[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Ribose Phosphates: Production from Nucleotides, Ion-Exchange Separation and Characterization[1]

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Ribose-2- and -3-phosphates have been formed by the hydrolysis of adenylic acids with a polystyrene sulfonic acid cation-exchange resin, separated by ion-exchange chromatography with borate complexing and by fractional crystallization of the brucine salts, and prepared in pure form as the sodium, barium and brucine salts. Methods are given for the preparation of the various salts with negligible contamination by the inorganic phosphate produced by alkaline decomposition, for the interconversion of the various salts, and for the ion-exchange separation of all five isomers from one another. The ribose phosphates are characterized by their optical and ion-exchange behaviors in the presence and absence of borate, by the differences in susceptibility of their methyl pyranosides to periodate oxidation, and by the differences in their decomposition rates in alkali. A new isomer which appears on prolonged acid treatment is identified as ribose-4-phosphate. A simple procedure for the preparation of barium ribose-5-phosphate is given. The preparation of these ribose phosphates concludes the degradative identification of the isomeric adenylic acids (and guanylic acids) *a* and *b* as the 2′- and 3′-phosphonucleosides, respectively, makes the ribose phosphates available for study, and demonstrates that the previous preparations of ribose-3-phosphate were primarily mixtures of ribose-2- and -3-phosphates.

Introduction[2]

The sugar component of muscle inosinic acid[2a] was identified as the then unknown D-ribose by Levene and Jacobs[2b] in 1910. Hammersten[2c] had described a pentose from ribonucleic acid (RNA) in 1894 but could not identify it. Kossel, using Altmann's protein-free nucleic acid preparations, had isolated a carbohydrate three years earlier.[2d]

Levene and Jacobs also demonstrated, in muscle inosinic acid, the attachment of phosphate to the sugar, rather than to the purine base, by alkaline hydrolysis to a nucleoside and inorganic phosphate, on the one hand and by acid hydrolysis to base plus ribose phosphate, on the other.[2b] The latter was shown to be ribose-5-phosphate, which was subsequently synthesized.[3] Muscle adenylic acid, found by Embden[2e] in 1927, was shown to be deaminated to this same inosinic acid by a specific enzyme (Schmidt)[2e] and thus also to contain ribose-5-phosphate.

Muscle adenylic acid was compared, with respect to the ease of acid hydrolysis of its phosphate group, with the guanylic, adenylic and pyrimidine nuleotides derived from (yeast) nucleic acid and found, as with the related inosinic acid, to behave like the resistant pyrimidine nucleotides rather than the (six- to tenfold) more labile yeast purine nucleotides.[2f] The pyrimidine compounds, if reduced, displayed the same lability[2g]; hence, they were grouped eventually with the other labile compounds as being phosphorylated in other than the 5′-position. (The 4′-position could not yet be eliminated since the ring structure of the sugar was not yet determined.)

Although ribose-5-phosphate had been prepared by the acid hydrolysis of muscle inosinic (or adenylic) acid, similar attempts to prepare the new sugar phosphate by acid hydrolysis of yeast purine nucleotides were initially unsuccessful, owing to simultaneous hydrolysis of the phosphate group. Xanthylic and inosinic acids derived from the yeast purine nucleotides by deamination proved more amenable to this approach[4]; glycosidolysis could be produced with less phosphate loss[4a] and a barium ribose phosphate differing from ribose-5-phosphate was isolated.[4b,4d] The 4-position was excluded as the point of phosphate attachment,[4b] leaving only the 2- and 3-positions. From the observation that the derived ribitol phosphate was optically inactive,[4c] the new ribose phosphate was considered to be ribose-3-phosphate. The possibility that a mixture of the 2- and 3-phosphates had been unwittingly fractionated in the course of preparation and purification, to yield only the 3-compound, was apparently never considered.

A second opportunity to consider ribose-2-phosphate as a constituent of the mononucleotides derived from RNA arose when possible methods of joining the mononucleotides were being reconsidered in the light of the 3′-phosphofuranose structure.[5] Titration and hydrolytic properties indicated a phosphodiester linkage. Because of the acid stability of ribose-5-phosphate, it was argued that this substance (or its derivatives) should be found in acid hydrolyzates of RNA if it existed in the nucleic acid. Acid-stable phosphates were indeed found—the pyrimidine nucleotides—but these had been shown not to be 5′-phosphates.[2f,2g] Hence only the 2′-position seemed to be available to fill the requirements. The supposed absence of 2′-nucleotides in RNA hydrolyzates (based entirely on the ribose phosphate identification just discussed) was argued to be due to a preferential splitting of the 2′-bond leaving only 3′-nucleotides, but this lability factor was not demonstrated independently.

The question of 2′-phosphate linkages was not further investigated at that time. Even when Gulland and Jackson[6] produced indirect evidence for a 5′-phosphate linkage in RNA, the 2′,3′-hypothesis was so firmly entrenched that Gulland subsequently abandoned the newer idea which ultimately was shown, essentially by his methods, to be correct. Gulland and Walsh[7] did, however, indi-

(1) Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

(2) See P. A. Levene and L. W. Bass, "The Nucleic Acids," The Chemical Catalog Co. (Reinhold Publ. Corp.), New York, N. Y., 1931: (a) p. 187; (b) p. 189; (c) pp. 129–130; (d) p. 246; (e) p. 194; (f) p. 209; (g) p. 210; also R. S. Tipson, in *Advances in Carbohydrate Chemistry*, **1**, 193 (1945).

(3) P. A. Levene and E. T. Stiller, *J. Biol. Chem.*, **104**, 299 (1934).

(4) (a) P. A. Levene and A. Dmochowski, *ibid.*, **93**, 563 (1931); (b) P. A. Levene and S. A. Harris, *ibid.*, **95**, 755 (1932); (c) **98**, 9 (1932); (d) **101**, 419 (1933).

(5) P. A. Levene and R. S. Tipson, *ibid.*, **109**, 623 (1935).

(6) J. M. Gulland and E. M. Jackson, *J. Chem. Soc.*, 1492 (1938).

(7) J. M. Gulland and E. O. Walsh, *ibid.*, 172 (1945).

cate the need for a direct investigation and characterization of the ribose-2-phosphate derivatives to provide positive evidence for the 2′,3′-hypothesis.

To follow up this recommendation, Gulland and Smith[8] attempted the synthesis of uridine-2′-phosphate and of cytidine-2′-phosphate. The uridylic acid, which is now known to be the 5′-phosphate, differed to some degree in hydrolytic properties from both the previously synthesized 5′-compound and the RNA hydrolysis product. This difference served as the chief proof of structure but it was not enough, as Gulland and Smith emphasized, to support the concept of a special lability of the 2′-phosphate group unless this lability were limited to the diester (polynucleotide) stage.

Elaborating on the basic concept that all twelve nucleotides should be synthesized for study, Michelson and Todd[9] synthesized four of the previously known nucleotides and four new ones, including the 2′-phosphates (supposedly) of adenosine and guanosine. Again, the alkali stability of these afforded no support for a 2′,3′-polynucleotide link. Here the matter rested. No attempts to prepare ribose-2-phosphate, by degradation or by synthesis, are recorded nor is the possibility of preparing this substance even mentioned in recent reviews.[10]

The discovery of isomeric nucleotides (*a* and *b*) in alkaline hydrolyzates of RNA[11] was first interpreted in the light of the 2′,3′-hypothesis and, since the *b*-isomers had properties conforming generally to those recorded for what were considered as 3′-compounds, the new (*a*) isomers were thought to be the long-lost 2′-nucleotides. The results of Levene and Harris[4] were then interpreted as being due to the unwitting selection of the *b*(3′)-nucleotide as a starting substance. Discrepancies between the properties of the new isolated substances (the *a*-isomers) and the presumed synthetic 2′-nucleotides led to the discovery that the latter were, in fact, 5′-nucleotides.[12] This removed the possibility of determining the structures of the *a*- and *b*-nucleotides by direct comparison with synthetic 2′- and 3′-nucleotides. Then followed the discovery that 5′-nucleotides were constituents of RNA,[13] as Gulland[6] had surmised earlier. This, together with other evidence (such as the interconvertibility of the *a*- and *b*-isomers in acid),[14,15] led to the conclusion, subsequently verified, that the internucleotide link in RNA is 3′,5′ and/or 2′,5′[16,17] and that the *a*- and *b*-nucleotides were the 2′ and the 3′ but not necessarily respectively, since they arose simultaneously from a common intermediate, the cyclic 2′,3′-phosphonucleoside.[17,18] These conclusions and demonstrations, in turn, invalidated completely the reinterpretation of the Levene and Harris experiment because isomerization and fractionation of isomers could have occurred at the nucleotide, the ribose phosphate, or the ribitol phosphate stage of their experiments.[15,16]

The problem of the structure of the alkali-produced RNA nucleotides, now eight in number instead of four, was, therefore, back to where it had been in 1932, before the Levene and Harris experiments.[4] However, one way of settling the problem remained as they had conceived of it, namely, the production and structural identification of the ribose phosphate moiety. In the light of the more recent knowledge of acid-catalyzed phospho-migration, the degradation had to be performed without too great a degree of isomerization. This need was met by the use of a cation-exchange resin as the acid catalyst.[15,19] Secondly, it was necessary to be able to separate the two ribose phosphates (the 2 and the 3). This was accomplished by the adaptation of a new method[20] for separating sugar phosphates by ion exchange in the presence of borate. Finally, ribose-2- and -3-phosphate, never before prepared free of each other and characterized, had to be identified and their structures proved.

The production, separation and characterization of ribose-2- and -3-phosphate and the coincidental discovery of ribose-4-phosphate are reported in this communication.

Experimental

Adenylic acids *a* and *b* (2′ and 3′) were prepared either by large-scale ion-exchange chromatography[11,21] or by crystallization, guanylic acids *a* and *b* (2′ and 3′) by the former method only.[21] Mixed adenylic acids (or guanylic acids) were obtained commercially[22] as was adenosine-5′-phosphate (AMP).[23]

Adenylic Acid Separation by Crystallization.—This was accomplished by evaporation at 50° *in vacuo* at *p*H 3.5, cooling intermittently to 20°, seeding alternately with one or the other isomer, and stirring for 24 hours. A typical procedure is the following: 50 g. of mixed (44% 2′:56% 3′) adenylic acids in 2250 ml. of H_2O was seeded with 3′ at 20°. The precipitate contained 26 g. of a 75% 3′ mixture. The supernatant was evaporated to 900 ml., cooled to 20° and seeded with 2′; 8.2 g. of pure 2′ precipitated. The supernatant, evaporated to 450 ml. and seeded with 3′, yielded 3 g. of an 80% 3′ mixture. The supernatant, concentrated to 200 ml. and seeded with 2′, yielded 2 g. of pure 2′. The 3′-rich fractions, reworked in this fashion, yielded 23 g. of 98% 3′ material on the first crystallization (from 1800 ml. containing 40 g. of a 71% mixture).

Ribose-5-phosphate.—This compound was prepared by heating 50 ml. of water containing an equal volume of Dowex-50-H^+ resin[24] to 100° and then adding 5 g. of adenosine-5′-phosphate to the rapidly stirred mixture. After 4 minutes, the vessel was cooled in an ice-bath to room temperature or below and filtered. The filtrate and water wash (total volume 128 ml.) contained 96–98% of the theoretical amount of ribose-5-phosphate, based on orcinol[25] and phosphorus

(8) J. M. Gulland and H. Smith, *J. Chem. Soc.*, 338 (1947).

(9) A. M. Michelson and A. R. Todd, *ibid.*, 2476 (1949).

(10) For example, L. F. Leloir, in "Fortschritte der Chemie organischer Naturstoffe," L. Zechmeister (ed.), Springer, Vienna, 1941, p. 47.

(11) W. E. Cohn, THIS JOURNAL, **72**, 1471 (1950); C. E. Carter, *ibid.*, **72**, 1466 (1950).

(12) D. M. Brown, L. J. Haynes and A. R. Todd, *J. Chem. Soc.*, 408, 3299 (1950).

(13) W. E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951); *J. Biol. Chem.*, **203**, 319 (1953).

(14) W. E. Cohn, THIS JOURNAL, **72**, 2811 (1950).

(15) J. X. Khym and W. E. Cohn, *ibid.*, **76**, 1818 (1954).

(16) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

(17) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552, 558, 565 (1952).

(18) D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, 2708 (1952).

(19) J. X. Khym, D. G. Doherty, E. Volkin and W. E. Cohn, THIS JOURNAL, **75**, 1262 (1953).

(20) J. X. Khym and W. E. Cohn, *ibid.*, **75**, 1153 (1953).

(21) W. E. Cohn and J. X. Khym, in "Biochemical Preparations," Vol. IV, W. W. Westerfeld (ed.), John Wiley and Sons, Inc., New York, N. Y., in press.

(22) Schwarz Laboratories, Mt. Vernon, N. Y.

(23) Sigma Chemical Co., St. Louis, Mo.

(24) Dow Chemical Co., Midland, Mich.

(25) See E. Volkin and W. E. Cohn, in "Methods in Biochemical Analysis," Vol. I, D. Glick (ed.), Interscience Publishers, New York, N. Y., 1954, pp. 298–299.

measurements, less than 0.4% of adenine or adenosine compounds (by ultraviolet spectrophotometry), and no detectable amount of inorganic phosphate. The solution next was titrated to a faint pink (phenolphthalein) with hot (70°), saturated, carbonate-free barium hydroxide and filtered, if cloudy, both before and after concentration under reduced pressure to 15–20 ml. Four volumes of absolute ethanol was added, whereupon crystallization began at once. After 1 hour at 4° the crystals were filtered, washed with absolute ethanol and acetone and finally dried *in vacuo* over phosphorus pentoxide for 5 days. This treatment changed the crystals to a white, powdery, anhydrous salt weighing 4.96 g. (about 95% yield). Less than 0.2% of the total phosphorus present was detectable as inorganic phosphate.

Anal. Calcd. for ribose-5-phosphate, $C_5H_9O_8PBa$ (365.5): P, 8.48; Ba, 37.6. Found: P, 8.40; Ba, 37.8.

Ribose-4-phosphate.—This previously unknown compound of ribose is formed by heating ribose-2- or -3-phosphate for 2 hours at 100° with Dowex-50-H^+ or for 45 minutes with 1 *N* H_2SO_4. Not enough material (yield ranged from 8 to 10%) has been isolated to permit characterization of a solid salt.

Ribose-1-phosphate.—Prepared by E. Volkin by the method of Friedkin.[26]

Ribose-2- and Ribose-3-phosphates.—A mixture of these two compounds was prepared by heating to 100° an equal volume (1000 ml.) of water and Amberlite IR-120-H^+ [27] to which was added rapidly with stirring 100 g. of solid adenylic acid. After 4 minutes the reaction mixture was cooled quickly to room temperature in an ice-bath and filtered with suction, and the resin was washed with a volume of water equal to the volume of resin. The filtrate and washings, containing the mixed ribose phosphates plus some inorganic phosphate, were titrated to a faint pink (phenolphthalein) with hot (70°) saturated, carbonate-free barium hydroxide. The precipitated barium phosphate was removed by filtration and washed with 500 ml. of hot water. The filtrate and water wash (total volume 3500 ml.) were distilled under diminished pressure to a volume of about 300 ml. and four volumes of absolute ethanol was added to precipitate the barium ribose phosphates. After chilling overnight, these were filtered, washed with absolute alcohol and acetone and finally dried *in vacuo* over P_2O_5 for 6 days. The yield was 79 g. and contained 10% inorganic phosphate calculated as barium phosphate. The barium salts were shown by ion-exchange analysis to be a 36:64 mixture, respectively, of ribose-2- and ribose-3-phosphates.[28]

Since many analytical runs were necessary to determine the percentage composition of ribose-2- and -3-phosphates in the fractionation methods to be described, a detailed description of the process is presented. Forty to fifty μmoles of a mixture (either barium or dibrucine salts) was dissolved in 10 ml. of water; barium was removed by stirring with Dowex-50-H^+ resin at room temperature, brucine by three extractions at *p*H 8.5 with equal volumes of chloroform (the free brucine which initially precipitates but which is subsequently extracted was ignored). The ribose phosphates were absorbed at *p*H 8.5 on a column of Dowex-1, 1 sq. cm. × 10 cm., chloride form, and separately eluted,[15] following a water wash, with 0.04 *M* ammonium chloride containing 0.004 *M* potassium (or sodium) tetraborate, about 1 liter being required to give two distinct peaks (detected by the orcinol colorimetric method).[25]

Isolation of Pure Ribose-3-phosphate by the Fractional Crystallization of the Dibrucine Salts.—Mixed barium salts (39.0 g.) were dissolved in 1000 ml. of water and, after the slightly cloudy solution was filtered, 140 ml. (dry volume) of Dowex-50-H^+ resin (or Amberlite IR-120-H^+) was added, the mixture was stirred for 5 minutes and filtered through a sintered glass funnel. The filtrate plus the wash solution (300 ml.) was diluted to about 2000 ml., heated to about 40°, and titrated with brucine dissolved in methyl alcohol to an apparent *p*H of 7.2. The mixture was stirred overnight at 4° and the crystals obtained were filtered, washed first with a small amount of absolute alcohol and then with a large excess of acetone, and air-dried, giving 32.3 g. of a 10:90 mixture[28] of the dibrucine salts. The filtrate (washes were excluded) was distilled under diminished pressure to about 1000 ml., heated (50°) to dissolve precipitated material and filtered while hot,[29] then allowed to cool overnight at 4° to obtain 30 g. of crystals which had the composition 23:77. In a similar manner, by halving the volume and then cooling overnight, 10 g. and 6.1 g. with the composition 60:40 and 70:30 were obtained. Two final fractions were obtained by the same concentration procedure, but the addition of excess acetone (5–8 volumes) was necessary to obtain a solid product. The acetone-precipitated fractions weighed 13 g. and 8 g. and had the composition 80:20 and 90:10 in the order obtained.

The first two crops of crystals (10:90 and 23:77) were recrystallized separately three times with hot (50°) water[29] and allowed to cool overnight with stirring at 4°. Both gave pure dibrucine ribose-3-phosphate in a combined yield of 26 g. An amount of solvent (*ca.* 600 ml. of water for the first recrystallization of 30 g.) was used so that about 70–75% of the salts was recovered at each recrystallization. Mother liquors were washed from the crystals with a small amount of absolute alcohol followed by a large excess of acetone; the salts were then air-dried. A value of $[\alpha]^{20}D$ −35.0° (*c* 5%, water–pyridine 1:1) was obtained for the optical rotation. The purified salt gave a value which corresponds to the theoretical amount of ribose present when weighed amounts were compared to standard ribose by use of the orcinol test.[25]

Anal. Calcd. for dibrucine ribose-3-phosphate hexahydrate, $C_{51}H_{75}O_{22}N_4P$ (1127): C, 54.5; H, 6.6; N, 4.96; P, 2.75. Found: C, 53.5; H, 5.87; N, 5.12; P, 2.36.

This salt, on dehydration, lost weight corresponding exactly to 6 molecules of water, simultaneously darkening to a considerable degree. Hence, for analysis, attention was turned to the barium salt.

Ten grams of dibrucine ribose-3-phosphate (hexahydrate) was converted to the barium salt according to a procedure detailed in a later section. The theoretical yield, 3.4 g., was obtained. Rotation observations were made on the barium salt, the sodium salt (preparation described in a later section), and on the latter diluted with an equal volume of saturated borax. The values of $[\alpha]^{20}D$, in the order listed, are: −6.8° (*c* 5%, water *p*H 7.2); −10.8° (*c* 3.75%, water *p*H 6.9); +50° (*c* 1.87%, borax *p*H 9.0).

Anal. Calcd. for barium ribose-3-phosphate $C_5H_9O_8PBa$ (365.46): C, 16.42; H, 2.48; P, 8.48; Ba, 37.6. Found: C, 16.52; H, 3.09; P, 8.40; Ba, 38.8, 38.3.

Pure ribose-2-phosphate could not be obtained by the fractional crystallization of the enriched dibrucine ribose-2-phosphate mixtures. Both the 90:10 and the 80:20 mixtures approached a 65:35 ratio when attempts to fractionate them were carried out. These enriched fractions were saved for final separation by the ion-exchange procedure described in the next section.

Ion-Exchange Separations. (a) Small Scale.—These separations were designed for analytical and investigative experiments such as the effect of borate in relation to the structure of ribose-1-, -2-, -3-, -4- and -5-phosphates when all or any combination of the ribose phosphates were chromatographed by ion exchange. The small-scale separations were carried out on 1 sq. cm. × 10 cm. columns of Dowex-1 in the chloride or sulfate forms. Details have been presented in an earlier section and in a prior publication.[15]

(b) Large Scale.—These separations involved only ribose-2- and ribose-3-phosphates and were made for the purpose of procuring gram amounts of the compounds, particularly the 2-isomer. The large-scale preparations utilized a column of Dowex-1-sulfate (*ca.* 300 mesh) 33 sq. cm. × 13 cm. in size. The material (20–30 mmoles total ribose phosphate in about 1 liter) was absorbed at *p*H 8.5 and washed through with 6 liters of water to remove any free ribose present from the column. An amount of 38–43 liters of 0.0015 *M* $Na_2B_4O_7$ + 0.0015 *M* Na_2SO_4 was used[15] to arrive at the beginning of the ribose-2-phosphate peak; 40–50 liters more removed this substance. About 26 liters of 0.005 *M* Na_2SO_4 (the first 6 liters are discarded) was used to collect ribose-3-phosphate.[15]

Procedures for removing brucine or barium prior to column separation have been presented already.

(26) M. Friedkin, THIS JOURNAL, **74**, 112 (1952).

(27) Resinous Products Co., Philadelphia, Pa.

(28) Compositions of ribose-2- and -3-phosphate mixtures will be stated as simple ratios throughout.

(29) The susceptibility of ribose-3-phosphate to alkaline decomposition made it necessary to limit the heating period to 5–6 minutes, regardless of the completeness of solution of the solid, and to cool the filtered solution to room temperature.

TABLE I

SEPARATION OF 18 MILLIMOLES OF RIBOSE-2- AND -3-PHOSPHATES ON 33 SQ. CM. × 13 CM. COLUMN OF DOWEX-1-SULFATE

Fraction number	Volume of fraction, l.	Accum. vol. for each eluting ag., l.	O.D. in orcinol test, units	Units[a] per fraction	Eluting agent	Remarks
1	7	7	0.11	770	Water	Influent + water-wash discard
2	20	20	0.16	3,200	0.0015 *M* Na_2SO_4 + 0.0015 *M* $Na_2B_4O_7$	Discard
3	20	40	0.31	6,200	.0015 *M* Na_2SO_4 + 0.0015 *M* $Na_2B_4O_7$	Discard
4	20	60	1.2	24,000	.0015 *M* Na_2SO_4 + 0.0015 *M* $Na_2B_4O_7$	Ribose-2-PO_4
5	20	80	1.3	26,000	.0015 *M* Na_2SO_4 + 0.0015 *M* $Na_2B_4O_7$	Ribose-2-PO_4
6	6	6	0.85	5,100	.005 *M* Na_2SO_4	Discard
7	20	26	1.5	30,000	.005 *M* Na_2SO_4	Ribose-3-PO_4
			Total	95,300[a]		

[a] Conversion factor based on standard ribose: 6.3 units/μmole; the 95,000 units represent about 86% recovery; "units" = optical density × volume in milliliters.

Enriched dibrucine ribose-2-phosphate mixtures (either mother liquors or the solid salts) were obviously the best source of starting material to obtain high yields of the pure 2-isomer with a minimum number of column runs. One sequence consisted in making three runs in which an average of 20 mmoles of mixed ribose phosphates was used per run. Data pertaining to one of these runs are presented in Table I; the isolation procedure to give solid material follows.

Concentration of the Separated Ribose Phosphates and Removal of Borate.—The concentration of and removal of borate from the ribose phosphate solutions obtained by the ion-exchange separations just described were achieved by the reabsorption of each fraction on separate smaller columns (IRA-400-acetate, 8 sq. cm. × 4 cm.). In order to have maximum absorption while utilizing a minimum amount of resin, it was necessary to remove the sulfate ions and to replace them with monovalent acetate ions which then altered the exchange potential to favor the absorption process. This was accomplished by vigorously stirring each fraction for 5 minutes with 20% excess IR-120 (large-mesh size and in the hydrogen form) to remove the Na^+, allowing the resin to settle out, and siphoning off the supernatant solution. Sulfate was removed as barium sulfate by stirring in sufficient 10 *M* acetic acid to give a final *p*H of 4.0 (at more alkaline *p*H's, considerable material is lost by occlusion on the barium sulfate precipitate) after the addition of a 20% excess of barium acetate. After an overnight settling period, the supernatant solution was siphoned off, adjusted to *p*H 8.0 with ammonium hydroxide and put through the IR-400 acetate column. This process absorbs the ribose phosphates (and any inorganic phosphate present) but not the borate, because the phosphate compounds are essentially divalent at *p*H 8.0–8.5 while the acetate has a higher affinity and is present at a much higher concentration than the borate ion.

After a water wash, the ribose phosphates were eluted from their respective columns with 125 ml. of 1 *M* ammonium acetate (the first 15 ml. was discarded). The ammonium acetate was removed from the ribose phosphates by adding, with stirring (5 min.), a 20% excess of Dowex-50-H^+ fine-mesh cation exchanger to convert the acetate to acetic acid, which (after removing the resin) was eliminated by distillation *in vacuo* (about 30° inside of flask). Water was added and the distillation repeated until the distillate reached a *p*H of at least 4. The ribose phosphates remained in solution, were essentially free of acetate, and could be isolated as either the dibrucine or barium salts.

It was found more desirable to isolate the dibrucine salts first and then convert these to the barium salts instead of directly forming the latter compounds. Inorganic phosphate is always present, arising principally from the alkaline degradation (discussed later) of ribose-3-phosphate. This inorganic phosphate not only contaminates the parent compound but also ribose-2-phosphate since inorganic and ribose-2-phosphate are eluted close together in the ion-exchange separation. Since inorganic phosphate precipitates with the barium salts, its removal was difficult and was achieved only with large losses. Brucine phosphate is water soluble and alcohol soluble and is eliminated easily if the dibrucine ribose phosphate salts are recrystallized. The amount of inorganic phosphate produced in the systems described was a deciding factor in the yields and purity of final products. Thus those steps (separation and concentration) that involved alkaline conditions were performed as rapidly as possible.

Isolation of the Ion-exchange Separated Ribose Phosphates as their Dibrucine Salts and Conversion of these to the Corresponding Barium Salts.—After the acetate was removed, the volumes were adjusted to *ca.* 150 ml. and the ribose phosphates were titrated with brucine dissolved in methyl alcohol to an apparent *p*H of 7.5. The dibrucine ribose phosphate solutions were distilled under diminished pressure until almost all the material filled the flask as a solid, hot acetone (*ca.* 50°) was added to dissolve all the solid, and then additional hot acetone until the solution turned milky. After the mixture had been cooled with stirring at 4° for at least 6 hours, the precipitates were filtered off, washed with acetone and finally air-dried. The amounts of the dibrucine salts (1 g. is approximately 1 mmole) obtained from three column runs yielded a total of 23 g. of brucine ribose-2-phosphate and 13 g. for brucine ribose-3-phosphate. Total impurities (free brucine + inorganic phosphate as the brucine salt) in these crude salts ranged from 10 to 15%, based on comparison of weighed amounts with standard ribose solution in the orcinol test.

Dibrucine ribose-2-phosphate was recrystallized three times by dissolving in hot (*ca.* 50°) 75% acetone, then allowing the solution to cool gradually with stirring at 4° for at least 6 hours. The mother liquor was washed from the crystals with a small amount of cold 75% acetone, followed by several portions of acetone, and finally the salt was air-dried. Such recrystallized dibrucine ribose-2-phosphate showed an extinction corresponding to the theoretical amount of ribose when compared with standard ribose solution in the orcinol test, had a rotation of $[\alpha]^{22}D = -27.50$ (*c* 5%, water–pyridine 1:1) and a ratio of phosphorus to ribose of 1:1, m.p. 112–114° dec. (in bath at 110°, heated 2°/min.).

Anal. Calcd. for $C_{61}H_{63}O_{16}N_4P \cdot 6H_2O$ (1127): P, 2.75; N, 4.96. Found: P, 2.87; N, 4.38.

To form the barium salt, 5.2 g. of purified dibrucine ribose-2-phosphate was dissolved in 300 ml. of water and extracted with an equal amount of chloroform to remove the brucine. Three more extractions were carried out at *p*H 8.0 ignoring thr free brucine that precipitated initially when the solution was made alkaline. After the extractions, 10 ml. (wet volume) of Dowex-50-H^+ was added and was removed by filtration after a 5-minute stirring period. The solution was then titrated with hot (70°), saturated, carbonate-free barium hydroxide to a faint pink (phenolphthalein). The solution was filtered through a fine-porosity sintered glass disc, if cloudy, before and after concentrating under reduced pressure to about 15 ml. Then 4 volumes of alcohol was added rapidly with stirring, after which the solution was cooled at 4° for several hours. No reprecipitation was necessary; the yield of barium ribose-2-phosphate was 1.8 g., essentially the theoretical amount.

Anal. Calcd. for barium ribose-2-phosphate, $C_5H_9O_8PBa$ (365.5): C, 16.42; H, 2.48; P, 8.48; Ba, 37.59. Found: C, 16.10; H, 2.90; P, 8.57; Ba, 38.2.

Rotations were observed on the barium salt, the sodium salt and the sodium salt after dilution with an equal volume

of saturated borax. The values of $[\alpha]^{23}D$ obtained, in this order, were: −6.8° (*c* 5%, water, *p*H 6.8); −10.3° (*c* 3.75%, water, *p*H 8.0); −14.3° (*c* 1.87, 1:1 borax, *p*H 9.0). The sodium salt was formed by adding moist Dowex-50-Na^+ to the 5% solution of the barium salt and removing the resin by centrifugation.

The crude dibrucine ribose-3-phosphate obtained *via* the ion-exchange column method was recrystallized once from hot (50°) water[29] and once from hot 75% acetone. The purified salt gave an extinction value which corresponded to the theoretical amount of ribose when compared with standard ribose in the orcinol test.[25] It gave a value of $[\alpha]^{22}D$ −34.5° (*c* 5%, water–pyridine, 1:1) for the rotation (compared to −35° on the material prepared by crystallization) and the ratio of phosphorus to ribose found was 1:1; m.p. 114–117° dec. (in bath at 110°, heated 2°/min.).

Pure dibrucine ribose-3-phosphate (6.3 g.) was converted to the barium salt in exactly the same manner as described for the 2-isomer; the yields were comparable.

Anal. Calcd. for barium ribose-3-phosphate, $C_5H_9O_8PBa$ (365.5): P, 8.48; Ba, 37.59. Found: P, 8.50; Ba, 37.7.

Preparation of the Methyl-2- and -3-phosphoribopyranosides.—D-Ribose-3-phosphate (barium salt) in the amount of 300 mg. was added to 50 ml. of absolute methanol containing 1 g. of dry HCl in a pressure bottle and incubated at 50° for 40 hours. The alcoholic solution was poured into ice-water, neutralized with silver carbonate, filtered and washed with 50% methanol. The residual silver in the filtrate was removed with Dowex-50-H^+ and the filtered solution brought to *p*H 7.5 with barium hydroxide. The clear solution was evaporated *in vacuo* to dryness, and the solid barium methyl-D-riboside-3-phosphate removed with acetone and dried; yield 200 mg. (65%). A modified Kline–Acree titration[30] gave a value of 13% free aldehyde. Hydrolysis of a sample in 0.1 *N* HCl for 10 minutes at 100° yielded an increase of 7% in the free aldehyde present.[31] Thus the barium salt was composed of 80% pyranoside, 7% furanoside and 13% unmethylated ribose phosphate. The ratio of reducing power (orcinol)[25] to organic phosphate was 1:1.

This procedure was followed with 300 mg. of barium D-ribose-2-phosphate and essentially the same yield obtained. The product was 89% pyranoside, 4% furanoside and 7% unmethylated ribose phosphate with an orcinol:bound phosphate ratio of 1:1.

Periodate Titration.—The procedure followed was that of Jackson[32] in which 0.25 *M* sodium periodate, 0.01 *M* sodium arsenite and 0.01 *M* iodine were used. The methyl-3-phospho-D-ribopyranoside preparation described above consumed 0.46 mole of periodate per mole of phosphate in 24 hours. In a separate experiment, one mole of ribose phosphate took up 3.6 moles of periodate in 24 hours. Therefore, the entire periodate consumption of the 3-ribopyranoside preparation is accounted for by the 13% ribose phosphate impurity; none was consumed by the pyranoside (or furanoside), thus identifying it as the 3-compound.

The methyl-2-phospho-D-ribopyranoside preparation, under the same conditions, consumed 1.27 moles of periodate per mole of phosphate, of which 0.25 mole can be accounted for by the ribose phosphate present (7%). The remaining 1.02 moles of periodate are ascribed to the 0.89 mole of pyranoside, thus identifying it as the 2-compound.

Preparation of Ribitol-2- and -3-phosphates.—Each barium ribose phosphate (1.2 g. in 50 ml. of H_2O) was reduced by shaking with hydrogen (40 lb. pressure) in the presence of 0.2 g. of Adams catalyst. After 16 hours (32 in the case of the -2-phosphate) the suspension was filtered, evaporated *in vacuo* to dryness, dissolved in 10 ml. of H_2O, precipitated by dropping slowly into 200 ml. of acetone with vigorous stirring, filtered and dried; yield 1.0 g.

The total reducing power found by the orcinol procedure amounted to 3% of the original, in the case of the -2-phosphate (<1% in the case of the 3). Inorganic phosphate was less than 0.2% and the organic phosphate found equalled the theoretical amount predicted (8.3%, 8.4%). The rotations observed on the barium salts before and after diluting with an equal volume of saturated borax (and centrifuging off the precipitated barium borate) for the ribitol-2- and -3-phosphates were, in that order, $[\alpha]^{22}D$ +6.0°, 0.0° (*c* 2%, H_2O); +3.4°, 0.0° (*c* 1%, half-saturated borax).

(30) G. M. Kline and S. F. Acree, *Ind. Eng. Chem., Anal. Ed.*, **2**, 413 (1930).

(31) P. A. Levene, A. L. Raymond and R. T. Dillon, *J. Biol. Chem.*, **95**, 699 (1932).

(32) E. L. Jackson, in "Organic Reactions," John Wiley and Sons, Inc., New York, N. Y., 1944, p. 341.

Results and Discussion

Ion-exchange resins not only furnish a method of producing, by hydrolysis of purine nucleotides, and separating ribose-2- and -3-phosphates as pure substances but also, by interaction with the ribose phosphates in the absence as well as the presence of borate, furnish a means of identifying and characterizing these compounds.[15] This differentiation by ion exchange depends on the differences in ionization properties of the ribose phosphates and on the varying extents to which they form ionizable complexes with borate ion. These properties, as they apply to the identification and characterization of ribose phosphates, are discussed.

Acidic and Ion-exchange Properties of Ribose Phosphates.—Kumler and Eiler[33] have discussed the effect of hydrogen bonding in the sugar phosphates on the acidic strength of these compounds and point out that hydrogen bonding may act either to increase or to decrease the ionization of the phosphate group. Sugar phosphates in which there is bonding between the phosphate group and the ring oxygen atom (as in glucose-1- and ribose-1-phosphates) will lessen the degree of ionization (more so if a six-membered ring rather than a seven-membered one results, as in glucose-6- and ribose-5-phosphates), whereas those in which a hydrogen atom of an alcoholic group is attracted to an oxygen atom of an adjacent phosphoryl group will increase the degree of ionization (as in ribose-3-phosphate). In the former case, the cation is shared by two oxygens; in the latter case, two alcoholic hydrogens (from the 2-OH and 4-OH if in the pyranose form) or one such (from the 2-OH if in the furanose form) form a bond to the phosphate oxygen.

In Fig. 1 are shown the possibilities for forming ionization-increasing bonds (+) and ionization-decreasing bonds (−) and borate complexes in the ribose phosphates. Tabulation of these possibilities gives the "score" indicated in the last two columns of Table II. The order of increasing ionization, which should relate to the order of elution in the absence of complexing or complicating factors, is then 1, 5, 4, 3, 2. Since the forces are not large (and excluding 1 which was not tested in a non-borate system), the 5 and 4 compounds would be expected to be only slightly separable in the order given, with 3 and 2 not separable from each other but somewhat removed from the 5 and 4.

These predictions are borne out by the ion-exchange separations in the absence of borate (Fig. 2A, B). The position of ribose-4-phosphate, which follows the 5-compound, is consistent with the structure assigned to it.[15]

The presence of a purine or pyrimidine base at carbon atom 1 of the ribose (in the furanose form) reverses the (+) shown for ribose-2-phosphate, but has no similar effect on the ribose-3- or -5-phosphates because of the greater distance to the phosphate group. Thus the 2′-nucleotide should fall between the 3′ and 5′, which is consistent with the

(33) W. D. Kumler and J. J. Eiler, THIS JOURNAL, **65**, 2355 (1943).

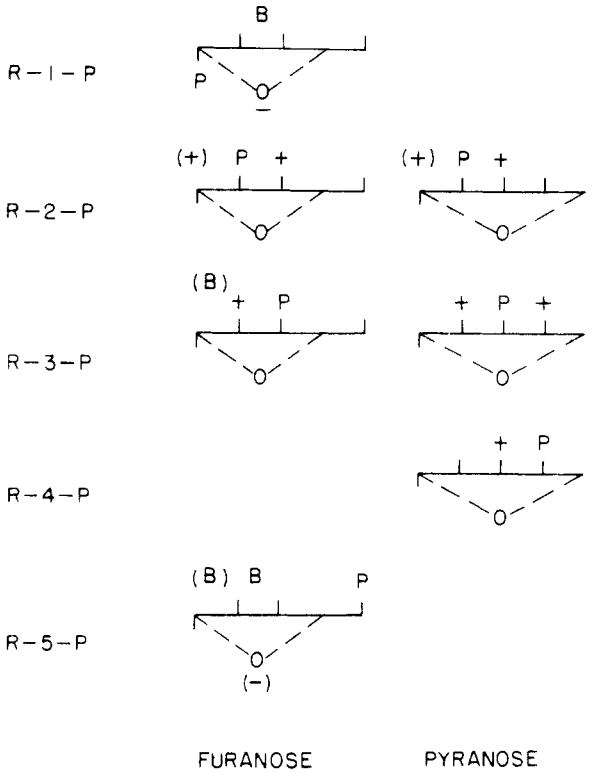

Fig. 1.—Forms of the (β) ribose phosphates, showing major sites and effects of hydrogen bonding affecting ionization and also the sites of strong complexing by borate ion. The carbon chain is shown by a solid horizontal line, the oxygen ring (assumed to lie in a plane perpendicular to the page and extending behind it) by a broken line, alcoholic hydroxyl groups by short vertical lines, the phosphoryl ($-PO_3H_2$ group) by P. + and − indicate ionization-increasing and ionization-weakening bonding possibilities, B a site of strong complexing by borate ion (pyranose-borate complex formation is negligible). Parentheses indicate sites which are available in the α form only, not in β (R-2-P, R-3-P), or are less favored because a seven-membered ring is required (R-5-P). Ribose-1-phosphate exists in the β form only; the others, in solution, exist in both forms (Leloir[11]).

older observations[13] that the order of elution of nucleotides in simple acid systems is 5′, 2′, and 3′.

Effect of Borate on Ion-exchange Properties of Ribose Phosphates.—The earlier work on borate–sugar interaction summarized by Boeseken[34a] and the ion-exchange observations of Khym and Zill,[34b] Zill, *et al.*,[34c] and Khym and Cohn[20] allow three generalizations concerning the reactions of borate with sugars: (a) *cis*-α-glycols are strongly complexed; (b) pyranose systems are not complexed relative to furanose systems; (c) the stronger the complex, the greater the ionization and thus the affinity for anion exchangers.

Of the four furanose structures listed in Fig. 1, only ribose-2-phosphate presents no *cis*-α-glycol arrangement to a borate ion and should, therefore, be no more affected in its ion-exchange behavior than ribose-4-phosphate which can exist only in the (uncomplexing) pyranose form (or inorganic phosphate or glucose-1-phosphate, which are similarly not complexed).[20] The other three structures

(34) (a) J. Boeseken, in "Advances in Carbohydrate Chemistry," Vol. IV, W. W. Pigman and M. L. Wolfrom (eds.), Academic Press, Inc., New York, N. Y., 1949, p. 189; (b) J. X. Khym and L. P. Zill, THIS JOURNAL, **74**, 2090 (1952); (c) L. P. Zill, J. X. Khym and G. M. Chenine, *ibid.*, **75**, 1339 (1953).

TABLE II

NUMBER OF OPPORTUNITIES FOR BONDING TO INCREASE (+) OR DECREASE (−) THE IONIZATION OF THE RIBOSE PHOSPHATES, IN THE PRESENCE AND ABSENCE OF BORATE

Ribose-	Furanose[a] (+)	Furanose[a] (−)	Pyranose[a] (+)	Pyranose[a] (−)	Σ Opportunities[e] Borate absent	Σ Opportunities[e] Borate present
1-Phosphate	0[b]	1			−1	−1 + B
2-Phosphate	2[c]	0	2[c]	0	2[c]	2[c]
3-Phosphate	1	0	2	0	1–2[d]	(B)[d]
4-Phosphate			1	0	1	1
5-Phosphate	0	<1[d]			0	0 + B

[a] Both furanose and pyranose forms, as well as small amounts of the open chain forms, exist in solution except where substituents prevent (see Leloir[10]). [b] β-Form. [c] 2 if in the α-form, 1 if in the β-form; both forms coexist in solution (Leloir[10]). [d] Seven-membered ring is less favored than the six-membered ring formed by the 1-phosphate; this is analogous to the glucose-1-phosphate, glucose-6-phosphate difference noted by Kumler and Eiler.[33] [e] B is used to designate the charge due to borate complexing, independently of bonding possibilities. Parentheses indicate complexing in α-form only. The degree of complex formation between borate and pyranoses is essentially zero compared to furanoses.

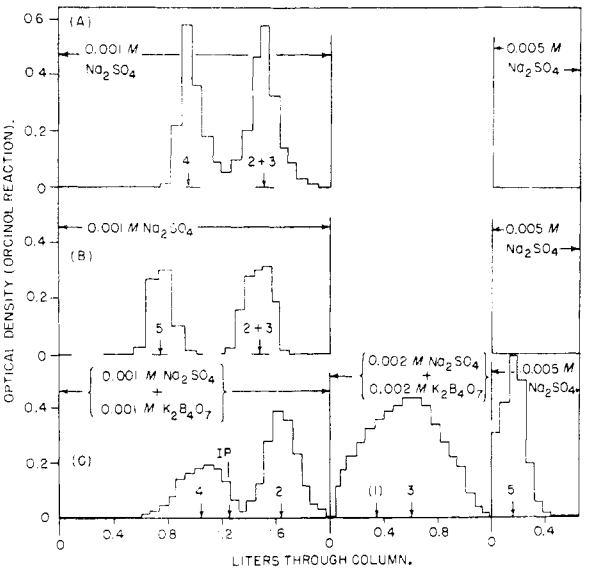

Fig. 2.—Ion-exchange separation of ribose phosphates in the presence and absence of borate; exchanger, Dowex-1-sulfate, *ca.* 300 mesh, 12 cm. × 0.86 sq. cm., flow rates, *ca.* 3.5 ml./min.: (A) 60 mg. of commercial adenylic acids heated with 6 ml. of Dowex-50-H^+ in 6 ml. of H_2O at 100° for 3 hours to yield ribose-2-, -3- and -4-phosphates (and free ribose, not shown), eluted with 2 liters of 0.001 *M* Na_2SO_4 followed by 700 ml. of 0.005 *M* Na_2SO_4. (B) 8 mg. of ribose-5-phosphate plus 4 mg. each of ribose-2- and -3-phosphates, eluted as in (A). (C) 3–10 mg. each of ribose-2-, -3-, -4- and -5-phosphates, eluted successively with 2 liters of 0.001 *M* Na_2SO_4 plus 0.001 *M* $K_2B_4O_7$, 1.1 liters of 0.002 *M* Na_2SO_4 plus 0.002 *M* $K_2B_4O_7$, and 0.005 *M* Na_2SO_4. (The probable position of the ribose-1-phosphate peak, from the separate experiment described in the text, is shown in parentheses.)

should complex to varying degrees, ribose-5-phosphate and ribose-1-phosphate more than ribose-3-phosphate which offers a *cis*-α-glycol system only in the α-form. These three should increase in ionization (anion-exchange affinity) in the presence of borate so that the 3 should now succeed the uncomplexed 2 and the strongly complexed 5 should follow the 3 and the 1 since the latter will still retain the ionization-decreasing bond discussed.

Figure 2C indicates, as predicted, that ribose-2- and -4-phosphates are unaffected by borate (but for the different reasons given), that ribose-3- and -5-phosphates are affected by borate in the direction of increased ionization, and that ribose-5-phosphate is more strongly affected than ribose-3-phosphate. It has been shown, in a separate experiment, that ribose-1-phosphate also is affected strongly, showing a peak at 1500 ml. in 0.0018 M Na_2SO_4 plus 0.0018 M $K_2B_4O_7$. This places it slightly ahead of ribose-3-phosphate (see Fig. 2C). However, since no evidence of its behavior in the absence of borate has been obtained, the magnitude of the borate effect on ribose-1-phosphate cannot accurately be estimated. Its position preceding the 5 is consistent with a greater residual ionization-weakening effect.

Thus the ion-exchange behavior of ribose-2- and -4-phosphates in the presence of borate affords further confirmation of the structures which we have assigned them, for only these two of the four possible formulas could remain unaffected by borate, while their order (4 preceding 2) serves to differentiate them from each other.

Effect of Borate on Optical Rotation.—A pronounced effect of borate on optical rotation is evidence of complex formation. It has been concluded and demonstrated that ribose-2-phosphate is relatively unaffected, whereas the -3-phosphate is quite strongly affected. The rotations of these two substances (and of the preparations of Levene and Harris)[4b,c,d] are given in Table III.

TABLE III

OPTICAL ROTATIONS OF RIBOSE PHOSPHATES

	Optical rotation $[\alpha]^{23}D$			
	Brucine salt (in 1:1 pyridine:H_2O)	Sodium salt		
		Borate absent	Borate present	Calcd. ratio of 2:3
Ribose-2-phosphate	−27.5	−10.3	−14.3	(100:0)[a]
Ribose-3-phosphate	−35.0	−10.8	+50	(0:100)[a]
L & H[b] 1		−8.9	+40.8	14:86
L & H[b] 2		−9.6	+35.5	22:78
L & H[b] 3		−9.7	+38.9	17:83
L & H[b] ?	−33.3			22:78

[a] By definition. [b] Levene and Harris.[4b,c,d]

From this it is evident that ribose-2-phosphate again is relatively inert to borate ion, in contrast to the 3-compound. Our values also permit a calculation of the 2:3 ratios in the Levene and Harris preparations. These indicate that their preparations contained amounts of each isomer which were slightly enriched in the less-soluble ribose-3-phosphate from the ratio of the two substances expected by acid hydrolysis (29:71 from adenylic acid *a* but 20:80 from adenylic acid *b*[15] which, to judge from the stated properties of their starting material, was used). Since little inorganic phosphate was produced in their hydrolysis (none in one case, 20% in another), it is likely that little ribose-4-phosphate was produced to invalidate our calculation.

One case in which ribose-4- and -2-phosphates were found to be formed in approximately equal amounts was in the ribose phosphates resulting from reductive debenzylation of benzyl ribose phosphate previously formed from adenosine phosphates in dry benzyl alcohol and dry hydrochloric acid.[35] The presence of the 2- and 4-compounds in this product in equal amounts offers an explanation of the absence of optical activity in the ribitol phosphates produced by reduction of the ribose phosphates formed from the adenosine-2′-phosphate as well as from the adenosine-3′-phosphate[35] for, as pointed out by Brown and Todd,[16] the 2- and 4-ribitol phosphates constitute a racemic mixture and the 3 is symmetrical.

Differentiation between Ribose-2- and -3-phosphate by Acid Treatment.—The heating of either ribose-2- or -3-phosphate with Dowex-50-H^+ for prolonged periods (*ca.* 2 hours) results in isomerization of each to a mixture of the 2, 3 and 4 isomers and a hydrolysis to ribose and inorganic phosphate.[15] The relative amounts of the 2, 3 and 4 isomers left after such treatment are: from ribose-2-phosphate, 11:12:7; from ribose-3-phosphate, 9:12:12 (in each case, 70% hydrolysis to free ribose was encountered). The much larger production of 4 from 3 (almost twice that from 2) is consistent with the proximity of the 4-group to the 3 and, therefore, with the structures proposed.

It may also be mentioned that acid hydrolysis of ribose phosphates to this extent yields only ribose as the sugar component. The sugar was identified in the pentose separation scheme of Khym and Zill[34]; no other sugar could be found in the acid-hydrolyzed solutions. Hence no other sugar need be considered in interpreting the results.

Alkali Lability of Ribose and Ribose Phosphates.—The degradation of sugars and sugar esters in alkali, discussed by Browne and Zerban,[36] involves the migration of hydrogen atoms from hydroxyl to aldehyde (or ketone) group of the non-cyclic form of the substance. The unstable 1,2- and 2,3-enediols that are formed in ribose are formulated as CH(OH)=C(OH)—CH(OH)—CH(OH)—CH_2-(OH) and CH_2(OH)—C(OH)=C(OH)—CH_2(OH) and coexist along with ribose and ribulose. However, they are unstable and are attacked by oxygen to form lower products. The presence of a substituent (such as a phosphate group), in place of a hydrogen at the second or third carbon atom inhibits hydrogen migration from that locale and thus minimizes the number of unstable enediols that may coexist in alkaline solution. Thus we would predict that ribose-2-phosphate, in which no enediol should exist, should be more stable in alkali than ribose-3-phosphate, in which only the 1,2-enediol can exist. Ribose-5-phosphate, in which both the 1,2- and 2,3-

(35) D. G. Doherty, Abstr. 118th Meet., Am. Chem. Soc., 56C (1950).

(36) C. A. Browne and F. W. Zerban, "Physical and Chemical Methods of Sugar Analysis," John Wiley and Sons, Inc., New York N. Y., 1941, p. 653.

enediols may exist, should be even less stable than the 3.

In Fig. 3 are plotted the residual reducing power and organic phosphate in ribose and ribose phosphates in 0.01 *M* NaOH at room temperature. The order of stability is clearly ribose-2-phosphate, which is scarcely attacked, ribose-3-phosphate, and ribose-5-phosphate (ribose-4-phosphate has not been examined), which is consistent with the prediction and thus with the structures proposed. The apparently anomalous position of ribose-3-phosphate with respect to ribose may be due to an enhancement of oxidation at the enediol linkage by the electronegative phosphate group. The discrepancy between loss of reducing power and of organic phosphate by ribose-5-phosphate is consistent with the mechanism of destruction discussed (enediol formation followed by oxidation) for it would be anticipated that oxidation of the 1,2- or 2,3-enediols could take place at a faster rate than loss of phosphate, which would probably not occur until after an attack on the 3,4-linkage.

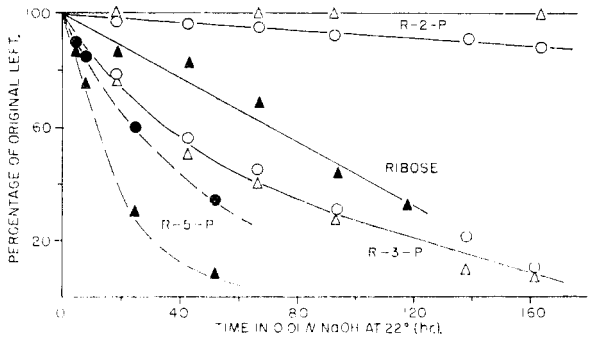

Fig. 3.—Loss in reducing power and organic phosphate by ribose phosphates and ribose in 0.01 *N* NaOH at room temperature: Δ, reducing power; O, organic phosphate remaining at end of stated time, in percentage of that at time = zero.

The Methyl Glycosides and their Susceptibility to Periodate Oxidation.—The susceptibility of the 1-methyl 2-phosphoribopyranoside to periodate (1 mole consumed per mole of ribopyranoside) distinguishes it from that derived from the 3-isomer (no periodate consumed) and indicates the presence of a *cis*-α-glycol system in the 2, 3, or 4 carbons. The 4-position is eliminated as a primary product of adenylic acid hydrolysis by the furanose nature of the nucleotide[37] as well as by the other evidence just discussed; this is further proof that the ribose-2- and -3-phosphates have the structures assigned to them.

The Ribitol Phosphates.—The absence of any rotation in the symmetrical ribitol-3-phosphate, before or after the addition of borate, is to be expected. The positive rotation observed in the ribitol-2-phosphate, which is significantly altered by borate (6.0° and 3.4°, respectively), is consistent with the assignment of structure. The rotations observed are of sufficient magnitude to have been observed by Levene and Harris.[4c] The absence of any rotation (or change thereof with borate) in their preparation, in spite of the evidence here and previously presented to indicate the probability that their ribose phosphate was a mixture, cannot be taken as indicating a pure ribitol-3-phosphate because of the possibility that ribose-4-phosphate was present also in amount sufficient to counterbalance, as ribitol-4-phosphate, the rotation of that ribitol-2-phosphate derived from ribose-2-phosphate.[16] Until ribose-4-phosphate is prepared and its rotations compared with those set forth in Table III, the latter possibility can neither be eliminated nor confirmed.

NOTE ADDED IN PROOF.—The reaction of the ribitol-2- and -3-phosphates with periodate has been measured. At the end point, which occurs within 40 hours, these consume about 2 moles and about six moles, respectively. Ribose-2- and -3-phosphates require about four days to reach an end point at about six moles each while ribose-5-phosphate consumes three moles. The ribose-2- and -3-phosphates and ribitol-3-phosphate are converted by two moles of periodate into a 1,3-dialdehyde glycero-2-phosphate which seemingly consumes four more moles of periodate with the liberation of ester phosphate as inorganic phosphate.[38] Neither ribose-5-phosphate or ribitol-2-phosphate can form this intermediate, proposed by Courtois and Ramet,[38] and hence are limited in periodate uptake to the number of glycol linkages present; they liberate essentially no inorganic phosphate. These findings further identify ribitol-2-phosphate, and through it ribose-2-phosphate, and support the hypothesis of the labile dialdehyde intermediate.

OAK RIDGE, TENNESSEE

(37) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **93**, 623 (1931); **94**, 809 (1932); **97**, 491 (1932).

(38) J. Courtois and N. Ramet, *Bull. soc. chim. biol.*, **27**, 610 (1945).