353. *The Configuration of the Ribitol Phosphate Residue in Cytidine Diphosphate Ribitol.*

By J. BADDILEY, J. G. BUCHANAN, and B. CARSS.

The ribitol cyclic phosphate (IV), obtained from cytidine diphosphate ribitol (CDP-ribitol) and hot ammonia, was oxidised with periodate and then bromine water to glyceric acid 2 : 3-(hydrogen phosphate) (V). On brief acid treatment this yielded a mixture (probably) of glyceric acid 2- and 3-phosphates (VI and VII), the configuration of which was determined with a mixed enzyme system from rabbit muscle where D-glyceric acid phosphates are known metabolites.

The ribitol cyclic phosphate from CDP-ribitol, like that from D-ribitol-5 phosphate, gave a product fully active in the enzyme system, whereas the L-isomer was inactive. It is concluded that the nucleotide contains a D-ribitol-5 phosphate residue. The biochemical significance of this is discussed.

The isomeric ribitol phosphates formed during acid-catalysed racemisation of the 5-phosphate have been identified.

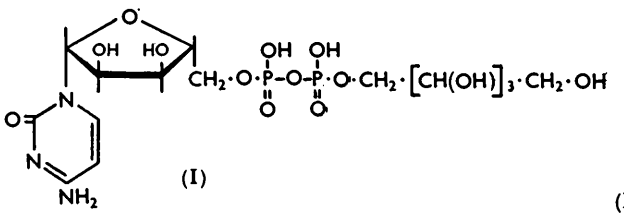
The glycerophosphate residue in cytidine diphosphate glycerol has the L-configuration.

ONE of the nucleotides isolated from *Lactobacillus arabinosus* [1,2] has been shown [3] to be P^1-cytidine-5′ P^2-ribitol-1 pyrophosphate (I), conveniently called cytidine diphosphate ribitol (CDP-ribitol). Although ribitol is symmetrical, its 1-phosphate is not, and there remained the problem of establishing the configuration of this residue in CDP-ribitol. The isomerism in the 1-phosphates of ribitol is similar to that in the α-glycerophosphates.

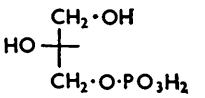
For the related nucleotide, cytidine diphosphate glycerol, it has now been shown that the α-glycerophosphate, produced from the nucleotide by the action of a rattlesnake venom enzyme,[4] is readily and completely oxidised to dihydroxyacetone phosphate by the enzyme glycerophosphate dehydrogenase (experiments were kindly performed by

[1] Baddiley and Mathias, *J.*, 1954, 2723.
[2] Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, 1956, **64**, 599.
[3] Baddiley, Buchanan, Carss, and Mathias, *J.*, 1956, 4583.
[4] Baddiley, Buchanan, Mathias, and Sanderson, *J.*, 1956, 4186.

Dr. D. E. Hughes and Mr. A. R. Sanderson). This enzyme is specific in its action on L-α-glycerophosphate (II). It is concluded that the α-glycerophosphate residue in CDP-glycerol has the L-configuration. The observation is consistent with the fact that α-glycerophosphate from other natural sources has the L-configuration.[5]



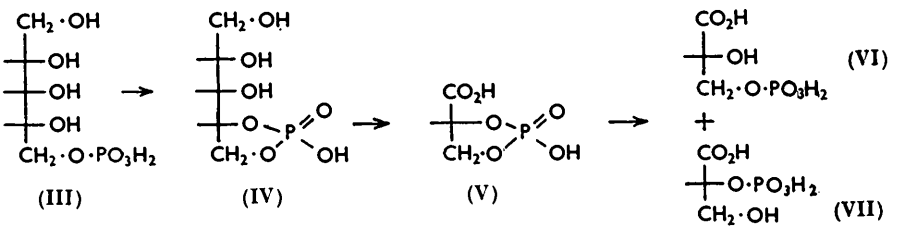
(I)



(II)

As in the case of the glycerol-containing nucleotide, very small amounts of CDP-ribitol were available, and it was considered unlikely that direct measurements of rotations on the ribitol phosphate derived from it would be possible by the conventional methods at our disposal. As ribitol phosphates had not been encountered hitherto in Nature no specific and direct enzymic method could be used for determining the configuration in the ribitol-containing nucleotide. A micromethod has now been developed for the degradation of ribitol-1 phosphates to glyceric acid phosphates and their subsequent determination by enzymic reactions. The degradation is outlined in the annexed formulæ.

D-Ribitol-1 phosphate (III),* prepared by reduction of D-ribose-5 phosphate with sodium borohydride, was cyclised to the 4 : 5-(hydrogen phosphate) (IV) through the action of trifluoroacetic anhydride.[3] A few milligrams were purified by paper chromatography; the material was eluted from the paper and oxidised with sodium metaperiodate, followed



(III) (IV) (V) (VI) (VII)

by bromine water. It is assumed that the periodate oxidation yields D-glyceraldehyde 2 : 3-(hydrogen phosphate), which is further oxidised by bromine to D-glyceric acid 2 : 3-(hydrogen phosphate) (V). Without isolation the cyclic phosphate was hydrolysed by brief treatment with Dowex-50 resin (H^+ form) and the resulting mixture of D-glyceric acid 3- (VI) and 2-phosphate (VII) was run on a paper chromatogram. It was important to demonstrate that no inorganic phosphates had been formed during the series of reactions. The proportion of the two glyceric acid phosphates in the mixture was unimportant (see below), but the ratio [6] of 3- to 2-isomer is probably about 4 : 1. The authenticity of the

* D-Ribitol 5-phosphate is, of course, the same substance as L-ribitol 1-phosphate. According to the standard rules of nomenclature, the name with the lower number should have preference, *i.e.*, L-ribitol 1-phosphate. Nevertheless, D-ribitol-5 phosphate is used in this paper because of its use in previous papers and because the substance is probably related metabolically to D-ribose 5-phosphate and D-ribulose 5-phosphate and is degraded to D-glyceric acid. Moreover, it was important to avoid the possible implication that L-ribitol 1-phosphate is in any way related to L-α-glycerophosphate. These two compounds are, in fact, in stereochemically opposite series. The correct name for the naturally occurring L-α-glycerophosphate (II), according to standard rules of nomenclature, is D-glycerol 1-phosphate (equivalent to L-glycerol 3-phosphate). However, probably for reasons concerning its biochemical origin, the L-α or L-3 nomenclature has always been adopted for this compound and we have retained it in this paper.

[5] Baer and Fischer, *J. Biol. Chem.*, 1939, **128**, 491.
[6] Ballou and Fischer, *J. Amer. Chem. Soc.*, 1954, **76**, 3188.

mixture was shown by enzymic dephosphorylation of a sample extracted from paper to give glyceric acid, identified by paper chromatography.

Both D-glyceric acid 2- and 3-phosphate are known intermediates in the alcoholic fermentation of glucose in yeast and in the conversion of glucose into lactic acid in muscle. It was thought that if the mixed phosphates obtained from the above degradation of D-ribitol-5 phosphate were added to a multi-enzyme system from yeast or muscle their incorporation into the glucose metabolic system would be measurable. A mixed enzyme preparation from rabbit muscle (kindly provided by Dr. G. R. Greenberg) was used in our experiments. Adenosine triphosphate (ATP) and magnesium ions were added to the enzyme solution and utilisation of glyceric acid phosphates was measured by the oxidation of reduced diphosphopyridine nucleotide (DPNH). The oxidation was observed spectrophotometrically by standard methods.[7]

When the above degradation and enzymic assay were carried out on D-ribitol-5 phosphate it was found that one mol. of DPNH was oxidised per mol. of glyceric acid phosphate (calculated as organic phosphate) added, and the same behaviour was observed with standard glyceric acid phosphate. The reaction products from this multi-enzyme system have not been identified, but it is probable that lactic acid is the principal one.

The nature of the products in the enzymic test and even the correct identity of the chemical degradation products are not important for our purposes, provided that the test can be shown to be specific for D-ribitol-5 phosphate. It was necessary then to show that L-ribitol-5 phosphate, after degradation, yielded products inactive in the enzymic assay. Pure L-ribitol-5 phosphate would be difficult to obtain, but the specificity of the test would be demonstrated if the DL-compound exhibited half of the activity of the D-5 isomer.

Racemisation of phosphates of symmetrical polyols is readily achieved by phosphate migration under acid conditions. In the studies leading to the structure of CDP-ribitol it was found that treatment of D-ribitol-5 phosphate with acid gave a mixture of phosphates, which were separable by paper chromatography.[3] The 1(5)-phosphate had the lowest R_F value of the phosphates in the *n*-propyl alcohol–ammonia solvent and could be isolated in pure form from chromatograms. Samples of D-ribitol-5 phosphate were treated with N-hydrochloric acid at 100° for different periods of time and the 1(5)-phosphate was re-isolated from a paper chromatogram. The degradation procedure and enzyme test were repeated and it was found (see Table 1) that in each case DPNH oxidation reached an end-point corresponding to less than one mol. per mol. of organic phosphate. This implied that the enzyme system was inactive with the L-glyceric acid phosphates, in confirmation of the work of Meyerhof and Kiessling.[8] The measurements indicated, moreover, that racemisation of the ribitol phosphates had been substantial after 30 minutes and was complete after 90 minutes. It followed that the test would be specific in determining the configuration of the ribitol phosphate from CDP-ribitol.

Ribitol 1 : 2-(hydrogen phosphate) can be obtained directly from CDP-ribitol by treatment with hot aqueous ammonia. A sample prepared in this way was isolated from a paper chromatogram, then degraded, and the products were tested by the enzymic method. One mol. of DPNH was oxidised for each mol. of organic phosphate (see Table 1) and it is concluded that the ribitol phosphate residue in CDP-ribitol corresponds to D-ribitol-5 phosphate.

The other phosphates produced by acid treatment of D-ribitol-5 phosphate have now been identified. The earlier work[3] had shown that, besides anhydroribitol and a phosphate with the same chromatographic properties as the original, two compounds were produced with R_F values slightly greater than that of ribitol-1(5) phosphate in *n*-propyl alcohol–ammonia. The unknown compound with the higher R_F value (" YS ") gave a yellow colour a few minutes after being sprayed with the periodate–Schiff reagents; that with the lower R_F value (" PS ") gave a purple colour with these reagents in the same way

[7] Colowick and Kaplan, " Methods in Enzymology," Vol. I, Academic Press, New York, 1955.
[8] Meyerhof and Kiessling, *Biochem. Z.*, 1935, **276**, 239.

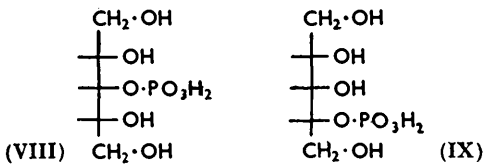
as did ribitol-1(5) phosphate. YS and PS could not be completely resolved and the experiments described below, which prove that YS is ribitol-3 phosphate (VIII) and PS is ribitol-2(4) phosphate (IX), were carried out on mixtures of the two.

TABLE 1. *Action of rabbit muscle enzymes on glyceric acid phosphates.*

Sample of glyceric acid phosphates (PGA)	PGA (mol.) (phosphate detn.)	D-PGA (mol.) (enzyme assay)	D-PGA (%) in mixture
A	0·50, 0·50	0·49, 0·50	98, 100
B	0·18, 0·18	0·13, 0·13	72, 72
C	0·58, 0·58	0·27, 0·28	47, 48
D	0·70, 0·69	0·33, 0·33	47, 48
E	0·53, 0·54	0·55, 0·55	104, 102

A, From D-ribitol-5 phosphate. B, From D-ribitol-5 phosphate, treated with acid for 30 min. C, From D-ribitol-5 phosphate, treated with acid for 90 min. D, From D-ribitol-2 and ribitol-3 phosphate, treated with acid for 90 min. E, From ribitol 1 : 2-(hydrogen phosphate) from CDP-ribitol.

A mixture of YS and PS, separated from ribitol-1(5) phosphate on a long chromatogram, was treated with prostate phosphatase. The original compounds disappeared and ribitol was produced, together with a trace of anhydroribitol. The significance of the anhydroribitol will be discussed later. Treatment of YS and PS with acid gave the typical mixture



of compounds produced by acid treatment of ribitol-1(5) phosphate. A sample of yeast adenylic acid, which had been shown by ion-exchange chromatography to be a mixture of the 2′- and 3′-isomers, was hydrolysed by Dowex-50 resin (H^+ form). The mixed ribose phosphates were reduced with sodium borohydride to the ribitol phosphates and examined on paper. Two spots were obtained, identical in R_P values and colour reactions with YS and PS. The effects of prostate phosphatase and of acid on these ribitol phosphates were the same as those on YS and PS with the exception that phosphatase action yielded no anhydroribitol. When the ribitol-2 and -3 phosphate obtained from yeast adenylic acid were heated with N-hydrochloric acid and the 1(5)-phosphate, which was produced, subjected to the chemical degradation and enzymic test to establish its configuration, it was found to be completely racemised. These observations proved that YS and PS were ribitol-2(4) and -3 phosphate. It remained only to decide which of these structures corresponded to each spot.

Ribitol 1 : 2(4 : 5)-(hydrogen phosphate) was readily hydrolysed in sodium hydroxide solution to a mixture of two ribitol phosphates. One of these corresponded with the 1(5)-phosphate and the other must be ribitol-2(4) phosphate (IX), since phosphate migration does not occur in alkali. The latter corresponded with PS. Hydrolysis of CDP-ribitol with dilute aqueous sodium hydroxide also gave the 1(5)-phosphate and PS. YS was not formed and is therefore the 3-phosphate. Compounds analogous to YS and PS are produced when mannitol-1(6) phosphate is treated with acid.

The trace of anhydroribitol produced when YS and PS were treated with prostate phosphatase probably arose from traces of anhydroribitol phosphate(s) in these spots. No ribitol can be detected during acid hydrolysis of ribitol phosphates under our conditions. Since free ribitol itself would yield some anhydro-compound under these conditions it is probable that some anhydroribitol phosphate would be present.

The configuration of the ribitol phosphate residue in CDP-ribitol is of some biochemical importance. Although the function of CDP-ribitol is still unknown, it probably

participates as a coenzyme or intermediate in the metabolism of ribitol and its phosphate. It is reasonable to assume that the ribitol phosphate residue will be related metabolically to some naturally occurring pentose. As it is now shown that this residue is D-ribitol-5 phosphate (equivalent to L-ribitol-1 phosphate) (III), it follows that it and CDP-ribitol are related to either D-ribose-5 phosphate or D-ribulose-5 phosphate. Both these pentose phosphates occur as interconvertible intermediates in glucose metabolism in many living cells and it is not yet possible to determine which is more directly related to the nucleotide. It is interesting that, although the reversible reduction of aldoses and ketoses to polyols by bacteria is well known, only recently have similar reductions been observed with their phosphoric esters. A microbial enzyme has now been described which catalyses the reversible reduction of fructose-6 phosphate to D-mannitol-1 phosphate,[9] and a similar but less specific enzyme present in silkworm blood readily reduced D-ribose-5 phosphate to ribitol phosphate in the presence of triphosphopyridine nucleotide.[10]

Experimental

Oxidation of D-*Ribitol* 4 : 5-(*Hydrogen Phosphate*).—D-Ribitol-5 phosphate was cyclised by the use of trifluoroacetic anhydride as described earlier.[3] The cyclic phosphate (*ca.* 2 mg.) was purified by paper chromatography in solvent system (1). Pure D-ribitol 4 : 5-(hydrogen phosphate) was obtained by eluting the appropriate area of the paper and evaporating the eluate to dryness *in vacuo*. The residue was dissolved in sodium metaperiodate solution (80 μl., containing 150 mg./ml.) and the pH was kept at 5—6 by the addition of micro-drops of dilute sodium hydroxide solution. After 30 min. bromine (5—10 μl.) was added to the mixture with stirring, together with dilute sodium hydroxide solution to pH 7. After a further 1 hr. the pH had fallen to *ca.* 4 and solid inorganic material was removed by centrifugation. The yellow supernatant solution was evaporated to dryness *in vacuo* on a Polythene plaque. The residue was dissolved in water (0·1 ml.), and some solid inorganic material was removed by centrifugation. The supernatant solution was passed through a Dowex-50 (H^+ form) resin column (2 × 0·5 cm.). The column was washed with water, and the acid eluate (*ca.* 0·5 ml.) was collected. After 30 min. it was neutralised with ammonia and after suitable adjustment of the volume the products were resolved by paper chromatography run in solvent system (1) (descending front, 16 hr.). The area of the paper containing the D-glyceric acid phosphates was eluted. In some experiments a trace of a slower-running phosphate was observed. No inorganic phosphate was detected. The solution of pure D-glyceric acid phosphate thus obtained (sample A) was adjusted to a volume of *ca.* 120 μl. and samples were used in the following experiments.

Action of Prostate Phosphatase on the Product of Oxidation of D-*Ribitol* 4 : 5-(*Hydrogen Phosphate*).—Solution A (40 μl., containing *ca.* 300 μg. of D-glyceric acid phosphate) was mixed with 0·4M-ammonium acetate buffer (40 μl., pH 5·5) and a preparation [2] of prostate phosphatase (20 μl.). The mixture was kept under toluene at 37° for 36 hr. A sample of authentic D-glyceric acid phosphate was treated similarly. After suitable adjustment of the volume the products were subjected to paper chromatography in solvent systems (1), (2), and (3), and after drying were sprayed with reagents for phosphates and α-glycols.[11, 12] The oxidation product and D-glyceric acid phosphate were indistinguishable (R_F values are given in Table 2).

These experiments were repeated with samples of the oxidation products obtained from racemic ribitol cyclic phosphate (see below) and the cyclic phosphate derived from CDP-ribitol. Results were identical in all cases.

Production of Racemic Glyceric Acid Phosphate.—The barium salt of D-ribitol-5 phosphate (8 mg.) was heated with N-hydrochloric acid (0·5 ml.) for 30 min. at 100° in a sealed tube. The solution was evaporated to dryness; the residue was dissolved in water (40 μl.) and passed through a Dowex-50 (H^+ form) resin column (2 × 0·5 cm.) which was then washed with water. The acid eluate and washings were collected, neutralised with ammonia, evaporated to 80 μl.,

[9] Wolff and Kaplan, *J. Biol. Chem.*, 1956, **218**, 849.
[10] Faulkner, *Biochem. J.*, 1956, **64**, 436.
[11] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[12] Buchanan, Dekker, and Long, *J.*, 1950, 3162; Baddiley, Buchanan, Handschumacher, and Prescott, *J.*, 1956, 2818.

and applied to the baseline of a chromatogram paper. After chromatography (16 hr., descending front) in solvent system (1) the paper was dried and the area containing ribitol-1(5) phosphate was eluted. After evaporation (to 40 μl.) the solution was passed through a Dowex-50 (H^+ form) resin column (2 × 0·5 cm.) and the eluate (*ca.* 0·3 ml.) was collected and freeze-dried in a bulb-tube (*ca.* 3 ml. capacity). To the syrupy residue was added trifluoroacetic anhydride (1·0 ml.). The bulb-tube was sealed and shaken for 1 hr. Excess of trifluoroacetic anhydride was removed *in vacuo* and the syrupy residue was treated with ammonia (*d* 0·88, 120 μl. in water, 40 μl.). The product was run on paper as a band in solvent system (1) (ascending front). The subsequent elution and oxidation procedure were as described above, except that 120 μl. of sodium periodate solution were used. Sample B of glyceric acid phosphate was thus obtained.

The procedure was repeated on the barium salt of D-ribitol-5 phosphate (25 mg.) racemised for 1·5 hr. in N-hydrochloric acid at 100° (when about 50% breakdown to phosphoric acid and anhydroribitol occurs) (glyceric acid phosphate, sample C), and with a similarly racemised mixture from D-ribitol-2 and -3 phosphate from yeast adenylic acid (glyceric acid phosphate, sample D).

Glyceric Acid Phosphate from CDP-ribitol.—CDP-ribitol solution (140 μl. containing 2·8 mg. of nucleotide) was heated in a sealed tube with ammonia (*d* 0·88, 150 μl.) for 30 min. at 100°. After evaporation to dryness the residue was dissolved in water (80 μl.) and run on paper as a band in solvent system (1) (ascending front). The cyclic phosphate was eluted from the appropriate area of the paper and was subjected to oxidative degradation to glyceric acid phosphate in the manner already described (sample E).

Determination of Phosphate in Samples A—E.—The phosphate content (in terms of glyceric acid phosphate) of each of the samples was determined in duplicate by Allen's method.[13] Control determinations were carried out on solutions which had passed through all the stages described. The average control value in all the runs was +0·024 μmole of phosphate per 10 μl. of sample. This is allowed for in values reported in Table 1.

Enzymic Assay of D-*Glyceric Acid Phosphate in Samples* A—E.—*Materials.* Enzyme: a freeze-dried sample of rabbit muscle enzyme was prepared by Racker's method,[14] the fraction being used which was precipitated between 50% and 72% ammonium sulphate saturation.[15] Reduced DPN: a sample supplied by C. F. Boehringer & Sons, Mannheim, and containing 64% of DPNH was used. Phosphate buffer: 0·01M-potassium dihydrogen phosphate solution was treated dropwise with concentrated potassium hydroxide solution until the pH had risen to 7·3. Magnesium sulphate: an aqueous solution containing 100 μmoles/ml. ATP: a 0·05M-solution of the Na/K salt. The rabbit muscle contains myokinase and other enzymes which convert ATP into the diphosphate required for the production of lactate from glyceric acid phosphate.

Method. In a 1 cm. silica cell were placed phosphate buffer (3·0 ml.), DPNH (*ca.* 1·4 mg.) to give an optical density of *ca.* 2·5 at 340 mμ, magnesium sulphate solution (0·05 ml.), ATP solution (0·05 ml.), and freeze-dried rabbit muscle enzyme (100—200 μg.). Variation in the amount of enzyme added merely altered the rate at which D-glyceric acid phosphate was consumed and had no effect on the amount of DPNH oxidised. The optical density of the solution at 340 mμ was recorded at convenient intervals. After a slight initial fall in optical density a steady reading was observed. The glyceric acid phosphate (10 μl.) was added and the fall in optical density at 340 mμ was recorded until the reading was again constant (10—30 min.). It was found in separate experiments that glyceric acid phosphate was not consumed unless both magnesium and ATP were present.

Samples A—E were examined in duplicate and the amount of D-phosphate present in 10 μl. was calculated on the assumption that one equiv. of it consumed one equiv. of DPNH, a value of ε 6220 at 340 mμ being used for DPNH. The results are shown in Table 1.

Action of Phosphatase on "PS" and "YS."—The barium salt of D-ribitol-5 phosphate (2 mg.) was heated with N-hydrochloric acid in a sealed tube for 15 min. at 100°. After removal of barium with a little Dowex-50 (H^+ form) resin and evaporation to dryness *in vacuo*, the hydrolysis products were resolved by paper chromatography as a band in solvent system (1) using a descending front (20 hr.). PS and YS were eluted from the appropriate area of the

[13] Allen, *Biochem. J.*, 1940, **34**, 858.
[14] Racker, *J. Biol. Chem.*, 1947, **167**, 843.
[15] Ratner and Pappas, *ibid.*, 1949, **179**, 1197.

paper on to a small Polythene plaque, with subsequent evaporation of solvent *in vacuo* (P_2O_5). In this way, although it was not possible to achieve a complete separation of PS and YS from each other, they were obtained free from the other products of acid action (ribitol-1(5) phosphate, anhydroribitol, and phosphoric acid).

A part of the mixture was incubated at 37° for 16 hr. with prostate phosphatase in 0·2M-ammonium acetate buffer (pH 5·5). A second part was heated with N-hydrochloric acid at 100° for 15 min. and solvent was removed *in vacuo*. The products of these reactions were examined, together with a sample of unchanged material in solvent system (1). Products were detected by spraying the dried paper with the molybdate reagent for phosphoric esters and the periodate–Schiff reagents for glycols. Phosphatase action completely destroyed PS and YS, yielding ribitol, inorganic phosphate, and a trace of anhydroribitol, indistinguishable in behaviour from the authentic materials. Short acid treatment of PS and YS produced the typical acid hydrolysis mixture observed with all ribitol phosphates.[3]

Synthesis of a Mixture of D-*Ribitol-2 and -3 Phosphate.*—Yeast adenylic acid (150 mg.) was added rapidly, with stirring, to Dowex-50 (H^+ form) resin (1·5 ml.) in water (1·5 ml.) at 100°. After 5 min. at this temperature the mixture was cooled in an ice-bath and the resin was filtered off and washed with water (2 ml.). The filtrate contained much organic phosphate, a little inorganic phosphate, and no ultraviolet-absorbing material. After adjustment to pH 8 with dilute ammonia, sodium borohydride (22 mg.) was added with stirring. The course of the reduction was followed at intervals by examining samples on filter paper with the aniline phthalate spray reagent.[16] Reduction was almost complete after 1 hr. but a further 2 hr. was allowed to complete the reaction. Acetic acid (0·15 ml.) was added to decompose the excess of borohydride, followed by water (to 60 ml.) and ammonia to pH 8. The solution which was N/24 with respect to ammonium acetate was passed through a column of Amberlite IRA-400 (acetate form) resin (6 × 1 cm.), and borate was eluted with M/24-ammonium acetate solution (50 ml.). The phosphates were eluted with M-ammonium acetate solution (20 ml.). The eluate was stirred with Dowex-50 (H^+ form) resin (12 ml.), then filtered, and the resin was washed with a little water. Filtrate and washings were evaporated to small volume *in vacuo* below 40°. Residual acetic acid was removed by distillation *in vacuo* below 40°. The remaining oil was distilled with 5 portions (each of 20 ml.) of anhydrous methanol to remove traces of boric acid. The residue was dissolved in water (5 ml.) and 0·1N-barium hydroxide was added to pH 9. Excess of barium was precipitated by the addition of a little solid carbon dioxide. After evaporation *in vacuo* to 5 ml., barium phosphate and carbonate were removed by centrifugation. Alcohol (5 ml.) was added to the supernatant liquor, and the precipitated barium salt was centrifuged off, washed with acetone, and dried. It was purified by redissolving it in water (5 ml.), centrifuging off a small insoluble residue, and treating the supernatant liquid with acetone (10 ml.). The precipitated salt was washed with acetone and dried *in vacuo* (Found : P, 8·2. Calc. for $C_5H_{11}O_8PBa$: P, 8·4%).

According to Khym, Doherty, and Cohn [17] the salt should consist of *ca.* 60% of ribitol-3 phosphate and 40% of D-ribitol-2 phosphate. These authors hydrolysed yeast adenylic acid by a method similar to that described above and reduced the ribose phosphates by catalytic hydrogenation.

Experiments with the Mixture of Ribitol-2 and -3 Phosphate.—Barium ions were removed from a small sample of the salt by treatment with a little Dowex-50 (H^+ form) resin. The sample was examined by chromatography in solvent system (1), a descending front being used and the solvent allowed to drip (20 hr.). A typical acid-treated sample of ribitol-1(5) phosphate was examined concurrently. Papers were sprayed with reagents for the detection of phosphates and α-glycols. Ribitol-2(4) and -3 phosphate were incompletely separated and were indistinguishable from PS and YS. The synthetic mixture contained no detectable ribitol-1(5) phosphate or inorganic phosphate. Short acid treatment of the mixture gave results identical with those already described for a similar treatment of a mixture of PS and YS. Enzymic dephosphorylation of the synthetic mixture yielded ribitol and inorganic phosphate. (No trace of anhydroribitol was detected.)

Alkaline Hydrolysis of CDP-ribitol.—CDP-ribitol (400 μg.) in water (20 μl.) was heated with 2N-sodium hydroxide (20 μl.) in a sealed tube at 100° for 30 min. Sodium ions were removed with a little Dowex-50 (H^+ form) resin, ammonia was added to neutrality and, after suitable

[16] Partridge, *Nature*, 1949, **164**, 443.
[17] Khym, Doherty, and Cohn, *J. Amer. Chem. Soc.*, 1954, **76**, 5525.

adjustment of the volume, the mixture was examined by paper chromatography with descending front in solvent system (1) (20 hr.). The products were cytidine-5′ phosphate, ribitol-1(5) phosphate, and PS. The ribitol phosphates were present in comparable amounts, with the 1(5)-phosphate slightly predominating. No YS or inorganic phosphate was detected.

Alkaline Hydrolysis of Ribitol 1 : 2-(Hydrogen Phosphate).—This was carried out in a manner similar to that described for CDP-ribitol. The ribitol phosphates formed were indistinguishable from those obtained from the nucleotide.

The Behaviour of Ribitol Phosphates with the Periodate–Schiff Reagents.—Ribitol-1(5) and -2(4) phosphate behave similarly towards the periodate–Schiff spray reagents, *i.e.*, a purple-red colour is rapidly formed which later intensifies and deepens to purple. This behaviour is typical of acyclic polyols. Only a little of the 2(4)-isomer is produced by acid treatment of ribitol-1(5) phosphate—sufficient to give a weak phosphate reaction and a weak purple colour with the glycol reagents after several hours, but insufficient to produce the initial purple-red colour. Ribitol-3 phosphate also gives the initial purple-red colour, but after a few minutes a strong yellow colour is superimposed on the purple-red area and is seen as a yellow halo. The amount of ribitol-3 phosphate formed during acid treatment of ribitol-1(5) phosphate is too small for detection by the phosphate spray reagents applied to paper chromatograms containing normal amounts (*ca.* 100 μg.) of total phosphates. However, the yellow colour reaction with the periodate–Schiff reagents is clearly shown on such papers.

Inositol phosphate gives a yellow colour with the latter reagents.

Paper Chromatography.—Whatman No. 4 paper was washed with 2N-acetic acid, then water. The following solvent systems were used :

(1) *n*-Propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1); (2) *n*-butyl alcohol–acetic acid–water (organic layer) (4 : 1 : 5); (3) *n*-butyl alcohol–ethanol–water (organic layer) (4 : 1 : 5). Ascending front chromatography was employed.

TABLE 2.

	R_F in solvents :		
	(1)	(2)	(3)
CDP-ribitol	0·33	—	—
Ribitol-1 phosphate	0·34	—	—
Ribitol-2 phosphate (PS)	0·39	—	—
Ribitol-3 phosphate (YS)	0·39	—	—
Ribitol 1 : 2-(hydrogen phosphate)	0·53	—	—
Anhydroribitol	0·87	—	—
Glyceric acid phosphate	0·20	0·23	0·07
Glyceric acid	0·53	0·34	0·12
Ribitol	0·76	0·34	0·34

The following distances of migration were observed in solvent system (1), when a descending front was used and the solvent allowed to drip : ribitol-1 phosphate, 21·6 cm.; ribitol-2 phosphate, 25·1 cm.; ribitol-3 phosphate, 26·1 cm.

We are grateful to Dr. G. R. Greenberg for helpful advice on the enzyme experiments. We thank the Nuffield Foundation and the Rockefeller Foundation for grants and the Council of King's College for the award of a George Angus Studentship (to B. C.).

KING'S COLLEGE, UNIVERSITY OF DURHAM,
NEWCASTLE UPON TYNE.

[*Received, November 15th,* 1956.]