5-Deoxy-6-*O*-methylhexulose Phenylosazone.—The osazone was obtained in a 60% crude yield by the method outlined above. Two recrystallizations from benzene yielded yellow needles melting at 128–129°. The substance underwent mutarotation in pyridine: ethanol (3:2) solution (*c* 0.93) from +3.5° to an equilibrium value of −24.1° (in 16 hours at 25°).

Anal. Calcd. for $C_{19}H_{24}O_8N_4$: C, 64.02; H, 6.79. Found: C, 63.90; H, 6.60.

Acknowledgment.—Supported by grants from Ben May, Mobile, Alabama, National Institutes of Health, and the Nutrition Foundation, Inc.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY]

The Periodate Oxidation of Ribose-5-phosphate in Acid and Alkaline Solution

BY G. V. MARINETTI AND G. ROUSER[1]

RECEIVED MAY 6, 1955

The periodate oxidation of ribose-5-phosphate was studied in acid and alkaline solutions. In acid solution there was a rapid initial uptake of 2 moles of periodate per mole of ribose-5-phosphate followed by a slow uptake approaching a third mole. In alkaline solution there was a rapid initial uptake of 3 moles of periodate per mole of ribose-5-phosphate followed by a slow uptake approaching a fourth mole. These results support the existence of the formic acid ester of 3-phosphoglyceraldehyde which appears to be fairly stable in acid solution but unstable in alkaline solution. The periodate consumption was followed spectrophotometrically at 310 mμ. Studies on the effect of *p*H, temperature and periodate concentration on the absorption of periodate in the range 280–310 mμ are given. Although the absorption maximum of periodate is at 222 mμ, the spectral range 280–310 mμ has been found to be of practical use and suffers less interference by other ions.

Although the standard method for the determination of periodate consumption has utilized volumetric analysis,[2] recently a spectrophotometric method has been found applicable.[3] Crouthamel and co-workers[4] have studied the effect of *p*H and temperature on the periodate absorption at 222 mμ and have provided useful data for the development of an analytical method. Dixon and Lipkin[3] have made use of the absorption of periodate at 224–230 mμ to study the periodate oxidation of purine and pyrimidine ribosides.

The useful spectral range for the determination of a compound need not necessarily be limited to the region of maximum absorption. Although maximum sensitivity is usually attained at the wave length of maximum absorption, this wave length may not always be of practical use because of interfering absorption by other compounds in the system being studied. An interest in the periodate oxidation of adenylic acid led us to study the 280–310 mμ spectral range (since adenylic acid absorbs strongly in the region 250–270 mμ, with a maximum at 260 mμ) for following the periodate consumption spectrophotometrically. Lower wave lengths were avoided because of interference by certain ions constituting the buffer systems. Extensive studies showed that the range 280–310 mμ was useful for the measurement of periodate uptake during the oxidation of adenosine-5′-phosphate, sugars, sugar phosphates, ethylene glycol and other compounds. Interference by ions such as pyrophosphate, acetate, citrate, nitrate and many complex ions has been shown by Buck and co-workers[5] to be small in the 280–310 mμ spectral range whereas in the 220–260 mμ range, interference by these ions is considerable and indeed may exclude their use. Therefore, the 280–310 mμ region should permit the study of the periodate oxidation of a larger number of compounds and allow the use of buffers containing relatively high concentrations of phosphate, acetate and other ions. In addition, interference by iodate, a product of the periodate reaction, is more significant at 220–230 mμ (about 10%) than at 280–310 mμ (about 3%). The absorption spectra of periodate and iodate in the region 250–350 mμ are given in Fig. 1.

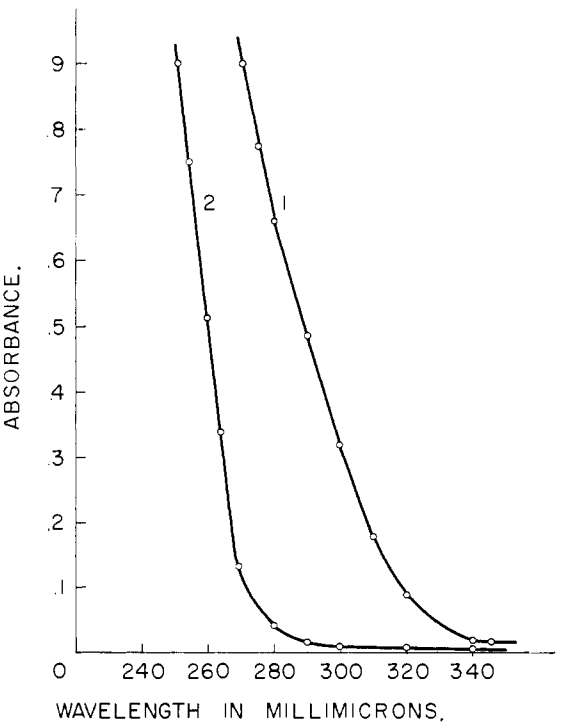


Fig. 1.—Absorption spectra of $NaIO_4$ and KIO_3 in distilled water: curve 1, 0.002 *M* $NaIO_4$; 2, 0.005 *M* KIO_3.

(1) U. S. Public Health Post-doctoral Fellow.

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

(3) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

(4) (a) C. E. Crouthamel, H. V. Meek, D. S. Martin and C. V. Banks, THIS JOURNAL, **71**, 3031 (1949); (b) C. E. Crouthamel, A. M. Hayes and D. S. Martin, *ibid.*, **73**, 82 (1954).

(5) R. P. Buck, S. Singhadeja and L. B. Rogers, *Anal. Chem.*, **26**, 1240 (1954).

The calibration curves shown in Fig. 2 demonstrate that the periodate absorption at 280 and 310 mμ obeys Beer's law over a wide range of *p*H at a concentration of 0.002–0.01 *M*. Further studies showed that the periodate absorption obeyed Beer's law also at 290 mμ. The absorption was found to increase steadily with an increase in *p*H, there being a sharp rise between *p*H 7 and *p*H 8. At *p*H values higher than 12.0 the absorption was found to decrease sharply. Crouthamel, *et al.*,[4] have observed a similar phenomenon at 222 mμ.

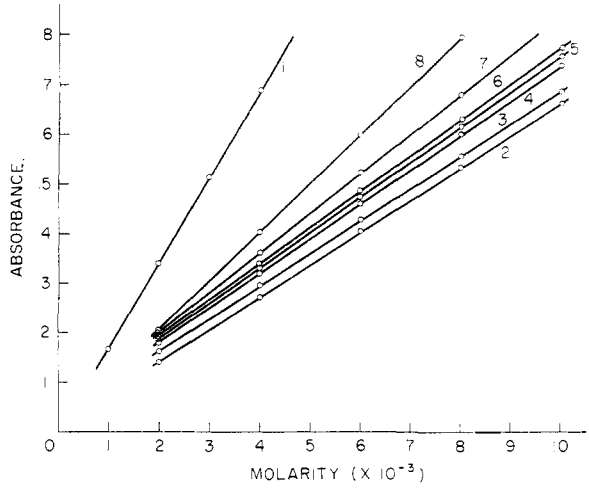


Fig. 2.—Calibration curves of $NaIO_4$ at room temperature at various *p*H values: curve 1, 280 mμ; curves 2–8, 310 mμ. The following *p*H values were used: curve 1, 0.54; 2, 2.3; 3, 2.7; 4, 4.1; 5, 5.0; 6, 5.1–5.3; 7, 6.8; 8, 8.0.

The absorption of periodate at 310 mμ was also found to be dependent on temperature. As shown in Fig. 3, in acid solution the temperature effect was quite pronounced whereas in alkaline solution the effect was essentially negligible. The influence of *p*H and temperature on the absorption of periodate has been shown to be related to the ionization of periodate and to the ionic species which exist in solution.[4]

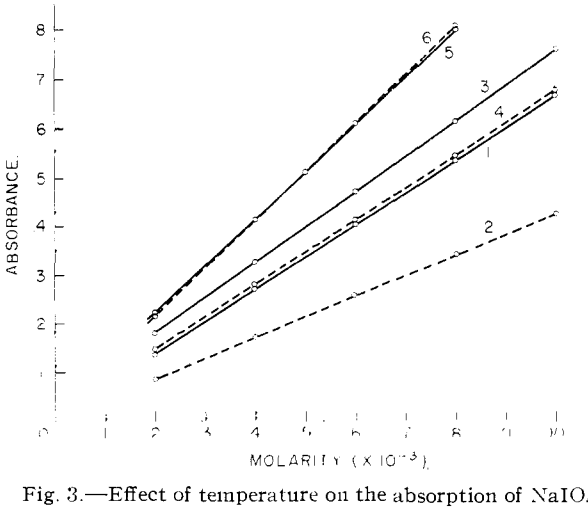


Fig. 3.—Effect of temperature on the absorption of $NaIO_4$ at 310 mμ at various *p*H values: —, room temperature; ----, 0°. The following *p*H values were used: curve 1, 2.3; 2, 2.3; 3, 5.0; 4, 5.0; 5, 8.0; 6, 8.0.

Since ultraviolet light has been shown to cause a photochemical decomposition of periodate[6] and to accelerate the periodate oxidation of formic acid[3] the reactions must be run in the dark. Control experiments demonstrated that the short exposure of ultraviolet light at 280–310 mμ used to take the readings did not cause significant decomposition of periodate or periodate oxidation of formic acid.

After preliminary studies had demonstrated that the spectrophotometric determination of periodate in the 280–310 mμ range was feasible, the periodate oxidation of adenosine-5′-phosphate, ethylene glycol and glucose was carried out and theoretical yields of periodate consumption were obtained.

The spectrophotometric method was then used to study the periodate oxidation of ribose-5-phosphate at various *p*H values. The production of a formic acid ester of glyceraldehyde-3-phosphate, as well as products of a similar nature from fructose-6-phosphate, fructose-1,6-diphosphate and glucose-6-phosphate, has been reported by Morrison, Rouser and Stotz.[7] The measurement of periodate consumption by the usual methods was not possible because the labile products underwent varying degrees of decomposition as a result of changes in *p*H of the unbuffered solution and because of interfering substances. The spectrophotometric method thus appeared to offer an excellent approach to this problem since conditions could be chosen to maintain constant *p*H and also the periodate consumption could be followed con-

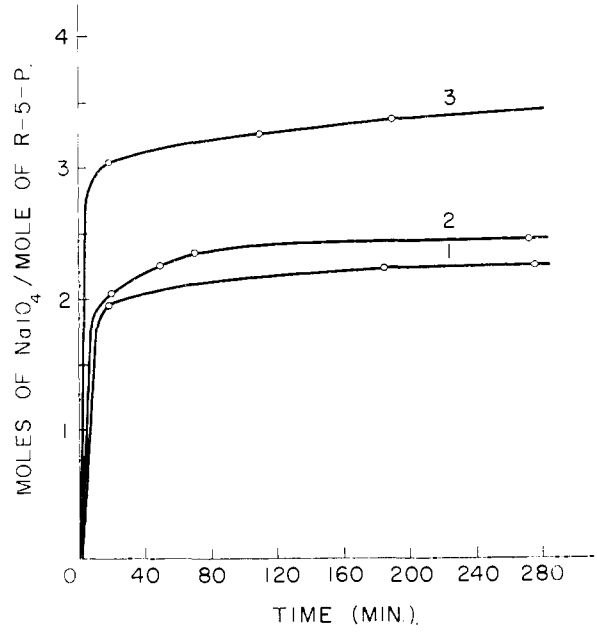


Fig. 4. The periodate oxidation of ribose-5-phosphate at various *p*H values. The following *p*H values were used: curve 1, 0.54; 2, 5.0; 3, 8.0. After 20 hours curve 1 and 2 leveled off at 2.50 and curve 3 at 3.56 moles of $NaIO_4$ per mole of ribose-5-phosphate.

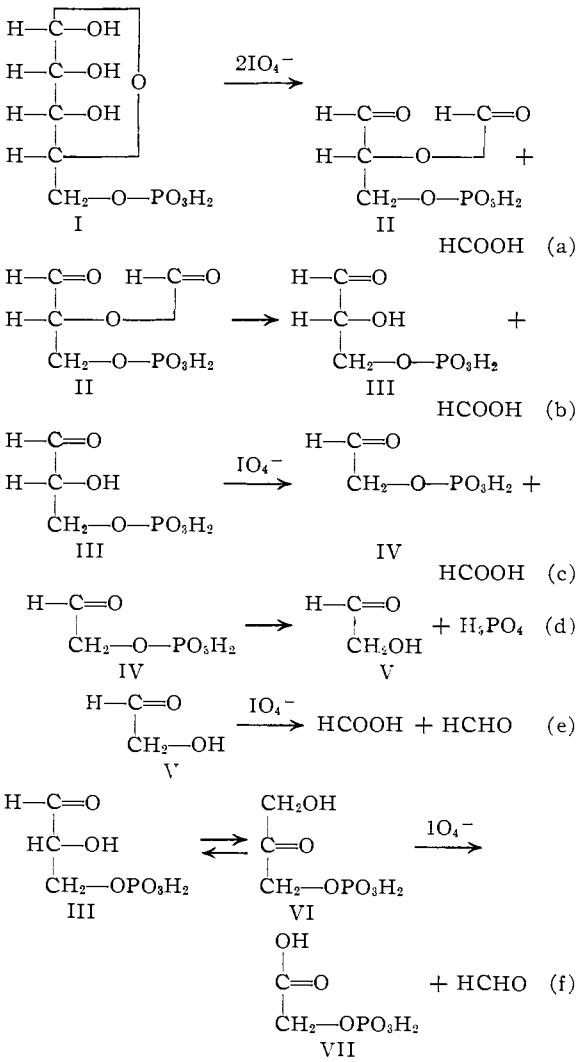
(6) F. S. H. Head and H. A. Standing, THIS JOURNAL, **74**, 1457 (1952).

(7) M. Morrison, G. Rouser and E. Stotz, *ibid.*, **77**, 5156 (1955).

tinuously. Ribose-5-phosphate was chosen for study because a very pure sample was readily obtainable from commercial sources. This sample was tested chromatographically before conducting the periodate oxidation and found to be free from other phosphate esters or free sugars.

In strong acid solution (*p*H 0.54) there was observed a rapid initial uptake of 2.0 moles of periodate per mole of ribose-5-phosphate followed by a slow oxidation until after 275 minutes 2.26 moles of periodate were consumed (Fig. 4). After 20 hours 2.50 moles of periodate had been consumed per mole of ribose-5-phosphate. In acetate buffer at *p*H 5.0 the periodate uptake was similar to that in the HCl solution *p*H 0.54. However, in an alkaline solution of phosphate buffer *p*H 8.0 there was a rapid initial uptake of 3.0 moles of periodate per mole of ribose-5-phosphate followed by a slow oxidation until after 190 minutes 3.38 moles of periodate were consumed. After 20 hours 3.56 moles of periodate were taken up.

The experimental results presented are in good agreement with the following sequence of reactions



In acid solution ribose-5-phosphate (I) reacts in the ribofuranoside form with 2 moles of periodate to yield the formic acid ester of 3-phosphoglyceraldehyde (II) and free formic acid (reaction a). The formic acid ester II is more stable in acid solution than in alkaline solution but appears to undergo a slow hydrolysis to yield 3-phosphoglyceraldehyde (III) and free formic acid (reaction b). This hydrolysis occurs rapidly in alkaline solution and slowly in acid solution and thereby explains the rapid initial uptake of 2 moles of periodate at *p*H 0.54 and 5.0 but a rapid initial uptake of 3.0 moles of periodate at *p*H 8.0. The 3-phosphoglyceraldehyde (III) now reacts with one more mole of periodate and accounts for the further uptake of periodate approaching the third mole (reaction c). Glycolaldehyde phosphate (IV) which is formed is stable in acid solution but does undergo hydrolysis in alkaline solution to yield glycolaldehyde (V) as shown in reaction d. The stability of glycolaldehyde phosphate (IV) in acid solution would explain the findings that at *p*H 0.54 and *p*H 5.0 the periodate uptake levelled off when 2.50 moles had been consumed. However, in alkaline solution glycolaldehyde phosphate (IV) is slowly hydrolyzed to yield glycolaldehyde (V) which then reacts with one more mole of periodate (reaction e). This would offer an explanation of the finding that at *p*H 8.0 the periodate uptake has reached a value of 3.56 moles. The periodate consumption data presented in this paper confirm the work of Morrison and co-workers.[7] Supporting evidence for the intermediate compounds given in the sequence of reactions a-f has been obtained by paper chromatography.[8]

The failure to obtain the theoretical three moles of periodate consumption in acid solution may be accounted for by the stability of the formic acid ester II in solutions of low *p*H. On the other hand, the reason for not obtaining the theoretical four moles of periodate consumption in alkaline solution may be further explained by the known chemical properties of glyceraldehyde phosphate (III) which in solution is in equilibrium with dihydroxyacetone phosphate (reaction f). The latter compound VI on periodate oxidation would yield glycolic acid phosphate (VII) which is considerably more stable in alkaline solution than glycolaldehyde phosphate (IV). Whereas the glycolaldehyde phosphate (IV) would be hydrolyzed to glycolaldehyde (V) which then would react with one more mole of periodate, glycolic acid phosphate (VII) would not be appreciably hydrolyzed to glycolic acid and therefore the consumption of the fourth mole of periodate would not be complete. Thus in contrast to the phosphorylated sugars, the free sugars consume the theoretical amount of periodate since with the free sugars any glycolic acid formed from dihydroxyacetone is completely oxidized by periodate. In addition it should be noted that a large excess of periodate was not used to drive the reaction to completion.

It is of interest that Schopf and Wied[9] have recently isolated the formic acid ester of glyceraldehyde by the periodate oxidation of glucose. This ester is apparently more labile than the cor-

(8) G. Rouser and M. Morrison, work to be published.
(9) C. Schopf and H. Wied, *Chem. Ber.*, **87**, 1571 (1954).

responding ester of glyceraldehyde phosphate but can be isolated by utilizing the proper amount of periodate. In our hands the periodate oxidation of glucose at *p*H 5.0 either at 0° or at room temperature gave a gradual uptake of the theoretical 5 moles, there being no indication of a leveling off when 3 moles of periodate had been consumed which would be the case if the formic acid ester of glyceraldehyde were stable at *p*H 5.0 (Fig. 5).

The formation of formic acid esters of periodate oxidized sucrose[10] and lactose[11] have also been reported. Furthermore, Lindstedt[12] has observed a greater periodate consumption by glucose phosphate and glyceraldehyde phosphate in neutral solution than in acid solution. The work presented in this paper indicates that the reason for this finding is the greater stability of the sugar phosphates in acid solution.

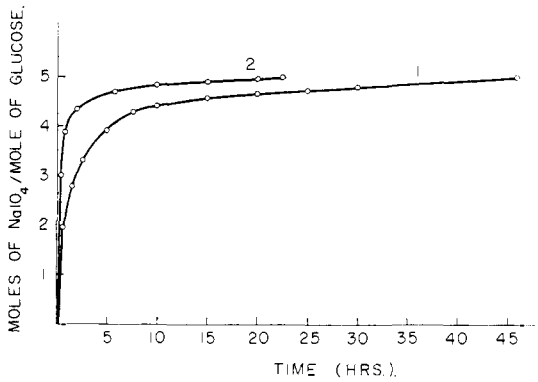


Fig. 5.—The periodate oxidation of D-glucose in acetate buffer *p*H 5.0: curve 1, 0°; 2, room temperature.

Experimental

A. **Periodate Oxidation of Ribose-5-phosphate in HCl Solution at *p*H 0.54.**—Into a 25-ml. volumetric flask were pipetted 5.0 ml. of 0.01 *M* ribose-5-phosphate (Schwartz, chromatographically pure), 2.0 ml. of 0.1 *M* $NaIO_4$ (G. Frederick Smith, reagent grade) and 1 *N* HCl added to the mark. A blank solution was prepared containing only 5.0 ml. of 0.01 *M* ribose-5-phosphate, 2.0 ml. of water, and 1 *N* HCl to a volume of 25 ml. The optical densities of the solutions were determined with a Beckman DU spectrophotometer at 280 mμ (slit, 0.78 mm.) against distilled water, using 1.0 cm. quartz cells. The optical density reading of the blank solution was subtracted from the optical density readings of the reaction solution. It was found that the optical density of the blank solution remained constant over the period of the experiment. All reactions were run in the dark at room temperature unless otherwise specified. The *p*H of the solutions was determined with a Beckman *p*H meter. The results are given graphically in Fig. 4.

B. **Periodate Oxidation of Ribose-5-phosphate in Acetate Buffer at *p*H 5.0.**—Into a 50-ml. volumetric flask were pipetted 3.03 ml. of 0.033 *M* ribose-5-phosphate, 5.0 ml. of 0.1 *M* $NaIO_4$, 20 ml. of 0.2 *M* sodium acetate–acetic acid buffer *p*H 5.0, and water added to the mark. A blank flask was prepared containing 3.03 ml. of 0.033 *M* ribose-5-phosphate, 20 ml. of 0.2 *M* acetate buffer *p*H 5.0, and water to a volume of 50 ml. The optical densities of the solutions were read at 310 mμ (slit, 0.52 mm.) against water. The blank solution gave a constant optical density reading over the period of the experiment which was subtracted from the readings of the reaction solution. The results are plotted graphically in Fig. 4.

C. **Periodate Oxidation of Ribose-5-phosphate in Phosphate Buffer *p*H 8.0.**—Into a 25-ml. volumetric flask were pipetted 5.0 ml. of 0.01 *M* ribose-5-phosphate, 2.0 ml. of 0.1 *M* $NaIO_4$, 10 ml. of 0.1 *M* phosphate buffer *p*H 8.0 and water added to the mark. A blank solution was prepared containing 5.0 ml. of 0.01 *M* ribose-5-phosphate, 10 ml. of 0.1 *M* phosphate buffer *p*H 8.0, and water to a volume of 25 ml. The optical densities of the solutions were read at 310 mμ (slit, 0.62 mm.) against distilled water. The blank solution gave a constant reading over the period of the experiment. The reading was subtracted from the optical density readings of the reaction flask. The results are given graphically in Fig. 4.

D. **Calibration Curves for Periodate at Various *p*H's, Temperatures and Wave Lengths.**—Several aliquots of from 1 to 5 ml. of 0.1 *M* $NaIO_4$ were pipetted into a series of 50-ml. volumetric flasks. The following procedure was used to determine the absorption curves at various *p*H values.

1. *p*H 0.54—to each aliquot was added 5 ml. of water and 1 *N* HCl to the mark.
2. *p*H 2.3—to each aliquot was added 1.0 ml. of 90% formic acid and water to the mark. The *p*H of the solution varied slightly depending on the final concentration of periodate.
3. *p*H 2.70—to each aliquot was added 20 ml. of glacial acetic acid and water to the mark.
4. *p*H 4.1—to each aliquot was added 20 ml. of 0.2 *M* acetate buffer *p*H 4.1 and water to the mark.
5. *p*H 5.0—to each aliquot was added 20 ml. of 0.2 *M* acetate buffer *p*H 5.0 and water to the mark.
6. *p*H 5.1–5.3—to each aliquot was added water to the mark. The *p*H of periodate alone in water varies slightly with the concentration of periodate.
7. *p*H 6.8—to each aliquot was added 20 ml. of 0.1 *M* phosphate buffer *p*H 6.8 and water to the mark. The *p*H of the solution varied slightly depending on the concentration of periodate.
8. *p*H 8.0—to each aliquot was added 20 ml. of 0.1 *M* phosphate buffer *p*H 8.0 and water to the mark. The *p*H of the solution varied slightly depending on the final concentration of periodate.

Blank solutions were also prepared containing all ingredients except the periodate. The optical density readings were read against distilled water and corrected by subtracting the blank readings.

Since the absorption of periodate varies with *p*H it is recommended that enough buffer be used to minimize *p*H changes due to varying the concentration of periodate and to formation of formic acid during the oxidation of the compound under investigation.

In HCl solution the absorption of periodate at 310 mμ was considerably diminished. Therefore, the calibration curve was determined at 280 mμ (Fig. 2). Calibration curves in acetate buffer at *p*H 5.0 were determined at 280 and 310 mμ. It was found that in the range 280–310 mμ the absorption of periodate obeys Beer's law.

In order to study the effect of temperature on the periodate absorption, calibration curves were determined at 0° and at room temperature at *p*H 8.0, 5.0 and 2.3. The results shown graphically in Fig. 3 demonstrate that the temperature effect is almost negligible at higher *p*H but that at lower *p*H values the absorption decreases at the lower temperature. The temperature effect was most pronounced at *p*H 2.3. This effect is undoubtedly due to repression of the ionization of periodate since the absorption of periodate is dependent on the ionic species.[4,5] In alkaline solution the periodate is essentially all in the ionized form and thus the temperature exerts little effect since the ionic species remain unchanged.

E. **Periodate Oxidation of D-Glucose in Acetate Buffer *p*H 5.0.**—In order to compare the oxidation rates of a sugar phosphate with a free sugar the oxidation of D-glucose (Eastman Kodak Co. anhydrous D-glucose) was carried out. The reaction flask contained 1.0 ml. of 0.1 *M* D-glucose, 5.0 ml. of 0.1 *M* $NaIO_4$, 20 ml. of 0.2 *M* acetate buffer *p*H 5.0 and water to a volume of 50 ml. A blank solution was prepared containing the same amount of glucose, buffer and water added to a volume of 50 ml. Optical density readings were taken at 310 mμ (slit, 0.52 mm.) and corrected by subtract-

(10) M. Morrison, A. C. Kuyper and J. M. Orten, THIS JOURNAL, **75**, 1502 (1953).

(11) K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta*, **31**, 1540 (1948).

(12) G. Lindstedt, *Nature*, **156**, 448 (1945).

ing the blank reading. The rates of glucose oxidation at room temperature and at 0° are shown graphically in Fig. 5. At room temperature the theoretical 5.0 moles of periodate was consumed after 23 hours and at 0° the theoretical amount of periodate consumed after 46 hours. Additional studies on other sugars and sugar alcohols revealed that the rates of reaction with periodate are dependent on the structure of the compound. The spectrophotometric method is a very simple one for following the periodate consumption and therefore for determining reaction velocity curves.

ROCHESTER, N. Y.

[CONTRIBUTION FROM THE BRITISH COLUMBIA RESEARCH COUNCIL, UNIVERSITY OF BRITISH COLUMBIA]

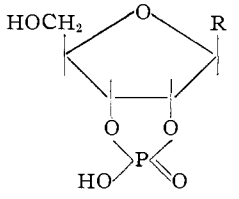
Cyclic Phosphates. II. Further Studies of Ribonucleoside 2′:3′-Cyclic Phosphates[1]

BY G. M. TENER AND H. G. KHORANA

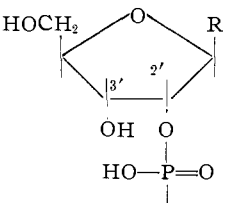
RECEIVED JUNE 2, 1955

A satisfactory method for the synthesis of monoalkyl esters of the ribonucleoside 2′- and 3′-phosphates is described which utilizes the facile, acid-catalyzed transesterification reaction between alcohols and ribonucleoside 2′:3′-cyclic phosphates. The latter compounds are prepared readily by the treatment of the yeast ribonucleotides with dicyclohexylcarbodiimide.

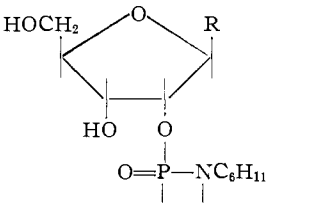
Pyrimidine ribonucleoside 2′:3′-cyclic phosphates (I, R = cytosine, uracil) were first encountered by Markham and Smith[2] as intermediates in the ribonuclease-catalyzed hydrolysis of ribonucleic acid (RNA) and their identification was accomplished through comparison with samples of these substances synthesized by Todd and collaborators.[3] Markham and Smith[4] were also able to isolate the cyclic phosphates corresponding to all the four ribonucleosides after mild alkaline hydrolysis of RNA. Recently, Lipkin and Talbert[5] have shown that the cyclic esters can be obtained in markedly improved yield by treatment of RNA with potassium *t*-butoxide in anhydrous *t*-butyl alcohol.



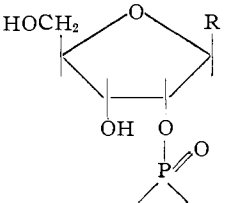
I



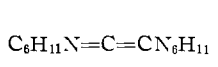
IIa



IIIa



IVa



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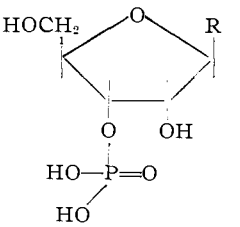
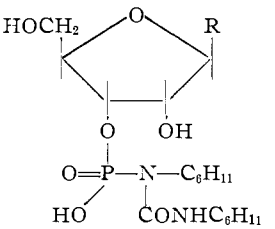
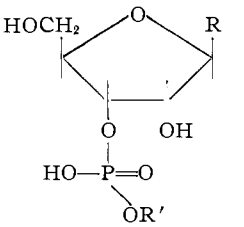


IIb



IIIb



IVb

The method employed by Brown, Magrath and Todd[3] for the chemical synthesis of the cyclic phosphates involved the reaction of the yeast ribonucleotides (II) (mixtures of ribonucleoside 2′- and 3′-phosphates) with trifluoroacetic anhydride and the yields reported were low. Recently a simple method for the purpose was reported[1] which consisted in treating the yeast ribonucleotides (II) with dicyclohexylcarbodiimide (DCC) (V) in aqueous pyridine at room temperature. The reaction converted the parent nucleotides quantitatively to mixtures of the cyclic phosphates (I) and phosphorylureas of the type III, the former predominating in the short period reactions. Compounds of the type III are reconverted to the cyclic phosphates under acidic or alkaline conditions and are, for all practical purposes, equivalent to the cyclic phosphates. Thus the method described[1] makes the cyclic phosphates readily available in quantity.

Thorough chemical study of the properties of the cyclic phosphates is desirable since the process of cyclization (II → I) represents a simple means for the activation of the phosphate group present in the yeast ribonucleotides (II). The energy stored in these labile esters of phosphoric acid (I), it is hoped, may be used to bring about, through transesterification reactions, the synthesis of 2′,5′- and 3′,5′-dinucleoside phosphates (IVa and IVb, respectively; R = purine or pyrimidine, R′ = purine or pyrimidine nucleoside) and polynucleotides. Ample enzymatic as well as chemical evidence has

(1) For the previous paper see C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

(2) R. Markham and J. D. Smith, *Nature*, **168**, 406 (1951).

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

(4) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(5) D. Lipkin and P. T. Talbert, *Chemistry and Industry*, 143 (1955).