side with a methanolic solution of barium hydroxide.[19] Treatment of the crude product in benzene (100 ml.) with charcoal (2 g.) followed by removal of charcoal and solvent gave a sirup which crystallized spontaneously. When recrystallized from the smallest volume of benzene–Skelly C (1:1), pure methyl 2,4-di-*O*-methyl-β-D-glucoside was obtained; yield 0.115 g. (27% of the theoretical amount), m.p. 124°, $[\alpha]^{25}D$ −16° (acetone, *c* 1). A mixed melting point with an authentic specimen of methyl 2,4-di-*O*-methyl-β-D-glucoside prepared by Reeves[5] gave no depression.

Anal. Calcd. for $C_9H_{18}O_6$: C, 48.7; H, 8.2; OCH_3, 41.9. Found: C, 48.9; H, 8.2; OCH_3, 41.7.

N-*p*-Nitrophenyl-2,4-di-*O*-methyl-D-glucosylamine.—To a solution of 2,4-di-*O*-methyl-D-glucose (0.112 g.) in absolute ethanol (3 ml.) was added *p*-nitroaniline (0.108 g., 50% excess) and 1 drop of glacial acetic acid. During 1 hour of refluxing the crystalline product separated. From the cooled reaction mixture was obtained, by filtration and washing of the product with a little absolute ethanol, N-*p*-nitrophenyl-2,4-di-*O*-methyl-D-glucosylamine (0.161 g., or 91% of the theoretical amount). Recrystallized from anhydrous ethyl acetate (150 ml.) it had m.p. 250–251° dec. If heated slowly the crystals had a lower melting point. $[\alpha]^{25}D$ −252° (10 min.) → −268° (equil.) (pyridine, *c* 0.5).

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Anal. Calcd. for $C_{14}H_{20}O_7N_2$: C, 51.2; H, 6.1; N, 8.5; OCH_3, 18.9. Found: C, 51.4; H, 6.1; N, 8.6; OCH_3, 19.0.

N-*p*-Bromophenyl-2,4-di-*O*-methyl-D-glucosylamine.—To a solution of 2,4-di-*O*-methyl-D-glucose (0.104 g.) in absolute ethanol (3 ml.) was added *p*-bromoaniline (0.130 g., 50% excess) and the mixture was refluxed for 4 hours. The product which crystallized during the refluxing was separated from the cooled reaction mixture by filtration and was washed with a little ethanol; yield 0.162 g. or 90% of the theoretical amount. Recrystallized from anhydrous ethyl acetate (200 ml.), pure N-*p*-bromophenyl-2,4-di-*O*-methyl-D-glucosylamine was obtained, m.p. 243–244° (sl. dec.), $[\alpha]^{25}D$ −147° (pyridine, *c* 0.5). No mutarotation was observed.

Anal. Calcd. for $C_{14}H_{20}O_5NBr$: C, 46.4; H, 5.6; N, 3.9; Br, 22.1; OCH_3, 17.1. Found: C, 46.4; H, 5.8; N, 3.9; Br, 22.3; OCH_3, 17.7.

Acknowledgment.—We wish to thank Dr. R. E. Reeves for an authentic specimen of methyl 2,4-di-*O*-methyl-β-D-glucoside and Mr. C. H. Van Etten and Mrs. Clara E. McGrew for performing the microanalyses.

PEORIA, ILLINOIS

[CONTRIBUTION FROM THE BEN MAY LABORATORY FOR CANCER RESEARCH, UNIVERSITY OF CHICAGO]

Enzymatic Preparation of Two Ketohexose 1-Phosphates

BY ALBERT L. LEHNINGER[1] AND JEAN SICE

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Propionaldehyde and 3-methoxypropionaldehyde each undergo enzymatic aldol condensation with dihydroxyacetone phosphate in muscle extracts containing aldolase and triose phosphate isomerase. The products, presumably 5,6-dideoxy-D-fructose 1-phosphate and 5-deoxy-6-*O*-methyl-D-fructose 1-phosphate, were isolated in good yield, thus extending the list of ketoses and ketose 1-phosphates which may be easily prepared enzymatically.

Aldolase catalyzes the reversible reaction

D-glyceraldehyde 3-phosphate + dihydroxyacetone phosphate ⇌ D-fructose 1,6-diphosphate (1)

It has been found[2-4] that a variety of aldehydes may replace glyceraldehyde 3-phosphate in this reaction; however, dihydroxyacetone phosphate is a specific reaction partner. When "foreign" aldehydes react enzymatically with dihydroxyacetone phosphate corresponding ketose 1-phosphates are formed; for instance, D-fructose 1-phosphate is formed from D-glyceraldehyde[2,3] and D-xylulose 1-phosphate is formed from glycolaldehyde.[4]

Dialyzed rabbit muscle extracts contain, in addition to aldolase, triose phosphate isomerase, catalyzing the reaction

D-glyceraldehyde 3-phosphate ⇌ dihydroxyacetone phosphate (2)

In such extracts the readily available D-fructose 1,6-diphosphate may be used as starting material for the relatively simple enzymatic preparation of new ketose 1-phosphates, in the presence of a large excess of a "foreign" aldehyde. In this way, D-fructose 1-phosphate,[2,3] L-sorbose 1-phosphate,[2,3] 5-deoxyxylulose 1-phosphate,[2] and D-xylulose 1-phosphate[5] have been prepared. With a specific aldolase of liver, erythrulose 1-phosphate has also been prepared.[5] Extracts of peas contain phosphatases in addition to aldolase and isomerase and with such preparations Jones and colleagues have prepared a series of free (dephosphorylated) ketoses.[6] In all enzymatic aldolization products which have been studied to this end, the configuration of hydroxyl groups at carbon atoms 3 and 4 has been found to be D-*threo* (*trans*-glycol). These products are thus homologs of D-xylulose, and D-fructose or L-sorbose, etc. The enzymatic formation of the L-*threo* or D- or L-*erythro* configurations by aldolase has not been observed, although D-tagatose 1,6-diphosphate (an *erythro* form) is very slowly cleaved by aldolase.[3]

In this paper the enzymatic preparation of 5,6-dideoxyhexulose 1-phosphate and 5-deoxy-6-*O*-methylhexulose 1-phosphate from the corresponding aldehydes (propionaldehyde and 3-methoxypropionaldehyde) is described. The naming of the products is non-committal with respect to configuration at carbons 3 and 4, which was not directly determined. However, it is probable that the products consist largely if not entirely of 5,6-dideoxy-D-fructose 1-phosphate and 5-deoxy-6-*O*-methyl-D-

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fructose 1-phosphate in view of the enzyme specificity.

The enzymatic method employed[2] is simple and capable of yielding fairly large quantities of ketose phosphates which are otherwise rather difficult to prepare by purely chemical procedures. Measurements of the rate and extent of the enzymatic aldolization showed that the preparation of 5,7-dideoxyheptulose 1-phosphate from 3-hydroxybutyraldehyde and 5-deoxy-D-fructose 1-phosphate from 3-hydroxypropionaldehyde was also feasible by this method.

Experimental

Materials.—Commercial D-fructose 1,6-diphosphate (FDP) was purified chromatographically by a modification of the method of MacCready and Hassid.[7] The product contained less than 0.5% hexose 6-phosphates as determined enzymatically.[8] The 3-methoxypropionaldehyde was obtained from Shell Development Co.

Rabbit skeletal muscle extracts were prepared by stirring one part of finely minced muscle with two parts of cold water for 30 minutes. After straining and centrifugation the extracts were dialyzed 24 hours against 0.01 *M* $NaHCO_3$ and again clarified by centrifugation.

Inorganic P, total P and alkali-labile P were determined colorimetrically. The melting points were determined in evacuated capillaries and are corrected. The optical rotations were determined in a 1 dm. tube, *c* being the concentration in g./100 ml. of solution. The microanalyses were performed by Mr. W. Saschek.

5,6-Dideoxyhexulose 1-(Disilver-Phosphate).—A 0.08 *M* solution of FDP (350 ml.) at *p*H 7.6 was incubated with 200 ml. of dialyzed muscle extract in the presence of a final concentration of 0.002 *M* sodium iodoacetate at 25°. The aldolase–isomerase equilibrium was established within one minute, as determined by the formation of an equilibrium level of alkali-labile P (3.51 mmoles). To this mixture was then added 250 ml. of a 1.0 *M* aqueous solution of propionaldehyde (250 mmoles). The alkali-labile P rose to a plateau of 51.6 mmoles at about 25 minutes (indicating approximately 91% conversion of the initial FDP to the newly synthesized ketose phosphate, assuming both trioses were utilized *via* the isomerase equilibrium). To stop the reaction 50 ml. of 100 g./100 ml. aqueous trichloroacetic acid was added. After complete mixing the proteins were filtered off. The filtrate was reduced in volume to 490 ml. by evaporation from the frozen state. For each 10.0-ml. volume, 0.65 ml. of 2 *M* $AgNO_3$ was slowly added dropwise in the cold with thorough stirring. The precipitated inorganic silver salts were removed by centrifugation. To the clear supernatant, *p*H 5.5, was added 2.0 ml. of 2 *M* $AgNO_3$ per 10.0 ml. of solution in the cold, slowly, with stirring. The white precipitate was recovered by centrifugation, washed with ethanol and ether and dried in a darkened vacuum desiccator; yield 11.1 grams of white powder, containing 6.14% total P, all of which was present as alkali-labile P (theoretical for monohydrated disilver salt 6.74%). There was no inorganic phosphate or inorganic chloride. The silver salt was suspended in 400 ml. of cold water and brought into solution by dropwise addition of concentrated nitric acid. Silver ion was removed by addition of 24.0 ml. of 2 *M* NaCl, followed by fast filtration in the dark. Sufficient 2 *M* $AgNO_3$ was added (about 4.0 ml.) to remove the excess chloride. After filtration the solution was adjusted to *p*H 8.9 with concentrated ammonium hydroxide under efficient stirring. Fractional precipitation of the silver salt was then begun. The addition of 1.0 ml. of 2 *M* $AgNO_3$ yielded a slight, yellow precipitate, removed by centrifugation. Addition of 2.0 ml. of 2 *M* $AgNO_3$ to the clear centrifugate gave no precipitate. Addition of a further 2.0-ml. portion of 2 *M* $AgNO_3$ yielded a pure white micro-crystalline precipitate with pronounced "creeping" tendency and with relative insensitivity to light. The bulk of this material was then precipitated by slow addition of a total of 60.0 ml. of 2 *M* $AgNO_3$. After standing in the cold for 9 hours, the white precipitate was recovered by centrifugation. It was washed with 50% ethanol, 75% ethanol, twice with absolute ethanol and then with ether. After drying *in vacuo* over phosphorus pentoxide for 2 days this product weighed 7.9 g., an isolation yield of about 33%. The compound appeared quite stable when stored at −15° in a darkened desiccator.

Anal. Calcd. for $C_6H_{11}O_7PAg_2 \cdot H_2O$: total P, 6.74; alkali-labile P, 6.74; Ag, 46.8. Found: total P, 6.85; alkali-labile P, 6.69; Ag, 47.2.

5,6-Dideoxyhexulose 1-Dicyclohexylammonium Phosphate.—This was obtained in crystalline form by a procedure similar to that used by Friedkin.[9] The silver salt (92 mg., 0.2 mmole) was suspended in 0.5 ml. of water and to it was added 0.3 ml. of 2 *M* NaCl. After thorough mixing, the precipitated silver chloride was removed by centrifugation; to the clear supernatant was added 0.3 ml. of 20% aqueous cyclohexylamine sulfate. To this was added 15.0 ml. of *n*-butanol and the mixture shaken vigorously until the aqueous phase dissolved in the *n*-butanol. The material which precipitated on standing overnight at 0° was removed by centrifugation and discarded. To the clear supernatant was added an equal volume of diethyl ether, producing a slight turbidity. On standing overnight in the refrigerator the dicyclohexylammonium salt crystallized as fine needles. A second crystallization yielded 29.4 mg. of needles arranged in sheaves (69% of calculated).

Anal. Calcd. for $C_{18}H_{39}O_7N_2P$: P, 7.26; N, 6.57. Found: P, 7.07; N, 6.40; $[\alpha]^{25}D$ +7.2° (*c* 1.04, H_2O), unchanged after 20 hours.

The phosphorus was entirely in the form of alkali-labile P.

5,6-Dideoxyhexulose Phenylosazone.—The silver salt of 5,6-dideoxyhexulose 1-phosphate (0.48 g.) was dissolved in 5.0 ml. of 40% acetic acid and allowed to stand overnight at 20°. The silver ion was removed by adding a small excess of sodium chloride. To the clear colorless supernatant was added 0.5 ml. of a saturated aqueous solution of sodium bisulfite and 0.45 g. of recrystallized phenylhydrazine hydrochloride, and the *p*H was adjusted to 5.0 with sodium carbonate. The solution was placed in a boiling water-bath for 15 minutes. After cooling, the precipitate was removed by filtration, washed with water and dried (0.18 g.). Four recrystallizations from benzene yielded yellow needles melting at 126–128° dec. The substance underwent mutarotation in pyridine:ethanol (3:2) solution (*c* 0.74) from −1.4° to an equilibrium value of −14.9° (in 20 hr. at 26°).

Anal. Calcd. for $C_{18}H_{22}O_2N_4$: C, 66.23; H, 6.79. Found: C, 66.53; H, 6.97.

5-Deoxy-6-*O*-methylhexulose 1-(Disilver Phosphate).—The enzymatic condensation was carried out as described above, starting with 38.5 mmoles of FDP and 350 mmoles of 3-methoxypropionaldehyde, yielding 64.5 mmoles of product in a volume of 800 ml. The reaction was stopped by the addition of 50 ml. of 100 g./100 ml. aqueous trichloroacetic acid and the precipitated proteins were removed by filtration. To the cold filtrate was added 37.5 ml. of 2 *M* $AgNO_3$ and the precipitated silver salts were removed by filtration. The cold supernatant was then carefully brought to *p*H 7.5 with concentrated ammonium hydroxide and clarified by filtration. To the filtrate was added 170 ml. of 2 *M* $AgNO_3$ and the slight precipitate formed was removed by centrifugation and discarded. To the clear, slightly yellow supernatant was slowly added an equal volume of cold ethanol with stirring. After standing 3 hours at 0° the heavy granular silver salt was recovered and reprecipitated, essentially by a repetition of the above procedure. The product was washed with ethanol and ether and dried, yielding 3.00 g. of material nearly insoluble in cold water but readily soluble in 0.1 *M* $NaNO_3$, as was the silver salt of 5,6-dideoxyhexulose 1-phosphate.[2]

The substance was dried in high vacuum over phosphorus pentoxide for 18 hours at room temperature.

Anal. Calcd. for $C_7H_{15}O_8PAg_2 \cdot H_2O$: total P, 6.30; alkali labile P, 6.30; Ag, 43.86. Found: total P, 6.41; alkali-labile P, 6.14; Ag, 44.41.

5-Deoxy-6-*O*-methylhexulose 1-Dicyclohexylammonium Phosphate.—The crystalline salt (fine needles) was prepared as described in the case above.

Anal. Calcd. for $C_{19}H_{41}O_8N_2P$: P, 6.79; N, 6.14. Found: P, 6.73; N, 6.04; $[\alpha]^{25}D$ +12.6° (*c* 1.43, H_2O), unchanged after 20 hours.

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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.

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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]

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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.

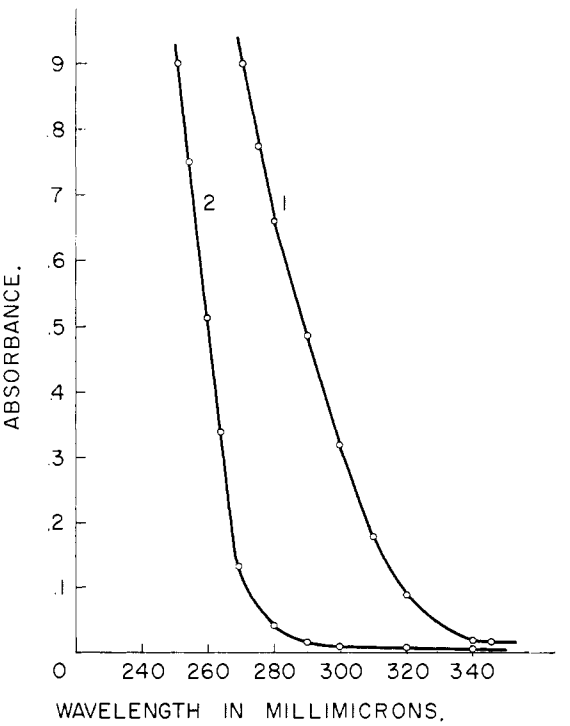

Fig. 1.—Absorption spectra of $NaIO_4$ and KIO_3 in distilled water: curve 1, 0.002 *M* $NaIO_4$; 2, 0.005 *M* KIO_3.

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