

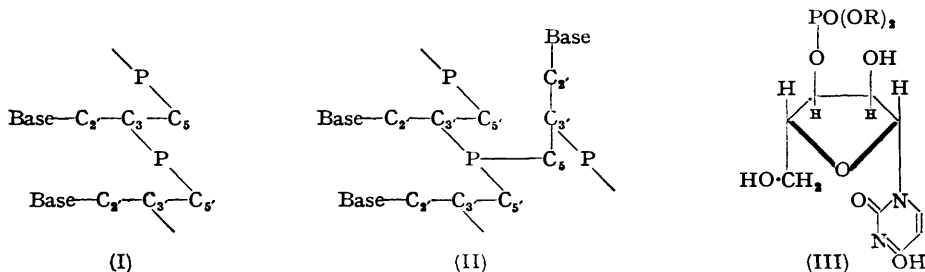
Nucleotides. Part XXXIV. The Hydrolysis of Dialkyl Esters of Uridine-3' Phosphate and its Relevance to the Question of Phosphotriester Linkages in Ribonucleic Acids.*

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Dimethyl and dibenzyl uridine-3' phosphate have been prepared by the action of the corresponding diazoalkanes on uridine-3' phosphate. In aqueous solvents they are unstable over the whole pH range, decomposing to a mixture of the monoesters of uridine-2' and uridine-3' phosphate together with traces of uridine-2' : 3' phosphate. It is considered from these findings that the occurrence of phosphotriester groupings in ribonucleic acids is unlikely.

EARLIER papers in this series have stressed the close analogy which exists between the hydrolytic behaviour of the monoalkyl esters of ribonucleoside-2' and -3' phosphates and that of the ribonucleic acids. The simplicity of the former compounds permitted a more precise study of the mechanism of their hydrolysis and this led to an acceptable structural hypothesis (Brown and Todd, *J.*, 1952, 52; 1953, 2040) in which the ribonucleic acids were formulated as polynucleotides (I), where the main chain was made up of nucleoside residues linked together in the 3'- and the 5'-positions by phosphodiester groups. Earlier, suggestions had been made that ribonucleic acids contained some phosphotriester linkages (Fletcher, Gulland, and Jordan, *J.*, 1944, 34); these suggestions rested on electrometric titration studies but no direct chemical evidence was adduced to support them. Such groupings if present would represent branching points in a polynucleotide chain such as (II). We pointed out (Brown and Todd, *J.*, 1952, 52) that a neutral triester group such as that present in (II) would be expected to be as readily hydrolysed as the normal internucleotidic linkage and hence, if present, would not affect the final composition of ribonucleic acid hydrolysates, but whether or not it did occur was left open.



In later work on the methylation of uridine-3' phosphate (III; R = H) with methyl iodide and silver oxide (Brown, Magrath, and Todd, *J.*, 1954, 1442), intermediate products were observed which were considered to be neutral dimethyl esters (*e.g.*, III; R = Me); these products were surprisingly labile, being apparently converted into monomethyl esters in very weakly alkaline solution. In view of the fact that esters such as (III; R = Me) should be analogous in their behaviour to triester groups in polynucleotide structures such as (II) it was decided to study them more closely.

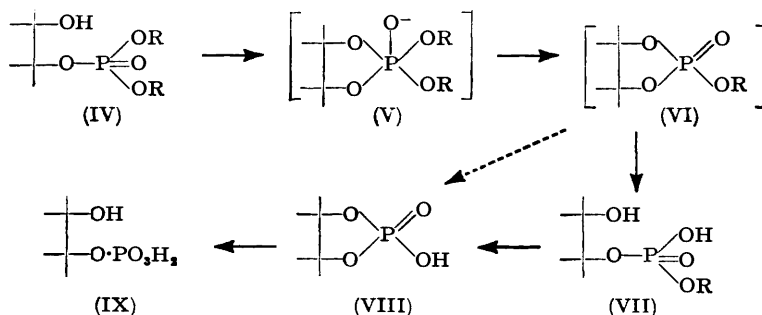
When uridine-3' phosphate, dissolved in methanol, is treated with cold ethereal diazomethane until neutral, a crystalline uridine-3' dimethyl phosphate (III; R = Me) is obtained. By using phenyldiazomethane the corresponding dibenzyl ester (III; R = CH₂Ph) is obtained as a gum from which final traces of phenyldiazomethane decomposition products are very difficult to remove. That the ester is essentially uridine-3' dibenzyl phosphate follows from its smooth hydrogenolysis to uridine-3' phosphate; this also demonstrates that no phosphoryl migration occurs during its preparation, and it can therefore be reasonably assumed that the substance obtained by using diazomethane is indeed

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(III; R = Me). The two esters behave as fast-running substances on paper chromatograms in the neutral propan-2-ol-water system. When chromatographed in the same system containing a little ammonia (pH 8–9) the compounds do not run as such, but as the monomethyl and monobenzyl esters respectively. A trace of the cyclic uridine-2' : 3' phosphate is also observed, but under these mildly alkaline conditions the hydrolysis which occurs leads almost exclusively to the monoesters. A qualitative study was made of the stability of the dimethyl ester at 37° over the pH range 0–9 in buffer solutions. Hydrolysis to the monomethyl ester and a small amount of cyclic phosphate occurred over the whole range (cf. Table 2 in Experimental section). At all pH values hydrolysis was complete in 16 hours. The half-life of the dimethyl ester was approximately 15 minutes at pH 0 and pH 9, and 75 minutes at pH 7. The ester showed maximum stability at pH 2–3 where it had a half life of 4–5 hours. At extreme pH values (*i.e.*, <2 and >11) further hydrolysis of the initially formed monomethyl ester and cyclic phosphate to free nucleotide became important; hydrolysis to uridine did not occur under any of the conditions studied.

The remarkable ease of hydrolysis of these dialkyl esters, especially under mildly alkaline conditions clearly indicates that the vicinal hydroxyl at C_{2'} is involved. Thus, in contrast, deoxyadenosine-5' dibenzyl phosphate, which contains no vicinal hydroxyl group, can be prepared from its 3'-acetyl derivative by treatment with methanolic ammonia during 100 hours (Hayes, Michelson, and Todd *J.*, 1955, 808). The monoesters of uridine-2' and uridine-3' phosphate are not readily distinguishable by paper chromatography but when uridine-3' dibenzyl phosphate is dissolved in the propan-2-ol-ammonia solvent and the solution set aside for some minutes the uridine benzyl phosphate produced can be shown by hydrogenation to be a mixture of approximately equal parts of the 2'- and the 3'-isomer. Additional evidence is provided by the action of ribonuclease on the hydrolysis product. This enzyme attacks esters of 3'- but not 2'-phosphates of the pyrimidine nucleosides (Brown and Todd *J.*, 1953, 2040); only half of the monobenzyl ester was converted into uridine phosphate, the rest being unaffected. A similar result was obtained with the uridine methyl phosphate obtained from uridine-3' dimethyl phosphate under similar conditions; ribonuclease did not appear to have any action on the unhydrolysed dimethyl ester. There is thus very strong evidence that hydrolysis of the dialkyl to the monoalkyl esters is accompanied by phosphoryl migration.

In discussing the hydrolysis of monoesters (VII) of the 2'- and 3'-ribonucleotides with alkali or ribonuclease it was concluded (Brown and Todd, *loc. cit.*) that the mechanism involves a transesterification yielding the nucleoside-2' : 3' phosphate (VIII) which then breaks down to a mixture of the free 2'- and 3'-nucleotides (IX). Evidently the dialkyl esters undergo a similar form of alkaline hydrolysis but even more readily by virtue of their being neutral triesters. The conversion of the diester (IV) into the monoester (VII) must occur by a two-stage process if the oxygen of the vicinal hydroxyl enters into covalent linkage with the phosphorus, as the evidence of migration indicates. A possible mechanism is indicated below.



The first step would lead, perhaps *via* (V), to the cyclic triester (VI). Further hydrolysis of (VI) would be expected to proceed mainly by fission of a cyclic O–P bond giving the

monoester (VII) although a small amount of (VIII) might be formed directly by fission of the exocyclic O-P bond. This scheme accords with the observed products. No evidence for the occurrence of (VI) during the hydrolyses was found, but it might well be very unstable in view of the ready hydrolysis of the cyclic nucleoside-2' : 3' phosphates (VIII) compared with simple acyclic dialkyl phosphates (Brown and Todd, *J.*, 1952, 52). It may be recalled that Baddiley and Thain (*J.*, 1953, 903) noted a ready reaction between *cyclohexylamine* and pantoil-lactone-2 diphenyl phosphate, yielding the *cyclohexylamide* of the 2 : 4-phosphate of pantothenic acid. This reaction, seemingly analogous to the conversion of (IV) into (VII) and (VIII), led to the cyclic phosphate rather than the acyclic monophenyl phosphate, presumably because of the greater stability of a six-membered as opposed to a five-membered cyclic phosphate ring (Baddiley and Thain, *J.*, 1952, 3783). A final decision on the mechanism of hydrolysis of the diesters must await quantitative kinetic studies.

Another point of some interest was noted during these investigations. Although alkylation with a diazoalkane is probably the best method for preparing the nucleoside dialkyl phosphates it was observed that when cytidine-2' and -3' phosphates were treated with trifluoroacetic anhydride, and anhydrous ethanolic ammonia added subsequently, a mixture containing cytidine-2' : 3' phosphate and, apparently, cytidine diethyl phosphate was obtained. Although insufficient of the latter compound was obtained for full characterisation, its identity seems in little doubt since it underwent very ready hydrolysis (*e.g.*, in the propan-2-ol-ammonia solvent) to a substance chromatographically identical with cytidine-2' (or -3') ethyl phosphate. This observation suggests that the monoethyl ester isolated as a by-product in the preparation of cytidine-2' : 3' phosphate by the trifluoroacetic anhydride route (Brown, Magrath, and Todd, *J.*, 1952, 2708) may have originated from the diethyl ester, initially formed by a reaction between ethanol and a mixed anhydride of the type (RO)PO(O-CO-CF₃)₂.

It is pertinent to discuss the possibility of chain-branching in ribonucleic acids through triester linkages as in (II), in the light of the investigations reported in this paper. This type of branching was originally postulated in order to permit an unexpectedly high ratio of monoesterified to diesterified phosphate in a polynucleotide of high molecular weight, since in effect it increased the number of end groups in the molecule. Fletcher, Gulland, and Jordan (*loc. cit.*) found ratios up to 0.25 in their electrometric titration studies, and similar titration results have been reported by other workers using a variety of ribonucleic acid preparations (*cf.* Jordan, *Progr. Biophysics Biophys. Chem.*, 1951, 2, 51). Other results seeming to support this postulate have come from studies on dye-binding and enzymic end-group assay but more recent reports have tended to minimise the proportion of monoesterified phosphate possibly because isolation techniques for ribonucleic acids have improved [for references see Jordan (*loc. cit.*); "The Nucleic Acids," Vol. I, p. 447, edited by Davidson and Chargaff, Academic Press, New York, 1955; Brown and Todd, *op. cit.*, p. 409]. Clearly the necessity or otherwise of invoking phosphotriester linkages on the basis of end-group assays by titration or by enzyme action must depend on the validity of molecular-weight determinations on the sample of ribonucleic acid used. Aside from the heterogeneity of the samples used by earlier workers it now appears that past estimates of the molecular weight of, for example, yeast ribonucleic acid have erred on the high side. No recent studies including both molecular-weight determination and electrometric titration seem to have been reported.

No direct and unequivocal evidence for the presence of phosphotriester groupings in ribonucleic acids has thus ever been given. It may be noted too that solutions of ribonucleic acids have been maintained at pH values up to 10.6 without an observable increase in acidic groups (Zittle, *J. Franklin Inst.*, 1946, 242, 221; Brown, Fried, and Todd, *J.*, 1955, 2206) whereas under these conditions the uridine dialkyl phosphates are rapidly hydrolysed. If the presence of phosphotriester groupings in isolated ribonucleic acids is to be maintained it will be necessary to account for their apparent stability in the polynucleotide in contrast to their marked instability in a mononucleotide system. Since no abnormal behaviour of the phosphodiester linkages is observed in ribonucleic acids there seems no reason to suppose that any phosphotriester groupings would behave in an unexpected manner. In these circumstances unless and until some quite incontrovertible evidence is given for their

presence we prefer to assume that phosphotriester linkages do not exist in isolated ribonucleic acids. The presence or absence of triester branching in native nucleic acids within the living cell is a purely hypothetical question at the present time and is irrelevant to the discussion.

The experimental observations reported in this paper do not bear directly on the suggestions of Lee and Peacocke (*J.*, 1951, 3361) and of Cavalieri and Angelos (*J. Amer. Chem. Soc.*, 1950, **72**, 4686) that phosphotriester branching must be assumed to occur in deoxyribonucleic acids (cf. also Jordan, *loc. cit.*). Since no vicinal hydroxyl is present at C_(3') in these acids any phosphotriester grouping in them should show the stability normally associated with a trialkyl phosphate. That trialkyl phosphates undergo ready hydrolysis to diesters is well known, so that in view of the well-recognised stability of deoxyribonucleic acids towards alkali it seems very unlikely that they could contain any phosphotriester groupings. The evidence bearing on this point has been discussed at greater length elsewhere (Brown and Todd, *Ann. Rev. Biochem.*, 1955, **24**, 311) but it may be noted here that Frick (*Biochim. Biophys. Acta*, 1952, **8**, 625) has found that treatment of a deoxyribonucleic acid with 0.1N-sodium hydroxide yields only traces of dialysable, ultraviolet-absorbing material; this argues strongly against the presence of short branches involving phosphotriester groups.

EXPERIMENTAL

Paper Chromatography of Uridine Phosphate Derivatives.—Two solvent systems were used throughout, *viz.*: A, propan-2-ol-water (70 : 30 v/v); B, propan-2-ol-water-ammonia (*d* 0.88) (70 : 25 : 5). The comparative *R_F* values of the materials studied in the two systems are recorded in Table 1. Spots were detected by photography in ultraviolet light and where necessary by spraying for phosphate. Substances were identified in all instances by comparison with authentic materials.

TABLE 1. *R_F* values in solvent systems.

Substance	A	B
Uridine-3' phosphate	0.33	0.16
Dimethyl ester	0.76	—
Monomethyl ester	0.44	0.41
Dibenzyl ester	0.91	—
Monobenzyl ester	0.66	0.62
Uridine-2' : 3' phosphate	0.39	0.37

Uridine-3' Dimethyl Phosphate.—Anhydrous uridine-3' phosphate (0.1 g.) in dry methanol (3 c.c.), cooled in ice-water, was titrated to *ca.* pH 4 with ethereal diazomethane. A small amount of flocculent precipitate was removed and the solution was concentrated to small bulk, *in vacuo*, during which the product crystallised. A small amount of ethereal diazomethane was added to neutrality and, after addition of a little ether, the colourless needles of *uridine-3' dimethyl phosphate* (0.066 g.), *m. p.* 147–148° with gas evolution, were collected and washed with ether. The substance was immediately transferred to a vacuum-desiccator for storage. It was very soluble in water (Found : C, 37.6; H, 4.85; N, 8.1. C₁₁H₁₇O₆N₂P requires C, 37.6; H, 4.85; N, 8.0%).

Uridine-3' Dibenzyl Phosphate.—Uridine-3' phosphate (0.165 g.) was dissolved in methanol (3 c.c.), and a solution of phenyldiazomethane in ether added dropwise with cooling in an ice-bath. Addition was continued until the disappearance of red colour was slow. A small amount of amorphous material was removed at a centrifuge, and the clear solution evaporated under reduced pressure. The residual gum was dissolved in chloroform (5 c.c.) and the solution quickly washed with ice-cold sodium hydrogen carbonate solution and water, and then dried (Na₂SO₄). Removal of the chloroform *in vacuo* gave a gum which was triturated successively with ether (3 × 4 c.c.) and thiophen-free benzene (2 × 4 c.c.) and then dried at 30° over phosphoric oxide at 10⁻⁴/mm. (Found : C, 56.6; H, 5.3; N, 5.45. C₂₂H₂₄O₆N₂P requires C, 54.9; H, 4.7; N, 5.6%). The product was still contaminated with traces of phenyldiazomethane degradation products but was essentially the desired substance as shown by its reactions (below). The ether and the benzene extracts contained considerable quantities of the dibenzyl ester, contaminated with phenyldiazomethane degradation products.

The dibenzyl ester (200 mg.) in methanol (1 c.c.) was hydrogenated over a mixture (5 mg.) of palladium oxide and palladised charcoal. After 24 hr. paper chromatography showed that complete conversion into uridine phosphate had occurred. The solution was brought to pH 8

with dilute aqueous ammonia, and an aliquot part (containing 5 mg. of uridine phosphate) was run on to a column (1 × 10 cm.) of Dowex-2 resin (200—400 mesh; formate form) and the nucleotide eluted with sodium formate-formic acid buffer, as described earlier (Brown, Dekker, and Todd, *J.*, 1952, 2715). Only one peak was obtained corresponding in position (fraction 78) and optical density ratio (280/260 m μ , 0.33) to uridine-3' phosphate.

Hydrolysis of Uridine-3' Dibenzylophosphate.—The ester (25 mg.) was dissolved in a mixture (2 c.c.) of propan-2-ol, water, and ammonia (*d* 0.88) (70 : 25 : 5), set aside in the ice-chest overnight, and then evaporated to dryness *in vacuo*. The residue was shown by paper chromatography to consist of ammonium uridine benzyl phosphate with a trace of uridine-2' : 3' phosphate. The same result was observed on spotting the dibenzyl ester at the origin of a chromatogram and running in the ammoniacal solvent.

The ammonium salt (7.0 mg.) was hydrogenated in water (2 c.c.), and the nucleotide content of the solution examined by ion-exchange chromatography, as above. Two peaks of equal height corresponded to uridine-2' and -3' phosphate, demonstrating that the uridine benzyl phosphate was a mixture of approximately equal parts of the 2'- and 3'-isomers.

Another sample of the ammonium salt (3.6 mg.) in water (0.36 c.c.) was mixed with a solution (0.1 c.c.) of ribonuclease (10 mg./c.c.) and the pH of the solution was adjusted to 8.5 with very dilute aqueous ammonia (0.1 c.c.). After two days the solution was analysed on paper chromatograms. Only two spots were present, corresponding to uridine benzyl phosphate and uridine phosphate. Elution of the spots with 0.1N-hydrochloric acid (4 c.c.) and determination of the optical density of the solutions at 260 m μ gave the ratio, uridine phosphate : benzyl ester, 1.1. After 7 days no change in the amount of remaining benzyl ester had occurred. Added uridine-2' : 3' phosphate (2.1 mg.) was hydrolysed overnight to uridine-3' phosphate, showing that the enzyme was still active.

Hydrolysis of Uridine-3' Dimethyl Phosphate.—(a) *Propan-2-ol-water-ammonia.* It was initially observed that the methyl ester, when spotted on a chromatogram and run in the ammoniacal solvent, gave one spot corresponding to authentic uridine methyl phosphate (R_F 0.44; Brown, Magrath, and Todd, *loc. cit.*).

The dimethyl ester (2.0 mg.) was dissolved in the above solvent and set aside for several hr. at room temperature, the solution evaporated under reduced pressure, and phosphate buffer (0.2 c.c.; pH 7) containing ribonuclease (2.5 mg./c.c.) added. After incubation at 37° overnight the solution was chromatographed on paper. Two spots were present corresponding to uridine phosphate and unchanged methyl ester. These were present in about equal quantity (visually estimated). That the enzyme was still active was shown by its ability to convert added uridine-2' : 3' phosphate into uridine-3' phosphate.

(b) *Buffer solutions.* The dimethyl ester (3 mg.) was dissolved in the following solutions (0.2 c.c.): phosphate-citrate buffers (Vogel, "Quantitative Inorganic Analysis," Longmans Green and Company, London, 1951, p. 869) of pH 2.2, 3.0, 5.0, 7.0, in a standard buffer pH 9.0, and in 0.1N- and N-hydrochloric acid. The solutions were incubated at 37° and drops were removed at 0.25, 1.25, 4.25, and 16 hr. intervals. These were spotted on chromatograms which were run in solvent systems A and B. The results are collected in Table 2 where the symbols +, ++, and +++ refer to <10%, *ca.* 50% and >90% hydrolysis of uridine dimethyl phosphate, as judged visually from the intensity of the spots on photographs of the chromatograms in ultraviolet light. In the Table, pH 0 and 1 refer to the N- and 0.1N-hydrochloric acid solutions. In all cases the product of hydrolysis was uridine methyl phosphate together with some uridine-2' : 3' phosphate. Further hydrolysis to uridine phosphate occurred below pH 3.

TABLE 2. *Hydrolysis of uridine-3' dimethyl phosphate.*

Time (hr.)	pH						
	0	1	2.2	3.0	5.0	7.0	9.0
0.25	++	+			+	+	++
1.25	+++	++	+	++	++	++	+++
4.25	+++	+++	+	++	+++	+++	+++
16	+++	+++	+++	+++	+++	+++	+++

Action of Ribonuclease on Uridine-3' Dimethyl Phosphate.—In view of the great lability of the triester it was impossible to work at the pH optimum of the enzyme (*ca.* pH 8). Massive amounts of the enzyme were therefore used at a low pH. The dimethyl ester (2.0 mg.) in phosphate-citrate buffer (0.2 c.c., pH 3 and 4) containing 5 mg. of enzyme per c.c., was kept at 18°, and drops were applied at intervals to chromatograms. Controls consisted of (1) solutions without the

enzyme and (2) uridine-2' : 3' phosphate (2.0 mg.) with and without enzyme. The rate of disappearance of the dimethyl ester was the same with and without enzyme although further degradation by the enzyme of the liberated monomethyl ester and cyclic phosphate (including cyclic phosphate added at the end of the experiment) was evident. That the enzyme was active at pH 3 was confirmed by the complete hydrolysis of uridine-2' : 3' phosphate in 5 hr. The experiment showed that the enzyme was not active against the dimethyl ester.

Action of Trifluoroacetic Anhydride on Cytidine-2' (and -3') Phosphate.—Anhydrous cytidylic acid (0.1 g.) and trifluoroacetic anhydride (0.6 c.c.) were shaken together. Dissolution was complete in 15 min. and next morning the excess of anhydride was removed *in vacuo* with the strict exclusion of moisture. The residual glass was treated with dry ethanolic ammonia and kept stoppered for 15 min. at 0°. The solid product, which consisted of *ca.* 70—80% of cytidine-2' : 3' phosphate, was removed at a centrifuge and discarded. The ethanolic solution was evaporated *in vacuo*, to give a gum. Paper chromatography in propan-2-ol–water showed it to consist of *ca.* 70% cytidine-2' : 3' phosphate together with a fast-running neutral material (R_F 0.72). The latter product was freed entirely from cyclic phosphate by chromatography on Whatman No. 3. paper, followed by elution with absolute ethanol of the relevant section of the paper. Insufficient material was isolated for characterisation (it appeared to crystallise in needles) but the substance evidently was cytidine diethyl phosphate since paper chromatography in the propan-2-ol–water–ammonia converted it entirely into a material having the same characteristics as cytidine ethyl phosphate (R_F 0.46) (Brown, Magrath, and Todd, *J.*, 1952, 2708).

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