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FURTHER CHARACTERIZATION OF THE GALACTOSYLTRANSFERASES IN CHICK CARTILAGE

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SUMMARY

The substrate specificity of the galactosyltransferase system in microsomal particles from embryonic chick cartilage has been investigated further. In addition to the previously described galactosyltransferases, involved in the biosynthesis of the chondroitin sulfate–protein linkage region, a third galactosyltransferase was detected in the particulate enzyme preparation, which transferred galactose from UDP-galactose to *N*-acetylglucosamine (*N*-acetyllactosamine synthetase). Mixed substrate experiments as well as heat inactivation studies with galactose acceptors derived from chondroitin sulfate indicated that the catalytic center involved in the *N*-acetyllactosamine synthetase reaction is distinct from those catalyzing the synthesis of the two galactose moieties of chondroitin sulfate.

The addition of α-lactalbumin to the enzyme stimulated the synthesis of lactose from glucose and UDP-galactose, in agreement with similar observations on the A subunit of lactose synthetase from milk as well as UDP-galactose:*N*-acetyl-D-glucosamine galactosyltransferases from other sources.

Partial acid hydrolysis of [^{14}C]galactose-labeled endogenous acceptor from the cartilage homogenate yielded a fragment with the chromatographic properties of *N*-acetyllactosamine, suggesting that a portion of the galactose incorporated into the endogenous acceptor may be transferred to *N*-acetylglucosamine residues and not exclusively to precursors of the chondroitin sulfate–protein complex.

INTRODUCTION

Previous investigations concerning the galactosyltransferase activity in embryonic chick cartilage have dealt with the biosynthesis of the chondroitin sulfate–protein linkage region where galactose is a component[1]:

Abbreviations: Gal, D-galactose; Xyl, D-xylose; GlcUA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; LacNAc, *N*-acetyllactosamine (4-*O*-β-D-galactosyl-*N*-acetyl-D-glucosamine); Gal-4-Xyl, 4-*O*-β-D-galactosyl-D-xylose; Gal-3-Gal-4-Xyl, 3-*O*-β-D-galactosyl-4-*O*-β-D-galactosyl-D-xylose.

$(\text{GalNAc–GlcUA})_n$–Gal–Gal–Xyl–protein
|
SO_4

Transfer of galactose from UDP-galactose to an endogenous acceptor in the cartilage homogenate[2,3] as well as to fragments derived from the linkage region, such as D-xylose and Gal-4–Xyl, have been reported. Inhibition experiments indicated that the two galactose moieties were transferred sequentially and by two distinct catalytic centra.

The present report describes the transfer of galactose from UDP-galactose to *N*-acetylglucosamine, to form *N*-acetyllactosamine in a particulate enzyme preparation from chick cartilage. Evidence is presented which indicates that this reaction is catalyzed by an enzyme independent from the two galactosyltransferase activities described above.

MATERIALS AND METHODS

UDP-[^{14}C]galactose (240–280 μC/μmole) was obtained from Amersham Nuclear Centre. D-[^{14}C]Glucosamine (55 μC/μmole) was supplied by Amersham. Unlabeled UDP-*N*-acetylglucosamine and UDP-galactose were purchased from Sigma Chemical Co. D-Galactose oxidase was a product of Kabi, Sweden. α-Lactalbumin was obtained from Koch–Light Industries, England. β-D-Galactosidase *(Escherichia coli)* was a gift from Dr. Arne Dahlqvist, University of Lund, Sweden. Keratan sulfate was a gift from Professor Torvard C. Laurent of this institute.

Analytical methods

Hexosamine was determined by a modification of the method of Boas[4] with omission of the resin treatment, or as *N*-acetylglucosamine following reacetylation[5]. Disaccharide samples were hydrolyzed in 4 M HCl for 4 h at 100° prior to hexosamine analyses. The hexosamine content of a microsomal enzyme preparation from embryonic chick cartilage was estimated with an automatic amino acid analyzer following hydrolysis in 6 M HCl for 24 h. Galactose was assayed by the D-galactose oxidase assay[6]. Protein was determined according to the method of Lowry *et al.*[7].

Digestion with β-galactosidase was carried out in 0.1 M potassium acetate buffer, pH 7.3, containing 1% albumin, enzyme (10 μg) and substrate (about 1000 counts/min) in a total volume of 0.1 ml. Incubations were carried out at 30° for 15 h and the products were analyzed by paper chromatography (Solvent C; see below).

Paper chromatography was carried out in (A) *n*-butanol–ethanol–water (10:1:2, by vol.), (B) ethyl acetate–acetic acid–water (3:1:1, by vol.) or (C) ethyl acetate–pyridine–water (8:2:1, by vol.). Whatman No. 3 MM papers were run for 96 h (Solvent A) or for 24 h (Solvents B and C). With Schleicher and Schuell (No. 589; green ribbon) papers, adequate separations were obtained after 48 h and 15 h, respectively. Paper electrophoresis was performed in buffer (D), 0.046 M acetic acid–0.080 M pyridine, pH 5.3, at 80 V/cm for 60 min. The guide strips were stained with aniline hydrogen phthalate[8] or with a silver dip reagent[9].

Paper chromatograms or electrophoretograms were analyzed for radioactivity with a Packard Model 7201 strip scanner. Radioactive products were eluted with

water and quantitated with a Beckman Model LS 250 liquid scintillation spectrometer.

Isolation of N-acetyllactosamine from keratan sulfate

A 0.1% solution of 20 mg of keratan sulfate was adjusted to pH 1.5 with HCl and heated at 100° for 5 h. After neutralization with NaOH, the hydrolysate was passed through columns of Dowex 1-X2 (acetate form, 200–400 mesh) and Dowex 50-X2 (H^+ form, 200–400 mesh). The effluent and a water wash were combined, concentrated and applied to a washed Whatman No. 3 MM paper for preparative chromatography (Solvent A). This procedure led to the isolation of a fraction (R_{Gal} = 0.50; *cf.* ref. 10) containing galactose and *N*-acetylglucosamine in the molar proportions 1.00:0.89. Treatment with potassium borohydride[1] destroyed 93% of the hexosamine component, leaving the galactose intact. Digestion with β-galactosidase yielded galactose and *N*-acetylglucosamine as the only products (paper chromatography, Solvent A). The *N*-acetylglucosamine value of the unhydrolyzed fraction was 2% of that of the hydrolyzed and reacetylated material, indicating substitution at C-4 of the hexosamine component[11]. These results, including the chromatographic behaviour of the isolated compound, seem to establish its identity with *N*-acetyllactosamine. The yield was 1.4 mg.

Preparation of N-acetyl-[^{14}C]glucosamine

[^{14}C]Glucosamine (55 μC/μmole; 0.02 μmole) was mixed with unlabeled glucosamine (1 μmole) in 0.5 ml of water, and 0.1 ml of a 1.5% solution of acetic anhydride in acetone was added. After the addition of 0.1 ml of saturated $NaHCO_3$, the mixture was shaken for 10 min, passed through a column (2 cm × 6 cm) of Dowex 50-X2 (H^+ form, 200–400 mesh) and concentrated. On paper chromatography (Solvent B) all the radioactivity of the effluent fraction migrated similarly to *N*-acetylglucosamine.

Preparation of galactosyltransferases from embryonic chick cartilage

The particulate enzyme was prepared from homogenates of epiphyseal cartilage derived from 20 dozen 13-day-old chick embryos as described previously[3]. The 100 000 × *g* pellet fraction was suspended in 5 ml of 50 mM Tris–acetate buffer, pH 7.0, containing KCl (70 mM), EDTA (1 mM) and $MnCl_2$ (6 mM) to give a protein concentration of 4 mg/ml.

Transfer of galactose to low-molecular-weight substrates

In testing for the UDP-galactose:GlcNAc galactosyltransferase activity, the procedure previously described[3] for the UDP-galactose:D-xylose galactosyltransferase was modified slightly. Unless indicated otherwise, incubations were carried out at 37° for 60 min in the presence of UDP-[^{14}C]galactose (0.1 μC; 1 μC/μmole), particulate enzyme (0.2 mg of protein) and substrate (0.5 μmole) in a total volume of 0.06 ml. The reactions were stopped by immersing the tubes into a boiling-water bath for 2–3 min, after which the materials were transferred to Whatman No. 3 MM or Schleicher and Schuell paper. Subsequent electrophoresis (Buffer D) removed charged derivatives of [^{14}C]galactose, whereas free galactose and neutral products remained at the site of application. Separation of *N*-acetyllactosamine from free galactose was

accomplished by subjecting the dried papers to chromatography in Solvent A. In mixed substrate experiments involving *N*-acetylglucosamine and either one of xylose, Gal-4-Xyl or glucose, the separation of products was achieved after sequential chromatography in Solvents A and B.

Transfer of galactose from UDP-galactose to endogenous acceptor

[^{14}C]Galactose-labeled endogenous acceptor was obtained by incubation of the particulate enzyme (0.4 ml; 2.0 mg of protein) and UDP-[^{14}C]galactose (1.0 μC; 240 μC/μmole) at 37° for 3 h. The reaction was stopped by addition of one volume of cold 10% trichloroacetic acid, and the precipitate was washed 4 times with 5% trichloroacetic acid, twice with ethanol–ether (1:1, by vol.) and once with ether. To characterize the product, the precipitate was suspended in 2 ml of water which were adjusted to pH 1.5 by addition of HCl. After heating the mixture at 100° for 5 h, the pH was adjusted to 5.0 and the solution was passed through columns (1 cm × 4 cm) of Dowex 1-X2 (acetate form, 200–400 mesh) and Dowex 50-X2 (H^+ form, 200–400 mesh). The effluent was concentrated to dryness with several additions of methanol and subjected to paper chromatography in Solvent B.

RESULTS

Transfer of galactose to N-acetylglucosamine

Incubation of the particulate enzyme with UDP-[^{14}C]galactose and *N*-acetylglucosamine resulted in the formation of a compound which migrated as *N*-acetyl-

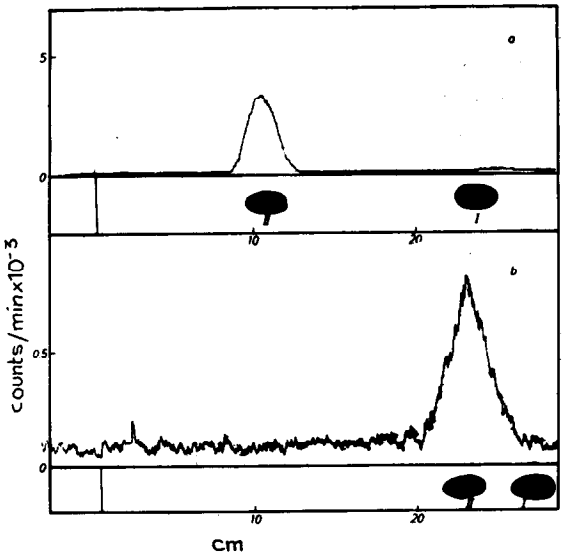

Fig. 1. Transfer of galactose to *N*-acetylglucosamine. (a) Distribution of radioactivity after paper chromatography (Solvent A) of an incubation mixture prepared as described in MATERIALS AND METHODS, with the exception that the specific activity of UDP-[^{14}C]galactose was 20 μC/μmole. The radioactive material from the peak migrating similarly to *N*-acetyllactosamine was eluted with water, and a portion was subjected to paper chromatography in Solvent B (b). The locations of (I) galactose and (II) *N*-acetyllactosamine are indicated on the guide strips below each tracing.

lactosamine on paper chromatography in Solvents A and B. In the absence of exogenous substrate, no radioactivity moved to this position of the chromatogram (Fig. 1).

To characterize the product, a large-scale incubation with UDP-[^{14}C]galactose and *N*-acetylglucosamine was carried out as described in the legend to Table I. The product (yield, 40 000 counts/min) was isolated by paper chromatography in Solvent A. Analytical data (Table I) revealed that galactose and glucosamine were present

TABLE I

ANALYTICAL DATA FOR *N*-ACETYL[^{14}C]LACTOSAMINE

The ^{14}C-labeled product was synthesized by incubation of *N*-acetylglucosamine (3 μmoles) with UDP-[^{14}C]galactose (0.4 μC; 0.32 μC/μmole) and particulate enzyme (2 mg of protein) for 3 h at 37° in a total volume of 0.5 ml. After addition of 2 vol. of ethanol, the precipitate was removed by centrifugation and the supernatant was diluted 10-fold with water and passed through ion-exchange columns. The effluent was lyophilized, dissolved in a small amount of water and applied to washed Whatman No. 3 MM paper for chromatography (Solvent A). The product which migrated at the rate of *N*-acetyllactosamine gave the analytical data indicated below.

^{14}C-labeled product	*Galactose* (μg)*	*GlcNAc** (μg)*	*Molar ratio Gal:GlcNAc*
Analysis before hydrolysis		0.3	
Analysis after hydrolysis	5.05	5.30	1.00:0.86

* Galactose oxidase assay.
** Morgan–Elson reaction after re-acetylation[5].

in equimolar amounts. The absence of color obtained in the Morgan–Elson reaction of the unhydrolyzed compound suggests that the hexosamine moiety was substituted at C-4 (ref. 11). Digestion with β-galactosidase or acid hydrolysis released galactose as the only radioactive product (paper chromatography, Solvent C). Furthermore, incubation of unlabeled UDP-galactose (0.25 μmole) with *N*-acetyl-[^{14}C]glucosamine (0.5 μmole; see MATERIALS AND METHODS) and the particulate enzyme in a total volume of 0.12 ml resulted in the production of a substance (3800 counts/min) with chromatographic properties indistinguishable from those where ^{14}C-labeled UDP-galactose was incubated with unlabeled GlcNAc.

TABLE II

SUBSTRATE SPECIFICITY OF GALACTOSYLTRANSFERASE

For experimental details, see MATERIALS AND METHODS. The specific activity of UDP-[^{14}C]-galactose was 20 μC/μmole.

Acceptor	*^{14}C-labeled product (counts/min)*
GlcNAc	32 200
GalNAc	0
Glucose	480
Xylose	5 800
Gal-4-Xyl	2 300

These data establish that the product formed on incubation of UDP-galactose and *N*-acetylglucosamine with the particulate enzyme from chick cartilage was identical with *N*-acetyllactosamine.

Some properties of the UDP-galactose:GlcNAc galactosyltransferase in chick cartilage

Table II summarizes the galactose acceptor activity of some exogenous substrates that were tested. Of all the compounds examined, *N*-acetylglucosamine was

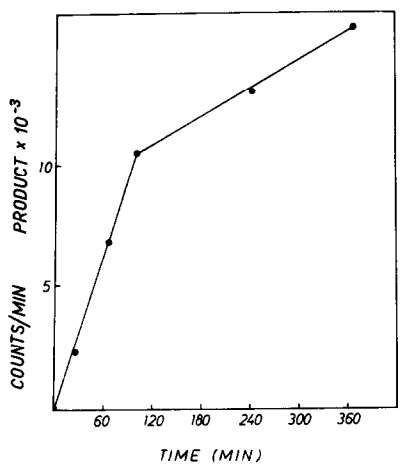

Fig. 2. Formation of *N*-acetyllactosamine as a function of time. The incubations were carried out under standard assay conditions (see MATERIALS AND METHODS) during the periods of time indicated.

by far the most active substrate. *N*-Acetylgalactosamine possessed no acceptor activity, whereas glucose exhibited a small but significant activity. The product migrated as lactose on paper chromatography (Solvents A and B) and released galactose as the sole radioactive substance on treatment with β-galactosidase (paper chromato-

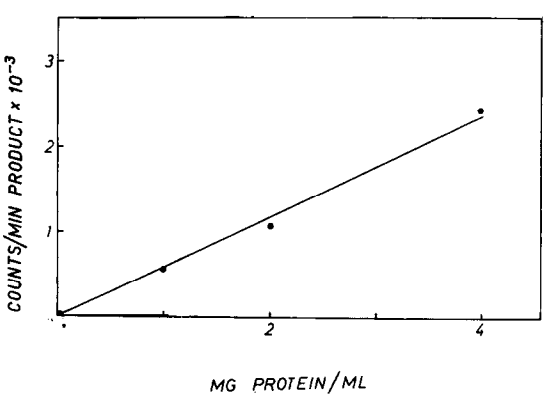

Fig. 3. Formation of *N*-acetyllactosamine as a function of protein concentration. The standard procedure was employed, except that half the usual amount of UDP-[^{14}C]galactose was included in each reaction mixture.

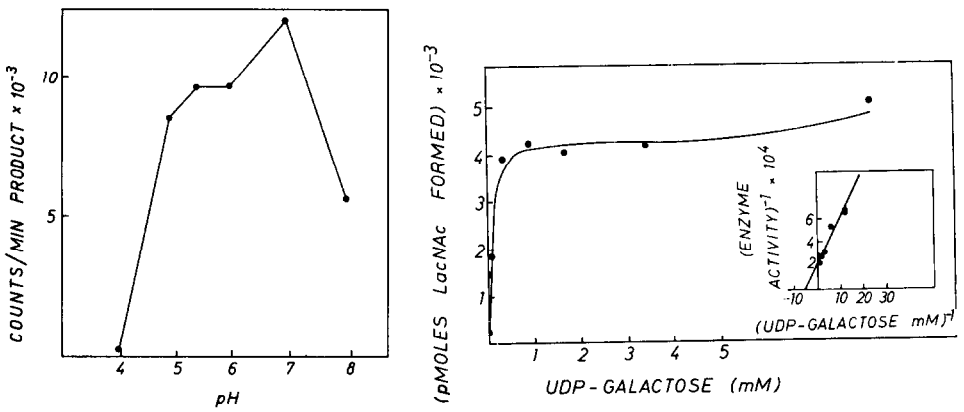

Fig. 4. Transfer of galactose to *N*-acetylglucosamine as a function of pH. The enzyme was prepared by suspending the particulate fraction from 2 dozen embryos in the buffer of appropriate pH and recentrifuging in the ultracentrifuge. The pellets were taken up in 1 ml of buffer and incubated as described in MATERIALS AND METHODS.

Fig. 5. Formation of *N*-acetyllactosamine from *N*-acetylglucosamine and UDP-galactose as a function of the nucleotide concentration.

graphy, Solvent C). The acceptor activities of xylose and Gal-4–Xyl have been discussed previously[3].

With *N*-acetylglucosamine as acceptor, product formation increased linearly with time for 90 min (Fig. 2), and it was also proportional to the concentration of protein within the range tested (Fig. 3). The enzyme was active over a wide pH range with an optimum around pH 7.0 (Fig. 4). Determinations of the K_m values for UDP-galactose and *N*-acetylglucosamine gave $1.5 \cdot 10^{-4}$ M and $1.7 \cdot 10^{-3}$ M, respectively (Figs. 5 and 6).

Product formation was greatly stimulated by the presence of Mn^{2+}. A plateau

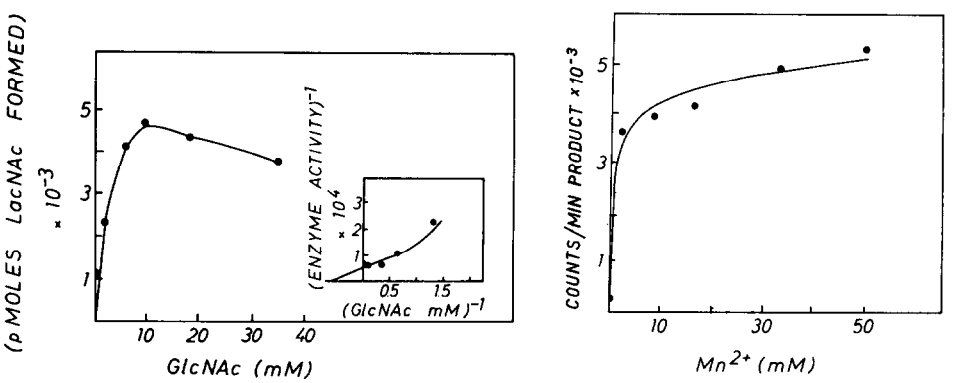

Fig. 6. Formation of *N*-acetyllactosamine from *N*-acetylglucosamine and UDP-galactose as a function of acceptor concentration.

Fig. 7. Transfer of galactose to *N*-acetylglucosamine as a function of Mn^{2+} concentration. The enzyme was prepared in modified Tris–acetate buffer with the omission of $MnCl_2$. Incubations were carried out under standard assay conditions with additions of $MnCl_2$ as indicated.

TABLE III

METAL REQUIREMENTS OF UDP-GALACTOSE:GlcNAc GALACTOSYLTRANSFERASE

The enzyme was prepared in a modified Tris–acetate buffer, pH 7.0, with omission of $MnCl_2$. Each tube contained the metal ion indicated (final concentration, 50 mM), UDP-[^{14}C]galactose, *N*-acetylglucosamine and particulate enzyme. After 90 min at 37°, the products were analyzed by the standard assay procedure.

Metal added (50 mM)	*Activity (%)**
Mn^{2+}	100
Co^{2+}	40
Mg^{2+}	6
Cd^{2+}	0.2
Ca^{2+}	12

* Expressed as percent activity obtained with Mn^{2+}.

was reached at about 1.5 mM concentration (Fig. 7). Among other metals tested, Co^{2+} and Ca^{2+} stimulated the enzyme significantly (Table III).

Mixed substrate experiments with N-acetylglucosamine and fragments from the chondroitin sulfate–protein linkage region

Since the enzyme preparation utilized for the present study has previously been shown to contain galactosyltransferase activities related to the biosynthesis of the two galactose residues occurring in the carbohydrate–protein linkage region of chondroitin sulfate[2,3], it was of interest to establish whether these enzymatic activities were independent from the UDP-galactose:GlcNAc galactosyltransferase described here. As none of the galactosyl transferase activities have been solubilized, substrate

TABLE IV

MIXED SUBSTRATE EXPERIMENT WITH GlcNAc, AND XYLOSE OR Gal-4-Xyl

Each tube contained the amount of exogenous substrate indicated, UDP-[^{14}C]galactose (0.1 μC; 20 μC/μmole) and particulate enzyme (pH 5.4). After incubating the tubes under otherwise standard conditions, the reactions were stopped and the products were determined after chromatography in Solvents A and B.

Substrate (μmoles)			*Product (counts/min)*		
GlcNAc	*Xylose*	*Gal-4-Xyl*	*LacNAc from GlcNAc*	*Gal-4–Xyl from xylose*	*Gal-3–Gal-4–Xyl from Gal-4-Xyl*
	0.50			5 830	
0.05	0.50		9 263	5 880	
0.10	0.50		14 215	5 427	
0.50	0.50		32 198	5 522	
0.05			9 732		
0.05	1.00		9 100	9 155	
0.05	2.00		9 620	14 232	
		0.50			2 360
0.05		0.50	9 357		2 240
0.50		0.50	32 137		2 236

competition experiments were carried out. Conceivably, if the enzyme responsible for the synthesis of *N*-acetyllactosamine also catalyzed the formation of Gal-4–Xyl from xylose, or Gal-3–Gal-4–Xyl from Gal-4–Xyl, it should be possible to demonstrate a competitive effect between the *N*-acetylglucosamine and either of the other two substrates (*cf.* ref. 3). Table IV illustrates an experiment of this type where two galactose acceptors were simultaneously incubated at varying molar ratios. As is seen, neither xylose nor Gal-4–Xyl suppressed product formation from *N*-acetylglucosamine. Similarly, the latter substrate did not impede the transfer of galactose to xylose or to Gal-4–Xyl.

These results suggest that the UDP-galactose:GlcNAc galactosyltransferase reaction demonstrated in the chick cartilage preparation is mediated by a catalytic centre which is distinct from those involved in the biosynthesis of the galactose units in chondroitin sulfate. Further proof of this contention was obtained by heat inactivation studies. By pre-incubation of the enzyme for 5 min at 50°, over 90% of the UDP-galactose:GlcNAc galactosyltransferase activity disappeared, whereas the formation of product from xylose or from Gal-4–Xyl proceeded essentially unaffected (Table V).

TABLE V

HEAT INACTIVATION OF UDP-GALACTOSE:GlcNAc GALACTOSYLTRANSFERASE

Each tube contained xylose (0.5 μmole), Gal-4–Xyl (0.5 μmole) or *N*-acetylglucosamine (0.05 μmole) and the particulate enzyme. After pre-incubation of such mixtures at 50–55° as indicated below, the tubes were cooled on ice. The reactions were started by adding UDP-[^{14}C]galactose (0.05 μC; 280 μC/μmole), and the products were analyzed as described in MATERIALS AND METHODS.

Temperature of preincubation	*Time of preincubation (min)*	*Activity (%)**		
		Gal-4–Xyl from xylose	*Gal-3–Gal-from Gal-4–Xyl*	*LacNAc from GlcNAc*
4°	5	100	100	100
50°	1	101	115	65
50°	5	84	95	7
55°	1	93	98	2

* Counts/min product formed without prior incubation of the enzyme at the higher temperatures = 100%.

Effect of α-lactalbumin on the substrate specificity of UDP-galactose:GlcNAc galactosyltransferase

Recent work has shown that lactose synthetase from milk may be separated by gel chromatography into two protein components (A and B components; ref. 12). The B protein has been found to be identical with α-lactalbumin[13,14]. BREW *et al.*[15] showed that the A protein, although essentially devoid of lactose synthetase activity in the absence of the B protein, catalyzed the transfer of galactose from UDP-galactose to *N*-acetylglucosamine with the formation of *N*-acetyllactosamine. Upon addition of α-lactalbumin, LacNAc synthesis decreased while the formation of lactose from glucose and UDP-galactose was stimulated. These workers also showed that

TABLE VI

EFFECT OF α-LACTALBUMIN ON THE SUBSTRATE SPECIFICITY OF UDP-GALACTOSE:GlcNAc GALACTOSYLTRANSFERASE

Each tube received the amount of α-lactalbumin indicated, *N*-acetylglucosamine (1 μmole) or glucose (1 μmole), UDP-[^{14}C]galactose (0.1 μC; 240 μC/μmole) and particulate enzyme (0.15 mg of protein) in a total volume of 0.07 ml. These mixtures were incubated and assayed under the standard conditions described in MATERIALS AND METHODS.

α-Lactalbumin added (μg)	*Counts/min*	
	LacNAc from GlcNAc	*Lactose from glucose*
0	24 709	
20	23 944	
100	25 660	
200	24 124	
0		247
20		2 322
100		9 698
200		14 400

addition of α-lactalbumin to a UDP-galactose:GlcNAc galactosyltransferase from rat liver promoted the synthesis of lactose, indicating a similarity between this enzyme and the A protein of lactose synthetase.

To test the effect of α-lactalbumin on the LacNAc synthetase under study, glucose and *N*-acetylglucosamine were incubated separately with varying amounts of α-lactalbumin. The results given in Table VI indicate that the synthesis of lactose was stimulated by α-lactalbumin, whereas no significant effect on the transfer of galactose to *N*-acetylglucosamine was observed.

In mixed substrate experiments in the presence of α-lactalbumin, *N*-acetylglucosamine extensively impeded the formation of lactose from UDP-galactose and glucose (Table VII). By contrast, glucose but slightly affected the synthesis of *N*-acetyllactosamine from UDP-galactose and *N*-acetylglucosamine.

TABLE VII

MIXED SUBSTRATE EXPERIMENT WITH GlcNAc AND GLUCOSE IN THE PRESENCE OF α-LACTALBUMIN

Each tube contained the amount of GlcNAc and glucose indicated, UDP-[^{14}C]galactose (0.1 μC; 1 μC/μmole), α-lactalbumin (200 μg) and particulate enzyme (0.15 mg of protein) in a total volume of 0.07 ml. Separation of products was achieved by sequential paper chromatography in Solvents A and B.

Substrate (μmoles)		*^{14}C-labeled product (counts/min)*	
GlcNAc	*Glucose*	*LacNAc from GlcNAc*	*Lactose from glucose*
	2.5		4390
0.5	2.5	1935	612
0.5		2138	

Transfer of galactose from UDP-galactose to endogenous acceptor

The demonstration of the UDP-galactose:GlcNAc galactosyltransferase in chick cartilage prompted further studies to obtain information as to whether this reaction may reflect the synthesis of a similar linkage between galactose and endogenous *N*-acetylglucosamine residues present in the microsomal fraction. Hexosamine analysis (amino acid analyzer) revealed that glucosamine constituted 12% of the total hexosamine in the particulate enzyme preparation. Provided that some of these residues are located at non-reducing, terminal positions, it is conceivable that they might serve as acceptors for the transfer of galactose.

Incubation of the particulate enzyme with UDP-[^{14}C]galactose as described above resulted in incorporation of galactose (26 500 counts/min; 2% of added radioactivity) into trichloroacetic acid-precipitable material. After hydrolysis of the labeled precipitate, a neutral fraction was obtained by passage of the material through columns of Dowex 1 and Dowex 50. On chromatography of the effluent (8200 counts/min) in Solvent B, small amounts of material migrating similar to fragments from the chondroitin sulfate–protein linkage region, notably Gal-4–Xyl, were observed (Fig. 8a; *cf.* ref. 3). In addition, however, a major peak was located at the position of *N*-acetyllactosamine. Elution of this fraction with water and chromatography in Solvent A showed the presence of a compound (800 counts/min; 3% of the radioactivity incorporated into the trichloroacetic acid-precipitable material) with an R_F-value identical to that of *N*-acetyllactosamine (Fig. 8b). Treatment with β-galactosidase released all the radioactivity as galactose (paper chromatography, Solvent C).

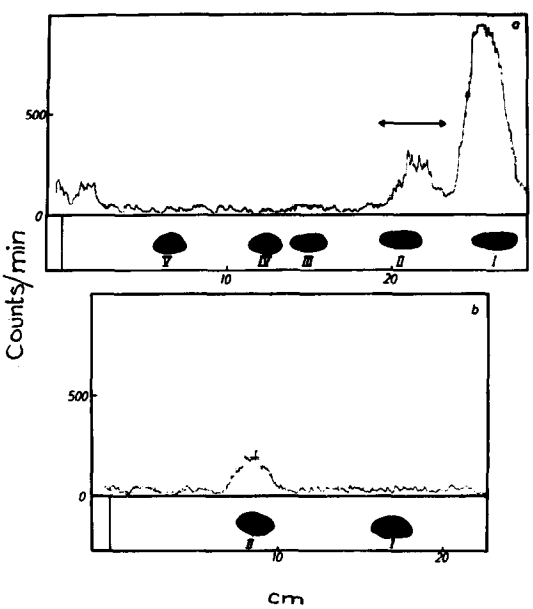

Fig. 8. Transfer of galactose from UDP-[^{14}C]galactose to endogenous acceptor. (a) Distribution of radioactivity obtained after paper chromatography (Solvent B) of a deionized hydrolysate of the labeled trichloroacetic acid precipitate. The material eluted from the section indicated was subjected to paper chromatography in Solvent A (b). The standards shown on the guide strips are: I, galactose; II, *N*-acetyllactosamine; III, Gal-4-Xyl; IV, Gal-3-Gal; V, Gal-3-Gal-4-Xyl.

Due to the small amounts of material available, a more detailed characterization of this hydrolytic fragment was not carried out, but its identity with *N*-acetyllactosamine was also indicated by the fact that it appeared at the same effluent volume as did the latter compound after chromatography on a column (2 cm × 140 cm) of Sephadex G-25.

DISCUSSION

Transfer of galactose from UDP-galactose to *N*-acetylglucosamine has previously been reported to occur in a number of tissues including colostrum, rat liver, thyroid and chick brain[10,16,17]. The study of an enzyme catalyzing the same reaction in chick cartilage was desirable since previous work on the biosynthesis of chondroitin sulfate in this system has established the presence of two independent catalytic centers for galactosyl transfer[3].

The results obtained in the present study suggest that the synthesis of *N*-acetyllactosamine in chick cartilage is mediated by a third distinct catalytic center, independent from the two galactosyl transfer reactions which complete the biosynthesis of the neutral trisaccharide in the chondroitin sulfate–protein linkage region. This conclusion is based on substrate competition experiments as well as on heat inactivation studies.

The demonstration of the LacNAc synthetase as an independent catalytic center in this system raised the question of whether transfer of galactose to endogenous *N*-acetylglucosamine residues occurs in chick cartilage. Although a significant portion of the galactose incorporated into the endogenous acceptor present in the microsomal fraction of the chick cartilage homogenate certainly represents the synthesis of the chondroitin sulfate–protein linkage region[2,3], it is likely that other types of galactosyl transfer also occur. For example, the yield of [^{14}C]Gal-4-Xyl from a partial acid hydrolysate of [^{14}C]galactose-labeled endogenous acceptor is considerably lower than may be expected for the transfer of galactose to chondroitin sulfate precursors only (about 1.5%; *cf.* refs. 1 and 3). It is thus possible that the UDP-galactose:GlcNAc galactosyltransferase being investigated may reflect the synthesis of a similar linkage to a moiety of *N*-acetylglucosamine, bound to an as yet unidentified structure. The presence of a compound with the chromatographic properties of *N*-acetyllactosamine in a partial acid hydrolysate of [^{14}C]galactose-labeled endogenous acceptor supports this contention.

Although the structures of the galactose acceptors in chick cartilage are largely unknown, some observations pertaining to this matter should be considered. ROBINSON *et al.*[2] found no evidence for the presence of collagen precursors as acceptors for galactose. Furthermore, it is possible that synthesis of keratan sulfate, which consists of alternating galactose and *N*-acetylglucosamine residues, may account for some of the galactose incorporated into embryonic chick cartilage. However, the quantity of keratan sulfate present in other types of embryonic cartilage tissue has been shown to be negligible[18]. Furthermore, no appreciable synthesis of keratan sulfate was observed on incubating the particulate enzyme preparation with UDP-[^{14}C]galactose and unlabeled UDP-*N*-acetylglucosamine. After treatment of the incubation mixture with papain and chromatography of the digest on a column of Sephadex G-50, practically none of the added radioactivity appeared with the void volume fractions.

These observations do not exclude the possibility that the enzyme might be involved in the synthesis of keratan sulfate present in the cartilage tissue of grown animals. It seems likely, however, that the endogenous galactose acceptors of embryonic chick cartilage consist of proteins to which low-molecular-weight carbohydrate prosthetic groups are attached.

In view of recent reports[13–15] concerning the relationship between the A subunit of lactose synthetase and LacNAc synthetase activity, it was of interest to study the effect of α-lactalbumin on the substrate specificity of the system from chick cartilage. In agreement with the observation by BREW *et al.*[15] on the LacNAc synthetase from rat liver, addition of α-lactalbumin to the chick cartilage enzyme greatly stimulated the synthesis of lactose from glucose and UDP-galactose. In contradiction to the results obtained with the rat-liver enzyme, however, the transfer of galactose to *N*-acetylglucosamine was not impeded as the concentration of α-lactalbumin was increased. The reason for this discrepancy is unknown but the lack of inhibition may be due to the particulate nature of the cartilage enzyme, or possibly to species differences. As is seen from Table VII, *N*-acetylglucosamine appeared to be the preferred substrate even in the presence of α-lactalbumin. Whereas the presence of a molar excess of glucose over *N*-acetylglucosamine impeded the synthesis of *N*-acetyllactosamine but slightly, the formation of lactose decreased sharply in mixed substrate experiments with glucose and *N*-acetylglucosamine.

The further characterization of the enzyme investigated in the present study, particularly with regard to its relationship with the A subunit of lactose synthetase, awaits the solubilization and purification of the LacNAc synthetase in embryonic chick cartilage.

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