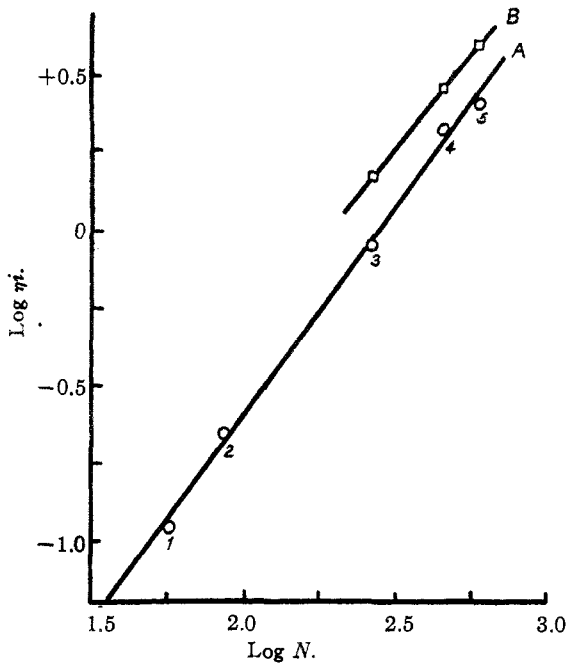

Fig. 3.—Relation between intrinsic viscosity and degree of polymerization for amyloses in ethylenediamine (A) and amylose acetates in chloroform (B): 1, amylodextrin 4; 2, synthetic starch; 3, corn; 4, tapioca and 5, potato amylose.

that most of these materials are prepared from natural sources with little if any degradation it does not seem unlikely that their homogeneity approaches that of materials prepared by fractional precipitation of originally highly polydisperse materials such as synthetic polymers (or even such natural polymers as cellulose where considerable degradation is involved in the preparation). Also the fact that these different materials can be titrated quantitatively with iodine in mixtures[5,21] would seem to indicate that the molecular weight distributions are fairly small relative to the magnitude of the differences between them. For the present, however, it is perhaps best to consider the value 1.5 as an upper limit for α in this series. There would seem to be little doubt that the value is greater than unity confirming the expectations from the rigidity of Fischer–Hirschfelder models of amylose.

The fact that α for the acetates in chloroform is the same, within the experimental error, as for the amyloses in ethylenediamine (as shown by the similar slopes in Fig. 3) is somewhat surprising. This would seem to indicate the molecules to be equally rigid in the two cases.

Summary

1. Osmotic pressure values for corn and tapioca amylose are given and molecular weights of other members of this series estimated from other considerations.

2. A dependence of intrinsic viscosity on a power of molecular weight greater than unity is indicated, confirming expectations from the comparative rigidity of Fischer–Hirschfelder models of amylose.

3. The rigidity of acetylated amylose molecules in chloroform appears to be the same as that of amylose in ethylenediamine.

(21) The implications of this observation are being further investigated. It does not seem unlikely that the iodine titration method may provide a means for the direct determination of molecular weight distribution in amylose preparations.

Ames, Iowa Received August 20, 1943

[Contribution from the Division of Plant Nutrition, College of Agriculture, University of California]

The Preparation and Purification of Glucose 1-Phosphate by the Aid of Ion Exchange Adsorbents

By R. M. McCready[1] and W. Z. Hassid

The establishment of glucose-1-phosphate (Cori ester) as an important intermediary product in the synthesis and breakdown of glycogen and starch[2,3] contributed much toward the stimulation of further study of the mechanism of carbohydrate formation in general. Since studies along these lines require an available supply of glucose-1-phosphate, it is important to have a satisfactory method whereby this ester can readily be prepared. Although Hanes' method[3] of preparing the ester from starch in fairly large quantities is satisfactory, the product is usually contaminated with small amounts of dextrins, which are very difficult to remove, and which are objectionable in certain types of experiments. Green and Stumpf[4] for example, in studying the influence of different carbohydrates on the synthesis of starch from glucose-1-phosphate *in vitro*, were obliged to use synthetic glucose-1-phosphate, which is laborious to prepare. Hanes' procedure can be very much shortened and improved by using ion exchange adsorbents,[5,6] whereby the following steps are eliminated: concentration of large volumes of

(1) Present address, Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, Calif.

(2) C. F. Cori, *Endocrinology*, **26**, 285 (1940).

(3) C. S. Hanes, *Proc. Roy. Soc.* (London), **B129**, 174 (1940).

(4) D. E. Green and P. K. Stumpf, *J. Biol. Chem.*, **142**, 355 (1942).

(5) The synthetic resins IR-100 and IR-4 were obtained from the Resinous Products and Chemical Company, Inc., Philadelphia, Penna.

(6) For a review on the use of ion exchange adsorbents see: R. J. Myers, *Ind. Eng. Chem.*, **35**, 858 (1943).

solution, precipitation of the ester with considerable quantities of alcohol, addition of trichloroacetic acid to precipitate the proteins, and the enzymatic hydrolysis of dextrin impurities.

In the present procedure the phosphorolysis of starch in the presence of phosphate buffer and the precipitation of the inorganic phosphate were carried out according to a modified procedure of Hanes, but the purification of the ester was accomplished by ion exchange adsorbents. The reaction mixture containing the ester was first passed through a column of cation exchange adsorbent, which removed the cations from solution, exchanging them for hydrogen ions. The effluent was then run through an acid adsorbing column. In this operation the Cori ester, being a strong acid with a high exchange capacity, was adsorbed, while the soluble impurities, including dextrins, proteins and weak organic acids, passed through unchanged. The glucose-1-phosphate was then eluted from the adsorbent with dilute alkali and isolated as the crystalline dipotassium salt.

It was found that the same general method could be applied to the purification of other hexosephosphates, such as fructose-1,6-diphosphate (Harden–Young ester), fructose-6-monophosphate (Neuberg ester), and glycerophosphate which are difficult to purify by the usual methods,[7,8,9] It is probable that this method may be advantageously used in isolating and purifying other phosphorylated products, such as dihydroxyacetone phosphate, glyceric acid phosphates, and also organic acids.

Experimental

Preparation of the Ion Exchange Columns.—The resin exchange materials as obtained from the manufacturer are in moist and swollen condition and little swelling or disintegration occurs on immersion in water. The columns containing the resins are constructed as shown in Fig. 1. The larger cylinder A consists of a 100 × 1000 mm. tube containing a 1200 ml. Amberlite IR-100 bed and some Pyrex glass wool at the bottom. Tubes cc_1 are attached to distilled water; aa_1 are outflow tubes for backwashing; bb_1 are effluent outflow tubes. Cylinder B is a 35 × 1000 mm. tube containing a 200 ml. bed of Amberlite IR-4 and a glass wool plug at the bottom. C is a 1-liter flask arranged to give a constant level of influent. After filling the columns the exchange material should be "classified" to size by washing before use in a particular process. The classification is accomplished by backwashing with water at such a rate as to produce a 50% bed volume expansion.[10] Flow resistance is thus reduced by the elimination of fines and the classification to a desirable particle size. The bed should be "broken in" by three or four exchange cycles followed by regeneration and washing to eliminate any color discharge and fines.

In the present work 4% hydrochloric acid was used to regenerate a 1200-ml. bed volume of Amberlite IR-100.

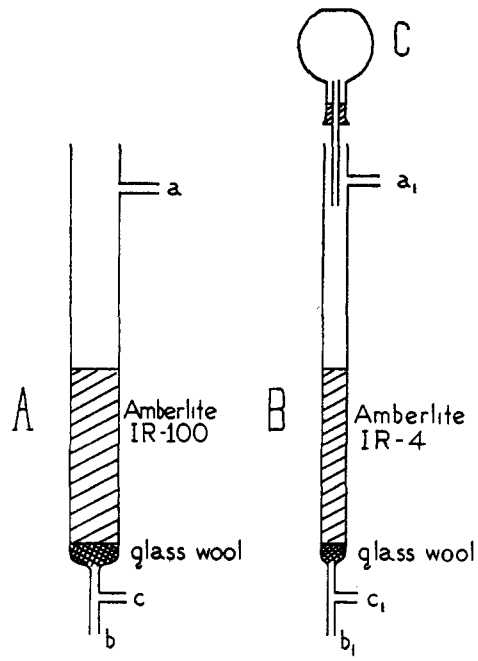

Fig. 1.

The acid was passed through the resin at the rate of seven bed volumes per hour, followed by thorough washing until the titratable acidity was negligible. Under these conditions the wash water effluent did not rise above *p*H 3. The resin in the form of the hydrogen derivative was treated with 4% sodium chloride solution to exchange the hydrogen of the resin for the cation of the sodium chloride solution. After 3000 ml. of the sodium chloride solution was allowed to pass through the resin at the rate of ten bed volumes per hour, the resin was completely saturated with sodium ions. The resin was then backwashed with distilled water and again regenerated with 3000 ml. of 4% hydrochloric acid solution by allowing it to pass through the column at the rate of 10 bed volumes per hour. The titratable acidity of the effluent then became the same as that of the influent, and the regeneration was considered complete. The column was put through three more exchange cycles as previously described, before it was ready for use.

The acid adsorbing Amberlite IR-4 was "broken in" in the same manner as the IR-100, except that ammonium hydroxide was used in the regeneration and 4% hydrochloric acid to saturate its acid binding capacity. In treating the IR-4 resin with ammonium hydroxide, its regeneration was considered complete when the reaction of the effluent became *p*H 11. When the column was subsequently treated with hydrochloric acid in the "breaking in" treatment, the *p*H value of 4.0 was used as an index of saturation of the resin with acid. Four exchange cycles were carried out on the Amberlite IR-4 before it was put into operation.

Preparation of the Digest.—Two kilograms of fresh potatoes were peeled and ground through a meat grinder fitted with a peanut butter cutter and the juice pressed through cheesecloth. Approximately 1200 ml. of the juice obtained from this weight of raw potatoes was then filtered through a canvas precoated with a thin layer of filter aid[11] on a Buchner funnel, using suction.

One hundred grams of air-dried potato starch was stirred into 600 ml. of cold water, then poured with stirring into 3500 ml. of boiling water and maintained at 100° for half an hour. This treatment completely ruptures the

(7) M. Kobel and C. Neuberg, "Handbuch der Pflanzenanalyse," edited by G. Klein, Julius Springer, Wien, 1932, Vol. II, part 1, pp. 546–577.

(8) C. Neuberg and H. Lustig, THIS JOURNAL, **64**, 2722 (1942).

(9) K. P. DuBois and V. R. Potter, *J. Biol. Chem.*, **147**, 41 (1943).

(10) The bed volume of a resin is known as the backwashed and drained volume and is measured by backwashing to 50% bed volume expansion, followed by draining the wash water to one inch of the top of the resin bed.

(11) "Hyflo-Super-Cel" obtained from Johns-Manville Co., New York, was employed. Other diatomaceous silica of similar grade may be used.

starch granules, forming a thick paste. The starch paste was cooled to room temperature before using.

A phosphate solution of *p*H 6.7 was prepared by dissolving 1 mole of $NaH_2PO_4 \cdot H_2O$ (138 g.) and 1 mole of $Na_2HPO_4 \cdot 12H_2O$ (358 g.) in 3 liters of water. The filtered potato juice, the starch solution and the phosphate buffer were mixed well, and treated with about 50 ml. of toluene and the mixture allowed to remain for a period of twenty-four hours at 25°. The amount of glucose-1-phosphate formed from the starch was determined by analyzing a portion of the digest, after removing the inorganic phosphate with magnesium ammonium chloride mixture[12] for ten-minute hydrolyzable phosphate with *N* sulfuric acid at 100°. The yield of ester, calculated as the dipotassium dihydrate salt of glucose-1-phosphate present in the total digest, is usually between 40 and 100 g., depending upon the potency of the phosphorylase. According to analysis, the present preparation contained 43 g. of the potassium salt of the ester. The digest was heated to boiling in order to coagulate the proteins and inactivate the enzymes. After cooling to room temperature, two moles of $MgAc_2 \cdot 4H_2O$ (428 g.) was introduced, followed by the addition of concentrated ammonium hydroxide to bring the *p*H of the solution to 8.5. The magnesium ammonium phosphate precipitate and the proteins were filtered with suction on a Buchner funnel through a cloth filter precoated with filter aid. The filtrate, amounting to 8000 ml., was now ready to be treated with the ion exchange materials for the recovery of glucose-1-phosphate. Besides the ester, the solution contained sodium, ammonium, magnesium and acetate ions, and also starch, proteins and other impurities from the potato juice.

Treatment of the Digest with Amberlite IR-100.—The solution containing the glucose-1-phosphate and impurities was passed through a column of 1200-ml. bed volume of Amberlite IR-100 at the rate of 10 bed volumes per hour, taking the *p*H of the effluent as an index of saturation capacity of the resin. The cations in the solution were exchanged for hydrogen ions by this treatment. When the *p*H value rose to 4 the resin was considered to be near its saturation capacity and was regenerated with acid. To pass eight liters of solution through this column required four regenerations with hydrochloric acid followed by washing as previously described.

Adsorption of the Glucose-1-phosphoric Acid on Amberlite IR-4.—The solution was run through a column of Amberlite IR-4 of a 200-ml. bed volume at the rate of seven bed volumes per hour. When the reaction of the effluent from the Amberlite IR-4 falls to a value of *p*H 4 its saturation capacity has been reached and the Amberlite should be regenerated. However, in the case of glucose-1-phosphoric acid, it is still being adsorbed, although according to the *p*H value, the resin appears to be saturated. The glucose-1-phosphoric acid, having a stronger affinity for combination with this resin than acetic acid, is preferentially adsorbed, thus facilitating the removal of the glucose-1-phosphoric acid present in relatively low concentration as compared to the acetic acid. Analysis of samples of effluent for ten-minute hydrolyzable phosphate showed that practically all the glucose-1-phosphoric acid was removed from the solution. After the eight liters of the solution containing the glucose-1-phosphoric acid passed through the Amberlite IR-4, the resin was washed with three liters of water to remove soluble impurities such as starch or protein.

Recovery of Glucose-1-phosphate and Regeneration of the Amberlite IR-4.—A 4% solution of ammonium hydroxide was passed through the Amberlite IR-4 at a rate of five bed volumes per hour, and the *p*H of successive 50-ml. portions of effluent was determined. When 500 ml. of the solution had passed through the resin the reaction of the effluent became *p*H 11. An additional 200 ml. of 4% ammonium hydroxide was passed through this column to ensure complete regeneration of the resin, but it was not combined with the first 500 ml. of effluent, containing at least 99% of the glucose-1-phosphate. A concentration of 8000 ml. to 500 ml. in terms of glucose-1-phosphate had thus been effected by the use of the exchange materials.

The solution containing the glucose-1-phosphate was treated with 50 g. of potassium acetate and the reaction adjusted to *p*H 12 with 10% potassium hydroxide. One and a half volumes of methanol was then added to the solution and the potassium salt allowed to crystallize at 4°. After twenty-four hours the crystals of dipotassium glucose-1-phosphate dihydrate were removed by filtration, washed with methanol and ether, and dried *in vacuo* at 50°. A yield of 40.5 g. was obtained. This yield constitutes 94% recovery of the ester, formed from starch after treatment with potato phosphorylase, shown to be present in the digest according to analysis of acid hydrolyzable phosphorus. The product was free of inorganic phosphorus, nitrogen and dextrins. Its reducing value in terms of glucose, after hydrolysis with *N* sulfuric acid, was 48.4% (calculated reducing value, 48.38%).

Anal. Calcd. for $C_6H_{11}O_5OPO_3K_2 \cdot 2H_2O$: P, 8.33. Found: P, 8.3. Specific rotation: $[\alpha]_D$ +78° (in water, $c = 2$).

Adsorption Capacity of Amberlite IR-4 with Various Acids at their "Break through" Points.—When the resin is saturated with acid to its full capacity, the concentration of the effluent becomes equal to that of the influent. When quantitative adsorption of an acid is desired, it is of interest to determine the point at which the resin is not fully saturated, but only to the degree where some of the acid begins to appear in the effluent. This point is termed by Myers, *et al.*,[13] as the "break through" capacity of the resin.

The adsorption capacities of Amberlite IR-4 with respect to hexose phosphate esters were determined as follows: a 500-ml. solution, containing two hundred milli-equivalents of hexose phosphate, was passed through a 300-ml. bed of Amberlite IR-100 to remove cations, and then through a bed of Amberlite IR-4 to adsorb the phosphoric acid ester. The weight of the Amberlite IR-4 bed in the column was equivalent to 10 g. of resin on the dry weight basis. The results of the adsorption capacities are expressed in terms of grams of acid adsorbed per gram of dry resin. Table I shows the break through capacities of the resins when treated with various hexose phosphoric acids and also of glycerophosphoric acid.

TABLE I

ADSORPTION CAPACITY OF AMBERLITE IR-4

Acid	Influent concn., millimoles/liter	Capacity at break through: g. acid/g. dry resin	Capacity at break through: millimoles acid/g. dry resin
Hydrochloric[a]	11.1	0.174	4.77
Nitric[a]	5.9	.495	7.86
Sulfuric[a]	5.1	.389	3.97
Phosphoric[a]	15.3	.508	5.18
Acetic[a]	33.3	.231	3.85
Glycerophosphoric	200	1.59	9.25
Glucose-1-phosphoric	200	2.28	8.77
Fructose-6-phosphoric	200	2.34	9.00
Glucose-6-phosphoric	200	2.34	9.00
Fructose-1,6-diphosphoric	100	1.49	4.38

[a] Data presented by Myers[6] and recalculated to compare with the hexose phosphoric acids.

The resin exchange adsorbents have been very successfully used for the purification of crude preparations of fructose-6-phosphate and fructose-1,6-diphosphate. A crude preparation of fructose-1,6-diphosphate prepared

(12) The magnesium mixture is made up as follows: 55 g. of $MgCl_2 \cdot 6H_2O$ and 70 g. of NH_4Cl are dissolved in 650 ml. of water; 350 ml. of 10% ammonium hydroxide is added and allowed to remain at room temperature for two or three days, until the solution becomes clear, and then filtered.

(13) R. J. Myers, J. W. Eastes and D. Urquhart, *Ind. Eng. Chem.*, **33**, 1270 (1941).

according to Kobel and Neuberg[7] was treated with the resin exchange adsorbents and recovered as the amorphous calcium salt by precipitation with alcohol. Analysis of this hexosediphosphate before treatment with the ion exchange adsorbents showed only 70% purity, the chief contaminants being proteins and calcium phosphate. After purification by the aid of the resin exchange adsorbents the calcium salt of the fructose diphosphoric acid was obtained in pure condition. *Anal.* Calcd. for $C_6H_{10}O_6(PO_3Ca)_2$: P, 14.9; Ca, 19.2. Found: P, 15.3; Ca, 19.4.

Crude fructose-6-monophosphate (75% purity) was similarly treated with the resin exchange adsorbents and the ester recovered as the barium salt. *Anal.* Calcd. for $C_6H_{11}O_6(PO_3Ba)$: P, 7.8; fructose, 45.5. Found: P, 8.0; fructose, 45.5.

The authors wish to thank Drs. J. R. Matchett, R. R. Le Gault and J. F. Carson for their interest and valuable suggestions.

Summary

A method for preparation of dipotassium glucose-1-phosphate dihydrate is described. The glucose-1-phosphate is obtained by digesting a starch with crude potato phosphorylase in the presence of inorganic phosphate and the purification of the ester is accomplished by using ion exchange adsorbents.

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

The Autoxidation of β-Eleostearic Acid. The Application of the Spectrophotometer to the Study of the Course and the Kinetics of the Reaction[1,2]

BY RALPH W. BRAUER[3] AND LUVILLE T. STEADMAN

The course and mechanism of the reactions between unsaturated fatty acids and molecular oxygen have remained obscure in spite of a vast amount of careful investigation. The solution of this important problem has been held up by a dilemma which the conventional approach has been unable to evade: Either the autoxidation takes a complicated destructive course, with the formation of numerous secondary products, or there are several centers within the molecule capable of undergoing reaction. In either case the methods hitherto employed measure a number of simultaneously occurring reactions which cannot be disentangled for theoretical studies.

Of the known fatty acids, those which contain a conjugated system of unsaturation show the least breakdown and the most clearly defined oxygen uptake. Due to their conjugated double bonds, these compounds all show strong absorption of light in the middle or remote ultraviolet region. This property permits the determination, impracticable by other methods, of a particular grouping throughout the course of the autoxidation reaction. Thus, in spite of the fact that more than one center in these molecules is capable of absorbing oxygen, it becomes possible to dissociate the various reactions from one another to some degree.

The work reported in this paper has been performed upon β-eleostearic acid, an octadecatrienoic acid of structure

$$CH_3(CH_2)_3(CH{=}CH)_3(CH_2)_7COOH$$

which is readily obtained in a state of high purity from tung oil. The autoxidation of this material was followed by spectrophotometric means and by simultaneous oxygen uptake measurements.

In order to attain a satisfactory degree of accuracy and reproducibility, it was deemed desirable to carry out the reactions in liquid solutions in an inert solvent. This required the development of a special technique for the oxygen uptake measurements, which will be described below in some detail, not only as an integral part of the spectrographic studies, but also because the procedure permits the investigation of the relation between the concentration of autoxidizable substrate and the rate of autoxidation. In the opinion of the authors such studies are of vital importance to the further elucidation of the mechanism of autoxidation of ethylenic systems.

Experimental

β-Eleostearic Acid.—Sulfur isomerized tung oil[4] was saponified according to the method of Dann, *et al.*,[5] and the non-saponifiable matter was extracted with ether from the alkaline mixture diluted 1:1 with water. The aqueous layer was separated and acidified after heating to about 90°. The liberated fatty acids were washed repeatedly with hot water, and finally solidified by the addition of ice water, and were then separated by filtration. β-Eleostearic acid was isolated from the mixed fatty acids by crystallization, once from methyl alcohol and three times from petroleum ether (b. p. 40–60°) at 0°.

The β-eleostearic acid thus obtained consisted of white platelets of m. p. 71.5–71.8°. The entire isolation was performed under nitrogen. The final product was stored in the dark in nitrogen-flushed containers, kept evacuated to less than 1 mm. of mercury.

***n*-Amyl Acetate.**—The commercial product (Eastman Kodak Co., C. P. grade) was purified by two different pro-

(1) Submitted by Ralph W. Brauer in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the Division of Graduate Studies of the University of Rochester.

(2) Read in part before the Division of Biological Chemistry at the 104th meeting of the American Chemical Society, Detroit, April, 1943.

(3) Present address: Distillation Products, Inc., Rochester, N. Y.

(4) The authors hereby acknowledge the kindness of Mr. C. F. Goodyear, President of the Bogalusa Tung Oil Corp., Bogalusa, La., in providing the oil employed in this work.

(5) Dann, Moore, Booth and Kon, *Biochem. J.*, **29**, 133 (1935).