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Preparation of Crystalline α-D-Galactosamine-1-Phosphoric Acid and Its Conversion to UDP-*N*-Acetylgalactosamine*

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A simple method has been developed for the enzymatic preparation of α-D-galactosamine-1-phosphoric acid in gram quantities, and the product has been crystallized. The compound, obtained in 60% yield, has an $[\alpha]_D^{25} = +142.6°$ ($c = 2.0\%$ in H_2O). After *N*-acetylation with acetic anhydride, the resulting *N*-acetyl-α-D-galactosamine-1-phosphate was crystallized as the potassium salt which had an $[\alpha]_D^{25} = +112.4°$ ($c = 2.9\%$ in H_2O). UDP-*N*-acetylgalactosamine was prepared by reacting mono-(tri-*n*-octylammonium)-*N*-acetylgalactosamine-1-phosphate with uridine-5′-phosphoromorpholidate in anhydrous pyridine. As the product, UDP-*N*-acetylgalactosamine, is synthesized by an unambiguous route from the α anomer of galactosamine-1-phosphate, it also must be the α anomer. A new procedure has been devised for the assay of galactokinase that is generally applicable to enzyme-catalyzed reactions where reducing sugars are converted to glycosides. This assay utilizes sodium borohydride for reduction of excess substrate to the corresponding sugar alcohol. The glycosidic bond, the aglycone being phosphate in the present case, is cleaved with acid, and the resulting sugar is measured by conventional techniques.

N-Acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactose) is found in many macromolecular substances (Kent and Whitehouse, 1955); a fungal polysaccharide (Distler and Roseman, 1960) is exceptional in that it contains D-galactosamine as well as the *N*-acetyl derivative. The precursor of the macromolecules is probably UDP-*N*-acetylgalactosamine, enzymatically derived from UDP-*N*-acetylglucosamine (Maley and Maley, 1959; Glaser, 1959). Although both enzymatic and chemical methods can be used for the synthesis of UDP-*N*-acetylgalactosamine (Davidson and Wheat, 1963; Kim and Davidson, 1963), these procedures are not convenient for preparing pure, specifically labeled material in substrate amounts.

Sugar-nucleotides similar to UDP-*N*-acetylgalactosamine can be synthesized by a general method (Roseman *et al.*, 1961), but only if the corresponding 1-phosphate derivative of sugar is readily available. The problem of obtaining galactosamine-1-phosphate in adequate amounts, specifically the α anomer, was therefore the primary purpose of this investigation. A simple method has been developed for the enzymatic synthesis of D-galactosamine-1-phosphate in gram quantities and, in addition, the pure compound has been crystallized. The hexosamine-phosphate was quantitatively *N*-acetylated by the usual procedure (Distler *et al.*, 1958), and the *N*-acetylgalactosamine-1-phosphate was converted to the desired UDP-*N*-acetylgalactosamine.

The original procedure for the enzymatic synthesis of galactosamine-1-phosphate (Cardini and Leloir, 1953) was repeated and gave only small amounts of material, a result explained by the observation that yeast galactokinase exhibits a high K_m for galactosamine (Alvardo, 1960). By modifying the original conditions and applying methods previously used for isolating the crystalline 6-phosphate esters of glucosamine, galactosamine, and mannosamine (Distler *et al.*, 1958; Jourdian and Roseman, 1962), we have obtained crystalline galactosamine-1-phosphate in better than 60% yield.

Since the enzyme incubations required high galactosamine concentrations, it was not practical to assay the crude reaction mixtures by the usual method, i.e., determining substrate disappearance. A new assay technique was therefore devised that made it possible to measure product formation. In this assay, the excess substrate is reduced with sodium borohydride, to which the product is resistant because it contains a protected carbonyl group. This principle is generally applicable to enzyme reactions where reducing sugars are converted to nonreducing products such as glycosides. The product is measured, in the case of galactosamine-1-P, by *N*-acetylation, hydrolysis, and a modified Morgan-Elson method. With galactose, the product is measured as reducing sugar after the borohydride treatment, hydrolysis, and deionization.

Experimental Procedure

Materials.—The yeast used in this study was a galactose-adapted strain of *Saccharomyces fragilis*, purchased from the Sigma Chemical Co. Galactosamine-hydrochloride was prepared as previously described (Roseman and Ludoweig, 1954). Phosphoenolpyruvate, 3-phosphoglyceric acid, UMP, and ATP were commercial materials. UMP-morpholidate was prepared as previously described (Moffatt and Khorana, 1961).

Methods.—The following substances were determined by the indicated procedures: protein by the

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method of Lowry *et al.* (1951); *N*-acetylgalactosamine by a modified Morgan-Elson procedure (Spivak and Roseman, 1959); reducing sugar by the method of Somogyi (1952;) phosphorus by the methods of Telep and Ehrlich (1958) and Fiske and Subbarow (1925).

Enzyme Preparation.—A crude enzyme preparation was obtained from galactose-adapted *S. fragilis* by autolyzing 2 g of yeast in 6 ml of 0.1 M $NaHCO_3$ for 12 hours at 25°. After centrifugation at 32,000 × *g* for 30 minutes the supernatant fluid was decanted. The precipitate was washed with 6 ml of bicarbonate solution, the mixture was centrifuged, and the supernatant fluids were combined. This crude extract contained approximately 50 mg protein per ml.

Assay.—The assay mixture contained 200 μmoles of phosphate buffer at *p*H 7.8, 2 μmoles of $MgCl_2$, 40 μmoles of ATP, 40 μmoles of galactosamine-HCl, 10 μmoles of phosphoenolpyruvate, 10 μmoles of phosphoglyceric acid, and 0.01 ml of the crude galactokinase preparation in a total volume of 0.14 ml. After incubation at 30° for 15 minutes the reaction mixture was heated at 100° for 2 minutes. Following centrifugation, 0.05 ml of the supernatant fluid was assayed for galactosamine-1-P as described in the following paragraphs.

(1) Reduction of the Remaining Substrate with $NaBH_4$.—One drop of capryl alcohol was added followed by 25 μl of 1.0 M $NaBH_4$. After being mixed, the solution was allowed to stand at room temperature for 5 minutes with occasional shaking, and the borohydride addition was repeated. After 5 minutes 25 μl of 2.0 M acetone was added and again the solution was thoroughly mixed and allowed to stand at room temperature for 5 minutes. Finally, the excess borohydride was completely destroyed by heating the mixture at 100° for 3 minutes.

(2) Acetylation.—While galactosamine-1-P is extremely resistant to acid hydrolysis (Cardini and Leloir, 1953), the *N*-acetyl derivative is much more labile (Leloir *et al.*, 1958). The acetylation procedure was that previously described (Distler *et al.*, 1958) and involved the addition of 0.1 ml of 0.5 M aqueous acetic anhydride while the *p*H was maintained between 7 and 9 with saturated $NaHCO_3$. The reaction was complete in about 10 minutes.

(3) Hydrolysis.—The *N*-acetylgalactosamine-1-P was hydrolyzed by adding 0.2 ml of 2 N HCl followed by heating at 100° for 10 minutes. After cooling, the reaction mixture was neutralized with 2 N NaOH using phenolphthalein as an indicator. Water was added to a final volume of 1.0 ml and 0.5 ml of the final mixture was assayed directly for *N*-acetylgalactosamine by the modified Morgan-Elson method.

The validity of the assay method was determined by measuring the recovery of added galactosamine-1-P. These experiments showed that excess substrate did not interfere with the assay procedure, and that recovery of the product averaged better than 90%. The method can be used for kinetic studies as indicated in Figure 1, which presents the reaction rate as a function of time and protein concentration.

A similar assay procedure can be used to follow the phosphorylation of galactose by the crude enzyme preparations. After incubating the enzyme with the substrates as described by Leloir and Trucco (1955), 0.10-ml aliquots of the reaction mixtures were treated with $NaBH_4$ and the borohydride was destroyed by addition of 0.10 ml of 2 N H_2SO_4. The product of enzymatic phosphorylation, galactose-1-P, was hydrolyzed by heating at 100° for 10 minutes. After cooling, the mixture was neutralized with $KHCO_3$, adjusted to 4 ml with water, and deionized with mixed-bed ion-exchange resin (Dowex 1-HCO_3^- and Dowex 50, H^+). Finally, 2-ml aliquots were removed for reducing sugar determinations. Control and recovery experiments with added galactose-1-P showed that this substance was quantitatively determined by the assay procedure.

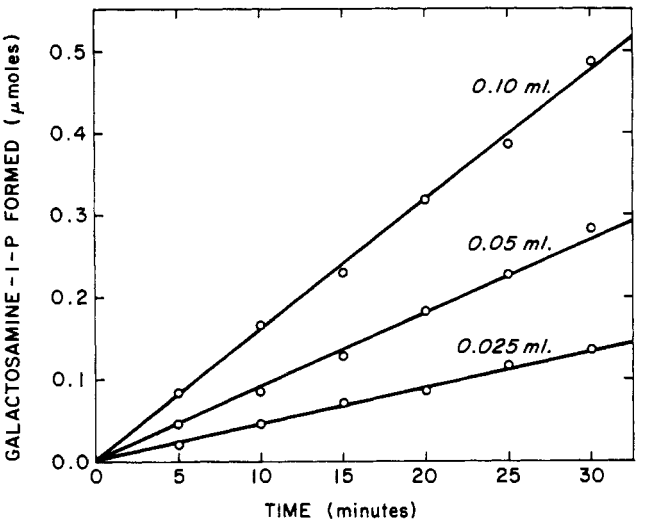

FIG. 1.—Effect of time and protein concentration on galactokinase reaction. Galactosamine was the substrate in incubation mixtures identical to those described under *Assay* in the text except that all components were increased 10-fold. As shown in the figure, 0.025–0.100 ml of crude extract was added to the incubation mixtures to final volumes of 1.40 ml. Aliquots (0.20 ml) were removed at the indicated times, denatured by heating at 100° for 2 minutes, and assayed as described in the text.

Preparation of Galactosamine-1-phosphate.—A typical incubation mixture for a large-scale preparation of galactosamine-1-P consisted of the following substances in a final volume of 140 ml; 6 mmoles galactosamine-HCl; 10 mmoles phosphoenolpyruvate; 6 mmoles ATP; 10 mmoles 3-phosphoglyceric acid; 30 mmoles potassium phosphate buffer, *p*H 7.8; 3 mmoles $MgCl_2$; and 30 ml of the crude galactokinase preparation. The *p*H was maintained by adding 1 N NaOH as required. After 6 hours at 30°, 0.2 ml of toluene was added and the mixture was maintained at 30° for an additional 12 hours. The reaction was terminated by heating at 100° for 5 minutes, and the precipitate was removed by centrifugation. Assay of the supernatant fluid showed the presence of 4.8 mmoles of galactosamine-1-P. The solution was placed on a column of Dowex-50, H^+ (420 ml, 100–200 mesh) and the column was washed with water to elute the desired product (Jourdian and Roseman, 1962). The product was eluted after the nucleotides and sugar phosphates, and was located by assaying the fractions for total P and galactosamine-1-P. The fractions containing the product were pooled, assayed by the usual procedure, and were found to contain 3.9 mmoles. After lyophilization, 1.1 g of a white powder was obtained, dissolved in 20 ml of water, and treated with 20 ml of 95% ethanol. Crystals slowly formed in the solution, and were harvested after 4–6 days. The first-crop material contained 0.95 g; approximately 0.1 g was obtained in a second crop of crystals. After drying to constant weight over P_2O_5 *in vacuo* at 56°, the crystals (Fig. 2) showed the analyses given in Table I.[1]

[1] Elementary analyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Michigan.

TABLE I
ELEMENTAL ANALYSES AND OPTICAL ROTATIONS

	Galactosamine-1-P, H_2		Acetylgalactosamine-1-P, K_2		UDP-acetylgalactosamine, Li_2, $3H_2O$	
	Calculated for $C_6H_{14}O_8NP$	Found	Calculated for $C_8H_{14}O_9NPK_2$	Found	Calculated for $C_{17}H_{33}O_{20}N_3P_2Li_2$	Found
C	27.81	27.67	25.46	25.44	30.33	31.00
H	5.45	5.64	3.74	4.10	4.64	5.01
N	5.41	5.56	3.71	3.77	9.20	9.76
P[a]	11.95	11.88	8.21	8.05	6.24	5.80
$[\alpha]_D^{25}$	142.6° (c = 2.0% in H_2O)[b]		112.4° (c = 2.9% in H_2O)[b]		81.7° (c = 0.8% in H_2O)[c]	

[a] None of the compounds contained inorganic P. The expected ratios of organic P to hexosamine (as *N*-acetylgalactosamine) were obtained after acid hydrolysis. [b] The concentrations were based on the organic P content of the solutions. [c] The concentration (*p*H 7) was calculated from the absorbance at 262 mμ, using 10,000 as the extinction coefficient.

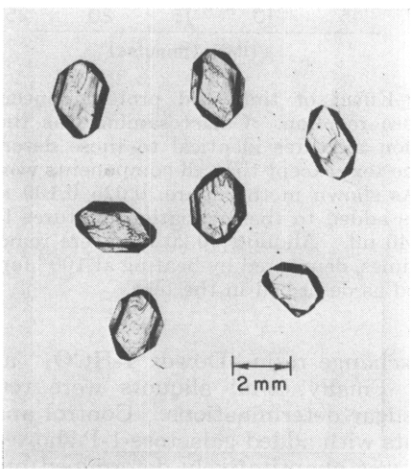

FIG. 2.—Photomicrograph of galactosamine-1-phosphoric acid crystals.

Preparation of N-Acetylgalactosamine-1-phosphate.—Crystalline galactosamine-1-P was *N*-acetylated by the general procedure previously described (Distler *et al.*, 1958). A solution containing 259 mg of galactosamine-1-P, an equivalent of $NaHCO_3$, and 3 ml of methanol in 20 ml of water was cooled in ice. Acetic anhydride (0.47 ml) was added over a 30-minute period with continuous stirring, while the *p*H was maintained between 6.5 and 8.0 by adding $NaHCO_3$. After 1 hour the solution was stirred with 50 ml of Dowex-50, H^+, 200–400 mesh, to remove Na^+, and filtered, and the resin was washed with water. The acetic acid was removed by repeated extraction with 3 volumes of ether. Control experiments with added ^{14}C-acetate showed that six or seven extractions were required for complete removal of acetic acid. The product was converted to its potassium salt by passage of the solution through a column of Dowex-50 K^+, 200–400 mesh. After adjusting the *p*H to 9.0 with KOH, the product was concentrated *in vacuo* to a colorless syrup which was insoluble in 95% ethanol, but very soluble in methanol. The methanolic solution was treated with a mixture containing equal volumes of ethanol and acetone until a faint persistent turbidity was observed. The addition of the ethanol-acetone mixture was repeated over a period of 3 weeks. The resulting crystals were harvested by centrifuging, washed twice with 95% ethanol, twice with absolute ethanol, and finally with ether. After drying to constant weight over P_2O_5 *in vacuo*, the product gave the analyses shown in Table I. While the *N*-acetylation step was quantitative, the first crop of crystals represented a 35–40% yield.

Preparation of Uridine Diphosphate N-Acetylgalactosamine.—The procedure previously described for the synthesis of UDP-*N*-acetylglucosamine (Roseman *et al.*, 1961) was applied to the preparation of UDP-*N*-acetylgalactosamine. Thus, 1.0 mmole of the potassium salt of *N*-acetylgalactosamine-1-P was converted to the bis-(tri-*n*-octylammonium) salt, and this product reacted with uridine-5′-phosphoromorpholidate (0.5 mmole) in anhydrous pyridine (20 ml) for 5 days. The reaction mixture was treated as described and fractionated on Dowex-1, Cl^- with LiCl in dilute HCl. The yield of purified UDP-*N*-acetylgalactosamine was 65%; analysis of this substance gave the results shown in Table I. The isolated product was shown to be homogeneous in several of the paper chromatographic and electrophoretic systems used for other sugar nucleotides (Roseman *et al.*, 1961). After heating at 100° for 10 minutes in 0.1 N HCl, 1.02 moles of *N*-acetylgalactosamine were released per mole of uridine nucleotide.

Preparation of ^{14}C-labeled UDP-N-acetylgalactosamine.[2]—Galactosamine-1-P was *N*-acetylated with ^{14}C–acetic anhydride. When this material was treated as described above, without crystallization of the *N*-acetylgalactosamine-1-P salt, ^{14}C-acetyl-UDP-*N*-acetylgalactosamine was isolated in 50% yield (based on 0.10 mmole of UMP-morpholidate). This product was found to be active in a system that enzymatically synthesized acid mucopolysaccharides (Perlman and Dorfman, 1963).

DISCUSSION

Anomeric Configuration.—Galactokinase is known to catalyze the synthesis of the α anomer of D-galactose-1-P (Wilkinson, 1949). While Cardini and Leloir (1953) used the kinase to prepare galactosamine-1-P, they did not report the optical rotation of their product. They *N*-acetylated the compound chemically, however, and reported optical rotations of the *N*-acetylgalactosamine-1-P, (Cardini and Leloir, 1957). A similar substance was isolated by direct phosphorylation of *N*-acetylgalactosamine with an animal kinase (Leloir *et al.*, 1958). The rotations indicated that the phosphate esters were the α anomers. In addition, Davidson and Wheat (1963) reported the optical rotation of their preparation of *N*-acetylgalactosamine-1-P as shown in Table II. The molar optical rotations reported by the previous workers differ significantly and also disagree with the values observed with our preparation.

For purposes of comparison, the molar rotations of D-galactosamine-1-P and its *N*-acetyl derivative as

[2] The labeled UDP-*N*-acetylgalactosamine was prepared by Mr. Jack Distler.

TABLE II
OPTICAL ROTATIONS OF SOME α-GLYCOSIDES AND α-GLYCOSIDE-1-PHOSPHATES

Compound	$[\alpha]^a$	$[M]^a$	Reference
Glucose-1-P, K_2, $2H_2O$	78	29,000	Krahl and Cori, 1949
Glucosamine-1-P, K	100	29,700	Maley *et al.*, 1956
N-Acetylglucosamine-1-P, K_2	79	29,800	Maley *et al.*, 1956
Methyl D-glucoside	157	30,500	Hudson, 1909
Methyl-D-glucosaminide, HCl	127	29,000	Neuberger and Rivers, 1939
Methyl-*N*-acetylglucosaminide	105	24,600	Neuberger and Rivers, 1939
Galactose-1-P, Ba	96	37,900	Hansen *et al.*, 1955
Galactosamine-1-P, H_2	142	38,900	[b]
Methyl-D-galactoside	179	38,000	Dale and Hudson, 1930
Methyl-*N*-acetylgalactosaminide	183	44,600	[b]
Methyl-*N*-acetylgalactosaminide	187	45,600	Masamune *et al.*, 1951
N-Acetylgalactosamine-1-P, K_2	112	44,000	[b]
N-Acetylgalactosamine-1-P, H_2	178	54,000	Cardini and Leloir, 1957
N-Acetylgalactosamine-1-P, H_2		52,000	Leloir *et al.*, 1958
N-Acetylgalactosamine-1-P, Li_2, $2H_2O$	189	66,000	Davidson and Wheat, 1963[c]
N-Acetylgalactosamine-1-P, K_2	118	46,300	[b]

[a] All rotations are given at the sodium D line except the last two. Davidson and Wheat report $[\alpha]^{25}_{578}$ for their preparation of *N*-acetylgalactosamine-1-P. Our sample was therefore also examined at this wavelength for purposes of comparison (temp. 23°). The $[\alpha]_D$ of our preparation was checked with the crystalline K salt and immediately after quantitative *N*-acetylation of the crystalline galactosamine-1-P (without isolating the product). The values agreed within 3%. [b] The blank spaces indicate compounds prepared in this laboratory. The methyl α-*N*-acetyl-D-galactosaminide was prepared according to the method of Stacey (1944) and recrystallized, with large losses until the mp was constant at 218–220° (decomp, uncorr). [c] The concentration of the sugar in solution was determined by analysis for "acid-labile" phosphate according to a personal communication from Dr. E. Davidson. Incomplete hydrolysis would give a high $[\alpha]^{25}_{578}$.

determined in this laboratory are given in Table II along with the values previously reported for *N*-acetylgalactosamine-1-P and of a variety of α-glycosides. If the first six compounds are regarded as derivatives of α-D-glucopyranose, excellent agreement in the molar rotations is observed, with the possible exception of one case. Thus the correlation is apparently independent of the nature of the aglycone group, methyl or phosphate, or whether the group attached to C-2 is hydroxyl, amino, or acetamido. Similarly, the molar rotation for galactosamine-1-P reported here, 38,900, agrees very well with the values found for the α anomers of galactose-1-P and methyl galactopyranoside, 37,900 and 38,000. While the value of 43,100 obtained with our preparation of *N*-acetylgalactosamine-1-P shows some disparity with these constants, it agrees with the two values found for methyl-α-*N*-acetylgalactosaminide, approximately 44,000. These comparisons therefore show that the derivatives reported here are the α anomers. Since the UDP-*N*-acetylgalactosamine is synthesized by an unambiguous route from the galactosamine-1-P, we conclude that the sugar nucleotide is also the desired α anomer.

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