511. *Nucleotides. Part XII.* The Preparation of Cyclic 2′ : 3′-Phosphates of Adenosine, Cytidine, and Uridine.*

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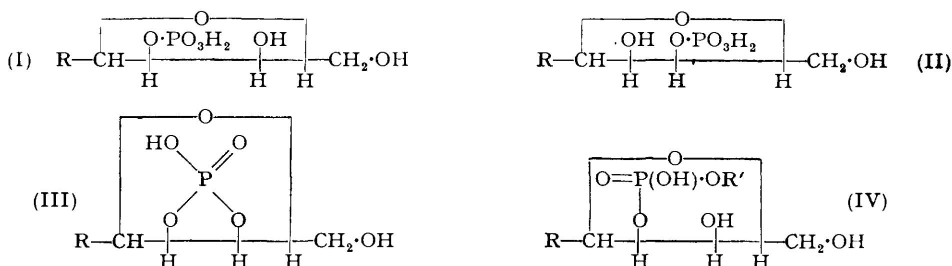
The preparation of the cyclic 2′ : 3′-phosphates of adenosine, cytidine, and uridine is described, and some of their properties are recorded. The cytidine and uridine derivatives have been identified as products of incomplete ribonuclease digestion of ribonucleic acids.

In Part IX of this series (*J.*, 1952, 44) Brown and Todd described the synthesis of the isomeric adenylic acids *a* and *b* originally isolated by Carter and Cohn (*Fed. Proc.*, 1949, **8**, 190) from alkaline hydrolysates of yeast ribonucleic acid and presented evidence for the view that they are the 2′- and 3′-phosphates of adenosine (I and II; R = adenine residue) although not necessarily respectively. It was concluded by analogy that the other isomeric pairs of *a* and *b* nucleotides derived from guanosine, uridine, and cytidine, which are also obtained from alkaline hydrolysates of ribonucleic acids (Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 1471, 2811; Loring, Luthy, Bortner, and Levy, *ibid.*, p. 1471) were similarly 2′- and 3′-phosphates of the respective nucleosides. The interconversion of the isomeric *a* and *b* nucleotides in acid media (Brown, Haynes, and Todd, *J.*, 1950, 2299; Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 2811) was ascribed by Brown and Todd (*loc. cit.*) to a facile phosphoryl migration analogous to that already well-known for the monophosphates of glycerol. This migration was considered to proceed *via* an intermediate cyclic nucleoside-2′ : 3′ phosphate (III). In the specific case of the adenylic acids *a* and *b* it was shown that their monobenzyl



esters did not show the normal stability of phosphodiesters towards alkali, but were readily hydrolysed by acid or alkali to yield in each case benzyl alcohol and a mixture of the free *a* and *b* nucleotides (Part IX, *loc. cit.*); the hydrolysis in these cases was postulated to occur by an initial cyclisation and to yield as an intermediate the cyclic adenosine-2′ : 3′ phosphate which then underwent fission in two directions giving both the *a* and *b* acids (Part X, Brown and Todd, *J.*, 1952, 52). Based on these observations a simple explanation was derived for the hydrolytic behaviour of the nucleic acids and certain necessary structural consequences were pointed out (Part X, *loc. cit.*). It was clearly desirable that efforts should be made to synthesise cyclic nucleoside-2′ : 3′ phosphates in order to ascertain whether their properties were in agreement with those which we predicted and whether their stability was such that, if our interpretation of the hydrolytic behaviour of the ribonucleic acids was correct, they might actually occur among the products of enzymic hydrolysis of these acids. The present paper records the preparation of the cyclic 2′ : 3′-phosphates of adenosine (III; R = adenine residue), cytidine (III; R = cytosine residue), and uridine (III; R = uracil residue). All three have been isolated in substance as their barium salts and some of their properties are described. Publication of these results on the cyclic nucleoside phosphates is desirable at this stage since certain of them have already been identified in ribonuclease digests of ribonucleic acid (Brown and Todd, Part X, *loc. cit.*; Markham and Smith, *Nature*, 1951, **168**, 406; *Biochem. J.*, 1952, in the press) and their importance in nucleic acid chemistry has become such that a description of their synthesis

* Part XI, 1952, 2530.

is required so that they can be made more generally available for comparative purposes in degradative studies.

During exploratory studies on possible routes to the synthesis of the oxidation–reduction coenzyme flavin-adenine dinucleotide (FAD) it had been observed that attempts to apply exchange reactions (cf. Mason and Todd, *J.*, 1951, 2267; Corby, Kenner, and Todd, *J.*, 1952, in the press) between riboflavin-5′ phosphate and pyrophosphates yielded the cyclic riboflavin-4′ : 5′ phosphate (Forrest, Mason, and Todd, *J.*, 1952, 2530). While seeking to prevent this undesirable reaction by prior acylation of the riboflavin-5′ phosphate, it was observed that treatment with trifluoroacetic anhydride followed by removal of trifluoroacetyl groups gave the same riboflavin-4′ : 5′ phosphate in excellent yield. It seemed therefore likely that a similar procedure applied to nucleoside-2′ or -3′ phosphates would give the cyclic 2′ : 3′-phosphates and it was decided to apply it first in the adenosine series. The production of cyclic phosphates from such compounds as riboflavin-5′ phosphate or adenosine-2′ and -3′ phosphates by means of trifluoroacetic anhydride proceeds without doubt through initial formation of a mixed anhydride of the phosphate with trifluoroacetic acid (*e.g.*, IV; R = adenine residue; R′ = $CO{\cdot}CF_3$). The mixed anhydride can then react as a phosphorylating reagent towards the adjacent hydroxyl group, or it might, under appropriate conditions, react with more phosphate to yield a pyrophosphate which could phosphorylate in an analogous manner liberating a molecule of the original phosphate. In addition to intramolecular reaction to produce cyclic phosphate, intermolecular phosphorylation by the mixed anhydride might also occur yielding, in the case of the adenylic acids, di- or poly-nucleotides. Since increased dilution of the reaction solution should favour intramolecular as opposed to intermolecular phosphorylation and since, moreover, excess of trifluoroacetic anhydride should tend to minimise pyrophosphate production it was decided that preparation of adenosine-2′ : 3′ phosphate (III; R = adenine residue) should be attempted by allowing adenylic acid *a* or *b* to react with excess of trifluoroacetic anhydride in the cold.

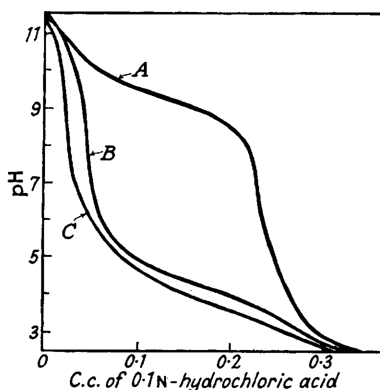
When this reaction was carried out with either adenylic acid *a* or adenylic acid *b* and the mixture treated with ethanolic ammonia to remove trifluoroacetyl groups, the product was found by paper chromatography to contain, in addition to some unchanged starting material, a new substance as its major constituent. This new substance—identical whether the starting material was the *a* or *b* isomer—showed the general behaviour expected of a cyclic 2′ : 3′-phosphate in that it behaved chromatographically like a monoester of a nucleotide, travelling rather faster than the original free nucleotide in *iso*propyl alcohol–water–ammonia and rather slower in aqueous disodium hydrogen phosphate–*iso*amyl alcohol; moreover on treatment with acid or alkali it was converted into a mixture of *a* and *b* nucleotides. The same preparative procedure applied to uridylic acids *a* and *b*, cytidylic acids *a* and *b*, and guanylic acids *a* and *b* yielded analogous products. In these preliminary experiments the products, although separated chromatographically and examined in an apparently homogeneous condition, were not isolated in the solid state. In a larger-scale experiment, in which yeast adenylic acid (containing both the *a* and *b* isomers) was used as starting material, the crude product after ammonia treatment was purified by precipitation from solvents till free from starting material and then converted into a barium salt. After purification by precipitation from aqueous solution with ethanol, barium adenosine-2′ : 3′ phosphate was obtained as a white microcrystalline powder; the salt was hydrated and water of crystallisation could only be removed with difficulty. The free acid did not appear to crystallise readily and its isolation in solid form was not pursued, the barium salt being adequate for general use as a source of solutions of the acid when required.

Adenosine-2′ : 3′ phosphate is stable to periodate, and electrometric titration (see Figure) shows that it contains no secondary phosphoryl dissociation, a fact which disposes of the possibility that the product might have been a dinucleotide. Cryoscopic determination of the molecular weight of the barium salt in water gave a value of 674 on the assumption that it would yield three ions in solution. This figure is a good deal lower than the theoretical figure of 793, but despite the apparent inaccuracy of the method the result definitely supports the cyclic phosphate structure rather than the conceivable diadenosine pyrophosphate structure; the stability of the product towards cold alcoholic ammonia also speaks

against a pyrophosphate structure. In aqueous solution between pH 4 and pH 9 the cyclic phosphate appears to be stable but treatment with 0·1N-hydrochloric acid for 1 hour at room temperature or 0·5N-sodium hydroxide at 37° for 12 hours caused hydrolysis to a mixture of adenylic acids *a* and *b*; the limiting conditions for hydrolysis have not been determined.

Cytidine-2′ : 3′ phosphate (III ; R = cytosine residue) was prepared and isolated as its barium salt by an analogous reaction of mixed cytidylic acids *a* and *b* with trifluoroacetic anhydride followed by treatment with ethanolic ammonia. In this case the product contained another substance (in amount 10—20%) whose nature is discussed below. It was rather similar to the cyclic phosphate and its separation from the ammonium salt of the latter required careful chromatography on a cellulose column. A similar by-product was encountered in the preparation of uridine-2′ : 3′ phosphate (III; R = uracil residue) which was also separated chromatographically, thereby permitting the isolation of the pure cyclic phosphate, finally as its barium salt.

The properties of the two pyrimidine cyclic phosphates towards hydrolytic agents are similar to those of adenosine-2′ : 3′ phosphate, since they are converted under the same mild



Electrometric titrations of :

A, barium uridine-2′ : 3′ phosphate.
B, barium cytidine-2′ : 3′ phosphate.
C, barium adenosine-2′ : 3′ phosphate.

conditions into the corresponding nucleotide (presumably a mixture of the *a* and the *b* isomer). Their electrometric titration curves (see Figure) likewise show no evidence of a secondary phosphoryl dissociation. Amino- and hydroxyl functions titrated normally, as was to be expected, since the ultra-violet light absorption characteristics showed that the aglycone moiety was unaffected by the preparative procedure. It is clear that the properties of the nucleoside-2′ : 3′ phosphates are in every way consistent with the hypothesis (Brown and Todd, Part X, *loc. cit.*) that they are involved in the hydrolytic degradation of ribonucleic acids; their non-recognition in the past is to be attributed to their lack of stability under the hydrolytic conditions usually employed, leading to the isolation of only the *a* and the *b* isomers of the corresponding nucleotides.

The structures of the two by-products mentioned above have been investigated. They were hydrolysed to the corresponding nucleotide under conditions only slightly more vigorous than those required to hydrolyse the cyclic phosphates. Ultra-violet light absorption measurements showed that the pyrimidine residues were unaltered and electrometric titrations demonstrated that the substances behaved as ammonium salts of strong monobasic acids; in particular secondary phosphoryl dissociation was absent. Paper-strip electrophoresis experiments lent support to this conclusion. In a preparation of cytidine-2′ : 3′ phosphate, the ethanolic ammonia used in working up was replaced by ethanol solutions of triethylamine and of *cyclo*hexylamine. The same by-product was again obtained in each case, showing that the nature of the base did not affect the constitution of the products.

After consideration of the above evidence it was concluded that the substances were best formulated as esters of uridylic and cytidylic acids. Their ready hydrolysis accords well with our earlier observations on the benzyl esters of adenylic acids *a* and *b* (Brown and

Todd, Part IX, *loc. cit.*). This conclusion was confirmed by treatment of uridylic acid with ethanol containing a little hydrogen chloride and study of the products on paper chromatograms. In addition to much unchanged nucleotide another product was observed which corresponded exactly to the by-product from the uridine-2′ : 3′ phosphate preparation. Furthermore, by dissolving uridylic acid in trifluoroacetic anhydride and working up, this time with a solution of ammonia in benzyl alcohol, there was obtained in addition to cyclic phosphate another substance corresponding exactly on paper chromatograms to uridine benzyl phosphate prepared by phenyldiazomethane esterification (unpublished work). The proportion of ester was very small when ammoniacal benzyl alcohol was employed; this suggests that in the preparation of the cyclic phosphates the use of a less reactive alcohol, *e.g.*, benzyl alcohol or *tert.*-butyl alcohol, instead of ethanol during the working up would be advantageous.

The substances are therefore regarded as the ammonium salts of uridine ethyl phosphate and cytidine ethyl phosphate (IV; R = uracil or cytosine residue respectively, R′ = Et), probably being in each case a mixture of the *a* and the *b* isomer. Although analytical values are not satisfactory, difficulty has been experienced in this respect with other ammonium salts during the investigation. The production of esters of this type is not surprising since reaction between a mixed anhydride (IV; R′ = $CO{\cdot}CF_3$) and an alcohol in presence of base should yield an ester, particularly if prior trifluoroacetylation of the hydroxyl group vicinal to the phosphoryl group prevented intramolecular phosphorylation.

Experimental

Adenosine-2′ : 3′ Phosphate.—Trifluoroacetic anhydride (5 c.c., 12 mols.) was added to anhydrous yeast adenylic acid (mixture of *a* and *b* isomers; 1 g., 1 mol.) and the resulting solution was set aside overnight at room temperature in a stoppered flask. The solution was evaporated under reduced pressure and the residual pale yellow resin triturated with dry ether. The white powder so obtained was added rapidly to a stirred ice-cold saturated solution of ammonia in ethanol (25 c.c.), and the mixture set aside for 30 minutes. Solid material (0·3 g.; mainly adenylic acid) was removed by centrifugation and washed with ethanol. The combined centrifugate and washings were evaporated under reduced pressure, and the residual resin was thoroughly washed with acetone, then redissolved in methanol. Addition of ether gave the ammonium salt of the cyclic phosphate as a white deliquescent powder; it gave a single ultraviolet absorbing spot when chromatographed on paper with disodium hydrogen phosphate–*iso*amyl alcohol. On account of its very hygroscopic character satisfactory analyses could not be carried out on this material and it was therefore dissolved in water and heated on the steam-bath for 40 minutes with a slight excess of barium carbonate. The mixture was filtered, the filtrate concentrated to small bulk under reduced pressure, and excess of ethanol was added. The microcrystalline precipitate of *barium adenosine-2′ : 3′ phosphate* was purified by re-precipitation from aqueous solution with ethanol. For analysis samples were dried for varying periods over phosphoric oxide at 110—115°/0·1 mm.; the dried materials were very hygroscopic and difficult to burn [Found, in material dried for: (*a*) 1½ hours: C, 27·2; H, 4·0. $(C_{10}H_{11}O_6N_5P)_2Ba,4H_2O$ requires C, 27·7; H, 3·5. (*b*) 20 hours: C, 29·0; H, 3·1; N, 17·0; P, 7·4. $(C_{10}H_{11}O_6N_5P)_2Ba,2H_2O$ requires C, 28·9; H, 3·2; N, 16·9; P, 7·5. (*c*) 50 hours: C, 30·7; H, 3·5; N, 17·5. $(C_{10}H_{11}O_6N_5P)_2Ba$ requires C, 30·2; H, 2·8; N, 17·7%]. Light absorption in water: $\lambda_{max.}$ 260 mμ; $\lambda_{min.}$ 225 mμ (ε, 13,150, 1830, for M = 397). The same product was obtained by starting from either of the pure adenylic acids *a* and *b* as was obtained from the mixture used in the preparation described.

Cytidine-2′ : 3′ Phosphate.—The cyclic phosphate was obtained both from cytidylic acid *a* and cytidylic acid *b*; for preparative purposes it was convenient to employ a preparation of cytidylic acid obtained by hydrolysis of yeast ribonucleic acid without separationof th e pure isomers.

Trifluoroacetic anhydride (6 c.c., 14 mols.) was added to anhydrous yeast cytidylic acid (1 g., 1 mol.). The mixture became warm and the acid dissolved completely within 20 minutes, giving a clear solution which was left overnight at room temperature, then evaporated under reduced pressure; the residue was triturated with dry ether (30 c.c.), falling to a white powder. The product was collected by centrifugation and washed with ether, and traces of solvent were removed in a vacuum-desiccator; it was then added to ice-cold saturated ethanolic ammonia (35 c.c.), and the mixture left for 30 minutes and centrifuged. The solid which was a mixture

of cytidylic acid and some of the cyclic phosphate was washed with cold ethanol (10 c.c.) and the combined supernatant liquid and washings were concentrated to small bulk *in vacuo* at 20°. The product (0·47 g.) was precipitated by ether (*ca.* 30 c.c.), collected, washed once with ether containing a little ethanol (9 : 1), and dried in a desiccator; it was free from nucleotide but in addition to the ammonium salt of the cyclic phosphate it contained another substance (in amount 10—20%) which on paper chromatography travelled faster than the latter in the *iso*propyl alcohol–ammonia–water system. Attempts to remove this impurity by fractional precipitation as either the ammonium or the barium salt or by adsorption on charcoal were unsuccessful and recourse was had to chromatography on a cellulose column. The column (29·5 × 5·5 cm.) was packed with cellulose powder (90 mesh Whatman; 220 g.) by allowing the latter to settle from acetone suspension, washed with a solution of 8-hydroxyquinoline (1 mg./g. of cellulose) in acetone, then with acetone followed by *iso*propyl alcohol, and finally the solvent system used for chromatography, *viz.*, *iso*propyl alcohol–water–ammonia (*d* 0·880) (80 : 18 : 2). The crude ammonium salt (0·36 g.) was dissolved in a small amount of solvent mixture to which more water had been added and put on the column, and the chromatogram was developed (8 hours) with the above solvent system. The first 500 c.c. of eluate passing through the column were discarded, the next 150 c.c. collected in 3 × 50-c.c. fractions, and subsequently 50 fractions of 20 c.c. each were collected. The contents of each fraction were ascertained by chromatography on paper strips. The peak of elution of the secondary component (for isolation see below) occurred in nos. 12—15 of the 20-c.c. fractions and of the cyclic phosphate in fraction 45. There was thus good separation, with negligible overlap.

Fractions 28—50 (of the 20-c.c. fractions) were combined and evaporated as above and the residue was converted into a white powder (0·22 g.) by trituration with acetone. It was purified by dissolution in methanol and reprecipitation with ether; the final product was extremely hygroscopic and perhaps for this reason gave unsatisfactory analytical values for the ammonium salt of cytidine-2′ : 3′ phosphate (Found, in material dried at room temperature/0·1 mm. over phosphoric oxide for 16 hours: C, 34·9; H, 5·7; N, 15·1. Calc. for $C_9H_{15}O_7N_4P$: C, 33·5; H, 4·7; N, 17·4%).

The above ammonium salt (0·1 g.) in ice-cold solution was treated with a slight excess of 0·1N-barium hydroxide. The excess of barium ions was removed with carbon dioxide, and the filtered solution concentrated to small bulk under reduced pressure, again filtered, and evaporated to dryness. The residual glass was redissolved in a little water, filtered, and concentrated, and excess of methanol added. *Barium cytidine-2′ : 3′ phosphate* separated as a white amorphous powder (35 mg.) which was chromatographically homogeneous [Found, in material dried for 24 hours at 110°/0·1 mm. over phosphoric oxide: C, 29·5; H, 3·4; N, 11·4; P, 8·6. $(C_9H_{11}O_7N_3P)_2Ba$ requires C, 29·0; H, 3·0; N, 11·3; P, 8·3%]. Light absorption in water: Max. at 268 and 232 mμ (ε, 8400, 8150); min. at 250 and 223 mμ (ε, 6900, 7750 for $M = 373$).

Uridine-2′ : 3′ Phosphate.—Trifluoroacetic anhydride (6 c.c., 14 mols.) was added to anhydrous yeast uridylic acid (1 g., 1 mol.). The mixture became warm and the acid dissolved completely within 15 minutes. The flask was stoppered and set aside at room temperature overnight. The solution was then evaporated under reduced pressure and the residual gum triturated with dry ether (30 c.c.). The resulting white powder was collected by centrifugation, dried under vacuum, and then added rapidly, with stirring, to ice-cold saturated ethanolic ammonia (35 c.c.). After 30 minutes at 0° the solid, which consisted of unchanged starting material together with some cyclic phosphate, was collected in a centrifuge and washed with ice-cold ethanolic ammonia (10 c.c.). The combined supernatant solution and washings were evaporated under reduced pressure, the residue was dissolved in methanol (2 c.c.), and the product (0·61 g.) precipitated with ether. It was collected by centrifugation, washed with ether containing a little ethanol (9 : 1; 10 c.c.), and dried *in vacuo*.

This product, on chromatographic evidence, was free from unchanged nucleotide but contained, in addition to cyclic phosphate, a by-product (10—15%). It (0·4 g.) was therefore chromatographed on a column (5·5-cm. diameter) of powdered cellulose (240 g.) prepared as described for the cytidine derivatives above. The first 500 c.c. of eluate were discarded and the remainder was collected in 90 fractions of approx. 20 c.c. each. Paper chromatography showed that practically all the by-product was in fractions 14—34 (peak at 30) (see below) and the cyclic phosphate in fractions 44—85 (peak at 65).

Fractions 44—85 were combined and evaporated under reduced pressure and the residue converted into a white powder by trituration with acetone. This was purified by dissolving it in methanol and precipitating it with ether. *Ammonium uridine-2′ : 3′ phosphate* separated as a very hygroscopic white powder (0·25 g.) which was chromatographically homogeneous (Found,

in material dried at room temp./15 mm. over phosphoric oxide: C, 33·6; H, 4·3; N, 12·1; P, 8·7. $C_9H_{14}O_8N_3P$ requires C, 33·4; H, 4·4; N, 13·0; P, 9·6%).

The above ammonium salt (0·1 g.) was dissolved in water and the calculated volume of barium hydroxide solution (0·1N) added rapidly to the ice-cooled solution. After neutralisation by passage of carbon dioxide the solution was concentrated, filtered, and further concentrated, and *barium uridine-2′ : 3′ phosphate* (0·045 g.) was precipitated by addition of methanol. It was chromatographically homogeneous. A further quantity of the product (0·045 g.) was obtained by concentration of the mother-liquors [Found, in material dried at 110°/0·1 mm. for 24 hours over phosphoric oxide: C, 29·4; H, 3·0; N, 7·8; P, 8·0. $(C_9H_{10}O_8N_2P)_2Ba$ requires C, 28·9; H, 2·7; N, 7·5; P, 8·3%]. Light absorption in water: $\lambda_{max.}$ 258—259 mμ; $\lambda_{min.}$ 228—229 mμ (ε, 9570, 2290, for M = 374).

Isolation and Nature of the By-products.—(a) *In the preparation of cytidine-2′ : 3′ phosphate.* The by-product was isolated by combining the three 50-c.c. fractions and Nos. 1—15 of the 20-c.c. fractions (obtained above) and evaporating them at 30° under reduced pressure. The residual gum crumbled to a white hygroscopic powder (0·066 g.) when triturated with acetone. This product was dissolved in a mixture of ethanol and methanol (it is much more soluble in the latter) and reprecipitated with ether and finally washed with acetone. The white amorphous solid so obtained was very hygroscopic (Found, in material dried at room temperature/15 mm. for 48 hours over phosphoric oxide: P, 7·7%). Light absorption in water: $\lambda_{max.}$ 270 mμ; $\lambda_{min.}$ 249 mμ ($E^{1\%}_{1\,cm.}$ 226, 164 respectively). The molecular weight = 372 calculated on light absorption data on the assumption of one absorbing unit/mole and $\varepsilon_{max.}$ 8400 for barium cytidine-2′ : 3′ phosphate. The substance is considered to be ammonium cytidine ethyl phosphate, probably a mixture of the *a* and the *b* isomer (Calc. for $C_{11}H_{21}O_8N_4P$: P, 8·4%, M; 368).

Cytidylic acid was treated with trifluoroacetic anhydride and the product added to solutions of triethylamine and *cyclo*hexylamine in ethanol instead of to ethanolic ammonia as described above. In both cases paper chromatography showed that cytidine-2′ : 3′ phosphate was produced together with the same by-product as before. Thus the nature of the base did not alter the constitution of either of the reaction products.

(b) *In the preparation of uridine-2′ : 3′ phosphate.* The by-product was isolated as its ammonium salt by evaporation of the combined fractions 14—34 (above) at 30° under reduced pressure. The residual glass, when triturated with acetone, formed an amorphous powder which was dissolved in a little methanol and reprecipitated with acetone–ether. The cream-coloured powder (0·054 g.) was hygroscopic and ran as a single spot in the *iso*propyl alcohol–ammonia system (Found, in material dried at 110°/0·1 mm. for 24 hours over phosphoric oxide: N, 10·1; P, 7·3%). Absorption of light in water: $\lambda_{max.}$ 260 mμ; $\lambda_{min.}$ 230 mμ ($E^{1\%}_{1\,cm.}$, 248, 55 respectively). The molecular weight = 386 calculated on light-absorption data with the assumptions of one absorbing unit/mole and $\varepsilon_{max.}$ 9570 for barium uridine-2′ : 3′ phosphate. The substance is considered to be ammonium uridine ethyl phosphate, probably a mixture of the *a* and the *b* isomer, on this and other evidence given below (Calc. for $C_{11}H_{20}O_9N_3P$: N, 11·4; P, 8·4%. M, 369).

After a solution of uridylic acid in ethanol containing a little hydrogen chloride had been kept for 48 hours it was found that there was present, in addition to unchanged nucleotide, a small amount of material having the same characteristics on chromatograms as the above by-product, thus affording evidence for its formulation as an ethyl ester.

When, in a small-scale preparation of uridine-2′ : 3′ phosphate as above, a solution of ammonia in benzyl alcohol instead of in ethanol was used, and the product analysed on paper chromatograms, no product was found corresponding to the ethyl ester; instead a small amount of substance was observed having a higher R_F value, corresponding exactly to that of uridine benzyl phosphate, prepared by the action of phenyldiazomethane on uridylic acid (unpublished work).

Action of Acid and Alkali on the Nucleoside-2′ : 3′ Phosphates.—Adenosine-2′ : 3′ phosphate was completely hydrolysed when dissolved in 0·1N-hydrochloric acid at 18° for 1 hour or at 100° for 15 minutes and in 0·5N-sodium hydroxide solution for 12 hours at 37°. The products in each case were adenylic acids *a* and *b*, identified by chromatography in the *iso*propyl alcohol–ammonia and sodium phosphate–*iso*amyl alcohol solvent systems.

Cytidine- and uridine-2′ : 3′ phosphate were completely hydrolysed to the corresponding nucleotides by 0·1N-hydrochloric acid for 1 hour at 18° or 15 minutes at 100° and by 0·5N-sodium hydroxide solution for 12 hours at 37°. These compounds were stable over the pH range 4—9 in phosphate–citric acid buffers at room temperature for at least 18 hours. At pH 2 hydrolysis proceeded to the extent of 5—10%.

The two ethyl esters, described above, were hydrolysed to the corresponding nucleotide but under somewhat more vigorous conditions than the cyclic phosphates. With 0·1N-hydrochloric acid at 100° for 15 minutes the materials were hydrolysed to the extent of about 70%, but unaffected at 16° for 12 hours. 0·5N-Sodium hydroxide solution caused about 80% hydrolysis after 12 hours at 16° and about 90% after 5 minutes at 100°. They were stable to concentrated ethanolic ammonia at 0° for 30 minutes.

Electrometric Titrations.—The general procedure employed was to titrate the barium or ammonium salt up to pH 11·5 with 0·1N-sodium hydroxide and back-titrate with 0·1N-hydrochloric acid to pH 2·5. One c.c. of a solution, containing approx. 0·62 mg. P (*i.e.*, 0·01M for the barium salts), was used in each case. A Cambridge pH meter in combination with a glass electrode was used for the pH determinations. The titration curves of the barium nucleoside-2′ : 3′ phosphates are given in the Figure. It is clear that no group is present titrating in the pH range 5—8, characteristic of the secondary phosphoryl dissociation (pK 5·9; Levene and Simms, *J. Biol Chem.*, 1925, **65**, 519). The amino- and hydroxyl groups in the respective compounds titrated normally.

The titration curves of the two ethyl esters (above), which are not included in the Figure, showed that they behaved as the ammonium salts of strong monobasic acids. No secondary phosphoryl dissociation was observed. The amino-function in the cytidine derivative and the hydroxyl group in the uridine derivative titrated normally, indicating that the pyrimidine moiety in these compounds was unaltered. Results relative to, and in accord with the titration data, were obtained by submitting cytidine-2′ : 3′ phosphate and cytidine ethyl phosphate to electrophoresis on paper strips (Markham and Smith, *loc. cit.*). Both compounds migrated as negative ions (*i.e.*, to the positive pole) in buffers in the pH range 3·5—7 and in 1M-ammonia, but at a slower rate than cytidylic acid, consistently with their formulation as monobasic acids.

Paper Chromatography of the Nucleotide Derivatives.—Paper chromatography was used throughout the investigation for routine analysis of reaction products. The solvent found most useful was the *iso*propyl alcohol–ammonia–water system of Markham and Smith (*Nature*, 1951, **168**, 406), slightly modified in composition (70 : 5 : 25 v/v). Typical R_F values are given in the Table for upward-run chromatograms after equilibration, together with those of the substances run in 5% disodium hydrogen phosphate–*iso*amyl alcohol (Carter, *J. Amer. Chem. Soc.*, 1950, **72**, 1466). Standard substances were always used simultaneously for identification purposes. Spots were detected by photographing the chromatograms in ultra-violet light, or, in some cases, by use of a phosphate spray reagent.

Substance	R_F value: *iso*Propyl-alcohol–ammonia–water	R_F value: Sodium phosphate–*iso*amyl alcohol	Substance	R_F value: *iso*Propyl-alcohol–ammonia–water	R_F value: Sodium phosphate–*iso*amyl alcohol
Adenylic acid *a*	0·12	0·71	Guanosine-2′ : 3′ phosphate	0·23	0·67
Adenylic acid *b*	0·12	0·63	Uridine-2′ : 3′ phosphate	0·30	0·82
Guanylic acid	0·03	0·74	Cytidine-2′ : 3′ phosphate	0·36	0·81
Uridylic acid	0·09	0·85	Uridine ethyl phosphate	0·43	0·86
Cytidylic acid	0·10	0·85	Cytidine ethyl phosphate	0·48	0·84
Adenosine-2′ : 3′ phosphate	0·43	0·57			

[*Added in proof,* 28.5.52]*: Barium guanosine-*2′ : 3′ *phosphate* has now been isolated in substance and its properties studied. It was obtained by a method completely analogous to that used in the preparation of the other nucleoside-2′ : 3′ phosphates and was purified by reprecipitation by acetone from aqueous solution [Found, in material dried at 110°/0·1 mm. over phosphoric oxide : C, 28·9; H, 3·4; N, 17·3; P, 7·2. $(C_{10}H_{11}O_7N_5P)_2Ba$ requires C, 29·1; H, 2·7; N, 17·0; P, 7·5%]. Light absorption in water : $\lambda_{max.}$ 251—253 mμ (ε 12,150 for $M = 413$). Electrometric titration demonstrated the absence of secondary phosphoryl dissociation. The substance behaved towards acid and alkali in the same way as the other nucleoside-2′ : 3′ phosphates.

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