512. *Nucleotides. Part XIII.* The Action of Ribonuclease on the 2′ : 3′-Phosphates of Uridine and Cytidine.*

By D. M. Brown, C. A. Dekker, and A. R. Todd.

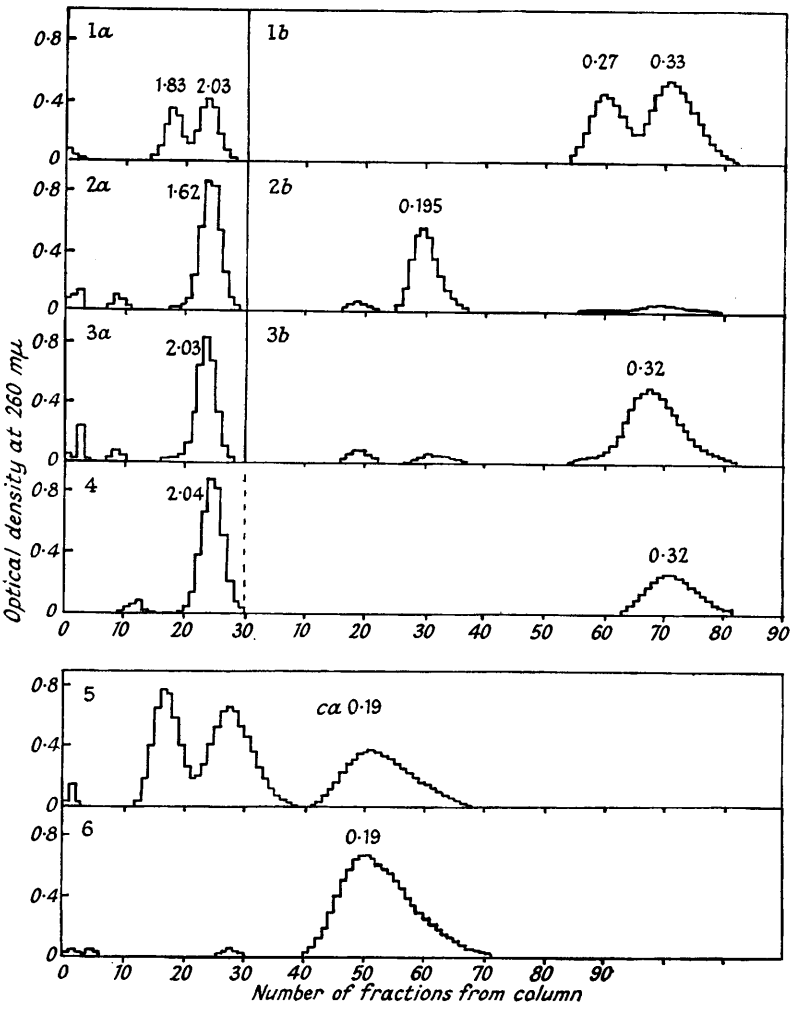
Ribonuclease is shown to convert cytidine-2′ : 3′ phosphate into cytidylic acid *b*, and uridine-2′ : 3′ phosphate into uridylic acid *b*; it has no action on adenosine-2′ : 3′ phosphate. Alkaline deamination of cytidylic acid *b* yields uridylic acid *b*, indicating that the phosphoryl group in these two compounds occupies the same position in the ribofuranose residue. Some features in the chemistry of the ribonucleic acids are discussed in the light of these observations.

In the structural study of the macromolecular nucleic acids it is evident that enzymic degradation must play an important part. This is especially true of the ribonucleic acids since chemical hydrolysis degrades them directly to mononucleotides without yielding the larger fragments (oligonucleotides) containing more than one nucleotide unit which are essential to a study of the sequence of nucleotide residues and the intimate detail of the internucleotidic linkage. The crystalline enzyme ribonuclease (Kunitz, *J. Gen. Physiol.*, 1940, **24**, 15) is a valuable tool in this respect since it is known to act on ribonucleic acids, yielding products of widely varying molecular weight, and further information on its specificity would be of considerable value. The present experiments were designed to correlate and clarify two apparently related observations on its action. Cohn and Volkin (*Nature*, 1951, **167**, 483) digested calf-liver ribonucleic acid, first, with ribonuclease and then with a phosphatase preparation from intestinal mucosa, arsenate being added to inhibit phosphomonoesterase activity (cf. Klein, *Z. physiol. Chem.*, 1933, **218**, 164); they found that, in addition to the 5′-mononucleotides and some unidentified materials, the digest contained substantial amounts of cytidylic acid *b* and uridylic acid *b*. The last two acids apparently arose directly from the initial action of the ribonuclease and remained unaffected by the intestinal phosphatase (Cohn, Oak Ridge Symp. Nucleic Acids, *J. Cell. Comp. Physiol.*, 1951, Suppl. 1, **38**, 21). Other workers, too, had reported the formation of large amounts of pyrimidine mononucleotides in ribonuclease digests without concomitant production of purine mononucleotides (Carter and Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 2604; Magasanik and Chargaff, *Biochim. Biophys. Acta*, 1951, **7**, 396; Schmidt, Cubiles, and Thannhauser, Oak Ridge Symp. Nucleic Acids, *J. Cell. Comp. Physiol.*, 1951, Suppl. 1, **38**, 61). Markham and Smith (*Research*, 1951, **4**, 344) observed that, with several ribonucleic acids, a short period of digestion with ribonuclease gave, together with other products, two substances which could be further degraded by the slow action of ribonuclease or by chemical hydrolysis and yielded in this way uridylic and cytidylic acids respectively. These initial products of ribonuclease digestion we were able to identify as the cyclic 2′ : 3′-phosphates of uridine (III) and cytidine (I) by direct comparison with synthetic specimens of these acids (Brown and Todd, *J.*, 1952, 52; Brown, Magrath, and Todd, preceding paper; see also Markham and Smith, *Nature*, 1951, **168**, 406). The production of such cyclic phosphates as intermediates in the hydrolytic breakdown of ribonucleic acids is in accord with the mechanism of hydrolysis already advanced (Brown and Todd, *loc. cit.*) and it seemed to us that the production of only uridylic acid *b* and cytidylic acid *b* without the corresponding *a* isomers in the above-mentioned experiments of Cohn and Volkin might indicate that ribonuclease brought about a unidirectional cleavage of the cyclic phosphoryl groupings present in the initially liberated uridine- and cytidine-2′ : 3′ phosphates.

Synthetic cytidine-2′ : 3′ phosphate (I) (Brown, Magrath, and Todd, *loc. cit.*) was allowed to stand in solution with crystalline ribonuclease at pH 7·5 and 37° overnight. Paper-chromatography of the solution after this time showed that complete conversion into cytidylic acid had occurred, no cyclic phosphate being present; in a control experiment under the same conditions but without addition of ribonuclease the cyclic phosphate was

* Part XII, preceding paper.

unchanged. Since we have found no solvent system which will separate the *a* and *b* isomers of the pyrimidine nucleotides satisfactorily on paper chromatograms, we examined the solution obtained by ribonuclease action by means of ion-exchange columns (Dowex-2 resin) (see, *inter al.*, Cohn, *loc. cit.*). In this way it was established that the solution con-



Ion-exchange analysis diagrams of:

Cytidylic acids *a* and *b* (1*a*); uridylic acids *a* and *b* (1*b*); cytidine-2′ : 3′ phosphate (2*a*); uridine-2′ : 3′ phosphate (2*b*); product of action of ribonuclease on cytidine-2′ : 3′-phosphate (3*a*) and on uridine-2′ : 3′ phosphate (3*b*); products from alkaline deamination of cytidylic acid *b* (4); adenosine-5′ phosphate, and adenylic acids *a* and *b*, in that order (5); adenosine-2′ : 3′ phosphate (6).

The ordinates of (2*b*) and the cytidylic acid fraction in (4) are drawn, for convenience, to half-scale. Figures relative to the peaks represent, in all cases, the ratios of optical densities at 280 and 260 mμ.

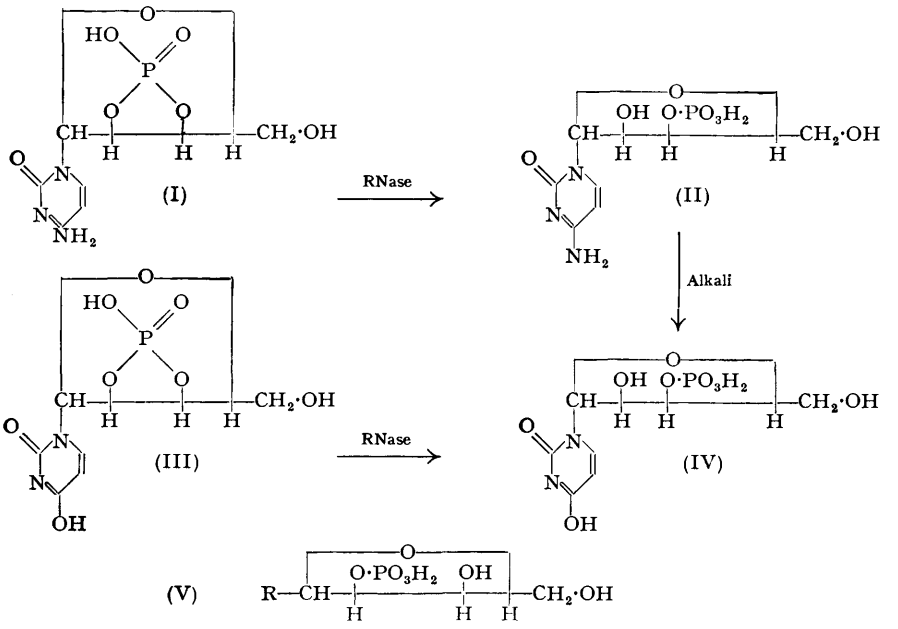
tained only one substance which in its position on the ion-exchange analysis diagram (see Fig. 3*a*) corresponded to cytidylic acid *b*. Moreover the characteristic ratio of the optical densities of this substance at 280 and 260 mμ (cf. Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 2811) was identical with that of cytidylic acid *b* but differed markedly from that of the *a* isomer. It will be noticed from the analysis diagram (Figure) that the cyclic cytidine-2′ : 3′ phosphate appeared on ion-exchange chromatography in the same position as cytidylic acid *b*. It had, however, a distinctly different optical-density ratio and it passed

through the column without decomposition. There can therefore be no doubt that when ribonuclease acts on cytidine-2′ : 3′ phosphate it yields exclusively cytidylic acid *b*. In a similar series of experiments the ribonuclease hydrolysis of the cyclic uridine-2′ : 3′ phosphate (III) was found to yield exclusively uridylic acid *b*, as shown by its position on ion-exchange analysis diagrams and its optical-density ratio.

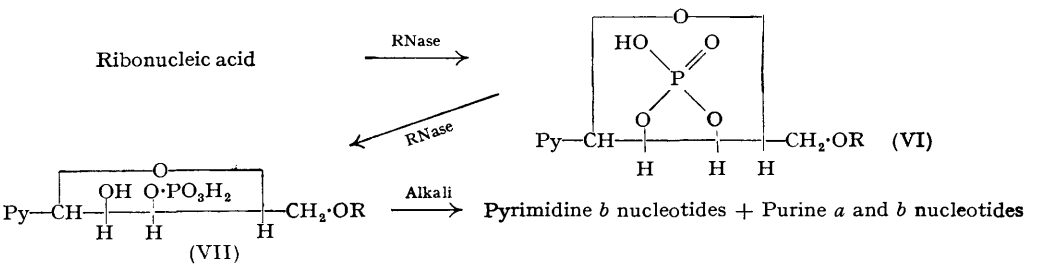
In distinction from the cyclic phosphates of the pyrimidine nucleosides, synthetic adenosine-2′ : 3′ phosphate is unaffected by ribonuclease treatment; this observation, already made by Markham and Smith (*Nature*, 1951, **168**, 406), we confirm. It accords well with the absence of purine nucleotides in the mononucleotide fraction of ribonuclease digests of ribonucleic acids discussed above, and the very small amounts of them (from end groups?) found by Markham and Smith (*Nature*, 151, **168**, 406). It should be noted, however, that Loring and Carpenter (*J. Biol. Chem.*, 1943, **150**, 381) isolated both pyrimidine and purine mononucleotides from ribonuclease digests. The presence of traces of other related enzymes in some samples of ribonuclease might perhaps account for this apparent contradiction. Hirs, Stein, and Moore (*J. Amer. Chem. Soc.*, 1951, **73**, 1893) separated two components from a sample of crystalline ribonuclease, both of which had enzymic activity, and Schmidt, Cubiles, and Thannhauser (*loc. cit.*) have also commented on possible variations in materials with ribonuclease activity.

In an earlier paper (Part IX; Brown and Todd, *J.*, 1952, 44) the nature of the isomeric *a* and *b* nucleotides isolated from alkaline hydrolysates of ribonucleic acids has been discussed and both adenylic acid *a* and adenylic acid *b* were synthesised by phosphorylating 5′-trityl adenosine and then removing protecting groups. The evidence is overwhelmingly in favour of the view that the isomerism is positional, *i.e.*, that the *a* and the *b* acids are 2′- and 3′-phosphates of their various nucleosides but which of them is the 2′- and which the 3′-phosphate has in no case been established, nor is there any definite evidence to show whether the location of the phosphoryl group in the four *a* nucleotides is the same, *i.e.*, whether the group in adenylic acid *a* occupies the same position as it does in, say, uridylic acid *a*. From the standpoint of the work described in the present communication it is clearly necessary to know whether the phosphoryl group in uridylic acid *b* occupies the same position in the ribofuranose residue as that in cytidylic acid *b*. From the results of our ribonuclease experiments it was to be expected that this would be the case. Loring and Luthy (*J. Amer. Chem. Soc.*, 1951, **73**, 4215) deaminated the cytidylic acid of $[\alpha]_D$ +49° (the *b* isomer) and obtained an uridylic acid in good yield, isolated as its highly insoluble brucine salt, which had $[\alpha]_D$ −58·9°, characteristic of the dibrucine salt of the uridylic acid usually isolated from nucleic acid hydrolysates (uridylic acid *b*; Cohn, *J. Cell. Comp. Physiol.*, 1951, Suppl. 1, **38**, 21). However since acidic conditions are known to lead to isomerisation of the yeast pyrimidine nucleotides (Cohn, *loc. cit.*) and since the deamination was carried out in acetic acid solution, we have sought to obviate this possibility by deamination under conditions which preclude isomerisation, and thus establish unequivocally the relation between the cytidylic and uridylic acids. Marrian, Spicer, Balis, and Brown (*J. Biol. Chem.*, 1951, **189**, 533) showed that sodium hydroxide slowly deaminates cytidylic acid, and, since we had found that adenylic aids *a* and *b* and hence by analogy the other *a* and *b* nucleotides were stable without undergoing interconversion in presence of alkali, this reaction seemed well suited to our purpose. Treatment of cytidylic acid *b* with N-sodium hydroxide for 40 hours at 37° caused considerable deamination (*ca.* 25%) and gave, in addition to unchanged cytidylic acid, uridylic acid and some nucleosidic material. Ion-exchange analysis showed that the nucleotidic material contained only cytidylic acid *b* and uridylic acid *b*; no trace of either *a* isomer could be detected (see Fig. 4). It follows that, not only are the pyrimidine nucleotides stable in alkali like the adenine nucleotides, but that the phosphoryl groups in the *b* isomers of uridylic and cytidylic acids occupy the same position in the sugar residues. If, for the purposes of formulation, we make the arbitary assumption that the phosphate residue in the pyrimidine *b* nucleotides (II and IV) occupies the 3′- rather than the 2′-position, then the reactions described can be formulated as in the accompanying scheme. Uridylic acid *a* and cytidylic acid *a* would then be the 2′-phosphates (V; R = uracil or cytosine residue). The assignment of the actual position of the phosphoryl group in each case is the object of other work at present in progress.

The fact that ribonuclease reacts with the cyclic phosphates of the pyrimidine nucleosides and not with the corresponding purine derivatives is of interest in connexion with the specificity of ribonuclease toward the pyrimidine nucleotide residues in ribonucleic acid which has been postulated by other workers. Relevant to this is the evidence of Schmidt,



Cubiles, and Thannhauser (*loc. cit.*; see also Schmidt *et al.*, *J. Biol. Chem.*, 1951, **192**, 633) that the oligonucleotides ("limit polynucleotides") produced by ribonuclease action carry a terminal pyrimidine nucleotide residue. Markham and Smith's observations (*Nature*, 1951, **168**, 406), as they have pointed out, are consistent with the hypothesis (Part X;



Brown and Todd, *loc. cit.*) that the oligonucleotides as initially formed carry a cyclic phosphoryl group on the terminal pyrimidine nucleoside residue (VI; R = remainder of oligonucleotide) which is then opened to yield the true oligonucleotide (VII; R = remainder of oligonucleotide). This opening of the cyclic phosphoryl group by ribonuclease is strictly analogous to the opening in the simple nucleoside-2′ : 3′ phosphates and should yield a terminal *b* nucleotide.

In agreement with this view it has been observed that treatment of ribonucleic acid with ribonuclease followed by alkali yields only the *b* isomers of the pyrimidine nucleotides together with the *a* and *b* isomers of the purine nucleotides (Cohn, private communication). These considerations and results can be accommodated on the basis of the ribonucleic acid structures and the mechanism of their hydrolytic breakdown proposed in Part X of this series (*loc. cit.*). It may be observed that the liberation of pyrimidine mononucleotides (*via*

their cyclic derivatives) by the action of ribonuclease on ribonucleic acid does not of itself indicate a specificity of the enzyme for pyrimidine nucleotide linkages; it would if the polynucleotide chain were unbranched, but not if the chain were branched. The types of branching which could be expected have been discussed in Part X (*loc. cit.*), but in the absence of further degradative evidence the degree to which it may occur in ribonucleic acids cannot be assessed. In the work reported in this paper we have examined only the secondary action of ribonuclease, *i.e.*, its action on the cyclic phosphoryl groups of nucleoside-2′ : 3′ phosphates. Since these cyclic phosphates appear to be the primary products of the action of the enzyme it is clear that a decision between $C_{(2')}$ and $C_{(3')}$ as one of the points of attachment of the internucleotidic link (the other being $C_{(5')}$) in the main polynucleotide chain is unlikely to be reached by mere examination of ribonuclease digests. We are, however, studying the chemical and enzymic hydrolysis of simpler esters of the isomeric pairs of uridylic and cytidylic acids whose structure can be closely defined and will report our findings later.

An observation of some interest in connexion with the action of enzymes on nucleoside-2′ : 3′ phosphates was made incidentally to the main investigation. A solution of phosphatase prepared from a glycerol extract of intestinal mucosa (Klein, *loc. cit.*) had no effect on cytidine-2′ : 3′ phosphate at pH 8 during 20 hours; under the same conditions a preparation of yeast cytidylic acid (containing both the *a* and the *b* isomer) was completely converted into cytidine, identified by paper chromatography. The enzyme preparation employed had strong phosphodiesterase activity since it was in use currently in the laboratory for nucleoside preparation from deoxyribonucleic acids.

Experimental

Paper-chromatography of Uridine and Cytidine Derivatives.—For the paper-chromatography of the compounds discussed in this paper several solvent systems were used, generally in ascending chromatograms (Whatman No. 1 paper). *iso*Propyl alcohol–ammonia–water (70 : 5 : 25 v/v) gave good separation of the nucleoside-2′ : 3′ phosphates from the corresponding nucleotides (for R_F values see Part XII, preceding paper). The system *n*-propanol–2N-hydrochloric acid (65 : 35 v/v) (cf. Wyatt, *Biochem. J.*, 1951, **48**, 584) was used for analysis of mixtures of cytidine, uridine, cytidylic acid, and uridylic acid. For the separation of cytidine (R_F, 0·3) from cytidylic acid and cytidine-2′ : 3′ phosphate, 90% acetone proved a convenient solvent since the phosphates did not move from the origin. None of the systems used gave any separation of uridylic acids *a* and *b* or of cytidylic acids *a* and *b* from each other.

Anion-exchange Analyses.—A column (10 × 1 cm.) of Dowex-2 resin (200—400 mesh) in the formate form was used with a flow-rate of approx. 0·3—0·4 c.c./min. The same column was used throughout the investigation without any apparent change in its absorption characteristics; the resin was occasionally re-suspended when the flow-rate became too slow. Fractions were collected by means of an automatic fraction collector (syphon type, giving fractions of *ca.* 11·5 c.c.) and their optical densities at 260 and 280 mμ were determined with a Beckman ultraviolet spectrograph. For the elution of the cytidine derivatives 0·02N-formic acid was used, while for uridine derivatives we used a solution of 0·01N-formic acid which was 0·05M with respect to sodium formate (Cohn and Volkin, *loc. cit.*). Collection of fractions was started as soon as the eluting solvent was put on the column. The elution diagrams are shown in the Figure; the products were identified by the number of fractions to the peak of the diagram together with their optical density ratios (280 mμ/260 mμ) over the peak. A correction was applied in some instances in calculation of the optical density ratio to compensate for any constant background absorption; where significant, this absorption, which in no case exceeded 0·015 unit, was subtracted from the optical-density readings at the peak before calculation of the ratio.

Action of Ribonuclease on Nucleoside-2′ : 3′ Phosphates.—(a) *Cytidine-2′ : 3′ phosphate.* Ammonium cytidine-2′ : 3′ phosphate (19·1 mg.) was dissolved in water (1·0 c.c.), and the solution adjusted to pH 7·5 by very dilute ammonia solution. To 0·2 c.c. of this solution was added a solution (0·1 c.c.) of crystalline ribonuclease (0·5 mg./c.c.) and water (0·2 c.c.). The solution was incubated at 37° for 20 hours, as was another aliquot (0·2 c.c.) of substrate diluted to 0·5 c.c. with water, but without enzyme. Both solutions were protected from bacterial contamination by addition of one drop of toluene. Paper-chromatographic examination of the solutions after incubation, with the *iso*propyl alcohol–ammonia system, showed that complete conversion

of the cyclic phosphate into nucleotide had been effected by the enzyme; the solution without enzyme was unchanged. The enzyme hydrolysate was adjusted to pH 7·5, run on to the ion-exchange column, and washed in with two portions (2 c.c.) of water. This procedure was used in all subsequent experiments, except where otherwise stated. Elution was then commenced with 0·02N-formic acid. The result is shown in the Fig. (3*a*): only one peak was observed, corresponding to that of cytidylic acid *b* (Fig. 1*a*) in position and in the optical-density ratio (2·03) of the peak fractions.

The elution diagram of cytidine-2′ : 3′ phosphate (Fig. 2*a*) showed that this substance occupied the same position as cytidylic acid *b*, but it had a different optical ratio. The peak fractions were neutralised with ammonia and reduced to small bulk under a vacuum; paper chromatography, with the *iso*propyl alcohol–ammonia system, showed that only the cyclic phosphate was present in the eluate.

(b) *Uridine-2′ : 3′ Phosphate.*—This experiment was performed in the same way as that with cytidine-2′ : 3′ phosphate, except that the pH was adjusted to 8 and 0·05 c.c. of enzyme solution was used for 2·5 mg. of substrate. Solutions were incubated for 14 hours at 37°. The control solution was unaffected under these conditions. The ion-exchange characteristics of uridylic acids *a* and *b* (Fig. 1*b*) and uridine-2′ : 3′ phosphate (Fig. 2*b*) were compared with that of the enzymic hydrolysis product. The latter was found to give only one peak (Fig. 3*b*), corresponding to that of uridylic acid *b*, both in the position of the peak and in the optical-density ratio (0·33) of the peak fractions.

(c) *Adenosine-2′ : 3′ Phosphate.*—Ammonium adenosine-2′ : 3′ phosphate (1·6 mg.) in water (0·2 c.c.) was treated with crystalline ribonuclease (0·06 mg.) at pH 8 for 16 hours at 37°. Paper chromatography in the *iso*propyl alcohol–ammonia solvent showed that no appreciable reaction had occurred. Under identical conditions ammonium uridine-2′ : 3′ phosphate (1·6 mg.) was completely converted into uridylic acid *b*.

For completion, the ion-exchange characteristics of adenosine-2′ : 3′ phosphate (Fig. 6) were compared by Mr. D. Magrath with those of adenosine-5′ phosphate and adenylic acids *a* and *b* (Fig. 5), 0·1N-formic acid being used as eluant. The cyclic phosphate occupied the same position as adenylic acid *b*. It was shown to be unaltered by passage through the column, since neutralisation of the peak fractions, evaporation of these to small bulk under a vacuum and paper-chromatography of the residue revealed only the cyclic phosphate. The optical-density ratios (280 mμ/260 mμ) of all these adenine derivatives are *ca.* 0·19.

Deamination of Cytidylic Acid b.—In initial experiments cytidylic acid (5 mg.) was heated at 100° for 2 hours in saturated barium hydroxide solution (0·2 c.c.). It appeared, from chromatograms, that the substance was largely converted into uridine and so the reaction was not further studied (cf. Marrian, Spicer, Balis, and Brown, *loc. cit.*). Cytidylic acid was not appreciably affected by heating with 0·1N-sodium hydroxide at 37° for 36 hours. With N-sodium hydroxide partial conversion into uridylic acid was observed.

Yeast cytidylic acid (300 mg.) was heated under reflux with 0·1N-hydrochloric acid for 15 minutes, to convert it into a mixture of the *a* and the *b* acid. Ion-exchange chromatography (Cohn, *loc. cit.*) permitted separation into the crystalline isomers, cytidylic acid *a* (103 mg.) and *b* (81 mg.).

Pure cytidylic acid *b* (6·1 mg.) was dissolved in sodium hydroxide solution (0·5 c.c. of N) and heated at 37° for 40 hours. The solution was then diluted, brought to pH 8 and a final volume of 50 c.c., and run on to the ion-exchange column. After washing with water (50 c.c.) elution was begun with 0·2N-formic acid. Thirty fractions were taken to ensure complete elution of unchanged cytidylic acid and then the solvent was changed to the sodium formate–formic acid buffer to elute the uridylic acid. Only two peaks were observed (see Figure) corresponding to cytidylic acid *b* and uridylic acid *b* (25% of total nucleotide) in position and in optical-density ratios (2·04 and 0·32 respectively) over the peaks.

Action of Intestinal Mucosa Phosphatase on Cytidine-2′ : 3′ Phosphate.—The solution of ammonium cytidine-2′ : 3′ phosphate used in the above experiments with ribonuclease (0·1 c.c.) was treated with a solution of intestinal phosphatase, prepared by Klein's method (*loc. cit.*), in presence of magnesium sulphate. Incubation at pH 8 for 20 hours at 37° left the compound unaffected, as evinced by paper chromatography of the solution before and after incubation, in both the *iso*propyl alcohol–ammonia and the 90% acetone system. Under the same conditions yeast cytidylic acid (1·6 mg.) was converted completely into cytidine.

[*Added in proof*, 28.5.52]: Guanosine-2′ : 3′ phosphate, like adenosine-2′ : 3′ phosphate, is not affected by ribonuclease under the conditions used above. It is eluted from a Dowex-2 resin column (12 × 1 cm.) by 0·1M-sodium formate–0·1N-formic acid buffer at a slightly slower rate

than guanylic acid *b* (nos. of fractions to peaks: guanylic acid ***a***, **63**; guanylic acid ***b***, **99**; guanosine-2′ : 3′ phosphate, **109**).

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