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Galactosyltransferase from buffalo milk: Further characterization

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Abstract. Buffalo milk galactosyltransferase is a single poly-peptide of molecular weight 55,000 to 56,000. The enzyme is specific for glucose as an acceptor substrate in the presence of ∝-lactalbumin, L-Arabinose. L-xylose, D-ribose and D-fructose did not serve as acceptor substrates even at concentration as high as 0.13 M, while N-acetylglucosamine and ovalbumin served as good acceptors of galactosyl moiety in the absence of ∝ -lactalbumin. UDP-galacturonic acid did not serve as a donor substrate; on the contrary, it inhibited the reaction. Lactose synthetase reaction was inhibited by D-ribose, L-arabinose and L-xylose, whereas D-fructose did not show any inhibition. Buffalo milk ∝ -lactalbumin enhanced the synthesis of lactose but inhibited the synthesis of N-acetyllactosamine. Cations like Ca^{2+} , Mg^{2+} , Cu^{2+} , Ba^{2+} and Co^{2+} could not replace Mn^{2+} in the N-acetyllactosamine synthetase reaction. Except Co^{2+} , these cations had no effect on this reaction. Co^{2+} was found to be a competitive inhibitor of Mn^{2+} . The observed inhibition of the reaction by·EDTA also confirmed the absolute requirement of Mn^{2+} for the reaction. Lactose synthetase reaction had an optimum pH of 8.5, whereas N-acetyllactosamine synthetase reaction was maximal at pH 8.0.

Keywords. Galactosyltransferase; buffalo milk; lactose synthetase.

Introduction

Galactosyltransferase (EC 2.4.1.38) catalyzes the formation of N-acetyllactosamine from UDP-galactose (UDP-gal) and N-acetylglycosamine (GlcNAc). Besides, the enzyme also synthesizes lactose using glucose as an acceptor of galactosyl moiety in presence of ∝ -lactalbumin (Brew *et al*., 1968). Extensive studies have been carried out on galactosyltransferases from bovine (Hill *et al*., 1968; Morrison and Ebner, 1971a, b, c; Ebner, 1973) and human milk (Andrews, 1970; Khatra *et al*., 1974). Galactosyltransferases from other sources like human serum (Kim *et al*., 1972), human cerebrospinal fluid (Ko *et al*., 1973), rat liver (Fraser and Mookerjee, 1977), chloroplast envelopes of soybean cotyledons (Dalgarn *et al*., 1979) and *Neurospora crassa* (Forsthoefel and Mishra, 1977) have also been studied. The Purification and some kinetic properties of the buffalo milk enzyme have already been reported (Mahajan *et al*., 1979). In this communication, we report further characterization of the buffalo milk galactosyltransferase.

Abbreviations used: GlcNAc: N-acetylglucosamine, UDP-gal: UDP-galactose, SDS: sodium dodecyl sulphate.

Materials and methods

Materials

NADH, phosphoenol pyruvate, pyruvate kinase type I (EC 2.7.1.40), containing 30 units of lactate dehydrogenase (EC 1.1.1.27) activity per mg protein, GlcNAc, bovine serum albumin and UDP-gal were purchased from Sigma Chemical, Co. St. Louis, Missouri, USA. UDP-galacturonic acid was from Calbiochem, Richmond, California, USA. Tris and 2-mercaptoethanol were purchased from E. Merck, Dermstadt, Germany and ∝ -chymotrypsin was from Nutritional Bio-Chemical Corporation, Cleveland, Ohio, USA. All other reagents used were of analytical grade and were purchased either from BDH or S. Merck, India.

Methods

Galactosyltransferase was purified from buffalo milk by ammonium sulphate precipitation, hydrophobic chromatography on norleucine-Sepharose and affinity chromatography on ∝ -lactalbumin-Sepharose. Concurrently ∝ -lactalbumin was also isolated and purified from buffalo milk (Mahajan *et al*., 1979).

Galactosyltransferase was assayed spectrophotometrically using Beckman spectrophotometer model 25, by the method of Ebner *et al*., (1972). When glucose was used as an acceptor substrate, ∝ -lactalbumin was included in the assay mixture. This reaction has been designated as the 'lactose synthetase' activity of the enzyme. N-acetyllactosamine synthetase activity of the enzyme was assayed using GlcNAc as an acceptor substrate in the absence of ∝ -lactalbumin. Concentrations of different reagents in the assay mixture (1 ml) were as follows: glycine, 50 mM pH 8.5; $MnCl_2$, 5 mM; NADH, 0.083 mM; phosphoenol pyruvate, 0.8 mM; pyruvate kinase, 21.0 units; ∝ -lactalbumin, 200 μg; UDP-gal, 0.23 mM; and either glucose 20 mM or GlcNAc, 20 mM. About 8-10 μg of enzyme, obtained after chromatography on ∝ -lactalbumin-Sepharose column, was used in each assay mixture. One unit of enzyme activity is defined as the amount of enzyme needed for the formation of 1 μ mol of UDP per min at 25°C.

Polyacrylamide gel electrophoresis of galactosyltransferase was performed using 7.5% gels, prepared according to the method of Davis (1964). Fifty mM potassium phosphate buffer pH 8.0 (Gomori, 1955), was used in the buffer chambers. The gels were stained with either Coomassie Brilliant Blue for protein detection or with periodate-Schiff's base stain for the detection of glycoproteins (Zacharius *et al*., 1969).

Molecular weight of galactosyltransferase was estimated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Weber and Osborn, 1969), as well as by using gel filtration on Sephadex G-100. K_{av} was calculated as described by Reiland (1971).

The different kinetic properties of the enzyme, namely, substrate specificity, effect of varying concentrations of ∝-lactalbumin, effect of various cations and effect of pH were studied using the spectrophotometric assay. The inhibition of lactose synthetase reaction by various sugars as well as the inhibitory effects of UDP-galacturonic acid, EDTA and Co^{2+} on the N-acetyllactosamine synthetase reaction were also studied.

Results

Molecular weight estimation

Using a calibrated Sephadex G-100 column, the molecular weight of the enzyme was estimated to be 56,000. The data, when replotted in the form of log of molecular weight against K_{av}, gave the same value (figure 1). SDS-gel electrophoresis under non-denaturing conditions gave a value of 55,000.

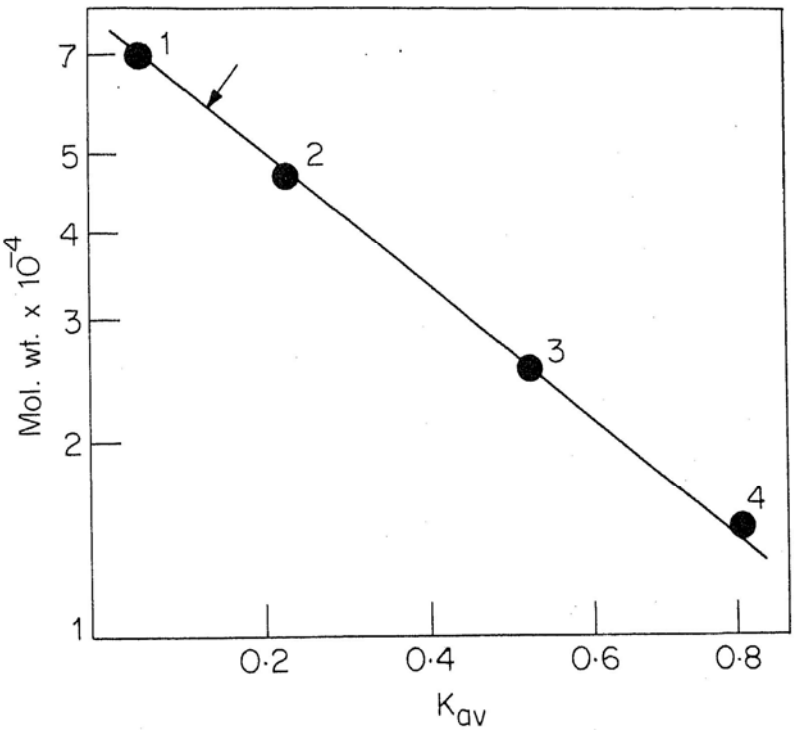

Figure 1. Estimation of molecular weight of buffalo milk galactosyltransferase using Sephadex G-100.
Sephadex G-100 column (0.9×100 cm) was equilibrated and developed with 20 mM Tris-HCl buffer, pH 7.5 containing 0.1 M KCl. The column was calibrated using (i) bovine serum albumin (68,000), (ii) ovalbumin (45,000), (iii) ∝-chymotrypsin (24,000) and (iv) bovine ∝-lactalbumin (14,500) as marker proteins. The numbers in paranthesies indicate molecular weights. Arrow indicates the position of galactosyltransferase.

Substrate specificity

Buffalo milk galactosyltransferase was specific for glucose as an acceptor of galactosyl moiety in the presence of ∝-lactalbumin. L-Arabinose, L-xylose, D-ribose and D-fructose could not replace glucose at concentrations even as high as 130 mM. In the absence of ∝ -lactalbumin, however, the enzyme was found to be more specific for GlcNAc as an acceptor substrate. Ovalbumin, a glycoprotein, could also accept galactose in the absence of ∝ -lactaibumin. UDP-galacturonic acid could not replace UDP-gal in the reaction mixture.

Inhibition by substrate analogues

None of the sugars studied inhibited lactose synthetase reaction at lower concentrations. However, at 130 mM concentration, L-arabinose showed 39% inhibition, D-ribose showed 46% inhibition, whereas L-xylose showed only 16% inhibition, and the enzyme D-fructose did not inhibit. UDP-galacturonic acid, an analogue of UDP-gal, inhibited the N-acetyllactosamine synthetase reaction. A maximum of 48% inhibition was obtained at 0.5 mM UDP-galacturonic acid concentration (figure 2).

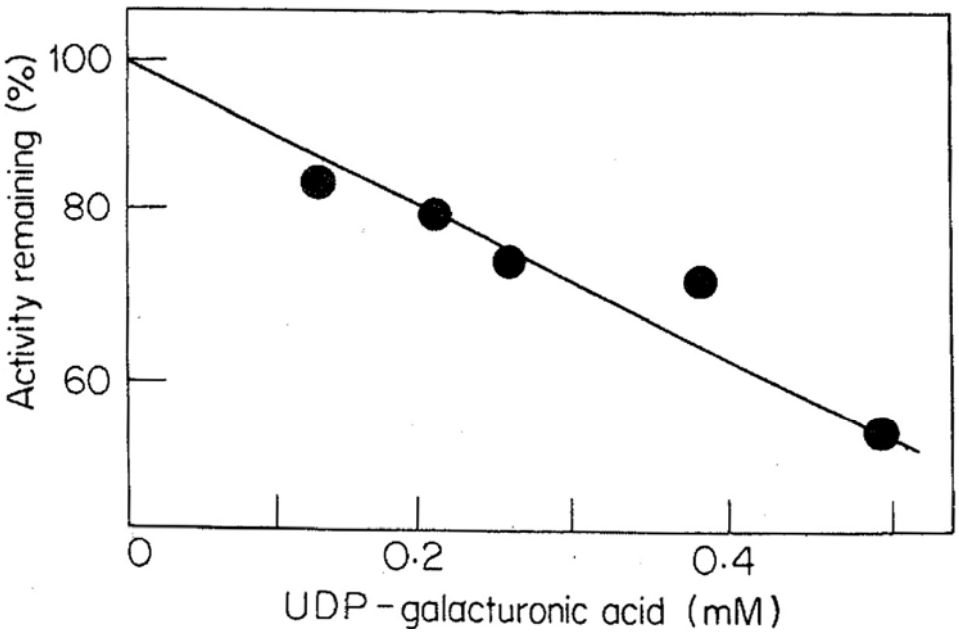

Figure 2. Effect of varying concentrations of UDP-galacturonic acid on N-acetyllactosamine synthetase of buffalo milk galactosyltransferase.
Reactions were carried out in the absence of ∝-lactalbumin and at fixed concentrations of UDP-gal (0.23 mM). Concentrations of other reagents in the reaction mixture were as described in the text.

Effect of ∝ -lactalbumin

The rate of lactose synthetase reaction increased with increasing concentrations of ∝ -lactalbumin in the reaction mixture, when the reaction was studied at 20 mM glucose concentration. However, the N-acetyllactosamine synthetase reaction was inhibited by ∝ -lactalbumin. A maximum of 82% inhibition was obtained at 200 μg/ml ∝ -lactalbumin (figure 3).

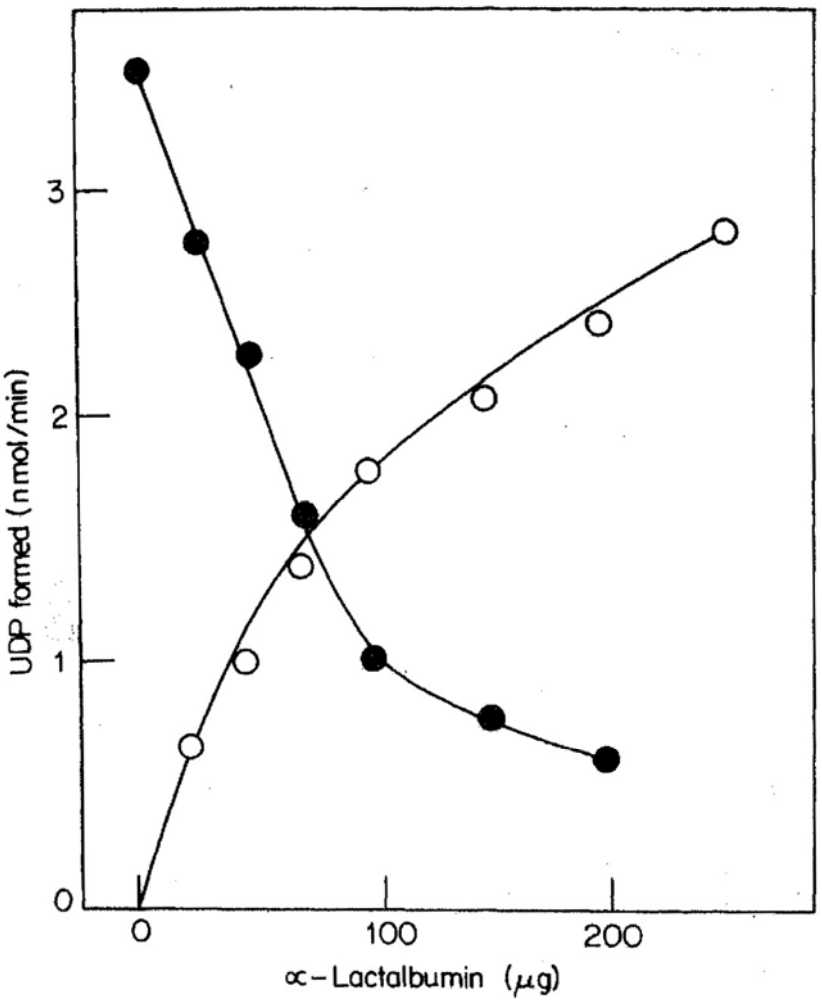

Figure 3. Effect of buffalo milk ∝ -lactalbumin on reactions of buffalo milk galactosyltransferase. Lactose synthetase (O) reaction was studied in the presence of different concentrations of buffalo milk ∝ -lactalbumin with 20 mM glucose; N-acetyllactosamine synthetase (●) reaction was studied with 20 mM GlcNAc. Concentrations of all other reagents in the reactions were as described in the text.

Effect of cations

Of the various cations studied, none could replace Mn^{2+} in the N-acetyllactosamine synthetase reaction. A negligible reduction in the rate of reaction was observed when either Ca^{2+} or Mg^{2+} was present at 5 mM concentration in the reaction mixture, while Cu^{2+} or Ba^{2+} at this concentration did not affect the rate of the reaction. Addition of 5 mM Hg^{2+} caused precipitation of the reaction mixture. A marked inhibition of the reaction was observed with Co^{2+} (table 1). Approximately 50% inhibition was observed when the concentrations of Co^{2+} and Mn^{2+} were equal in the reaction mixture (figure 4, inset). In another set of experiments, reactions were

Table 1. Effect of various cations on the N-acetyllactosamine synthetase reaction of buffalo milk galactosyltransferase.

Cation (5 mM)	nmol UDP formed min^{-1}
None	2.96
Ca^{2+}	2.80
Mg^{2+}	2.80
Ba^{2+}	2.90
Co^{2+}	1.60
Cu^{2+}	2.90
Hg^{2+}	caused precipitation

Effect of various cations on the N-acetyllactosamine reaction of buffalo milk enzyme was studied using GlcNAc as the acceptor substrate. Reactions were carried out in the presence of 5 mM Mn^{2+} No reaction was observed in the absence of Mn^{2+} and in the presence of 5 mM of individual cation. Concentrations of other reagents in the reaction mixture were as described in the text.

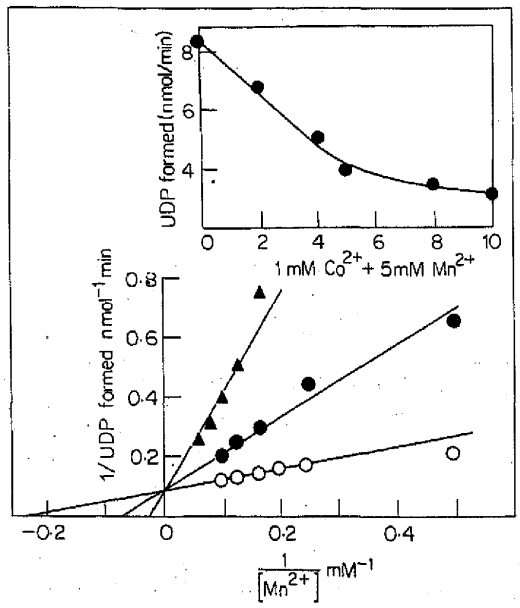

Figure 4. Competitive inhibition of Mn^{2+} by Co^{2+} in the N-acetyllactosamine synthetase reactions of buffalo milk galactosyltransferase. Co^{2+} , O; 4 mM Co^{2+} ,●; 8 mM Co^{2+} , ▲. Inhibitory effect of Co^{2+} on the reaction was studied at different fixed concentrations of Co^{2+} and at varying concentrations of Mn^{2+} , GlcNAc (20 mM) was used as an acceptor substrate in the absence of ∝ -lactalbumin. Inset shows the effect of varying concentrations of Co^{2+} on N-acetyllactosamine synthetase reaction in presence of 5 mM Mn^{2+} . Concentrations of all the other reagents in the reaction mixture were as described in the text.

carried out at different fixed concentrations of Co^{2+} and at varying concentrations of Mn^{2+}. A family of straight lines was obtained in the double reciprocal plot. Upon extrapolation, these lines intersected at a point on Y axis but the slopes of these lines were different (figure 4).

Inhibition by EDTA

EDTA inhibited N-acetyllactosamine synthetase reaction of the buffalo enzyme. Complete inhibition of the reaction was observed when the concentrations of EDTA and Mn^{2+} in the reaction mixture were equal. The inhibitory effect could be reversed by increasing Mn^{2+} concentrations in the reaction mixture (figure 5).

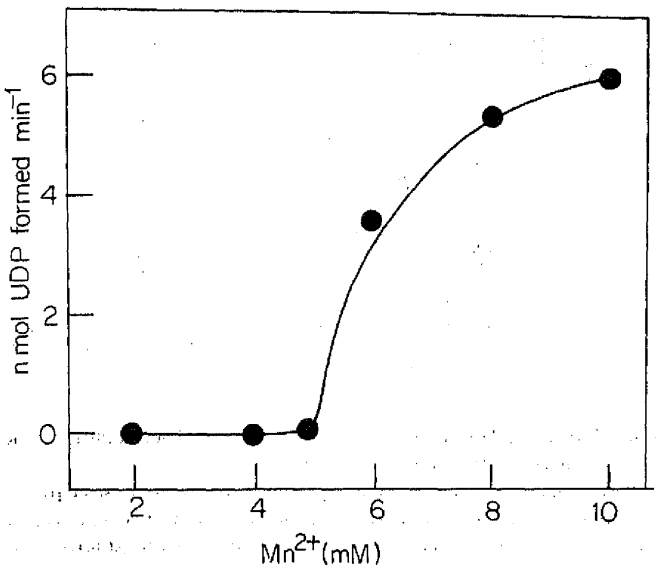

Figure 5. Reversible inhibition of N-acetyllactosamine synthetase reaction by EDTA.
Effect of EDTA on N-acetyllactosamine synthetase reaction was studied at a fixed concentration of EDTA (5 mM) and at varying concentrations of Mn^{2+} . Concentrations of other reagents in the reaction mixture were as described under assay.

Effect of pH

Effect of varying hydrogen ion concentration on the reactions of galactosyltransferase was studied using 50 mM glycine-KOH buffer in the pH range 6.2 to 9.5 (figure 6). N-Acetyllactosamine synthetase reaction was maximum at pH 8.0, whereas the rate of lactose synthetase reaction was optimal at pH 8.5.

Discussion

Galactosyltransferase from buffalo milk was made up of a single polypeptide as determined by polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis (figure 7). Trayer and Hill (1971) have reported three different

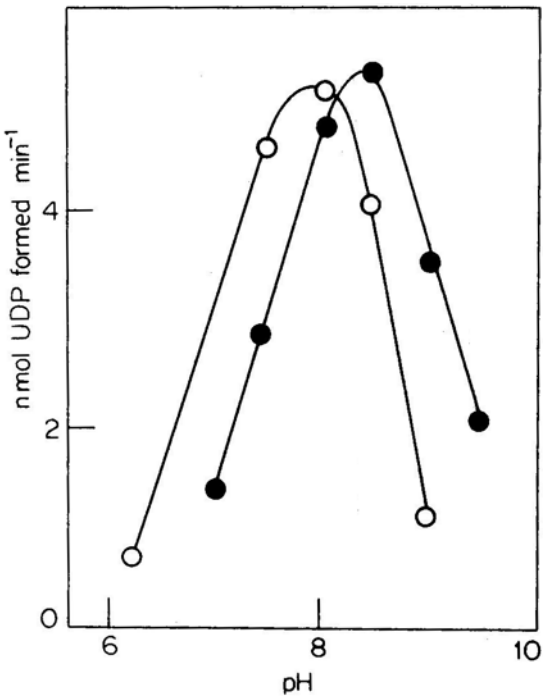

Figure 6. Effect of pH on lactose synthetase and N-acetyllactosamine synthetase reactions of buffalo milk galactosyltransferase.
Reactions were carried out using 50 mM glycine-KOH buffer of desired pH. Lactose synthetase reaction (●) was studied using 20 mM glucose and N-acetyllactosamine synthetase reaction (O) in absence of ∝ -lactalbumin and using 20 mM GlcNAc. Concentrations of other reagents in the reaction mixture were as described in the text.

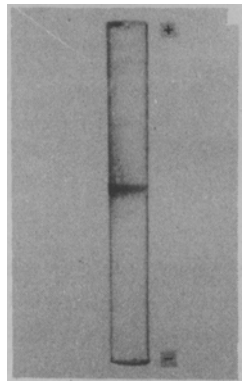

Figure 7. SDS-polyacrylamide gel electrophoresis of buffalo milk galactosyltransferase.
Electrophoresis was performed using 10% gels prepared in 0.2 M sodium phosphate buffer, pH 7.2 containing 0.2% SDS and 0.1% 2-mercaptoethanol. Buffer in the electrophoretic chamber was 0.1 M phosphate, pH 7.2, containing 0.1% SDS. About 80 μg of SDS treated protein was loaded on each gel and electrophoresis was done at 10 mA per gel for 4 h. Direction of migration was from top to bottom.

forms of bovine enzyme with molecular weights 54,000,48,000 and 42,000. However, Magee *et al*. (1974) isolated only two active forms of bovine enzyme with molecular weights 55,000-59,000 and 42,000-44,000. The higher molecular weight form of the enzyme is believed to undergo proteolysis (Magee *et al*., 1976) giving rise to lower molecular weight forms. The enzyme in colostrum, however remained in the higher molecular weight form only, due to the presence of protease inhibitors in the colostrum (Powel and Brew, 1974). Human milk enzyme also exhibited heterogeneity (Prieels *et al*., 1975). Our studies with the limited number of samples surveyed from buffalo milk did not show any heterogenity of the enzyme. Its molecular weight as estimated by gel exclusion chromatography was 56,000, while a value of 55,000 was obtained by SDS-gel electrophoresis. The red coloured protein bands observed on staining the polyacrylamide gels with periodate-Schiff' s base (Zacharius *et al*., 1969) suggested that the buffalo milk enzyme was a glycoprotein.

The enzyme was specific for UDP-gal as the donor substrate and was similar to the galactosyltransferases from bovine milk (Ebner *et al*, 1972), rat serum (Fraser and Mookerjea, 1977), reproductive organs of rodent and human males (Tadolini *et al*., 1977) and chick embryo (Risteli, 1978). In the present studies, we found that UDP-galacturonic acid could not replace UDP-gal in the reaction, indicating the requirement of an essential—CH_2OH group at the C-6 position of galactosyl moiety. On the contrary, UDP-galacturonic acid inhibited the N-acetyllactosamine synthetase reaction. Similar observations were reported for the enzyme from chick embryo (Risteli, 1978). Recently, a model for the UDP-gal binding site of the enzyme has been proposed (Andree and Berliner, 1978; Berliner and Andree, 1978). The binding site near the C-4 position has been proposed to play a crucial role in determining the substrate specificity. The buffalo enzyme is highly specific for glucose as an acceptor of galactose in the presence of ∝ -lactalbumin. L- xylose, L-arabinose, D-ribose and D-fructose did not serve as acceptor substrates even in the presence of ∝ -lactalbumin and at concentrations upto 130 mM. However, the enzyme was inhibited by L-arabinose, L-xylose and D-ribose at concentrations above 30 mM. L-Arabinose, L-xylose, D-ribose and UDP-galacturonic acid did not inhibit the actions of pyruvate kinase and lactate dehydrogenase, the enzymes used in coupled assay system used to estimate galactosyltransferase. Our results are in good agreement with those reported for the bovine enzyme (Morrison and Ebner, 1971b). In the absence of ∝ -lactalbumin, the enzyme transferred galactose to GlcNAc very efficiently. Ovalbumin was also a good acceptor of galactose in the absence of ∝-lactalbumin. Thus, the buffalo milk enzyme was very similar to the bovine milk enzyme (Schanbacher and Ebner, 1970).

The modifier role of buffalo milk ∝ -lactalbumin was studied using buffalo milk galactosyltransferase. Lactose synthetase activity of the enzyme, when studied at millimolar concentrations of glucose, was enhanced by ∝ -lactalbumin. However, the rate of N-acetyllactosamine synthesis decreased with increasing concentrations of ∝ -lactalbumin. Similar observations were reported for the bovine (Ebner, 1970) and human enzymes (Andrews, 1970). Galactosyltransferases from sources other than milk exhibited lactose synthetase activity in the presence of exogenously added ∝ -lactalbumin. The exception being the cancer-associated isoenzyme of galactosyltransferase which was insensitive to the presence of ∝ -lactalbumin (Podolsky and Weiser, 1979), and might be of some biological significance.

Galactosyltransferases in general require Mn^{2+} for their catalytic activity, but the colostrum enzyme was activated by Zn^{2+} , Cd^{2+} , Co^{2+} ,Fe^{2+} or Ca^{2+} (Powel and Brew, 1976). These workers showed that this enzyme had two metal binding sites, one of them was specific for Mn^{2+} . In the present studies, complete inhibition of the reaction was observed at EDTA concentrations equal and above the concentration of Mn^{2+} in the reaction mixture.This inhibitory effect could be reversed by increasing the concentrations of Mn^{2+} above the total concentration of EDTA in the reaction mixture. This confirms our earlier observations that the bufflo milk enzyme is absolutely dependent on Mn^{2+} for its activity (Mahajan *et al*., 1979).

We also observed that none of the metal ions studied could replace Mn^{2+} in the reactions catalyzed by the enzyme. Morrison and Ebner (1971a) have reported similar observations for the purified bovine enzyme. Although Mg^{2+} and Ca^{2+} were reported to replace Mn^{2+} for the crude bovine enzyme (Ebner *et al*., 1972), the divalent metal ions like Ca^{2+} , Mg^{2+} and Cu^{2+} had no effect at all on the rate of the N-acetyllactosamine synthetase reaction of the purified buffalo enzyme. A heavy metal ion, like Hg^{2+} caused precipitation of the reaction mixture. Only Co^{2+} showed a significant inhibition. Mg^{2+} , Co^{2+} and Mn^{2+} , which can form both octahedral and tetrahedral complexes, are activators of pyruvate kinase (Mildvan and Cohn, 1965) while these and other metal ions have no effect on lactate dehydrogenase (Hayaishi, 1966).

A detailed kinetic analysis was carried out to understand the inhibitory effect of Co^{2+} on the N-acetyllactosamine synthetase reaction. From the typical kinetic pattern obtained, we conclude that Co^{2+} acts as a competitive inhibitor of Mn^{2+} , consistent with the observations of Risteli (1978) with the chick embryo enzyme. This conclusion is further supported by the fact that Co^{2+} and Mn^{2+} have very close ionic radii and have the same charge (Lange, 1964).

Buffalo milk enzyme showed a broader pH profile. The lactose synthetase activity was maximum at pH 8.5, whereas the N-acetyllactosamine synthetase reaction showed an optimum pH of 8.0. Fitzgerald *et al*., (1970) have reported similar values for the bovine enzyme.

In conclusion it can be said that though galactosyltransferases from cow and buffalo milk differ in the number of their molecular forms and their molecular weights, most of their kinetic properties are similar.

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