from 2,3,4-tri-*O*-benzoyl-β-D-ribopyranosyl fluoride as described in the preceding paper

Anal. Calcd. for $C_{19}H_{17}O_6F$: C, 63.33; H, 4.76. Found: C, 63.53; H, 4.82.

(b) **From 2,3,4-Tri-*O*-benzoyl-β-L-arabinopyranosyl Fluoride (II).**—2,3,4-Tri-*O*-benzoyl-β-L-arabinopyranosyl fluoride (500 mg.) was dissolved in 5 ml. of liquid hydrogen fluoride and the solution kept at room temperature for 6 hr. The hydrogen fluoride was then removed with a stream of air, the residue dissolved in methylene chloride and washed successively with saturated aqueous sodium bicarbonate and water. Moisture was removed with sodium sulfate, the solution filtered through decolorizing carbon and then concentrated *in vacuo* to give 360 mg. of sirup. Dissolved in ether–pentane, seeded and cooled, the product, 170 mg. (44%), crystallized. It rotated $[\alpha]^{20}D$ +52.0° ($CHCl_3$, *c* 0.36) and melted at 147–149° dec., no depression in melting point being observed when mixed with the product obtained in (a) above.

2,3,4-Tri-*O*-benzoyl-β-L-ribopyranosyl Fluoride (VI) from 3,4-Di-O-benzoyl-β-L-ribopyranosyl Fluoride (V).—3,4-Di-*O*-benzoyl-β-L-ribopyranosyl fluoride (360 mg.) was added to an ice-cold solution of 0.230 ml. of benzoyl chloride in 3 ml. of anhydrous 2,4,6-trimethylpyridine and the mixture kept at room temperature for 18 hr. A few drops of water were then added and, after 15 min., the solution was diluted with methylene chloride (25 ml.) and washed successively with ice-cold 3 *N* sulfuric acid (3 × 25 ml.), saturated aqueous sodium bicarbonate (3 × 25 ml.) and water (3 × 25 ml.). The solution was dried with sodium sulfate, filtered through decolorizing carbon and concentrated *in vacuo* to a sirup which crystallized upon the addition of a little ether. Recrystallized from a mixture of ether (5 ml.) and pentane (15 ml.) the product (360 mg., 78%) melted at 138.5–140°. One further recrystallization from ether gave pure material which rotated $[\alpha]^{20}D$ +51.8° ($CHCl_3$, *c* 0.65) and melted at 139.5–140.5°. The infrared spectrum of the substance was identical with that of its enantiomorph, described in the preceding paper.

Anal. Calcd. for $C_{26}H_{21}O_7F$: C, 67.24; H, 4.56. Found: C, 67.48; H, 4.77.

Methyl β-L-Ribopyranoside (IV) from 3,4-Di-O-benzoyl-β-L-ribopyranosyl Fluoride (V).—3,4-Di-*O*-benzoyl-β-L-ribopyranosyl fluoride (400 mg.) was dissolved in a mixture of anhydrous methanol (5 ml.) and methylene chloride (5 ml.) in a 1-dm. all-glass polarimeter tube. A solution of sodium methoxide in methanol (1.74 *N*) was then added dropwise. The solution became neutral a few minutes after the addition of each drop, indicating the lability of the fluorine in the substance. After 0.60 ml. of the sodium methoxide had been, added the rotation of the solution was observed at 20° until it was constant (4 hr.). The solution was then evaporated *in vacuo* to dryness and the residue extracted with pentane (3 × 15 ml.) to remove the methyl benzoate. The remainder was then extracted with hot ethyl acetate (3 × 5 ml.) and the extracts concentrated *in vacuo* to a sirup which crystallized when dissolved in ether and cooled to −5°. Recrystallized from a mixture of ether (5 ml.) and pentane (5 ml.) the product (110 mg., 60%) melted at 75–78° and rotated $[\alpha]^{20}D$ +140° ($CHCl_3$, *c* 0.47). Two recrystallizations from ether–pentane gave 50 mg. of pure methyl β-L-ribopyranoside melting at 80–81° and showing $[\alpha]^{20}D$ +142° ($CHCl_3$, *c* 0.20). Methyl β-D-ribopyranoside melts at 83°[8] and we have found it to show $[\alpha]^{20}D$ −142.8° in chloroform (*c* 0.57). The infrared spectrum of the product obtained here was identical with that of its pure enantiomorph.

3,4-Di-O-benzoyl-2-O-methylsulfonyl-β-L-ribopyranosyl Fluoride.—3,4-Di-*O*-benzoyl-β-L-ribopyranosyl fluoride (360 mg.) was added to an ice-cold mixture of 0.16 ml. of methanesulfonyl chloride and 5 ml. of anhydrous 2,4,6-trimethylpyridine and the reaction mixture kept at room temperature for 2 hr. when some darkening was observed. A little water was then added and, 15 min. later, 25 ml. of methylene chloride. The solution was then washed successively with cold 3 *N* sulfuric acid (3 × 25 ml.), cold, saturated aqueous sodium bicarbonate (2 × 25 ml.) and water (2 × 25 ml.). Moisture was removed with sodium sulfate, the solution filtered through decolorizing carbon and concentrated *in vacuo* to give a sirup which crystallized on treatment with a little ether. The ether was evaporated and the product recrystallized from a mixture of ethyl acetate (5 ml.) and pentane (15 ml.) to give 380 mg. (87%) of colorless prisms melting at 157–158° and rotating $[\alpha]^{20}D$ +41.3° ($CHCl_3$, *c* 0.59). One further crystallization from ether–pentane gave 300 mg. of pure 3,4-di-*O*-benzoyl-2-*O*-methylsulfonyl-β-L-ribopyranosyl fluoride melting at 157–158° and rotating $[\alpha]^{20}D$ +41.8° in chloroform (*c* 0.98).

Anal. Calcd. for $C_{20}H_{19}O_8FS$: C, 54.79: H, 4.37; S, 7.31. Found: C, 54.55; H, 4.46; S, 7.34.

Acknowledgment.—We are indebted to the Analytical Services Unit of this Laboratory, under the direction of Dr. W. C. Alford, for analyses and infrared absorption measurements.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY MEDICAL CENTER]

The Mechanism of Glucose-6-phosphatase[1]

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Hepatic microsomal glucose-6-phosphatase, prepared from normal and alloxan-diabetic rats, was found to catalyze the following exchange reaction: G-6-P + C^{14}-glucose ⇄ C^{14}-G-6-P + Glucose. When glucose inhibition of glucose 6-phosphatase was correlated with the exchange reaction, it was found that the number of μmoles of orthophosphate whose liberation was prevented by glucose was approximately equal to the number of μmoles of C^{14}-glucose incorporated into G-6-P. Under identical conditions, P^{32}-orthophosphate was not incorporated; however, C^{14}-fructose was an acceptor at approximately one-twentieth the rate of glucose. These observations were found to be consistent with the mechanism: (1) Enz + G-6-P ⇄ E(G-6-P) ⇄ E-P + Glucose, (2) E-P + H_2O → Enz + Orthophosphate. Lineweaver–Burk plots of enzymatic activity at different concentrations of glucose yielded a series of lines with increasing ordinate intercepts and slightly increasing slopes. Steady-state kinetic treatment of the proposed mechanism gave a reciprocal velocity expression which was in good agreement with the described plots. No difference was found between the kinetic parameters (K_m, K_s and K'_1 for glucose) of the enzymes prepared from normal and diabetic animals. Orthophosphate was found to be a competitive inhibitor of glucose-6-phosphatase activity.

In contrast to previously studied non-specific phosphatases[2,3] phosphoserine phosphatase specifically catalyzes the exchange of C^{14}- serine with the serine moiety of phosphoserine.[4,5] The purpose of the present publication is to extend these observa-

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service. This work is part of a dissertation presented by L. F. Hass for the degree of Doctor of Philosophy at Duke University.

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tions to another specific phosphatase, glucose-6-phosphatase. The description of a detailed mechanism, based on the work to be described, is now possible for glucose-6-phosphatase. This mechanism is in agreement with that proposed for phosphoserine phosphatase,[6] and, by analogy, the data on glucose-6-phosphatase furnish kinetic justification for the phosphoryl–enzyme intermediate suggested previously for phosphoserine phosphatase. The enzyme preparation used in these studies was rat liver microsomes[7] which, according to de Duve,[8] contain essentially all the glucose-6-phosphatase activity present in the original homogenate. These studies, when it becomes possible, should be repeated using a purified soluble enzyme. but the purification appears to be very difficult.[9] Microsomal glucose-6-phosphatase has the advantage that the data which have been obtained with microsomes from normal and alloxan-diabetic rats may be used to achieve a better understanding of the increased activity of microsomal glucose-6-phosphatase in the diabetic rat observed by Ashmore, *et al*,[10] and Langdon, *et al.*[11] The only enzyme, known to be present in microsome preparations, which would interfere with the experiments to be described is phosphohexose isomerase, and minimal interference was established by determining its activity in representative microsome preparations. A preliminary report of these observations has been presented.[12]

An extensive review on glucose-6-phosphatase by Ashmore and Weber[13] is available; however, detailed studies of the mechanism of glucose-6-phosphatase have not been described. Broh-Kahn, *et al.*,[14] prior to the characterization of glucose-6-phosphatase, reported that glucose inhibited the conversion of glucose-1-phosphate to glucose in liver extracts. In a preliminary report Langdon and Weakley[15] mention the competitive inhibition of glucose-6-phosphatase by glucose. Independently of our work, Segal, *et al.*, have described certain kinetic parameters of glucose-6-phosphatase prepared from normal and diabetic rats.[16] They described the inhibition by glucose and noted that "the inhibition was of a non-competitive nature." The discovery by Hass and Byrne[12] that glucose-6-phosphatase catalyzes the exchange of C^{14}-glucose with the glucose moiety of G-6-P has been confirmed by Nemer and Ashmore.[17] More recently, a manuscript on the mechanism of glucose-6-phosphatase, which included our proposed mechanism and the accompanying rate equation, was made available to Dr. Harold L. Segal. Dr. Segal has, qualitatively and quantitatively, confirmed the exchange activity of glucose-6-phosphatase and has discussed certain modifications of our rate equation.[18] These modifications are largely based on his classification of the inhibition by glucose as "classical non-competitive inhibition." Our results do not agree with "classical non-competitive inhibition," or any other classical type, and the observed kinetic parameters of glucose-6-phosphatase are best described by the mechanism and its rate equation presented in this paper.

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Experimental

Materials.—D-Galactose, D-xylose, D-arabinose, D-ribose and D-lyxose were purchased from the Nutritional Biochemicals Corporation. D-Sodium glucuronate, D-glucosamine hydrochloride, N-acetyl-D-glucosamine and 2-deoxy-D-glucose were obtained from the California Foundation for Biochemical Research. D-Fructose was purchased from the Eastman Kodak Co. and D-mannose from the Pfanstiehl Laboratories. All sugars and sugar analogs were tested for contaminating sugars by paper chromatography using the ethyl acetate–glacial acetic acid solvent recommended by Jermyn and Isherwood.[19] The chromatograms were developed by the method of Chernick, *et al.*[20] In all cases there was little ($< 1\%$) or no contamination of one sugar by another. Glucose-6-phosphate was purchased as the disodium salt from the Sigma Chemical Co. and found to be free of contaminating orthophosphate. Fructose-6-phosphate was purchased as the barium salt from the Schwarz Laboratories and freed from contaminating hexose and orthophosphate by ion-exchange column chromatography. Elon (*p*-methylaminophenol sulfate) and alloxan monohydrate were obtained from the Eastman Kodak Co., perfluoroöctanoic acid from the Matheson Co., and crystalline bovine serum albumin (lot P67403) from the Armour Laboratories. Glucose oxidase reagents (Glucostat) were purchased from the Worthington Biochemical Corporation. Carrier-free P^{32} orthophosphate was obtained from the Oak Ridge National Laboratory and treated by the method of Meyerhof and Green[21] to eliminate contamination by metaphosphate, pyrophosphate and phosphite. D-Glucose-u-C^{14} (0.41 μc./mg.) was purchased from Atomlab Inc. and D-fructose-u-C^{14} (1.06 μc./mg.) was obtained from Tracerlab Inc. Carrier reagents were added to all radioactive preparations to give compounds of known specific activity and solutions of known concentration.

Analytical Procedures.—Reducing sugars were determined by the method of Nelson[22] and ketohexoses by the method of Roe, *et al.*[23] Appropriate reference standards were used with each sugar assay. Microsomal protein was determined by the method of Lowry, *et al.*,[24] using Armours' crystallin bovine serum albumin as a reference standard. The amount of protein in the standard was determined by measuring optical density at 280 mμ and comparing this with the value reported by Cohn, *et al.*[25]

All radioactivity measurements were carried out by plating at infinite thinness and counting in an internal gas flow counter. C^{14} and P^{32} samples were plated on 2.4 cm. stainless steel and nickel planchets, respectively. With small volumes, uniform plating was ensured by the addition of a few drops of ethyl alcohol. In most cases, sufficient counts were recorded to give a probable error of 5%.

Preparation of Diabetic Animals.—Young male rats, weighing between 180 and 220 g. were used. The rats were fasted for 48 hr. and then injected subcutaneously with one ml. of a 2% solution of alloxan monohydrate per 100 g. of body weight. In one to three weeks following injection, those animals having blood glucose levels in excess of 400 mg. % were used.[22]

Buffer Preparation.—The buffer preparation employed throughout consisted of succinic acid (0.1 *M*), maleic acid (0.1 *M*) and Tris-(hydroxymethyl)-aminomethane (0.1 *M*) and was adjusted to the proper *p*H with HCl or NaOH. Since some of the experiments were carried out over a wide *p*H range, a mixed buffer preparation was selected in order to mask small variations in glucose-6-phosphatase activity with different buffers.

Preparation of Microsomes.—Excised livers were minced and immediately chilled by immersing in 0.25 *M* sucrose solution at 0°. Exactly 3 g. of tissue was weighed, added to 27 ml. of sucrose solution, and homogenized for 2 min. with a glass Potter–Elvehjem homogenizer. The various subcellular fractions (nuclear, mitochondrial and microsomal) were separated from the homogenate by differential centrifugation according to the procedure described by Schneider.[7] The microsomal fraction, sedimenting after centrifugation at 80,000 × *g* for 30 min., was collected and resuspended in exactly 30 ml. of cold sucrose solution. Appropriate aliquots of this suspension were used in the experiments described.

Enzyme Assays.—Phosphatase activity was followed by analyzing for either phosphate or glucose release from G-6-P. All assays were made under conditions in which hydrolysis was linear with time.

a. **Phosphate Method.**—The reaction was initiated by adding enzyme after allowing for temperature equilibration at 37°. At zero time, 10 and 20 minutes, 0.5-ml. aliquots were withdrawn from the incubation mixture and deproteinized with trichloroacetic acid. Orthophosphate was determined using the method of Gomori[26] with a 30 minute color development time.[27]

b. **Glucose Method.**—Incubation procedures were the same as those described above. At appropriate time intervals, 0.5-ml. aliquots were removed from the incubation mixture and added to 0.5 ml. of 0.5 *N* HCl. The samples were then treated with 0.5 ml. of 0.5 *N* KOH and clarified by centrifugation. Two ml. of glucose oxidase reagent (buffered at *p*H 7.0) were added to 1.0-ml. aliquots of the clarified solutions and allowed to incubate at room temperature for 20 min. The reaction was terminated by adding one drop of 4 *N* HCl. Optical density was determined in a Beckman DU spectrophotometer at 401 mμ using a zero time sample in the reference cell. The procedure used is that recommended by the Worthington Biochemical Corporation for their Glucostat reagents except that sufficient phosphate buffer, *p*H 7.0, was included to make the final concentration of added phosphate 0.05 *M*. This procedure is very similar to that of McComb, *et al.*[28]

Ion-exchange Chromatography.—The following procedures were routinely used in connection with isotope exchange experiments.

a. **Separation of Glucose-6-Phosphate from C^{14}-Glucose.**—Reactions were terminated by adding 2.0 ml. of 0.5% perfluoroöctanoic acid directly to the incubation mixture and adjusting the *p*H to 3.0–3.5 with 0.5 *N* HCl.[29] The precipitate was removed by centrifugation. The supernatant fluid was adjusted to *p*H 7.0 with *N* KOH and quantitatively transferred onto a Dowex-1 × 2(200 – 400 mesh) column (1 × 16 cm.) in the chloride form with the aid of approximately one column volume of water. Water was passed through the column until no radioactivity, due to non-phosphorylated C^{14}-hexose, could be detected in the effluent. Glucose-6-phosphate was eluted with 0.03 *N* HCl using a flow rate of 0.7 ml./min./cm.^{2} of column cross section. Approximately 3.0-ml. fractions were collected and analyzed for radioactivity and reducing sugar.

b. **Separation of Glucose-6-Phosphate and P^{32}-Orthophosphate.**—Reactions were terminated with perfluoroöctanoic acid in the manner described above. After centrifugation, the deproteinized supernatant fluid was adjusted to *p*H 8.0 with *N* KOH and one ml. of 10% barium acetate was added to precipitate most of the orthophosphate (approximately 90%); glucose-6-phosphate remained in solution. The precipitate was removed by centrifugation and the supernatant fluid washed onto a Dowex-1 × 2 (200 – 400 mesh) column (1 × 20 cm.) in the chloride form. The column was then washed with three column volumes of water. Separation of glucose-6-phosphate from labeled orthophosphate was accomplished with a gradient elution system[30] which gave a linear increase in HCl concentration between 0.01 and 0.04 *N*. The flow rate through the column was 0.63 ml./min/cm.^{2} of column cross section. Approximately 3.0-ml. fractions were collected and analyzed for radioactivity and reducing sugar.

Results

It was observed that glucose-6-phosphatase catalyzes the incorporation of glucose-C^{14} into glucose-6-phosphate (G-6-P). Table I describes the incorporation of glucose-C^{14} and, for comparison, the hydrolytic activity in the presence and absence of glucose. It should be noted that per unit of time, under these conditions, the number of micromoles of orthophosphate whose formation is prevented by glucose is approximately equal to the number of micromoles of glucose-C^{14} incorporated into G-6-P.

TABLE I

GLUCOSE INHIBITION AND EXCHANGE[a]

Hydrolytic acitivity[b]				Exchange	
Uninhibited	Inhibited	Inhibition[c]	Exchange[d] activity	Inhibition	Microsomal protein, mg./g. liver
Normal rat					
5.15	3.48	1.67	1.38	0.83	27.1
7.17	5.05	2.12	2.12	1.00	33.0
Diabetic rat					
7.40	5.25	2.15	1.95	0.91	26.4
11.40	8.03	3.37	2.48	0.74	33.7
11.60	8.55	3.05	2.25	0.74	30.4

[a] Incubations were conducted at 37° and *p*H 6.0. Mixtures contained 20 μmoles of G-6-P, 1.0 ml. of buffer, and 0.2 ml. of microsomal preparation in a final volume of 2.5 ml. [b] Hydrolytic activity is in μmoles of orthophosphate liberated per g. of liver per min. Phosphate was determined by the method described under Enzyme assays. The uninhibited reaction mixture contained no glucose and the inhibited mixture contained 180 μmoles of glucose. [c] Difference between the inhibited and uninhibited activity. [d] Exchange activity is in μmoles of C^{14}-glucose incorporated into G-6-P per g. of liver per min. The exchange reaction mixture contained 180 μmoles of C^{14}-glucose (sp. act. 4700 c.p.m./μ mole). G-6-P was isolated from the incubation mixture by method (a) described under Ion exchange chromatography. The no. of μmoles of glucose incorporated into G-6-P was calculated by dividing the total counts in the isolated G-6-P by the sp. act. of glucose.

Incorporation of glucose-C^{14} into G-6-P suggested reversal of the hydrolytic reaction 1 or an exchange reaction 2.

$$\text{G-6-P} + H_2O \rightleftharpoons \text{Glucose} + \text{Orthophosphate} \quad (1)$$

$$\text{G-6-P} + \text{Glucose} - C^{14} \rightleftharpoons \text{G-6-P-}C^{14} + \text{Glucose} \quad (2)$$

If the first mechanism is valid, then equimolar amounts of glucose and orthophosphate should be incorporated into G-6-P. An exchange experiment was carried out in the presence of orthophosphate, see column No. 2 in Fig. 1, and glucose-C^{14} was incorporated into G-6-P. However, if

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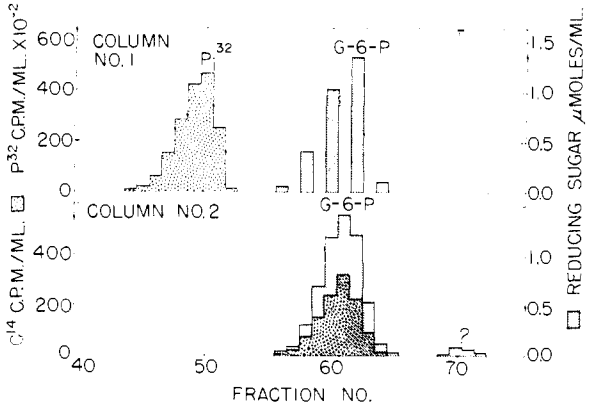

Fig. 1.—The exchange activity of glucose-6-phosphatase: incubations were conducted at 37° and *p*H 6.0. All mixtures contained 20 μmoles of G-6-P, 1.0 ml. of buffer and 0.2 ml. of microsomal preparation in a final volume of 2.5 ml. Mixtures added to column 1 contained 180 μmoles of unlabeled glucose and 180 μmoles of P^{32}-orthophosphate (24,000 c.p.m./μmole). Mixtures added to column 2 contained 180 μmoles of glucose-u-C^{14} (5,700 c.p.m./μmole) and 180 μmoles of unlabeled orthophosphate. Separation of orthophosphate from G-6-P was accomplished by method b described under Ion-exchange chromatography.

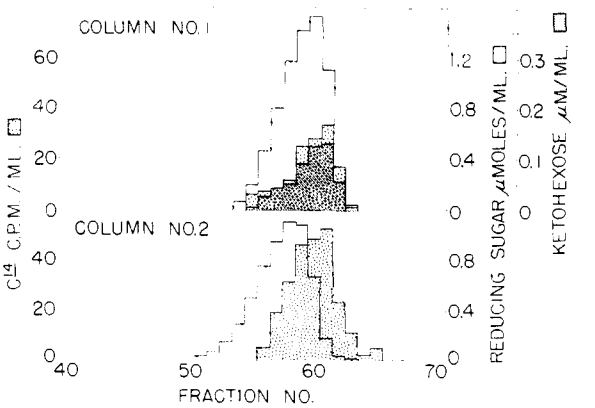

Fig. 2.—The incorporation of isotopically labeled fructose into F-6-P by glucose-6-phosphatase. Incubation conditions were the same as those described in Fig. 1. Mixtures contained 180 μmoles of fructose-u-C^{14} (21,00 c.p.m./μmole). Columns 1 and 2 represent the results obtained from 10 and 20 min. incubations, respectively. Method b, described under Ion exchange chromatography, was used.

P^{32} labeled orthophosphate was used instead of glucose-C^{14}, see column No. 1 in Fig. 1, there was no detectable incorporation of radioactivity into G-6-P. It can be concluded that under the conditions used there is no detectable contribution by reversal of the hydrolytic reaction 1 and that glucose-C^{14} is incorporated into G-6-P by an exchange reaction 2 where the phosphoryl group is transferred from glucose to glucose-C^{14}. In parallel experiments it was observed that there was no detectable incorporation of glucose-C^{14} into G-6-P if the enzyme or G-6-P was omitted.

With phosphoserine phosphatase[6] there is a close agreement between the structural and configurational requirements for substrates, inhibitors, and acceptors. Since fructose 6-phosphate (F-6-P) is a substrate for glucose-6-phosphatase,[31] it seemed likely that F-6-P would act as a donor or fructose as an acceptor in the exchange reaction. After ion-exchange column chromatography of incubation mixtures containing fructose-C^{14}, G-6-P and microsomes, a radioactive component was observed which appeared to be F-6-P. The peak concentration of radioactivity coincided with the concentration of ketohexose, see column No. 1 in Fig. 2, and the peak concentration of radioactivity was different from that of the peak concentration of reducing sugar which represents G-6-P. The relative position in the elution pattern of G-6-P and the radioactive ketohexose are those predicted for G-6-P and F-6-P. It can be tentatively concluded that fructose acts as an acceptor to form F-6-P, and the rate of formation of F-6-P is proportional to the time of incubation as can be seen from Fig. 2 where columns No. 1 and 2 represent 10 and 20 min. incubations, respectively. The rate of formation of F-6-P is approximately 1/20 of that observed for the exchange reaction with glucose-C^{14}. It should be pointed out that the greater portion of F-6-P formed during these experiments resulted from the conversion of G-6-P to F-6-P by phosphohexose isomerase. The F-6-P formed by isomerase activity, therefore, served as a carrier for the F-6-P resulting from transferase activity. Analogous experiments to show that F-6-P is a donor have not been carried out since the experiment would be complicated by the presence of even small quantities of phosphohexose isomerase.

Glucose-6-phosphatase activity in the presence and absence of glucose was examined for two purposes: (1) to establish corroborating evidence for an enzymatic mechanism and (2) to determine and compare kinetic parameters of the enzyme prepared from normal and diabetic animals. The inhibitory effect of different glucose levels on the initial hydrolysis rates of G-6-P at various concentrations was determined and the experimental data incorporated into the reciprocal plots[32] illustrated in Fig. 3. Inspection of Fig 3 reveals that for each increase in glucose concentration the corresponding plot manifests an increase in ordinate intercept and a slight increase in slope. The type of inhibition[33] is obviously neither competitive nor uncompetitive, and since extensions of the plots do not result in convergence at a common point on the negative abcissa, non-competitive inhibition is also eliminated. A consideration of appropriate nomenclature and certain alternate mechanisms will be presented in the Discussion. However, if further consideration is to be given to the data in Fig. 3, presentation of the proposed mechanism for glucose-6-phosphatase is required. This mechanism is

$$\text{Enz} + \text{G-6-P} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E(G-6-P)} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E-P} + \text{Glucose} \quad (3)$$

$$\text{H}_2\text{O} \downarrow k_5$$

$$\text{Enz} + \text{Orthophosphate}$$

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(33) P. W. Wilson, "Respiratory Enzymes," ed. by H. A. Lardy, Burgess Publ. Co., Minneapolis, Minn., 1949, pp. 16-56.

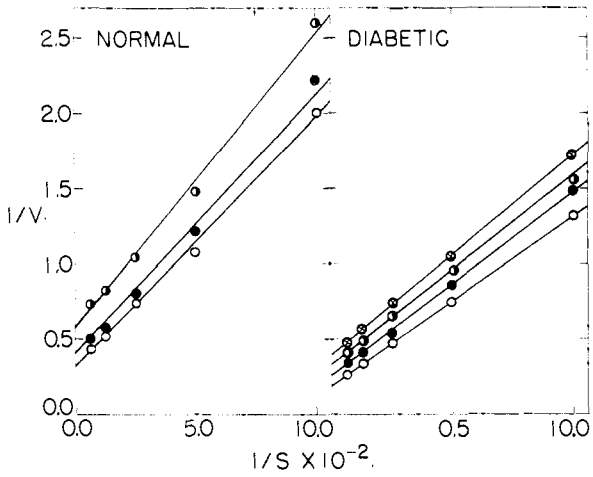

Fig. 3.—The influence of glucose on the reciprocal initial reaction velocity (reciprocal μmoles of orthophosphate released/incubation mixture/20 min.) of glucose-6-phosphatase at 37° and various reciprocal substrate concentrations (reciprocal moles of G-6-P/liter. O represents data obtained in the absence of glucose: ●, ◑ and ⊗ represent data obtained in the presence of 3.2×10^{-2} M, 7.2×10^{-2} M and 11.2×10^{-2} M glucose, respectively.

E(G-6-P) represents the Michaelis complex. and E-P represents the phosphoryl enzyme. The above mechanism is consistent with all of the experimental observations. It takes into account the irreversibility of the over all reaction by proposing a unidirectional step between the phosphoryl enzyme and the formation of free enzyme. It also takes into account glucose inhibition and exchange by proposing a reversible step involving the phosphoryl enzyme intermediate.

Steady-state treatment of the proposed mechanism leads to the following reciprocal velocity expression

$$\frac{1}{v} = \left[1 + \frac{I}{K'_I}\right]\frac{1}{V_m} + \left[\frac{k_2}{k_1} \times \frac{I}{K'_I} \times \frac{1}{V_m} + \frac{K_m}{V_m}\right]\left(\frac{1}{S}\right) \quad (4)$$

where

$$K_m = \frac{k_3(k_2 + k_3)}{k_1(k_3 + k_3)},\ K'_I = \frac{k_3 + k_3}{k_4},\ \text{and}\ V_m = \frac{k_3 k_3 e}{k_3 + k_3}$$

I = inhibitor concn. (glucose and structurally related inhibitors)
v = velocity at any substrate concn. (S). At (S) = ∞ and $I = 0$, $v = V_m$
e = total concn. of active enzymic centers
K_m = substrate concn. giving 50% of V_m
K'_I = inhibitor concn. giving 50% of V_m

In the absence of inhibitor (I), equation 4 reduces to the reciprocal form of the Michaelis-Menten expression[34]

$$\frac{1}{v} = \frac{1}{V_m} + \frac{Km}{V_m}\left(\frac{1}{S}\right) \quad (5)$$

In equation 5 when $1/v$ is plotted *vs.* $1/S$, the ordinate intercept is equal to the reciprocal of V_m, and K_m is equal to the slope divided by the intercept. Using equation 4, K'_I was determined by dividing the ordinate intercept of the reciprocal plot in the presence of inhibitor (Intercept-I) by the ordinate intercept of the reciprocal plot in the absence of inhibitor (Intercept-O).

$$\frac{\text{Intercept-I}}{\text{Intercept-O}} = \frac{(1 + I/K'_I)1/V_m}{1/V_m} = 1 + \frac{I}{K'_I}$$

therefore

$$K'_I = \frac{I}{\dfrac{\text{Intercept-I}}{\text{Intercept-O}} - 1} \quad (6)$$

Calculation of k_2/k_1, the dissociation constant of the enzyme-substrate complex (K_s), may be carried out by substituting values for K_m, K'_I, and V_m into the slope term of equation 4.

Inspection of equation 4 illustrates that as I is increased both the ordinate intercept and the slope increase by the disproportionate terms $(1 + I/K'_I) \times 1/V_m$ and $(k_2/k_1 \times I/K'_I + K_m)$ $1/V_m$, respectively. Furthermore, if the data obtained by using a single level of inhibitor are substituted into equation 4 in order to predict the slope and intercept for other levels of inhibitor, the predicted and observed values are in excellent agreement, see Table II.

Average values for K_m, K_S and K'_I, determined from Fig. 4 using equations 4, 5 and 6 are listed in Table III. There is no significant difference between the results obtained with normal and diabetic

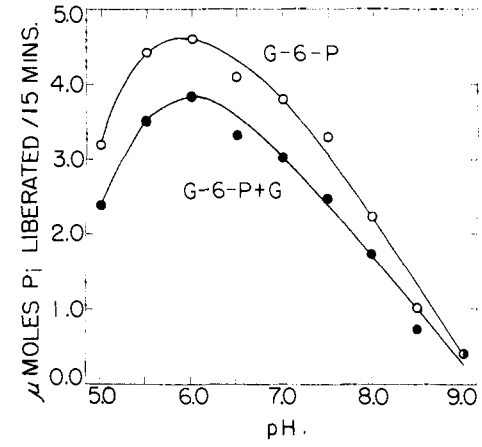

Fig. 4.—The influence of pH on the inhibition of glucose-6-phosphatase activity by glucose. Incubations were conducted at 37°. Mixtures contained 20 μmoles of G-6-P, 90 μmoles of glucose, 1.0 of buffer and 0.4 ml. of microsomal preparation in a final volume of 2.5 ml. Incubation mixtures contained 0.2 ml. of microsomal preparation and 1.0 ml. of buffer (pH 6.0) in a final volume of 2.5 ml. There was no significant alteration in G-6-P concentration due to phosphohexose isomerase activity.

TABLE II

CALCULATED AND OBSERVED INTERCEPTS AND SLOPES[a]

$I \times 10^2$ (M)	Intercept (normal) Calcd.	Obsd.	$I \times 10^2$ (M)	Intercept (diabetic) Calcd.	Obsd.
7.2	0.56	0.55	7.2	0.34	0.32
			11.2	0.43	0.39

$I \times 10^2$ (M)	Slope (normal) Calcd.	Obsd.	$I \times 10^2$ (M)	Slope (diabetic) Calcd.	Obsd.
7.2	1.84×10^{-3}	1.97×10^{-3}	7.2	1.29×10^{-3}	1.26×10^{-3}
			11.2	1.38×10^{-3}	1.34×10^{-3}

[a] The calculated slopes and intercepts are based on the data in Fig. 4. The value of k_2/k_1 was calculated, for the normal and the diabetic, from the data for 3.2×10^{-2} M glucose. The observed slopes and intercepts are also from Fig. 4.

(34) J. B. S. Haldane, "Enzymes," Longman Green and Co., New York, N. Y., 1930, p. 39.

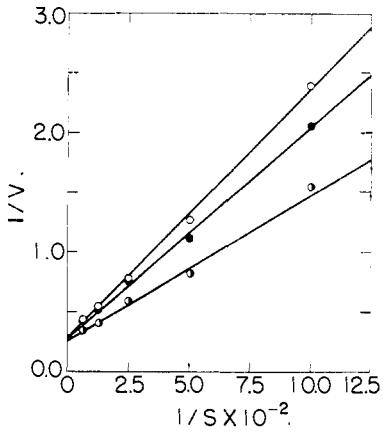

Fig. 5.—The influence of orthophosphate on the reciprocal initial reaction velocity (reciprocal μmoles of glucose released/incubation mixture/20 min.) of glucose-6-phosphatase at 37° and various reciprocal substrate concentrations (reciprocal moles of G-6-P/liter). ◑ represents data obtained in the absence of orthophosphate. ● and ○ represent data obtained in the presence of 0.8×10^{-2} *M* and 1.6×10^{-2} *M* orthophosphate, respectively. Incubation mixtures were similar to those described in Fig. 4.

animals. Segal, *et al.*, have reported[16] a careful study of apparent Michaelis constants using homogenates prepared from the livers of normal and diabetic rats. They reported that the average apparent Michaelis constant for glucose-6-phosphatase from diabetic animals was approximately twice the normal value. They later confirmed this difference with washed microsome preparations, but in solubilized preparations this difference disappeared.[35] In contrast to Segal, *et al.*, the kinetic constants in Table III are identical for the normal and diabetic. The conflicting results are perhaps explained by differences in experimental conditions.

TABLE III

THE KINETIC PARAMETERS OF MICROSOMAL GLUCOSE-6- PHOSPHATASE

	$K_m \times 10^3$ *M*	$K_S \times 10^4$ *M*	K'_I for glucose $\times 10^2$ *M*
Normal	5.9	7.8	8.6
Diabetic	6.3	8.7	9.0

A detailed study of the effect of *p*H on the kinetic constants was not carried out, but Fig. 4 shows that the per cent. inhibition (21%) by 0.036 *M* glucose is essentially constant over a *p*H range from 5.0 to 8.0.

Inhibition studies with various sugars and sugar derivatives were performed to demonstrate that glucose-6-phosphatase exhibits inhibitor specificity. The data in Table IV lists only the per cent. inhibition for a single level of inhibitor, but it is quite clear that there is inhibitor specificity. The inhibitor specificity may be compared with the substrate specificity data of Crane[36] and Maley, *et al.*,[37] for the following compounds: D-glucose, 2-deoxy-D-glucose, D-mannose, D-galactose, D-glucosamine, D-fructose, D-ribose and N-acetyl-D-glucosamine. The data available do not justify a precise comparison of inhibitor and substrate specificity, but the phosphorylated form of the best inhibitors appear to be the best substrates, and there is no obvious exception to this correlation of inhibitor and substrate specificity. This correlation suggests that the substrate and inhibitor use the same site or that there is an inhibitor site which is similar to the substrate site.

(35) H. L. Segal and M. E. Washko, *Federation Proc.*, **18**, 321 (1959).

(36) R. K. Crane, *Biochim et Biophys Acta*, **17**, 443 (1955).

(37) F. Maley and H. A. Lardy, THIS JOURNAL, **78**, 1393 (1956).

TABLE IV

THE INHIBITION OF GLUCOSE-6-PHOSPHATASE BY VARIOUS SUGARS AND SUGAR ANALOGS

Incubations were conducted at 37° and *p*H 6.0. Mixtures contained 40 μmoles of G-6-P, 280 μmoles of inhibitor, 1.0 ml. of buffer and 0.2 ml. of microsomal preparation in a final volume of 2.5 ml. Orthophospliate liberation in 20 min. was determined by the method described under Enzyme assays.

Inhibitor	Inhibition, %	Inhibitor	Inhibition, %
D-Glucose	43	D-Glucuronate	0
2-Deoxy-D-glucose	30	N-Acetyl-D-glucosamine	0
D-Mannose	26	D-Ribose	7
D-Galactose	20	D-Arabinose	2
D-Glucosamine	16	D-Xylose	0
D-Fructose	14	D-Lyxose	0

Beaufay, *et al.*,[38] demonstrated that, at a given concentration, orthophosphate was a more potent inhibitor of glucose-6-phosphatase than glucose. Since it was shown that P^{32}-labeled orthophosphate did not incorporate into G-6-P, it was decided to examine the mechanism of phosphate inhibition. A reciprocal plot[32] of G-6-P hydrolysis in the presence of different levels of orthophosphate illustrates that the latter compound, unlike glucose, inhibits in a competitive manner (Fig. 5). Thus, orthophosphate competes with G-6-P for the active site of the enzyme and in doing so forms an enzyme–phosphate complex. This complex should not be confused with the phosphoryl enzyme intermediate proposed in equation 3. The application of steady-state kinetics to the mechanism of orthophosphate inhibition leads to the velocity expression given by Alberty[39] for classical competitive inhibition. In employing the reciprocal form of this velocity expression for the determination of K_I for orthophosphate (K_I equals the enzyme inhibitor dissociation constant), an average value of 1.96×10^{-2} *M* was obtained.

Discussion

Equation 3 is the proposed mechanism for glucose-6-phosphatase. An adequate mechanism must account for inhibition by glucose and structurally related compounds, it must account for the transfer of a phosphoryl group from a donor to an acceptor (exchange or transfer activity), and the type of inhibition predicted from the mechanism must agree with the type which has been observed, see Fig. 3.

The following scheme, which is a single displacement reaction, accounts for glucose inhibition, and it accounts for the transfer activity.

(38) H. Beaufay, H. G. Hers, J. Berthet and C. de Duve, *Bull. soc. chim. biol.*, **36**, 1539 (1954).

(39) R. A. Alberty, *Adv. Enz.*, **17**, 1 (1956).

$$\text{Enz} + \text{G-6-P} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E(G-6-P)} \xrightarrow[H_2O]{k_3} \text{Enz} + \text{Glucose} + \text{Orthophosphate} \quad (7)$$

$$\text{E(G-6-P)} + \text{Glucose} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \left\{ \begin{matrix} \text{E(G-6-P)G*} \\ \uparrow\downarrow \\ \text{E(G*-6-P)G} \end{matrix} \right\} \text{inhibited complex}$$

The steady-state reciprocal velocity expression for the above scheme is

$$1/v = (1 + I/K_1)\, 1/V_m + K_m/V_m(1/S) \quad (8)$$

where

$$K_m = \frac{k_2 + k_3}{k_1},\ K_1 = \frac{k_5}{k_4},\ \text{and}\ V_m = k_3 e$$

This reciprocal velocity equation predicts uncompetitive inhibition,[40] and, therefore, a single displacement mechanism is not compatible with the experimental observations.

A double displacement mechanism, quite analogous to the scheme proposed for glucose-6-phosphatase, but not involving the formation of a binary complex is

$$\text{Enz} + \text{G-6-P} \rightleftharpoons \text{E-P} + \text{Glucose}$$
$$\downarrow H_2O \quad (9)$$
$$\text{Enz} + \text{Orthophosphate}$$

This mechanism accounts for glucose inhibition and exchange, but an examination of a steady-state treatment of this mechanism[6] indicates that it would predict data which would be classified as competitive inhibition. Previously, Slater had pointed out to Zatman, *et al.*,[41] that a mechanism which does not include a Michaelis complex fails to account for the noncompetitive nature of the nicotinamide inhibition of spleen DPNase.

Of the mechanisms considered, the only feasible one is represented by equation 3. The same mechanism has been proposed for phosphoserine phosphatase,[6] but the final choice of the double displacement mechanism in equation 3 over the single displacement mechanism in equation 7 has thus far only been possible with G-6-Pase where the type of inhibition clearly eliminates the latter mechanism. A double displacement mechanism which is identical to equation 3 has been proposed for non-specific phosphatases,[42] but the evidence for a double *vs.* a single displacement mechanism is only suggestive.[43,44] Many of the kinetic parameters which have been used to justify the mechanism for phosphoserine phosphatase[6] and glucose-6-phosphatase have been sought with non-specific phosphatases,[3] but the acceptors and inhibitors which have been studied, apparently introduce multiple interactions.

The mechanism of action and inhibition of glucose-6-phosphatase may be visualized as follows: hydrolysis of G-6-P is initiated through the formation of a binary complex between enzyme and substrate. *In situ*, a group at the active enzyme site (possibly an undetected divalent cation) enhances polarization of the phosphoryl group, thereby rendering it susceptible to nucleophilic attack by a second reactive enzymic group. If enzymic attack is followed by a concomitant depolarization of the phosphoryl group and cleavage of the P–O ester bond in G-6-P, a primary bond between enzyme and phosphate is established. A second polarization of the phosphoryl group renders it susceptible to nucleophilic attack by either water or glucose. Attack by water results in rupture of the phosphoryl enzyme and completion of the hydrolytic reaction. Attack by glucose re-establishes the G-6-P molecule which may be released from the enzyme surface. The latter action, therefore, provides an explanation for glucose inhibition and exchange. The fact that glucose inhibits at relatively low concentrations must mean that the molecule is preferentially attracted to the enzyme site, possibly through hydrogen bonding. The orientation and significance of the hydrogen bonding is shown by the specificity requirements of the substrates[36,37] and the specificity requirements of the inhibitors.

The assignment of a name to designate the type of inhibition has been avoided since the inhibition does not represent one of the three "classical" types: competitive, non-competitive or uncompetitive. In the proposed mechanism 3 the inhibitor combines preferentially with the phosphoryl-enzyme intermediate, and inspection of the slope term in the reciprocal rate equation 4 shows that the observed slope is a function of $k_2/k_1 \times I/K'_I$. If k_2/k_1 is small, relative to I/K'_I, the slope change with different concentrations of I will be small or perhaps undetectable, and the inhibition would be classified as uncompetitive.[40] If k_2/k_1 equals K_m, then the slope term becomes $(I/K'_I + 1)\, K_m/V_m$, and the inhibition would be classified as non-competitive.[32] A variety of names have been used to describe inhibition data similar to that observed for the inhibition of glucose-6-phosphatase by glucose.[45,46] Coupling inhibition[45] has been commonly used, but this erroneously implies that there is evidence for a changed affinity of the enzyme for its substrate. As far as we are aware, nomenclature which is adequate to describe the inhibition of glucose-6-phosphatase by glucose is not available, and a precise description is conveyed only by the proposed mechanism 3 and its rate equation 4.

Segal, *et al.*,[16] have reported that the inhibition of glucose-6-phosphatase by glucose is non-competitive, and their data were presented as a plot of v *vs.* v/s. When this plot is used, the slope, according to Eadie,[47] equals $-K_m$ if the inhibitor is non-competitive. This method of plotting makes it difficult to observe disagreement with non-competitive kinetics, and if our data are plotted

(40) E. R. Ebersole, C. Guttentag and P. W. Wilson, *Arch. Biochem. and Biophys.*, **3**, 399 (1943).

(41) L. J. Zatman, N. O. Kaplan, S. P. Colowick and M. M. Ciotti, *J. Biol. Chem.*, **209**, 467 (1954).

(42) R. K. Morton, *Disc. Faraday Soc.*, **20**, 154 (1955).

(43) D. E. Koshland, Jr., *ibid.*, **20**, 142 (1955).

(44) D. E. Koshland, Jr., *ibid.*, **20**, 271 (1955).

(45) J. S. Friedenwald and G. D. Maengwyn-Davies, "The Mechanism of Enzyme Action," ed. by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954, pp. 154–179.

(46) H. L. Segal, "The Enzymes," Vol. 1, ed. by P. D. Boyer, H. Lardy, K. Myrbäck, Academic Press, Inc., New York, N. Y., 1959, pp. 1–48.

(47) G. S. Eadie, *Science*, **116**, 688 (1952).

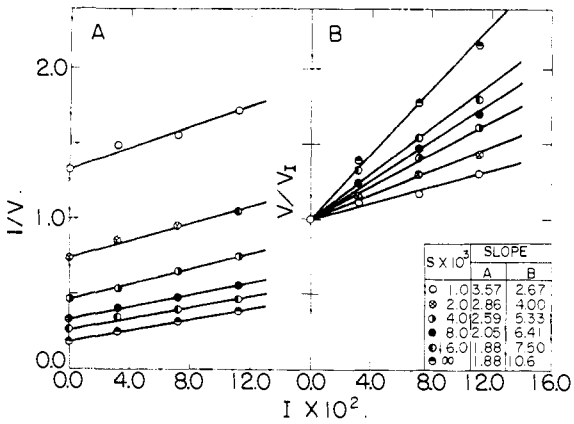

Fig. 6.—The influence of substrate concentration on Dixon (A) and Wilson (B) plots. The graphs represent different plots of the data appearing in Fig. 4 (diabetic). The values at infinite substrate concentration (S) were obtained from the ordinate intercept of Fig. 4. Similar plots may be obtained with microsomes from normal animals.

using v *vs.* v/s, they might be considered non-competitive. However, the plot of $1/v$ *vs.* $1/s$ according to Lineweaver and Burk[32] in Fig. 3, a plot of $1/v$ *vs.* I according to Dixon[48] in Fig. 6A and a plot of v/v_I *vs.* I according to Wilson[33] in Fig. 6B clearly show that our data cannot be classified as non-competitive inhibition. Equation 4 can be rearranged for the Dixon plot, equation 10, and the Wilson plot, equation 11

$$\frac{1}{v} = \left(1 + \frac{K_m}{s}\right)\frac{1}{V_m} + \left(1 + \frac{k_2}{k_1 S}\right)\frac{I}{K'_I V_m} \quad (10)$$

$$\frac{v}{v_1} = 1 + \frac{\left(1 + \frac{k_2}{k_1 S}\right)\frac{I}{K_I}}{1 + K_m/S} \quad (11)$$

As was previously pointed out, if k_2/k_1 equals K_m, equation 4 reduces to the equation for non-competitive inhibition, and of course this is equally true for alternative forms of equation 4 such as (10) or (11). However, k_2/k_1 equals approximately 8.3×10^{-4} M and K_m equals 6.1×10^{-3} M. This difference between k_2/k_1 and K_m is consistent with the disagreement between the plots in Fig. 6 and the plots expected for non-competitive inhibition. The magnitude of the disagreement is best seen in Fig. 6B where the substrate concentration should have no effect on the slope if the inhibition is of the non-competitive type.

K'_I for non-competitive inhibitors can be readily calculated from the Wilson plot since the slope equals $1/K'_I$, but the use of this method to determine K'_I for the inhibition of glucose-6-phosphatase by glucose would be in error except at high substrate concentrations where $k_2/k_1 \times 1/S \rightarrow 0$. Equation 11 and the Wilson plot (Fig. 6B) could be used for finite substrate concentrations if values for k_2/k_1 and K_m were included. The K'_I for glucose, reported in this paper, was calculated from the ordinate intercept in the Lineweaver–Burke plot, see Fig. 3, which, according to equation 4, requires only a knowledge of V_m and I to calculate K'_I.

The relatively specific inhibition of glucose-6-phosphatase by glucose could be of physiological importance. However, the K_I' for glucose is 0.088 M (0.088 M equals 1600 mg. %), and the normal blood and liver glucose concentration in the rat is *ca.* 80 mg. %.[49] The exchange or transfer activity has no known physiological function, but it should be pointed out that *in vivo* or in a complex *in vitro* system, the exchange activity would complicate the interpretation of experiments using isotopically labeled compounds. For example, Cahill, *et al.*,[50] have reported a series of studies where it was assumed that the conversion of glucose to G-6-P was carried out by an enzymatic phosphorylation using ATP, The application, insofar as possible, of the properties of microsomal glucose-6-phosphatase to hepatic carbohydrate metabolism and to the liver slice experiments of Cahill, *et al.*,[50] will be the subject of a future communication.

Acknowledgments.—We wish to thank Professor G. W. Schwert and Dr. Francis C. Neuhaus for their helpful advice.

(48) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

(49) K. F. Gey, *ibid.*, **64**, 145 (1956).

(50) G. F. Cahill, Jr., J. Ashmore, A. E. Renold and A. B. Hastings, *Am. J Med.*, **26**, 264 (1959).

DURHAM, NORTH CAROLINA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, WASHINGTON UNIVERSITY]

The Condensation of D-Galactose with Nitromethane

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The condensation of D-galactose with nitromethane in alkaline methanol provides the corresponding, epimeric deoxynitroheptitols in 50% combined yield. The configurations of the nitroalcohols were established by converting them to the corresponding aldoheptoses (D-*glycero*-L-*manno*-heptose and D-*glycero*-L-*gluco*-heptose).

The nitromethane synthesis[1] has been applied with varying degrees of success to certain aldose sugars of the tetrose, pentose, hexose, heptose and octose[2] series. In the aldohexose series, D-mannose combines with nitromethane in alkaline methanol to yield 55% of the corresponding deoxynitroheptitols,[3] whereas D-glucose, under similar reaction conditions, combines to only a very limited extent.[4]

(1) J. C. Sowden, *Adv. in Carbohydrate Chem.*, **6**, 291 (1951).

(2) The condensation of D-*erythro*-L-*manno*-octose with nitromethane will be described in a forthcoming communication.

(3) J. C. Sowden and R. Schaffer, THIS JOURNAL, **73**, 4662 (1951).

(4) J. C. Sowden and H. O. L. Fischer, *ibid.*, **69**, 1048 (1947).