Tetrahedron, 1958, Vol. 2, pp. 333–344. Pergamon Press Ltd., London

THE ORGANIC CHEMISTRY OF DEOXYNUCLEOSIDES AND DEOXYNUCLEOTIDES

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(*Received* 23 *October* 1957)

Abstract—The organic chemistry of the naturally occurring deoxynucleosides and their phosphate esters is reviewed.

THE chemistry of the degradation products of deoxyribonucleic acid has been somewhat neglected until recent years, largely due to the relative inaccessibility of the deoxyribonucleosides and their derivatives. To date there is no convenient method of preparing large quantities of the deoxyribonucleosides, though their isolation after enzymic digestion of polydeoxyribonucleotides has been somewhat simplified by the application of ion-exchange chromatography.[1]

Treatment of DNA with a pancreatic deoxyribonuclease produces a complex mixture which has been examined by Markham and Smith[2] using paper chromatography and paper electrophoresis, and by Sinsheimer[3] using ion-exchange chromatography. Both types of analysis indicate the presence of a number of different dinucleotides, together with tri-and higher-nucleotide material and in the case of Sinsheimer's results, some small amount of mononucleotide (ca. 1 per cent). Further degradation of this mixture using an intestinal phosphatase preparation[4] yields the deoxynucleosides, or if the monoesterase activity is inhibited, the deoxynucleotides. Carter[5] has shown these latter are not analogous to the ribonucleotides isolated ofter alkaline degradation of ribonucleic acid, in that all the deoxyribonucleotides isolated were smoothly dephosphorylated by a 5′-nucleotidase.

When DNA is treated with dilute acid under mild conditions, rupture of the purine glycosidic linkages occurs, to give a purine-free product, apurinic acid.[6] Under more vigorous conditions, however, acid hydrolysis of DNA gives rise to the 3′:5′-diphosphates of thymidine and deoxycytidine[7] together with some pyrimidine mononucleotide material (both 3′-and 5′-phosphates), dinucleoside triphosphate and larger pyrimidine nucleotide fractions.[8] Since the benzyl esters of the various deoxynucleotides are analogous in structure to deoxyribonucleic acid, their acid hydrolysis was investigated. Both deoxygaunosine-3′ benzyl phosphate and deoxyadenosine-3′ benzyl phosphate were rapidly hydrolysed to the respective purine and 2-deoxyribose-3-benzyl phosphate. This sugar derivative then decomposed to give monobenzyl

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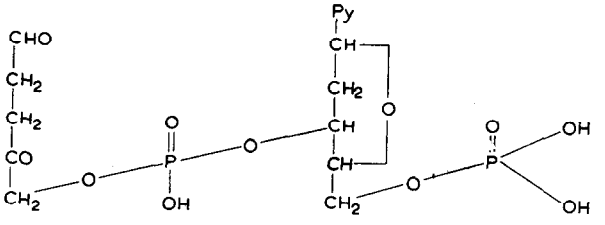
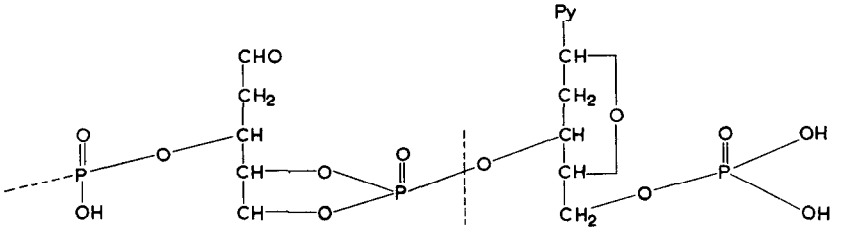
[1] W. Andersen, C. A. Dekker and A. R. Todd *J. Chem. Soc.* 2721 (1952).
[2] R. Markham and J. D. Smith *Biochim. Biophys. Acta* **8**, 350 (1952).
[3] R. L. Sinsheimer *J. Biol. Chem.* **208**, 444 (1954).
[4] W. Klein *Hoppe-Seyl. Z.* **207**, 125 (1932).
[5] C. E. Carter *J. Amer. Chem. Soc.* **73**, 1537 (1951).
[6] A. Kossel and A. Neumann *Ber. Dtsch. Chem. Ges.* **26**, 2753 (1893).
H. Bredereck and G. Müller *Ber. Dstch. Chem. Ges.* **72**, 115 (1939).
[7] C. A. Dekker, A. M. Michelson and A. R. Todd *J. Chem. Soc.* 947 (1953).
[8] A. M. Michelson and A. R. Todd Unpublished work; W. E. Cohn and E. Volkin *Biochim. Biophys. Acta* **24**, 359 (1957); S. K. Shapiro and E. Chargaff *Ibid.* **23**, 451 (1957).

phosphoric acid as the only identifiable product. Likewise the 2-deoxyribose-5-benzyl phosphate initially produced by the action of acid on deoxyadenosine-5′ benzyl phosphate, was hydrolysed to benzyl phosphoric acid. While the two monobenzyl phosphates of 2-deoxyribose were hydrolysed at different rates, the 3 isomer being more rapidly decomposed than the 5 compound, both were completely converted to monobenzyl phosphoric acid after 3 hr at 100° in 0·1 N HCl. Hydrolysis of the pyrimidine nucleotide benzyl esters followed quite a different pattern however. After even prolonged heating in 0·1 N HCl at 100°, the products from the 3′- and 5′-benzyl phosphates of thymidine and deoxycytidine were: unchanged benzyl ester, free nucleotide and only traces of other degradation products.[9] These results are in agreement with a mechanism involving preferential fission or elimination of the phosphate (β to an aldehyde group) from C_3 of the deoxyribose units flanking pyrimidine nucleotide residues or tracts, in the acid hydrolysis of apurinic acid.[10] It is known that acid treatment of 2-deoxyribose gives rise to ω-hydroxylaevulic aldehyde,[11] and this being the case, one might also expect ready fission of phosphate from C_5 due to the presence of the α-keto group.[12]



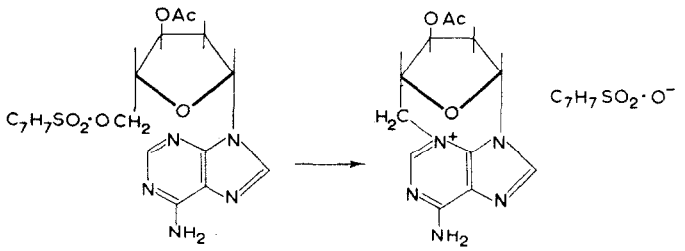
Some alternative fission of phosphoric acid from the pyrimidine nucleoside residue could also occur, giving rise to both 3′- and 5′-monophosphates of the pyrimidine nucleosides, via the 3:4 or 4:5 cyclic intermediates.[13]



Alkaline degradation of DNA has not been examined since Fischer[14] claimed to obtain oligonucleotide mixtures corresponding to 3·2–4·0 nucleotides in size, by the action of hot alkali. The stability of DNA towards alkali is of course the result of two factors (a) the phosphate linkage is stabilised by glycosidation of the sugar[15] and (b) lack of an OH group at C_2'.

[9] D. H. Hayes, A. M. Michelson and A. R. Todd Unpublished work.
[10] A. B. Foster, W. G. Overend and M. Stacey *J. Chem. Soc.* 980, 987 (1951); O. Meyerhof and K. Lohmann *Biochem. Z.* **271**, 98 (1934); D. M. Brown and A. R. Todd *The Nucleic Acids* (Edited by E. Chargaff and J. N. Davidson) Vol. 1, p. 409. Academic Press, New York (1955).
[11] R. E. Deriaz, M. Stacey, E. G. Teece and L. F. Wiggins *J. Chem. Soc.* 1222 (1949).
[12] P. Fleury, J. Courtois and A. Desjobert *Bull. Soc. Chim. Fr.* 694 (1948).
[13] C. Tam, H. S. Shapiro, R. Lipshitz and E. Chargaff *J. Biol. Chem.* **203**, 673 (1953).
[14] F. G. Fischer *Naturwissenschaften* **30**, 377 (1942).
[15] A. B. Foster, W. G. Overend and M. Stacey *J. Chem. Soc.* 987 (1951).

Since the deoxyribonucleosides do not contain an α-glycol system, the periodate oxidation method of Davoll *et al.*[16] cannot be used to establish the configuration of the glycosidic carbon atom. An independent proof of the β-configuration of the ribonucleosides however, lies in the discovery of the 5′-*cyclo*nucleosides derived from cytidine and adenosine.[17] Cyclisation from the 5′-position can only occur with a β-nucleoside; the preparation of *cyclo*nucleosides from deoxyadenosine and deoxycytidine thus provides a proof of their β-configuration. Treatment of 3′-acetyl deoxyadenosine with toluene-*p*-sulphonyl chloride gave a covalent toluene-*p*-sulphonyl derivative, which on heating in acetone, rearranged to the toluene-*p*-sulphonate of 3′-acetyl-2′-deoxy -3:5′ *cyclo*adenosine.[18]

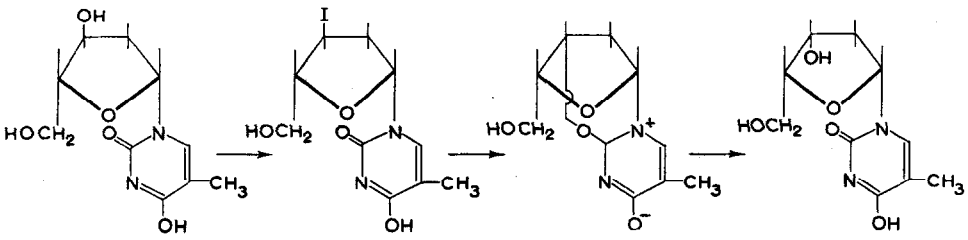


The analogous 3′-acetyl 5′-toluene-*p*-sulphonyldeoxyguanosine could not be obtained. Treatment of 3′-acetyldeoxyguanosine with toluene-*p*-sulphonyl chloride, methane sulphonyl chloride and methane sulphonic anhydride was completely unsuccessful. Low reactivity of the hydroxyl groups in guanosine derivatives towards certain reagents has been previously observed. While 2′:3′-*iso*propylidene guanosine is readily acetylated and tritylated, toluene-*p*-sulphonylation could not be effected, and phosphorylation was successful only when phosphoryl chloride was used.[19]

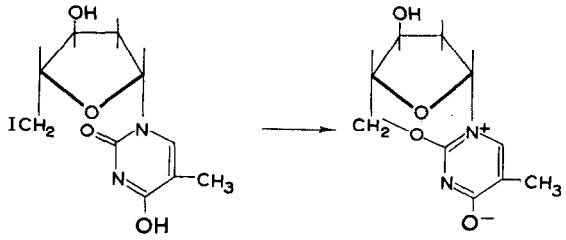
Toluene-*p*-sulphonylation of N^6:3′-diacetyldeoxycytidine gave a covalent toluene-*p*-sulphonyl derivative, which was somewhat resistant to cyclisation, as would be expected, since the formation of *cyclo*nucleoside salts is to some extent dependent on the basic nature of the purine or pyrimidine residue, and N^6-acetylation of the cytosine residue might well prevent its occurrence. Deacetylation with cold methanolic ammonia gave 5′-toluene-*p*-sulphonyldeoxycytidine, which on heating in acetone solution, gave a complex mixture containing cytosine, toluene-*p*-sulphonate ion and sugar derivatives of unidentified nature. While the expected 3′-acetyl O^2:5′-*cyclo*deoxycytidine toluene-*p*-sulphonate was not isolated, the observation of toluene-*p*-sulphonate ion was a strong indication that an unstable *cyclo*nucleoside salt was formed, and that deoxycytidine is likewise to be regarded as a 2′-deoxy-β-D-ribofuranoside. It is likely that the positive charge on N_3, with a β—CH_2 group leads to instability of the N_3—C_1' linkage as well as the O_2—C_5' bond, thus giving complete fission of sugar from pyrimidine. Further evidence of the unstable character of the N_3—C_1' bond when a β—CH_2 is also present, is found in the behaviour of the *cyclo*thymidines—mild acid hydrolysis yields thymine—and not the nucleoside.[17] These *cyclo* derivatives, formation of which proves the β-configuration of thymidine, were first obtained from the

[16] J. Davoll, B. Lythgoe and A. R. Todd *J. Chem. Soc.* 833 (1946).
[17] V. M. Clark, A. R. Todd and J. Zussman *J. Chem. Soc.* 2952 (1951).
[18] W. Andersen, D. H. Hayes, A. M. Michelson and A. R. Todd *J. Chem. Soc.* 1882 (1954).
[19] A. M. Michelson and A. R. Todd *J. Chem. Soc.* 521 (1949); R. W. Chambers, J. G. Moffatt and H. G. Khorana *J. Amer. Chem. Soc.* **79**, 3747 (1957).

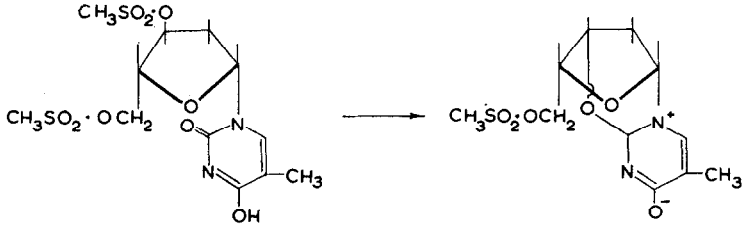
iodo-deoxythymidines. Treatment of 3′-iodo-3′-deoxythymidine with silver acetate in dry methyl cyanide containing a little base, yielded a highly crystalline compound, with however, none of the properties of the expected 3′-acetyl thymidine. Analysis and its physical properties indicated that it was a *cyclo*nucleoside. Acid hydrolysis gave thymine and 2-deoxyxylose, while alkaline hydrolysis gave thymine-3 β-D-2′-deoxy-xyloside, indicating inversion at C_3'.



Similarly 5′-iodo-5′-deoxythymidine yielded O^2-:5′-*cyclo*thymidine together with a little 5′-acetylthymidine, while cyclisation of 3′-acetyl-5′-iodo-5′-deoxythymidine gave the corresponding 3′-acetyl-O^2:5′-*cyclo*thymidine.[20]



Similar derivatives were obtained by the action of alcoholic ammonia on the 3′-sulphonyl derivatives—e.g. 3′:5′-dimethanesulphonyl thymidine gave 5′-methane-sulphonyl-O^2:3′-*cyclo*thymidine, the 5′-sulphonyl derivatives being quite stable under the conditions used.

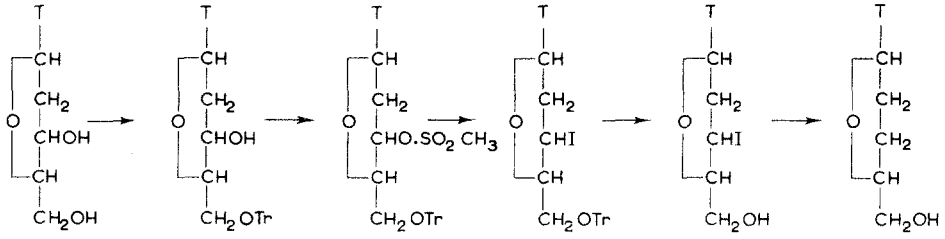


Molecular models indicate that the ease of formation of these compounds is in the order O^2:$C^{3'} > O^2$:$C^{5'}$, the former involving inversion, and that in the ribose series an O^2:$C^{2'}$ cyclisation would be even more facile.[21]

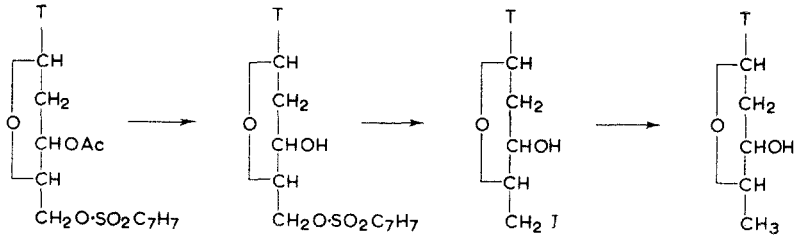
The complete structure of thymidine as thymine 3-β-D-2′-deoxyribofuranoside has been confirmed by an X-ray crystallographic examination of 5′-bromo-5′-deoxythy-midine, by W. Cochran and M. Huber of the Cavendish Laboratory.

[20] A. M. Michelson and A. R. Todd *J. Chem. Soc.* 816 (1955).
[21] D. M. Brown, A. R. Todd and S. Varadarajan *J. Chem. Soc.* 2388 (1956).

The preparation of the above-mentioned iodo-deoxythymidines and other derivatives is of some interest. Tritylation of thymidine under standard conditions gave 5′-tritylthymidine. On treatment with methane sulphonyl chloride, this yielded 3′-methanesulphonyl-5′-tritylthymidine which on heating with sodium iodide or lithium bromide in acetone, was converted to the 3′-halogeno-3′-deoxy-5′-tritylthymidine. Detritylation of 3′-iodo-3′-deoxy-5′-tritylthymidine gave 3′-iodo-3′-deoxythymidine, which was readily reduced by catalytic hydrogenation, to 3′-deoxythymidine.

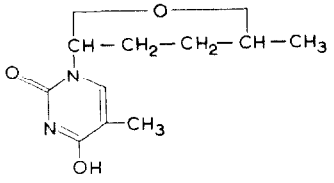


A similar series of reactions culminated in 5′-deoxythymidine. Acetylation of 5′-tritylthymidine followed by detritylation gave 3′-acetylthymidine, which was toluene-*p*-sulphonylated and the product deacetylated to give the highly crystalline 5′-toluene-*p*-sulphonylthymidine. Treatment with lithium bromide yielded 5′-bromo-5′- deoxythymidine, while sodium iodide produced 5′-iodo-5′-deoxythymidine, smoothly



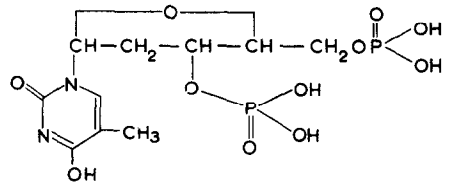
reduced to 5′-deoxythymidine. The 5′-deoxy- and 3′-deoxythymidines are of some interest as possible inhibitors of DNA metabolism.

Treatment of 3′:5′-dimethanesulphonylthymidine (obtained by methanesulphonylation of thymidine) with lithium bromide yielded 3′:5′-dibromo-3′:5′dideoxythymidine. Hydrogenation with a palladium–barium sulphate catalyst gave a monobromo derivative, shown to be 5′-bromo-3′:5′-dideoxythymidine, by an X-ray crystallographic examination of the compound, by Mr. M. M. Woolfson of the Cavendish Laboratory. Further hydrogenation gave 3′:5′-dideoxythymidine.



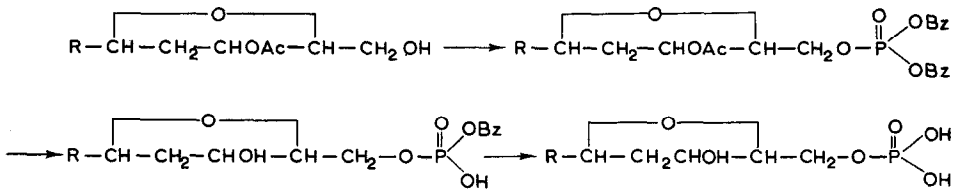
The fairly easy replacement of secondary sulphonyloxy groups by halogen, in these nucleoside derivatives is possibly due to the absence of the C_2'-hydroxyl group in the deoxyribonucleosides.

While the synthesis of the natural deoxynucleosides is as yet unreported,* the next step—phosphorylation of protected nucleosides to give the mononucleotides—has been completely achieved. Phosphorylation of thymidine with excess dibenzyl phosphorochloridate, followed by catalytic hydrogenation to remove benzyl groups, gave largely thymidine-3':5'-diphosphate, together with some thymidine-5'-phosphate but no 3'-phosphate. The diphosphate, purified by ion exchange chromatography, or by boiling an aqueous solution of the dibarium salt, was identical in all respects with the product obtained by the acid hydrolysis of DNA.[7]



An analogous phosphorylation of deoxycytidine yielded deoxycytidine-3':5' diphosphate, though in rather small yield. The main product was deoxycytidine-5'-phosphate. As in the phosphorylation of thymidine, no 3'-phosphate was isolated, probably because under the conditions employed, the lower reactivity of the 3'-hydroxyl group (more pronounced in the case of deoxycytidine than in the case of thymidine) leads to the formation of 5'-phosphate which is then slowly phosphorylated at the 3'-position.

Thymidine-5'-phosphate was readily obtained from 3'-acetylthymidine. Phosphorylation with dibenzylphosphorochloridate gave 3'-acetylthymidine-5'-dibenzyl phosphate, which on alkaline treatment yielded thymidine-5'-benzylphosphate. Catalytic hydrogenation gave the nucleotide, identical with the natural material isolated from enzymic digests of DNA. This unambiguous synthesis is the final proof of the postulated structure for thymidylic acid.[22]



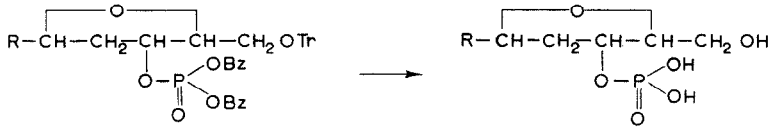
Thymidine-5'-phosphate can also be prepared by the preferential phosphorylation of thymidine, using dibenzyl phosphorochloridate.

The isomeric thymidine-3'-phosphate was prepared by phosphorylating 5'-trityl-thymidine and hydrolysing the product to thymidine-3'-benzyl phosphate, which on

* A recent communication by D. M. Brown, D. B. Parihar C. B. Reese and A. R Todd [*Proc. Chem. Soc.* 321 (1957)] describes the synthesis of 2'-deoxyuridine from 2'-toluene-*p*-sulphonyluridine, in excellent yield.

[22] A. M. Michelson and A. R. Todd *J. Chem. Soc.* 951 (1953).

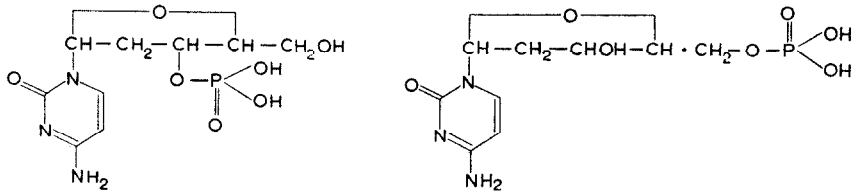
hydrogenation, yielded thymidine-3′-phosphate, quite different from the natural nucleotide.



Like thymidine-3′:5′diphosphate, calcium and barium salts of thymidine-3′-phosphate are much more soluble in cold water than in hot, and both can be isolated by boiling an aqueous solution. This reverse solubility effect is not shown by salts of thymidine-5′-phosphate, and is seemingly characteristic of the 3′-position. Thymidine-5′-phosphate was also obtained by treating 3′-acetyl-5′-iodo-5′-deoxythymidine (obtained from 3′-acetyl-5′-toluene-*p*-sulphonylthymidine by the action of sodium iodide in acetone) with silver dibenzyl phosphate; protecting groups were removed from the product in the usual way.[20]

The isomeric thymidine mononucleotides can be distinguished by paper chromatography in a *n*-propanol (3 vol):2 N HCl (1 vol) system, or by ion exchange chromatography using Dowex-2 (formate form) and eluting with a solution of 0·01 M formic acid, 0·05 M with respect to sodium formate.

The deoxycytidine mononucleotides were prepared by analogous methods. Tritylation of deoxycytidine gave a very good yield of 5′-trityldeoxycytidine, which was phosphorylated with dibenzylphosphorochloridate. Acid hydrolysis of the crude product yielded deoxycytidine-3′-benzylphosphate, which was catalytically hydrogenated to deoxycytidine-3′-phosphate. Acetylation of 5′-trityldeoxycytidine yielded N^6:3′-diacetyl-5′-trityldeoxycytidine, which on acid hydrolysis gave a mixture of N^6:3′-diacetyldeoxycytidine, 3′-acetyldeoxycytidine and a little N^6-acetylcytosine. The diacetate was separated by counter-current distribution, phosphorylated and converted to deoxycytidine-5′-phosphate, identical with the nucleotide obtained by the action of DNAase and diesterase on DNA.[23]



The deoxycytidine mononucleotides can be conveniently separated by ion exchange chromatography on a Dowex-2 column (formate form) using 0·015 M formic acid as eluting solvent. As with the thymidylic nucleotides, the 5′-phosphate is eluted prior to the 3′-isomer.

The properties of the deoxycytidine phosphates are of considerable interest, due to their bearing on the orientation of the *a*- and *b*-ribonucleotides. A comparison of the infra-red spectra of the phosphates of deoxycytidine and cytidine provided strong evidence that cytidylic acid *b* is cytidine-3′-phosphate and hence that cytidylic acid *a* is the 2′-phosphate, a view which was further confirmed by a comparison of optical rotation and ultra-violet absorption data in the two series. Since uridylic acid *b* has

[23] A. M. Michelson and A. R. Todd *J. Chem. Soc.* **34** (1954).

been prepared by deamination of cytidylic acid *b*, this evidence would apply equally to the orientation of uridylic acids *a* and *b*.

The problem of synthesis of the purine deoxynucleotides was complicated by the extreme instability of the purine deoxynucleosides and their derivatives to acid, and in the case of deoxyguanosine, by low reactivity of the nucleoside towards certain acylating agents. The methods adopted were however, essentially similar to those used for the pyrimidine deoxyribonucleotides.

Preparation of 3′-acetyldeoxyadenosine by tritylation of deoxyadenosine followed by acetylation and removal of the trityl group by hydrogenation gave such low yields (due to rupture of the glycosidic linkage) that the route was useless for preparative purposes. Partial acetylation of deoxyadenosine with acetic anhydride in pyridine, gave reasonable yields of the 3′- and 5′-monoacetates, separated from each other and from 3′:5′-diacetyldeoxyadenosine and unreacted deoxyadenosine by counter-current distribution. Comparison of the two monoacetates with the small amount of authentic 3′-acetyldeoxyadenosine available, identified the monoacetate obtained in smaller yield as 3′-acetyldeoxyadenosine and thus that the other was 5′-acetyldeoxyadenosine.[18] The two monoacetates were best prepared by partial deacetylation of 3′:5′-diacetyldeoxyadenosine, which gave a 60 per cent yield of the mixed monoacetates. With this method, the yield of monoacetate was fairly constant over a reasonable time base; variation occurred in the ratio of diacetylnucleoside to completely deacetylated nucleoside.[24]

Treatment of 3′-acetyldeoxyadenosine with dibenzylphosphoro chloridate in pyridine gave 3′-acetyldeoxyadenosine-5′-dibenzyl phosphate, which, with methanolic ammonia under suitable conditions, yielded deoxyadenosine-5′-benzyl phosphate. Removal of the benzyl group was effected by hydrogenolysis in aqueous solution, buffered to pH7 with sodium acetate to avoid fission of the glycosidic linkage. The deoxyadenosine-5′-phosphate so obtained was identical with that from natural sources.[24]

Phosphorylation of 5′-acetyldeoxyadenosine with dibenzyl phosphorochloridate was not satisfactory. However, with O-benzylphosphorous-O:O-diphenyl phosphoric anhydride,[25] the monoacetate gave a reasonable yield of 5′-acetyldeoxyadenosine-3′-benzyl phosphite. This was chlorinated with N-chlorosuccinimide and the nucleoside phosphorochloridate hydrolysed to 5′-acetyl-deoxyadenosine-3′-benzyl phosphate. Deacetylation and hydrogenolysis yielded deoxyadenosine-3′-phosphate, isolated as its calcium salt. The two deoxyadenosine phosphates were readily distinguished by ion exchange and paper chromatography.

For the synthesis of the corresponding deoxyguanosine phosphates, the isomeric monoacetates were prepared either by partial acetylation of deoxyguanosine, or by partial deacetylation of 3′:5′-diacetyldeoxyguanosine, the latter method being more convenient and also producing a better ratio of the two monoacetates. Since no reference compound corresponding to 3′-acetyl-5′-tritydeoxyadenosine was available, orientation of the two acetates was achieved by a direct comparison of the monoacetyldeoxyribose liberated on mild acid hydrolysis of 3′-acetyldeoxyadenosine, 5′-acetyldeoxyadenosine, and the two deoxyguanosine monoacetates. Differentiation of 3-acetyl-2-deoxyribose was possible by paper chromatography, in several solvent systems. Correspondence with the acetyldeoxyribose from each acetyl deoxyguanosine,

[24] D. H. Hayes, A. M. Michelson and A. R. Todd *J. Chem. Soc.* 808 (1955).
[25] N. S. Corby, G. W. Kenner and A. R. Todd *J. Chem. Soc.* 3669 (1952).

identified the monoacetate produced in larger amount as 5′-acetyldeoxyguanosine and the other as 3′-acetyldeoxyguanosine. An interesting observation noted during these experiments on the acid hydrolysis of the acetylated nucleosides, was the stabilising influence of sugar acetyl groups on the glycosidic linkage. Using 0·02 N hydrochloric acid at 100°, the results may be summarised as follows:

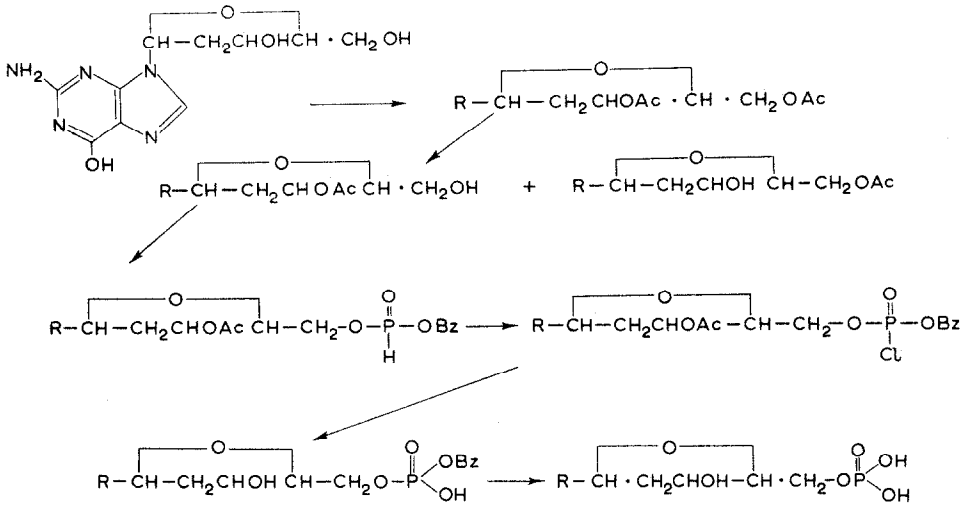
(1) The nucleosides are completely hydrolysed to purine and sugar after ca. 2 min.

(2) Glycosidic rupture of the monoacetyl nucleosides, independent of the position of the acetyl group in the sugar, is complete after 5 min.

(3) The glycosidic linkage in the 3′:5′-diacetyl derivatives is completely hydrolysed after 10 min, i.e. the effect of each acetyl group is approximately additive.

(4) Deoxyadenosine and its acetates are slightly more stable than the corresponding deoxyguanosine derivatives.

The two monoacetyldeoxyguanosines were phosphorylated with O-benzyl-phosphorous-O:O-diphenylphosphoric anhydride and the products converted to the corresponding nucleotides, by the methods described for the synthesis of deoxyadenosine-3′-phosphate.



The action of several enzymes on the deoxynucleotides and their esters was examined. Prostate monoesterase dephosphorylated all the mononucleotides (including thymidine-3′:5′-diphosphate), while rattlesnake (*Crotalus atrox*) venom dephosphorylated thymidine-5′-phosphate, deoxyadenosine-5′-phosphate, deoxycytidine-5′-phosphate, deoxyguanosine-5′-phosphate and their monobenzyl esters, but had no action on the corresponding 3′-phosphates and their benzyl esters or on thymidine-3′:5′-diphosphate and deoxycytidine-3′:5′-diphosphate. None of the compounds was affected by deoxyribonuclease or ribonuclease.[23]

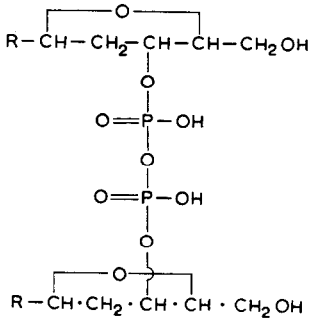
The next phase of synthetic nucleotide work was the synthesis of small polynucleotides for comparison with the natural nucleic acids and some of their larger breakdown products. Thymidine was acetylated and the resultant 3′:5′-diacetyl thymidine submitted to a partial deacetylation treatment with dilute methanolic ammonia. The products—thymidine, 5′-acetyl-thymidine, 3′-acetylthymidine and unchanged 3′:5′-diacetylthymidine—were separated by counter-current distribution, to give good

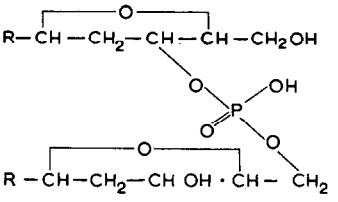
yields of the two monoacetates. The ratio of monoacetates formed was even more favourable than in the purine series, where a fairly marked selectivity is evident. Under optimum conditions for total monoacetate formation, the results may be summarised in Table 1:

TABLE 1

	Partial acetylation of nucleoside		Partial deacetylation of diacetyl nucleoside	
	% 5′-acetyl	% 3′-acetyl	% 5′-acetyl	% 3′-acetyl
Deoxyadenosine	73·5	26·5	66·0	34·0
Deoxyguanosine	90·6	9·4	66·6	33·4
Thymidine			57·0	43·0

Treatment of 5′-acetylthymidine with O-benzyl phosphorous-O:O-diphenylphosphoric anhydride yielded 5′-acetylthymidine-3′-benzyl phosphite. This was chlorinated with N-chlorosuccinimide to give 5′-acetylthymidine-3′-benzyl phosphorochloridate. While phosphorylation of 3′-acetylthymidine with 5′-acetylthymidine-3′-benzyl-P_1:P_2-diphenyl pyrophosphate (i.e. the mixed anhydride prepared from the phosphorochloridate and the triethylamine salt of diphenylphosphoric acid) or the trifluoroacetyl anhydride was unsuccessful, direct phosphorylation was achieved on treating 3′-acetylthymidine with 5′-acetylthymidine-3′-benzyl phosphorochloridate in the presence of base. After the removal of protecting groups from the crude product, a mixture containing thymidine-3′-phosphate, di(thymidine-3′)-P_1:P_2-pyrophosphate and thymidine-3′-thymidine-5′-phosphate, was obtained. Separation by ion-exchange chromatography gave the individual components.



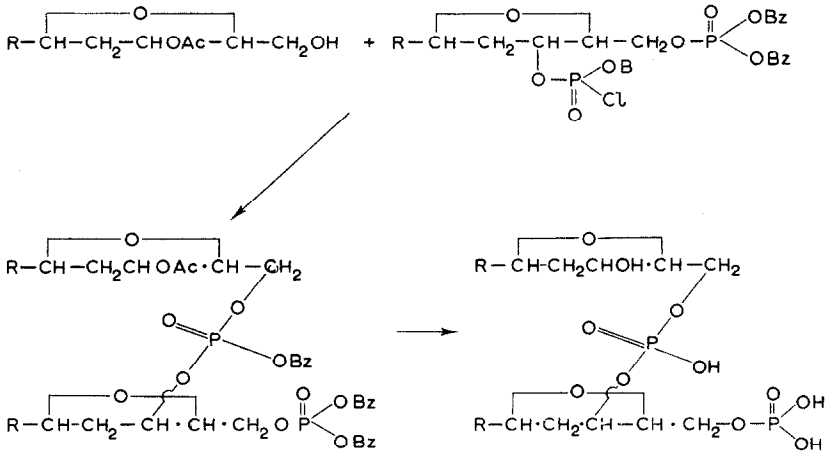


Dithymidine-3′-P_1:P_2-3′-pyrophosphate probably arises from slight hydrolysis of the nucleoside phosphorochloridate, to give the benzyl phosphate, which then rapidly reacts with the phosphorochloridate.

The synthesis of a true dinucleotide was accomplished by an analogous route.[26] Phosphorylation of 3′-acetylthymidine with dibenzyl phosphorochloridate yielded 3′-acetylthymidine-5′-dibenzylphosphate which was deacetylated and the thymidine -5′-dibenzylphosphate treated with O-benzyl phosphorous-O:O-diphenylphosphoric

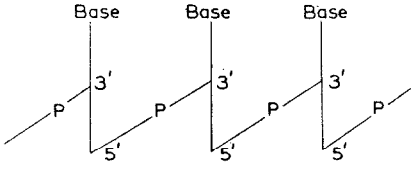
[26] A. M. Michelson and A. R. Todd *J. Chem. Soc.* 2632 (1955).

anhydride to give the 3′-phosphite. Chlorination gave thymidine-3′-benzylphosphochloridate-5′-dibenzyl phosphate, which was used to phosphorylate 3′-acetylthymidine in the presence of base, to give the fully protected dinucleotide. Acid hydrolysis to remove tertiary benzyl groups, followed by alkaline hydrolysis to remove the acetyl group yielded the monobenzyl dinucleotide. Catalytic hydrogenation gave 5′-phosphothymidine-3′-thymidine-5′-phosphate, isolated as its crystalline calcium salt. By-products of the reaction also isolated were thymidine-3′:5′-diphosphate and di-5′-phosphothymidine-3′-P_1:P_2-3′-pyrophosphate.



The various thymidine phosphates can be characterised by paper chromatography; a better method however, is paper electrophoresis in 0·1 M potassium dihydrogen phosphate and in 0·1 M disodium hydrogen phosphate. While the former solvent suppresses dissociation of secondary phosphoryl groups, so that migration is dependent on the number of primary dissociations only, (apart from the effect due to increase in molecular weight) with the alkaline system, both primary and secondary dissociations are of importance. Complete identification can therefore be established by running in both solvents.

As expected, purified prostate monoesterase removed the terminal phosphate from the dinucleotide, giving thymidine-3′-thymidine-5′-phosphate, identical with the material prepared synthetically. Similarly, dithymidine-3′:3′-P_1:P_2-pyrophosphate



was obtained from di(5′-phosphothymidine)3′-P_1:P_2-pyrophosphate. Purified rattlesnake venom diesterase split the synthetic thymidine-3′-thymidine-5′-phosphate to 1 mol. of thymidine and 1 mol. of thymidine-5′-phosphate, while the dinucleotide gave 2 mol. of thymidine-5′-phosphate only.

These results are in accord with expectation and are in complete agreement with those obtained with solutions of dinucleotides prepared by enzymic degradation of DNA and so provide direct synthetic support for the structures assigned to them, and hence to the 3′:5′-linked polynucleotide structure of DNA.

The outstanding chemical problems in deoxyribonucleic acid work concern the synthesis of the naturally occurring deoxynucleosides, development of methods[27] for the synthesis of a number of purine and pyrimidine di- and tri-nucleotides, and the polymerisation of deoxynucleotides or nucleotide derivatives to a degree when the product could legitimately be called a deoxyribonucleic acid. As far as the gross molecule is concerned, a suitable method for the determination of sequence in the polynucleotide chain has yet to be devised.

[27] H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol *J. Amer. Chem. Soc.* **79**, 1002 (1957).