Studies on the Particulate Lactose Synthetase of Mouse Mammary Gland and the Role of α -Lactalbumin in the Initiation of Lactose Synthesis

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1. Some of the kinetic properties of the particulate lactose synthetase of mouse mammary gland were investigated and shown to resemble those that have been reported for the soluble enzyme. Typical values for intact preparations were $2.3 \mu\text{M}$ for the apparent K_m for α -lactalbumin at 40 mM-glucose and 1.7 mM for the apparent K_m for glucose at the endogenous concentration of α -lactalbumin. 2. Digitonin treatment increased total assayable activity approximately twofold but almost eliminated the endogenous activity found in the absence of α -lactalbumin, these findings being consistent with the location of endogenous activity within Golgi vesicles. 3. From the properties of the particulate fraction from lactating mice it was deduced that the effective endogenous α -lactalbumin concentrations were in the range $1\text{--}10 \mu\text{M}$. 4. The concentration of α -lactalbumin was not significantly different in particles isolated at various stages of pregnancy and early lactation. 5. The implications of these results for the control of lactose synthetase *in vivo* are discussed.

Lactogenesis, which may be defined as the initiation of the synthesis and secretion of milk by the mammary gland, is ultimately under hormonal control (see Cowie, 1969), but this control is mediated by changes in the metabolic activity of the mammary gland, which are themselves of great interest. In ruminants, where the characteristic milk constituent lactose is detectable in the blood and urine before parturition (Wheelock & Rook, 1966), the onset of synthetic activity in the mammary gland is a gradual process occurring in parallel with structural development. In rodents it is much more abrupt and in the rat, for example, significant lactose synthesis only commences at 12 h *pre partum* (Kuhn & Lowenstein, 1967). Brew (1969) suggested that this sudden onset of synthetic activity could be explained on the basis of the properties of the enzyme lactose synthetase (UDP-galactose-D-glucose 1-galactosyltransferase, EC 2.4.2.22), which catalyses the reaction:

As originally shown by Brodbeck & Ebner (1966a; see also Brew, 1970), lactose synthetase consists of two components, the A and B proteins, the B protein being subsequently identified as α -lactalbumin (Brodbeck & Ebner, 1966b). By itself the A protein, which is apparently widely distributed, acts as a galactosyltransferase utilizing a variety of acceptors (Brew *et al.*, 1968; Ebner & Schanbacher, 1970) but only in the presence of α -lactalbumin does glucose act as an acceptor at physiological concentrations (Fitzgerald *et al.*, 1970; Klee & Klee, 1970). As the A

protein is membrane-bound (Watkins & Hassid, 1962) and located in the Golgi apparatus (Coffey & Reithel, 1968a,b; Keenan *et al.*, 1970), Brew (1969) postulated that the rate of lactose synthesis in the mammary gland is controlled by the flux of α -lactalbumin through the Golgi apparatus. This theory is consistent with the known role of the Golgi apparatus in the secretion of protein synthesized on the rough endoplasmic reticulum and was supported by the work of Turkington *et al.* (1968), who showed that in homogenates of mouse mammary gland the concentration of A protein was high during the second half of pregnancy but that the concentration of α -lactalbumin remained low until the day of parturition. However, Palmiter (1969b) detected an increasing rate of lactose synthesis in mouse mammary-gland homogenates in the absence of added α -lactalbumin from the tenth day of pregnancy onwards, suggesting that some endogenous α -lactalbumin must be present. This apparent discrepancy could be explained by the fact that measurements of α -lactalbumin in free solution in mammary homogenates are largely irrelevant, as it is the concentration within the Golgi apparatus that actually determines lactose synthetase activity. The increase in soluble α -lactalbumin at the time of parturition might reflect increased availability as milk constituents are secreted into the alveoli.

The work described in the present paper was designed to determine the properties of the particulate lactose synthetase of mouse mammary gland in more detail and, in the light of these properties, to obtain insight into the changes occurring immediately before

and after parturition. The most interesting finding was that there was no significant change in the concentration of α -lactalbumin in Golgi vesicles isolated between the 16th day of pregnancy and the 6th day of lactation.

Materials and Methods

Animals

Nulliparous albino mice from the departmental colony were used. Day 1 of pregnancy was taken as the day on which the appearance of the vaginal plug was noted and day 1 of lactation as the day after the overnight birth of the litter. All mice were killed between the 8th and 15th days of lactation.

Preparation of particulate fractions

The mice were killed by cervical dislocation and all easily accessible mammary-gland tissue was removed and placed in cold MSE medium (0.25 M-mannitol–0.028 M-sucrose–1.1 mM-EDTA, pH 7.4) (Chance & Hagihara, 1960). After being minced with scissors and washed with more medium the tissue was homogenized in a glass homogenizer with a motor-driven nylon pestle with a clearance of approx. 0.2 mm, 10–15 strokes being used. This and all subsequent operations were performed below 5°C. The homogenate was made up to a known volume (about 4 ml) with MSE medium and 1 ml removed for determination of DNA and lactose. The remainder was centrifuged for 5 min at 600 g_{av} in the 40.2 rotor of a model L2 Spinco ultracentrifuge to remove intact tissue and debris, and the supernatant was centrifuged for 30 min at 57000 g_{av} . The resultant supernatant was kept and the pellet resuspended in MSE medium, centrifuged for 15 min at 57000 g_{av} , and finally resuspended by hand homogenization in MSE medium, and the volume noted. In experiments in which the effect of varying the glucose concentration on activity was studied the final resuspension was carried out in sucrose-free medium, i.e. 0.28 M-mannitol–1.1 mM-EDTA, pH 7.4. This preparation is subsequently referred to as the washed particulate preparation. The digitonin-treated preparation was prepared from it by the addition of an equal volume of 1% (w/v) digitonin in 0.1 M-tris-HCl, pH 7.4, 30 min before assay.

Membrane-bound A protein was prepared from the digitonin-treated preparation by centrifugation for 30 min at 57000 g_{av} , discarding the pellet, and re-centrifuging the supernatant for 2 h at 165000 g_{av} in a Spinco 50 Ti rotor. The red translucent pellet was washed by re-suspension in MSE medium, centrifuged for 1 h at 165000 g_{av} , and finally resuspended in MSE medium.

Assay of lactose synthetase activity

Lactose synthetase was assayed by a method similar to that of Coffey & Reithel (1968a) in which the lactose produced is hydrolysed with β -galactosidase and the resultant galactose determined by measuring the reduction of NAD⁺ in the presence of galactose dehydrogenase.

The assay medium comprised 50 mM-tris-HCl, 40 mM-D-glucose, 10 mM-MnCl₂, 1.0 mM-UDP-galactose, bovine α -lactalbumin at the stated concentration, and enzyme (total volume 0.05 ml, pH 7.4). The molarity of α -lactalbumin solutions was calculated by assuming $E_{1cm}^{1\%}$ 20 at 280 nm (Kronman & Andreotti, 1964) and mol.wt. 14437 (Brew *et al.*, 1967). The reaction was carried out at 37°C and started by the addition of enzyme. It was ended by the addition of 0.05 ml of 0.167 M-HClO₄; 0.4 ml of water was then added and precipitated protein removed by centrifugation. A known volume of supernatant was neutralized with a previously determined volume of 1.5 M-K₂CO₃ and, after chilling, the precipitated KClO₄ removed by centrifugation. Two 0.1 ml samples of the supernatant were removed and to one 0.05 ml of 0.1 M-potassium phosphate, pH 7.0, containing 1 μ g (0.03 unit) of β -galactosidase [Boehringer Corp. (London) Ltd., London W.5, U.K.] was added, the other receiving buffer alone. After incubation for 45 min at 37°C each of the tubes received 0.45 ml of a solution containing 40 μ mol of tris, 2 μ mol of NAD⁺ and 1 μ g (0.005 unit) of galactose dehydrogenase [Boehringer Corp. (London) Ltd.], pH 9.5. After a further 45 min at 37°C the fluorescence of 0.5 ml of this solution was measured in a E.I.L. 27A-2 fluorimeter (Electronic Instruments Ltd., Richmond, Surrey, U.K.) adapted for use with small samples by replacement of the standard cuvette with a small Pyrex test tube held in a fixed position and filled and emptied *in situ*. The fluorimeter was standardized by using a solution of NADH of known concentration in an identical buffer mixture. From the difference in the amounts of galactose present before and after hydrolysis the lactose present in the sample can be calculated and, by knowing the lactose present initially, the amount synthesized can be determined.

When *N*-acetylglucosamine was used as galactosyl-group acceptor, the procedure was identical, except that 4 mM-*N*-acetylglucosamine replaced 40 mM-glucose in the assay medium. The product, *N*-acetylgalactosamine, was hydrolysed exactly as for lactose and the resultant galactose determined.

In an alternative procedure used with pre-lactating glands when low activities were expected, the final galactose dehydrogenase reaction was monitored continuously by using a Servoscribe Potentiometric Recorder (Smith Industries Ltd., Wembley, London, U.K.) attached across the terminals of the fluorimeter galvanometer. The conditions were the same as in

the end-point assay except that the galactose dehydrogenase concentration was doubled to ensure that the reaction was complete within 15min, and the temperature was not controlled.

The synthesis of lactose in this assay was shown to be dependent on the presence of both substrates, glucose and UDP-galactose, and to be linear with respect to time and the amount of enzyme added, provided no more than half of the UDP-galactose present was utilized.

Other assays

Acetyl-CoA carboxylase (EC 6.4.1.2) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed as previously described (Jones, 1967). Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of King (1967). DNA was assayed by the method of Burton (1956) as modified by Munford (1963) by using calf thymus DNA as standard, lactose essentially by the procedure of Kuhn & Lowenstein (1967) with the modification that *Escherichia coli* β -galactosidase [Boehringer Corp. (London) Ltd.] replaced the yeast enzyme, and protein by a micro-Kjeldahl procedure (Markham, 1942).

Materials

Bovine α -lactalbumin was generously provided by Dr. T. E. Barman (this Institute) and UDP-galactose by Dr. P. Andrews (this Institute). Other reagents were obtained from normal commercial sources.

Results

Intracellular distribution of lactose synthetase

The lactose synthetase of mouse mammary gland, like that of other species, is largely particle-bound, as revealed by differential centrifugation (Table 1). Brew & Campbell (1967) have pointed out that the fractionation of mammary subcellular organelles by centrifugation gives a less clear separation than it does for some other tissues. This is illustrated here by the occurrence of the mitochondrial enzyme, succinate dehydrogenase, in the conventionally designated nuclei and cell debris (S_1) and microsomal (S_3) fractions as well as in the mitochondrial (S_2) fractions. Particulate lactose synthetase, assayed in the presence of added α -lactalbumin, is only present as traces in the S_1 fraction and is distributed between the S_2 and S_3 fractions, which is consistent with its presumed location in the Golgi apparatus. In the work reported in the present paper the combined S_2 and S_3 fractions, whose preparation as the washed particulate fraction is described in the Materials and Methods section, were used without attempting to characterize further the location of the particulate enzyme. The organelles with endogenous lactose synthetase activity will be subsequently called vesicles without implying any particular location within the Golgi system.

Properties of the washed particulate fraction

The lactose synthetase activity of the washed particulate fraction is stable for several days if kept at 4°C suspended in MSE medium, and survives

Table 1. Intracellular distribution of lactose synthetase and succinate dehydrogenase in a mammary-gland homogenate

The homogenate was prepared from the mammary glands of a lactating mouse as described in the Materials and Methods section. Fractions S_1 , S_2 and S_3 were produced by successive centrifugations at 600g_{av.} for 5min, 10000g_{av.} for 10min and 104000g_{av.} for 45min respectively in the 40.2 rotor of a model L2 Spinco ultracentrifuge. The pellets were resuspended in MSE medium without washing and lactose synthetase activity was assayed under standard conditions in the presence of 30 μ M- α -lactalbumin. Enzyme activities are expressed as μ mol of product formed/min for the whole fraction.

		Whole homogenate	Fraction S_1	Fraction S_2	Fraction S_3	Supernatant	Total recovered
Lactose synthetase	Activity (μ mol/min)	0.330	0.003	0.087	0.143	0.063	0.296
	Percentage of whole homogenate	100	1	26	43	19	89
Succinate dehydrogenase	Activity (μ mol/min)	2.79	0.28	2.04	0.24	0	2.56
	Percentage of whole homogenate	100	10	73	9	0	92

Fig. 1. Dependence of particulate lactose synthetase activity on α -lactalbumin concentration

Activity was assayed under standard conditions at the concentration of α -lactalbumin indicated and is expressed as nmol of lactose synthesized/30 min per $10\mu\text{l}$ of preparation. (a) Direct relationship of total activity (v) to concentration of α -lactalbumin; (b) double-reciprocal plot of total activity (v) minus activity in the absence of added α -lactalbumin (v_0) against concentration of α -lactalbumin.

freezing and thawing. The dependence of the activity of a typical preparation on added α -lactalbumin is shown in Fig. 1(a). If the activity in the absence of added α -lactalbumin, subsequently referred to as endogenous activity, is subtracted from the total activity, the resultant difference is the activity of that part of the A protein exposed to added α -lactalbumin. The double-reciprocal plot of exposed A protein activity against the concentration of α -lactalbumin (Fig. 1b) is linear and gives an apparent K_m for α -lactalbumin of $2.3\mu\text{M}$ at 40 mM-glucose and 1 mM-UDP-galactose. This is in the same range as the values found for the soluble A protein from human (Andrews, 1969) and cow (Ley & Jenness, 1970), which suggests that this property of the enzyme is not greatly affected by binding.

Fig. 2 shows the relationship between the lactose synthetase activity of the particulate preparation assayed in the presence or absence of $30\mu\text{M}$ - α -lactalbumin and glucose concentration. In both cases linear double-reciprocal plots were obtained, yielding

Fig. 2. Dependence of particulate lactose synthetase activity on glucose concentration

The activity of a washed particulate preparation was assayed under standard conditions except that the glucose concentration was varied as shown. Reaction velocity is expressed as nmol of lactose formed/30 min per $10\mu\text{l}$ of preparation. ○, Activity in the absence of added α -lactalbumin; ●, activity in the presence of $30\mu\text{M}$ - α -lactalbumin.

apparent K_m values of 1.7 mM at the endogenous concentration of α -lactalbumin and 2.1 mM in the presence of $30\mu\text{M}$ - α -lactalbumin, the UDP-galactose concentration being 1 mM in both cases. These are typical values, and though the difference may not be significant, it was consistently observed that the value in the presence of α -lactalbumin was the higher of the two. These values are lower than those in the region of 5 mM reported for the bovine enzyme (Klee & Klee, 1970; Fitzgerald *et al.*, 1970), which may indicate a species difference or the influence of membrane binding on the properties of the enzyme. As a routine lactose synthetase was assayed at 40 mM-glucose to ensure that the glucose concentration was not rate-limiting.

Effect of digitonin on particulate lactose synthetase

To determine the total A protein in a homogenate it is necessary to disrupt the vesicular structure so that all the galactosyltransferase activity is assayed at a known uniform α -lactalbumin concentration. Palmiter (1969a) used sonication for this purpose with mammary homogenates and Coffey & Reithel (1968b) studied the effects of various detergents on the activity of cow udder preparations. Preliminary experiments using washed particulate preparations indicated that of the various treatments tried, exposure to digitonin produced the largest and most reproducible increase in lactose synthetase activity. Activity assayed with $30\mu\text{M}$ - α -lactalbumin was increased by approx. 100% by prior exposure to

Table 2. *Effect of pretreatment with various concentrations of digitonin on particulate lactose synthetase activity*

Portions of a washed particulate preparation were diluted with equal volumes of digitonin solutions in 0.1 M-tris-HCl buffer, pH 7.4, to give the final concentrations shown. Then 30 min later lactose synthetase was assayed under standard conditions in the presence of 30 μ M- α -lactalbumin.

Digitonin (%)	0	0.25	0.5	0.75	1.0
Lactose synthetase activity (nmol of lactose/30 min per 10 μ l)	10.3	10.6	18.1	18.6	19.6

Table 3. *Effect of digitonin on the dependence on α -lactalbumin of particulate lactose synthetase activity*

A solution of 1% digitonin in 0.1 M-tris-HCl buffer, pH 7.4 was added to an equal volume of a washed particulate preparation 30 min before assay under standard conditions. The control was diluted with an equal volume of 0.1 M-tris-HCl, pH 7.4.

α -Lactalbumin (μ M)	Lactose synthetase activity (nmol of lactose/30 min per 10 μ l)	
	Control	Digitonin-treated
0	6.48	1.37
30	14.73	31.02

digitonin at concentrations of 0.5% or greater (Table 2). The activity assayed after treatment with 0.5% digitonin will be referred to as total A protein activity, though it is possible that further inaccessible material remains. As expected, this increase in available A protein was accompanied by a large decrease in endogenous activity (Table 3), suggesting that the association between α -lactalbumin and A protein had been extensively disrupted. Digitonin does not solubilize A protein to any great extent (Table 4), as much of the activity still sediments and the specific activity of the supernatant is much lower than that of the starting material. Recoveries of total activity after differential centrifugation of digitonin-treated material were low, of the order of 50–60%, but the reasons for this were not explored. The material sedimented after 2 h at 165 000g_{av} had a slightly higher specific activity than the starting material and was completely devoid of lactose synthetase activity in the absence of added α -lactalbumin. It was used for studies of the properties of membrane-bound A protein, its preparation being described in more detail in the Materials and Methods section.

Effect of α -lactalbumin on membrane-bound A protein activity

It has been shown that the effect of the binding of α -lactalbumin to the A protein component of lactose synthetase is to lower its K_m for the acceptor sub-

strates, glucose and *N*-acetylglucosamine (Fitzgerald *et al.*, 1970; Klee & Klee, 1970). Because of the relative affinity of these substrates and the occurrence of substrate inhibition this has the effect, at the substrate concentrations commonly used, of making α -lactalbumin an activator of galactosyl transfer to glucose and an inhibitor of transfer to *N*-acetylglucosamine (Brew *et al.*, 1968). As it seemed possible that this phenomenon might be used to obtain an estimate of the effective concentration of α -lactalbumin within particulate material, it was investigated by using membrane-bound A protein prepared as described in the Materials and Methods section. Fig. 3 shows the results obtained when this preparation is assayed with glucose or *N*-acetylglucosamine as acceptor in the presence of various concentrations of α -lactalbumin. To obtain a reliable basis for subsequent calculations results from five preparations are included, and as the specific activities vary widely the values have been normalized by setting the activity of each preparation under the specified conditions as 100. As found with partially purified soluble enzyme from cow's milk (Brew *et al.*, 1968) increasing concentrations of α -lactalbumin elevate activity with glucose and decrease activity with *N*-acetylglucosamine and the 'cross-over' point, where the rates with both substrates are equal, is approx. 6 μ M- α -lactalbumin for both systems. This suggests that, in this respect, the membrane-bound enzyme has similar properties to the free enzyme. The ratio of activities with the two substrates (Fig. 3) can be used to estimate the concentration of α -lactalbumin in an isolated system, provided assays are carried out with the concentrations of glucose and *N*-acetylglucosamine used here, and with the reservation that there are differences in the binding of A protein and α -lactalbumin from different species (Ley & Jenness, 1970).

Concentration of α -lactalbumin within isolated vesicles

If it is accepted that the endogenous lactose synthetase activity of the particulate fraction is due to the physical enclosure of dissolved α -lactalbumin within vesicles, rather than to the existence of a stabilized complex of the type suggested by Palmiter (1969a), the concentration of α -lactalbumin within these

Table 4. *Distribution of lactose synthetase activity after digitonin treatment of a washed particulate preparation*

After the addition of an equal volume of 1% digitonin in 0.1M-tris-HCl buffer, pH7.4, the preparation was subjected to successive centrifugation as shown under the conditions given in the Materials and Methods section. The unwashed pellets were resuspended in MSE medium and lactose synthetase activity was assayed under standard conditions in the presence of 30 μ M- α -lactalbumin

Lactose synthetase (nmol of lactose/30min)	(total activity) (activity/mg of protein)	Material sedimenting after			Supernatant
		Whole fraction	30min at 57000g _{av.}	120min at 165000g _{av.}	
4030		141.5	1380	397	344
			140.0	165.5	36.7

Fig. 3. *Dependence of membrane-bound galactosyltransferase activity on α -lactalbumin concentration*

Membrane-bound galactosyltransferase (prepared by digitonin treatment as described in the Materials and Methods section) was assayed under standard conditions with 40mM-glucose ($\circ$) or 4mM-*N*-acetylglucosamine ($\square$) as acceptor and in the presence of the concentration of α -lactalbumin shown. The results are the means of those obtained with five preparations, normalized by setting the activity of each preparation with glucose as acceptor in the presence of 6 μ M- α -lactalbumin as 100. Δ , Ratio of activity with glucose (v_{Glc}) to that with *N*-acetylglucosamine (v_{GlcNAc}).

vesicles can be estimated from the relative rates of galactosyl transfer to glucose and *N*-acetylglucosamine, assuming that the intact isolated system has properties similar to those found after digitonin treatment (Fig. 3).

It is necessary to postulate that the measured galactosyltransferase activity of a particulate preparation (T) is the sum of two activities, one occurring inside intact vesicles and independent of added α -lactalbumin (I) and the other catalysed by A protein exposed to added α -lactalbumin (E). Activity is assayed with as acceptor 40mM glucose (g) or 4mM-*N*-acetylglucosamine (n) and in the presence (+) or

absence (–) of 30 μ M- α -lactalbumin. So, for example, Tg^+ represents the activity measured with glucose as acceptor in the presence of α -lactalbumin.

It follows from these definitions that Ig and In are independent of addition of α -lactalbumin and from the properties of the system (Fig. 3) that Eg^- is zero, i.e. that at the concentration used glucose does not act as a substrate in the absence of α -lactalbumin.

$$\therefore Tg^- = Ig \quad (1)$$

The required internal ratio, Ig/In , which is a function of the internal concentration of α -lactalbumin, can be calculated in two ways, depending on two different relationships derived from Fig. 3.

$$(a) \text{ Taking } En^+ = 0.23 Eg^+ \quad (2)$$

$$\text{By definition } Tg^+ = Ig + Eg^+ \quad (3)$$

$$\text{Combining eqns. (1), (2) and (3)}$$

$$En^+ = 0.23 (Tg^+ - Tg^-) \quad (4)$$

$$\text{By definition } In = Tn^+ - En^+ \quad (5)$$

$$\text{Substituting from eqn. (4)}$$

$$In = Tn^+ - 0.23 (Tg^+ - Tg^-) \quad (6)$$

$$\therefore \frac{Ig}{In} = \frac{Tg^-}{Tn^+ - 0.23 (Tg^+ - Tg^-)} \quad (7)$$

$$(b) \text{ Taking } En^+ = 0.30 En^- \quad (8)$$

$$\text{Substituting in eqn. (5)}$$

$$In = Tn^+ - 0.30 En^- \quad (9)$$

$$\text{By definition } In = Tn^- - En^- \quad (10)$$

$$\text{Eliminating } En^- \text{ from eqns. (9) and (10) gives}$$

$$In = 1.43 Tn^+ - 0.43 Tn^- \quad (11)$$

$$\therefore \frac{Ig}{In} = \frac{Tg^-}{1.43 Tn^+ - 0.43 Tn^-} \quad (12)$$

Table 5 shows results from three experiments carried out with washed particulate preparations from mice in the second week of lactation, together with the derived Ig/In ratios and the values for the endogenous concentration of α -lactalbumin read off from Fig. 3. The ratios calculated from the two equations

Table 5. Apparent concentration of α -lactalbumin in washed particulate preparations

Washed particulate preparations from lactating mice were assayed for galactosyltransferase activity with 40mm-glucose or 4mm-*N*-acetylglucosamine as acceptor and in the presence or absence of 30 μ M- α -lactalbumin. Other experimental details are given in the Materials and Methods section. The symbols used and details of the calculation are explained in the text.

Preparation	Galactosyltransferase activity (nmol of product/30 min per 10 μ l of preparation)						Endogenous α -lactalbumin (μ M)
	Glucose		<i>N</i> -Acetylglucosamine		Ig/In ratio		
	α -Lactalbumin absent	α -Lactalbumin present	α -Lactalbumin absent	α -Lactalbumin present	From eqn.	From eqn.	
	(Tg ⁻)	(Tg ⁺)	(Tn ⁻)	(Tn ⁺)	(7)	(12)	
1	6.23	18.09	16.16	8.78	1.03	1.02	5.6
2	10.39	17.70	19.58	18.04	0.64	0.60	3.5
3	12.38	21.46	17.66	13.26	1.11	1.08	6.4

show good agreement, which provides some support for the underlying theory, and the values for the concentration of α -lactalbumin are of the same order in the three experiments. These concentrations are subject to error because of uncertainties about the accuracy of the ratio line in Fig. 3. In addition, because α -lactalbamins from different species show quantitative differences of behaviour in the lactose synthetase reaction (Ley & Jenness, 1970; Brew, 1970), the derived concentrations in Table 5 are not the concentrations of mouse α -lactalbumin actually present in the vesicles but the concentrations of bovine α -lactalbumin that would produce equivalent effects. Despite these reservations the values calculated by this procedure provide an estimate of the order of magnitude of the mean α -lactalbumin concentration in isolated vesicles and a basis for the comparison of the properties of different preparations.

Changes in enzyme activities at the time of parturition

On the basis of the properties of the lactose synthesis system described above it was decided to assay four different types of lactose synthetase activity in a series of mice killed at different stages of late pregnancy and early lactation. The activities assayed were: (1) particulate activity after treatment with 0.5% digitonin and in the presence of 30 μ M- α -lactalbumin, i.e. total A-protein activity; (2) particulate activity in the absence of added α -lactalbumin, i.e. endogenous activity; (3) particulate activity in the presence of 30 μ M- α -lactalbumin; (4) supernatant activity in the presence of 30 μ M- α -lactalbumin.

The difference between activities (2) and (3) is a measure of the bound A protein accessible to added α -lactalbumin, i.e. exposed A-protein activity.

6-Phosphogluconate dehydrogenase and acetyl-CoA carboxylase activities were also assayed in the

Table 6. Gestation period of mice

This table records the gestation periods of those mice used in this work that were killed *post partum* and others from the same colony that littered during the same period.

Gestation (days)	16	17	18	19	20	21
Number of mice	5	18	33	18	2	1

supernatant, and lactose and DNA in the whole homogenate.

The colony of mice used in this work did not have a constant gestation period (Table 6), so that mice killed *pre partum* cannot be classified in relation to a known date of parturition. For this reason in the presentation of the results, pre-partum mice are identified by the day of pregnancy on which they were killed.

Fig. 4 shows the results obtained between the 16th day of pregnancy and the 6th day of lactation, all measurements being expressed on the basis of the DNA content of the homogenate. The values for lactose synthetase have high standard errors, precluding a detailed analysis of day-to-day changes, but certain trends are apparent. The supernatant lactose synthetase does not increase to a significant extent and this is consistent with its presumed lack of role in lactose synthesis *in vivo*. The three particulate activities all increase in a roughly linear manner and none shows an abrupt increase at the time of parturition. In this they resemble the other two enzymes assayed, 6-phosphogluconate dehydrogenase and acetyl-CoA carboxylase, both of which play an important role in mammary-gland metabolism (see Jones, 1969) and differ sharply from lactose itself

Fig. 4. Lactose synthetase activities of the mouse mammary gland before and after parturition

Experimental details are given in the Materials and Methods section. (a) ○, Total particulate activity assayed after treatment with 0.5% digitonin and in the presence of $30\mu\text{M}$ - α -lactalbumin; ●, supernatant activity assayed in the presence of $30\mu\text{M}$ - α -lactalbumin; (b) ○, endogenous particulate activity assayed in the absence of added α -lactalbumin; ●, exposed activity calculated by subtracting endogenous particulate activity from particulate activity assayed in the presence of $30\mu\text{M}$ - α -lactalbumin. Mean values $\pm$ S.E.M. are shown.

(Fig. 5), the gland content of which shows a large increase on the day of parturition.

The relatively constant relationship between the three types of particulate lactose synthetase activity is demonstrated more clearly in Table 7, where the means of ratios calculated for individual mice are presented. As total and exposed A-protein activities were assayed under the same conditions, including the presence of $30\mu\text{M}$ - α -lactalbumin, their ratio is of

Fig. 5. Metabolic changes in the mammary gland during pregnancy and lactation

See the Materials and Methods section for experimental details. □, 6-Phosphogluconate dehydrogenase and ●, acetyl-CoA carboxylase activities assayed in the supernatant and expressed as μmol of substrate utilized/min per mg of DNA; ○, homogenate lactose content (mg/mg of DNA).

actual amounts of A protein, not merely activities, if it is assumed that digitonin treatment has not altered the kinetic properties of the enzyme. The proportion of total A protein that is exposed to added α -lactalbumin will increase if the Golgi apparatus suffers disruption during preparation. Table 7 shows that this ratio is reasonably constant at the different stages studied and shows no marked trend between the 16th day of pregnancy and the 6th day of lactation. Thus it seems that the fragility of the Golgi apparatus does not change at parturition and possible artifacts arising from this cause can be ignored.

If the assumption is made that all the A protein in a particulate preparation is exposed either to the external medium or to the interior of the vesicles then the size of the latter fraction can be obtained by subtracting exposed-A from total-A-protein activities. The ratio of this vesicular A-protein activity to the measured endogenous lactose synthetase activity should therefore be the ratio of the activities of the same fraction of A protein at two different α -lactalbumin concentrations, $30\mu\text{M}$ and the endogenous value respectively. So this ratio can be taken as a function of the mean concentration of α -lactalbumin

Table 7. Relationships between different types of particulate lactose synthetase activity before and after parturition

See the text for definitions of the different types of activity and details of the calculation of concentration of α -lactalbumin. The values are derived from the results shown in Fig. 4. The ratios are presented as the means $\pm$ S.E.M. of the ratios for individual mice.

	Day of pregnancy			Day of lactation			
	16 4	17 5	18 5	1 4	2 3	3 4	6 5
No. of mice							
Exposed A-protein/total A-protein activity ratio	0.286 ± 0.035	0.222 ± 0.053	0.390 ± 0.048	0.331 ± 0.026	0.252 ± 0.085	0.371 ± 0.022	0.399 ± 0.022
(Total A protein – exposed A protein)/Endogenous activity	3.40 ± 0.23	3.98 ± 0.35	4.23 ± 0.69	4.81 ± 1.70	3.83 ± 1.26	3.02 ± 0.27	3.04 ± 0.71
Calc. [α -lactalbumin] (μ M)	2.6	2.2	1.9	1.7	2.2	3.1	3.0

within the vesicles of a particulate preparation, and by using the relationship between lactose synthesis and concentration of α -lactalbumin shown in Fig. 3 an estimate of this concentration can be derived. As shown in Table 7 the observed ratios vary between 3.04 and 4.81 and correspond to concentrations of α -lactalbumin in the range 1.7–3.0 μ M. It must be stressed that because of the various assumptions made these values can only be approximate and, like those in Table 5, represent the equivalent concentration of bovine α -lactalbumin. However, the important point is that the ratios do not show a marked downward trend during the period studied and so provide no evidence for a large increase in the effective concentration of α -lactalbumin at the time of parturition.

Discussion

The relationship between the activities of enzymes assayed *in vitro* and their actual metabolic role *in vivo* is always difficult to establish, and this is particularly so for lactose synthetase, which has the unusual property of being formed by the reversible association of two protein components. The intracellular distribution of these components, A protein and α -lactalbumin, will be of crucial importance *in vivo*. Keenan *et al.* (1970) have confirmed that the lactose synthetase activity of rat mammary-gland homogenates is located in the Golgi fraction and the results of Coffey & Reithel (1968*a,b*) suggest that the distribution is similar in the cow. Radioautographic studies of mouse mammary gland (Wellings & Philp, 1964; Verley & Hollmann, 1966) have shown that the alveolar epithelial cells resemble other types of secretory cells in that proteins destined for secretion pass through the Golgi apparatus in transit from their site of synthesis on the rough endoplasmic reticulum to the secretory vesicles, which eventually discharge into the alveolar lumina. It is therefore highly probable that the Golgi apparatus is the site of the interaction between membrane-bound A protein

and α -lactalbumin to produce a functional lactose synthetase. If this cell organelle can be isolated in a reasonably intact condition a study of its properties should illuminate the control of lactose synthesis *in vivo*.

The Golgi fraction isolated by Keenan *et al.* (1970) from rat mammary gland possessed the characteristic morphology of stacks of flattened cisternae surrounded by peripheral tubules, but by biochemical criteria it appeared to have suffered some damage during preparation. The ratio of lactose synthetase activities in the presence and absence of added α -lactalbumin rose from approx. 3 in the whole homogenate to 16 in the isolated Golgi fraction. This ratio is a rough measure of the intactness of the system, as leakage of α -lactalbumin from the cisternae will cause it to rise. In the work reported in the present paper this ratio was about 2 in the washed particulate fraction, suggesting that this crude preparation, which contains all types of cell organelles apart from nuclei, is more stable than the purer Golgi fraction. However the lactose-synthesizing fraction that was isolated from cow udder by Coffey & Reithel (1968*b*) consisted mainly of large electron-dense particles and membranous fragments and it appeared that the Golgi structure had been considerably distorted during isolation. Similar changes in structure may have occurred during the preparation of the washed particulate fraction and this possibility must be considered in discussing the present results. The occurrence of artifacts owing to the enclosure of α -lactalbumin in vesicles formed spontaneously after homogenization is unlikely, as the cytosol is greatly diluted by medium, and the possibility suggested by Palmiter (1969*a*), that the relationship of α -lactalbumin and A protein is maintained, not by sub-cellular compartmentation but by the presence of an additional stabilizing factor, has little evidence to support it and seems an unnecessary complication.

For the rest of the discussion, therefore, it will be assumed that the particulate lactose synthetase

activity found in mammary homogenates is due to the presence of Golgi vesicles containing membrane-bound A protein exposed to dissolved α -lactalbumin. These vesicles maintain their structure during storage in the cold and survive freezing, but are almost totally disrupted by digitonin treatment, which evidently allows α -lactalbumin to escape but which only solubilizes a small proportion of bound A protein. In the absence of any measure of the effective volume within these vesicles the concentration of α -lactalbumin can only be estimated indirectly and expressed as the equivalent concentration of bovine α -lactalbumin, but the results of the three different indirect approaches show quite good agreement considering the various uncertainties involved. The two approaches based on the rates with glucose and *N*-acetylglucosamine as acceptors show excellent agreement, providing support for the model used in their derivation, and lie in the range 3.5–6.4 μM for preparations from mice in the second week of lactation. The third approach, used with mice killed around parturition, involved additional assumptions and the values of 1.7–3.1 μM are probably less reliable. A calculation of this third type performed on the results in Table 3, obtained with a mouse in the second week of lactation, gives a value of 2.5 μM - α -lactalbumin, confirming that the rise between parturition and mid-lactation is small or non-existent. These concentrations are far lower than the presumed concentration of α -lactalbumin in mouse milk which, by analogy with the milk of other species, is probably in the range 0.1–1.0 mM (Ley & Jenness, 1970), but as the contents of the Golgi cisternae undergo concentration during the formation of secretory vesicles (Hollmann & Verley, 1971) this difference is not surprising. If concentrations in the range 3.5–6.4 μM exist *in vivo* they will be sufficiently near to the K_m of particulate lactose synthetase for α -lactalbumin of 2.3 μM to allow the concentration of this substance to control the rate of lactose synthesis as suggested by Brew (1969).

The results reported in the present paper do not lead to any positive conclusion about the control of lactose synthetase at the time of parturition. The finding by Turkington *et al.* (1968) that the concentration of A protein in mouse mammary tissue does not increase suddenly at the time of parturition has been confirmed and shown to apply also to total A protein measured after digitonin treatment. The concentration of the other lactose synthetase component, α -lactalbumin, also shows no increase at parturition when assayed in vesicles isolated from the mammary gland. If the concentration of α -lactalbumin within these vesicles is approximately equal to the functional concentration *in vivo* or if it bears a constant relationship to that concentration then an increase in the flux of α -lactalbumin through the Golgi apparatus cannot initiate lactose synthesis.

This conclusion holds true even if the concentration *in vivo* is underestimated *post partum*, owing, perhaps, to the swelling of vesicles containing a high concentration of protein. This follows from the fact that the concentration of α -lactalbumin present *pre partum* is high enough to allow lactose synthetase to function at one-quarter of its maximum rate (Table 7), and thus an increase after parturition could at most produce a fourfold increase in synthesis. This argument would not be valid if the concentration of α -lactalbumin *in vivo* before parturition was much lower than that in the isolated vesicles, but this circumstance, though meriting further investigation, does not appear very probable. Hence the results reported in the present paper, though not conclusive, do cast doubt on the role of α -lactalbumin in initiating lactose synthesis. Certainly the increase in free α -lactalbumin in mammary homogenates reported by Turkington *et al.* (1968) does not provide support for this role. Palmiter (1969b), who assayed lactose synthesis in mouse mammary homogenates in the absence of added α -lactalbumin at different stages of pregnancy and lactation obtained results essentially similar to those reported in the present paper, confirming that the functional concentration of α -lactalbumin in these homogenates is already high before parturition. In the rat, Kuhn (1968), using whole mammary homogenates, found that both endogenous lactose synthetase activity and activity in the presence of added α -lactalbumin remained very low until the last day of pregnancy, when both activities began to increase. McKenzie *et al.* (1971) have confirmed these differences in the time of appearance of endogenous lactose synthetase activity in mouse and rat. The very low activities in late pregnancy in the rat may be due to the high UDP-galactose-hydrolysing activity, which interferes with the assay, but there are probably species differences in rates of development; Girardie (1968) showed histologically that secretory activity commenced earlier in the mouse mammary gland than in the rat and Mills & Topper (1970) found a proportion of well-developed alveolar-epithelial cells in the mid-pregnant mouse. Thus it may be that the appearance of both A protein and α -lactalbumin is delayed in the rat compared with the mouse.

It appears that the phenomenon requiring explanation in the mouse is not the initiation of lactose synthesis at parturition but its suppression in late pregnancy. From the results in Figs. 4 and 5 it can be calculated that although the potential rate of endogenous lactose synthesis of a mouse mammary gland on the 17th day of pregnancy is 4.43 mg/mg of DNA per day the actual gland content is only 0.057 mg/mg of DNA. In view of the lack of reports of β -galactosidase activity in mammary gland it is unlikely that lactose is synthesized and rehydrolysed. The above rate of synthesis is based on activities measured under

optimum conditions and will undoubtedly be lower *in vivo*, though the actual concentrations of substrates are not known. The glucose concentration in the mammary gland of lactating rat has been estimated as 0.3–1.5mM (Baldwin & Cheng, 1969) and if in the same range in the mouse would be lower than the apparent K_m for glucose of about 2mM but not sufficiently low to account for the small amount of lactose found. Even less is known of UDP-galactose concentrations, but the high activities of the enzymes involved in its synthesis in the rat (Baldwin & Milligan, 1966) and rabbit (Heitzman, 1968) compared with the actual rate of lactose production suggests that, at least in these species, its rate of formation is not rate-limiting. It is possible that the UDP-galactose concentration is also controlled by its rate of hydrolysis, which as mentioned above is high in the pregnant rat (Kuhn, 1968) but which is very low in the mouse. (The rate of galactose production, i.e. the sum of UDP-galactose hydrolase and β -galactosidase activities, is incidentally measured in the lactose synthetase assay procedure described in the present paper and was always undetectable or very low compared with the lactose synthetase activity.) Obviously before possible control mechanisms *in vivo* can be usefully assessed it will be necessary to measure the concentrations of glucose and UDP-galactose at different stages of pregnancy and lactation, to investigate the potentially complex kinetic inter-relationship of the substrates and ligands of lactose synthesis in more detail and to determine the extent of the changes undergone by the Golgi vesicles during their isolation.

The role of α -lactalbumin in the control of lactose synthesis remains problematical. As an essential component of lactose synthetase it obviously exerts an overall control in determining that the mammary gland is the only site of lactose synthesis, but it has been argued in this discussion that it may not be responsible for the abrupt onset of synthesis just before parturition. Kuhn (1969) proposed that, at least in the rat, lactogenesis is triggered by a decrease in circulating progesterone and Turkington & Hill (1969) showed that progesterone inhibits the synthesis of α -lactalbumin but not A protein in cultured mouse mammary explants. Taken together these observations appear to support the Brew (1969) hypothesis of control by changing α -lactalbumin flux, but again the available α -lactalbumin in homogenates was measured with the resultant difficulties of interpretation. If, as has been suggested in the present paper, the functional concentration of α -lactalbumin, as distinct from the total α -lactalbumin content of the mammary gland, does not change greatly at the time of parturition, it is possible that the onset of lactose synthetic activity that occurs at this time. The fundamental cause of this remains to be elucidated.

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