Agarose Derivatives of Uridine Diphosphate and N-Acetylglucosamine for the Purification of a Galactosyltransferase*

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SUMMARY

The applicability of affinity chromatography for the purification of UDP-galactose:N-acetylglucosamine galactosyltransferase has been evaluated. Three different types of specific adsorbents have been synthesized by reaction of a ligand structurally related to portions of the substrates of the transferase with cyanogen bromide-treated agarose. The synthesis of the ligands, \( P^1-(6\text{-amino-1\-hexyl})-P^2-(5^-\text{uridine}) \text{ pyrophosphate}, \) \( 6\text{-amino-1\-hexyl-2\-acetamido-2\-deoxy-\(\beta\text{-D-glucopyranoside} \) and \( P^1-(6\text{-amino-1\-hexyl})-P^2-(\beta\text{-D-galactopyranosyl})\text{pyrophosphate} \) is described.

The galactosyltransferase from bovine milk has been found to bind to two of the adsorbents and some of the parameters influencing the binding have been evaluated. The affinity of the transferase for the UDP-hexanolamine-agarose adsorbent was enhanced by manganous ions whereas the affinity was decreased by EDTA, urea, borate, or magnesium ions. The affinity of the enzyme for N-acetylglucosamine hexanolamine-agarose was enhanced considerably by UDP or UMP and was decreased by borate, urea or N-acetylglucosamine. N-Acetylglucosamine-agarose also served as an acceptor substrate for the transferase. The galactosy phosphosphate-agarose was found to have a low capacity and specificity of binding for the galactosyltransferase.

On the basis of the properties of the adsorbents, it has been possible to purify the galactosyltransferase from bovine milk to constant specific activity solely by affinity chromatography. The enzyme from the whey of bovine milk was first adsorbed on UDP-agarose and the eluate from this adsorbent then chromatographed on either N-acetylglucosamine-agarose or \( \alpha\text{-lactalbumin-agarose} \).

A major group of glycosyltransferases functions to transfer a glycosyl moiety from a nucleoside diphosphate glycose to an acceptor molecule that may also be a carbohydrate, releasing a nucleoside diphosphate as shown in Reaction 1.

\[
\text{Glycosyl-P-P-nucleoside + glycose} \rightarrow \text{glycosylglycose} + \text{nucleoside diphosphate}
\]

Such enzymes function in the synthesis of lactose, fructose, glycogen, starch, and the complex oligosaccharides of glycoproteins, glycolipids, and bacterial cell walls (1). Many of the enzymes are present in relatively small amounts in tissues and are associated with complex structures which make them difficult to purify. In many cases membrane-bound enzymes cannot be obtained in free form.

If sufficiently specific adsorbents with functional groups resembling either glycose, glycose or the nucleoside moieties of the substrates and products could be prepared, they should be useful for isolation of a specific transferase from a complex mixture. To test this possibility we have synthesized agarose derivatives with ligands resembling parts of the substrates of a typical galactosyltransferase and report here on their utility in the purification of the galactosyltransferase of lactose synthetase (2, 3). A preliminary report of these studies was made earlier (4).

EXPERIMENTAL PROCEDURE

Materials and Methods

Enzyme Assay—The galactosyltransferase of lactose synthetase was assayed as previously described with N-acetylglucosa.
mine as substrate unless indicated (2, 3). Glycogen synthetase activity was assayed by a similar procedure in which the transfer of glycosyl residues from UDP-[14C]glucose to glycogen was estimated as follows. A solution (50 μl) of 2 mm UDP-[14C]-glucose, 8 mm in glucose 6-phosphate, containing 1.3 mg of glycogen ml⁻¹ in 50 mM glyceraldehyde phosphate buffer, pH 8.2, containing 2 mM EDTA, and 40 mM 2-mercaptoethanol was incubated with 50 μl of the enzyme solution for 30 min at 37°C. The reaction was stopped by chilling the sample in an ice bath, diluting with 0.5 ml of water (5') and transferring the sample to a small column (0.5 ml) of Dowex 1-X8 (Cl⁻ cycle) contained in a disposable Pasteur pipette. The incubation tube was rinsed with 0.5 ml of water and the rinse applied to the column which was then washed with 1.0 ml of water. Eluent and washings were collected in a scintillation vial and then 14 ml of scintillation fluid as reported earlier (3) was added. Samples were counted at ambient temperature. Phosphorylase activity was determined as described earlier (9).

Analytical Procedures—Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Melting points are corrected. Concentration of solvents was performed on a rotary evaporator at water aspirate pressure unless otherwise specified. Optical rotations were determined with a Cary 60 recording spectropolarimeter.

Ascending paper chromatography for the organic compounds was carried out on Whatman No. 1 with isobutyrine acid-concentrated ammonium hydroxide-water (66:1:33, v/v) as Solvent 1 and isopropyl alcohol-1.3 mM aqueous acetate buffer pH 5.0 (7:3, v/v) as Solvent 2. Descending paper chromatography was performed on Whatman No. 1 with ethyl acetate-pyridine-water (10:1:4, v/v) as Solvent 3. Chromatograms were developed with a molybdic acid spray (6) for phosphates, ninhydrin (7) for amines, and silver nitrate (8) for carbohydrates.

Electrophoresis was performed on 7.5% polyacrylamide gels in sodium dodecyl sulfate by the method of Weber and Osborne (9) or by the slight modification of this method employed by Schwartz et al. (10). The latter method differs principally in that 6 M urea is added to the gel during polymerization. About 10 μg of protein were applied to each gel. Gels were stained with protein with Coomassie brilliant blue and for carbohydrate with a periodic acid-Schiff reagent (11).

Protein concentrations in impure enzyme preparations were estimated routinely by the biuret method (12) or where indicated by the Lowry method (13). The concentration of highly purified galactosyltransferase was estimated from the extinction coefficient reported earlier (3).

Chromatographic Procedures with Specific Adsorbents—All experiments were performed at 4°C unless otherwise stated. Columns were packed with a slurry of the adsorbent and allowed to settle under gravity. Prior to application of the sample, columns were washed with at least 3 bed volumes of the buffer in which the sample was contained. After use, UDP-Sepharose and N-acetylglucosamine-Sepharose were removed from the columns, washed with 3 volumes of 6 M urea (deionized) and then exhaustively washed with water or buffers saturated with chloroform. α-Lactalbumin-Sepharose was washed in the same way after use except that 2 M instead of 6 M urea was used. The latter columns were operated as described earlier (3) except that N-acetylglucosamine replaced glucose during development of the column.

Concentration of Dilute Solutions of Galactosyltransferase—The dilute solutions of enzyme obtained after chromatography on the specific adsorbents were concentrated by pressure dialysis at 4°C with a 15-cm Amicon membrane (PM-30). During concentration of the enzyme care was taken to maintain the concentration of N-acetylglucosamine in the enzyme solution at 0.005 M. As noted earlier (3) the enzyme is very unstable in the absence of its substrates but under these conditions it is stable for at least 3 months when stored at 4°C in the presence of chlo-roform. The purified enzyme after chromatography on α-lactalbumin- or N-acetylglucosamine-Sepharose begins to precipitate at concentrations greater than about 0.7 mg per ml.

Preparation of Whey—Raw, skim bovine milk (3.8 liters) was made 9 mm with respect to β-mercaptoethanol (3.0 ml) and 0.03 M with respect to EDTA (250 ml of 0.5 M EDTA, pH 7.2). The milk was then adjusted to pH 4.2 by the addition over a 10-min period of 5 M HCl (50 ml). The preparation was centrifuged in a Sorvall RC-3 centrifuge in a type HG-4L swinging bucket rotor at 5250 rpm (7000 X g) at 10°C for 10 min to remove most of the casein. The pooled supernatant solution, after filtration through glass wool, was adjusted to pH 6.7 to 6.8 with 2 M Tris, made 0.12 M with respect to magnesium chloride (75 g), adjusted again to pH 6.7 to 6.8 with 2 M Tris, and after standing for 4 to 5 hours centrifuged and as described above to remove any insoluble material. The resulting characteristically amber-colored whey was stored at 4°C in the presence of chloroform (1.0 ml) for periods of up to 2 weeks without any apparent loss of galactosyltransferase activity.

Reagents—Reagent grade organic solvents dried over 4 A molecular sieves (Fisher), were used without further purification. Tri-n-octylamine, tributylamine, diphenylchlorophosphate, ethanamine, and 1,1-carbonyldiimidazole, were purchased from Aldrich Chemical Co. N-Acetyl-D-glucosamine was purchased from Pfannstiehl Laboratories, nucleosides and nucleotides from P-L Biochemicals, ethyl trifluoroacetate from Pierce Chemical Co., and crystalline phosphoric acid from K and K Laboratories. Methyl tri-n-octylammonium hydroxide was synthesized as described earlier (14). Pancreatic α-amylase was obtained from Sigma Chemical Co.

Synthetic Procedures

6-Amino-1-hexanol Phosphate (I)—6-Amino-1-hexanol (11.7 g, 0.1 mole) was mixed with 9.8 g (0.1 mole) of crystalline phosphate, ethanolamine, and 1,1-carbonyldiimidazole, were purified by chromatography on Sephadex G-50. 

The following names will be used throughout the text for the compounds described here and the Sepharose derivates: UDP-hexanolamine refers to P1-(6-amino-1-hexyl)-P1-P1-uridine)pyrophosphate (VI). UDP-Sepharose is the derivative formed by the reaction of compound VI with cyanogen bromide-treated Sepharose 4B. Galactosyl pyrophosphate-hexanolamine refers to P1-(6-amino-1-hexyl)-P1-P1-galactopyranosyl pyrophosphate (VII). UDP-Sepharose-Sepharose is the derivative formed by reaction of VI with cyanogen bromide-treated Sepharose 4B. N-Acetylglucosamine-Sepharose is the derivative formed by reaction of 6-amino-1-hexyl-2-acetamido-2-deoxy-α-D-glucopyranoside (XII) with cyanogen bromide treated Sepharose 4B.
and the course of the reaction was monitored by spotting was adjusted intermittently to pH 9.5 with 5 N lithium hydroxide mixture was stirred vigorously with a magnetic stirrer to produce trifluoroacetate to the starting amine as judged by chromatography in Solvent 1 (product, \( R_f 0.54 \); 6-amino-1-hexanol, \( R_f 0.85 \); inorganic phosphate, \( R_f 0.45 \). In Solvent 2 the compound has \( R_f 0.35 \).

Reccrystallization from water by the addition of ethyl alcohol gave material which has m.p. 245°; yield 10.0 g, 51%. The compound has \( pK_a \) values of 2.0, 6.3, and 11.0.

\[
\text{C}_9\text{H}_{19}\text{NO}_7\text{P} (197)
\]

Calculated: C 36.55, H 8.18, N 7.10

Found: C 36.18, H 8.20, N 7.06

**N-Trifluoroacetyl-6-Amino-1-hexanol Phosphate (II)**—To 2 g of 6-amino-1-hexanol phosphate, dissolved in 20 ml of water at 0°, was added 1 ml of ethyl trifluorothiol acetate. The reaction mixture was stirred vigorously with a magnetic stirrer to produce a fine dispersion of the ethyl trifluorothiol acetate and the temperature kept below 4° with an ice bath. The reaction mixture was adjusted intermittently to pH 9.5 with 5 N lithium hydroxide and the course of the reaction was monitored by spotting an aliquot on paper, spraying with ninhydrin and heating at 100° for 5 min. After approximately 1 hour a second 1.ml portion of ethyl trifluorothiol acetate was added and the reaction was judged to be complete (45 min) when a pink color was obtained with ninhydrin. The reaction mixture was adjusted to pH 5 with trifluoroacetic acid, and then concentrated to dryness at 45°. The residue was dissolved in water and then concentrated several times to remove residual reagents and finally dissolved in 50 ml of water, cooled, and brought to pH 1.5 with trifluoroacetic acid. This solution was passed over a column containing 25 ml of Dowex 50-X8 (II+ cycle; 20 to 50 mesh) to remove unreacted amine. The acidic eluate was concentrated to dryness under a drying tube for 6 hours and then held at room temperature for 24 hours at which time the solution was chromatographed as shown in Fig. 1. The absorbance of the eluate fractions was measured at 262 nm by diluting 10 µl samples to 1.0 ml. The desired product (N-trifluoroacetyl-UDP-hexanolamine; Compound IV) accounted for approximately 70% of the starting absorbance and was eluted between tubes 70 and 110. The material in these fractions can be used for the preparation of the UDP-Sepharose adsorbent without further purification after incubation (10 to 20 pmoles per ml) at pH 11 for 4 hours at 25° to hydrolyze the trifluoroacetyl derivative to the free amine (VI).

Compound IV was isolated from the eluate and characterized as follows. Fractions were combined and adjusted to pH 8.0 with 1 N lithium hydroxide and then concentrated to dryness at 45°. The syrupy residue is extracted five to seven times by centrifugation with 50 ml of ethanol-ethyl ether (1:2, v/v) to remove lithium chloride. The resulting residue is ninhydrin-negative but gives a strong positive reaction after incubation at pH 11 to 12 for a few minutes at 25°. Its molar extinction at 262 nm is 10° based on a molecular weight of 611 for the dilithium salt. It has \( R_f 0.45 \) in Solvent 1 and 0.5 in Solvent 2. Hydrolysis with 1 N hydrochloric acid at 100° for 1 hour yields a mixture of Compound II and UMP. Hydrolysis at pH 12 gives a product that is eluted from Dowex 1 (Cl-) as a single band in the same region as UMP as shown in Fig. 1 but with a strong positive ninhydrin reaction. This product after acid hydrolysis yields a mixture of Compound I and UMP as determined by chromatography in Solvents 1 and 2.

![Absorbance vs. Fraction Number](http://www.jbc.org/fig.png)

**Fig. 1.** The purification of trifluoroacetylhexanolamine-UDP and UDP-hexanolamine by chromatography on Dowex 1 (Cl- cycle). Trifluoroacetylhexanolamine-UDP, the reaction mixture from 1 mmole of UMP was applied to a column (1 X 25 cm) of Dowex 1-X2 (Cl- cycle; 200 to 400 mesh) and eluted with 230 ml of 50% aqueous methanol and then with a linear gradient of 300 ml of 0.01 N HCl as starting solvent and 500 ml of 0.01 N HCl containing 0.4 N lithium chloride as the limit solvent. Fractions (12 ml) were collected automatically. The column was operated at 25° at a flow rate of about 60 ml per hour. The elution pattern is shown by the solid line. UDP-hexanolamine, the alkaline hydrolysisate of the trifluoroacetylhexanolamine-UDP, which was prepared as described in the text, was chromatographed exactly as described above. The elution pattern is shown by the dashed line.
The reaction of 0.22 mmole of the methyl tri-n-octylammonium solution was mixed with a 5-fold excess of Dowex 50 (H⁺ form) for 5 min and filtered. The filtrate was dried, dissolved in hesanolamine phosphate. After 1 hour at 25°C the pyridine was added to the reaction mixture and washed several times with water. To the combined filtrate and washings was added 1 molar equivalent of methyl tri-n-octylammonium chloride and then the solvents were removed in vacuo at 45°C. The residue melted from 149-158°C with decomposition; [α]D = +94° ± 4° (5.2 g per 100 ml of H₂O).

C₆H₁₃NO₃ (119.17)
Calculated: C 64.70, H 7.57
Found: C 64.52, H 7.66

Preparation of Sepharose Adsorbents—The procedure described by Caturencas (18) was used with modification as follows. Sepharose 4B (Pharmacia) was washed with 10 or more bed volumes of distilled water on a coarse sintered glass funnel. The bulk of the water was removed and the firm gel (500 g) was mixed with water (300 ml). Cyanogen bromide (from 150 to 200 mg per g of gel) was pulverized in a mortar in a hood and then added in small batches over a period of 1 to 2 min, with stirring to the gel suspension. When all of the cyanogen bromide had been added, the mixture was brought to pH 11.0 ± 0.2 by the addition of 4 N sodium hydroxide. The temperature was kept at 20°C by the addition of chilled ice. The consumption of base was rapid over a 15-min period at which time all of the cyanogen bromide was in solution. The reaction was then stopped by the addition of 400 to 500 ml of chilled ice to the mixture of ice and gel filtered with suction on a 2-liter coarse sintered glass funnel. The gel was washed during 3 to 4 min with 10 to 12 bed volumes of ice-cold water. The residual ice...
was separated from the gel and the gel quickly mixed with a solution containing 1 to 2 mmoles of the appropriate ligand in 200 to 300 ml of water at pH 10.0. The mixture was adjusted to pH 10 if necessary and the slurry stirred at 4° for 12 to 18 hours. The gel was then packed in a column and washed with water at room temperature. The ninhydrin-positive or ultraviolet-absorbing washings were collected and the amount of unreacted ligand estimated.

The procedure gave adsorbents with 1 to 6 mmoles of ligand per ml of packed, wet gel. The concentrations of the ligands in the adsorbents used in this study were estimated to be as follows. UDP-Sepharose contained 3 to 6 mmoles of UDP per ml. This was estimated on the basis of the loss of material adsorbing at 262 nm during coupling. Usually 70 to 80% of the ligand added is coupled. Estimation of the extent of substitution by acid-catalyzed hydrolysis of the pyrophosphate bond to the adsorbent is complicated by the concomitant degradation of the Sepharose to ultraviolet-absorbing material. Galactosyl pyrophosphate-Sepharose contained 2 mmoles of galactose per ml. This was estimated as the loss of organic phosphate in an aliquot of the Sepharose-free reaction mixture during coupling. Hydrolysis was performed in 0.5 N hydrochloric acid at 100° for 1 hour. Similar values were obtained by hydrolysis of the final adsorbent under the same conditions. N-Acetylglucosamine-Sepharose contained 3 to 4 mmoles of N-acetylglucosamine per ml. This was estimated as glucoseamine by ion exchange chromatography after hydrolysis of the gel with 6 N HCl at 110°. The capacity of the adsorbents and the pattern of affinity chromatography will probably be a function of the amount of the ligand per ml of adsorbent but this has not been examined thoroughly at present.

**EXPERIMENTAL RESULTS**

**Synthesis of Specific Adsorbents**

In order to test the applicability of specific adsorbents for affinity chromatography of glycosyltransferases, ligands were synthesized which were structurally related to the substrates or inhibitors of the UDP-galactose:N-acetylgalactosyltransferase of bovine milk. Three types of ligands were synthesized containing either a uridine pyrophosphate, a galactosyl pyrophosphate or an N-acetylglucosamine substituent. In each ligand one of these functional groups was linked to the hydroxyl group of 6-amino-1-hexanol. Each ligand was then coupled to cyanogen bromide-treated Sepharose through the free amino group in the hexanolamine moiety.

**Synthesis of UDP-hexanolamine and Galactosyl Pyrophosphate-Hexanolamine**—Synthesis of these two ligands involved the formation of an unsymmetrically substituted pyrophosphate with uridine or galactose and an alkyl amine. The latter moiety contained the amino group required for coupling to activated agarose particles. Two methods of synthesis that appeared to have general applicability were explored.

The anion displacement method (14), which involves activation of a phosphate group by conversion to the diphenylpyrophosphoryl derivative as shown in Fig. 2, was utilized to synthesize N-trifluoroacetyl-UDP-hexanolamine (IV) in 60% yield from UMP. The product as isolated by chromatography on Dowex 1 (Cl− cycle) was contaminated with the symmetrical compound P2P-diuridine 5′-pyrophosphate. This compound can be produced in the first step of the synthesis and its occurrence presents a major difficulty with this method. Pure material can be obtained by removing the trifluoroacetyl group by hydrolysis above pH 11.5 for 4 hours at 25° and redchromatographing the material on Dowex 1 (Cl−). The desired product has gained a positive charge compared to Compound IV and is well separated from material that elutes in the region where simple monophosphates are found, while uridine 5′-pyrophosphate is unchanged by the hydrolysis step. The purified material can be used for the preparation of affinity columns after the first Dowex 1 column without being isolated from the eluent. Fractions containing Compound IV and uridine 5′-pyrophosphate were pooled, concentrated to a convenient volume, and adjusted to pH 12.0. After 4 hours the solution is adjusted to pH 10 and used directly for coupling. Neither uridine 5′-pyrophosphate nor lithium chloride interferes with the coupling reaction.

The imidazolide method (19) provides a more convenient and more generally applicable approach to the synthesis of pyrophosphoryl ligands. The sequence of reactions is shown in Fig. 3. This procedure allows the synthesis of N-trifluoroacetyl-6-amino-1-hexanol phosphate imidazolide (III) which is apparently quite stable if protected from moisture and which reacts with phosphate or pyrophosphate esters to form the corresponding di- and triphosphates in high yield (60 to 80%). The synthesis of the 5′-uridine diphosphate and 5′-d-galactopyranosyl pyrophosphate derivatives are presented under "Experimental Procedure." The synthesis of the corresponding adenosine, guanosine, and 5′-d-glucopyranosyl derivatives has also been accomplished2; thus, the synthetic methods employed here may provide other useful specific adsorbents.

**Synthesis of 6-Amino-1-hexyl-2-acetamido-2-deoxy-β-D-glucopyranoside**—This compound was synthesized as shown in Fig. 4. N-Trifluoroacetyl-6-amino-1-hexanol was condensed with 3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl chloride in dimethylformamide solution in the presence of mercuric cyanide. The condensation product was a mixture of α and β anomers.

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2 R. Barker, unpublished studies.
Fig. 3. The synthesis of UDP-hexanolamine by the imidazolide method (19).

which were separated by fractional crystallization from ethyl acetate-hexane mixtures. Deacylation of the β anomer with barium methylate in methanol afforded the desired product as its trifluoroacetyl salt. The ligand was reacted with Sepharose 4B as described under “Experimental Procedure” to give the specific adsorbent.

Fig. 4. The synthesis of 6-amino-1-hexyl-2-acetamido-2-deoxy-β-d-glucopyranoside. DMF, dimethylformamide.

Fig. 5 shows that the transferase bound to UDP-Sepharose can be eluted by several means. EDTA, which was required to combine with manganous ions, and N-acetylglucosamine which stabilizes the enzyme (3) were used under all conditions tested. Dilute solutions of urea (1.5 M) were most effective in eluting the enzyme in a small volume. Elution with urea solutions, however, gave about half the expected yield of enzyme. Borate buffers at pH 8.5 containing EDTA, eluted the enzyme in better yields, but the enzyme emerged in a somewhat larger volume than that required for elution with urea. Although the exact basis for the action of borate has not been established it is possible that it forms an addition compound with the ribose moiety of UDP and thereby weakens the binding of the enzyme. Elution with buffers containing magnesium ions in the absence of EDTA was unsuccessful, presumably by competing with manganous ions, but the enzyme was eluted in a large volume. The most satisfactory yields of enzyme were obtained on elution with cacodylate buffers containing EDTA, despite the fact that the enzyme was
FIG. 5. Elution of the galactosyltransferase from UDP-Sepharose under different conditions. Four separate columns (0.6 X 3 cm) of UDP-Sepharose (4 to 5 pmoles of UDP per ml) were equilibrated with buffer as described in Fig. 6. Whey (50 ml) was applied to each column and the columns washed at A (arrow) with 25 ml of equilibration buffer. The columns were then washed at B (arrow) with different buffers as follows. Top, Column 1, 0.025 M sodium cacodylate (pH 7.4), containing 1.5 M urea, 0.01 M mercaptoethanol, 0.025 M EDTA, and 0.005 M N-acetylglucosamine (O); Column 2, 0.025 M sodium cacodylate (pH 7.4), containing 0.01 M mercaptoethanol and 0.025 M magnesium chloride (O). Bottom, Column 3, 0.025 M sodium tetraborate (pH 8.5), containing 0.025 M EDTA and 0.01 M mercaptoethanol (O); Column 4, 0.025 M sodium cacodylate (pH 7.4), containing 0.025 M EDTA, 0.01 M mercaptoethanol, and 0.005 M N-acetylglucosamine (O).

eluted in a larger volume than with borate buffer or buffered solutions of urea.

Based upon the results of the above preliminary studies, it was found that columns of UDP-Sepharose could be used routinely on a preparative scale to purify the enzyme from whey under the conditions shown in Fig. 6. The enzyme was adsorbed from whey containing manganous ions whereas the majority of the whey proteins passed unretarded through the column. After washing the column with buffer, the active enzyme was eluted in buffer containing EDTA and N-acetylglucosamine. Although an extremely dilute solution of enzyme is obtained, it can be concentrated readily by ultrafiltration or readily adsorbed on N-acetylglucosamine-Sepharose as described below.

The extent of purification of the transferase can be judged by polyacrylamide gel electrophoresis of appropriate fractions in sodium dodecyl sulfate. Unfortunately, as noted earlier (3), the transferase will not give sharp, discrete bands on electrophoresis under nondenaturing conditions. Fig. 7 shows the electrophoretic pattern of whey, of the proteins in whey not adsorbed to UDP-Sepharose and the proteins adsorbed to UDP-Sepharose. Recrhomatography of the adsorbed proteins did not result in further purification.

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FIG. 6. The purification of the galactosyltransferase from whey with UDP-Sepharose. A column (3 X 15 cm) of UDP-Sepharose (4 to 5 pmoles of UDP per ml) was equilibrated at 5° with 0.025 M sodium cacodylate buffer, pH 7.4 containing 0.025 M MnCl2 and 0.01 M mercaptoethanol. Whey (1.8 liters) was applied to the column at a flow rate of about 100 ml per hour at 5°. The column was then washed at A (arrow) with 1 liter of the equilibration buffer. The enzyme was then eluted by washing the column at B (arrow) with 0.025 M sodium cacodylate, pH 7.4, containing 0.025 M EDTA, 0.01 M mercaptoethanol, and 0.005 M N-acetylglucosamine. The column was washed and eluted at about 100 ml per hour. Fractions (25 ml) were collected automatically. Absorbance at 280 nm (O). Galactosyltransferase activity (O).

Chromatography on N-Acetylglucosamine-Sepharose.—It was found that the galactosyltransferase in whey or in partially purified preparations from UDP-Sepharose could be effectively adsorbed to N-acetylglucosamine-Sepharose. It was also observed, however, that the binding of the transferase to this adsorbent is enhanced considerably by UDP and manganous ions, as shown in Fig. 8. Essentially identical results were obtained when UMP was used instead of UDP.

Fig. 9 shows the effectiveness of N-acetylglucosamine, borate buffer and buffered solutions of urea in eluting the transferase from N-acetylglucosamine-Sepharose. Urea and borate are much more effective than N-acetylglucosamine for elution of the enzyme, but the yields of enzyme appear to be somewhat lower. In addition, as shown by electrophoretic analyses in Fig. 10, urea and borate also appear to elute proteins which are not found on elution with N-acetylglucosamine. Thus, the most suitable conditions for chromatography of the transferase utilize UMP and Mn++ during adsorption of the enzyme and buffers containing N-acetylglucosamine and EDTA for elution of the enzyme. The use of N-acetylglucosamine-Sepharose for purification of the transferase under these conditions is shown in Fig. 11.

Chromatography on Galactosyl Pyrophosphate-Hexanolamine-Sepharose.—This adsorbent was completely ineffective in binding the transferase when tested under a variety of conditions. Neither the enzyme in whey nor in preparations partially purified on UDP-Sepharose was adsorbed to a significant extent at pH 7.4 in either the presence or absence of manganous ions, N-acetyl-
Fig. 7. Gel electrophoretic patterns of fractions from chromatography of whey on UDP-Sepharose. Appropriate fractions from the chromatogram in Fig. 5 were analyzed electrophoretically on gels containing sodium dodecyl sulfate by the method of Schwartz et al. (10). Gel 1, whey; Gel 2, proteins from whey not adsorbed to UDP-Sepharose (pooled fractions between 0.05 and 2 liters in Fig. 5); Gel 3, proteins from whey adsorbed to UDP-Sepharose (pooled fractions between 2.9 and 6 liters in Fig. 5); Gel 4, the galactosyltransferase obtained by affinity chromatography of the proteins adsorbed to UDP-Sepharose on a column of α-lactalbumin-Sepharose. The latter column was operated as described in Table I. M.W., molecular weight.

Fig. 8. The effect of UDP on the binding of the galactosyltransferase to N-acetylglucosamine-Sepharose. Two columns (0.6 X 3 cm) of N-acetylglucosamine-Sepharose at 5° were equilibrated with 0.025 M sodium cacodylate, pH 7.4, containing 0.001 M mercuriethanol, 0.025 M manganese chloride, and 5 X 10^{-4} M UMP. A solution (30 ml) of partially purified enzyme from a UDP-Sepharose column as described in Fig. 5 and dialyzed against the equilibration buffer was applied to each column at 5°. After the enzyme was applied, the columns were washed with 200 ml of the equilibration buffer at 5° and then one of three buffers at 0°. Columns 1 and 2 were eluted with 0.025 M sodium cacodylate, pH 7.6, containing 1.5 M urea, 0.005 M N-acetylglucosamine, 0.025 M EDTA, and 0.001 M mercaptoethanol (○). Column 2 was eluted with 0.025 M sodium tetraborate, pH 8.3, containing 0.025 M EDTA and 0.001 M mercaptoethanol (■). Column 3 was eluted with 0.025 M sodium cacodylate, pH 7.6, containing 0.001 M mercaptoethanol and 0.005 M N-acetylglucosamine (●). Fractions (10 ml) were assayed for transferase activity.

Fig. 9. Elution of galactosyltransferase from N-acetylglucosamine-Sepharose. Three columns (2.2 X 10 cm) of N-acetylglucosamine-Sepharose were equilibrated with 0.025 M sodium cacodylate, pH 7.4, containing 0.001 M mercaptoethanol, 0.025 M manganese chloride, and 5 X 10^{-4} M UMP. A solution (200 ml) of partially purified enzyme from a UDP-Sepharose column as described in Fig. 5 and dialyzed against the equilibration buffer was applied to each column at 5°. After the enzyme was applied, the columns were washed with 200 ml of the equilibration buffer at 5° (arrow) and then with one of three buffers at 0° (arrow). Column 1 was eluted with 0.025 M sodium cacodylate, pH 7.6, containing 1.5 M urea, 0.005 M N-acetylglucosamine, 0.025 M EDTA, and 0.001 M mercaptoethanol (○). Column 2 was eluted with 0.025 M sodium tetraborate, pH 8.3, containing 0.025 M EDTA and 0.001 M mercaptoethanol (■). Column 3 was eluted with 0.025 M sodium cacodylate, pH 7.6, containing 0.001 M mercaptoethanol and 0.005 M N-acetylglucosamine (●). Fractions (10 ml) were assayed for transferase activity.

glucosamine, uridine, or UMP. Small amounts of the whey proteins or partially purified transferase were retarded by this adsorbent but because no purification was observed, the adsorbed enzyme may have been bound only through nonspecific ionic interactions.

Purification of Galactosyltransferase from Whey by Affinity Chromatography—On the basis of the behavior of the galactosyltransferase with the specific adsorbents described here it was possible to devise a purification of the transferase from whey based solely on affinity chromatography. This procedure is summarized in Table I. The enzyme from whey was adsorbed onto, and eluted from a column of UDP-Sepharose (Step 3) as described in Fig. 6. Because it has a much larger capacity for the enzyme than the other adsorbents, UDP-Sepharose seemed best suited for adsorbing the enzyme from whey, although it is less specific than the other adsorbents. α-Lactalbumin-Sepharose and N-acetylglucosamine-Sepharose are also capable of adsorbing enzyme directly from whey but are best used at later stages of the purification procedure. As shown in Table I, the enzyme from the UDP-Sepharose column can be purified further on a column of α-lactalbumin-Sepharose. The enzyme obtained at this step has a constant specific activity on rechromatography on any of the specific adsorbents and gave the gel pattern shown in Fig. 7. It is noteworthy that this transferase preparation from α-lactalbumin-Sepharose columns with a constant specific activity gives two bands on the sodium dodecyl sulfate gels pre-then applied to each column. One enzyme solution also contained 2.5 X 10^{-4} M UDP as indicated in the figure by the closed circles. After the enzyme was applied the columns were washed with buffered urea solutions (arrow) as described in Fig. 9. Fractions (1 ml) were collected and assayed for transferase activity. Enzyme applied in the absence and presence of UDP are indicated by the open and closed circles, respectively.
pared by the method of Schwartz et al. (10). The major band gives an average molecular weight of about 51,000, and the minor band a molecular weight of about 43,000. In contrast, the same preparation gives three distinct bands as noted below (Fig. 12) when analyzed by the method of Weber and Osborne (9).

N-Acetylglucosamine-Sepharose can also be used on a preparative scale to purify the transferase. This is indicated by the results in Table II which summarizes the purification of the enzyme from whey. After the enzyme was partially purified with UDP-Sepharose (Step 3) it was then applied to a column of N-acetylglucosamine-Sepharose (Step 4) as shown in Fig. 11. Clearly, this specific adsorbent is not as effective as α-lactalbumin-Sepharose since the specific activity of the enzyme from Step 4 increased slightly after rechromatography on N-acetylglucosamine-Sepharose (Step 5a) or on α-lactalbumin-Sepharose (Step 5b).

It is noteworthy that either α-lactalbumin-Sepharose or N-acetylglucosamine-Sepharose can adsorb the enzyme from very dilute solutions, such as those obtained after elution of the enzyme from UDP-Sepharose (Fig. 6), however, better adsorption is obtained if the dilute solutions are concentrated about 5-fold before being applied to these specific adsorbents. The binding constants for the enzyme to these two adsorbents have not been determined and the relationship between concentration of enzyme and binding to these adsorbents cannot be stated quantitatively at present.

Properties of Galactosyltransferase Purified by Affinity Chromatography

Repeated chromatography of the galactosyltransferase on α-lactalbumin-Sepharose as described in Table I does not in-
increase the specific activity of the transferase. The specific activity of different preparations from α-lactalbumin-Sepharose columns varies from about 11 to 15 units per mg (3) but the preparations are indistinguishable electrophoretically and in amino acid composition. It appears, however, that the transferase purified solely by affinity chromatography (Tables I and II) is heterogeneous in size as shown by the electrophoretic analyses in sodium dodecyl sulfate (Fig. 7). The gel patterns given by preparations purified on either α-lactalbumin-Sepharose or N-acetylglucosamine-Sepharose are compared in Fig. 12 with those of the enzyme prepared by the method of Trayer and Hill (3). These gels show that the enzyme prepared solely by affinity chromatography contains at least three different species with approximate molecular weights of 54,000, 49,000, or 43,000. Three species are also noted in the enzyme prepared by N-acetylglucosamine-Sepharose affinity chromatography, but the species with a molecular weight of 54,000 appears to be present in larger amounts. Chromatography of this preparation on α-lactalbumin-Sepharose columns reduced the amount of the higher molecular weight species to give a pattern essentially identical to that on Gel 3 in Fig. 12 and the specific activity increased slightly. In contrast, a preparation of enzyme prepared by the method of Trayer and Hill (3) gave only one major band with a molecular weight of about 45,000, and three bands in minor amounts which do not correspond to other species of the transferase.

The basis for the differences among these species is unknown, but the amino acid composition of the enzyme prepared as described in Table I and containing three components is indistinguishable from that reported earlier (3) for a species of 42,000 molecular weight. In addition, these compositions are indistinguishable from those for each of the bands shown in Fig. 12 (these compositions were obtained by analysis of acid hydrolysates of gel slices containing the appropriate band as described earlier (10)). It is possible that the three species differ in carbohydrate content although each species on a gel gives stains positively for carbohydrate with the periodic acid-Schiff reagent (11).

Conversion of N-Acetylglucosamine-Sepharose to N-Acetyllactosamine-Sepharose by Galactosyltransferase

N-Acetylglucosamine-Sepharose proved to be an acceptor substrate for the galactosyltransferase. The adsorbent (1.5 ml) was saturated with enzyme as shown in Fig. 9, washed with 0.025 M cacodylate buffer, pH 7.4, containing 0.02 M manganese chloride and 0.02 M mercuric chloride, and then incubated with 25 μmoles of UDP-[14C]galactose (175,000 cpm total) in a volume of 2.5 ml at 37°C for 45 min. After the incubation, the supernatant solution contained 142,000 cpm, which is equivalent to 20 μmoles of UDP-[14C]galactose. The supernatant also contained synthetase activity approximately 4 times that in the supernatant before incubation. The adsorbent was packed in a small column, as described in Table I.

Table II

| Steps         | Volume | Concentration | Activity | Total activity | Yield | Specific activity | Purification |
|---------------|--------|---------------|----------|----------------|-------|------------------|--------------|
|               | ml     | mg/ml         | unit/ml  | Total          | %     | Step             |              |
| 1. Skim milk  | 7270   | 35.5          | 0.0882   | 601.8          | 100   | 100              | 0.0023       |
| 2. Whey       | 5860   | 3.72          | 0.0066   | 386.8          | 64.3  | 64.3             | 0.018        |
| 3. UDP-Sepharose | 600  | 0.185         | 0.0406   | 243.4          | 40.4  | 62.9             | 2.19         |
| 4. NAG-Sepharose | 218 | 0.089         | 0.0068   | 132.5          | 22.0  | 54.4             | 6.83         |
| 5a. NAG-Sepharose | 68  | 0.057         | 0.0051   | 35.0           | 17.5  | 79.9             | 9.04         |
| 5b. α-Lactalbumin-Sepharose | 80  | 0.041         | 0.0047   | 38.0           | 18.9  | 80.7             | 11.6         |

* A unit is 1 μmole of galactose incorporated per min.
* Total activity is activity times volume and is expressed in units.
* Specific activity is expressed in units per mg of protein.
* One-third of the active fraction from Step 4 was applied to a second NAG-Sepharose column, and one-third to an α-lactalbumin-Sepharose column, as described in Table I. NAG is an abbreviation for N-acetylglucosamine.
column and washed with 200 bed volumes of water. The eluent was checked for radioactivity and none was found after 20 bed volumes had passed through the column. The adsorbent was then dispersed in an equal volume of 1 N hydrochloric acid and heated at 145° for 20 min to give a slightly amber solution. Aliquots were counted and the whole sample was calculated to contain approximately 37,000 cpm, equivalent to 5.0 μmoles of UDP[14C]glactose, or 3.3 μmoles of galactose incorporated per ml of resin. This value is in close agreement with the amount of N-acetylglucosamine estimated to be contained in the adsorbent.

When N-acetylglucosamine and UDP[14C]galactose are both added to the enzyme adsorbed to N-acetylglucosamine-Sepharose, the incorporation of [14C]galactose into the adsorbent and the release of enzyme into the medium are both inhibited. The conversion of the unbound N-acetylglucosamine to N-acetyllactosamine was obtained in good yield.

**UDP-Sepharose for Purification of Glycogen Synthetase**

In order to test whether another UDP-glycosyltransferase could be purified with the aid of UDP-Sepharose, studies were performed with glycogen synthetase. The partially purified enzyme (44-fold) for these experiments was obtained from rabbit muscle by differential centrifugation as described earlier (20). This was the glycogen-rich fraction containing 70 to 80% of the synthetase activity of the muscle. The impure preparation was treated with pancreatic α-amylase to reduce the glycogen content and then chromatographed as shown in Fig. 13. A small amount of synthetase emerged unretarded from this column and was associated with opalescent material. Inactive protein was washed from the column after the sample was applied with buffer containing EDTA. Synthetase activity did not appear during washing but was eluted only when glycogen was added to the wash buffer. In a separate experiment, the synthetase activity was not removed from the column on washing with buffer containing 10 mM UDP. Analysis of the synthetase from the column by gel electrophoresis in sodium dodecyl sulfate revealed one major band with an apparent molecular weight of about 90,000. No attempts were made to purify further the synthetase on this column or to assess its chemical and physical properties. The synthetase was, however, free of glycogen phosphorylase activity.

**DISCUSSION**

The synthesis of agarose derivatives useful in enzyme purification can frequently be accomplished by reacting a suitable ligand such as a substrate or inhibitor containing an amino function with cyanogen bromide activated agarose as described by Porath and Axon (21), and Cuatrecasas (18). In some instances a ligand attached directly to the agarose matrix is ineffective and it is necessary to allow it to extend from the matrix through a linear side chain and thereby enhance the affinity of the enzyme (22). Ligands without amino functions can be attached by a variety of methods (18) but in all cases the derivatization involves reaction of agarose with an amine as an initial event. Of the ligands of interest in this study only some of the nucleoside bases have an amino function capable of reaction with activated agarose and reaction of these would not only mask groups that may be essential for binding the enzyme but also place ligands very close to the agarose matrix. The syntheses described here were designed to generate ligands with a side chain containing a terminal amino group suitable for direct condensation with cyanogen bromide-activated agarose and of sufficient length to decrease steric interference for binding of proteins. Direct coupling of a previously formed ligand of suitable structure also assures that the agarose derivative possessed a single kind of functional group. Synthesis of adsorbents by reaction of a ligand with a preformed arm on the agarose may lead to mixed function adsorbents because of incomplete reactions. Such adsorbents would have less specificity because of nonspecific adsorption, particularly by ionic interactions.

Unfortunately, attempts to synthesize UDP-hexanolamine by this method were unsuccessful. The imidazole method (19) also gave adequate yields of both UDP-hexanolamine and the galactosylpyrophosphate-hexanolamine and appears to be the more generally applicable method. The N-trifluoroacetyl-6-amino-1-hexanol phosphate imidazolide can react with many other phosphate derivatives and has been used successfully for synthesis of GDP- and ADP-hexanolamine. It is anticipated that the specific adsorbents of the type used in this study can be applied generally for the purification of a wide variety of enzymes. In principle, any enzyme with a uridine phosphate derivative as a substrate or a product could be purified with the aid of UDP-Sepharose. Many enzymes of this type have $K_m$ or $K_I$ values for uridine derivatives of the same order of magnitude as those for the galactosyltransferase studied here. UDP and UDP-glucose, two inhibitors of the transferase, have $K_I$ values of $7 \times 10^{-3}$ M (23, 24) and $10^{-4}$ M (25), respectively, and the $K_m$ for UDP-galactose, its normal

![Fig. 13. The glycogen-rich fraction containing glycogen synthetase (20) from 600 g of rabbit muscle was incubated with pancreatic α-amylase (2 mg) in 50 ml of 0.05 μM glycerol phosphate buffer, pH 7.8, containing 0.004 μM EDTA, 0.04 μM mercaptoethanol, and 0.005 μM sodium chloride. After incubation at 37° for 2 hours the digest was centrifuged at 30,000 × g and the resulting supernatant solution was applied to a column (1 × 10 cm) of UDP-Sepharose at 25°. After the sample was applied the column was washed with a (arrow) with the glycerol phosphate buffer (500 ml) and then with the same buffer containing glycogen (20 mg per ml) at B (arrow). The adsorbance is indicated by the solid line and the glycogen synthetase activity by the dashed line.](http://www.jbc.org/)

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substrate, is about $6 \times 10^{-2}$ m (23, 24). Thus, especially low $K_m$ or $K_I$ values are not necessary for effective binding. It should be noted, however, that the galactosyltransferase is not bound by the galactosyl pyrophosphate-Sepharose. This suggests that the major binding energy of UDP-galactose is contributed by the UDP moiety. In addition, the enzyme is poorly bound to N-acetylgalactosamine-Sepharose, in accord with the observation that the $K_m$ for N-acetylglucosamine is only about $5 \times 10^{-3}$ m. It has been shown, however, that the $K_m$ for N-acetylglucosamine is decreased by UDP-galactose (23), and as expected, the transferase is adsorbed effectively by N-acetylgalactosamine-Sepharose in the presence of UDP or UMP. Thus, it would appear possible in the case of glycosyltransferases to effect considerable purification, first on a specific adsorbent with a ligand resembling the nucleotide portion of the substrate and then on another adsorbent containing a ligand resembling the acceptor substrate. Studies to test this possibility for other glycosyltransferases are now in progress. The preliminary studies with glycosyltransferase reported here indicate that in some cases successful purification can be achieved.

The fact that N-acetylgalactosamine-Sepharose serves as an acceptor substrate for the galactosyltransferase is of interest in three major respects. First, the N-acetylgalactosamine-Sepharose that is formed on reaction of the enzyme saturated adsorbent with UDP-galactose, should serve as an effective specific adsorbent for other transferases which use N-acetylgalactosamine as a substrate. Secondly, it should be possible to synthesize polysaccharides of known sequence beginning with an appropriate monosaccharide-Sepharose derivative if other glycosyltransferases are available.

Further insight into the nature of the galactosyltransferase of lactose synthetase has also been obtained from the studies presented here. Although it is evident that the specific adsorbents can be employed to obtain a highly purified transferase it is of interest that the properties of the adsorbents reflect the order of binding as well as the interactions among the transferase substrates. Earlier studies (23, 24) have shown that the transferase acts by an ordered mechanism and that it must bind its substrates in the sequence mannose ion, UDP galactose, and N-acetylgalactosamine for catalysis to occur. The enhancement of the binding of enzyme to UDP-Sepharose by manganese ions supports this mechanism. In addition, the fact that UDP and manganese ions enhance the binding of enzyme to N-acetylgalactosamine-Sepharose agrees with the observed kinetic interactions and supports the view that several so-called dead end complexes can occur (23) in addition to those formed with $\alpha$-lactalbumin (3). More precise studies must be made with these adsorbents, however, to obtain the quantitative relationships for substrate and product interactions.

The fact that the galactosyltransferase purified solely by affinity chromatography appears to be heterogeneous in size also raises problems for future studies. It is of interest, however, that earlier methods for purification of the transferase (3) gave one major species with a molecular weight of about 42,000. In contrast, at least three species with apparent molecular weights of about 54,000, 49,000, and 42,000, respectively, are clearly observed on purifying the enzyme solely by affinity chromatography. It is probable that species with molecular weights greater than 42,000 were removed on purification by the methods reported earlier. In this respect, it is of interest that the transferase prepared by methods which do not employ affinity chromatography is also reported to be homogeneous with a molecular weight of about 44,000 but containing only about 5% carbohydrate (27). Each species isolated as described here appears to be closely related since each has an amino acid composition very similar to one another as well as to that reported earlier (3) for the species of 42,000 molecular weight. In addition, the species of 42,000 molecular weight has essentially the same specific activity and $K_m$ for UDP-galactose and N-acetylglucosamine as preparations which contain three species. Magee et al. (28) have also observed two distinct species of the transferase after electrophoresis under non-denaturing conditions. Both species were enzymically active and differed slightly in molecular weight. Since each species purified as described here is a glycoprotein, they may vary in carbohydrate content. It is also possible that they are the result of limited proteolysis; however, further studies will be required to establish the exact basis for the heterogeneity. Isolation and characterization of the enzyme directly from mammary glands may shed considerable light on this problem.

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