders containing carbon, hydrogen, oxygen, nitrogen, and phosphorus, and this amorphous character greatly increases the difficulties of investigation because of uncertainty as to the homogeneity of the material.

Until recently it was believed that without exception the nuclei of plant cells all contain one nucleic acid, whereas another nucleic acid, similarly constituted but differing in details, characterises the nuclei of animal cells. This generalisation, however, is no longer accurate. Feulgen and Rossenbeck (*Z. physiol. Chem.*, 1924, **135**, 203), Feulgen, Behrens, and Mahdihassan (*ibid.*, 1937, **246**, 203), Belozerski *et al.* (*Biochimia*, 1936, **1**, 134, 255, 665), and Milovidov (*Planta*, 1936, **25**, 197) have found the so-called animal nucleic acid in plants, and Hammarsten, Hammarsten, and Olivecrona (*Acta med. scand.*, 1928, **68**, 215), Jorpes (*Biochem. J.*, 1934, **28**, 2102), and Steudel (*Z. physiol. Chem.*, 1936, **241**, 84) have found a nucleic acid of the plant type in pancreas. More recently, Behrens (*Z. physiol. Chem.*, 1938, **253**, 185) has shown that in the rye germ, deoxyribonucleic acid occurs in the cell nuclei and ribonucleic acid in the protoplasm, and Delaporte and Roukhelman (*Compt. rend.*, 1938, **206**, 1399) have isolated deoxyribonucleic acid from the nuclei of yeast cells, and ribonucleic acid from the metachromatic granules. It is therefore best to describe a nucleic acid by referring both to its origin and to its type. First, therefore, there are ribonucleic acids, of which the sugar component is *d*-ribose and of which "yeast nucleic acid" and *allo*nucleic acid of pancreas are the chief examples. Ribonucleic acids were formerly regarded as occurring solely in plants. Secondly, there are deoxyribonucleic acids, in which the sugar component is *d*-2-deoxyribose and of which "thymus nucleic acid" is the classical example. Such nucleic acids were formerly regarded as occurring solely in animal tissues.

Nucleotides of Yeast Nucleic Acid.

Cold alkaline hydrolysis (Levene, *J. Biol. Chem.*, 1923, **55**, 9; Jones and Perkins, *ibid.*, p. 567; 1924, **62**, 557; Calvery and Jones, *ibid.*, 1927, **73**, 73; Buell and Perkins, *ibid.*, 1927, **72**, 21; Calvery and Remsen, *ibid.*, 1927, **73**, 593) or enzymic hydrolysis (Jones and Perkins, *loc. cit.*, p. 557; Makino, *Z. physiol. Chem.*, 1934, **225**, 154) transforms yeast nucleic acid into four nucleotides, namely, guanylic (I), yeast adenylic (II), cytidylic (III), and uridylic (IV) acids, of which the constitutions are now regarded as established for the reasons outlined below.

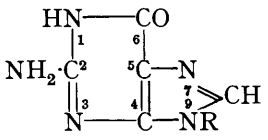
Complete chemical or enzymic hydrolysis of these nucleotides yields phosphoric acid, *d*-ribose or its degradation product furfural, the purine bases guanine and adenine, and the pyrimidine bases cytosine and uracil.

* This communication summarises and also extends the lecture. The bibliography is not complete.

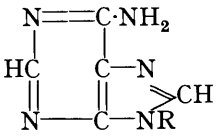
The pentose is united directly to the nitrogenous constituent, since the nucleotides undergo simple dephosphorylation to the corresponding nucleosides (base–sugar derivatives) guanosine, adenosine, cytidine, uridine, either chemically (Levene and Jacobs, *Ber.*, 1909, **42**, 2471, 2476; 1910, **43**, 3154; Levene and La Forge, *Ber.*, 1912, **45**, 608) or enzymically (Bielschovsky, *Z. physiol. Chem.*, 1930, **190**, 15; Gulland and Macrae, J., 1933, 662; Makino, *Z. physiol. Chem.* 1934, **225**, 147; Bredereck, Beuchelt, and Richter, *ibid.*, 1936, **244**, 102). The union must be glycosidic, since the nucleosides have no reducing properties until the sugar component is liberated by hydrolysis.

The ribose and phosphoric acid may be obtained combined as an ester and free from the nitrogenous constituent either by the spontaneous fission of certain nucleotides (see below) or by the use of a specific pancreatic enzyme, " nucleotide-*N*-ribosidase " (Ishikawa and Komita, *J. Biochem. Japan*, 1936, **23**, 351; Komita, *ibid.*, 1937, **25**, 405; 1938, **27**, 23).

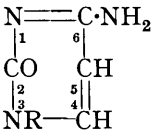
The outstanding points as regards the constitutions of the nucleotides prepared from yeast nucleic acid are (*a*) the position of the glycosidic linkage of the pentose to the purine or pyrimidine; (*b*) the existence of a furanose, not a pyranose, structure for the sugar; (*c*) the position of the phosphoric acid on the C_3 atom of the sugar. These questions are discussed below.



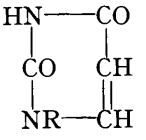
(I.)



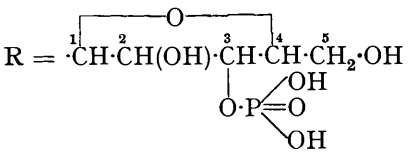
(II.)



(III.)



(IV.)



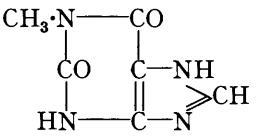
(i) *Position of the Glycosidic Linkage.—Pyrimidines.* The amino-group cannot be the point of attachment of the pentose, because nitrous acid transforms cytidylic acid into uridylic acid (Bredereck, *Z. physiol. Chem.*, 1934, **224**, 79) and cytidine into uridine (Levene and Jacobs, *Ber.*, 1910, **43**, 3159); C_4 and C_5 are also excluded since such compounds, having a C–C linkage, would not be glycosides. The correctness of the remaining alternative (III and IV) was confirmed (Levene and Tipson, *J. Biol. Chem.*, 1934, **104**, 385) by conversion of uridine into 1-methyluridine and hydrolysis of this to 1-methyluracil.

Purines. Deamination by nitrous acid transforms guanosine and adenosine into xanthosine and inosine respectively, both of which retain the pentose radical, and hence the amino-groups cannot be the point of attachment. A direct C–C linkage is excluded for the reason given above, and the remaining alternatives are N_1, N_3, N_7, N_9 of guanine and N_7, N_9 of adenosine.

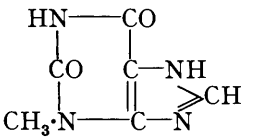
The choice between these positions was made by comparison of the ultra-violet absorption spectra of the glycoside in various solvents with the spectra of methyl derivatives of the corresponding purine, where the methyl group occupies successively the possible positions for the sugar. The assumption is made that the methyl group and the sugar radical occupy the same position in the molecule when the spectra are closely similar and mutually unlike those of other methylated derivatives, it being recognised (Goos, Schlubach, and Schröter, *Z. physiol. Chem.*, 1930, **186**, 148) that the effect of the carbohydrate group on such spectra is negligible. Thus, the spectra of guanosine closely resemble those of 9-methylguanine but are quite unlike those of 7-methylguanine (Gulland and Story, this vol., p. 692), and the spectra of xanthosine, when compared with those of the four monomethyl xanthines (V, VI, VII, VIII) closely resemble the spectra of 9-methylxanthine (VIII) but not those of the three other compounds (Gulland, Holiday, and Macrae, J., 1934, 1639).

Similarly, the spectra of adenosine and inosine are practically identical with those of

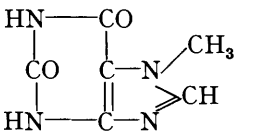
9-methyladenine and 9-methylhypoxanthine, respectively, and different from those of the corresponding 7-methyl derivatives (Gulland and Holiday, J., 1936, 765). Other



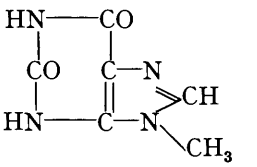
(V.)



(VI.)



(VII.)



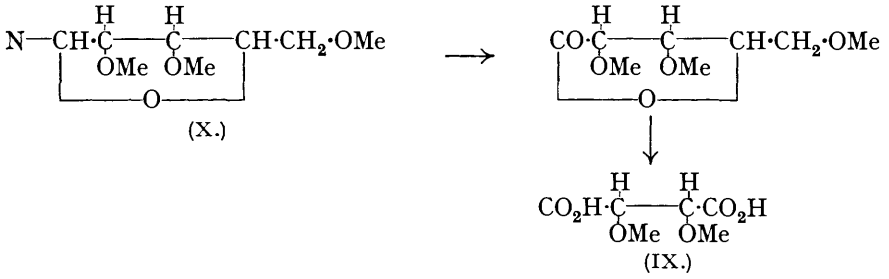
(VIII.)

adenine derivatives are also 9-substituted—muscle adenylic acid (XXVI; Gulland and Holiday, *loc. cit.*) and adenine thiomethyl pentoside (Falconer and Gulland, J., 1937, 1912).

It is clear that 9-substitution is apparently the rule in the purine ribonucleosides and, as will be shown below, it has also been found to occur in the deoxyribose series.

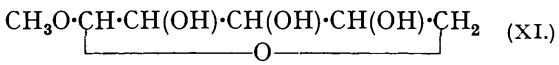
(ii) *Ring Structure of Pentose.*—The demonstration of the furanose structure of the sugar follows two methods: (*a*) methylation and oxidation, and (*b*) interaction with triphenylmethyl chloride.

Methylation and oxidation. Levene and Tipson methylated guanosine (*J. Biol. Chem.*, 1932, **97**, 491), adenosine (*ibid.*, 1932, **94**, 809), and uridine (*ibid.*, 1933, **101**, 529) with methyl sulphate and alkali, split off the methylated sugar by hydrolysis, and oxidised it in each case to *i*-dimethoxysuccinic acid (IX) under conditions in which the isomeric 2 : 3 : 4-trimethylribose gave only trimethoxyglutaric acid. The methylated sugar of the nucleosides (X) was therefore 2 : 3 : 5-trimethylribose with a furanose ring. Thus all four nucleotides are furanosides, since each nucleotide may be transformed into the corresponding nucleoside and since uridylic acid may be prepared by deamination of cytidylic acid.

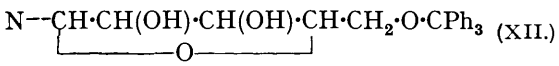


(X.) (IX.)

Interaction with trityl chloride. Bredereck (*Ber.*, 1932, **65**, 1830; 1933, **66**, 198; *Z. physiol. Chem.*, 1934, **223**, 61) showed polarimetrically that a mixture of α- and β-methylribopyranosides (no $-CH_2OH$) (XI) failed to react with trityl chloride, whereas he could prepare trityl derivatives of adenosine, uridine, and cytidine. He concluded that interaction with trityl chloride may be regarded as evidence of the presence of a primary alcoholic group in the sugar, and hence in the case of pentosides, of a furanose ring (XII). Evidence which might be contrary to this conclusion was provided by Hockett and Hudson (*J. Amer. Chem. Soc.*, 1931, **53**, 4456), who showed that in various methylglycosides trityl chloride may react with secondary alcoholic groups. Bredereck's observations with the methylribopyranosides, however, seem to dispose of such possible objections.



(XI.)

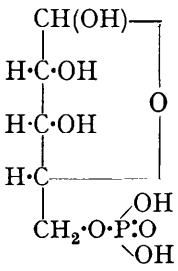


(XII.)

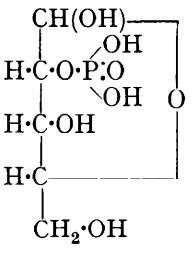
(iii) *Position of Phosphoric Acid Group.—Purine nucleotides.* Xanthylic acid (Levene and Dmochowski, *J. Biol. Chem.*, 1931, **93**, 563; Levene and Harris, *ibid.*, 1932, **95**, 755) and inosine phosphoric acid (*idem, ibid.*, 1933, **101**, 419), prepared by deamination of

guanylic and adenylic acids respectively, undergo spontaneous hydrolysis at the p_H of their aqueous solutions, yielding xanthine and hypoxanthine, respectively, and a ribose phosphoric acid, which is the same in both cases. The two samples have the same rotation, which is similarly affected by the presence of boric acid, and the same hydrolysis curve for the fission of the phosphoric acid group by acid (*idem, ibid.*).

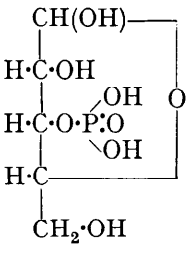
This ribose phosphoric acid could theoretically have any of the structures (XIII), (XIV), and (XV); (XIII) is excluded, however, because an acid of that constitution was isolated from muscle adenylic acid (XXVI) (inosinic acid) by Levene and Jacobs (*Ber.*, 1908, **41**, 2703; 1911, **44**, 746), was synthesised by Levene and Stiller (*J. Biol. Chem.*, 1934, **104**, 299), and was shown to be dephosphorylated much more slowly than the ribose phosphoric acid under consideration. This difference between the properties of 3- and 5-phosphoribose is further illustrated by the ease with which adenosine-3-phosphate (II) is broken down by acid hydrolysis into adenine, furfural, and phosphoric acid, and the relative stability of adenosine-5-phosphate (XXVI) under the same conditions (Emden and Schmidt, *Z. physiol. Chem.*, 1929, **181**, 130; 1931, **197**, 191; Steudel, *ibid.*, 1933, **216**, 77; Kobayashi, *J. Biochem. Japan*, 1932, **15**, 261).



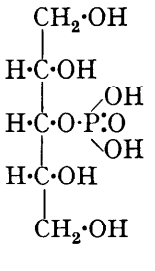
(XIII.)



(XIV.)



(XV.)



(XVI.)

Levene and Harris (*J. Biol. Chem.*, 1932, **98**, 9) decided most ingeniously in favour of (XV) and not (XIV) by reducing the aldehyde group catalytically to a primary alcoholic group. The resulting ribitol phosphoric acid was optically inactive, a condition which can only occur if the phosphoric acid group is situated symmetrically as in (XVI). In confirmation of the soundness of this line of argument, Levene, Harris, and Stiller (*ibid.*, 1934, **105**, 153) reduced (XIII) to ribitol-5-phosphoric acid and observed that this acid was optically active.

Pyrimidine nucleotides. A discussion has already been given of the argument that a nucleotide contains a primary alcoholic group if it reacts with trityl chloride. Using this line of reasoning, Bredereck (*Z. physiol. Chem.*, 1934, **224**, 79) condensed uridylic acid with trityl chloride and concluded that the phosphoric acid group cannot be attached to the terminal C_5 of the pentose but must esterify the hydroxyl at C_2 or C_3. The latter position was preferred by analogy with the purine nucleotides, but there is no clear-cut evidence on which to base a decision.

(iv) *Purine and Pyrimidine Nucleotides present in Equal Numbers.*—Jones ("Nucleic Acids," 1920, p. 40, Longmans, Green and Co., London) recounts that in acid hydrolyses of yeast nucleic acid 50% of the total phosphorus was rapidly liberated as inorganic phosphoric acid, whereas the remainder was only slowly hydrolysed. He was also able to construct a similar hydrolysis curve for an artificial mixture of a purine nucleotide and a pyrimidine nucleotide. In view of the fact that purine nucleotides are rapidly and completely dephosphorylated by acid hydrolysis whereas pyrimidine nucleotides are hydrolysed very slowly, Jones concluded that in yeast nucleic acid the number of purine nucleotides is equal to the number of pyrimidine nucleotides.

The same conclusion was reached by Hoffman (*J. Biol. Chem.*, 1927, **73**, 15) from estimations of the amount of furfural formed in acid hydrolysis of yeast nucleic acid; purine nucleotides give the theoretical quantities of furfural whilst the pyrimidine nucleotides yield none (Steudel, *Z. physiol. Chem.*, 1936, **242**, 100).

Both the preceding methods were used in combination by Kobayashi (*J. Biochem.*

Japan, 1932, **15**, 261) to confirm the conclusions. From estimations of furfural and phosphate, he observed a marked retardation in the rate of hydrolysis by acid or alkali when 50% of the phosphorus or ribose had been liberated.

Ellinghaus (*Z. physiol. Chem.*, 1927, **164**, 308) supported these views from a comparison of the combined heats of combustion of the components of the four nucleotides on the one hand and of yeast nucleic acid itself on the other.

The Nucleotides of Thymus Nucleic Acid.

Thymus nucleic (thymonucleic) acid, prepared from thymus gland, is characteristic of the nucleic acids of many animal tissues (for examples, see Levene and Bass, " Nucleic Acids," 1931, p. 294, Chemical Catalog Co., New York). Like yeast nucleic acid, it is composed of four nucleotides, but, although readily broken down by acids, it is more resistant to alkaline fission, a fact which indicates some difference in the nature of the unions of the nucleotides of the two nucleic acids.

The investigations of Steudel (*Z. physiol. Chem.*, 1906, **49**, 406) and of Levene and Mandel (*Biochem. Z.*, 1908, **10**, 215) showed that two purine bases, guanine and adenine, and two pyrimidine bases, cytosine and thymine, are present in equimolecular proportions (for historical discussion see Levene and Bass, *op. cit.*, p. 252).

After acid hydrolyses, Levene and Mandel (*Ber.*, 1908, **41**, 1905) and Thannhauser and Ottenstein (*Z. physiol. Chem.*, 1921, **114**, 39) succeeded in isolating the two pyrimidine nucleotides in what was probably an impure state, the two more labile purine nucleotides having been destroyed in the process. Other investigators (see later) have also prepared both diphosphopyrimidine nucleosides. Hydrolysis by chemical means, however, was too drastic, and Levene and London (*J. Biol. Chem.*, 1929, **81**, 711; **83**, 793) first prepared the four nucleosides, of guanine, hypoxanthine, thymine, and cytosine respectively, by the action of the enzymes of intestinal juice. The occurrence of the hypoxanthine nucleoside, instead of the corresponding adenine derivative, was the result of enzymic deamination.

For many years the sugar component of thymonucleic acid was thought to be a hexose, but ultimately it was shown to be *d*-2-deoxyribose (*idem, loc. cit.*, p. 711; Levene and Mori, *J. Biol. Chem.*, 1929, **83**, 803; Levene, Mikeska, and Mori, *ibid.*, 1930, **85**, 785).

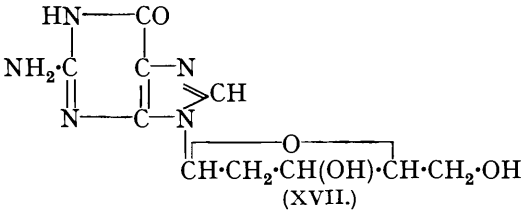
Finally, Klein (*Z. physiol. Chem.*, 1933, **218**, 164) observed that the action of the nucleotidase of intestinal mucosa was inhibited by arsenate, thus allowing the fission of thymonucleic acid by intestinal thymonucleinase to take place without dephosphorylation of the resulting nucleotides. He also found (*ibid.*, 1934, **224**, 244) that silver ions selectively inhibit the deaminase of the intestine, thus permitting the isolation of the adenine, instead of the hypoxanthine derivative. As a result, Klein and Thannhauser (*ibid.*, 1933, **218**, 173; 1934, **224**, 252; 1935, **231**, 96) were able to isolate pure deoxyribo-guanylic, deoxyribo-adenylic, deoxyribo-cytidylic, and thymosine-phosphoric acids, and to dephosphorylate these to the corresponding nucleosides, the deoxyribosides of guanine (XVII), adenine (XVIII), hypoxanthine, cytosine (XIX), and thymine (XX).

Using the ultra-violet absorption spectra method, Gulland and Story showed that adenine deoxyriboside (this vol., p. 259) and guanine deoxyriboside (*ibid.*, p. 692) are both 9-, not 7-, substituted purine derivatives, and the arguments used in the allocation of the position of the ribose radical in the pyrimidine nucleotides of yeast nucleic acid also hold good in determining the position (N_3) of the sugar in the pyrimidine derivatives of thymonucleic acid.

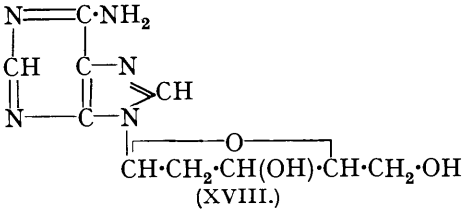
Levene and Tipson (*Science*, 1935, **81**, 98; *J. Biol. Chem.*, 1935, **109**, **623**) concluded that the deoxyribose nucleosides have a furanose, not a pyranose, structure after demonstrating that thymine deoxyriboside reacts with triphenylmethyl chloride to form the trityl derivative of the terminal $-CH_2OH$ group. This conclusion was confirmed by the failure of boric acid to alter the specific rotation, a fact which implies the absence of two hydroxyls on adjacent carbon atoms (*idem, Z. physiol. Chem.*, 1935, **234**, V; Makino, *Biochem. Z.*, 1935, **282**, 263).

The deoxyribose nucleosides are therefore formulated as in (XVII)—(XX), but the positions of the phosphoric acid groups of the corresponding nucleotides have not yet been determined.

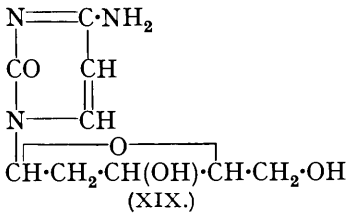
Hammarsten (*Biochem. Z.*, 1924, **144**, 383) has studied the conductivity, hydrogen-ion concentration, osmotic pressure, and viscosity of thymonucleic acid, and has concluded



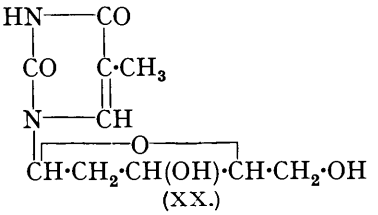
(XVII.)



(XVIII.)



(XIX.)



(XX.)

that each of the four phosphorus atoms has one dissociable hydrogen atom, from which it might be inferred that it has a cyclic structure (compare yeast nucleic acid, below). In view of the molecular-weight determinations on thymus nucleic acid (section below), it is inopportune to discuss further the question of the union of the nucleotides in the thymonucleic acid molecule, except to point out that, as each deoxyribose radical has only two available hydroxyls at C_3 and C_5, each must be esterified by phosphoric acid in any diester structure.

Nucleic Acids of Pancreas.

Pancreas contains a deoxyribonucleic acid and a ribonucleic acid. The former was shown by Steudel (*Z. physiol. Chem.*, 1935, **231**, 273) to be probably thymonucleic acid, since on hydrolysis by acid it yielded phosphoric acid, guanine, adenine, cytosine, thymine, and lævulic acid (from deoxyribose).

The ribonucleic acid, known as *allo*nucleic acid, is the chief nucleic acid of the nucleoprotein of the pancreas, and according to Hammarsten, Hammarsten, and Olivecrona (*Acta med. scand.*, 1928, **68**, 215) it occurs principally in the secretory epithelium of the gland. Its constitution has been investigated by Jorpes (*Biochem. J.*, 1934, **28**, 2102) and by Steudel (*Z. physiol. Chem.*, 1936, **241**, 84), but it is still unsettled. Guanylic and adenylic acids are present in the ratio 4 : 1 (Steudel) or 2 : 1 (Jorpes), and cytidylic and possibly uridylic acids also occur.

Molecular Weights of Nucleic Acids.

Yeast-Nucleic and allo*Nucleic Acids.*—Determinations of the molecular weight of yeast nucleic acid by a diffusion method show that the molecule is composed of four nucleotides only, since Myrbäck and Jorpes (*Z. physiol. Chem.*, 1935, **237**, 159) found that the molecular weight lies between 1300 and 1700, and nearer to 1300. The sum of the molecular weights of the four nucleotides, guanylic, adenylic, cytidylic, and uridylic acids, is 1357.

By the same method Myrbäck and Jorpes found the molecular weight of *allo*nucleic acid to be 3000, showing that it is not merely a mixture of yeast nucleic and guanylic acids.

Thymonucleic Acid.—From a study of the viscosity and double refraction of flow of a specially prepared sample of thymonucleic acid in water, Signer, Caspersson, and Hammarsten (*Nature*, 1938, **141**, 122) concluded that in solution the molecules of thymonucleic acid have the form of thin rods, the length of which is approximately 300 times their width, and that the molecular weight lies between 500,000 and 1,000,000. Astbury and Bell (*ibid.*, p. 747) found that films of the sodium salt of a similar sample of thymonucleic acid, when stretched 250% gave an *X*-ray fibre photograph in which the most prominent reflection corresponded to a spacing along the axis of 3·3 A., which is almost

identical with that of a fully extended polypeptide chain. They concluded that there is a close succession of about 2000 flat, or flattish nucleotides standing out perpendicularly to the long axis of the molecule to form a relatively rigid structure, and they confirmed the molecular weight of 500,000—1,000,000.

It is not yet clear whether these conclusions would also apply to less meticulously prepared specimens and whether ordinary samples of thymonucleic acid, on which investigations have hitherto been made, may not have undergone hydrolytic fission, or depolymerisation, in the course of their preparation.

Mode of Union of the Nucleotides of Yeast Nucleic Acid.

The determination of the mode of union of the nucleotides in the molecule of yeast nucleic acid provides problems which are less straightforward than those described above. The chief contributing factor to these difficulties is the fact that the first degradation products which have been isolated after chemical treatment are the nucleotides themselves. With one exception, no compounds have been isolated which are composed of two nucleotides or portions thereof. As will be seen below, this marked sensitivity to chemical reagents has directed attention to the use of specific enzymes. In this connection it should be noted that enzyme experiments always suffer from the disadvantage that the phenomena observed may not be caused by the enzyme under consideration but may result from the action of some unknown or unlooked-for enzyme in the preparation.

It is generally accepted that the four nucleotides of yeast nucleic acid are united by elimination of the elements of water, and since alkaline fission into nucleotides results in a neutralisation of alkali, it is evident that some or all of the phosphoric acid groups are involved in these unions. Chiefly on the basis of this conclusion a considerable number of formulæ for nucleic acids have been postulated (Steudel, *Z. physiol. Chem.*, 1912, **77**, 497; Levene, *Biochem. Z.*, 1909, **17**, 120; Levene and Jacobs, *J. Biol. Chem.*, 1912, **12**, 411; Jones and Perkins, *ibid.*, 1919, **40**, 415; 1923, **55**, 557; Thannhauser and Dorfmüller, *Z. physiol. Chem.*, 1917, **100**, 121; Jones *et al.*, *J. Biol. Chem.*, 1914, **17**, 71; 1916, **25**, 93; 1917, **29**, 111, 123; **31**, 39). All these alternatives, however, have either subsequently been abandoned by their promoters or may be discountenanced by recognition of the experimental facts that yeast nucleic acid when titrated either electrometrically (Levene and Simms, *ibid.*, 1926, **70**, 327; Baker, Gulland, and Prideaux, unpublished observation) or by using phenolphthalein as an indicator (hence titrating both primary and secondary phosphoric acid dissociations; Makino, *Z. physiol. Chem.*, 1935, **232**, 229) exhibits four primary phosphoric acid dissociations. Levene and Simms believed that there is also one secondary phosphoric acid dissociation, but this view is not shared by the other authors.

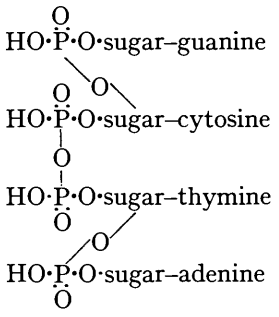
In the light of these facts, three formulæ remain to be considered, namely, those of Feulgen (*Z. physiol. Chem.*, 1918, **101**, 288) (XXI), of Levene and Simms (*J. Biol. Chem.*, 1926, **70**, 327) (XXII), and of Takahashi (*J. Biochem. Japan*, 1932, **16**, 463) (XXIII).

It should be noted that in none of these formulæ are the purine or pyrimidine radicals involved in the unions of the nucleotides, and that the relative positions of the nucleotides were selected arbitrarily.

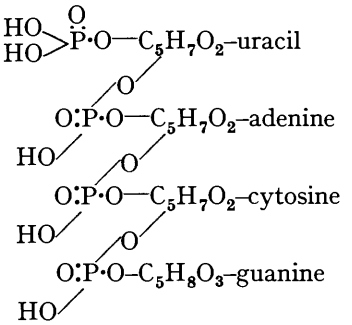
In Feulgen's formula (XXI) two phosphoryl groups are combined in a pyrophosphate radical and two are doubly esterified by sugar hydroxyls. The formula of Levene and Simms (XXII), containing three doubly esterified phosphoryl groups and one in the form of a mono-ester, was based on a comparison of electrometric titration curves of yeast nucleic acid with a theoretical curve constructed from the titration constants of the constituent nucleotides and their components. The resemblance between experimental and theoretical curves was not as close as could be desired, and, as mentioned above, later investigators do not agree with the presence of a secondary phosphoric acid dissociation. It is, however, within the bounds of possibility that the material handled by Levene and Simms might have exhibited one secondary dissociation as a result of hydrolytic fission of the molecule during the preparation of the samples.

Takahashi's formula (XXIII), containing four doubly esterified phosphoryl groups and

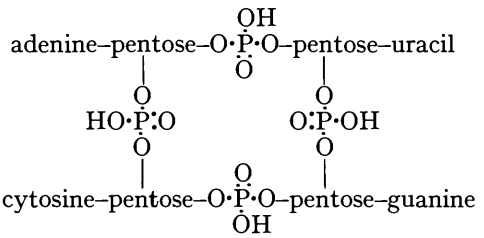
no mono-ester, was based on the results of experiments with the following phosphatases. (i) Phosphomonoesterase, prepared from kidney (Kurata, *J. Biochem. Japan*, 1931, **14**, 25; Takahashi, *ibid.*, 1932, **16**, 447) and freed from phosphodiesterase by absorptive methods, liberates inorganic phosphate from phenyl phosphate and many other mono-esters of phosphoric acid; it is highly non-specific (Hotta, *ibid.*, 1934, **20**, 343) towards mono-esters but does not attack phosphodiesters. (ii) Diphenyl pyrophosphatase, or pyrophosphatase in the presence of an activator, splits diphenyl pyrophosphate into two molecules of phenyl phosphate but does not liberate inorganic phosphate from these (Kurata, *loc. cit.*; Takahashi, *loc. cit.*). (iii) Phosphodiesterase, present without phosphomonoesterase in the venom of the snake *Trimeresurus flavoviridis*, liberates phenol but not phosphoric acid, from diphenyl phosphate by acting on only one of the two ester linkages (Uzawa, *J. Biochem. Japan*, 1932, **15**, 19). It has no action on phosphomonoesters.



(XXI.)



(XXII.)



(XXIII.)

According to Takahashi (*ibid.*, 1932, **16**, 463), phosphoric acid was not set free from yeast nucleic acid by phosphomonoesterase or by pyrophosphatase acting either separately or jointly in the presence of the diester-pyrophosphatase activator. These results, therefore, apparently disposed of the formulæ of Feulgen and of Levene and Simms, since the non-specific phosphomonoesterase would liberate phosphoric acid from the singly-bound phosphate group of the latter, and the combined action of this enzyme and activated diester-pyrophosphatase would set free two phosphoric acid groups from the former. Takahashi also observed that all the phosphoric acid of yeast nucleic acid was liberated in the inorganic state after the prolonged joint action of phosphomonoesterase and phosphodiesterase, and he therefore concluded that all the phosphate groups are doubly esterified and that there are no mono-ester groups (XXIII).

Makino (*Z. physiol. Chem.*, 1935, **232**, 299; **236**, 201) supported Takahashi's conclusion by estimating only four acidic groups in yeast nucleic acid when titrated to phenolphthalein and by determining that four additional acidic groups are liberated by cold alkaline fission. In further support of a cyclic structure, electrometric titrations of yeast nucleic acid by Baker, Gulland, and Prideaux show the presence of four primary, but no secondary phosphoric acid groups.

Klein and Rossi (*ibid.*, 1935, **231**, 104), on the other hand, attempted to repeat Takahashi's experiments, but failed to effect the necessary separation of phosphomono-

esterase and phosphodiesterase of kidney, liver or intestine, and thus obtain the necessary diesterase-free monoesterase. They also found that a specimen of *Trimeresurus flavoviridis* venom obtained from Japan contained both phosphomonoesterase and phosphodiesterase, whereas both Uzawa and Takahashi had used this venom as a source of monoesterase-free diesterase. Klein and Rossi therefore concluded with somewhat little justification that Takahashi's cyclic formula had no experimental basis. In this connection it may be remarked that the enzyme content of a snake venom may well vary with its age and the conditions under which it has been dried and stored.

In the enzymic investigation of the mode of union of the nucleotides, the author decided to use bone phosphatases, rather than those of kidney, liver, bran, or taka-diastase as employed by others, partly because their action towards yeast nucleic acid had not previously been studied in detail and partly because their use would allow an approach to the conflicting results of Takahashi and of Klein and Rossi by a different path.

Gulland and Jackson (*Biochem. J.*, 1938, **32**, 590) observed that, though sheep and guinea-pig bone phosphatase contained both phosphomonoesterase and phosphodiesterase when prepared according to Martland and Robison (*Biochem. J.*, 1929, **23**, 237), yet purification by charcoal absorption removed the diesterase completely and left the non-specific monoesterase, which readily dephosphorylated the nucleotides of yeast nucleic acid and had no action on phospho-diesters. Attempts to recover the diesterase were unsuccessful, but six out of twelve different snake venoms were found to contain diesterase but no non-specific monoesterase; these therefore hydrolysed phosphodiesters without the liberation of phosphoric acid and had no action on the nucleotides prepared from yeast nucleic acid.

Neither the purified bone phosphomonoesterase nor copperhead venom, which contained diesterase but not non-specific monoesterase, liberated phosphoric acid from yeast nucleic acid. Dephosphorylation was readily effected, however, by various mixtures of monoesterases and diesterases (Gulland and Jackson, preceding paper). Taking into account these experiments, the results of Takahashi, and the titrations of Makino and of Baker, Gulland, and Prideaux, it may be regarded as firmly established that in yeast nucleic acid there are no singly-linked phosphoric acid groups, and that each is present as a diester or similar structure. The degree of hydrolysis accomplished by the mixtures of monoesterases and diesterases is discussed below.

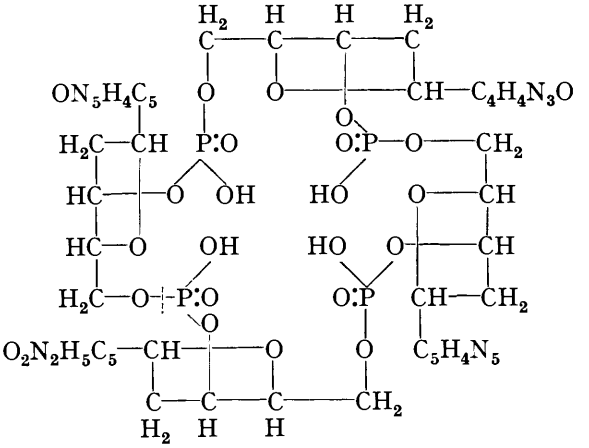
The allocation of positions to the doubly-linked phosphoryl radicals in yeast nucleic acid is still under discussion, and here it is necessary to introduce conclusions which have been drawn as regards the structure of thymonucleic acid.

Levene and Tipson (*J. Biol. Chem.*, 1935, **109**, 623) and also Makino (*Z. physiol. Chem.*, 1935, **236**, 201) suggest that in thymonucleic acid the positions occupied by the phosphoryl groups are C_3 and C_5 of adjacent deoxyribose radicals, whereas in yeast nucleic acid the positions are C_2 and C_3. The diagrammatic expressions of the views of these authors are virtually the same, since the cyclic structures of Makino for thymonucleic (XXIV) and yeast nucleic (XXV) acids may be transformed into the open-chain formulæ of Levene by hydrolytic fission at the dotted lines.

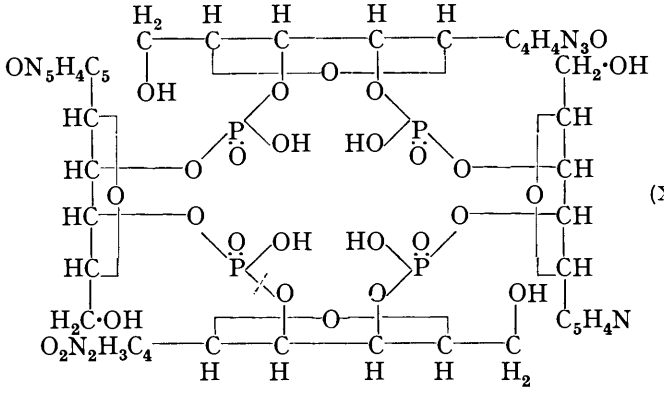
It is believed by these authors that these structures explain the undermentioned differences in the properties of the two nucleic acids. Yeast nucleic acid is labile towards dilute alkali, and when hydrolysed by dilute mineral acids yields the pyrimidine nucleotides, cytidylic and uridylic acids, having one phosphoryl radical only and that attached at C_3, whereas thymonucleic acid is relatively stable towards alkali and under similar conditions of acid hydrolysis yields pyrimidine nucleotides with two phosphoryl radicals, which must of necessity be at C_3 and C_5 (Levene and Jacobs, *J. Biol. Chem.*, 1912, **12**, 411; Levene, *ibid.*, 1921, **48**, 119; Thannhauser and Ottenstein, *Z. physiol. Chem.*, 1921, **114**, 39; Thannhauser and Blanco, *ibid.*, 1926, **161**, 116; Makino, *ibid.*, 1935, **236**, 201; Bredereck and Caro, *ibid.*, 1938, **253**, 170).

Levene and Tipson also point out that as the phosphoryl radical in 5-phosphoribose is more resistant to acid hydrolysis than that in 3-phosphoribose (Yamagawa, *J. Biol. Chem.*, 1920, **43**, 339; Emden and Schmidt, *Z. physiol. Chem.*, 1929, **181**, 130; 1931, **197**, 191; Levene and Jorpes, *J. Biol. Chem.*, 1929, **81**, 575; Steudel, *Z. physiol. Chem.*, 1933, **216**,

77), it may be assumed that the hydroxyl at C_5 is not substituted by the phosphoryl group in yeast nucleic acid, and that since substituents at C_2 are characterised by greater



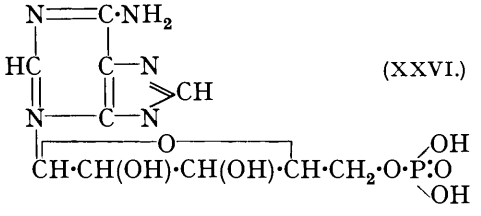
(XXIV.)



(XXV).

instability than in the other positions, it is the phosphoric ester linkage at C_2 which undergoes hydrolysis by alkali. Thus the resulting nucleotides remain phosphorylated in the more stable position at C_3.

These formulæ may require modification in the light of recent experiments. Gulland and Jackson (*Biochem. J.*, 1938, **32**, 597) have shown that the venoms of certain snakes, for example, Russell's viper and the water moccasin, contain a phosphodiesterase but no specific phosphomonoesterase. These venoms also contain 5-nucleotidase, a highly

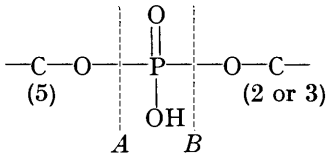


(XXVI.)

specific enzyme which dephosphorylates adenosine(inosine)-5-phosphate (XXVI), but has no action whatsoever on fifteen other phosphomonoesters, amongst which are esters with primary and secondary alcoholic groups and also the nucleotides prepared by alkaline fission of yeast nucleic acid (phosphoryl group at C_3).

These venoms liberate 35% only of the total phosphorus of yeast nucleic acid as inorganic phosphate. This suggests that at least two, or more, phosphoryl groups may be attached at C_5 of the ribose molecules, the unexpected (between 25 and 50%) degree of

hydrolysis being the result of simultaneous competitive diesterase action at *A* and *B* (XXVII) on two or more phosphoryl groups; hydrolysis at *A* would not be followed by liberation of phosphate (linked at C_2 or C_3) whereas that at *B* would allow dephosphorylation by 5-nucleotidase.



(XXVII.)

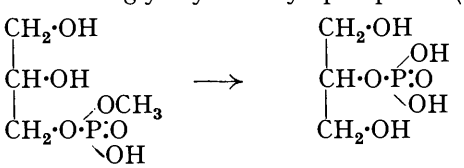
The incorporation of these facts in the construction of possible formulæ for yeast nucleic acid is not yet feasible, largely owing to present lack of knowledge of the behaviour of 5-nucleotidase towards the 5-phosphoric esters of guanosine, cytidine, and uridine (analogous to XXVI). It is hoped that this knowledge will soon be available, and in the meantime certain implications may be considered.

Although it is suggested that two or more nucleotides may be united in the yeast nucleic acid molecule by 5-phospho-linkages, no 5-phospho-nucleotide has ever been isolated after hydrolysis. Thus the four nucleotides of alkaline fission, and also the pyrimidine nucleotides from acid hydrolysis (Levene and Jacobs, *Ber.*, 1911, **44**, 1027), are all 3-phospho-derivatives. This is not the result of inferior stability of the 5-phospho-linkage, because this bond has long been known to be more stable to acid than the 3-phospho-linkage, as has already been mentioned, and because Gulland and Jackson (this vol., p. 1492) have shown that both adenosine-3- and -5-phosphate are remarkably stable to alkaline dephosphorylation.

It must be inferred, therefore, that a transference or migration of a phosphoryl radical may occur during hydrolysis of yeast nucleic acid, and the two alternative possibilities are discussed below. First, if the doubly-phosphorylated pentose radicals are substituted at C_2 and C_3, as suggested by Levene and by Makino, then enzymic transference to C_5 of one of these phosphate groups must precede dephosphorylation by 5-nucleotidase. Such a change would be analogous to the enzymic conversion of 3-phosphoglyceric acid into pyruvic acid by way of 2-phosphoglyceric acid (Lohmann and Meyerhof, *Biochem. Z.*, 1934, **273**, 60; Meyerhof and Kiessling, *ibid.*, 1935, **276**, 239; **280**, 99), but it would probably have to take place before the hydrolysis by the accompanying diesterase of snake venom, since enzymic transfer does not occur in the case of the 3-phosphonucleosides obtained from yeast nucleic acid.

In the second alternative, where the pentose is phosphorylated at C_5, migration of the phosphate group must take place, and this migration must be to C_3, not to C_2, for stereochemical reasons; it may be shown by means of models of the Haworth type that an unstrained cyclic di-ester could exist as an intermediate stage. This implies that in such doubly phosphorylated nucleosides the second phosphate group must be at C_2, and that simultaneous substitution at C_3 and C_5 is not possible. This migration must take place in alkaline solution, because the nucleotides obtained from yeast nucleic acid by alkaline hydrolysis are phosphorylated at C_3. It must also occur in acid hydrolysis, because 50% of the phosphorus of yeast nucleic acid is acid-labile, whereas both the pyrimidine nucleotides and 5-phosphorylated purine nucleosides (adenosine-5-phosphate) are relatively slowly dephosphorylated by hot acids, although the 3-phosphorylated purine nucleosides are readily hydrolysed. An excellent analogy for such migrations is to be found in the transformations of α-glyceryl methyl phosphate (XXVIII) to β-glycero-

(XXVIII.)



phosphate by means of alkali and of β-glyceryl methyl phosphate to α-glycerophosphate by means of alkali or of acid (Bailly and Gaumé, *Compt. rend.*, 1934, **198**, 1932, 2258; **199**,

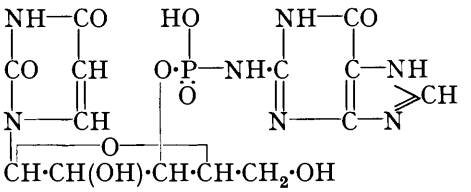
793; *Bull. Soc. chim.*, 1935, **2**, 354); these changes apparently occur only when the diesters are hydrolysed and not with α- or β-glycerophosphate.

A similar migration is recorded by Macfarlane and Robison (*Enzymologia*, 1937, **4**, 125), in which the phospho-group of 6-phosphofructo-furanose is transferred to another carbon atom under the influence of hydrochloric acid, and in the xylose series Levene and Raymond (*J. Biol. Chem.*, 1933, **102**, 347; 1934, **107**, 75) observed that phosphorylation of 5-acetyl-, 5-benzoyl-, or 5-carbobenzyloxy-mono*iso*propylidene xylose led ultimately to the production of xylose-5-phosphate instead of the expected xylose-3-phosphate, presumably as the result of a migration of the phosphoryl group at some stage of the reactions.

Continuing their study of the action of enzymes on yeast nucleic acid, Gulland and Jackson (*loc. cit.*) found that the following mixtures of monoesterases and diesterases effected the liberation of 75%, but not more, of the total phosphorus as inorganic phosphate, and that this constancy is a true figure and not merely the result of experimental conditions: (*a*) diesterase and non-specific monoesterase of crude bone phosphatase; (*b*) diesterase of copperhead venom and the purified non-specific monoesterase of bone; (*c*) the diesterase and 5-nucleotidase of Russell's viper or water moccasin venoms and the purified non-specific monoesterase of bone; the monoesterase and diesterase of the venoms of *Naja naja* or Wagler's pit viper; Russell's viper venom and kidney phosphatase (Albers and Albers, *Z. physiol. Chem.*, 1935, **232**, 165, 171). This cessation of dephosphorylation at 75% has not received attention by other investigators, although it has previously been recorded without special comment in enzymic hydrolyses of yeast nucleic acid (see, *e.g.*, Bielschowsky, *Z. physiol. Chem.*, 1933, **190**, 15; Schmidt, *Enzymologia*, 1936, **1**, 135). It suggests either that one phosphoryl group is linked differently from the other three, which need not be identical amongst themselves, or that all four are similarly constituted and that the specificity of the enzymes does not allow fission of one of them.

One obvious possibility for the structure of this enzyme-stable group is the mixed phospho-amide-ester of guanine uridylic acid (XXIX) which was isolated by Bredereck and Richter (*Ber.*, 1936, **69**, 1129) from the products of the aqueous hydrolysis of yeast nucleic acid. This question is under examination and will form the subject of a future publication. The isolation of this substance reveals fresh problems in the chemistry of nucleic acids, since other phosphoryl groups may be similarly constituted, as these authors point out.

(XXIX.)



Synthesis of Nucleic Acid Derivatives.

(i) *Nucleotides by Phosphorylation of Nucleosides.*—Fischer (*Ber.*, 1914, **47**, 3193) phosphorylated theophylline glucoside with phosphorus oxychloride, the phospho-group being assigned without proof to the terminal primary alcoholic group. Subsequently, Levene and Tipson blocked the hydroxyls at C_2 and C_3 of uridine and inosine with the *iso*propylidene group and prepared uridine-5-phosphate (*J. Biol. Chem.*, 1934, **106**, 113) and inosine-5-phosphate (*ibid.*, 1935, **111**, 313), identical with muscle inosinic acid from natural sources.

Jachimowicz (*Biochem. Z.*, 1937, **292**, 356) obtained adenosine-5-phosphate, identical with muscle adenylic acid (XXVI), by phosphorylation of adenosine without protection of the hydroxyl groups.*

Finally, by adding adenosine to living yeast, Ostern and Terszakoweć (*Z. physiol. Chem.*,

* Levene and Tipson (*J. Biol. Chem.*, 1937, **121**, 131), however, found that phosphorylation of 2:3-*iso*propylidene and -diacetyl adenosines may have produced *N*- and *O*-phospho-derivatives with acid-labile P—N linkages.

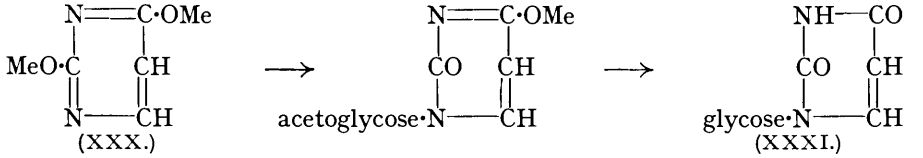
1937, **250**, 155) have effected its enzymic phosphorylation to adenosine-5-phosphate through the stage of adenosine triphosphate.

Up to the present, a 2- or 3-phospho-nucleoside has not been synthesised.

(ii) *Nucleosides.*—Fischer and Helferich (*Ber.*, 1914, **47**, 210) first synthesised nucleosides and obtained theophylline-*d*-glucoside and theobromine-*d*-glucoside from the interaction of the silver derivatives of the purines and acetobromoglucose. Since then, their method has been widely adopted both in the purine and in the pyrimidine series; theophylline rhamnoside (Fischer and Fodor, *ibid.*, p. 1058), *d*-galactoside and *l*-arabinoside (Helferich and Kühlewein, *Ber.*, 1920, **53**, 17), *d-iso*rhamnoside (Fischer, Helferich, and Ostmann, *Ber.*, 1920, **53**, 873), *d*-xyloside and *d*-riboside (Levene and Sobotka, *J. Biol. Chem.*, 1925, **65**, 463), *d*-arabinoside (Pryde and Williams, J., 1933, 640), *d*-glucodesoside (Levene and Cortese, *J. Biol. Chem.*, 1931, **92**, 53), 5-methyl-*l*-rhamnofuranoside (Levene and Compton, *ibid.*, 1936, **114**, 9), and *d*-allomethyloside (*idem*, *ibid.*, 1937, **117**, 37); 2-thiouracil-di-(tetra-acetylglucoside) and 2-ethylthiouracil-tetra-acetylglucoside (Fischer, *Ber.*, 1914, **47**, 1377); xylosides of 2-ethylthiouracil, 1-methyluracil, and 5-nitrouracil (Levene and Sobotka, *J. Biol. Chem.*, 1925, **65**, 469).

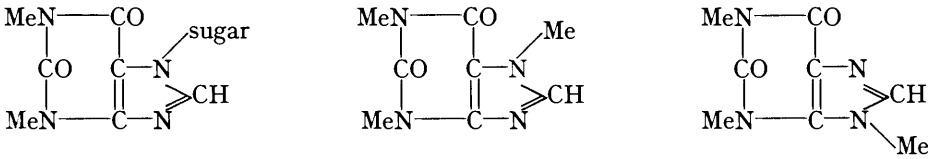
The pyrimidine nucleosides thus obtained, however, are not analogous in constitution with the natural pyrimidine nucleosides because the ready fission of their glycosidic linkage by hydrolysis, in contrast to its stability in the natural compounds, indicates that the sugar is not attached to either of the nitrogen atoms.

Hilbert and Johnson (*J. Amer. Chem. Soc.*, 1930, **52**, 4489), on the other hand, obtained 3-glucosido-uracil by the interaction of 2 : 6-dimethoxypyrimidine (XXX) with aceto-bromoglucose followed by deacetylation and demethylation with alcoholic hydrogen chloride, and Hilbert and Rist (*J. Biol. Chem.*, 1937, **117**, 371) and Hilbert (*J. Amer. Chem. Soc.*, 1937, **59**, 330) extended the method to the preparation of various glycosido-uracils (XXXI). These substances resemble uridine in their behaviour towards hydrolytic agents.



(XXX.) (XXXI.)

Theophylline glycosides prepared by the action of theophylline silver and acetobromo-sugars are also not analogous with the natural nucleosides, since Gulland, Holiday, and Macrae (J., 1934, 1639) showed that these synthetic compounds are 7-substituted derivatives, by comparing the ultra-violet absorption spectra of theophylline glucoside and arabinoside (XXXII) with those of caffeine (XXXIII) and *iso*caffeine (XXXIV); the natural purine nucleosides are all 9-substituted derivatives.



(XXXII.) (XXXIII.) (XXXIV.)

Recently, however, a route to the synthesis of the natural purine nucleosides has been found. Trichloropurine-tetra-aceto-*d*-glucoside, prepared from trichloropurine silver and acetobromoglucose, was converted by Fischer and Helferich (*Ber.*, 1914, **47**, 210) into the glucosides of adenine, hypoxanthine, and guanine, and Gulland and Story (this vol., p. 259) have shown by the ultra-violet absorption spectra method that this adenine glucoside is a 9-substituted adenine. It should therefore be possible to synthesise the natural purine nucleosides by the use of the appropriate acetobromoribose and acetobromodeoxyribose and trichloropurine silver.

I wish to acknowledge my indebtedness to Miss E. M. Jackson, B.Sc., with whom I have discussed many points in this paper.