Nucleotides. Part XXXIII. The Structure of Cytidylic Acids* a *and* b.

By (Mlle.) F. Baron and D. M. Brown.

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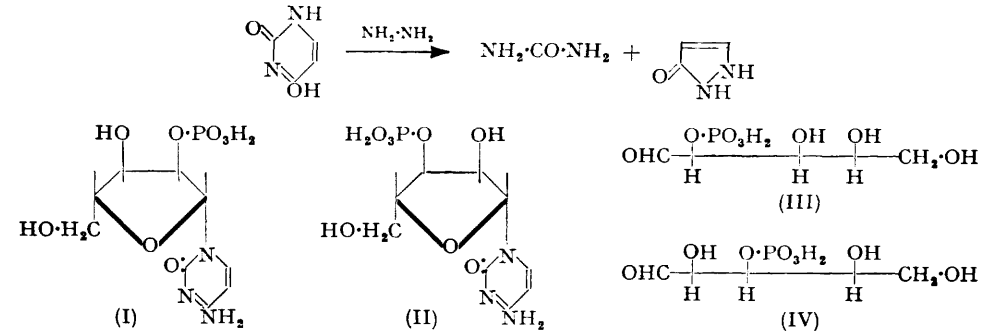
Fission of cytidylic acid *a* and *b* by hydrazine yields ribose 2- and 3-phosphate respectively, characterised by, *inter alia*, comparison with the sugar phosphates derived from adenylic acid *a* and *b* by acid hydrolysis. Uridylic acid *b* yields the same ribose phosphate as does cytidylic acid *b*. The *a* and the *b* isomers of these nucleotides are therefore the 2′- and 3′-phosphates, respectively, of the ribonucleosides.

Fosse, Hieulle, and Bass (*Compt. rend.*, 1924, **178**, 811) described a reaction between hydrazine and uracil whereby pyrazolone and urea were formed and most conveniently isolated as xanthhydryl derivatives. Levene and Bass (*J. Biol. Chem.*, 1927, **71**, 167) later showed that uridine and hydrazine hydrate afforded pyrazolone and, they presumed, ribosylurea, their inability to isolate the latter being attributed to the high solubility of its xanthhydryl derivative. We saw in these observations a possible solution to the problem of the structure of the pyrimidine mononucleotides. Meanwhile, physical evidence has been given, based on ultraviolet and infrared spectra, pK values, and solution densities, supporting the view that cytidylic acid *a* and *b* are the 2′ and the 3′ phosphate of cytidine (I and II), respectively (Cavalieri, *J. Amer. Chem. Soc.*, 1952, **74**, 5804; Fox, Cavalieri, and Chang, *ibid.*, 1953, **75**, 4315; Michelson and Todd, *J.*, 1954, 34). The present work affords confirmation and was carried through since it was felt that rigid chemical proof was necessary.



For the orientation of the phosphoryl group in the monoribonucleotides derived from nucleic acids, degradative procedures generally applicable to the sugars are not feasible. Methylation leads to phosphoryl migration (Brown, Magrath, and Todd, *J.*, 1954, **1442**) and acylation leads to cyclic phosphates (*idem*, *J.*, 1952, 2708). Removal of the aglycone, effected by acid hydrolysis, is accompanied by phosphoryl migration (cf. Brown and Todd, *J.*, 1952, 44). Khym *et al.* (*J. Amer. Chem. Soc.*, 1953, **75**, 1262; 1954, **76**, 1818, 5523) showed that during the treatment of adenylic acids *a* and *b* with the sulphonic acid resin Dowex-50 in the hydrogen form, phosphate migration proceeds more rapidly that hydrolysis of the glycosidic linkage so that a mixture of ribose 2-phosphate (III) and ribose 3-phosphate (IV) is obtained from either the *a* or the *b* acid. The liberated ribose phosphates are not significantly isomerised by the acidic resin, so that by using short hydrolysis times they were able to relate each ribose phosphate to its parent nucleotide. This procedure is inapplicable to the pyrimidine nucleotides since the *N*-glycosidic linkage in these compounds is very stable to hydrolysis.

No migration of the phosphate residue occurs in the isomeric nucleotides in alkaline solutions, a property they share with other polyhydroxyalkyl dihydrogen phosphates

* Part XXXII, *J.*, 1955, 2632.

(Brown and Todd, *loc. cit.*). In addition glycosylureas are unstable, decomposing to urea and a sugar under very mild conditions (Schoorl, *Rec. Trav. chim.*, 1903, **22**, 35). It seemed, therefore, that if fission by hydrazine could be applied to the isomeric uridylic or cytidylic acids, the corresponding ribose phosphates should be readily obtained, uncontaminated by products resulting from migration.

As a preliminary, uridine and cytidine were treated with hydrazine. The former, as reported by Levene and Bass (*loc. cit.*), yielded pyrazolone, and cytidine gave apparently 3-aminopyrazole. In neither case was free ribose produced although a material giving sugar reactions was present. A probable explanation is that further condensation of hydrazine with the liberated sugar or sugar ureide had occurred. Treatment of the product with benzaldehyde yielded a material indistinguishable on chromatograms from ribose, although this sugar was not produced by treatment with mineral acid or nitrous acid.

Reaction of hydrazine with the nucleotides was first studied with the readily accessible mixtures of the *a* and *b* isomers of uridylic and cytidylic acids. The reaction of hydrazine on uridylic acid was readily followed spectrophotometrically at 260 mμ, as the pyrazolone formed showed little or no absorption at that wavelength. With hydrazine hydrate at 70° reaction was complete in 10 minutes, and with 15% aqueous hydrazine in one hour. Cytidylic acid was more resistant, reaction being complete only in 70 minutes when hydrazine hydrate was used. 3-Aminopyrazole was probably the heterocyclic product in the latter case.

From the reaction mixture from either uridylic acid or cytidylic acid a phosphorus-containing acid was isolated as the barium salt. The product was clearly not barium diribosylurea phosphate, although it contained nitrogen. Paper chromatography indicated that this product was a sugar phosphate containing nitrogenous impurities. In accord with this, purification by absorption on an anion-exchange resin reduced the nitrogen content to zero.

Cytidylic acid *a* and *b* (Loring, Bortner, Levy, and Hammel, *J. Biol. Chem.*, 1953, **196**, 807) were next treated individually with hydrazine, and the crude barium ribose phosphate from each purified by treatment with an ion-exchange resin, followed by precipitation of barium and lithium salts. The salts were hygroscopic and extremely unstable at pH values above neutrality, the latter fact explaining the low yields from the hydrazine reaction. Acid hydrolysis yielded a sugar, indistinguishable from ribose on paper chromatograms.

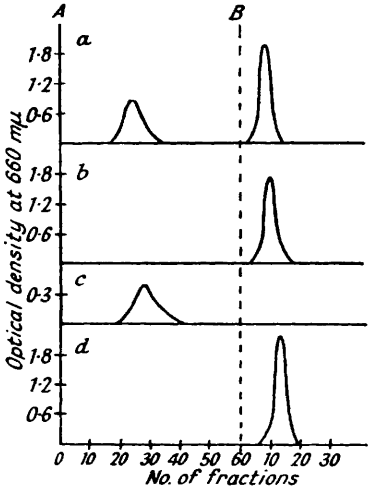
The two ribose phosphate preparations were compared with the ribose phosphates produced by hydrolysis of adenylic acids *a* and *b* (Khym *et al.*, *locc. cit.*) on Dowex-50. The results are shown in Fig. 1, which represents an ion-exchange analysis, in which a borate-containing eluant was used. Both acids were eluted at the same rate if borate was omitted. It is clear that one ribose phosphate is produced from cytidylic acid *a* (Fig. 1*c*) and another from cytidylic acid *b* (Fig. 1*b*). No cross contamination was observed, in accord with expectation. Uridylic acid *b* gave a sugar phosphate showing only one peak on the elution diagram (Fig. 1*d*) corresponding to that from cytidylic acid *b*. This confirms earlier work which showed that the phosphate residue occupies the same (although undefined) position in the ribofuranose residue of both nucleotides (Brown, Dekker, and Todd, *J.*, 1952, 2715). Khym *et al.* (*locc. cit.*), on the basis of ion-exchange characteristics and acceptable structural studies, show that the first eluted acid (Fig. 1*a*) is ribose 2-phosphate (III) while the second is ribose 3-phosphate (IV) (elution retarded owing to formation of an anionic 1 : 2-borate complex; cf. Khym and Cohn, *J. Amer. Chem. Soc.*, 1953, **75**, 1153). We have also reached the same conclusion. Paper chromatography in borate solutions afforded corroboration of the ion-exchange experiments. More important however was the action of alkali on the two ribose phosphates, illustrated in Fig. 2. Khym, Doherty, and Cohn (*J. Amer. Chem. Soc.*, 1954, **76**, 1818, 5523) also find that ribose 3-phosphate is degraded more rapidly than the 2-isomer. This order of instability is to be expected since the 3-phosphate should undergo β-elimination, a course not directly open to the 2-phosphate. Although this explanation differs from that of Khym, Doherty, and Cohn (*loc. cit.*), an easy base-catalysed elimination is shown by phosphate esters of other β-hydroxycarbonyl compounds which have been discussed earlier (Brown, Fried, and Todd, *Chem. and Ind.*, 1953, 352; *J.*, 1955,

2206). We are seeking confirmation of this view by a study of the products of alkaline fission of these and other sugar phosphates.

When treated with acid the sugar phosphates yielded ribose, the isomer considered to be ribose 2-phosphate (from cytidylic acid *a*) reacting faster than the other isomer. This is the order of hydrolysis rates found for the analogous glucose phosphates (Farrar, *J.*, 1949, 3131).

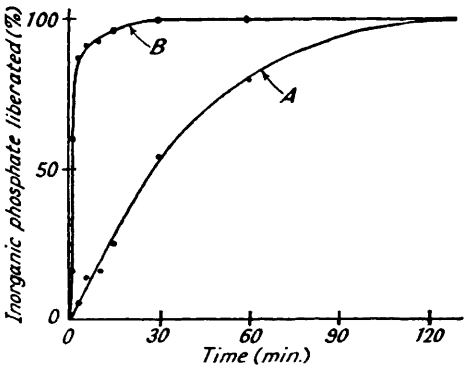
The above evidence points to the fact that cytidylic acid *a* yields ribose 2-phosphate, and cytidylic acid *b* yields ribose 3-phosphate. Cytidylic acids *a* and *b* are therefore cytidine-2′ and -3′ phosphate respectively. This conclusion can be reached independently, if it be accepted that the kinetic study by Khym *et al.* (*J. Amer. Chem. Soc.*, 1954, **76**, 1818) truly relates the first eluted acid (Fig. 1*a*) to adenylic acid *a* and the second to adenylic acid *b*. From the present study cytidylic acids *a* and *b* stand in the same relation to the

FIG. 1. *Ion-exchange analysis of ribose phosphates.*



a, Ribose 2- and 3-phosphate from adenylic acids *a* and *b*. *b*, *c*, and *d*, Ribose phosphates from cytidylic acid *b* and *a* and uridylic acid *b* respectively.

FIG. 2. *Rate of liberation of inorganic phosphate from the ribose phosphates by 0·2N-sodium hydroxide at 100°*



A, Ribose phosphate from cytidylic acid *a* (ribose 2-phosphate). *B*, Ribose phosphate from cytidylic acid *b* (ribose 3-phosphate).

derived phosphates so that the phosphate group in, for example, cytidylic acid *a* must occupy the same relative position in the ribofuranose residue as that in adenylic acid *a*. Since three independent methods (Cavalieri, *loc. cit.*; Brown, Fasman, Magrath, Todd, Cochran, and Woolfson, *Nature*, 1953, **172**, 1184; Brown, Fasman, Magrath, and Todd, *J.*, 1954, 1448), in addition to that of Cohn and his co-workers, show unambiguously that adenylic acids *a* and *b* are adenosine-2′ and -3′ phosphate, it follows that cytidylic and uridylic acids *a* and *b* are the -2′ and -3′ phosphate of cytidine and uridine respectively. Further confirmation is being sought in collaboration with Dr. S. Varadarajan, by unambiguous synthesis in the uridylic acid series.

The bearing of these results on the orientation of the internucleotidic linkage in ribonucleic acids is apparent from earlier studies (*inter al.*, Brown and Todd, *J.*, 1953, 2040; Brown, Heppel, and Hilmoe, *J.*, 1954, 40; Whitfeld, *Biochem. J.*, 1954, **58**, 390).

EXPERIMENTAL

Cytidylic Acids a *and* b.—These were prepared by fractionation of yeast cytidylic acid according to Loring *et al.* (*loc. cit.*). The *a* isomer had $[\alpha]_D^{19}$ −4°, and the *b* isomer +47°, measured as the sodium salts at pH 7. Loring *et al.* give $[\alpha]_D^{24.5}$ −8° and +46°, respectively, for

the disodium salts. The purity of the two acids was confirmed by ion-exchange chromatography on Dowex-2 resin in the formate cycle (cf. Brown, Dekker, and Todd, *loc. cit.*).

Uridylic Acid b.—The crystalline sample was kindly supplied by Dr. D. I. Magrath (cf. Brown, Magrath, and Todd, *J.*, 1954, 1442). Its purity was confirmed by ion-exchange chromatography.

Ribose 2- and 3-Phosphate from Adenylic Acid a.—Adenylic acid *a* was hydrolysed in aqueous solution with Dowex-50 at 100° for 1 min. After filtration the solution was neutralised with barium hydroxide, and the mixed barium salts of ribose 2- and 3-phosphate were precipitated by addition of ethanol (cf. Khym and Cohn, *loc. cit.*). This preparation served as a standard for comparison with the ribose phosphates obtained by hydrazinolysis, including ion-exchange and paper-chromatographic methods.

Paper Chromatography.—When the solvent system propan-2-ol–1% aqueous ammonium sulphate (2 : 1) was used, ribose 2-, 3-, and 5-phosphate had almost identical R_F values (*ca.* 0·34). Later it was found that use of propan-2-ol–1% aqueous boric acid–10% ammonia (50 : 25 : 1) permitted the separation of the sugar phosphates with R_F values increasing in the order ribose 5-, 3-, 2-phosphate (*e.g.*, 0·05, 0·10, and 0·18 respectively). The R_F values were variable but the ratios R_F of 2-phosphate/R_F of 3-phosphate lay close about 1·5. Sugar phosphates were detected both with the aniline hydrogen phthalate (Partridge, *Nature*, 1949, **164**, 443) and the phosphate spray reagent (Hanes and Isherwood, *ibid.*, p. 1107).

Fission of Uridine and Cytidine Derivatives by Hydrazine; Preliminary Experiments.—(a) *Spectroscopic.* Uridine (10 mg.) was heated in 15% aqueous hydrazine (10 c.c.) at 65°. The optical densities of aliquot parts were measured at 260 mμ. Disappearance of light absorption after 80 min. indicated completion of reaction. Under these conditions uridylic acid was degraded in 50 min., while cytidylic acid was unaffected. With hydrazine hydrate at 65°, uridylic and cytidylic acid had reacted completely in 10 and 60 min. respectively.

(b) *Paper chromatographic and isolation experiments.* (1) Cytidine (540 mg.) and hydrazine hydrate (1·5 c.c.) were heated at 65° for 90 min. After addition of water (2 c.c.) the solution was extracted with ethyl acetate. The extract, on concentration, yielded a colourless oil which when treated with xanthhydrol in aqueous acetic acid yielded a crystalline product, presumably 3-*amino*-xx-*dixanthhydrylpyrazole*. Crystallised from acetone–light petroleum (b. p. 60—80°) it had m. p. 216° (decomp.) (Found : C, 77·5; H, 5·4; N, 9·3. $C_{29}H_{21}O_2N_3$ requires C, 78·4; H, 4·8; N, 9·4%). In other experiments products giving analyses closer to those of the trixanthhydryl derivative were obtained.

Evaporation *in vacuo* of the aqueous phase gave an oil which was studied on paper chromatograms with ethyl acetate–pyridine–water (2 : 1 : 2). The material which reacted with the aniline phthalate reagent had R_F 0·38 but did not correspond to ribose (R_F 0·46). 0·1N-Hydrochloric acid at 100° did not affect the substance, but heating its aqueous solution with benzaldehyde, and then ether-extraction, gave a product in the aqueous phase corresponding on chromatograms to ribose. Similar chromatographic observations were made on the product derived from uridine.

(2) Uridylic acid *b* (1 g.) was heated at 65° for 90 min. with 15% aqueous hydrazine (15 c.c.). After concentration *in vacuo* the dried residual gum was dissolved in water and neutralised with barium hydroxide solution, and the barium salt precipitated by addition of ethanol (4 vol.). After reprecipitation the barium salt was dried at 50° *in vacuo* (Found : C, 18·3; H, 3·6; N, 7·5%). Other samples had various nitrogen contents. This material was studied further together with the corresponding product from cytidylic acid (see below).

The aqueous-ethanol filtrate from the barium salt was reduced to small bulk, and acetic acid added, followed by methanolic xanthhydrol. Next morning the solid product was collected, and washed with ethanol and then with hot water. Extraction with hot acetone, and concentration of the extract, gave dixanthhydrylpyrazolone, m. p. 215—217° (Found, in material dried at 80° *in vacuo* : C, 78·2; H, 4·7; N, 6·4. Calc. for $C_{29}H_{20}O_3N_2$: C, 78·3; H, 4·5; N, 6·3%). Dixanthhydrylpyrazolone was also obtained in corresponding reactions with uridine and 3-methyluracil. The material insoluble in acetone had m. p. 265—266° alone and in admixture with dixanthhydrylurea.

Cytidylic acid and hydrazine hydrate at 65° (90 min.) gave, after working up as in the case of uridylic acid, a barium salt (Found : C, 14·6; H, 3·1; N, 5·0%). This salt together with that derived from uridylic acid were studied on paper chromatograms with propan-2-ol–aqueous ammonium sulphate. The spots, detected with the aniline hydrogen phthalate or the phosphate reagent, corresponded in position to ribose 2-, 3-, or 5-phosphate.

In attempts to remove nitrogenous material from the barium salts they were treated in

aqueous solution with various reagents and then reprecipitated. Nitrogen content (N, 4%) was little reduced by nitrous acid or by overnight shaking at room temperature with benzaldehyde in di*iso*propyl ether (N, 3%) or with benzaldehyde at 100° for 15 min. (N, 4·6%). Treatment with Dowex-50 (H form) at 100° for 1 min. reduced the nitrogen content to *ca.* 1%. All of these treatments yielded salts showing only one sugar phosphate spot on paper chromatograms corresponding in its R_F value to ribose phosphate.

Anion-exchange Analyses of Ribose Phosphates.—For the ion-exchange analysis of the ribose phosphates from the hydrazinolysis a column (1 × 10 cm.) of the anion-exchange resin Dowex-2 (200—400 mesh) in the chloride form was employed, fractions (15 c.c.) being collected in an automatic fraction collector. Pentose was estimated in each fraction by Mejbaum's method (Brown, *Arch. Biochem.*, **1946**, **11**, 269) whereby 1 c.c. of solution and 3 c.c. of reagent were heated together at 100° for 20 min. and the optical density of the solution determined at 660 mμ (Fig. 1).

For elution of the ribose phosphates from the column, solvents closely similar to those used by Khym and Cohn (*locc. cit.*) were employed. Ribose 2-phosphate was eluted by a solution (*A*) containing ammonium chloride (0·05N), borax (0·01M), and ammonia (0·005M), which was followed by 0·05M-ammonium chloride (*B*) to elute ribose 3-phosphate. In other experiments the solvents, 0·0015M-sodium sulphate containing 0·0015M-borax, followed by 0·005M-sodium sulphate, were used, with the resin in the sulphate form. The results were similar to those with the chloride cycle.

The mixed barium salts of ribose 2- and 3-phosphate prepared from adenylic acid were used to standardise the column with respect to the position of elution of the ribose phosphates (Fig. 1*a*).

The following procedure was typical of that used with the cytidylic and uridylic acids. Cytidylic acid *b* (50 mg.) was heated with hydrazine hydrate (0·5 c.c.) at 70° for 60 min. and excess of hydrazine then removed by evaporation in a vacuum-desiccator. The residual gum was treated with saturated aqueous barium hydroxide (2·5 c.c.), and the solution immediately neutralised with carbon dioxide. The filtered solution was evaporated to dryness, redissolved in water with shaking, and after filtration treated with ethanol (4 vol.). The precipitated crude barium ribose phosphate was dissolved in water, stirred with Dowex-50, and filtered, and the solution then neutralised with ammonia to pH 8. After dilution to 25 c.c. the solution was put on the ion-exchange column, the column washed with water, and elution analysis carried out with the solutions named above. The elution diagram is shown in Fig. 1*b*. The only peak observed corresponded to that of ribose 3-phosphate.

Cytidylic acid *a* treated in the same way gave the elution diagram shown in Fig. 1*c*. The single peak corresponded to that of ribose 2-phosphate.

Uridylic acid *b* yielded a ribose phosphate corresponding to ribose 3-phosphate (Fig. 1*d*).

When mixtures of cytidylic acid *a* and *b* or uridylic acid *a* and *b* were employed, two peaks were always observed corresponding to ribose 2- and 3-phosphate.

In each case the fractions under the peak were pooled and percolated through a small Dowex-2 column (chloride form; 1 × 2 cm.), and the columns were eluted with 0·1N-hydrochloric acid. The effluent was neutralised with lithium hydroxide solution and concentrated *in vacuo*, and the product precipitated by ethanol. When run on chromatograms one spot was observed, corresponding to that of the ribose phosphate marker.

Ribose 2-Phosphate from Cytidylic Acid a.—The ammonium salt of cytidylic acid *a* (0·75 g.) and hydrazine hydrate (5 c.c.) were heated together at 65° for 60 min. After cooling, saturated barium hydroxide solution (10 c.c.) was added followed by acetone (200 c.c.), and the solution was kept overnight at 0°. The solid product was collected by centrifugation, washed with acetone, and dried. This barium salt was dissolved in water, barium removed by treatment with Dowex-50 resin (acid form), and the solution brought to pH 7·5 with dilute aqueous ammonia. After dilution to 300 c.c. the solution was percolated through a column of Dowex-2 (1 × 5 cm.; sulphate form). A ribose determination showed that 82% of the total reducing sugar had been retained on the column. Elution with 0·05M-sodium sulphate removed most of the product in the first 75 c.c. of eluate. This solution was treated with Dowex-50 to remove sodium ions, and then neutralised with aqueous barium hydroxide. Barium sulphate was centrifuged off, the solution concentrated under reduced pressure at 30°, and the barium ribose 2-phosphate precipitated by acetone. Part of this salt was further purified by reprecipitation twice from water, by ethanol (4 vol.). The hygroscopic material was dried *in vacuo* at 50° over phosphoric oxide (Found: C, 15·9; H, 4·4; P, 8·4. Calc. for $C_5H_9O_8PBa,H_2O$: C, 15·7; H, 2·9; P, 8·1%).

Another part of the crude barium salt was dissolved in water, the solution was clarified by

centrifugation, barium ions were removed by Dowex-50, and the lithium salt was prepared by adjustment to pH 7·0 with lithium hydroxide solution. The solution was concentrated *in vacuo* and *lithium ribose 2-phosphate* precipitated by addition of ethanol (4 vol.) in the cold. The very hygroscopic salt was centrifuged, washed with ethanol and ether and dried *in vacuo* at 30° (Found : C, 23·4; H, 4·6; P, 12·0. $C_5H_9O_8PLi_2,H_2O$ requires C, 23·1; H, 4·3; P, 11·9%).

Ribose 3-Phosphate from Cytidylic Acid b.—The above experiment was repeated with cytidylic acid *b*. The lithium salt was isolated and dried at 30° *in vacuo*. It was very unstable and hygroscopic, and still contained traces of lithium phosphate [Found : C, 22·6; H, 3·85; P(total), 12·5; P(organic), 11·3. Corrected for lithium phosphate : C, 23·7; H, 4·0; P, 11·9. Calc. for $C_5H_9O_8PLi_2,0{\cdot}5H_2O$: C, 23·9; H, 4·0; P, 12·2%].

Ribose 2- and 3-phosphate, as their lithium salts, ran as single spots on chromatograms and had *R*, 0·34 and 0·21 respectively in propan-2-ol–1% aqueous ammonium borate (2 : 1).

Hydrolysis of Ribose 2- and 3-Phosphate.—(a) *Acid.* The substances, as their lithium salts, were heated in 0·1N-hydrochloric acid at 100°. Aliquot parts were analysed at intervals for inorganic phosphate. After 15, 30, and 60 min. respectively ribose 2-phosphate released 22, 35, and 56%, and ribose 3-phosphate 11, 21, and 50% of inorganic phosphate.

Paper chromatograms with butanol–acetic acid–water and ethyl acetate–pyridine–water disclosed ribose as the only sugar produced after complete hydrolysis.

(b) *Alkali.* The two acids, as their lithium salts, were heated in 0·2N-sodium hydroxide at 100°. Aliquot parts were analysed at intervals for liberated phosphate. The results are recorded in Fig. 2.

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UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE. [*Received, March 24th*, 1955.]