THE SYNTHESIS OF UDP-GALACTOSAMINE AND UDP-N-ACETYLGALACTOSAMINE[1]

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SUMMARY

A commercially available preparation of galactose-1-phosphate-uridyl transferase was used in the synthesis of UDP-galactosamine and UDP-N-acetylgalactosamine. The method provides a relatively simple means for preparing these important biological materials in high yield and in an isotopic form of almost any desired specific radioactivity, labeled singly or in combination. Support for the route of metabolism of galactosamine in rat liver proposed previously is also presented.

Galactosamine appears to be metabolized in rat liver by the same route as galactose, as evidenced by the products formed on perfusion of this organ with galactosamine-1-^{14}C (1). One of the compounds isolated, UDPGalN[2], is probably produced as a consequence of the substitution of galN-1-P for gal-1-P in the gal-1-P-uridyl transferase reaction as discussed earlier (2). The availability of gal-1-P-uridyl transferase from a commercial supplier not only provided a means of verifying the above proposal, but also suggested a synthesis for radioactive UDPGalNAc, an important precursor of blood group substance A (3,4), other glycoproteins (5), and possibly of the gangliosides.

Although the synthesis of UDPGalNAc has been described by a combined enzymic and chemical method (6), the procedure presented in this paper offers the advantages of simplicity, high yield, and the potential

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[2]Abbreviation used: UDPGalN, uridine diphosphate galactosamine.

of obtaining UDPGalNAc labeled singly or in combination with ^{3}H, ^{14}C, and ^{32}P of almost any desired specific radioactivity. Unlike most chemical procedures where yields are best when conducted on a macro scale, the yields obtained by this method are comparable whether conducted at the micro- or millimole level.

MATERIALS AND METHODS

UDPG, NADP, glc-1,6-P_2, gal-1-P, phosphoglucomutase, glc-6-P dehydrogenase, and UDPGal-4-epimerase were purchased from the Sigma Chemical Co. Gal-1-P-uridyl transferase, partially purified from calf liver, was obtained from the Boehringer Mannheim Corp., and dithiothreitol from Calbiochem. Acetic anhydride-1-^{14}C was purchased from Amersham-Searle.

GalN-1-P was prepared enzymically from galactosamine with galactose-adapted yeast obtained from the Sigma Chemical Co. and isolated by the method of Carlson et al. (6). N-acetylgalactosamine was determined by the procedure of Reissig et al. (7) as was galactosamine following N-acetylation (8). The King method (9) was used to measure total phosphate. The location of radioactive compounds following electrophoresis was determined with a Nuclear Chicago Acti-graph III, 4π strip scanner.

K_m determination - Gal-1-P-uridyl transferase was assayed by a modification of the procedure described by Mayes and Hansen (10). The reaction mixtures (1 ml) contained the following components in a cuvette with a 1-cm lightpath: Tris-HCl (pH 8.0), 50 mM; dithiothreitol, 5 mM; UDPG, 0.47 mM; NADP, 0.16 mM; $MgCl_2$, 2.0 mM; glc-1,6-P_2, 5 μM; phosphoglucomutase, 2.75 units; glc-6-P dehydrogenase, 0.35 unit; gal-1-P-uridyl transferase, 0.032 unit.

To initiate the reaction, galN-1-P was added in a volume no greater than 50 μl, so that its final concentration in the cuvette varied from 0.3 to 8.9 mM. Reaction rates were measured at 30° in a Gilford model 2400 automatic recording spectrophotometer at a wave length of 340 mμ.

Large-scale preparation of UDPGalN - The reaction mixture consisted

of the following components in a volume of 14.0 ml: UDPG, 10 mM; galN-1-P, 7.85 mM; Tris-HCl (pH 8.0), 70 mM; dithiothreitol, 7 mM; glc-1,6-P_2, 14 μM; phosphoglucomutase, 110 units; gal-1-P-uridyl transferase, 3.3 units. Two drops of toluene were added to the mixture which was placed in a screw-top vial and incubated at 37°. To measure the extent of reaction, 0.1-ml aliquots were removed from the reaction mixture at the times indicated in Fig. 1, added to 0.9 ml of water, and heated at 100° for 2 min. Aliquots from this solution were added to a cuvette containing 50 mM Tris-HCl (pH 8.5), 0.2 mM NADP, and water to a volume of 1.0 ml; 5 μl (0.35 unit) of glc-6-P dehydrogenase were added to determine the amount of glc-6-P formed in the large-scale reaction. UDPGalN was isolated quantitatively by passing the reaction mixture through a column of Dowex-1-formate (5 x 2 cm) and eluting the column as follows: 50 ml of 0.1 N formic acid, 10 ml of 1 N formic acid, followed by five 20-ml fractions of 1 N formic acid. The latter fractions contained the major ultraviolet light absorbing compound and were combined and lyophilized to a white powder.

Synthesis of UDPGalNAc-1-^{14}C - To a 1-ml solution of 19.5 μmoles of UDPGalN in water were added 40 μl of 1 mM triethylamine in methanol and 36.8 μmoles of acetic anhydride-1-^{14}C (13.6 μc per μmole) in 1.5 ml of methanol. After 1 hr at room temperature the solution was placed at 4° overnight. The addition of 5 ml of water to this solution preceded its passage through a column (5 x 1 cm) of Dowex-50 in the pyridinium form. The column was eluted with 20 ml of water and the combined eluant containing 8.6×10^8 dpm was lyophilized, providing a residue with 3.1×10^8 dpm. Relyophilization of the residue did not remove additional radioactivity. Accurate specific activity determinations required the removal of the 260 mμ absorbing pyridine; therefore, a solution of the residue was passed through a Dowex-50 column in the cold and neutralized to the desired salt.

RESULTS AND DISCUSSION

The K_m and V_{max} values for galN-1-P were estimated from a double

reciprocal plot analysis over a concentration range of 0.56 to 8.9 mM and the values obtained were 4.9 mM and 4.5 mμmoles per min, respectively. An analogous plot for gal-1-P over a concentration range of 0.81 to 26.9 mμmoles yielded values for the K_m and V_{max} of 0.028 mM and 16.6 mμmoles per min, respectively. In recent independent studies with a similar Boehringer gal-1-P-uridyl transferase preparation, Keppler and Decker (11) reported respective K_m values for gal-1-P and galN-1-P of 0.17 mM and 15 mM. Although their values do not correspond with those reported here, possibly because of differences in assay conditions, the preference of the enzyme for gal-1-P in both studies is emphasized.

A slow but measurable reaction was observed prior to the addition of galN-1-P, suggesting that UDPG was being hydrolyzed to UMP and glc-1-P by a contaminating enzyme. Confirmation was obtained on electrophoresis of the products in 0.05 M ammonium formate (pH 3.8). Deletion of Mg^{++} from the preparative reaction mixtures, however, greatly reduced the problem of nonspecific hydrolysis which was found to diminish the yield of product by 40 percent. As seen in Fig. 1, 71 μmoles of UDPGalN were formed, repre-

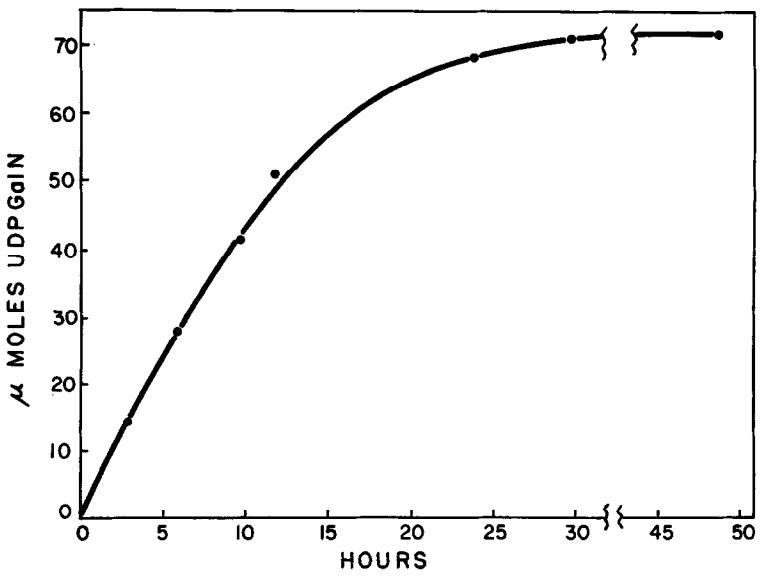

Figure 1. Rate of UDPGalN formation with the conditions presented in "Materials and Methods."

senting a 65 percent yield from the initial galN-1-P. Improved yields were not obtained on further addition of enzyme, suggesting product inhibition by UDPGalN, glc-6-P, or a combination of both. Since the N-acetylation to UDPGalNAc is almost quantitative, the over-all yield relative to galN-1-P is two-fold greater than that reported previously (6).

The isolated UDPGalN migrated on electrophoresis in 0.05 M ammonium formate (pH 3.8) 17.5 v/cm, exactly with enzymically prepared UDPGlcN and, on N-acetylation with an excess of acetic anhydride (8), it was converted quantitatively to a compound that migrated on electrophoresis with the faster moving UDPGlcNAc. Similar results were obtained on paper chromatography in ethanol-1 M ammonium acetate (7:3) (pH 7.5). Hydrolysis

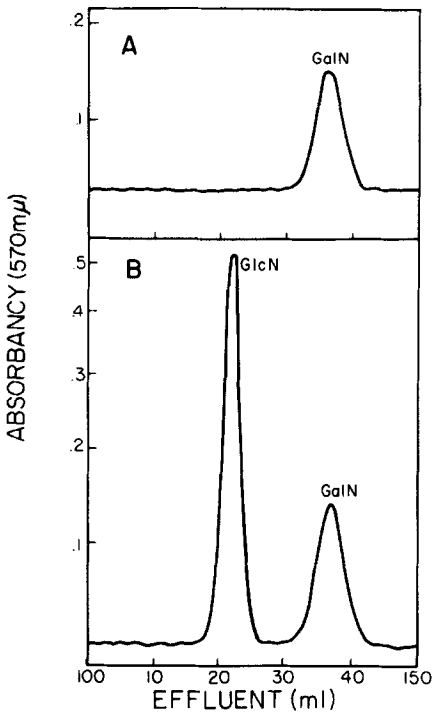

Figure 2. Effect of UDPGal-4-epimerase on UDPGalN. A, before epimerase treatment. B, after epimerase treatment. The reaction mixture contained 0.85 μmole of UDPGalN; 50 μmoles of Tris-HCl (pH 8.5); 10 μmoles of dithiothreitol; 0.1 μmole of NAD; and 0.02 unit of UDPGal-4-epimerase in a total volume of 0.67 ml. A drop of toluene was added and after incubation for 18 hr at 37°, the reaction mixture was hydrolyzed in 1 N HCl for 1 hr at 100°. Glucosamine and galactosamine were separated on an automatic amino acid analyzer (1).

of the N-acetylated compound at 100° for 10 min in 0.1 N HCl yielded 1.01 mole equivalents of N-acetylgalactosamine to uridine, as determined from the latter's absorbancy at 260 mμ. Total phosphate analysis provided 2.06 mole equivalents of organic phosphate. Final confirmation was obtained by treatment of the UDPGalN with UDPGal-4-epimerase, for, as described earlier (12), the epimerase will convert UDPGlcN to UDPGalN with a final equilibrium ratio of 73:27, respectively. As shown in Fig. 2, the acid hydrolyzed equilibrium mixture of UDPGalN and UDPGlcN was in a 71:29 ratio, as determined on the amino acid analyzer.

N-acetylation to UDPGalNAc-1-^{14}C is easily effected as described in "Materials and Methods," with most of the undesired radioactivity removed as volatile pyridine acetate. Electrophoresis of the resultant product (Fig. 3) revealed the presence of less than 10 percent unreacted UDPGalN and a nonvolatile radioactive contaminant at the origin of less than 5 percent of the total radioactivity. The UDPGalN content can be reduced even further by raising the ratio of acetic anhydride to UDPGalN from 2:1 to 3:1. The specific activity of the UDPGalNAc eluted from the elctrophoresis paper

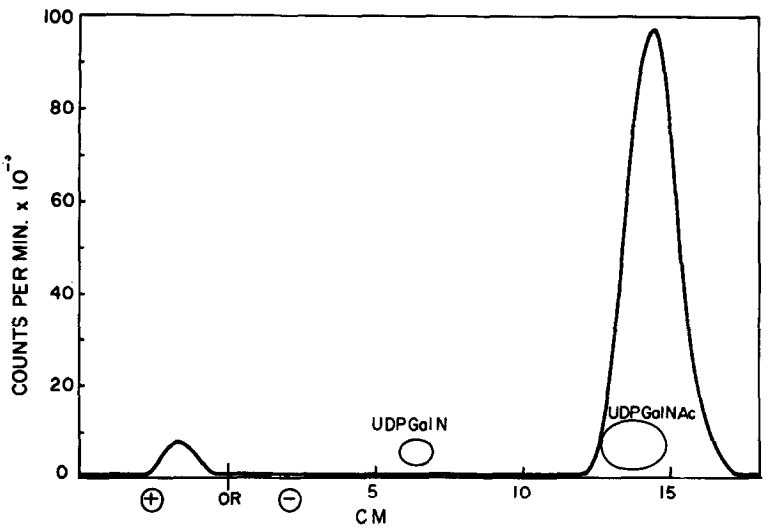

Figure 3. Electrophoresis of UDPGalNAc-1-^{14}C formed on N-acetylation of UDPGalN with acetic anhydride-1-^{14}C. The conditions of preparation and electrophoresis are presented in "Materials and Methods."

was 15.5 x 10^6 dpm, a value within experimental error of the initial acetic anhydride specific activity. If further purification is desired, gradient elution from Dowex-1-carbonate with the volatile triethylamine bicarbonate buffer system can be employed (13).

Although the major purpose of this report is to describe a new and improved procedure for the formation of UDPGalN and UDPGalNAc, it indirectly supports the thesis presented earlier (2) for the presence of UDPGalN and UDPGlcN in livers exposed to galactosamine; that is, galN-1-P displaces gal-1-P in the gal-1-P-uridyl transferase reaction and the resultant UDPGalN is converted by UDPGal-4-epimerase to UDPGlcN (Fig. 2). Similar observations were recently reported by Keppler and Decker (11), although their interpretation of our findings was obviously in error, since we did not state that UDPGalN was formed from UDPGalNAc. The hepatitis-like response provoked by galactosamine (14) could result from the substitution of UDPGalN and UDPGlcN for the corresponding UDPhexoses in glycosyl transferase reactions concerned with membrane and glycoprotein synthesis. A similar type of substitution of UDPGlcN for UDPGlc in the glycogen synthetase reaction was shown previously by us to result in the incorporation of glucosamine into glycogen (15).

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