THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 261, No. 12, Issue of April 25, pp. 5249-5255, 1986
Printed in U.S.A.

Purification and Characterization of UDP-*N*-Acetylgalactosamine: Polypeptide *N*-Acetylgalactosaminyltransferase from Bovine Colostrum and Murine Lymphoma BW5147 Cells*

(Received for publication, December 2, 1985)

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UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase has been purified from two sources. A soluble form, purified 517,000-fold to homogeneity from bovine colostrum, has a molecular mass of 70,000 daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 69,000 daltons by gel filtration. A membrane-bound form, partially purified 2,500-fold from BW5147 mouse lymphoma cells, has a molecular mass of 70,000 daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 71,500 daltons by gel filtration. The purified colostrum enzyme exhibits specificity for UDP-GalNAc, has its pH optimum between pH 7.2 and 8.6, and requires Mn^{2+} for activity. The K_m is 8 μM for UDP-GalNAc and 2.5 mg/ml for deglycosylated bovine submaxillary mucin.

Treatments with endo-β-*N*-acetylglucosaminidases H and F indicate that the colostrum enzyme is a glycoprotein containing two *N*-linked oligosaccharides. On most enzyme molecules, both oligosaccharides are of the complex type, but some molecules contain one complex type and one high mannose type. Antibodies raised against homogenous bovine enzyme cross-react on immunoblots with a single protein of 71,000 daltons in the partially purified preparation and in a crude microsomal extract from BW5147 cells.

A variety of proteins, including mucins, other secretory proteins, and integral membrane proteins, contain *O*-glycosidically linked oligosaccharides (see Ref. 1 for a recent review). In contrast to Asn-linked oligosaccharide synthesis which is initiated by the *en bloc* transfer of a preassembled oligosaccharide from a lipid carrier to the nascent protein (2), *O*-linked oligosaccharide synthesis begins with the transfer of *N*-acetylgalactosamine from its nucleotide sugar donor to serine or threonine residues on the protein (3). Additional sugars may then be added, one at a time, to form a great variety of oligosaccharide structures. The enzyme UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase has a key role in *O*-linked glycosylation, catalyzing the first step in the assembly of these structures. Although a number of kinetic studies have been performed with partially purified enzyme preparations from bovine, ovine, and porcine submaxillary glands, the enzyme has not been fully characterized (4–8). More recently, Sugiura *et al.* (9), using affinity chromatography on apomucin-Sepharose, reported a purification of the transferase to near homogeneity from ascites hepatoma AH66 cells. A number of the properties of this enzyme were studied.

In this paper we have used a slight modification of the procedure of Sugiura *et al.* to purify the soluble *N*-acetylgalactosaminyltransferase of bovine colostrum to electrophoretic homogeneity. Our preparation differs from the ascites hepatoma enzyme in several aspects. We also report a 2500-fold purification of an intracellular membrane-bound form of the enzyme from murine lymphoma BW5149 cells and describe an antibody raised against the purified colostrum transferase that cross-reacts with the intracellular murine lymphoma BW5149 enzyme.

EXPERIMENTAL PROCEDURES

RESULTS[1]

The procedures used to purify the *N*-acetylgalactosaminyltransferase from bovine colostrum and BW5147 lymphoma cells are described in the Miniprint Section. The following sections deal with some of the properties of the enzymes.

Enzymatic Properties of the Bovine Colostrum Transferase

Donor Specificity—The purified *N*-acetylgalactosaminyltransferase is highly specific for UDP-GalNAc, as shown in Fig. 6. Of the nucleotide sugars tested, only UDP-GalNAc competed effectively with the radiolabeled sugar nucleotide under the conditions used. A 50% decrease in counts incorporated was achieved at an added nucleotide concentration of ~18 μM similar to the theoretically expected value of 15 μM, the donor concentration used in the assay.

Effect of Substrate Concentration—The *N*-acetylgalactosaminyltransferase has an apparent K_m for UDP-GalNAc of 8 μM. The concentration of apomucin required for half-maximal

* This investigation was supported in part by Grant R01 CA08759 from the United States Public Health Service and by the Monsanto/Washington University Biomedical Research Grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] Portions of this paper (including "Experimental Procedures," part of "Results," Table I, and Figs. 1–8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3923, cite the authors, and include a check or money order for $8.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

reaction velocity was ~2.5 mg/ml. The acceptor concentration is expressed as mg of protein/ml rather than M due to the heterogeneity in the apomucin preparation.

pH Dependence—The assays to determine the pH optimum were performed in the absence of added Mn^{2+}, since this cation caused precipitates at pH >8. Three different buffers were used covering a pH range from 5.0 to 10.6. Under these conditions the purified enzyme was found to have a broad pH optimum between 7.2 and 8.6 (Fig. 7).

Ion Dependence—The purified transferase is dependent on divalent cations (Table II). The presence of a 0.5 mM EDTA completely inhibited the enzyme. This effect was suppressed by performing the assay in the presence of excess Mn^{2+}. In the absence of added Mn^{2+}, the enzyme retained ~60% of its activity, indicating that it contains some endogenous divalent cations (Fig. 8). Therefore, when the effect of different cations was measured, the enzyme was preincubated in 0.5 mM EDTA to chelate any endogenously bound cations. Table II shows that of the 9 cations tested, Mn^{2+} was the most effective in restoring the activity. Co^{2+} was almost as effective as Mn^{2+}, and Cd^{2+} and Ni^{2+} were able to restore the activity partially. Fig. 8 shows that maximum enzyme activity is reached at ~2.5 mM Mn^{2+}.

Physical Properties of the Bovine Colostrum Transferase

Amino Acid Composition—The amino acid composition of the bovine colostrum enzyme is shown in Table III. Since the analytical method used deaminates asparagine and glutamine, the values for aspartic acid and glutamic acid include also asparagine and glutamine, respectively. The overall composition agrees fairly well with the average composition of proteins as defined by Dayhoff (20). The major differences are in glycine, glutamic acid, and cysteine contents which are 208, 65, and 62%, respectively, of the average values.

Molecular Size—Comparison of the Sephadex G-100 superfine elution position of the purified *N*-acetylgalactosaminyltransferase with the elution positions of 5 protein standards yielded a molecular size of approximately 69,000 daltons (Fig. 4*A*). This is in good agreement with the molecular size of 70,000 daltons calculated from the migration distance of the enzyme on SDS-PAGE (Fig. 5*A*).

Evidence for N-Linked Glycosylation—Studies using affinity chromatography on various lectin columns indicated that the purified *N*-acetylgalactosaminyltransferase probably contained *N*-linked oligosaccharide units. The protein bound to and could be partially eluted with the appropriate hapten sugars from concanavalin A-Sepharose and lentil lectin-Sepharose (data not shown). When the ^{125}I-labeled enzyme was treated with endo-β-*N*-acetylglucosaminidases H and F and analyzed by SDS-PAGE[2]/autoradiography, a distinct shift in migration position was observed for the endo F-treated material (Fig. 9, *lanes 3, 4, 8,* and *9*). The magnitude of the shift corresponds to a M_r difference of approximately 5000. By contrast, most of the material was resistant to endo H with only a small fraction shifting to a lower M_r (Fig. 9, *lanes 2* and *7*). This pattern was not altered by increasing the incubation time and/or the amount of added endo H (data not shown). The faint 60,000-dalton band is a contaminant, most likely immunoglobulin heavy chain, present in the preparation used for these experiments.

TABLE II

Effect of cations on the activity of bovine colostrum UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase

The standard assay system and 10 milliunits of enzyme was used (see "Experimental Procedures"). Assay time was 10 min. Endogenous Mn^{2+} was removed from the purified enzyme by preincubation in 0.5 mM EDTA. All ions were added as chlorides to a final concentration of 10 mM.

Assay system	*pmol transferred*	*% of complete*
Complete	111	100
+EDTA, $-Mn^{2+}$	0	0
+EDTA, $+Mn^{2+}$	111	100
+EDTA, $+Co^{2+}$	96.4	87
+EDTA, $+Ca^{2+}$	3.6	3.3
+EDTA, $+Mg^{2+}$	8.5	7.7
+EDTA, $+Cd^{2+}$	50.0	45.1
+EDTA, $+Zn^{2+}$	0	0
+EDTA, $+Cu^{2+}$	12.3	11.1
+EDTA, $+Hg^{2+}$	0	0
+EDTA, $+Ni^{2+}$	33.6	30.3

TABLE III

Amino acid composition of bovine colostrum UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase

Values are based on replicate analyses of 24, 48, and 72 h, extrapolated to zero hydrolysis time and rounded to the nearest integer.

Amino acid	*residues/mol*[a]
Aspartic acid[b]	47
Threonine	27
Serine	55
Glutamic acid[c]	68
Proline	27
Glycine	83
Alanine	31
Cysteine[d]	8
Valine	29
Methionine[e]	8
Isoleucine	21
Leucine	35
Tyrosine	14
Phenylalanine	15
Histidine	12
Lysine	24
Arginine	22
Tryptophan	ND[f]

[a] Values corrected for 7% carbohydrate content.
[b] Includes asparagine.
[c] Includes glutamine.
[d] Determined as cysteic acid after performic acid oxidation.
[e] Determined as methionine sulfone after performic acid oxidation.
[f] ND, not determined.

Immunological Properties of the Transferase from Bovine Colostrum and Murine Lymphoma BW5147 Cells

Characterization of Antiserum—Rabbit antibodies raised against purified bovine colostrum *N*-acetylgalactosaminyltransferase precipitate transferase activity from a partially purified (Apomucin Sepharose II eluate) preparation of the colostrum enzyme (Fig. 10*A*). In this experiment, 72 μg of IgG (corresponding to 50 μl of antiserum) added to an incubation mixture containing 69 units of *N*-acetylgalactosaminyltransferase precipitated 81% of the activity or approximately 30 ng of transferase (based on a specific activity of 1860 units/mg). A purified IgG fraction was used rather than whole antiserum since rabbit serum contains interfering *N*-acetylgalactosaminyltransferase activity. When titrating the antiserum with purified ^{125}I-labeled bovine colostrum trans-

[2] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; α-MEM, minimal essential medium; Endo F and H, endo-β-*N*-acetylglucosaminidase F and H, respectively; l, liter.

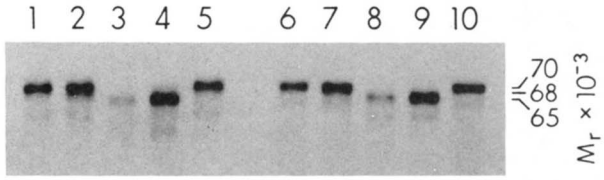

FIG. 9. **Endo-β-*N*-acetylglucosaminidase treatment of bovine colostrum *N*-acetylgalactosaminyltransferase.** 2.2 μg of purified bovine colostrum transferase in 20 μl of buffer G was iodinated with 50 μCi of $Na^{125}I$ in a tube coated with 10 μg of Iodogen at 4 °C for 30 min. Unreacted iodine was removed by gel filtration on a Sephadex G-25 column equilibrated with 20 mM imidazole, pH 7.2, 150 mM NaCl, 1 mg/ml bovine serum albumin, 0.1% taurodeoxycholate, 1 mg/ml KI followed by extensive dialysis against 20 mM imidazole, pH 7.2, 150 mM NaCl, 0.1% taurodeoxycholate. Aliquots of the iodinated enzyme (45 ng, ~100,000 cpm) were digested with endo H or endo F and analyzed on SDS-PAGE followed by autoradiography as described under "Experimental Procedures." Samples loaded on the gel contained 3600–4800 cpm. Autoradiographic exposure time was 5 h. The migration positions of molecular weight standards are indicated to the *right*. *Lanes 1, 5, 6,* and *10*, transferase incubated with endo H buffer but without endo H. *Lanes 2* and *7*, SDS-denatured (see "Experimental Procedures") transferase digested with endo H. *Lanes 3* and *8*, native transferase digested with endo F. *Lanes 4* and *9*, SDS-denatured transferase digested with endo F. *Lanes 1* through *5*, samples run under nonreducing conditions. *Lanes 6* through *10*, samples run under reducing conditions.

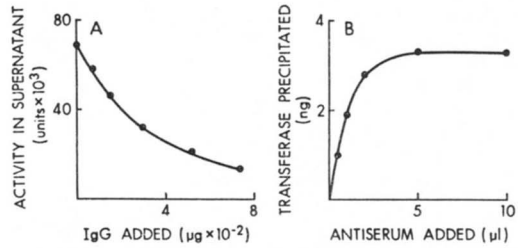

FIG. 10. **Immunoprecipitation of bovine colostrum *N*-acetylgalactosaminyltransferase.** *A*. 68 milliunits of purified bovine colostrum *N*-acetylgalactosaminyltransferase was incubated with increasing amounts of anti-*N*-acetylgalactosaminyltransferase IgG in PBS for 18 h on ice in a final volume of 50 μl. 100 μl of a 1:1 suspension of protein A-Sepharose in PBS was then added and the incubation continued for an additional 50 min on ice during which time the tube was vortexed every 5 min. Following incubation, the gel was sedimented at 10,000 × *g* and the supernatant was assayed for remaining *N*-acetylgalactosaminyltransferase activity. All values have been corrected for nonspecific adsorption. *B*, 4 ng (~9000 cpm) of ^{125}I-conjugated bovine colostrum *N*-acetylgalactosaminyltransferase was incubated with increasing amounts of anti-*N*-acetylgalactosaminyltransferase antiserum for 18 h on ice. Final volume was adjusted to 50 μl with PBS, and the incubation mixture also contained 0.1% taurodeoxycholate. 100 μl of a 1:1 suspension of protein A-Sepharose in PBS was added to precipitate antigen-antibody complexes and the incubation was continued on ice for 30 min with vortexing every 5 min. The gel was then sedimented at 10,000 × *g* for 5 min, washed 3 times with PBS containing 0.1% taurodeoxycholate, and counted. Radioactivity precipitable with preimmune serum has been subtracted from each value.

ferase, approximately 1.9 ng of transferase protein could be precipitated per μl of antiserum (Fig. 10*B*). More than 95% of the radioactivity in the ^{125}I-labeled enzyme preparation was precipitable with trichloroacetic acid.

Cross-reactivity between Bovine Colostrum Transferase and the Intracellular Enzyme of Murine Lymphoma BW5147 Cells—When the purified colostrum transferase was subjected to SDS-PAGE followed by immunoblotting with antiserum raised against this enzyme, a single band with a M_r of approximately 70,000 was observed (Fig. 11, *lane 3*). This was expected, based on the previous characterization of the purified

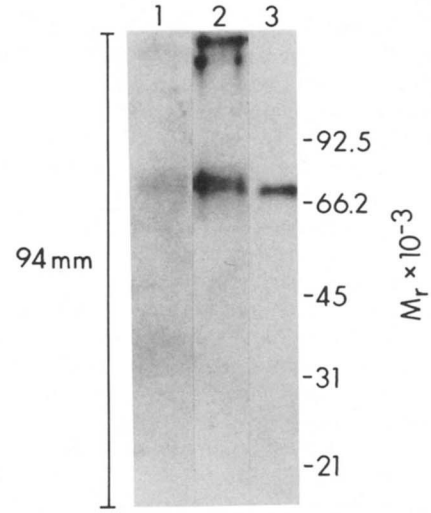

FIG. 11. **SDS-polyacrylamide gel electrophoresis and immunoblotting of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase from bovine colostrum and murine lymphoma BW5147 cells.** Triton X-114 extracts from microsomes isolated from murine lymphoma BW5147 cells, partially purified *N*-acetylgalactosaminyltransferase from murine lymphoma BW5147 cells, and purified *N*-acetylgalactosaminyltransferase from bovine colostrum were subjected to SDS-PAGE in 10% gels (16). The proteins were transferred electrophoretically to nitrocellulose membranes and incubated with 17 μl/ml anti-*N*-acetylgalactosaminyltransferase antiserum followed by 100 ng/ml (5.6×10^6 cpm) ^{125}I-Protein A. The nitrocellulose membranes were washed, dried, and processed for autoradiography as described under "Experimental Procedures." *Lane 1*, detergent phase from Triton X-114 extraction of 80 μg of mouse lymphoma BW5147 microsomes; *lane 2*, 0.22 unit of partially purified *N*-acetylgalactosaminyltransferase from murine lymphoma BW5147 cells; *lane 3*, 1.8 units of purified bovine colostrum *N*-acetylgalactosaminyltransferase.

enzyme. A similar analysis was performed on a Triton X-114 microsome extract and a partially purified preparation of the transferase, both from murine lymphoma BW5147 cells. As shown in *lanes 1* and *2* of Fig. 11, an immunoreactive species was observed which had a M_r of 71,000, slightly greater than that of the soluble bovine enzyme. The band at the top of the gel in *lane 2* most likely represents aggregated material although we cannot exclude the possibility that there is a very high molecular weight form of the enzyme.

DISCUSSION

UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase purified to homogeneity from bovine colostrum is a monomer of approximately 70,000 daltons as determined by both SDS-PAGE and gel filtration chromatography. An antibody raised against the purified 70,000-dalton enzyme precipitates the enzyme activity as well as the radiolabeled protein. The difference in the precipitation capacity of the antibody between the two experiments (~0.7 ng of enzyme precipitated/μl when calculated on the basis of activity *versus* ~1.8 ng/μl when calculated on the basis of the iodinated enzyme protein) is probably due to the presence of inactive enzyme. This would be expected since the enzyme loses activity upon storage. Isolation of the intracellular form of this enzyme from mouse lymphoma BW5147 cells yielded a preparation which, although not homogenous, eluted at a position corresponding to a molecular mass of 71,500 on gel filtration chromatography and contained a band of approximately 70,000 daltons on SDS-PAGE (Figs. 4*B* and 5*B*). Blotting of this preparation with anti-bovine colostrum *N*-acetylgalactosaminyltransferase antibody resulted in a band of approx-

imately 71,000 daltons. If one assumes that the soluble transferase is derived from a membrane-bound intracellular form by proteolytic cleavage, these results indicate either that the membrane-anchoring portion of the intracellular transferase is relatively small or that this part of the enzyme is very sensitive to proteases and is readily cleaved during the purification procedure. The former possibility seems more likely since even when a crude membrane extract prepared in the presence of protease inhibitors is blotted, no species of higher molecular mass could be detected. The fact that antibody raised against the soluble bovine enzyme cross-reacts with the membrane-derived murine enzyme lends further support to the conclusion that the soluble enzyme is a cleavage product.

In the purification of *N*-acetylgalactosaminyltransferase from ascites hepatoma AH66 cells, Sugiura *et al.* (9) reported an apparent M_r of 54,000–56,000. The specific activity of this enzyme was 390 units/mg as compared to 1,860 units/mg for the bovine colostrum enzyme. However, the assays of the ascites hepatoma enzyme were done at UDP-GalNAc concentrations below the K_m. The two enzymes differ in several other respects. While both enzymes are specific for UDP-GalNAc, the ascites hepatoma enzyme has a K_m of 42 μM for this nucleotide sugar whereas the apparent K_m of the bovine colostrum enzyme is 8 μM. Another major difference is in the cation requirement. Both enzymes prefer Mn^{2+}, but the bovine colostrum enzyme utilizes Co^{2+} almost as well as Mn^{2+} whereas Ca^{2+} is poorly utilized. By contrast, the ascites hepatoma enzyme uses Co^{2+} and Ca^{2+} equally well, each being 50% as effective as Mn^{2+}. The reasons for these differences in the properties of the two enzymes is not clear. One possiblity is that the two enzymes represent different proteins. Alternatively the ascites hepatoma enzyme may be a proteolytic fragment of the intracellular enzyme resulting in some alterations in its properties. Further studies will be required to resolve this discrepancy.

An intriguing finding is the presence of complex-type Asn-linked oligosaccharides on the bovine enzyme. This indicates that the enzyme has been exposed to the late stage oligosaccharide processing enzymes which have been immunolocalized to the middle and trans cisternae of the Golgi stack (2, 21, 22). Several lines of evidence indicate that the *N*-acetylgalactosaminyltransferase resides in the cis Golgi and/or the endoplasmic reticulum (13, 23–32). For example, Cummings *et al.* (33) have shown that the precursor form of the low density lipoprotein receptor of A431 cells has high mannose-type Asn-linked units and *O*-linked units containing only *N*-acetylgalactosamine. By contrast, the mature form of the receptor contains complex-type Asn-linked units plus *O*-linked oligosaccharides that contain sialic acid, galactose, and *N*-acetylgalactosamine. These findings demonstrate that the transfer of *N*-acetylgalactosamine residues occurs before the entry of the low density lipoprotein receptor into the regions of the Golgi that contain the late stage processing enzymes (*e.g.* middle and trans elements). In support of this, Roth has published an electron microscopy study that has localized GalNAc-containing glycoproteins to the cis Golgi cisternae of intestinal goblet cell (23). If the *N*-acetylgalactosaminyltransferase acts in the cis Golgi or the endoplasmic reticulum, how did the colostrum form of the enzyme acquire complex-type oligosaccharides? The simplest explanation is that the cleavage of the membrane-bound enzyme occurs within the Golgi (or endoplasmic reticulum) and that the formation of complex oligosaccharides procedes as the soluble enzyme is transported through the stack. A less likely explanation is that the enzyme initially passed through the Golgi before returning to reside in the cis cisternae (or the endoplasmic reticulum). In this case it could be initially present in the colostrum in a membrane-associated form, similar to galactosyltransferase (34). A final possibility is that the oligosaccharide processing occurs after the enzyme is released into the colostrum.

The availability of the antiserum described in this report will hopefully enable us to study further the relationship between the intracellular and secreted forms of this enzyme and also to immunolocalize it, thereby providing direct evidence for its subcellular location.

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SUPPLEMENTARY MATERIAL TO:

PURIFICATION AND CHARACTERIZATION OF UDP-GALNAC: POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASE FROM BOVINE COLOSTRUM AND MURINE LYMPHOMA BW5147 CELLS

BY

AKE ELHAMMER AND STUART KORNFELD

EXPERIMENTAL PROCEDURES

Materials - UDP-[1-^{3}H]N-acetylgalactosamine (10.7 Ci/mmol) was from New England Nuclear (Boston, MA). Na ^{125}I (13-17 Ci/μg) was from the Radiochemical Centre (Amersham, England). UDP-N-acetylgalactosamine, UDP, PMSF, chymostatin, leupeptin, antipain, pepstatin, aprotinin, bovine submaxillary mucin, NP-40, Triton X-100, Triton X-114, taurodeoxycholate, chloramine T and bovine hemoglobin were from Sigma Chemical Co., St. Louis, MO. DEAE-Sephacel, Sepharose 6B, and Protein A-Sepharose were from Pharmacia, Inc., Piscataway, NY. α-Minimal Essential Medium (α-MEM) was from Flow Laboratories, Rockville, MD. Fetal calf-serum (heat-inactivated) was from K.C. Biological, Inc., Kansas City, MO. Cryoprotective medium was from Whittaker M.A. Bioproducts, Walkersville, MD. Freunds complete adjuvant was from Gibco Grand Island, NY. Nitrocellulose membranes were from Schleicher and Schuell, Inc., Keene, NH. Endo-β-N-acetylglucosaminidase H (Endo H) was from Miles Biochemicals. Endo-β-N-acetylglucosaminidase F (Endo F) was a generous gift from Dr. Jacques Baenziger at Washington University, St. Louis, MO. Bovine colostrum was kindly supplied by Dr. John Mahoney at the Ralston-Purina Co., St. Louis, MO. All other reagents were from standard sources.

Cells - Mouse lymphoma BW5147 cells were grown in 10 liter spinner bottles to a density of ~1.5 x 10^6 cells/ml in α-MEM supplemented with 10% fetal calf serum and 10 mM HEPES, pH 7.0. The cells were harvested by centrifugation, washed in ice-cold phosphate buffered saline and the cell pellets were suspended in an equal volume of 50% fetal calf serum and 50% cryoprotective medium. The suspension was then frozen at -20°C for 3 hours, after which it was transferred to -70°C for 18 hours and finally to -270°C (liquid nitrogen) for storage.

Preparation of Apomucin and Apomucin-Sepharose - Apomucin (deglycosylated mucin) was prepared from bovine submaxillary mucin by the method of Hagopian and Eylar (5) with minor modifications. The carbohydrate content of the apomucin preparation was determined by the method of Reinhold (10). CNBr-activated Sepharose was prepared from Sepharose 6B essentially as described by Cautrecasas (11). The apomucin was coupled to the activated Sepharose in 0.1 M sodium carbonate buffer pH 9.2 at 4°C overnight. The protein concentration during the reaction was 2.5 mg/ml. All subsequent steps were carried out as in (12). The coupling efficiency was nearly 100% and the final apomucin-Sepharose contained ~5 mg of bound apomucin/ml sedimented gel.

Enzyme Assay - The standard assay for UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase contained the following components in a final volume of 80 μl:50 mM Imidazole pH 7.2, 10 mM $MnCl_2$, 0.5% Triton X-100, 15 μM UDP-GalNAc, UDP[1-^{3}H]GalNAc (27,000 cpm/assay), 0.15 mg/ml apomucin and varying amounts of enzyme (see individual experiments). The reaction mixture was incubated at 37°C for 5-10 min (see individual experiments) and the reaction product was TCA precipitated and radioactivity measured as described (13).

Endo-β-N-Acetylglucosaminidase Digestions - When denatured enzyme was digested, the material was first boiled for 5 min in the appropriate enzyme digestion buffer containing 1% SDS. The samples were then diluted with an equal volume 50 mM Imidazole pH 7.2 (Endo F) or 100 mM citrate pH 5.5 (Endo H) containing 100 mM EDTA, 10 μg/ml each of leupeptin, antipain, chymostatin and pepstatin, 0.1 TIU/ml aprotinin and 20 μM PMSF. Endo F (4 mU/ml) or Endo H (15 mU/ml) were added,and the samples were incubated for 16 hours at 37°C under toluene atmosphere. The digested samples were supplemented with an equal volume SDS-PAGE sample buffer and boiled for 10 min before loading on the gel. SDS-PAGE was done on 10% gels as described in (16) and autoradiography of the dried gels was performed at -70°C using Kodak X-Omat AR film. Digestion of native enzyme was done as for denatured enzyme but omitting the boiling in 1% SDS.

Purification of UDP-GalNAc:Polypeptide N-acetylgalactosaminyltransferase from Bovine Colostrum - All steps in the purification procedure were performed at +4°C and enzyme activity was assayed with the standard assay described above throughout the purification.

The following buffers were used. Buffer A: 25 mM Imidazole, pH 7.2, 6 mM $MnCl_2$, 30 mM NaCl; buffer B: 25 mM Imidazole pH 7.2, 1 M NaCl, 1% Triton X-100, 20 mM EDTA; buffer C: 25 mM Imidazole pH 7.2, 30 mM $MnCl_2$, 20 mM NaCl; buffer D: 25 mM Imidazole pH 7.2, 0.5 M NaCl, 20 mM EDTA; buffer E: 25 mM Imidazole pH 7.2, 10 mM $MnCl_2$, 20% glycerol; buffer F: 25 mM Imidazole pH 7.2, 30 mM $MnCl_2$, 100 mM NaCl; buffer G: 25 mM Imidazole pH 7.2, 80 mM NaCl, 0.1% taurodeoxycholate, 10% glycerol; buffer H: 25 mM Imidazole pH 7.2, 100 mM NaCl, 0.1% Triton X-100, 10% glycerol.

Step 1: Separation of lipid globules and particles. Crude frozen colostrum was thawed and centrifuged at 15,000 g for 30 min. The resulting yellowish lipid layer was removed and discarded. The colostrum was then dialyzed against 20 volumes of buffer A for 16 hours with two buffer changes. The dialyzed material was centrifuged at 100,000 g for 60 min. The upper lipid layer was removed and discarded and the clear supernatant was carefully collected. The pellet and fluffy layer at the bottom was discarded.

Step 2: DEAE-Sephacel chromatography. The supernatant from the 100,000 g centrifugation was loaded directly on a DEAE-Sephacel column equilibrated in buffer A. For optimum results, the bed volume of this column should be approximately equal to the amount of 100,000 g supernatant loaded (or ~750 ml/L crude colostrum). The run-through fractions were assayed for N-acetylgalactosaminyltransferase, and the fractions with activity were collected and pooled. Typically more than 90% of the applied activity can be recovered after passage through the column.

Step 3: Apomucin affinity chromatography I. The affinity chromatography steps were carried out on apomucin-Sepharose columns with a bed volume of ~60 ml. The columns were run by gravity at a pressure of ~30 cm H_2O during loading and ~60 cm H_2O during washing, elution and regeneration. Before loading, the column was washed with 400 ml buffer B (regeneration buffer) followed by 500 ml buffer C and 150 ml buffer C containing 0.25 mM UDP. Prior to loading the column the sample (~200U enzyme activity per 60 ml column in the first affinity step) was supplemented with $MnCl_2$ and UDP to final concentrations of 30 mM and 1.25 mM, respectively. The column was washed with 4 column volumes of buffer C containing 0.25 mM UDP and 6 40 ml fractions were collected. The column was then eluted with buffer D. Due to the specific elution pattern of these columns the eluate was routinely collected as follows: fractions 1 and 2: 25 ml each, normally contains no, or very little activity; fractions 3 and 4: 50 ml each, contains the bulk of the activity; fractions 5 through 7: 25 ml each, contains in some cases smaller amounts of activity (Figure 1A). The individual fractions were dialyzed against 4 liters of buffer E (2 changes) immediately after elution, and assayed for enzyme activity. Typically only fractions 3 and 4 were used in the subsequent purification.

Step 4: Apomucin affinity chromatography II. In this step the same type column was used as in the previous one. Before loading, the column was first washed with 400 ml buffer B followed by 500 ml buffer F and 150 ml buffer F, containing 0.25 mM UDP. Prior to running the column, dialyzed fractions 3 and 4 from step 3 were supplemented with 1 M $MnCl_2$, 4 M NaCl and UDP to achieve final concentrations of 30 mM, 100 mM and 1.25 mM respectively. Approximately 600U enzyme activity per run could be loaded during this step. After loading the sample, the column was washed with 2 column volumes of buffer F followed by two column volumes of buffer F containing 0.5 M NaCl and finally with two column volumes of buffer F containing 1 M NaCl. All the wash buffers contained 0.25 mM UDP. The washes were collected in 40 ml fractions. Elution was then carried out in the same manner as in step 3 but with buffer D containing only 100 mM NaCl. The eluted fractions were dialyzed and assayed for transferase activity as described for step 3.

Step 5: Gel filtration chromatography on Sephadex G-100 superfine. The dialyzed fractions from three step 4 runs were pooled, 1/50 volume 5% taurodeoxycholate was added, and the material was concentrated to 2.5 ml on an Amicon YM-10 filter under 40 psi pressure. Half of this material, 1.25 ml, was loaded on a Sephadex G-100 superfine column (1.5 x 100 cm) equilibrated in buffer G. The column was run by gravity at a pressure of 28 cm H_2O; 1 hour fractions (1.76 ml) were collected and assayed for activity. The fractions comprising the activity peak were pooled and concentrated as described above but without any further addition of detergent. Analytical gel filtration to determine the molecular weight of the transferase was carried out using the same procedure but with a smaller column (0.9 x 100 cm) and collecting 1.06 ml fractions.

Purification of UDP-GalNAc:Polypeptide N-Acetylgalactosaminyltransferase from Mouse Lymphoma BW5147 Cells - All steps were carried out at 4°C. The standard assay was used for monitoring activity containing fractions. Buffers A through F were used but containing 0.1% Triton X-100.

Step 1: Detergent extraction. 22 x 10^{10} frozen cells were thawed and suspended in 400 ml 100 mM Imidazole pH 7.2, 0.5% Triton X-100, 0.2 TIU aprotinin/ml. The cells were homogenized (30 strokes) in a Potter Elvehjem homogenizer, and the homogenate was centrifuged at 10,000 g for 20 min and the resulting supernatant saved. The pellet was rehomogenized in 215 ml 100 mM Imidazole, 0.5% Triton X-100, 0.2 TIU/ml Aprotinin and stirred on ice for 30 min. The homogenate was then centrifuged at 10,000 g for 20 min and the supernatant was collected. The two 10,000 g supernatants were pooled and dialyzed for 6 hours against 10 volumes buffer A containing 0.1% Triton X-100 with 3 changes. The dialyzed preparation was centrifuged at 95,000 g for 2 hours. After carefully decanting the supernatant, the pellet was reextracted with 180 ml buffer A, 0.1% Triton X-100, centrifuged again at 95,000 g for 2 hours and the supernatant was collected. The two 95,000 g supernatants were pooled and used in subsequent purification steps.

Steps 2 through 4: DEAE-Sephacel chromatography and apomucin affinity chromatography. These steps were essentially identical to the procedure used for the colostrum enzyme with the following differences: 1) all buffers contained 0.1% Triton X-100; 2) the active fractions from DEAE-Sephacel chromatography were supplemented with glycerol to a final concentration of 20% to stabilize the enzyme; and 3) no detergent was added to the eluate from the second apomucin affinity chromatography step.

Determination of molecular weight by analytical gel filtration was performed on a Sephadex G-150 column (0.9 x 100 cm) equilibrated in buffer H at a flow rate of 2.75 ml/hour. 0.92 ml fractions were collected.

Protein Determination - Protein was measured as described by Lowry, et al. (14).

Amino Acid Analysis - Amino acid analysis was performed as described in (15).

Preparation of Anti-N-Acetylgalactosaminyltransferase Antibodies - Antibodies against bovine colostrum N-acetylgalactosaminyltransferase were raised as follows. 35 μg of purified enzyme in 1 ml of a 1:1 mixture of Freunds complete adjuvant and buffer G was injected into the footpads of a female New Zealand rabbit. Four weeks later the rabbit was boosted with 22 μg of the purified enzyme using the above procedure. Blood was collected one week later. After an additional three weeks, 51 μg of the antigen (dissolved as above) was injected subcutaneously into six sites on the back of the rabbit. Serum collected after this last injection yielded the highest titer.

Preparation of IgG - IgG was isolated from whole serum by chromatography on Protein A-Sepharose. 1 ml of serum was loaded onto a column (~1.1 ml bed volume) equilibrated in 20 mM Tris-HCl pH 7.4. The column was then washed with 10 volumes of equilibration buffer followed by 15 volumes of 20 mM Tris HCl pH 7.4, 0.5% taurodeoxycholate, 0.5 M NaCl and finally another 15 volumes of equilibration buffer. The bound IgG was eluted with 100 mM Glycine-HCl pH 2.8. One ml fractions were collected and immediately neutralized by the addition of 50 μl 1 M Tris base. The IgG containing fractions (identified by measuring A_{280}) were pooled, dialyzed against 100 volumes phosphate buffered saline (PBS) (2 changes) and concentrated to ~1 ml on a YM-10 membrane. NaN_3 was added to a final concentration of 0.02% and the isolated IgG was stored on ice.

Preparation of ^{125}I-Labeled Protein A - 100 μg Protein A was reacted with 1 mCi Na ^{125}I and 0.2 mg/ml chloramine T in 100 μl 0.3 M phosphate buffer pH 7.3 for 2 min at room temperature. The reaction was stopped by the addition of 2.5 μl of a saturated solution of tyrosine in water and the iodinated product was separated from smaller radioactive molecules by chromatography on a 1 ml Sephadex G-25 column equilibrated in PBS containing 1% BSA.

Immunoreplica Experiments - Electrophoretic transfer of proteins from SDS-polyacrylamide slab gels to nitrocellulose membranes was carried out in a Bio Rad Trans Blot Cell using the procedure of Burnette (17). Transfer was for 14-16 hours at 30V. The section of the nitrocellulose membrane containing the molecular weight standards was cut off and stained with Amido black (0.1% w/v) in 45% methanol and 10% acetic acid. Destain was in 10% acetic acid. The rest of the membrane was incubated in PBS containing 1.25% w/v hemoglobin for 2 hours. The nitrocellulose sheet was then incubated for 2 hours in a sealed plastic bag containing 15 ml 1.25% hemoglobin in PBS and 250 μl anti-N-acetylgalactosaminyltransferase serum. After incubation with antiserum the membrane was washed once for 10 min in PBS followed by two washes of 10 min each in PBS containing 0.05% NP-40 and two washes 5 min each in PBS. The washed membrane was then incubated for 2 hours in a sealed plastic bag containing 20 ml 1.25% hemoglobin in PBS and 2 μg (5.6 x 10^6 cpm) ^{125}I-Protein A, after which it was washed once for 10 min in PBS, twice for 10 min each in PBS containing 0.2% NP-40 and finally twice for 5 min in PBS. Excess moisture was then removed from the membrane with paper towels and it was allowed to air dry on a filter paper. All washes and incubations were carried out at room temperature on a shaking platform. The dried nitrocellulose membranes were autoradiographed at -70°C using Kodak X-Omat AR film.

Triton X-114 Extraction of Microsomes Isolated from Mouse Lymphoma BW5147 Cells - Washing of Triton X-114 was done as described by Bourdier (18) and the concentration of the washed stock solution was estimated by measuring the absorption at 276 nm and using the extinction coefficient:Log ε = 3.16. The stock solution was diluted to 1% with 10 mM Tris-HCl pH 7.4, 150 mM NaCl. Microsomes were prepared from 4.2 x 10^8 cells using the procedure in (13) but with the following protease inhibitors present during the preparation: 0.2 TIU/ml aprotinin, 10 μM PMSF and 20 μg/ml each of antipain, leupeptin, chymostatin and pepstatin. The sedimented microsomes were suspended in 500 μl 10 mM Tris pH 7.4, 150 mM NaCl and the protein concentration was determined using the procedure of Lowry, et al. (14). 2 mg microsomal protein was sedimented at 100,000 g for 45 min. The resulting pellet was homogenized in 500 μl 1% Triton X-114, 10 mM Tris HCl, pH 7.4, 150 mM NaCl and incubated on ice for 30 min. The suspension was centrifuged at 12,000 g at 4°C for 10 min and the pellet was discarded. The supernatant was incubated at 30°C for 3 min and centrifuged at 12,000 g in room temperature for 3 min to achieve phase separation. The upper phase was removed and supplemented with concentrated Triton X-114 stock to 1% final concentration, and the lower detergent phase was suspended in 500 μl, 10 mM Tris HCl, pH 7.4, 150 mM NaCl. Both phases were incubated on ice until clear solutions were formed. Phase separation was then performed again as described above. The water phases were aspirated and pooled and the detergent phases were each dissolved in 500 μl 10 mM Tris HCl pH 7.5, 150 mM NaCl and pooled. Aliquots from the pooled phases were supplemented with an equal volume of SDS-PAGE sample buffer, boiled for 10 min and applied directly to SDS-PAGE.

RESULTS

Purification of UDP-GalNAc:Polypeptide N-Acetylgalactosaminyltransferase from Bovine Colostrum - Bovine colostrum was chosen as the source for UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase since it contains high levels of water soluble enzyme activity (900 units per liter) and is readily obtainable. The purification of this enzyme from 3.3 L colostrum is summarized in Table I. For the procedure to be successful attention had to be given to a number of points, which are discussed in the following paragraphs.

Step 1: Separation of lipid globules and particles. The crude colostrum was first delipidated by an initial low speed centrifugtion. The main purpose of the subsequent dialysis was to adjust the ionic content of the preparation for DEAE-Sephacel chromatography. This procedure however, also appeared to destabilize the remaining particles in the preparation, and thus facilitated their sedimentation during the subsequent high speed centrifugation. The product from Step 1 was a clear yellowish solution which could be directly loaded on the DEAE-Sephacel column. The step typically resulted in a 2.5-3 fold purification with a yield exceeding 96%.

Step 2: DEAE-Sephacel chromatography. Although the bovine colostrum N-acetylgalactosaminyltransferase does not bind to DEAE-Sephacel, even at pH's >8.5, this procedure served as a negative purification step, i.e., to remove contaminating proteins which bound to the column. This is of great importance since the capacity of the apomucin columns used in the subsequent affinity steps appears to be inversely proportional to the amount of contaminating protein loaded.

Step 3: Affinity chromatography on apomucin-Sepharose I. This step and the following one are the major basis for the purification. The procedure is adapted from that used by Sugiura, et al. (9). The capacity of the apomucin gel is low (~3 U/ml gel) in this first affinity step due to the large amounts of contaminating proteins. In addition, since the enzyme binds poorly at this step, the column was washed with a low ionic strength buffer containing UDP and Mn^{2+} which are essential for enzyme binding. The enzyme elutes in a broad peak (~two column volumes). The subsequent dialysis against buffer containing Mn^{2+} and glycerol removes the EDTA used in the elution (which is necessary to be able to assay the enzyme) and also concentrates the eluate ~1.7x. The concentration of UDP when loading and EDTA when eluting are optimal for this system. The sodium chloride concentration is not critical.

Step 4: Affinity chromatography on apomucin Sepharose II. At this point in the purification, the capacity of the affinity column is at least 5 times greater than that of the first affinity step. Furthermore, since the interaction of the enzyme with the bound apomucin is stronger, the column can be washed with up to 1 M NaCl (in the presence of Mn^{2+} and UDP) with less than 5% loss of the adsorbed enzyme. The eluted fractions must be dialyzed as soon as possible since the partially purified enzyme rapidly loses activity in the presence of high salt and in the absence of glycerol. Even with these precautions the preparation loses about 20% of its activity per week, particularly after concentration, unless a further stabilizing agent is added. To remedy this problem, a variety of proteins, reducing agents, glycerol concentrations and detergents were tried. We found that the addition of a low concentration of a non-denaturing detergent like Triton X-100 or sodium deoxycholate had the best stabilizing effect. Therefore the enzyme preparation was routinely supplemented with taurodeoxycholate to a final concentration of 0.1% before concentration and final purification by gel filtration chromatography. Taurodeoxycholate was chosen because it forms stable solutions at 4°C over a wide pH range and because it is dialysable. Typical elution profiles from the apomucin affinity chromatography steps are shown in Figure 1A and B.

Step 5: Gel filtration chromatography on Sephadex G-100 superfine. The concentrated eluate from the second affinity chromatography step contains two proteins: the N-acetylgalactosaminyltransferase and a larger protein which, based on its behavior on SDS-PAGE in the presence and absence of β-mercaptoethanol, is most likely an immunoglobulin. It runs large in the non-reduced lane and splits into smaller peptides, Mr 60,000 and 25,000 daltons respectively, under reducing conditions (see Figure 2A lanes 1 and 2).

The G-100 superfine chromatography provides baseline separation between these two proteins under the conditions used (see Experimental Procedures) and the enzyme elutes as a single symmetric peak (Figure 4A). When material from this peak was analyzed by SDS-PAGE only one single protein band could be detected by silver staining (19) (Figure 2B).

Purification of UDP-GalNAc:Polypeptide N-Acetylgalactosaminyltransferase from Mouse Lymphoma BW5147 Cells - Mouse lymphoma BW5147 cells contain ~0.065 units UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase activity per mg total cell protein. All the activity is in a membrane-bound form which necessitates the detergent extraction procedure outlined in step 1 of the Experimental Procedures. Aprotinin was added to the extraction buffers to prevent proteolytic breakdown of the enzyme. Approximately 55% of the total activity was recovered in the pooled detergent extracts. The following three steps (steps 2 through 4) were essentially the same as for the colostrum enzyme although all buffers contained 0.1% Triton X-100. The capacity of the apomucin columns is lower in this system, only ~1.2 U/ml gel in the first affinity step and the activity tends to elute as a broader peak than the colostrum enzyme. Compared to the colostrum enzyme, the purification of this enzyme yielded smaller amounts of activity. The total yield after the second affinity step was usually 8-10% which translates to 103 U for a preparation starting with 22 x 10^{10} cells. The presence of high concentrations of Triton (which does not dialyze well and thus increases during concentration) in the preparation and a very low protein concentration made accurate protein measurements difficult. Typical preparations however yielded a specific activity of >150 U/mg and a total purification of >2,300 fold. An SDS-PAGE profile of the semi-purified enzyme is shown in Figure 3. The bands on the gel are broadened and distorted by the presence of Triton. Two major bands are however clearly visible. One at a migration distance corresponding to ~70,000 daltons and another at ~20,000 daltons (Figure 3 and 5B).

Gel filtration chromatography on Sephadex G-150 yielded a symmetric peak of activity with a molecular size of 71,500 daltons (Figure 4B).

TABLE I

Purification of UDP-GalNAc:Polypeptide N-Acetylgalactosaminyltransferase from Bovine Colostrum

Step	Total Protein	Total Activity	Specific Activity	Purification Factor	Yield
	mg	units[a]	units/mg		%
Dialyzed colostrum[b]	762,300	2,758	0.0036	1	100
1. 95,000 x g supernatant	279,047	2,690	0.0096	2.7	97.5
2. DEAE-Sephacel	55,351	2,462	0.0445	12.4	89.3
3. Apomucin-Sepharose I	793	1,894	2.387	663	68.7
4. Apomucin-Sepharose II	7.5	1,542	205.6	57,111	55.9
5. Sephadex G-100 Superfine	0.28	522	1,860	516,667	18.9

a 1 unit equals 1 nmole N-acetylgalactosamine transferred to apomucin per minute under assay conditions
b total initial volume of colostrum: 3.3L

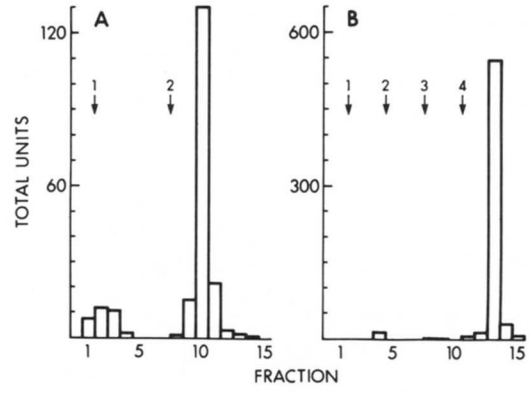

Figure 1 - Elution profiles (transferase activity) from apomucin-Sepharose. A. Apomucin-Sepharose I. B. Apomucin-Sepharose II. The columns were loaded, washed and eluted as described in Experimental Procedures. Application of wash - and elution buffers are indicated with arrows. A: 1, wash with buffer C containing 0.25 mM UDP; 2, elution with buffer D; B: 1-3, wash with buffer F containing 0.25 mM UDP alone and supplemented with NaCl to 0.5 and 1.0 M respectively; 4, elution with buffer D containing 100 mM NaCl.

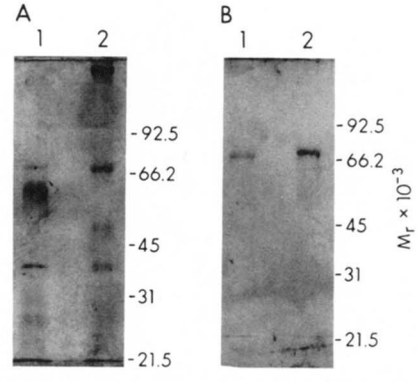

Figure 2 - SDS-Polyacrylamide gel electrophoresis of bovine colostrum UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase. Fractions containing enzyme activity from apomucin-Sepharose II and Sephadex G-100 superfine were concentrated as outlined in Experimental Procedures, mixed with an equal volume of SDS-PAGE sample buffer with or without β-mercaptoethanol, boiled for 10 min and analyzed on a 10% SDS polyacrylamide gel. After electrophoresis the gels were stained using the silver staining procedure (19). The migration positions of five molecular weight standards are shown to the right. Lane 1: sample run in the presence of β-mercaptoethanol. Lane 2: sample run in the absence of β-mercaptoethanol. A. Material eluted from apomucin-Sepharose II (10 μg). B. Aliquots of the pooled activity fractions from Sephadex G-100 superfine (1 μg).

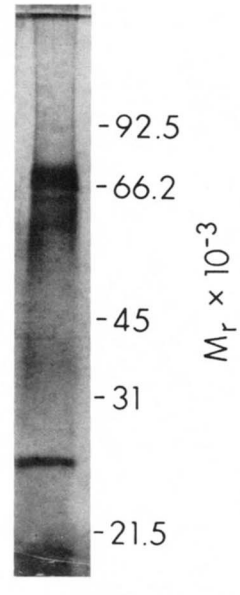

Figure 3 - SDS-polyacrylamide gel electrophoresis of affinity purified UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase from mouse lymphoma BW5147 cells. Transferase eluted from the second affinity step was concentrated as follows: 15 ml of elute was dialyzed against 20 volumes of 10 mM Tris maleate pH 6.0, 5 mM $MnCl_2$, 0.1% Triton X-100, 20% glycerol for 16 hours (2 buffer changes). The dialyzed sample was then loaded on a CM-cellulose column, 0.8 ml bed volume, equilibrated in the buffer used for dialysis. After washing with 5 column volumes of equilibration buffer, the column was eluted with 25 mM Imidazole pH 7.2, 5 mM $MnCl_2$, 0.1% Triton X-100, 20% glycerol, 500 mM NaCl. 250 μl fractions were collected, dialyzed against buffer E and assayed for activity. 40μl of the fraction containing the highest activity was mixed with an equal volume SDS-PAGE sample buffer and analyzed on a 10% SDS polyacrylamide gel.

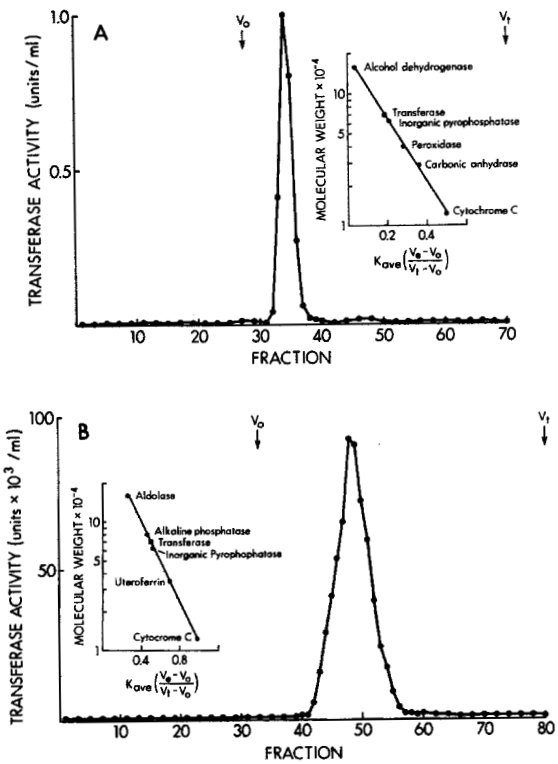

Figure 4 - Determination of molecular weight of N-acetylgalactosaminyltransferase on gel filtration. Affinity purified transferase from bovine colostrum and mouse lymphoma BW5147 cells was subjected to analytical gel filtration as described in Experimental Procedures. Standard proteins were: A, alcohol dehydrogenase (150,000), inorganic pyrophosphatase (63,000), peroxidase (40,000), carbonic anhydrase (29,000), and cytochrome c (12,400); B, aldolase (160,000), alkaline phosphatase (80,000), inorganic pyrophosphatase (63,000) uteroferrin (35,000), and cytochrome c (12,400). A. Bovine colostrum transferase chromatographed on Sephadex G-100 superfine. B. Mouse lymphoma BW5147 transferase chromatographed on Sephadex G-150.

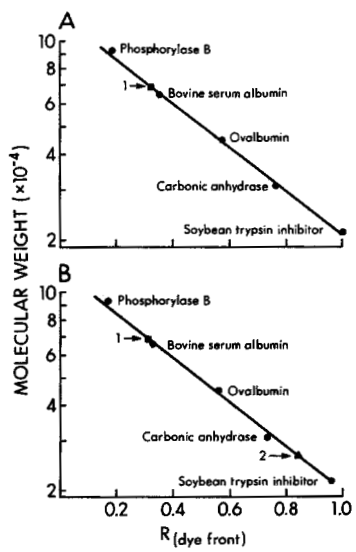

Figure 5 - Determination of molecular weight of N-acetylgalactosaminyltransferase by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Conditions for SDS-PAGE were as described in Figure 2. The standards used were: phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). The R_f's of the transferases were consistent during three separate determinations. A. Bovine colostrum transferase. B. Mouse lymphoma BW5147 transferase. (Note: The electrophoretic positionof the transferse bands in B are approximate due to the broadening and distortion caused by the presence of Triton X-100 in the BW5147 prepaPation.)

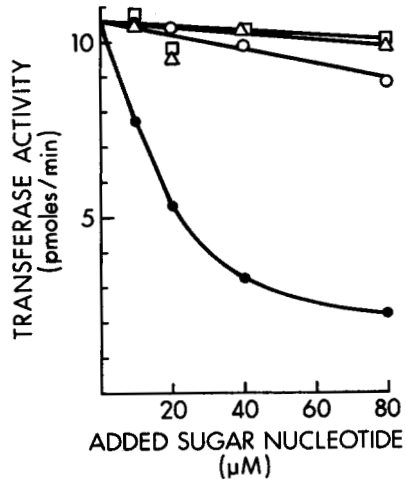

Figure 6 - Donor specificity of bovine colostrum N-acetylgalactosaminyltransferase. The nucleotide sugar specificity was determined using the standard assay procedure, but in the presence of different concentrations of unlabeled sugar nucleotides. 8.5 mU of enzyme was used and the assay time was 10 min. The competing effect of UDP-galactose (□), UDP-glucose (△), UDP-N-acetylglucosamine (○), and UDP-N-acetylgalactosamine (●) was determined.

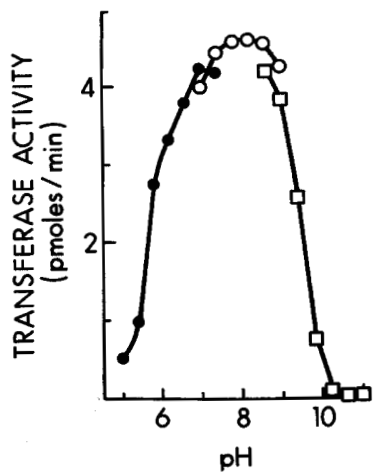

Figure 7 - Determination of pH optimum for bovine colostrum N-acetylgalactosaminyltransferase. Transferase activity was assayed for 10 min using 8.5 mU of enzyme and the standard assay procedure but in the absence of Mn^{2+} and with buffers of equal ionic strength but different pH substituted for the assay buffer. The buffers used were pH 5.4-7.4, cacodylate; pH 7.0-9.0, imidazole; and pH 8.6-10.6, glycine - NaOH.

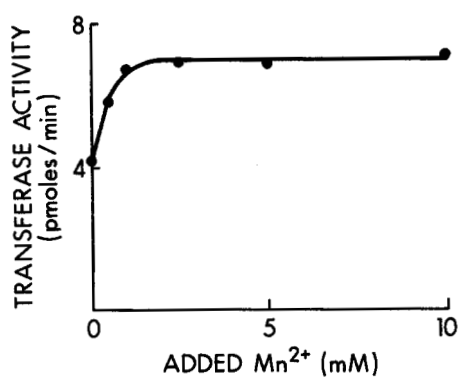

Figure 8 - Effect of increasing concentrations of Mn^{2+} on bovine colostrum N-acetylgalactosaminyltransferase. Transferase activity was measured using the standard assay procedure but varying the concentrations of added Mn^{2+}. 8.5 mU of enzyme was used and the assay time was 10 min.