The Role of Nucleoside Diphosphatase in a Uridine Nucleotide Cycle Associated with Lactose Synthesis in Rat Mammary-Gland Golgi Apparatus

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1. UDP-galactose utilization by isolated Golgi vesicles of rat mammary gland synthesizing lactose causes accumulation of UMP but not UDP, although UDP is the immediate product of lactose synthase (EC 2.4.1.22). 2. This can be ascribed to a nucleoside diphosphatase (EC 3.6.1.6), specific for UDP, GDP and IDP, activated by bivalent metal ions and apparently located on the luminal face of the Golgi membrane. 3. The uridine diphosphatase activity exceeds the total galactosyltransferase activity 5-fold, and is estimated to maintain UDP at about $14\mu\text{M}$ within the Golgi lumen. 4. Evidence is given that UMP, but not UDP, penetrates the membrane and that UMP is rephosphorylated to UDP by a UMP kinase located in the cytosol. 5. Golgi-cytosol relationships with respect to lactose synthesis are formulated in terms of a uridine nucleotide cycle which throws new light on the energy cost and possible regulation of lactose synthesis.

Studies on the formation of milk sugar have, in recent years, focused attention on the enzyme galactosyltransferase which, in the presence of α -lactalbumin, generates lactose (EC 2.4.1.22). Kinetic studies of the galactosyltransferase from bovine and human milk or colostrum have shown that UDP inhibits strongly, competitively with the substrate UDP-galactose (Khatra *et al.*, 1974). In view of the estimated intracellular concentrations of these nucleotides in rat mammary gland (UDP-galactose about $55\mu\text{M}$, UDP about $165\mu\text{M}$; Murphy *et al.*, 1973), it seemed to us likely that the transferase must normally be protected from the UDP present in the cytosol. We have previously shown that a similar inhibition by cytosolic UDP-glucose is apparently avoided by the location of the galactosyltransferase on the inner, luminal, face of the Golgi membrane and by the relative impermeability of this membrane to UDP-glucose (Kuhn & White, 1975, 1976). Although evidence is given below that cytosolic UDP also does not penetrate the membrane, a special situation arises, since UDP is actually generated within the Golgi lumen by the galactosyltransferase reaction itself:

$$\text{UDP-galactose} + \text{glucose} \xrightarrow{\text{M}^{2+}} \text{UDP} + \text{lactose}$$

where M^{2+} is a bivalent metal ion.

In the present paper we have followed up previous reports (see the Discussion section) that a nucleoside diphosphatase (EC 3.6.1.6), probably identical with thiamin diphosphatase, is associated with the Golgi apparatus of mammary gland. Evidence is presented for the efficient hydrolysis of the UDP generated in the reaction above to UMP, which then returns to the

cytosol where it is subsequently resynthesized to UDP, UTP, UDP-glucose and finally UDP-galactose. These changes constitute a uridine nucleotide cycle, the activity of which is linked to the synthesis of lactose. Recognition of this cycle permits a reassessment of the energy cost of lactose synthesis and opens up new possibilities for its metabolic control.

Methods

Purification of Golgi membranes

Chopped lactating mammary tissue (9–10 g) was suspended in 2.5 vol. of medium 1 (37.5 mM-Tris/maleate buffer, pH 6.5; 0.5 M-sucrose; 1% Ficoll-400 or Dextran B; 5 mM-MgCl₂) and divided into two portions. Each portion was homogenized at 0–4°C in a 25 ml glass beaker with the small probe of the Polytron homogenizer at setting 2 for 1 min. The combined homogenates were passed through two layers of muslin and centrifuged for 20 min at 0–4°C at 7000g_{av.} (MSE 18 centrifuge, 8 × 50 angle rotor). The loose buff-coloured upper part of the pellet was recovered, resuspended in the smallest possible volume of particle-free supernatant (prepared by further centrifugation of the above supernatant for 1 h at 100000g_{av.}) and layered on to 5 ml of medium 2 (the same as medium 1 but with 1.25 M- instead of 0.5 M-sucrose) in each of three 8 ml tubes. The tubes were centrifuged at 100000g_{av.} for 40 min at 0–4°C in a swing-out rotor. The Golgi membranes at the interface of the two media were collected in the mini-

imum volume of fluid with the aid of a bent syringe needle attached to a small hypodermic syringe and, after dilution with about 2ml of cold 0.25M-sucrose to lower the density, were washed two or three times by sedimentation at $2000g_{av.}$ for 20–30 min at 0–4°C and by resuspension in 0.25M-sucrose. The final pellet was resuspended with the aid of a vortex mixer in 2–4ml of 0.25M-sucrose. It contained typically 2–5mg of protein and about 10% of the galactosyltransferase activity in the original tissue. Protein was determined, after precipitation by 10% (w/v) $HClO_4$ and dissolution in 1M-NaOH, by the method of Lowry *et al.* (1964).

[^{14}C]UDP-galactose

This was synthesized from [^{14}C]UTP and galactose 1-phosphate in the presence of excess UDP-glucose pyrophosphorylase (EC 2.7.7.9). This enzyme shows an activity towards UDP-galactose about 3% of that towards UDP-glucose (Albrecht *et al.*, 1966).

Tes*/NaOH buffer, pH 7.5 (50mm), 2.4mm-[^{14}C]UTP (1.76 d.p.m./ μ mol), 2mm-MgCl₂, 60mm-D-galactose 1-phosphate and 5 units of UDP-glucose pyrophosphorylase in a final volume of 0.5ml were incubated at 25°C for 16h. The [^{14}C]UDP-galactose formed was isolated by chromatography for 23h on Whatman 3MM paper developed with ethanol/1M-ammonium acetate, pH 3.8 (2:1, v/v). Radioactivity yield was 1.21×10^7 d.p.m. Radiochemical purity was about 90% as monitored by rechromatography on thin layers of polyethyleneimine-cellulose MN 300 developed with 0.4M-LiCl. The major impurity appeared to be [^{14}C]UTP.

[β - ^{32}P]UDP

[γ - ^{32}P]ATP (3×10^6 c.p.m./ μ mol) was prepared by the method of Glynn & Chappell (1964). The [^{32}P]ATP (0.92mm) was mixed with 10mm-MgCl₂ and 10mm-UMP and, after the pH had been adjusted to 7.0, was incubated with 0.5 unit of nucleoside monophosphokinase (EC 2.7.4.4) in a final volume of 4.14ml at 26°C for 3h. The [^{32}P]UDP was isolated by chromatography for 45h on Whatman 3MM paper developed with isobutyric acid/1M-NH₃/0.1M-EDTA (250:150:4, by vol.) and eluted from the paper with water. The eluate was passed through a small column (1ml) of Dowex 1 resin (formate form; X8; 200 mesh), which was then washed with water and eluted with 0.5M-(NH₄)₂CO₃, pH 7.4. The first 6ml of eluate, which contained most of the radioactivity, was treated with Dowex 50 resin (H⁺ form; X8; 200 mesh) until no further CO₂ was evolved. The resin was removed by centrifugation at low speed and

* Abbreviations: Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid; UDPase, uridine diphosphatase.

the supernatant, together with five 1ml washings of the resin, was freeze-dried.

The product contained 4.8×10^6 c.p.m. of radioactivity and migrated as a single radioactive band identical with authentic UDP, and distinct from ATP and UTP, on Whatman no. 1 paper in the same solvent used above. However, 8% of the radioactivity was in the form of P_i, as judged by its extractability as the molybdate complex into 2-methylpropan-1-ol/toluene (1:1, v/v). The product contained 4.1 μ mol of UDP, assayed as P_i released by 15min hydrolysis in 0.5M-H₂SO₄ at 100°C.

Optical assay of UDPase activity

In the standard procedure 40mm-Tes/NaOH buffer, pH 7.0, 10mm-CaCl₂, 0.1% Triton X-100, 2mm-UDP and about 2–4 μ g of Golgi-membrane protein in a final volume of 0.5ml were incubated for 10min at 37°C. The reaction was stopped with 0.5ml of 10% (w/v) trichloroacetic acid, after which the tubes were chilled for 10min in ice and centrifuged at low speed to remove denatured protein. The P_i of the supernatants was extracted into 2-methylpropan-1-ol/benzene or 2-methylpropan-1-ol/toluene (both 1:1, v/v) and assayed by the method of Ernster *et al.* (1950). Zero-time incubations were routinely carried out to allow for P_i contamination of the UDP samples. The same procedure was adopted for assaying the release of P_i from other phosphate derivatives.

Radioisotopic assay of UDPase activity

This assay was used in place of the optical one to monitor UDPase activity in the presence of added P_i. MgCl₂ was used instead of CaCl₂ to avoid the precipitation of phosphate.

Reaction mixtures contained 40mm-Tes/NaOH buffer, pH 7.0, 10mm-MgCl₂, 20 μ M-[^{32}P]UDP (about 23000 c.p.m.), 0–40mm-sodium phosphate and 10 μ g of Golgi-membrane protein in a final volume of 1ml. After incubation for 10min at 37°C the reaction mixtures were shaken for 30min at 0–4°C with 50mg of activated charcoal (BDH, Poole, Dorset, U.K.; 'for cyclic AMP') suspended in 0.5ml of 1M-HCl to remove the [^{32}P]UDP. The charcoal was removed by centrifugation and the radioactivity of a portion (1ml) of clear supernatant was counted by liquid scintillation. Appropriate controls were carried out to correct for $^{32}P_i$ in the initial preparation of [^{32}P]UDP.

Assay of UMP kinase

Standard reaction mixtures contained 40mm-Tes/NaOH buffer, pH 7.0, 2mm-MgCl₂, 1mm-[^{14}C]UMP (0.74 μ Ci/ μ mol), 4mm-ATP and enzyme (about 0.25 μ g-equiv. of tissue) in a volume of 50 μ l, and were

incubated for 10 min at 37°C. Small portions were then mixed on a cold surface with equal volumes of solution containing 20 mM each of UMP and UDP, and 5 μ l of the mixture was spotted at the origin of a polyethyleneimine-cellulose thin-layer chromatogram. The chromatogram was developed with 0.75 M-LiCl over 18 cm, and the uridine nucleotides located with u.v. light were cut out and counted for radioactivity in the presence of 5 ml of toluene scintillator.

Assay of galactosyltransferase activity

Reaction mixtures contained 40 mM-Tes/NaOH buffer, pH 7.0, 15 mM-MnCl₂, 20 mM-N-acetylglucosamine, 0.5 mM-UDP-[¹⁴C]galactose (0.5 μ Ci/ μ mol), 0.5% Triton X-100 and enzyme in a volume of 50 μ l. N-Acetylglucosamine was omitted from control tubes. Reactions were carried out for 10 min at 37°C and then stopped by heating for 1.5 min at 100°C. The neutral sugars were then isolated on anion-exchange resin (Kuhn, 1968). This procedure assays the total galactosyltransferase activity of a tissue preparation.

Lactose synthase was assayed in the same manner except that N-acetylglucosamine was replaced by glucose. Where it was wished to assay 'intact' particle preparations, Triton was omitted and lactose was included at 250 mM final concentration; where lysed preparations were to be assayed, both 0.5% Triton and 0.04% bovine α -lactalbumin were included.

Other assays

Acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) were assayed by the method of Connock *et al.* (1971). L-Glutamate dehydrogenase (EC 1.4.1.3) was assayed as described by Schmidt (1963), but with the inclusion of 1.5 mM-ADP. RNA was determined by the method of Munro & Fleck (1967). The assay of UDP-glucose pyrophosphorylase (EC 2.7.7.9) and UDP-glucose 4-epimerase (EC 5.1.3.2) and the correction of their activities to pH 7.4 and 37°C have been described (Kuhn & Lowenstein, 1967).

Materials

Lactating rats (15–20 days *post partum*) were of a Wistar-derived strain bred in this Department.

The Polytron type PT 35 OD homogenizer was from The Northern Media Supply Ltd., Hull, U.K.

Dextran B was from Hopkin and Williams, Chadwell Heath, Essex, U.K., and Ficoll-400 was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Polyethyleneimine-cellulose thin layers (Polygram CEL 300 PEI) were from Macherey, Nagel and Co., supplied by Camlab, Cambridge, U.K.

Enzymes and nucleotides were from Sigma (London) Chemical Co., London S.W.6, U.K., or Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Nucleotides were purified by paper chromatography before use.

Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K., and radioactive samples were counted by liquid-scintillation with either a Philips liquid-scintillation analyser or a simple double-photomultiplier instrument constructed in the workshop. Solid samples from thin-layer chromatograms were counted for radioactivity as suspensions in toluene scintillator containing 2,5-diphenyloxazole (6 g/litre) and 1,4-bis-(5-phenoxazol-2-yl)benzene (0.12 g/litre). Aqueous samples (1 ml) were mixed with a scintillator (10 ml) containing 1 vol. of Triton X-100 added to 2 vol. of xylene containing 2,5-diphenyloxazole (6 g/litre) and 1,4-bis-(5-phenoxazol-2-yl)benzene (0.12 g/litre).

Results

Preparation of Golgi membranes

By the procedure of Morré *et al.* (1970) and Keenan *et al.* (1972a), mammary tissue was disintegrated with a Polytron homogenizer, and Golgi membranes were isolated by sucrose-density-gradient centrifugation in the presence of Mg²⁺ ions. We found, however, that, if the initial differential centrifugation was carried out at greater speed than that recommended by these authors, yields of Golgi membrane could be improved without sacrifice of specific galactosyltransferase activity. The isolated membranes were washed finally with 0.25 M-sucrose, instead of the recommended water, in an attempt to avoid osmotic rupture of the vesicles formed in the initial homogenization.

When such Golgi preparations, fixed with 4% (w/v) glutaraldehyde and stained with OsO₄, were viewed under the electron microscope they revealed extensively vesiculated membranes of various sizes. Some intact or partially intact dictyosomes were evident, and there were scattered pieces of rough endoplasmic reticulum and occasional mitochondria and nuclei. The specific activity of galactosyltransferase, assayed in the presence of Triton and with N-acetylglucosamine as acceptor, was 269 $\pm$ 31 nmol/min per mg of protein (mean $\pm$ S.E.M. of 11 preparations; range 106–464). Other values that have been published are 24 nmol/min per mg for rat liver (Morré *et al.*, 1970), 30 nmol/min per mg for rat mammary gland (Keenan *et al.*, 1972a), 6 nmol/min per mg for bovine mammary gland (Keenan *et al.*, 1972b), 150 nmol/min per mg for guinea-pig mammary gland and 170 nmol/min per mg for sheep mammary gland (Smith, 1975).

A number of preparations were assayed variously for galactosyltransferase activity, acid phosphatase,

Table 1. Comparison of the specific activities of enzymes, and amount of RNA, in Golgi membranes and whole homogenates of mammary tissue
Individual values from separate tissue preparations are shown.

Substance assayed	Specific activity (nmol/min, or mg of RNA, per mg of protein)		Mean enrichment in Golgi fraction (fold)
	Homo-genate	Golgi fraction	
Galactosyl-transferase	1.05	278	256
	1.15	286	
Acid phosphatase	3.3	4.7	1.72
	4.2	8.2	
	4.6	7.9	
Glutamate dehydrogenase	29	27	1.04
	16	20	
RNA	0.093	0.076	0.84
	0.102	0.088	
Alkaline phosphatase	275	58	0.34
	120	51	
	73	50

Fig. 1. Changing concentration of [¹⁴C]UDP-galactose (○), [¹⁴C]UMP (●) and [¹⁴C]UDP (■) associated with lactose synthesis by Golgi membrane vesicles
Reaction mixtures contained 18 mM-Tes/NaOH buffer, pH 7.5, 0.9 mM-[¹⁴C]UDP-galactose, 1.8 mM-ATP, 91 mM-glucose, 13.6 mM-MnCl₂, 318 mM-lactose and about 10 μg of Golgi protein in a final volume of 27.5 μl and were incubated at 37°C. Values have been corrected for small changes due to hydrolysis of [¹⁴C]UDP-galactose in reaction mixtures from which glucose was omitted.

alkaline phosphatase, L-glutamate dehydrogenase and RNA to compare the enrichment of each in the Golgi membranes over that in the starting homogenate. Table 1 shows that galactosyltransferase activity was enriched about 256-fold, whereas the other enzymes and RNA were scarcely enriched or not at all. It appears, however, that the preparations cannot be considered to be completely free of non-Golgi membranes.

Fate of uridine nucleotides during lactose synthesis

When [¹⁴C]UDP-galactose was incubated with Golgi membranes under conditions suitable for the synthesis of lactose, its disappearance was mirrored by the accumulation of [¹⁴C]UMP (Fig. 1). At no stage was any accumulation of [¹⁴C]UDP observed.

Properties of a UDPase in Golgi membranes

When UDP was incubated with Golgi membranes in the presence of a bivalent metal ion, P_i was released and could be extracted from the acidified reaction mixture into 2-methylpropan-1-ol/toluene for determination as the reduced molybdate complex. Since neither UMP nor PP_i could replace UDP in this respect, the phosphate released was taken as a measure of UDPase activity:

Fig. 2 shows that the UDPase activity of Golgi membranes increased markedly with the inclusion

Fig. 2. Activation of UDPase in Golgi membranes by Triton X-100
Standard reaction mixtures were used (see the Methods section) but with the inclusion of 300 mM-sucrose and various amounts of Triton X-100. The two curves show the results of two separate experiments.

of Triton in the reaction mixture, about 0.02% Triton giving maximum activity. The degree of activation varied somewhat from one preparation to another. Ernster & Jones (1962) used deoxycholate to show a

Fig. 3. Variation of UDPase activity with pH. Standard reaction mixtures were used, except for the buffers, which were: acetic acid/NaOH (pH 4.0–5.0), Tris/acetic acid (pH 6.5 and 7.0), Tris/HCl (pH 7.5 and 8.0) and glycine/NaOH (pH 9.0).

similar latency of UDPase activity in liver microsomal preparations, but in our case deoxycholate was only inhibitory. Freezing and thawing was ineffective in raising the activity. We interpret the latency of UDPase activity, as revealed by Triton, as evidence that the enzyme is exposed only to the lumen of the vesicles that were either originally present in the tissue or were formed during homogenization by pinching-off of the dictyosome membrane. Such a vesicular form of the membranes would accord with their electron-microscopic appearance and with the findings of Kuhn & White (1975, 1976) on a less-purified mammary preparation. That the UDPase activity is not completely latent may reflect the presence of damaged or leaky vesicles in the preparation.

In the presence of Triton the UDPase activity was greatest at about pH 6.5 (Fig. 3). The optimum pH was shifted to 5.5 when Triton was omitted, presumably because acid conditions favoured the breakdown of membrane impermeability. Most assays were carried out at pH 7.0, under conditions where the hydrolysis of UDP was linearly proportional to time and to amount of enzyme (Figs. 4a and 4b).

Table 2 shows that the Golgi membranes could also catalyse the release of P_i from IDP and GDP, less so from CDP, and hardly or not at all from UTP, UMP, ATP, ADP, AMP, PP_i, glucose 6-phosphate and

Fig. 4. Proportionality of UDP hydrolysis with (a) amount of Golgi protein and (b) time of incubation. Standard reaction mixtures were used.

Table 2. P_i release from different substrates as a percentage of that from UDP

Each substrate was incubated at 2mM under the conditions used for UDPase assay. UDP was included in each experiment for comparison.

Substrate	Mean P_i release (%)	No. of experiments
UDP	100	—
GDP	83	2
IDP	101	2
CDP	17	4
ADP	4	2
UTP	8	2
ATP	4	2
UMP	0	2
AMP	4	2
Glucose 6-phosphate	0	1
Glycerol 3-phosphate	0	1
PP_i	0	1

Fig. 5. Variation of UDPase activity with concentration of $CaCl_2$ or $MgCl_2$

Standard incubations were used with $CaCl_2$ (●) or $MgCl_2$ (○). Absolute UDPase activities with $CaCl_2$ and $MgCl_2$ are not directly comparable in this Figure, since different Golgi preparations were used. For comparative activities see the text.

L-glycerol 3-phosphate. In a separate experiment, the results of which are not given in detail, the release of P_i from ATP was not enhanced by Mg^{2+} , Ca^{2+} or both. Therefore we were unable to demonstrate the presence of a Ca^{2+} , Mg^{2+} -stimulated adenosine triphosphatase that might be involved in the secretion of calcium into milk. The data in Table 2 suggest that the UDPase activity of these membranes is due to a fairly specific nucleotide diphosphatase. We have not tested the possibility

Fig. 6. Inhibition of UDPase by P_i

The radioisotopic assay was used (see the Methods section).

that GDP, IDP and UDP may be hydrolysed by different enzymes.

In three experiments in which the concentration of UDP was varied, the mean ($\pm$ s.e.m.) apparent K_m was 0.38 ± 0.04 mM.

The initial studies on UDPase activity were carried out in the presence of Mg^{2+} , but it was soon found that other bivalent cations could also serve. In two experiments the following mean relative activities were found with different cations at 8 mM: Ca^{2+} , 100; Co^{2+} , 97; Mn^{2+} , 94; Mg^{2+} , 83; Ni^{2+} , 46; Zn^{2+} , 47; none, 6. Metal ions were added as their chlorides. The slight superiority of Ca^{2+} over Mg^{2+} led to its use in most of the UDPase assays reported in this paper.

Fig. 5 shows the variation of UDPase activity with concentration of Ca^{2+} and Mg^{2+} . The curves are clearly not hyperbolic and did not give straight-line double-reciprocal plots. In two such experiments with each ion, half-maximal rates were observed at 0.6 mM- and 0.6 mM- Ca^{2+} and at 0.4 mM- and 0.5 mM- Mg^{2+} respectively.

Specific UDPase activity

The mean activity of six different Golgi-membrane preparations, assayed at 2 mM-UDP, 10 mM- Ca^{2+} , pH 7.0 and 37°C, was 1.57 ± 0.17 μ mol/min per mg of protein. When both UDPase and galactosyltransferase activities were assayed on preparations of mammary tissue from five different rats, the UDPase/transferase activity ratio was 4.9 ± 0.42 in the Golgi membranes and 8.38 ± 1.62 in the whole homogenates

Fig. 7. UMP inhibition of lactose synthesis in intact (●) and lysed (○) particle preparations
Particle preparations (Kuhn & White, 1975) were incubated under the conditions described in the Methods section, with the inclusion of various concentrations of UMP.

(mean±S.E.M.). The lower ratio in the Golgi membranes is probably not simply due to partial solubilization and loss of UDPase activity during their preparation, since in separate experiments, not described in detail, we were unable to demonstrate any solubilization by moderately strong sonication or by osmotic shock. It appears more likely that the higher activity ratio seen in homogenates represents the presence of UDPase activity in fractions other than those derived from the Golgi apparatus.

Inhibition of UDPase activity by phosphate and arsenate

Since P_i is an inhibitor of many phosphatases, and because it occurs prominently both in the milk and the mammary cells of rats (Murphy *et al.*, 1973), it was decided to see if it also inhibited the UDPase activity of mammary Golgi membranes. For this purpose it was necessary to adopt the radioisotopic assay described in the Methods section. Fig. 6 shows that P_i could strongly inhibit UDPase activity, 50% inhibition being reached at about 20mM- P_i . In a single experiment, with duplicate incubations, the mean UDPase activity in the absence of P_i (100%) was decreased to 31% by 40mM- P_i and to 43% by 40mM-arsenate. The inhibition by P_i was not relieved by raising the Mg^{2+} concentration from 10 to 20mM. These experiments were conducted at a very low concentration (20μM) of UDP, which, it was hoped, might compare with the actual concen-

Table 3. Dependence of UMP kinase activity on ATP, $MgCl_2$ and homogenate supernatant

The results of three experiments are shown, where the composition of the complete reaction mixture (see the Methods section) was altered as shown. Homogenate was separated into supernatant and pellet by centrifugation for 1h at 105000g_{av.} at 0–4°C.

Alteration to complete incubations	UDP formed (nmol)		
	Expt. 1	Expt. 2	Expt. 3
None	12.2	37	9.5
Minus $MgCl_2$	4.5	6.5	—
Minus $MgCl_2$, plus EDTA	—	1.3	—
Minus ATP	2.6	1.8	—
Boiled homogenate	1.2	—	—
Homogenate supernatant only	—	—	9.5
Homogenate pellet only	—	—	1.1
Plus Triton X-100	—	—	7.5

tration of UDP within the Golgi lumen. A calculated value for this parameter is given in the Discussion section.

Evidence for the UMP penetration of Golgi membranes

The relative immunity of lactose synthesis in apparently intact mammary vesicles to inhibition by external UDP-glucose was previously interpreted as evidence for the impermeability of Golgi membranes to this nucleotide (Kuhn & White, 1976). Such immunity was destroyed by Triton X-100, which lysed the membrane. Fig. 7 shows the results of similar experiments with UMP as the inhibitory nucleotide. By contrast with UDP-glucose, however, UMP inhibited the lactose synthesis of intact preparations at least as well as that of lysed preparations. It appears therefore that UMP can readily penetrate the Golgi membrane. The slightly greater inhibition that was observed with intact than with lysed preparations might be due to intravesicular UDP accumulating as a result of some inhibition of UDPase activity; alternatively, UMP might inhibit the transport of UDP-galactose across the membrane.

UMP kinase in mammary cytosol

When mammary homogenates were incubated with [^{14}C]UMP, ATP and Mg^{2+} , there was a disappearance of [^{14}C]UMP and appearance of [^{14}C]UDP, as shown by the subsequent isolation of these nucleotides on t.l.c. Table 3 shows that the formation of [^{14}C]UDP was dependent on the presence of ATP, Mg^{2+} and intact homogenate. The activity was not increased by Triton X-100, and was located entirely in the soluble portion of the homogenate. Figs. 8(a)

Fig. 8. Enzymic phosphorylation of UMP to UDP as a function of (a) amount of homogenate and (b) time of incubation (a) UDP formed in 10 min in 50 μ l of standard reaction mixture containing the equivalent of 0–250 μ g of fresh mammary tissue. (b) UDP formed in 250 μ l of standard reaction mixture containing the equivalent of 1.25 mg of fresh mammary tissue, incubated for up to 10 min. Further details are given in the Methods section.

Table 4. Rate of lactose synthesis and activities of enzymes of the proposed uridine nucleotide cycle

UDP kinase activities are taken from Murphy *et al.* (1973). Lactose synthesis is the actual rate *in vivo* (Carrick & Kuhn, 1977). The activity shown for UDPase represents only the contribution of Golgi UDPase to the whole tissue activity, and is derived by multiplying the galactosyltransferase activity by 4.69 (see the text). Activities are expressed at pH 7.0 or 7.4 and 37°C.

Enzyme or reaction	Activity (μ mol/min per g of tissue)
Lactose synthesis	0.12 $\pm$ 0.01 (32)
Galactosyltransferase	0.78 $\pm$ 0.17 (7)
UDPase activity	3.66 $\pm$ 0.88 (7)
UMP kinase	16.1 $\pm$ 6.1 (7)
UDP kinase	37 $\pm$ 12 (4)
UDP-glucose pyrophosphorylase	34.4 $\pm$ 2.6 (5)
UDP-glucose epimerase	1.56 $\pm$ 0.06 (5)

and 8(b) show how the formation of UDP varied with the amount of homogenate and the time of incubation. The lack of complete linearity in the time course may have been due to some further phosphorylation of [14 C]UDP to [14 C]UTP, since there is also a very active UDP kinase (Murphy *et al.*, 1973). Values from 5 min incubations were used to obtain the tissue UMP kinase activities shown in Table 4.

A single experiment was performed to see how the activity of the kinase might vary with different added metal ions. The mean relative activities of duplicate incubations were: Mg^{2+} , 100%; Mn^{2+} , 93%;

Co^{2+} , 90%; Ni^{2+} , 41%; Ca^{2+} , 36%; none, 23%. Thus it appears that several bivalent metal ions can support the activity of this enzyme, but the pattern of response differs from that of UDPase activity.

Discussion

Role of UDPase

The earlier literature contains a number of reports of nucleoside diphosphatase, including UDPase, activity in liver and kidney preparations (e.g. Plaut, 1955; Gibson *et al.*, 1955). A more detailed study by Ernster & Jones (1962) located this activity in the microsomal fraction of rat liver homogenates. They interpreted its activation by deoxycholate or mechanical disruption as being possibly due to its inclusion within the lumen of membranous sacs and consequent inaccessibility to the substrate. Such a location was supported by Kuriyama's (1972) observation that antibody to the purified enzyme of bovine liver could inhibit the solubilized but not the particulate enzyme. Ernster & Jones (1962) suggested that the nucleotide diphosphatase might act within the 'endoplasmic canals' to remove the GDP formed during the incorporation of amino acids into proteins, and the UDP formed by various uridine nucleotide-linked conjugation reactions. The latter role has been emphasized in the context of glucuronidation (Berry & Hallinan, 1976). Fungal chitin synthesis from UDP-*N*-acetylglucosamine is similarly inhibited by UDP, and a role for UDPase activity, analogous to that proposed here, has been put forward by Gooday & de Rousset-Hall (1975).

Scheme 1. Proposed uridine nucleotide cycle associated with lactose synthesis, and its relationship to the Golgi and cytosol compartments of the mammary secretory cell

A, galactosyltransferase; B, α -lactalbumin. $\leftrightarrow$ denotes transport assumed to be of the facilitated type. Double-headed arrows indicate reactions believed to be close to equilibrium. UDPase, a nucleoside diphosphatase active with UDP.

A similar participation of UDPase activity in the synthesis of mammary lactose would be particularly acceptable, since the marked sensitivity of lactose synthase to inhibition by UDP has been well documented (Khatra *et al.*, 1974). Coffey & Reithel (1968) showed a thiamin pyrophosphatase activity in partially purified Golgi membranes of rat mammary gland, and since the work of Yamazaki & Hayashi (1968) on liver we can probably assume that this is due to the same enzyme as is UDPase activity. Thiamin pyrophosphatase activity was also reported for Golgi preparations of cow (Keenan *et al.*, 1972b) and sheep (Smith, 1975) udders.

The present paper describes the preparation of vesiculated membranes from rat mammary Golgi apparatus with a higher specific activity of galactosyltransferase than has hitherto been reported. This preparation possesses characterized nucleoside diphosphatase activity towards UDP, GDP and IDP. This enzyme effectively removes the UDP generated within the lumina of such vesicles by the lactose synthase reaction, but reacts fully with externally added UDP only after treatment with detergent. At pH 7.0 the measured activity of the UDPase exceeds that of galactosyltransferase about 5-fold. The proposed location of both these enzymes on the inner face of the Golgi membrane, and their co-operation in the synthesis of lactose, is illustrated in Scheme 1. Such an arrangement, together with the apparent impermeability of the membrane to UDP, serves to prevent both the inhibition of lactose synthase by UDP arising inside and outside

the Golgi lumen and the destruction of cytosolic UDP by the UDPase activity. The extra UDPase activity that was observed in whole homogenates may reside in other regions of the endoplasmic reticulum, serving to remove GDP generated in the course of milk-protein synthesis.

The question arises as to whether any of the bivalent cations shown to stimulate the UDPase activity normally occur in sufficient concentration to saturate the enzyme. The concentrations of free Ca^{2+} and Mg^{2+} in cow milk are about 2.2 mM and 0.8 mM respectively (Christianson *et al.*, 1954; van Kreveland & van Minnen, 1955; Muldoon & Liska, 1969), and that of free Ca^{2+} in goat milk is about 1–2 mM (M. Peaker, personal communication). If these values are any guide to the concentrations of these ions within the Golgi lumen of rat mammary gland, then it seems possible that Ca^{2+} and Mg^{2+} together essentially satisfy the metal ion requirement of the UDPase activity (see Fig. 5).

Yamazaki & Hayashi (1968) reported that the nucleoside diphosphatase of bovine liver was activated by ATP. We did not observe such an effect with the UDPase activity of rat mammary Golgi.

Calculated concentration of UDP within the Golgi lumen

If the UDPase within the Golgi lumen is indeed saturated with metal ion, then only the concentration of UDP may limit its rate of catalysis. If, further, it

behaves *in situ* with Michaelis–Menten kinetics, then the steady-state concentration of UDP within the Golgi lumen can be calculated from the equation

$$\frac{v}{V} = \frac{[S]}{[S] + K_m}$$

where v is the actual rate of UDP hydrolysis/g of tissue, which equals the rate of lactose plus neuraminyl-lactose synthesis/g of tissue. This value has been determined in intact anaesthetized lactating rats by a radioisotopic method and is 0.12 ± 0.01 (32) $\mu\text{mol/min per g of tissue}$ (Carrick & Kuhn, 1977). V is the total assayable UDPase activity/g of tissue that is accounted for by the Golgi apparatus, and is $3.66 \mu\text{mol/min per g of tissue}$ (Table 4). $[S]$ is the concentration of UDP, unknown, and K_m is here $K_m(\text{UDP}) = 0.4 \text{ mM}$. From the insertion of these values in the above equation, the concentration of UDP within the Golgi lumen is $14 \mu\text{M}$. When this value is compared with the intracellular concentration of UDP-galactose (about $55 \mu\text{M}$) and UDP ($165 \mu\text{M}$) determined at an earlier stage of lactation (Murphy *et al.*, 1973), with the half-saturating concentration of UDP-galactose for lactose synthase ($60 \mu\text{M}$) (Kuhn, 1968), and with the similar affinity that UDP has for this enzyme (Khatra *et al.*, 1974; Kitchen & Andrews, 1974; Clymer *et al.*, 1976), it is seen that the UDPase activity is apparently just enough to prevent UDP becoming grossly inhibitory to lactose synthase. The large apparent difference in UDP concentration in the cytosol and Golgi lumen is compatible with the suggestion, described above, that this nucleotide does not readily cross the Golgi membrane.

Phosphate as a possible regulator of UDPase

Notwithstanding the above conclusion, one can envisage conditions under which restraint of UDPase activity might result in concentrations of UDP that effectively inhibit the synthesis of lactose. This might occur at certain stages of lactation, or in Golgi vesicles that are due to be discharged from the cell and where overproduction of lactose would cause osmotic rupture. Such a restraint might possibly be exerted by P_i , which inhibits UDPase activity (Fig. 6). The concentration of P_i in rat milk ranges from about $33 \mu\text{mol/ml}$ at parturition (Murphy *et al.*, 1973) to about $60 \mu\text{mol/ml}$ in later lactation (N. J. Kuhn & A. White, unpublished work). Although it is not clear at present what the concentrations of soluble P_i in the aqueous phase of milk are, nor how far they may reflect intra-Golgi concentrations, the values in whole milk raise the distinct possibility that P_i may be a physiological regulator of UDPase activity, and hence of lactose synthesis.

We have also entertained the possibility that the hydrolysis of UDP within the Golgi lumen may actually provide the P_i of milk, since there is a growing

impression that the aqueous phase of milk arises from this compartment of the secretory cell (Linzell & Peaker, 1971; Saacke & Heald, 1974; Kuhn & White, 1975). But initial analyses of rat milk (N. J. Kuhn & A. White, unpublished work) show that the amount of P_i thus generated, which is stoichiometric with the lactose and neuraminyl-lactose made, exceeds up to 2-fold the amount of P_i actually found in milk. Therefore the fate of the P_i arising from UDP hydrolysis within the Golgi lumen must presently be regarded as unclear.

Proposed uridine nucleotide cycle

The UMP that arises from UDPase action within the Golgi lumen presumably re-enters the cytosol, for we have given evidence that it readily penetrates the Golgi membrane (Fig. 7). Since UDP and UDP-glucose apparently cannot cross this membrane, it is necessary to postulate a specific UMP carrier; carriers for UDP-galactose and for glucose have already been proposed (Kuhn & White, 1975, 1976). With the presence of an active UMP kinase in the cytosol, and the previously established presence of UDP kinase (Murphy *et al.*, 1973), UDP-glucose pyrophosphorylase and UDP-glucose epimerase (see Baldwin & Yang, 1974), the conditions are suitable for the resynthesis of UDP-galactose. Scheme 1 shows how this sequence of enzyme reactions and membrane transport constitutes a uridine nucleotide cycle, the function and rate of which is linked to the synthesis of lactose. Table 4 compares the mean activity of each enzyme in the cycle with the actual rate of lactose synthesis (which equals the rate of cycling) measured *in vivo* by the technique referred to above. Galactosyltransferase and UDP-glucose epimerase show the lowest activities, but these still exceed the actual rate of lactose synthesis by about 6.5-fold and 13-fold respectively. The transferase catalyses an effectively irreversible reaction and is therefore working at about 15% efficiency; how far this is due to limiting concentrations of glucose, UDP-galactose, α -lactalbumin or metal ion is not yet clear. UDP-glucose epimerase catalyses a reversible reaction, and the only evidence available suggests that during active lactation it can maintain its reactants near, though perhaps not quite at, equilibrium (Murphy *et al.*, 1973).

The uridine nucleotide cycle of Scheme 1 illustrates some of the metabolic interrelationships that we believe to exist between the Golgi apparatus and the cytosol, and that bear on the synthesis of lactose. The cycle further indicates that the synthesis of lactose from glucose requires 3 mol of ATP/mol of lactose, not 2 mol as has hitherto seemed to be the case. It seems likely that comparable cycles and membrane transport processes will prove to be associated with the formation of other milk sugars and with the glycosylation of the outer regions of glycoproteins.

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