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Cationic activation of galactosyltransferase from rat mammary Golgi membranes by polyamines and by basic peptides and proteins

Naveenan NAVARATNAM, Surjit S. VIRK,* Simon WARD and Nicholas J. KUHN†
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

1. Galactosyltransferase (EC 2.4.1.22) requires bivalent metal ions for its activity. However, preparations of this enzyme solubilized from Golgi membranes of lactating rat mammary gland were shown to be activated not only by Mn^{2+}, Ca^{2+} and Mg^{2+}, but also by spermine, spermidine, lysyl-lysine, ethylenediamine and other diaminoalkanes, and by a range of basic proteins and peptides, including clupeine, histone, polylysine, ribonuclease, pancreatic trypsin inhibitor, cytochrome *c*, melittin, avidin and myelin basic protein. Both *N*-acetyl-lactosamine synthetase and lactose synthetase activities were enhanced. 2. A basic protein fraction was isolated from bovine milk and shown to activate galactosyltransferase at low concentrations. 3. The polyanions ATP, casein, chondroitin sulphate and heparin reversed the activation of galactosyltransferase by several of the above substances. 4. Galactosyltransferase, assayed as a lactose synthetase, showed a 10-fold greater affinity for glucose when Mn^{2+} ions were replaced by clupeine or by ribonuclease as cationic activator. Evidence was obtained for the presence of an endogenous cationic activator in solubilized Golgi membrane preparations which evoked a similar low apparent $K_{m,glucose}$. 5. The findings are discussed in the light of cationic activations of glycosyltransferases generally, of the porous nature of the Golgi membrane, and of the unlikelihood of bivalent metal ions being the physiological activators of galactosyltransferase. It is suggested that the natural cationic activator of lactose synthetase may be a secretory protein acting in a manner analogous to the enzyme's activation by α-lactalbumin. 6. A scheme is proposed for the two-stage synthesis of lactose and phosphorylation of casein within the cell, to accommodate the apparent incompatibility of these two processes.

INTRODUCTION

Bivalent metal ions are an absolute requirement for the activity of galactosyltransferase (EC 2.4.1.22), a Golgi-located enzyme that attaches galactosyl β1-4 residues in glycoproteins and, in the presence of α-lactalbumin, in lactose. Maximum activity is elicited by Mn^{2+} ions, although their optimum concentration has variously been reported as 40–50 mM (Babad & Hassid, 1964; Khatra *et al.*, 1974), 10–13 mM (Spiro & Spiro, 1968; Babad & Hassid, 1966) or only 4–5 mM (Morrison & Ebner, 1971*a*,*b*; Paquet & Moscarello, 1984). However, recognizing that such concentrations of Mn^{2+} are unlikely to occur *in vivo*, Powell & Brew (1976) reinvestigated the galactosyltransferase purified from bovine milk and gave evidence for two distinct metal-ion activation sites. Site I could be satisfied by 10–20 μM-Mn^{2+}, a concentration range that might reasonably be attained *in vivo*. Alternative, but less effective, ions included Zn^{2+}, Cd^{2+} and Fe^{2+}, but not Ca^{2+}. Site II required about 10 mM-Mn^{2+}, -Ca^{2+} or -Sr^{2+}, but not Mg^{2+}. In view of the well-known abundance of calcium in milk, it was suggested that the Mn^{2+}/Ca^{2+} ion-pair might be the natural activators of sites I and II respectively. The Mn^{2+}/Ca^{2+}-activated enzyme reached the activity of the Mn^{2+}/Mn^{2+}-activated enzyme only at a high, extrapolated, UDP-galactose concentration. O'Keeffe *et al.* (1980) have broadly confirmed the existence of two metal-ion sites and have extended knowledge of their properties.

In apparent support for this role of Ca^{2+} ions, several authors have described the ATP-dependent uptake of Ca^{2+} by Golgi-enriched fractions of rat liver (Hodson, 1978), and of lactating mammary gland from cows (Baumrucker & Keenan, 1975), mice (Neville *et al.*, 1981) and rats (West, 1981; Virk *et al.*, 1985). The subject has been reviewed (Neville & Watters, 1983). The particular vesicles involved have not been characterized, but might be the secretory vesicles that, in several cell types, have been found to concentrate Ca^{2+} or Zn^{2+} (see Poisner & Trifaró, 1982). By contrast, glycosyltransferases are located in the Golgi stack, with galactosyltransferase having been particularly located in the *trans* cisternae of HeLa cells and hepatoma cells (Roth & Berger, 1982; Slot & Geuze, 1983).

It might be expected that the natural activator of galactosyltransferase could be identified by surveying the bivalent-metal-ion requirements of other glycosyltransferases, many of which also originate from the Golgi apparatus. However, Table 1 shows that, apart from the general superiority of Mn^{2+} ions, which are evidently unphysiological at the concentration required (about 10 mM), a quite variable pattern emerges. In one case Ni^{2+} is actually far superior to Mn^{2+}, although several other enzymes responded little or not at all to Ni^{2+}. Co^{2+}, Cd^{2+}, Mg^{2+}, and even Cu^{2+} failed to activate some enzymes, although being nearly as effective as Mn^{2+} ions

* Present address: Midland Centre for Neurosurgery and Neurology, Holly Lane, Smethwick, West Midlands B67 7JX, U.K.
† To whom correspondence and reprint requests should be sent.

Table 1. Bivalent-metal-ion requirements of glycosyltransferases and other enzymes on the secretory pathway

All activities are expressed relative to Mn^{2+} (100%). A dash (–) indicates where an ion was not tested. Acceptor substrates employed and author references are: (1) α1-3 mannoside (Oppenheimer & Hill, 1981); (2) β1-4 galactosides (Piller & Cartron, 1983); (3) ganglioside G_{M3} (K. Yanagisawa, personal communication); (4) mucin (W. Wilson & M. Weiser, personal communication); (5) asialofuco-(submaxillary mucin) (Beyer *et al.*, 1980); (6) afuco-(blood-group-A glycoprotein) (Jarkovsky *et al.*, 1970); (7) asialo-casein (Keller *et al.*, 1979); (8) glucose (Babad & Hassid, 1966); (9) asialogalacto-fetuin, *N*-acetylglucosamine or glucose (Fraser & Mookerjea, 1977); (10) high- and low-M_r hydroxylysylgalactose acceptors (Spiro & Spiro, 1971); (11/12) chick-embryo cartilage (Myllylä *et al.*, 1975); (13) skin collagen (Bosmann & Eylar, 1968); (14) lactoceramide (Taniguchi *et al.*, 1985); (15) tetrasaccharide pyrophosphoryl-lipid (Jensen & Schutzbach, 1981); (16) lysosomal enzyme precursor (Waheed *et al.*, 1982); (17) liver microsomes (Larson & Suttie, 1980); (18) asialo-(ovine submaxillary mucin) (Cheng & Bona, 1982).

Author reference	Glycosyltransferase	Metal ion...	Relative activity (%)												
			Mn	Co	Cd	Mg	Ca	Ni	Zn	Fe	Ba	Cu	None	EDTA	Others
1	β1-2-*N*-Acetylglucosaminyltransferase		100	91	78	61	11	34	19	13	0	0	–	0	
2	β1-3-*N*-Acetylglucosaminyltransferase		100	58	76	0	0	18	0	4	–	–	–	0	
3	β1-4-*N*-Acetylgalactosaminyltransferase		100	0	–	0	0	730	0	71	–	21	–	0	
4	β-?-Galactosyltransferase		100	26	49	41	47	–	0	38	36	–	9	0	Li 33
5	α1-2-Fucosyltransferase		100	66	0	82	69	12	0	–	0	0	32	10	
6	α1-4-Fucosyltransferase		100	–	–	84	–	–	–	–	–	–	60	0	
7	Sialyltransferase		100	28	–	46	48	–	14	–	55	38	11	11	
8	β1-4-Galactosyltransferase		100	0	25	25	–	–	–	–	–	–	0	0	
9	β1-4-Galactosyltransferase		100	32	–	17	–	–	4	–	–	11	–	16	
10	α1-2-Glucosyltransferase		100	24	0	41	40	30	22	0	–	0	0	–	
11	α1-2-Glucosyltransferase		100	33	–	23	26	0	0	0	–	0	0	–	
12	β1-*O*-Galactosyltransferase		100	5	–	12	0	0	0	3	–	0	0	–	
13	β1-*O*-Galactosyltransferase		100	86	0	4	6	–	–	–	–	0	4	–	
14	α1-4-Galactosyltransferase		100	16	–	4	–	50	0	10	–	84	–	0	
15	α1-3-Mannosyltransferase		100	9	–	232	137	0	0	–	–	–	–	0	
16	*N*-Acetylglucosamine-1-phosphotransferase		100	5	–	80	4	–	–	–	–	–	–	–	
17	Vitamin K-dependent carboxylase		100	0	0	24	29	–	0	0	–	–	–	–	
18	β1-3-Galactosyltransferase		100	32	90	8	7	–	–	29	10	–	1	1	

with others. Fucosyltransferases tend not to be absolutely dependent upon metal ions, whereas sialyltransferases, apart from that shown, are generally fully active without bivalent metal ions. Zn^{2+}, found in some secretory vesicles, was reported to be inhibitory for several glycosyltransferases. Vitamin K-dependent carboxylase is included in Table 1 as an enzyme on the secretory pathway whose metal-ion requirements seem to correspond interestingly with the others shown.

In the course of our investigations on lactose synthesis, doubts about the possible activating role of Ca^{2+} or Mg^{2+} arose from the observed permeability of the Golgi membrane to electrolytes and non- electrolytes of M_r less than 200–300 (White *et al.*, 1980, 1981*a*,*b*, 1982, 1984). This appears to be due to a proteinase- and heat-sensitive component of the membrane that can be extracted and reconstituted into phospholipid vesicles (Wallace & Kuhn, 1986). Such permeability seemed to imply equilibration between free Ca^{2+} and Mg^{2+} ions of the Golgi lumen and those of the cytosol. Cytosolic free [Ca^{2+}] and [Mg^{2+}] are likely to be about 1 μM and 200 μM respectively. We have therefore searched for other cations that might activate galactosyltransferase and thereby throw light on the natural activation of this enzyme and perhaps of glycosyltransferases generally.

This paper presents evidence for the activation of rat mammary galactosyltransferase by a wide range of organic cations, including spermidine, basic peptides and basic proteins. It is suggested that, in the intact cell, galactosyltransferase may be activated by a non-metal ion that could possibly be a peptide or protein of similar properties. Some of this work has been briefly reported (Kuhn *et al.*, 1985). In the course of, or after, the investigation we came across early reports of the similar activation of UDP-galactose:mucin galactosyltransferase of dog and human trachea (Baker & Hillegas, 1974; Cheng & Bona, 1982).

MATERIALS AND METHODS

Preparations of galactosyltransferase

Golgi membranes were purified from mammary glands of Wistar rats in the days 14–18 of their first lactation (Kuhn & White, 1977) and were solubilized by the procedure of Fleischer & Smigel (1978). The extract (4 ml) was made 75 mM or 150 mM in EDTA and dialysed against 1 litre of 25 mM-Tris/maleate buffer, pH 7.0, containing 1 mM-mercaptoethanol and 1 mg of Triton X-100/ml, with daily changes. The use of [^{14}C]EDTA in certain preparations showed that dialysis left a residual concentration of about 130 μM-EDTA in the enzyme preparation, resulting in not more than about 9 μM in the final assay.

During the later part of this work, variations in the ability of different enzyme preparations to be stimulated, especially by Ca^{2+} ions, prompted us to try varying the above procedures. The omission of EDTA, or the inclusion of 20 μM-$MnCl_2$, during dialysis made no apparent difference; nor did the use of tissue from starved rats, where less carry-over of casein or other milk products might have been expected. However the treatment of the final preparation with Chelex, to remove endogenous activating material, together with the inclusion of 20 μM-$MnCl_2$ in the assay, appeared to give an enzyme preparation that was more uniformly stimulated by Ca^{2+} ions or by clupeine.

Assays

Galactosyltransferase assays contained 40 mM-Tes/NaOH, pH 7.0, 5 mM-*N*-acetylglucosamine, 0.5 mM-UDP-[^{14}C]galactose (0.5 Ci/mol), enzyme (about 1–5 μg of solubilized Golgi membrane protein or 0.6 μg of purified bovine milk galactosyltransferase) and cationic activator as shown, in a final volume of 50 μl. After incubation at 37 °C for 10–60 min, reactions were terminated by cooling in ice and by addition of 50 μl of 0.25 M-lactose/20 mM-EDTA. The solutions were transferred quantitatively to columns of Dowex-1 anionic resin (0.5 ml, formate form) in cut-off Pasteur pipettes and the neutral sugars were eluted into vials with a total of 1.2 ml of water. After the addition of 10 ml of Triton X-100-containing scintillation fluid (Kuhn & White, 1977), each vial was shaken and analysed for ^{14}C on a Beckman LS 1800 liquid-scintillation counter.

Lactose synthetase assays were identical, except for the inclusion of α-lactalbumin (1 mg/ml) and the replacement of *N*-acetylglucosamine by glucose (4 mM unless otherwise stated). Solutions of proteins or other activators were adjusted to pH 7 before addition to the assays. $K_{m,glucose}$ values calculated by the methods of Eisenthal & Cornish-Bowden (1974) and of Lineweaver & Burk gave closely agreeing results.

Extraction of basic-protein fraction from milk

All operations were at 0–4 °C. Unpasteurized bovine milk (1 litre) was centrifuged for 2 h at 75000 *g* to sediment the casein. This was extracted by stirring with 0.1 M-H_2SO_4 (200 ml) for 3 h, collected by centrifugation and re-extracted in the same way. Each extract was mixed with ethanol (5 vol.) and kept overnight to allow precipitation of the insoluble protein. The combined, centrifuged, sediments were suspended in 25 ml of water and dialysed for 2 days against 10 μM-phenylmethanesulphonyl fluoride (5 litres, changed daily), after which the insoluble residue was removed by centrifugation. The supernatant was made 20 mM in Tris/HCl, pH 8.0, and loaded on to a column (approx. 2 cm × 7 cm) of phosphocellulose equilibrated with the same buffer. The column was washed with this buffer to remove unretained protein and then eluted with 1 M-NaCl (approx. 50 ml). The eluted material was dialysed overnight against 20 mM-Tris/HCl, pH 8.0, and loaded on to a similarly sized column of carboxymethylcellulose equilibrated with the same buffer. After being washed with this buffer (35 ml) to remove unretained material, the column was eluted with a gradient (0–1.0 M) of NaCl in this buffer. The single emerging peak of protein [measured by A_{280} and by the method of Lowry *et al.* (1951)] was dialysed against water and freeze-dried to yield a fluffy white residue containing about 2.6 mg of protein.

Preparation of casein

Casein was prepared (for the experiment shown in Fig. 6) by adjusting defatted bovine milk to pH 4.6 with acetic acid and collecting the flocculated material by centrifugation. After the material had been washed twice by resuspension in, and sedimentation from, water, the cycle was repeated. The final casein was taken up in water and the pH adjusted with NaOH to 7.0. The protein concentration was 6.7 mg/ml.

Metal-ion analysis

Samples of Golgi membrane were digested in 60% (w/v) $HClO_4$ containing a few drops of conc. HNO_3, and appropriately diluted portions were analysed by atomic-absorption spectroscopy (kindly carried out by Dr. Dave Jackson and Dr. Paramjit Mahi, Department of Minerals Engineering, University of Birmingham). Correction was made for traces of Mn^{2+} in the digestion medium.

Polyamine analysis

Polyamines were extracted from chopped mammary tissue, and determined with a Locarte automatic amino acid analyser (Takyi *et al.*, 1977).

Sources of materials

Wistar rats were bred in the department. Unpasteurized bovine milk was kindly provided by Mrs. Joanna Clarke of Birmingham Dairies. Clupeine sulphate was from BDH. Galactosyltransferase and other proteins, including poly-L-lysine type VI and histone type II-S, and fine chemicals were from Sigma. UDP-[^{14}C]galactose and [^{14}C]EDTA were from Amersham International or New England Nuclear. Phosphocellulose and carboxymethylcellulose were from Whatman Biochemicals. Metal chlorides were of AnalaR grade and were from Fisons. Myelin basic protein (about 90% pure) was kindly donated by Dr. N. A. Gregson, Department of Anatomy, Guy's Hospital Medical School, London.

RESULTS

Activation of lactose synthetase by Mn^{2+}, Ca^{2+} and Mg^{2+} ions

Fig. 1 shows the activity of lactose synthetase in lysed Golgi preparations with different concentrations of $MnCl_2$, $CaCl_2$ or $MgCl_2$. Each metal ion was optimally active at about 10 mM, with Ca^{2+} and Mg^{2+} about 20–25% as effective as Mn^{2+}. The Mg^{2+} activation contrasts with the finding of Powell & Brew (1976). Experiments reported below were mostly carried out with preparations of galactosyltransferase that had been solubilized from Golgi membrane with Triton X-100, treated with EDTA, and dialysed for 4 days against an EDTA-free medium (see the Materials and methods section). Such preparations were considered devoid of free metal ion arising from the tissue or from the $MgCl_2$-containing medium in which Golgi membranes are prepared. The omission of EDTA or the inclusion of 10 μM-$MnCl_2$ in the dialysis did not appear to affect the properties of the enzyme. However, dialysis for a further 3 days against deionized water generated preparations of galactosyltransferase that did not respond to added Ca^{2+} or Mg^{2+} ions unless 10–20 μM-Mn^{2+} was also added. It was presumed that such preparations had lost endogenous Mn^{2+} ions essential for site-I activity according to the model of Powell & Brew (1976). Activation of 4-day-dialysed galactosyltransferase by Ca^{2+}, Mg^{2+} and by the organic cations described below was ascribed to their occupation of site II.

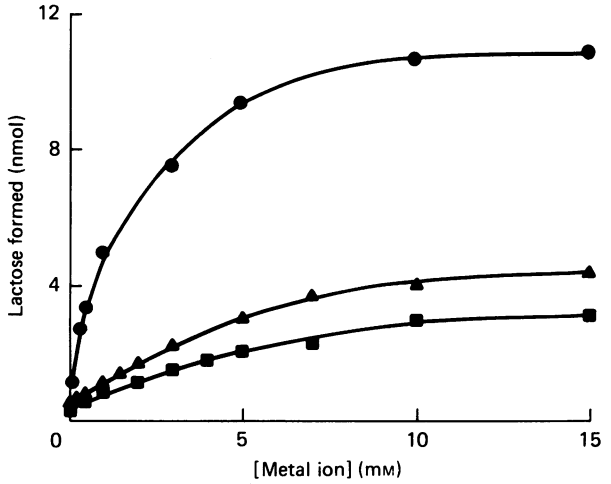


Fig. 1. Activation of galactosyltransferase by different concentrations of $MnCl_2$ (●), $CaCl_2$ (▲) and $MgCl_2$ (■)

Activation of galactosyltransferase by polyamines

Organic cations were examined in an attempt to find alternative activators. Fig. 2 shows that both spermidine and spermine activated the enzyme at concentrations similar to those used for activation by Ca^{2+} and Mg^{2+}. Some activation was also seen with lysine and arginine, albeit at higher concentrations, whereas methylamine activated significantly only at 50–100 mM. The diaminoalkane series $H_3N^+(CH_2)_nN^+H_3$ was examined in order to see how the ability to activate varied with the distance between two positive charges. Fig. 3 shows that maximal activation occurred with ethylenediamine ($n = 2$). Hydrazine ($n = 0$) was devoid of activity, but all other members of the series showed some activity. Ethylenediamine and putrescine activated maximally at 50 mM, L-lysyl-L-lysine activated as much as putrescine up to 20 mM, the maximum concentration at which it was tested.

Polyamine analysis revealed 2.7 μmol of spermidine, 0.21 μmol of spermine and 0.06 μmol of putrescine per g of mammary tissue in a 16-day lactating rat. The pooled milk of two lactating rats contained 7.0 μM-spermidine, 0.3 μM-spermine and 0.26 μM-putrescine.

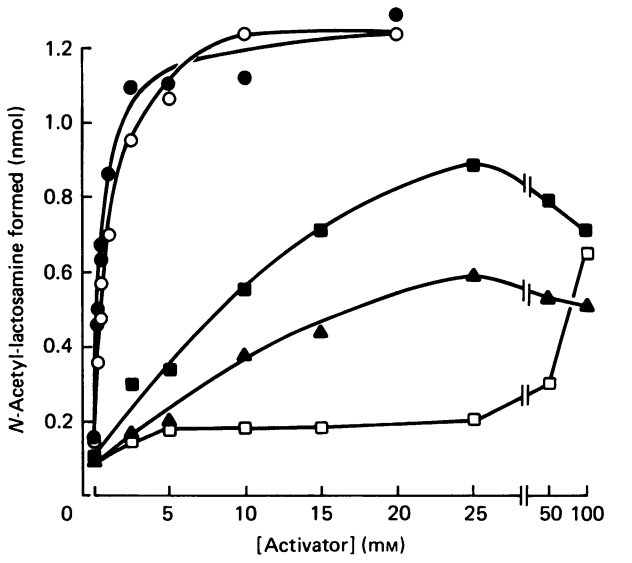


Fig. 2. Activation of galactosyltransferase by different concentrations of spermidine (○), spermine (●), L-lysine (■), L-arginine (▲) and methylamine (□)

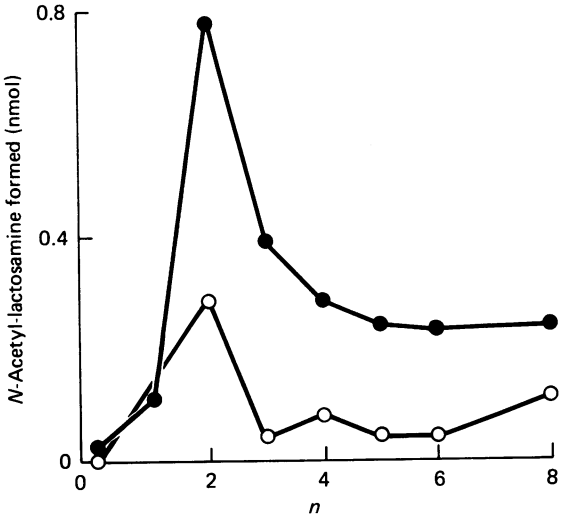


Fig. 3. Activation of galactosyltransferase by diaminoalkane homologues [H_3N^+-$(CH_2)_n$-N^+H_3]

Activation of 0.5 mM (○) and 5 mM (●) concentrations of each substance is shown.

Activation of galactosyltransferase by basic proteins and peptides

The above observations with polyamines and aliphatic amines naturally prompted an investigation with basic proteins and peptides, which are the major polycations of living cells. Clupeine, which we have extensively used, stimulated galactosyltransferase at a constant rate over 40–60 min. Such incubation times were useful, since the greatest fold stimulation was seen at low concentrations of enzyme. A wide range of proteins and peptides was surveyed in order to gain a perspective of the pattern of activation by proteins and its concentration-dependence. Fig. 4 shows the activations obtained with clupeine, poly-L-lysine, histone, pancreatic ribonuclease and trypsin inhibitor, avidin, bradykinin, cytochrome *c*, haemoglobin, fibrinogen, melittin and myelin basic protein. Although these were not all tested at the same time, there was evidently quite wide variation both in the maximal activation and in the concentration at which it was achieved.

Activation of galactosyltransferase by a basic-protein preparation from cow milk

Many of the proteins or peptides employed above are cellular secretory products. This suggested that the mammary gland might itself secrete into milk a basic protein that could possibly be a natural activator of the galactosyltransferase, or lactose synthetase, of the mammary-gland Golgi apparatus. It seemed likely that any such material would be found retained by the acidic casein fraction of milk. Therefore acid extracts of bovine milk casein were collected, dialysed free of acid, and further purified by simple ion-exchange chromatography (see the Materials and methods section). Fig. 5 shows that this material, which is likely to contain a mixture of proteins, gave a marked activation of galactosyltransferase at quite low concentrations of protein. The nature of this fraction of basic protein remains to be further explored, and it cannot yet be excluded that it is merely histone arising from mammary cells shed into the milk.

Comparative activations of galactosyltransferase and lactose synthetase by different substances

The different activators described above were necessarily studied over an extended period and with different enzyme preparations. Therefore experiments were carried out to compare directly the maximal activations given by a range of different activators. Table 2 shows that those substances which activated the Golgi membrane enzyme as an *N*-acetyl-lactosamine synthetase also activated it as a lactose synthetase. Activations in the latter assay were repeatedly found to be the greater, both absolutely and relative to clupeine. It may be noted that many of the proteins were superior to Mg^{2+} and Ca^{2+}. Galactosyltransferase purified from bovine milk was also stimulated by all these agents, although generally rather less.

Control experiments on several of these proteins showed that they did not contain endogenous galactosyltransferase activity even under the maximally stimulating influence of 10 mM-$MnCl_2$. Further, heating for 3 min at 100 °C, which destroys galactosyltransferase activity, modified, but did not destroy, their activating ability (results not shown). In addition to the proteins shown in Fig. 4 and Table 2, the trypsin inhibitors of soybean (*Glycine max*) and lima bean (*Phaseolus limensis*) also activated in the *N*-acetyl-lactosamine synthetase assay (results not shown). No activation was shown by oxytocin, insulin, bovine milk β-lactoglobulin, bovine pancreatic trypsinogen or chymotrypsinogen or amylase, human salivary amylase, γ-globulin, ovalbumin, conalbumin, conalbumin–iron complex, or by three different snake toxins. Little or no activation was shown by lysozyme from hen's eggs or human milk. Since a number of these proteins are strongly basic, it is evident that basicity alone is not a sufficient criterion for a protein to activate. The samples of trypsinogen and chymotrypsinogen, which might have been inactive due to endogenous proteolytic activity, did not prevent the activation of galactosyltransferase by clupeine.

Effect of polyanions on the cationic activation of galactosyltransferase

If the substances shown in Table 2 activate by virtue of their polycationic nature, then added polyanions would be expected to reverse the effect. In fact 5 mM-ATP completely abolished the activations due to Ca^{2+}, spermidine, clupeine, ribonuclease, and the basic-protein fraction of milk (results not shown). Fig. 6 shows that the activation of basic milk protein was progressively reversed by increasing concentrations of casein, heparin and chondroitin sulphate. Hyaluronic acid also inhibited, but less effectively (results not shown).

Activation of lactose synthetase at low glucose concentration

In the course of this work it was observed that although the organic cations employed above would often activate galactosyltransferase many fold, they rarely achieved more than 15–25% of the activity seen with Mn^{2+} ions. Moreover, certain preparations of enzyme responded very little to activation by cations other than Mn^{2+}, a variability reminiscent of the findings with the galactosyltransferase of dog trachea (Baker & Hillegas, 1974). However activation seemed greater when the enzyme was assayed, in the presence of α-lactalbumin, as a lactose synthetase. α-Lactalbumin is known to

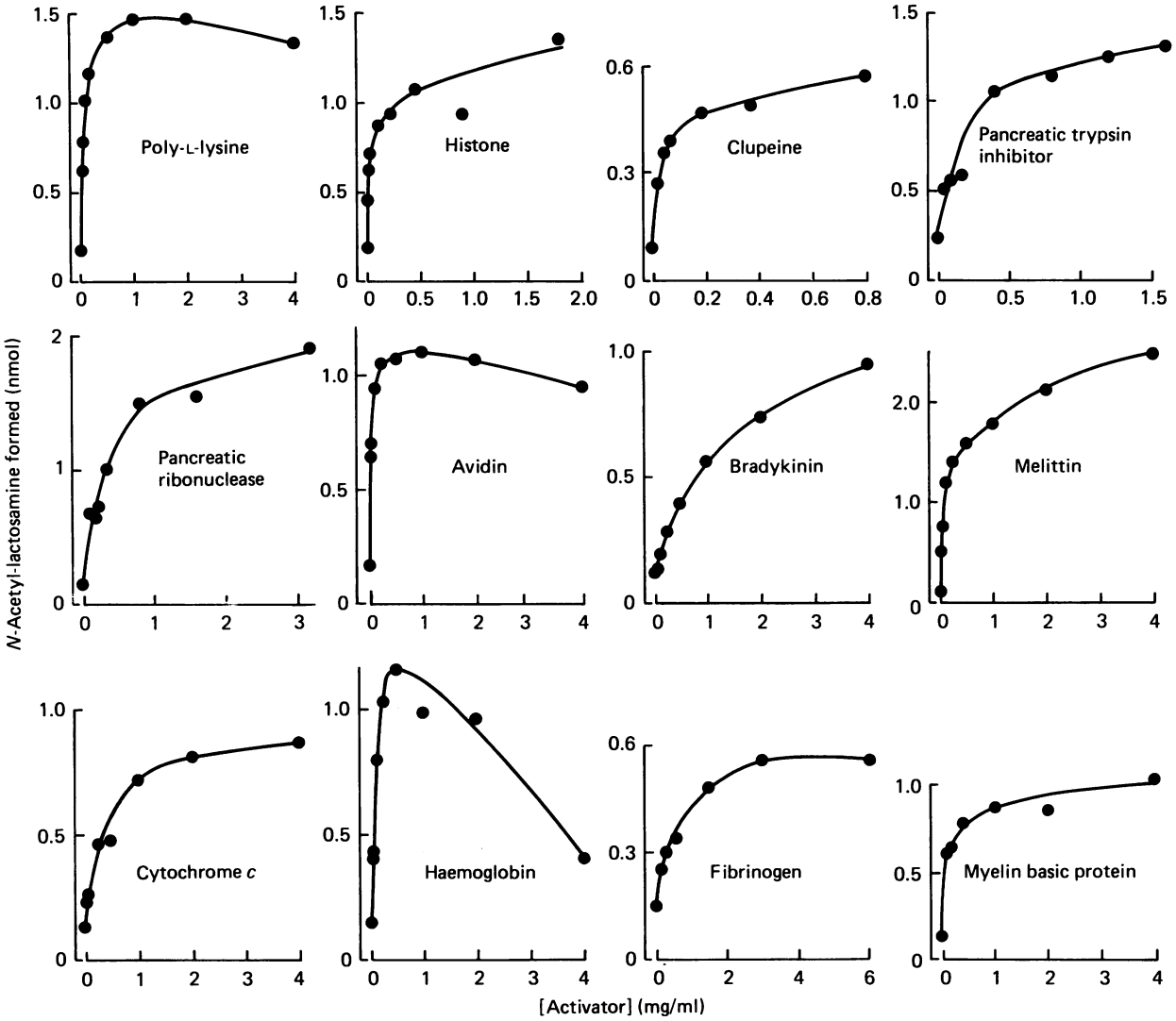


Fig. 4. Activation of galactosyltransferase by different proteins and peptides and the effects of different concentrations

Absolute activities should not be compared, since more than one enzyme preparation was used.

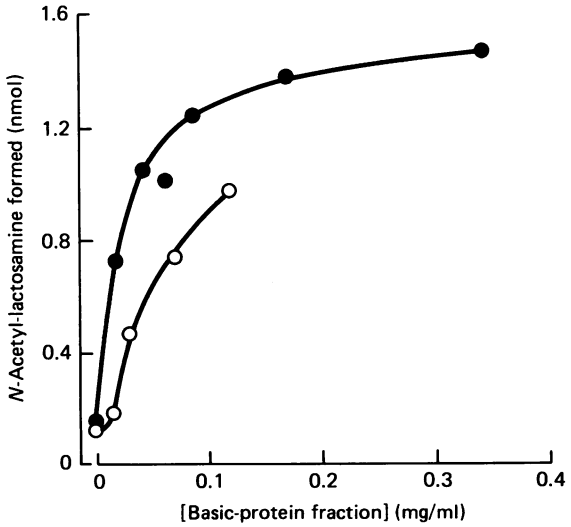


Fig. 5. Activation of galactosyltransferase by a basic-protein fraction from milk

Results are shown for two separate enzyme preparations.

greatly enhance the affinity of galactosyltransferase for glucose (Fitzgerald *et al.*, 1970), and it seemed possible that another protein activator might do the same, thereby stimulating more at low glucose concentration. It was observed that the clupeine stimulation of lactose synthetase, expressed relative to the stimulation by 15 mM-$MnCl_2$, rose from 20% over the glucose concentration range 4.0–0.4 mM to reach a maximal stimulation of 40–50% at 0.025 mM. It was for this reason that a low glucose concentration (0.05 mM) was employed for the assay in part of Table 3.

Three separate enzyme preparations from solubilized Golgi membranes, and one commercial preparation purified from bovine milk, were then employed to determine $K_{m,glucose}$ in the presence of clupeine (0.8 mg/ml) or $MnCl_2$ (10 mM). The results, which were not significantly different for the different enzyme preparations, gave mean ($\pm$S.E.M.) K_m values of 0.21 ± 0.01 mM ($n = 6$) and 2.14 ± 0.11 mM ($n = 7$) in the presence of clupeine and $MnCl_2$ respectively. One of these preparations gave a $K_{m,glucose}$ of 0.15 mM in the presence of ribonuclease (0.8 mg/ml).

Table 2. Relative activities of different cationic activators

Values are shown for one galactosyltransferase preparation solubilized from Golgi membranes and treated with Chelex to remove endogenous activator, assayed (*a*) as an *N*-acetyl-lactosamine synthetase and (*b*) as a lactose synthetase; also (*c*) for a galactosyltransferase, purified from bovine milk, assayed as a lactose synthetase. Results shown as a percentage of clupeine activation.

Activator (concn.)	Activation (%)		
	(*a*)	(*b*)	(*c*)
Pancreatic trypsin inhibitor (1.6 mg/ml)	138	68	53
Ribonuclease (3.2 mg/ml)	124	56	25
Cytochrome *c* (4 mg/ml)	117	59	33
Histone II-S (1.8 mg/ml)	113	15	57
Spermidine (10 mM)	104	48	25
Ethylenediamine (50 mM)	104	61	36
Poly-L-lysine (2 mg/ml)	102	59	33
Clupeine (0.8 mg/ml)	100	100	100
$CaCl_2$ (15 mM)	98	33	38
$MgCl_2$ (15 mM)	86	44	99
Haemoglobin (2 mg/ml)	68	8	2
Avidin (2 mg/ml)	23	27	18
Melittin (2 mg/ml)	23	56	6

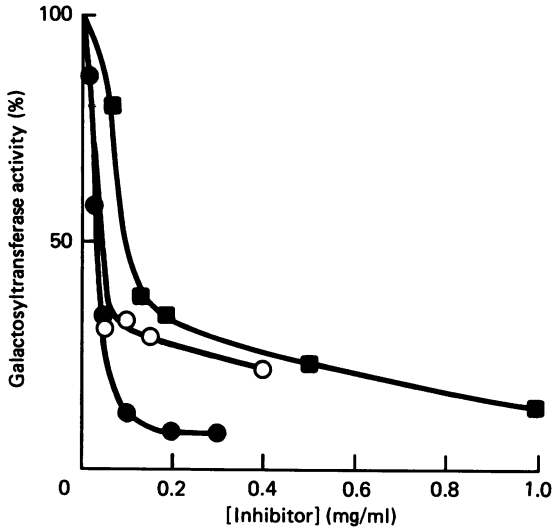


Fig. 6. Polyanion inhibition of galactosyltransferase activated by a basic-protein fraction from milk

Polyanions used were casein (■), chondroitin sulphate (○) and heparin (●). Milk basic protein was present at 65 μg of protein/ml.

Evidence for an endogenous activator in solubilized Golgi preparations

The solubilized Golgi preparations used in these investigations always showed certain enzymic activity, even when no cationic activator was added (see previous Figures). The partial inhibition of this residual activity by ATP that we had observed, comparable with the inhibition of stimulated activity, suggested the presence of some endogenous activator originating from the Golgi membranes themselves. This interpretation is consistent with the results in Table 3, where it is seen that the specific 'endogenous' activity decreased, and the fold stimulation due to added clupeine increased, with increasing dilution of enzyme preparation in the assay. For this reason most of the activations described above were studied with enzyme preparations diluted to the lowest acceptable transferase activity. Table 3 also shows that treatment of enzyme preparation with Chelex similarly lowered the endogenous activity and raised the fold stimulation by clupeine, implying the presence of a cationic endogenous activator. This was probably not Ca^{2+}, Mg^{2+} or Mn^{2+}, since the analysis of two separate Golgi membrane preparations revealed the following contents: Ca, 0.17 and 0.06 μg-atom/mg of protein; Mg, 0.10 and 0.10 μg-atom/mg of protein; Mn, 1.3 and 0.8 μg-atom/mg of protein respectively. In the presence of suitably high concentrations of one enzyme preparation, and employing only the putative endogenous activator, the $K_{m,glucose}$ was 0.11 mM.

DISCUSSION

The present discussion assumes the model of Powell & Brew (1976), according to which metal ion activators are bound tightly at site I, and less tightly at site II, of galactosyltransferase. It is further assumed that Ca^{2+}, Mg^{2+}– and all the organic amines and proteins studied here activate the transferase by occupation of site II, and that incomplete occupation of site I by endogenous cation (possibly Mn^{2+}) might partly explain why less-than-maximal activity is evoked by these substances. Mn^{2+} ions may evoke greater activity because they satisfy both sites. However the shortfall from full activity may also reflect the complexing of UDP-galactose, as in fact occurs with Ca^{2+} (Powell & Brew, 1976). It is beyond the scope of the present study to consider the full activation of galactosyltransferase, which now appears to involve two substrates, two activators, α-lactalbumin and phospholipids (Moscarello *et al.*, 1985). In view of the difficulties in accepting either Mn^{2+}, Ca^{2+} or Mg^{2+} as the physiological activator of lactose synthetase (see the Introduction), the activations by polyamines and by basic peptides and proteins described here can be interpreted as pointing to the nature of the true endogenous activator.

Table 3. Effect of enzyme dilution and of Chelex on endogenously stimulated activity, and of fold activation by clupeine, of galactosyltransferase or lactose synthetase

Four separate enzyme preparations were used.

Type of enzyme assay	Enzyme dilution	Endogenous specific activity (%)	Fold stimulation by clupeine
1. Galactosyltransferase	None	100	1.8
	2-Fold	50	4.0
	4-Fold	27	8.7
2. Lactose synthetase (4 mM-glucose)	None	100	1.1
	2.5-Fold	51	4.6
	5-Fold	39	5.5
3. Lactose synthetase (0.05 mM-glucose)	None	100	1.5
	2-Fold	87	2.4
	4-Fold	63	3.8
	8-Fold	71	3.9
4. Lactose synthetase (4 mM-glucose)	None (before Chelex)	100	1.5
	None (after Chelex)	20	11

The mechanism of cationic activation

So long as only bivalent metal cations were known to activate site II, it seemed likely that they acted by bridging the negatively charged pyrophosphate group of UDP-galactose with some negatively charged region at the active site of the enzyme. Kinetic studies have implicated an enzyme–Mn^{2+}–UDP-galactose complex (Morrison & Ebner, 1971*a*,*b*; Powell & Brew, 1976; O'Keeffe *et al.*, 1980), and n.m.r. studies have demonstrated the binding of Mn^{2+} at millimolar concentrations separately to both transferase and UDP-galactose (Berliner & Wong, 1975). A bridging role of metal ions would also be consistent with the Mn^{2+}-dependent retention of galactosyltransferase by columns of UDP-Sepharose (Barker *et al.*, 1972). However, a bridging role would be expected to show a strict steric requirement that a wide range of basic peptides and proteins, aliphatic amines, and especially methylamine, could hardly fulfil. Moreover, glycogen synthetase (Gold, 1970) and glucuronyltransferase (Marniemi & Hänninen, 1973) do not require bivalent metal ions, although they catalyse the same type of reaction. For this reason we favour a less specific role for the activating cation, such as the bridging or shielding of two mutually repulsive regions of negative charge on the enzyme, with consequent conformational change favourable to the binding of substrates at the active site. The enhanced enzyme affinity for glucose, brought about by clupeine, is consistent with this view.

An alternative possibility, that added cations might merely neutralize some endogenous acidic inhibitor, seems unlikely to account for stimulation by Ca^{2+} and clupeine, of galactosyltransferase highly purified from bovine milk (Powell & Brew, 1976; the present paper). Moreover, it is inconsistent with the effects of enzyme dilution, and of Chelex, reported in Table 3.

Polyamines as possible activators

The candidacy of polyamines as natural activators of galactosyltransferase stems from the occurrence of both spermidine and spermine in the mammary glands of lactating rats (Russell & McVicker, 1972) and mice (Oka *et al.*, 1978). Their concentrations rise at about the time of lactogenesis to reach about 5.0 and 0.5 μmol/g of tissue respectively in both species. However, these changes do not correlate closely with the onset of lactose synthesis (Kuhn & Lowenstein, 1967; Kuhn, 1969; McKenzie *et al.*, 1971). Moreover, the large changes in lactose synthesis that occur when lactating rats are starved and then re-fed (Carrick & Kuhn, 1978) are not accompanied by changes in tissue spermidine and spermine even though similarly large changes are seen in the activity of ornithine decarboxylase (Brosnan *et al.*, 1982, 1983). Finally, when the binding of polyamines to ribosomes, tRNA and other polyanions is considered, it is unlikely that their free concentration is more than a fraction of the total. We have detected total concentrations of only about 7 μM-spermidine and 0.3 μM-spermine in rat milk, and still lower concentrations have been reported for human and cow milk (Sanguansermsri *et al.*, 1975). Polyamines have been observed to activate casein kinase (West & Clegg, 1984*a*) and acetyl-CoA carboxylase (Munday & Hardie, 1985) from rat mammary gland, and the UDP-glucose epimerase of yeast (Darrow & Creveling, 1964), though not of mammary gland (Shatton *et al.*, 1965). They also reverse the inhibition by palmitoyl-CoA of glucose-6-phosphate dehydrogenase from yeast and sea-urchin eggs (Mita & Yasumasu, 1979, 1980), and activate the aminoacyl-tRNA synthetase of lupins (Jakubowski, 1980). Of particular interest is the activation by spermine, as well as by polylysine and protamine, of UDP-galactose:mucin galactosyltransferase from dog and human trachea (Baker & Hillegas, 1974;

Cheng & Bona, 1982). Taken as a whole, however, the present evidence does not argue strongly for polyamines being the natural activators of galactosyltransferase in the mammary gland.

Basic peptides or proteins as possible activators

The finding that galactosyltransferase can be activated by a wide range of mainly basic proteins immediately suggests that the natural activator in the mammary gland might itself be such a peptide or protein. This would circumvent the problem of reconciling Ca^{2+} activation with membrane permeability, described in the Introduction, since the activator might be a secretory protein within the Golgi lumen that associated reversibly with the membrane-bound transferase in just the manner envisaged for α-lactalbumin (Brew, 1970).

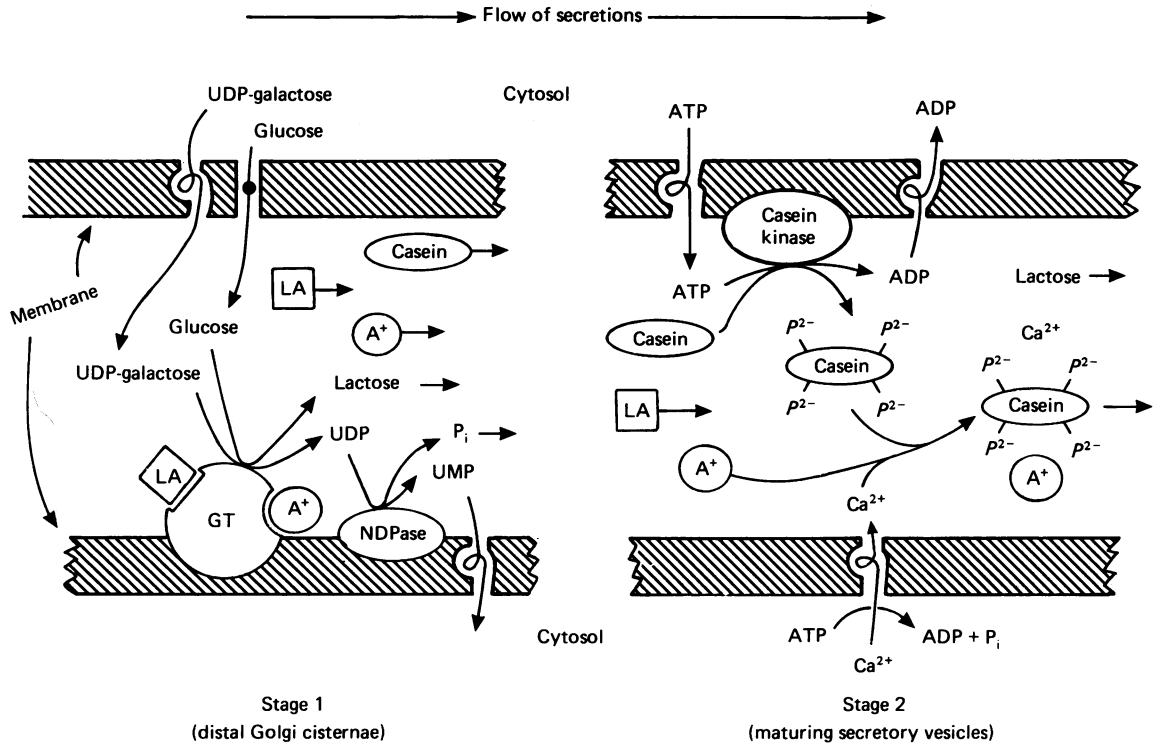
The survey of activators carried out in the present study may give some clues as to the nature of their natural counterpart. Most of the proteins and peptides that activated galactosyltransferase are basic, with relatively high isoelectric points, although it should be noted that trypsinogen, chymotrypsinogen, amylase, β-lactoglobulin and the snake toxins tried, are basic without being activators, whereas the trypsin inhibitors of soybean and lima bean were activators despite being acidic (pI 4.6 and less than 3.6 respectively). Thus the required feature seems likely to be a basic region on a secretory protein or peptide. The greater likelihood of such a region occurring on a basic protein guided our preliminary search for such an activator in milk (Fig. 5). That the natural activator might alternatively be a Golgi membrane protein, however, is suggested by the activity of myelin basic protein and of melittin, which is known to enter membranes. Our data do not, however, rule out an endogenous activator of quite different chemical nature.

Influence of clupeine upon $K_{m,glucose}$

The 10-fold decrease in apparent $K_{m,glucose}$, seen when clupeine or ribonuclease replaced Mn^{2+} as cationic activator, itself implies that these substances more nearly resemble the natural activator. It is therefore significant that a similar K_m value was obtained for lactose synthetase activated solely by the putative endogenous activator. Interestingly, Faulkner (1985) has reported a hyperbolic relationship between lactose production and milk glucose concentration in goats, yielding an apparent K_m for glucose of 0.16 mM. The concentration of glucose in milk (about 0.05–0.2 mM) is believed to reflect its concentration within the cytosol and Golgi lumen of the mammary secretory cell (Kuhn & White, 1975; Faulkner *et al.*, 1981), and it has long been puzzling that it lay so far below the apparent $K_{m,glucose}$ for lactose synthetase (1.5–2.5 mM in the presence of Mn^{2+}). It now seems possible that, in the lactating mammary gland of fed animals, the intracellular glucose concentration approximates to the K_m of both the hexokinase (type II) and the lactose synthetase that use it.

Proposed two-stage synthesis of milk products

The experiments showing inhibition of galactosyltransferase by ATP, casein and other polyanions (see the above text and Fig. 6) were undertaken not only to confirm the essentially ionic nature of the activation by large organic cations, but to illustrate the incompatibility of lactose synthesis and ATP-dependent phosphorylation of casein within a single compartment of the cell. Scheme



Scheme 1. Two-stage, two-compartment model for the synthesis of lactose and phosphorylation of casein

Symbols and abbreviations used: -●►, pore; -Ω►, carrier, GT, galactosyltransferase; [LA], α-lactalbumin; (A+), cationic activator; P^{2-}, phosphate group; NDPase, nucleoside diphosphatase.

1 depicts our proposal of two sequential secretory compartments within which the recently observed features of lactose synthesis (the present paper; for a review, see Kuhn, 1983), casein synthesis (West & Clegg, 1983, 1984*b*) and calcium accumulation (for references, see the Introduction) can be mutually accommodated. In compartment 1, probably represented by *trans*(distal) cisternae of the Golgi stack, α-lactalbumin and the cationic activator stimulate galactosyltransferase to form lactose, undisturbed by the presence of unphosphorylated casein. The membrane of this compartment must contain the pores (for references, see the Introduction) and carriers (Kuhn & White, 1976; Deutscher & Hirschberg, 1986) necessary for the admission of glucose and UDP-galactose. Compartment 2 is the site of casein phosphorylation and aggregation into calcium-containing micelles, its membrane containing carriers for the entry of ATP and Ca^{2+}. This compartment may correspond with the granule-containing (Hollman, 1959; Bargmann & Knoop, 1959; Wellings & DeOme, 1961; Wellings & Philp, 1964) and calcium-containing (Wooding & Morgan, 1978) vacuoles or vesicles that lie between the Golgi stack and the apical membrane, and that should perhaps be regarded as forming and maturing secretory granules. The proposed secreted cationic activator must be presumed to complex with phosphorylated casein and might even aid the nucleation of the micelles. This two-stage, two-compartment, model is consistent with the partial separation of galactosyltransferase and casein kinase activities in post-nuclear mammary supernatants sedimented through a sucrose gradient (Pascall *et al.*, 1981).

Glycosyltransferase activation in other tissues

To the extent that the present view of galactosyltransferase activation is valid, a similar type of activation could readily be envisaged for other glycosyltransferases in other cell types. To the extent that they are proteins, different ones might operate in each cell type. In addition to secretory ribonuclease, pancreatic trypsin inhibitor, fibrinogen, melittin and avidin, which are active in our system, basic proteins have been characterized in guinea-pig pancreatic secretion (Tartakoff *et al.*, 1974), human saliva (Saitoh *et al.*, 1983*a*,*b*), guinea-pig and rat semen (Notides & Williams-Ashman, 1967; Higgins *et al.*, 1976) and in the secretory granules of mast cells (Bergqvist *et al.*, 1971). Alternatively, the activation of galactosyltransferase by ethylenediamine and especially by lysyl-lysine raises the possibility that pairs of basic residues in secretory proteins destined to be proteolytically processed within secretory granules might serve as activators (Gainer *et al.*, 1985). Finally, integral membrane proteins destined for the plasma membrane might serve the same function. By analogy with Scheme 1 the assembly of polyanions by the phosphorylation or sulphation of macromolecules, or by the accumulation of ATP as in chromaffin granules, probably occurs downstream of glycosylation in maturing secretory granules. The concentration of secretory products, that has been so clearly described for the 'condensing granules' of pancreatic exocrine secretory cells and others (see Farquhar & Palade, 1981), could then reflect the ionic interaction between the basic proteins and accumulated Ca^{2+} or Zn^{2+} ions on the one hand and the polyanions generated there on the other (Reggio & Palade 1978).

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