416. *Nucleotides. Part XXI.* The Action of Ribonuclease on Simple Esters of the Monoribonucleotides.*

By D. M. Brown and A. R. Todd.

Preparation of uridine benzyl phosphates *a* and *b* as their mixed barium salts, barium uridine benzyl phosphate *b*, and the two crystalline isomers, cytidine benzyl phosphate *a* and *b* is described. Ribonuclease is found to effect hydrolysis of the *b* isomers only, yielding uridylic and cytidylic acids *b*, *via* the corresponding cyclic nucleoside-2′ : 3′ phosphates. Other comparable observations with methyl and ethyl esters are described. Benzyl esters of the adenylic acids and of uridine-5′ phosphate are not attacked by ribonuclease.

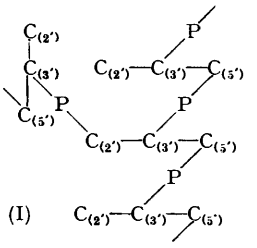
The mechanism and specificity of ribonuclease action are discussed in relation to chemical hydrolytic mechanisms. Structural features of the ribonucleic acids are discussed in the light of these observations.

The problem of the structure of macromolecular substances is complicated by the great number of theoretically possible isomers. In the case of the ribonucleic acids this isomerism might arise not only from the arrangement of the four constituent nucleotides, but also from the possible variety of phosphodiester internucleotidic linkages involving the 2′-, 3′-, and 5′-positions in the nucleoside residues. In an earlier publication (Brown and Todd, Part X, *J.*, 1952, 52) general structures for the ribonucleic acids were discussed and (I), in which the extent of chain-branching is unspecified, was put forward as a working hypothesis to be tested by further experiment. This structure was reached from consideration of the constitution of the mononucleotides derived from chemical and enzymic hydrolysates of nucleic acids and of the mechanism of the hydrolytic processes involved.

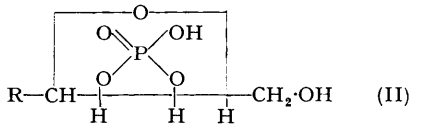
When ribonucleic acids are hydrolysed with alkali, the only products are the 2′ and 3′ phosphates of the nucleosides, adenosine, guanosine, uridine, and cytidine. These, it was held, were produced by way of the intermediate nucleoside-2′ : 3′ phosphates, by a mechanism for which adequate analogies were available. One important conclusion which emerged was that the hydrolysis of an ester of a mononucleotide (*e.g.*, III) should yield a mixture of the 2′ and the 3′ nucleotide regardless of the position of the phosphoryl group

* Part XX, *J.*, 1953, 951.

(2′ or 3′) in the original diester. Experimentally, adenosine benzyl phosphate *a* or *b* with sodium hydroxide yielded a mixture of adenylic acids *a* and *b* (Brown and Todd, Part IX, *J.*, 1952, **44**), and synthetic nucleoside-2′ : 3′ phosphates (Brown, Magrath, and Todd, *J.*, 1952, 2708) likewise yielded mixtures of the *a* and *b* mononucleotides under mild conditions. Because of these facts we pointed out (Part X) that structures for the ribonucleic acids differing from (I) in that $C_{(2')}$–$C_{(5')}$ internucleotide linkages are present, would in theory, be equally admissible, since the products of hydrolysis would be the same in either case.



(I)



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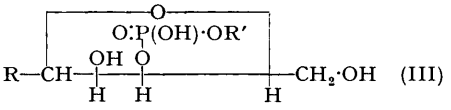


(III)

The true orientation of the phosphoryl group in the *a* and *b* isomers of the mononucleotides has not been fully established although physical evidence for the view that cytidylic acid *b* is cytidine-3′ phosphate has been advanced (Loring, Hammell, Levy, and Bortner, *J. Biol. Chem.*, 1952, **196**, 821; Cavalieri, *J. Amer. Chem. Soc.*, 1952, **74**, 5804). At present it will be most convenient to discuss whether *a*-$C_{(5')}$ or *b*-$C_{(5')}$ linkages, or both types, are present in the ribonucleic acids. Clearly, the arguments given above show that hydrolyses by acid or alkali at least in their present form do not permit any distinction to be made between the *a* and the *b* positions of the nucleoside residues as linkage points in the polynucleotides. We did, however, make the significant observation (Brown, Dekker, and Todd, *J.*, 1952, 2715) that both cytidine-2′ : 3′ phosphate and uridine-2′ : 3′ phosphate are hydrolysed by the enzyme ribonuclease, yielding only the *b* isomers of cytidylic acid and of uridylic acid. We discussed the relation between these observations and those made by others (*inter al.*, Markham and Smith, *Nature*, 1951, **168**, 406; Schmidt *et al.*, *J. Biol. Chem.*, 1951, **192**, 715) on the structure of the end-products of ribonuclease hydrolysis of ribonucleic acids, and their transformation products. The opening of the cyclic phosphoryl group was evidently the secondary action of the enzyme; the rupture of phosphodiester internucleotide linkages to yield products bearing the cyclic phosphoryl group was apparently its initial action. We decided to study this initial action, again using synthetic substrates whose structures could be closely defined. Particularly, it was considered necessary to prepare and study esters of the *a* and *b* nucleotides which could be related to the parent nucleotide by means other than hydrolysis. Benzyl esters were chosen since it was expected that hydrogenation would convert them into the free nucleotide without phosphoryl migration (cf. Brown, Abstr. Papers 2nd Internat. Congr. Biochem., Paris, 1952, p. 253; Todd, *Angew. Chem.*, 1953, **65**, 12).

By treating mixed adenylic acids *a* and *b* with phenyldiazomethane in dimethylformamide it was possible to prepare adenosine benzyl phosphates *a* and *b* in fair yield (Part IX). Application of this procedure to ordinary yeast uridylic acid and yeast cytidylic acid yielded analogously uridine benzyl phosphate and cytidine benzyl phosphate, isolated in the crude state as their barium salts. When treated with alkali they were hydrolysed to the corresponding mononucleotide mixture, as were the adenosine derivatives (Part IX).

When barium cytidine benzyl phosphate was treated with ribonuclease and the reaction followed on paper chromatograms it was found that hydrolysis of the ester occurred and cytidylic acid accumulated. Furthermore, cytidine-2′ : 3′ phosphate, which was present only in traces in the starting material, persisted in the solution at an approximately constant concentration during the hydrolysis and then disappeared when hydrolysis was complete. This was good evidence that cytidine-2′ : 3′ phosphate was an intermediate in the hydrolysis. In these experiments it was noticed that the spot on the chromatograms corresponding to the benzyl ester never disappeared completely, even after exhaustive

treatment with ribonuclease. Exactly comparable observations were made during the treatment of uridine benzyl phosphate with ribonuclease; uridine-2′ : 3′ phosphate was present in the solution during the hydrolysis and disappeared at the end, while some benzyl ester remained after prolonged treatment.

These phenomena were studied more closely by analysis of the materials before and after treatment with ribonuclease on a strong base anion-exchange column (Dowex-2). The crude preparation of barium cytidine benzyl phosphate, when analysed on the ion-exchange column with 0·02N-formic acid as eluant, showed several minor peaks, including two corresponding to cytidylic acid *a* and *b* (and the coincident cyclic phosphate). Further elution with 0·1N-formic acid removed the two major components, evidently the *a* and *b* benzyl esters (Fig. 1). Analysis of ribonuclease-treated material gave the elution diagram shown in Fig. 2. It was clear that one of the esters was hydrolysed and converted into cytidylic acid *b* since the position of the new peak and the optical density ratio over the peak (280/260 mμ) corresponded to that of the *b*-acid assessed by previous standardisation of the column with the pure *a*- and *b*-acids. Moreover, by integrating the optical-density units under each peak, it become clear that after the enzymic hydrolysis there was a correspondence between the increase in the cytidylic acid *b* peak and the disappearance of one of the benzyl ester peaks, while the other ester component did not change in quantity as estimated by this method. These estimations were semiquantitative and hence only indicative, since it was assumed that all the substances involved had the same extinction coefficient.

The mixed cytidine benzyl phosphates were prepared on a larger scale and by ion-exchange chromatography it was possible to isolate the two benzyl esters in pure crystalline form. The ester which was eluted first was oriented by hydrogenation over palladium oxide. It yielded a product which when analysed on the ion-exchange column was found to be cytidylic acid *a*; the ester was therefore cytidine benzyl phosphate *a*. It was found to be completely unaffected by ribonuclease. The second ester removed from the column gave the ion-exchange analysis diagram shown in Fig. 3. Hydrogenation gave cytidylic acid *b* (Fig. 4), showing that the ester was cytidine benzyl phosphate *b*. Treatment with ribonuclease also resulted in complete conversion into cytidylic acid *b* (Fig. 5). In the last experiment, paper chromatography showed that shortly after the start of the hydrolysis cytidine-2′ : 3′ phosphate was present in the solution although it could not be detected in the starting material or at the end of the hydrolysis. When cytidine benzyl phosphate *b* was set aside in 0·5N-sodium hydroxide at 37° overnight it yielded a solution which was shown by ion-exchange chromatography to contain only cytidylic acids *a* and *b* (*ca.* 40% and 60% respectively) in accord with the phosphoryl migration observed during the hydrolysis of adenosine benzyl phosphates *a* and *b* (Part IX).

When the barium salt of uridine benzyl phosphate was analysed on the ion-exchange column two peaks were observed, presumably corresponding to the ester components, together with a minor one due to contamination by uridine-2′ : 3′ phosphate (Figs. 6 and 8). Analysis of ribonuclease-treated material gave the elution diagram shown in Fig. 7, from which it could be seen that the major ester component (and the small amount of cyclic phosphate) had been converted into uridylic acid *b* (the position of which was known by prior standardisation of the column with uridylic acids *a* and *b*). The ester present in smaller amount (20—24%) was apparently unaffected by the enzyme. The difficulty of separating and isolating the two esters in a pure form, due mainly to the high ionic strengths of the buffers required to remove them from the ion-exchange resin, made it necessary to characterise them by other means. The mixed esters as their barium salts (20 mg.) were treated with ribonuclease and incubated until reaction was complete. The solution was applied to a paper strip and chromatographed in the *iso*propyl alcohol–water–ammonia system. Uridylic acid remained near the origin and the unchanged benzyl ester (R_F, 0·6; *ca.* 3—4 mg.) was eluted from the paper and the solution hydrogenated over palladium oxide. Ion-exchange analysis of the product showed that it was entirely uridylic acid *a*. It followed that the ester which was not attacked by ribonuclease was uridine benzyl phosphate *a* and that which was hydrolysed was uridine benzyl phosphate *b*. Confirmation of this was obtained by using another preparation of barium uridine benzyl phosphate

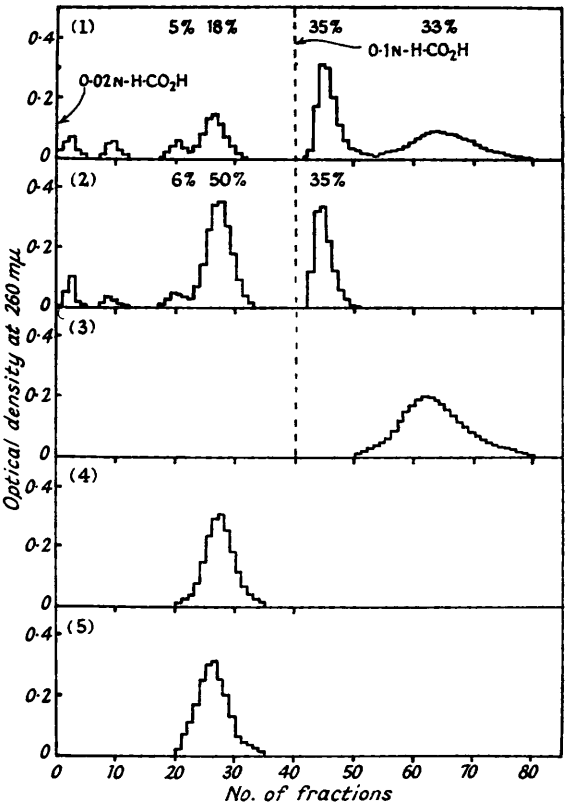


FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

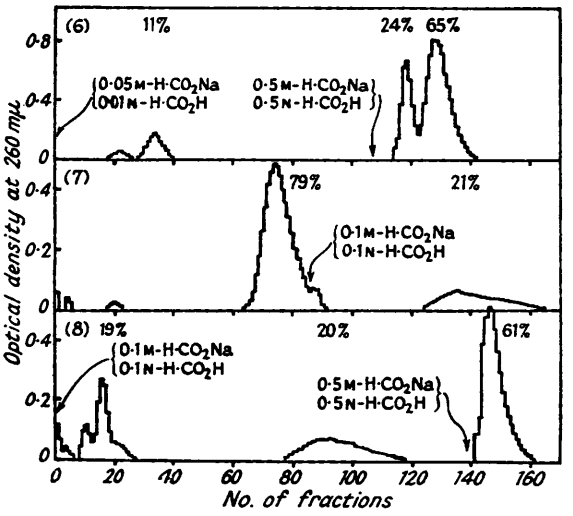


FIG. 6

FIG. 7

FIG. 8

Ion-exchange analysis diagrams of :

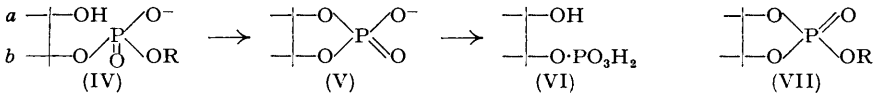
(1) Cytidine benzyl phosphates *a* and *b*. (2) Product of action of ribonuclease on (1). (3) Cytidine benzyl phosphate *b*. (4) Hydrogenation product from (3) (cytidylic acid *b*). (5) Product of ribonuclease action on (3). (6) and (8), Uridine benzyl phosphates *a* and *b*. (7) Product from ribonuclease action on (6).

Figures relative to peaks represent the optical density units in each peak as a percentage of total optical density units eluted. In standard runs cytidylic acids *a* and *b* had peaks at fractions 21 and 28 respectively, and uridylic acids *a* and *b* at fractions 60 and 71 respectively.

prepared under milder conditions from crystalline uridylic acid *b* (kindly provided by Mr. D. I. Magrath). This material was completely hydrolysed to uridylic acid by ribonuclease (paper-chromatographic examination) and on hydrogenation gave only uridylic acid *b* (ion-exchange analysis).

In addition to the above experiments using benzyl esters some observations have been made with other simple alkyl esters. Cytidine methyl phosphate (see below) was readily hydrolysed by the enzyme and showed the same phenomenon as the benzyl esters, *viz.*, a small amount was unaffected after exhaustive treatment. Mr. D. I. Magrath has found (in unpublished work) that uridine methyl phosphate and uridine ethyl phosphate (Brown, Magrath, and Todd, *loc. cit.*) are hydrolysed by ribonuclease, again leaving a small amount of material unaffected. It seems very probable that in these cases, too, the *b* isomer is hydrolysed, while the *a* isomer is stable towards the enzyme. The close analogy with the benzyl esters makes this likely and we feel justified in utilising this reactivity of esters of the *b*-pyrimidine nucleotides towards ribonuclease for the orientation of compounds of this type whose structures cannot otherwise be defined. To complete the model experiments on the specificity of the enzyme, uridine-5′ benzyl phosphate, adenosine-5′ benzyl phosphate, and adenosine benzyl phosphates *a* and *b* were treated with ribonuclease. In no case could evidence of reaction be obtained.

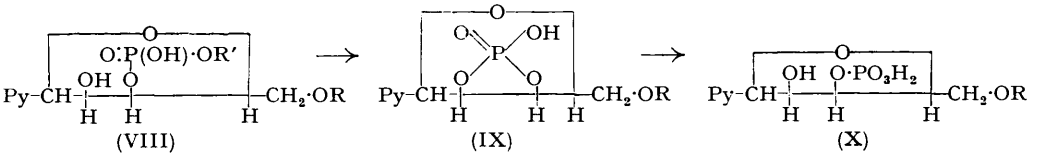
The implications of the above observations must now be assessed, first, regarding the intimate action of the enzyme and, secondly, as an aid to the further structural elucidation of the ribonucleic acids. Ribonuclease acts as a diesterase specifically hydrolysing esters of the *b*-isomers of the pyrimidine nucleotides. The observable sequence follows that given in the partial formulæ (IV—VI). The initial reaction (IV) ⟶ (V) is formally equivalent to a trans-esterification in which the $C_{(a)}$-O–P bond is formed with elimination of the esterifying group (R) as the alcohol, ROH. The second, slower reaction involves the hydrolysis of the cyclic phosphoryl group in this case by fission of the $C_{(a)}$-O–P bond. The nature of the esterifying group (R) does not appear to be important.



In essence the enzymic hydrolysis is similar to that which we proposed for the chemical hydrolysis of nucleotide esters (Part X) with the exception that the enzyme is more specific in that it attacks only esters of the *b*-isomers of the pyrimidine nucleotides. In our earlier discussion of the chemical hydrolysis we invoked for simplicity in representation an intermediate to which we gave the classical formulation (VII); this then broke down, yielding (V) and the alcohol, ROH. It seems likely that the chemical hydrolytic process depends on the attack by the vicinal hydroxyl group on the P=O bond, catalysed by hydrogen or hydroxyl ions, with simultaneous removal of R as an alkoxy-anion, possibly with the intervention of a quinquecovalent transition complex. The enzymic hydrolysis probably proceeds in a similar manner, the formation, and subsequent cleavage, of the $C_{(a)}$-O–P bond representing the fundamental catalytic steps. Since the first step proceeds without increase in the number of phosphoryl dissociations, this may represent the rapid initial "depolymerisation" observed in the action of the enzyme on ribonucleic acids during which no titratable groups are liberated (cf. Chantrenne, Linderstrøm-Lang, and Vandendriessche, *Nature*, 1947, **159**, 877).

When ribonuclease acts on ribonucleic acids the first products have properties consistent with the structure (IX; Py = uracil or cytosine residue; R = H or remainder of polynucleotide chain) (Markham and Smith, *loc. cit.*; *Biochem. J.*, 1952, **52**, 552, 558). As a further action, ribonuclease then opens the cyclic phosphoryl groups, yielding products of the general type (X; R = H or remainder of the polynucleotide chain) in which the terminal phosphoryl group occupies the *b*-position in the pyrimidine nucleoside residue (Brown, Dekker, and Todd, *loc. cit.*). The work described here leads to the conclusion that the first action of the enzyme is to cleave phosphodiester linkages to products of type (IX; Py= uracil or cytosine residue; R = H or remainder of polynucleotide chain). Furthermore,

the nature of the specificity as shown here with substrates of defined structure permits the conclusion that such cleavage should occur only at pyrimidine nucleotide residues which are linked at the *b*-position through a phosphoryl group to the next nucleoside residue (R′) as in (VIII); the identification of the *b* with the 3′-position in the pyrimidine nucleotides is supported by the physical data obtained by Loring, Hammell, Levy, and Bortner



(*loc. cit.*) and by Cavalieri (*loc. cit.*) for the isomeric cytidylic acids. For the reaction sequence (VIII) ⟶ (X) to be possible, the remainder of the polynucleotide chain (R in VIII) must be linked at the $C_{(5')}$-position of the pyrimidine nucleotide residue. The work of others (*inter al.*, Schmidt *et al.*, *J. Cell. Comp. Physiol.*, 1951, Suppl. 1, **38**, 61; *J. Biol. Chem.*, 1951, **192**, 715; Markham and Smith, *loc. cit.*; Cohn, personal communications) shows that all, or almost all, of the pyrimidine nucleotide residues involved in diester linkage in the ribonucleic acids are affected during ribonuclease action, so that at all these positions in the intact molecule the structure is of the form (VIII; R = R′ = polynucleotide chain). This does not preclude a similar situation existing at purine nucleotide sites in the nucleic acid molecule since the stability of the esters of the adenylic acids towards ribonuclease provides evidence that the previously assumed pyrimidine specificity is correct and does not depend on a difference in linkage point on the purine nucleoside residues. Other evidence supports the view that the purine nucleoside residues are linked at the *b*(or *a*)- and $C_{(5')}$-positions [VIII; Pu (=purine residue) in place of Py· (Part X, *loc. cit.*; Cohn and Volkin, *Nature*, 1951, **167**, 483; *Arch. Biochem. Biophys.*, 1952, **35**, 465; Merrifield and Woolley, *J. Biol. Chem.*, 1952, **197**, 521], and hydrolytic experiments with spleen nuclease suggest that here also the *b*-position is favoured (Volkin and Cohn, *Fed. Proc.*, 1952, **11**, 303).

It should, of course, be borne in mind that although the phosphoryl groups in uridylic acid *b* and cytidylic acid *b* occupy the same position (probably 3′) in the sugar residue (Loring and Luthy, *J. Amer. Chem. Soc.*, 1951, **73**, 4215; Brown, Dekker, and Todd, *loc. cit.*) no clear evidence exists as yet to show that the phosphoryl group is similarly located in the *b* isomers of the purine nucleotides.

The experiments described in this paper clarify the specificity and mode of action of crystalline pancreatic ribonuclease. They also provide additional confirmation for the general structural pattern (I) of the ribonucleic acids advanced by us in Part X, and limit the linkage of the pyrimidine nucleoside residues to the *b* position; they provide no further information about the position of linkage in the purine nucleoside residues. In Part X we discussed other types of internucleotidic linkage than that adopted in structure (I). Of these other types the exclusion of $C_{(5')}$–$C_{(5')}$ as a possibility on chemical grounds has since been justified by study of a synthetic dinucleoside-5′ : 5′ phosphate (Elmore and Todd, *J.*, 1952, 3681). As regards $C_{(2')}$–$C_{(3')}$ linkage, it was pointed out that in such a case alkaline hydrolysis, proceeding as it does through a cyclic intermediate, could only occur stepwise from the end of the chain. If we accept the findings of other workers on the nature of the end-products of ribonuclease action and the results obtained by Merrifield and Woolley (*loc. cit.*) by acid hydrolysis of ribonucleic acids, our own observations on ribonuclease action virtually exclude $C_{(2')}$–$C_{(3')}$ as an internucleotidic linkage save between a residue in the main chain and the first residue in a side-chain or branch. They do not, of course, permit any conclusion as to the occurrence or extent of chain-branching, but they do reinforce our earlier view (Part X) that if chain-branching (other than branching on phosphorus) occurs it can only do so in the manner indicated in (I), *i.e.*, the first residue in the branch must be a pyrimidine nucleoside linked through its *b* position to a residue in the main chain.

One observation has been made, unconnected with the main problem, which deserves

mention. When cytidylic acid was treated with phenyldiazomethane in dimethylformamide the reaction was very slow, owing, probably, to the very low solubility of the nucleotide. During the large-scale preparation of cytidine benzyl phosphates *a* and *b* a considerable amount of methanol was added and the reaction rate increased markedly. The solution, when submitted to ion-exchange chromatography, afforded a material in considerable yield which was eluted from the column before the other main products. It crystallised well and gave analytical values corresponding to cytidine methyl phosphate. Chromatographic behaviour and hydrolyses with sodium hydroxide and with ribonuclease were in accord with this formulation. This observation recalls the disputed claim that treatment of stilbœstrol with diazomethane in presence of *iso*propyl alcohol yields di*iso*propylstilbœstrol (Schonberg and Mustafa, *J.*, 1946, 746; Gerber and Curtin, *J. Amer. Chem. Soc.*, 1949, **71**, 1499; Weygand and Kirchner, *Angew. Chem.*, 1952, **64**, 203).

Experimental

Paper Chromatography of the Nucleotide Derivatives.—The solvent system most widely used in the present investigation was *iso*propyl alcohol–ammonia–water (70 : 10 : 20 v/v). This gave excellent separation of the nucleotide esters from the corresponding nucleotides and nucleoside cyclic phosphates (cf. Brown, Magrath, and Todd, *loc. cit.*). Other solvent systems used are mentioned at the appropriate place in the text. Spots were detected by photographing the chromatograms in ultra-violet light. Standard substances were always run simultaneously for identification purposes.

Ion-exchange Analyses.—A column (11·2 × 1 cm.) of Dowex-2 (200—400 mesh) in the formate form was used throughout. A flow rate of approx. 0·5—0·7 c.c./min. was maintained by application of pressure. Fractions were collected in an automatic apparatus (syphon type, giving fractions of *ca.* 11·5 c.c.). The solutions used for the analytical separations of the cytidylic and uridylic acids and the corresponding cyclic phosphates have been described before (Cohn and Volkin, *locc. cit.*; Brown, Dekker, and Todd, *loc. cit.*). Solutions of greater eluting power were required to remove the nucleotide benzyl esters from the column. These are recorded in the Figures, where some elution diagrams are shown. As before, substances were identified by the number of fractions to the peak and, where applicable, by the optical density ratios (280/260 mμ) over the peak.

Cytidine Benzyl Phosphates a *and* b.—*Initial preparation.* Cytidylic acid (0·3 g.; prepared from yeast nucleic acid) was suspended in dimethylformamide (3 c.c.) and treated with phenyldiazomethane (from 2·3 g. of benzaldehyde hydrazone). After the mixture had been shaken overnight little reaction had occurred. A small amount of methanol was added, and after 12 hr. the mixture was homogeneous. Ether (40 c.c.) was added and the solvent decanted from the gum which separated. The gum was shaken with chloroform and water, and the aqueous layer further extracted with chloroform and then reduced in bulk. Saturated barium hydroxide solution (0·6 c.c.) was added, and the solution brought to neutrality with carbon dioxide, boiled, filtered, and reduced to small bulk. Addition of ethanol (40 c.c.) precipitated the product (58 mg.) which was collected by centrifugation. This material, which was not pure enough for analysis, was a crude mixture of barium cytidine benzyl phosphates *a* and *b* on evidence detailed below. It was used for enzymic experiments. Ion-exchange analysis of the material (3·5 mg.) gave the diagram shown in Fig. 1. Paper chromatography showed that it consisted of a major component—the benzyl esters (R_F 0·7)—together with traces of cytidylic acid (R_F 0·1) and cytidine-2′ : 3′ phosphate (R_F 0·4). When the material (4·0 mg.) was treated with 0·5N-sodium hydroxide (0·2 c.c.) at 37° for 15 hr. complete conversion into cytidylic acid was shown by paper chromatography.

Large-scale preparation. Cytidylic acid (0·75 g.) was treated with phenyldiazomethane (from 3·0 g. of benzaldehyde hydrazone) in a mixture of dimethylformamide (25 c.c.) and methanol (25 c.c.). After 36 hr. reaction was complete and the product was precipitated by addition of ether (400 c.c.). The gum was shaken with chloroform, water, and barium carbonate, and the aqueous phase was washed with more chloroform and evaporated to small bulk. Addition of much ethanol precipitated the crude barium salt (0·64 g.). A solution of the material (18 mg.) was brought to pH 8, and analysed on the ion-exchange column. In addition to the two benzyl ester peaks (cf. Fig. 1) another major product was observed (peak at fraction 10). This product was isolated (see below) and shown to be cytidine methyl phosphate. For large-scale separation the crude product (0·62 g.) was dissolved in water (50 c.c.), and the solution brought to pH 8 and run on to a column (10·5 × 3 cm.) of Dowex-2 resin (formate form).

After washing of the column with water, elution was commenced with 0·02N-formic acid. The first component (cytidine methyl phosphate) was removed (volume to peak, 400 c.c.) and the fractions containing this material were bulked and worked up (see below). Elution was continued until all mononucleotide and cyclic phosphate had been removed from the column. 0·1N-Formic acid then removed cytidine benzyl phosphate *a* from the column, and then cytidine benzyl phosphate *b* was rapidly eluted with 0·5N-formic acid.

The fractions containing cytidine methyl phosphate were pooled and evaporated under reduced pressure; the residue crystallised from water containing a little ethanol. *Cytidine methyl phosphate* separated in colourless irregular prisms (52 mg.), slowly melting, with decomp. from 210° (Found : C, 36·0; H, 4·6; N, 12·6; P, 9·0. $C_{10}H_{16}O_8N_3P$ requires C, 35·7; H, 4·8; N, 12·45; P, 9·2%). The material (1·0 mg.) was analysed on the ion-exchange column in 0·02N-formic acid. Only one peak was observed (at fraction 10) with an optical density ratio of 1·96. The substance had R_P 0·5 in the *iso*propyl alcohol–ammonia–water system. The material (0·05 mg.) was dissolved in 0·5N-sodium hydroxide (0·1 c.c.) and kept at 37° for 15 hr. Paper chromatography showed complete hydrolysis to cytidylic acid.

The fractions containing *cytidine benzyl phosphate* a were pooled and evaporated to dryness under reduced pressure below room temperature. The product crystallised from water containing a little ethanol in colourless needles (48 mg.). The hydrated material was dried for analysis at 105° for 24 hr. over phosphoric oxide. It sintered at 168° and melted at 174° (Found, C, 46·4; H, 5·1; N, 10·4; P, 6·8. $C_{16}H_{20}O_8N_3P$ requires C, 46·6; H, 4·9; N, 10·2; P, 7·5%).

The material (1·1 mg.) was hydrogenated over palladium oxide for 2 hr. at room temperature and pressure. The product was then analysed on the ion-exchange column in the usual way. The elution diagram showed only one peak corresponding in position (fraction 20) and optical density ratio (1·74) to that given by authentic cytidylic acid *a*.

The pooled fractions containing *cytidine benzyl phosphate* b were evaporated under reduced pressure below room temperature, water being added in the later stages of the evaporation to reduce the formic acid concentration. The product crystallised from water in small prisms (66 mg.), which slowly decomposed from 170° with final m. p. 203° (Found, in material dried at 105°/vac. over phosphoric oxide : C, 46·7; H, 5·0; N, 10·2; P, 7·1%).

The substance (1·5 mg.) when analysed on the ion-exchange column showed only one peak on the diagram (Fig. 3) with an optical density ratio of 1·85 over the peak.

Hydrogenation of the material (1·5 mg.) over palladium oxide gave one product identical with cytidylic acid *b* in its ion-exchange characteristics (Fig. 4; peak at fraction 28 with an optical density ratio of 2·0).

Cytidine benzyl phosphate *b* (1·5 mg.) was treated with 0·5N-sodium hydroxide (0·2 c.c.) at 37° for 15 hr. Paper chromatography showed that complete hydrolysis to mononucleotide had occurred. After neutralisation with formic acid and dilution, the solution was analysed on the ion-exchange column. Two peaks were observed corresponding to cytidylic acids *a* and *b* (40% and 60% of total optical density units with optical density ratios of 1·8 and 2·0 respectively.

Barium Uridine Benzyl Phosphates a *and* b.—Uridylic acid (0·5 g.) was partially dissolved in dimethylformamide (3 c.c.), and phenyldiazomethane (from 1 g. of benzaldehyde hydrazone) added. The violent reaction was modified by cooling and next morning ether (40 c.c.) was added. The precipitated gum was washed with more ether. The gum was dissolved in a little ethanol and diluted with ether. A small amount of sticky solid separated and was discarded. The mother-liquors were evaporated to dryness, then shaken with water and chloroform, and the aqueous phase was washed again with chloroform. Saturated barium hydroxide solution (2 c.c.) was added to the aqueous solution, then carbon dioxide was passed in to neutrality. The solution was reduced to 5 c.c. and filtered through Hyflo Supercel. Addition of ethanol did not produce significant precipitation of the product, so the aqueous-ethanolic solution was evaporated under reduced pressure. The solid foam was very hygroscopic and gave poor analytical values, but was used as such for the enzymic experiments [Found, in material dried at 50°/0·1 mm. over phosphoric oxide : C, 38·2; H, 3·9; N, 6·9; P, 6·2; Calc. for $(C_{16}H_{18}O_9N_2P)_2Ba$: C, 39·8; H, 3·8; N, 5·8; P, 6·4%].

Paper chromatography showed that this material gave one spot due to the benzyl esters (R_P 0·7) and another corresponding to a very small amount of uridine-2′ : 3′ phosphate (R_P 0·35). Ion-exchange analysis of the product (Fig. 6) showed that in addition to uridine-2′ : 3′ phosphate (11%) two major products were present (24% and 65%) corresponding to uridine benzyl phosphates *a* and *b* respectively (see below). Another, better separation of the two isomers is shown in Fig. 8 (20% and 61% respectively). Treatment of the material with 0·5N-sodium

hydroxide at 37° overnight, and paper chromatography of the product, showed complete conversion into uridylic acid.

Uridine Benzyl Phosphate b.—Uridylic acid (0·5 g. of the crystalline *b*-isomer) was dissolved in dimethylformamide (5 c.c.) and treated dropwise with phenyldiazomethane (from 1 g. of benzaldehyde hydrazone) during 3 min. Ether was added to precipitate the product which was then shaken with chloroform, water, and barium carbonate. The aqueous layer was shaken with more chloroform, treated with charcoal, and evaporated under reduced pressure. The product was dissolved in water, and the solution filtered and freeze-dried. This crude barium salt (0·46 g.) was dissolved in water and converted into the free acid by passage through a column of Amberlite IRC-50 resin (hydrogen form). The product was dissolved in the *iso*-propanol–ammonia–water solvent (10 c.c.), containing added water to permit dissolution, and applied to a column (42 × 3·5 cm.) of cellulose powder (160 g. of 80-mesh). The column was developed with the *iso*propanol–ammonia–water and the fractions containing the product collected and evaporated. The residue was dissolved in water, ammonium ions were removed by passage through a column of Dowex-50 resin, and the eluate was freeze-dried. *Uridine benzyl phosphate* b was obtained as a fine hygroscopic powder (0·24 g.) (Found, in material dried at 60°/10^{-3} mm.: C, 45·6; H, 3·9; N, 7·3; P, 7·8. $C_{16}H_{19}O_9N_2P$ requires: C, 46·3; H, 4·6; N, 6·8; P, 7·5%).

A concentrated solution of the material, when set aside overnight at room temperature, was almost completely hydrolysed, under its own acidity, to uridylic acid. A small amount of uridine-2′ : 3′ phosphate was also present.

Hydrogenation of the above benzyl ester (3·7 mg. of the barium salt) in water (0·2 c.c.) over palladium oxide for 15 hr. yielded a solution which was analysed on the ion-exchange column. Only one main product was observed, corresponding to uridylic acid *b* (peak at fraction 69; optical density ratio, 0·32). Uridine-2′ : 3′ phosphate was present in traces (peak at fraction 30).

Action of Ribonuclease on Esters of the Mononucleotides.—Crystalline pancreatic ribonuclease (Armour) was used throughout. Ribonuclease hydrolyses were generally carried out with enzyme concentrations of 0·03 mg. per mg. of substrate in 0·1—0·2 c.c. of water at 37°. The pH was adjusted to *ca.* 8·3 before incubation by the addition of dilute ammonia solution. Buffers were not used, as this facilitated the analysis of the solutions. The materials studied were not affected by the slight changes of pH involved and were unchanged in control experiments in which they were incubated at the same pH but without enzyme. The activity of the enzyme was checked in each set of experiments by including a tube containing uridine-2′ : 3′ phosphate as substrate.

(*a*) *Cytidine derivatives.* The crude barium cytidine benzyl phosphates *a* and *b* (1·1 mg.) were treated as described above with ribonuclease (0·06 mg.) in water (0·4 c.c.). Drops were removed after 1, 2, and 4 hr. at 21° and after incubation overnight at 37°, and applied to paper chromatograms. The benzyl ester spot decreased but did not disappear. The spot corresponding to cyclic phosphate remained of constant intensity (up to 4 hr.) but had disappeared after overnight incubation. The amount of mononucleotide, only a trace at the beginning, increased with time.

The solution of ribonuclease-treated material (3·4 mg.) was analysed on the ion-exchange column. The analysis diagram (Fig. 2) showed that the component corresponding to cytidine benzyl phosphate *b* had disappeared and was replaced by another corresponding to cytidylic acid *b* (peak at fraction 28 with optical density ratio 2·04). Cytidine benzyl phosphate *a* (peak at fraction 45) was unaffected. Fig. 1 is an ion-exchange analysis diagram of untreated material (3·4 mg.).

Cytidine benzyl phosphate *b* (1·5 mg.) gave the ion-exchange diagram shown in Fig. 3. Treatment of this material (1·5 mg.) with ribonuclease (0·06 mg.), and paper chromatography of the solution showed that considerable amounts of cytidine-2′ : 3′ phosphate were present in the solution after 20 min. at room temperature and that after 15 hr. at 37° complete conversion into cytidylic acid had occurred. Analysis of this solution on the ion-exchange column showed that only cytidylic acid *b* was present (peak at fraction 27; optical density ratio 2·02).

Cytidine benzyl phosphate *a* (1·9 mg.) was treated in the usual way with ribonuclease (0·06 mg.). Paper chromatography showed that it was completely unaffected, even after 2 weeks.

Cytidine methyl phosphate (1·0 mg.), when treated with ribonuclease (0·03 mg.), was converted into cytidylic acid (presumably the *b* isomer). Paper chromatography showed that a small amount of the material having the same R_P value as the original ester was unaffected by

the enzyme, even after several days. This was due, undoubtedly, to a slight contamination of the crystalline *b* ester by cytidine methyl phosphate *a*.

(*b*) *Uridine derivatives.* The mixture of barium uridine benzyl phosphates *a* and *b* (2·0 mg.) was treated with ribonuclease (0·06 mg.) as in the case of the cytidine derivatives. Paper chromatography showed the presence of uridine-2′ : 3′ phosphate after 10, 30, and 90 min. but this disappeared after incubation at 37° overnight. Mononucleotide increased in amount during the hydrolysis. A comparison between ion-exchange diagrams of untreated (Fig. 6) and ribonuclease-treated material (6·0 mg.) (Fig. 7) showed that the benzyl ester, present in larger amount (*ca.* 65%), was converted into uridylic acid *b* (peak at 74; optical density ratio 0·33). The unchanged ester (*ca.* 24%) was shown, as follows, to be uridine benzyl phosphate *a*.

The barium salt of the mixed esters (18·2 mg.) was treated exhaustively with ribonuclease and then the solution was freeze-dried. After its dissolution in a small amount of water, the solution was applied to a paper strip (16-cm. line), and the chromatogram developed with the *iso*propyl alcohol–ammonia–water solvent. The strip containing the unchanged ester was eluted with water (0·2 c.c.), and the eluate hydrogenated over a mixed palladium oxide–palladium-charcoal catalyst for 24 hr. The solution was analysed on the ion-exchange column. The diagram showed only one component, corresponding to uridylic acid *a* in the position of the peak and the optical density over the peak (0·26). It followed that uridine benzyl phosphate *b* was alone hydrolysed by ribonuclease.

Barium uridine benzyl phosphate *b* (2·0 mg.) was treated with ribonuclease (0·06 mg.). After 12 hr. complete conversion into uridylic acid was shown by paper chromatography.

(*c*) *Other compounds.* Uridine-5′ (benzyl phosphate), adenosine-5′ (benzyl phosphate), and the mixed barium salts of adenosine benzyl phosphate *a* and *b* were each treated with ribonuclease in the usual way. In no case did paper chromatography disclose any action of the enzyme on these substances. In addition to the *iso*propyl alcohol–ammonia–water solvent, the butanol–acetic acid–water and disodium hydrogen phosphate–*iso*amyl alcohol solvents were used with the adenosine derivatives since these solvents effect separation of the *a* and *b* isomers of both the adenylic acids and their benzyl esters (Brown and Todd, Part IX, *loc. cit.*).

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