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Progesterone and the Metabolic Control of the Lactose Biosynthetic Pathway During Lactogenesis in the Rat

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1. Lactogenesis was initiated in pregnant rats by ovariectomy, thereby causing progesterone withdrawal, after which the mammary tissue was analysed for contents of enzymes and metabolites concerned with the biosynthesis of lactose. 2. Lactose synthesis increased about 126-fold with little or no accompanying change in the contents of most metabolic intermediates or in the adenine nucleotide energy charge. 3. Comparison of mass-action ratios with equilibrium constants showed that phosphoglucomutase (EC 2.7.5.1), UDP-glucose pyrophosphorylase (EC 2.7.7.9) and UDP-glucose epimerase (EC 5.1.3.2.) catalysed reactions close to equilibrium. Nucleoside diphosphokinase (EC 2.7.4.6.) activity was very high and probably equilibrates the UTP–UDP and ATP–ADP couples. Lactose synthetase and hexokinase (EC 2.7.1.1) appeared to catalyse rate-limiting reactions. 4. Large increases were seen of UDP-glucose pyrophosphorylase (5-fold), lactose synthetase A protein (3.8-fold) and α-lactalbumin (28-fold), but not of hexokinase, phosphoglucomutase, UDP-glucose epimerase, nucleoside diphosphokinase or glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activities. 5. It appeared that the increased lactose synthesis was largely accounted for by the increased lactose synthetase A protein activity and α-lactalbumin.

The hormonal control of lactogenesis in the rat involves high circulating concentrations of progesterone, which restrains mammary function during pregnancy. When these concentrations fall naturally at the end of pregnancy, or when the ovaries are removed, the mammary gland is released from inhibition and, in the presence of permissive hormones such as corticosterone and prolactin, embarks on a rapid synthesis and secretion of milk solids (for reviews see Cowie & Tindal, 1971; Denamur, 1971; Kuhn, 1971). Further understanding of this control by progesterone requires that the rate-limiting steps of biosynthetic pathways be identified, for it is through these that progesterone must exert its primary restraint.

In previous studies of the pathway of lactose biosynthesis it was noted that lactose synthetase is the only enzyme with sufficiently feeble activity during pregnancy and large enough increase during lactogenesis to account for the expected change in the rate of lactose synthesis (Kuhn & Lowenstein, 1967; Kuhn, 1968). Further, administered progesterone prevents the increase in tissue lactose and in lactose synthetase activity (Turkington & Hill, 1969; Kuhn, 1969). It was accordingly suggested that this enzyme might catalyse a rate-limiting reaction on the pathway (Kuhn, 1968).

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In the present more detailed study the increase in rate of lactose synthesis is estimated during lactogenesis induced by ovariectomy, and concurrent changes in tissue metabolites and enzyme activities are also measured.

Methods and Materials

Animals

Wistar-derived rats in their first pregnancy were kept in individual cages under alternating 12h periods of light and dark, and with food *ad lib.* They were about 13 weeks old and weighed about 300g.

Surgical procedures

Bilateral ovariectomy was performed under light diethyl ether anaesthesia on rats that were 2–3 days *pre partum*. When tissue was to be removed for enzyme assays, rats were exsanguinated under ether anaesthesia before dissection. When tissue was to be removed for metabolite analyses, the following precautions were taken to maintain its adequate oxygenation. Rats were anaesthetized with Nembutal (approx. 6mg/100g body wt.) and allowed to respire N_2+O_2 (60:40) while the tissue was freed from surrounding connective tissue, care being taken to leave intact its own main blood supply.

The increased O_2 concentration was used to counteract two possible deleterious effects. First, the respiratory rate under Nembutal is markedly less than normal. Secondly, one inevitably severs small blood vessels connecting the mammary tissue with the adjacent skin, which could lead to local hypoxia either directly or by the vasoconstriction of other vessels. With the above precautions, the mammary tissue appeared pink and well oxygenated. Metabolic criteria of integrity are discussed below. When extracellular water was to be measured, rats received [^{3}H]inulin (2μCi) or [^{3}H]sucrose (2.5μCi) in 0.2ml of 0.9% NaCl via the femoral vein 12min before removal of the gland. Immediately after dissection, one inguinal gland was freeze-clamped (Wollenberger *et al.*, 1960), severed and placed in liquid N_2. The animal was then decapitated and blood collected into a chilled beaker containing heparin for the measurement of plasma metabolites and radioactivity.

Milk was collected from parturient rats by expression from all nipples, after administration of 0.1i.u. of oxytocin subcutaneously.

Metabolite analyses

Frozen tissues were powdered under liquid N_2 in a mortar and pestle, and weighed samples were extracted at 0°C with 8vol. of 6% (w/v) $HClO_4$ or at −10°C with 8% (w/v) $HClO_4$ in aq. 40% (v/v) ethanol (Williamson & Corkey, 1969). No difference in the glucose 6-phosphate content of tissue was found when the two methods were compared. Extracts were neutralized with KOH to pH6–7 and the $KClO_4$ was removed by centrifugation. After their volumes had been recorded, the extracts were cleared of finely divided fat by passage through a Millipore filter (pore size 0.22μm). The residual opalescence of some samples could not be further removed.

Plasma was similarly extracted with an equal volume of 6% (w/v) $HClO_4$.

Enzymic–fluorimetric methods were used to determine glucose 6-phosphate (Hohorst, 1963*a*), glucose 1-phosphate (Bergmeyer & Klotzch, 1963), UDP-glucose (Mills & Smith, 1963, method B) and PP_i (Biochemica Service, Boehringer Mannheim, issue No. 1d, 1971) in a glass cuvette of internal dimensions 0.4cm×0.4cm×3.0cm. Measurements were made with a fluorimeter (Farrand Optical Co., New York, N.Y., U.S.A.) fitted with a double monochromator and modified to increase the electrical backing-off of the large baseline fluorescence caused by the tissue extracts. Care was taken to exclude dust, and all solutions were passed through Millipore filters. The general procedures followed the recommendations of Williamson & Corkey (1969). After each metabolite determination, the assay was internally calibrated by three successive additions (10μl each) of a solution of NADH (approx. 0.1mM) standardized by its extinction at 340nm.

Enzymic–spectrophotometric methods were used to determine glucose (Slein, 1963), lactose (Reithel, 1963), pyruvate (Bücher *et al.*, 1963), lactate (Hohorst, 1963*b*), ATP (Lamprecht & Trautschold, 1963), ADP and AMP (Adam, 1963), and the sum of UTP and UDP (Keppler *et al.*, 1970). P_i was determined chemically (Schultz *et al.*, 1967), and casein was determined as protein P (Kuhn, 1972*a*).

UDP-galactose was determined by a radioenzymic method in which, after isolation by adsorption on ion-exchange resin and charcoal, it was made to react with [^{3}H]glucose in the presence of lactose synthetase to give [^{3}H]lactose, which was separated and its radioactivity determined. Overall losses were corrected for by the use of tracer UDP-[^{14}C]galactose added to the original extracts. Tissue extract (10ml) was passed through a column (3cm×0.4cm diam.) of Dowex-1 resin (formate form; X8; 200 mesh) at 0–4°C. The column was washed with 25ml of water and the adsorbed nucleotides were eluted over 2h with 25ml of 0.8M-formic acid–2M-ammonium formate, pH4.1, into a thick-walled glass centrifuge tube containing a stirred slurry of 0.5g of Darco G charcoal in 5ml of water. After elution, the slurry was shaken mechanically for 10min and centrifuged for 10min at 2000*g*. The sedimented charcoal was washed five times by resuspension and sedimentation in 30ml batches of iced water. Then the nucleotides were eluted by shaking the charcoal with 30ml of 10% (v/v) redistilled pyridine in ethanol–water (1:1, v/v) at 37°C for 2h (Tsuboi & Price, 1959). After the addition of 3ml of 1M-acetic acid the charcoal was sedimented and the clear supernatant was freeze-dried. The residue was dissolved in 0.2ml of water. Duplicate samples (50μl) were each incubated for 2h at 37°C in a reaction mixture containing [6-^{3}H]glucose (20μmol, 0.057μCi), glycylglycine–KOH buffer, pH7.5 (20μmol), $MnCl_2$ (2μmol), lactose synthetase A protein (about 4munits) and α-lactalbumin (30μg) in a final volume of 0.16ml. After this period ATP (0.5μmol, $MgCl_2$ (1μmol) and hexokinase (280munits) were added and the incubation (0.21ml) was continued at room temperature for 30min. The reaction mixture was then passed through a column of Dowex resin as described above and the column was washed with 2ml of aq. 50% (v/v) dioxan, the eluate and washings being collected in a scintillation vial. Appropriate blank incubations were also carried out. Then 12ml of scintillation fluid was added and the samples were analysed for ^{3}H and ^{14}C by double-label liquid-scintillation counting. In a control experiment where 15nmol of UDP-galactose was added to the original tissue extract, 17.1nmol was measured by the assay

after subtraction of the measured endogenous UDP-galactose.

Determination of urinary lactose

Rats received 0.3ml of 0.9% NaCl containing [1-^{14}C]lactose (0.03μCi) via the femoral vein, and were then maintained with food and water for 24h in a metabolic cage. Urine and washings were filtered, de-ionized with a mixed-bed resin and freeze-dried. A portion of the residue was purified by descending paper chromatography on Whatman no. 4 paper with propan-1-ol–ethyl acetate–water (7:1:2, by vol.; Baar & Bull, 1953). The lactose region was located with the aid of standards run alongside, eluted with water and assayed for lactose (Reithel, 1963) and ^{14}C.

Enzyme assays

Mammary tissue was chopped (McIlwain & Buddle, 1953) and homogenized with 4vol of chilled 0.25M-sucrose in a glass tube fitted with Teflon–glass pestle. After centrifugation at 105000$g_{av.}$ for 1h, the clear, fat-free supernatant was withdrawn and assayed for UDP-glucose pyrophosphorylase (EC 2.7.7.9), UDP-glucose 4-epimerase (EC 5.1.3.2), phosphoglucomutase (EC 2.7.5.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) by methods given previously (Kuhn & Lowenstein, 1967). Hexokinase (EC 2.7.1.1) was assayed as described by Walters & McLean (1968). In the last three assays 6-phosphogluconate dehydrogenase (EC 1.1.1.44) was either added or shown to be already present in excess, and it was assumed that 1mol of glucose 6-phosphate gave rise to 2mol of NADPH. Nucleoside diphosphokinase (EC 2.7.4.6.) was assayed spectrophotometrically by the coupled reactions:

$$\text{UTP} + \text{ADP} \rightarrow \text{UDP} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{glucose 6-phosphate}$$

$$\text{Glucose 6-phosphate} + \text{NADP}^+ \rightarrow 6\text{-phosphogluconate} + \text{NADPH}$$

The standard assay contained Tris–HCl buffer, pH7.5 (25μmol), $MgCl_2$ (1μmol), glucose (1μmol), $NADP^+$ (0.35μmol), ADP (0.05μmol), UTP (0.5μmol), yeast hexokinase (280munits), glucose 6-phosphate dehydrogenase (280munits) and high-speed supernatant suitably diluted in bovine serum albumin (0.1mg/ml) in a final volume of 0.5ml. Reactions were initiated with supernatant and assayed at room temperature by the increase in extinction at 340nm. Appropriate controls were included to correct for endogenous adenylate kinase activity and for the phosphorylation of glucose by UTP.

For the assay of lactose synthetase A protein and of α-lactalbumin, chopped tissue was homogenized in 9vol. of 0.25M-mannitol and centrifuged at 105000$g_{av.}$ for 1h. The supernatant was retained and the drained pellet was resuspended in 2ml of a solution containing mannitol (0.125M), Tris–HCl, pH7.4 (25mM), and digitonin (0.5% and 2.0%, w/v, for extraction of α-lactalbumin and A protein respectively) at 0°C for 30min (Jones, 1972), after which the remaining particulate material was sedimented at 155000$g_{av.}$ for 2h. Repeated extraction in the same way, or with digitonin (4%, w/v) or Triton (1%, w/v) failed to reveal further α-lactalbumin or A protein. The A protein in the digitonin extracts was assayed as lactose synthetase activity in the presence of bovine α-lactalbumin (0.2mg/ml) by the radiochemical method of Fitzgerald *et al.* (1970). Control experiments showed that digitonin did not interfere with the assay, and that no significant activity was present in mammary supernatants from pregnant or lactogenic rats.

α-Lactalbumin was assayed in supernatants and in digitonin extracts by the method of Fitzgerald *et al.* (1970) in the presence of added bovine milk A protein (about 0.8munits/ml). A standard curve was prepared each time with bovine α-lactalbumin. Digitonin did not interfere with the assay.

Radioactivity determination

Samples were mixed with 10vol. of scintillation fluid prepared by mixing 1vol. of Triton with 2vol. of xylene containing 2,5-diphenyloxazole (6g/l) and 1,4-bis-(5-phenoxazol-2-yl)benzene (0.12g/l), and were counted for radioactivity in a suitably programmed Phillips liquid-scintillation analyser.

Materials

Lactose synthetase A protein was prepared from cow's milk up to step 3 of the method of Trayer & Hill (1971), with certain changes. In step 1 the skim milk was made 0.03M in $MnCl_2$ to aid precipitation of casein before centrifugation. Chromatography on cellulose phosphate, in step 3, was repeated once to remove traces of α-lactalbumin, and the eluted A protein was precipitated with 65%-satd. $(NH_4)_2SO_4$. The sediment was redissolved in 1–2ml of buffer containing Tris–HCl, pH7.4 (20mM), $MgCl_2$ (5mM) and 2-mercaptoethanol (1mM), dialysed overnight against the same buffer at 0°C, and finally stored in batches under liquid N_2.

Pure bovine α-lactalbumin was a previous gift from Dr. F. M. Robbins and Dr. M. J. Kronman of the U.S. Army Natick Laboratories, Natick, Mass., U.S.A. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Enzymes and nucleotides were from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., or from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.

Results

Rates of lactose synthesis

Lactogenesis was experimentally initiated in late-pregnant rats by bilateral ovariectomy (Shinde *et al.*, 1965; Kuhn, 1969). Accumulation of mammary lactose began 13h after ovariectomy and accelerated up to 30h, at which time the tissue contained 9.94±1.6μmol of lactose/g wet wt. (Fig. 1). This confirms the pattern found previously (Kuhn, 1969). Little further accumulation occurred. Casein accumulation also occurred (Fig. 2) but showed no lag period and was superimposed on the pre-existing content of tissue casein that was noted by Kuhn (1972*a*).

The rate of lactose synthesis at different times after ovariectomy was estimated from the sum of its accumulation rate in the tissue and its leakage rate from the tissue, via the bloodstream, into the urine. There appear to be no other fates of lactose, since the mammary tissue is virtually unable to hydrolyse it (Kuhn, 1968) and the young were not yet available for suckling. Conversion into *N*-acetylneuraminyl-lactose is unimportant at this very early stage of lactogenesis (Kuhn, 1972*b*). Rates of accumulation were estimated from the slopes of tangents drawn to the curve in Fig. 1. Rates of leakage into the blood were estimated from the 24h urinary lactose output of intact rats 2–3 days before parturition or of similar rats over the period 6–30h after ovariectomy. The recovery of intravenously injected [^{14}C]lactose permitted correction for losses owing to extra-mammary metabolism, bacterial action, collection and purification. Rates of lactose leakage for intact and ovariectomized rats were respectively 9.8±2.3 (7) and 12.9±3.3 (5) μmol/24h per rat, and the total fresh weights of mammary tissue at 0 and 30h were respectively 11.7±1.3 (4) and 13.5±0.8 (5) g/rat (means±S.E.M. with numbers of rats in parentheses). From these data the mean rates of lactose leakage are respectively 0.58 and 0.67nmol/min per g wet wt. of mammary tissue. These values are not significantly different, and a mean value of 0.62nmol/min per g wet wt. is used. Fig. 1 shows the summed rates of lactose synthesis, which increase from 0.62 to 80nmol/min per g wet wt. over the period 0–30h. Thus the flux through the lactose pathway increases about 126-fold.

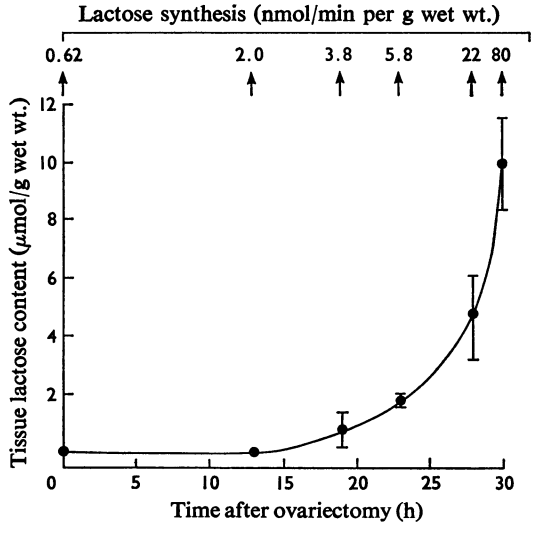


Fig. 1. *Lactose accumulation in mammary tissue of pregnant rats after ovariectomy*

Values are means±S.E.M. for five rats. Values for estimated mean rates of lactose synthesis (see the text) are given for the times shown by the vertical arrows.

Analysis of tissue metabolites

Tissue contents of metabolites were determined in freeze-clamped glands, and are expressed on a fresh-weight basis (Table 1). Further calculated values or ratios are given in Table 2, and their significance is discussed below. Intracellular water was measured to allow the calculation of the approximate intracellular concentrations of metabolites.

Intracellular water

This was calculated by subtracting the sum of interstitial water and milk water from total tissue water. Interstitial water was measured by comparing

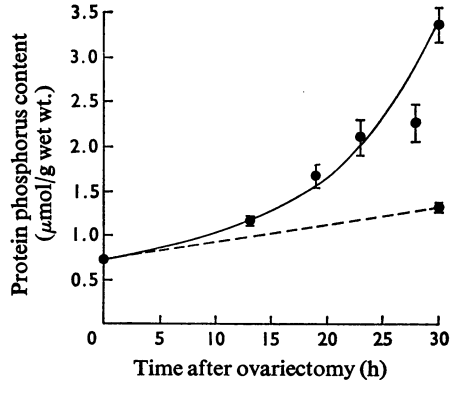


Fig. 2. *Casein accumulation in mammary tissue of pregnant rats after ovariectomy*

The effect of progesterone administration at ovariectomy (----) is indicated. Values are means±S.E.M. for five rats.

Table 1. *Contents of metabolites in mammary tissue or plasma at different times after ovariectomy of pregnant rats*

For details see the Methods and Materials section. Values are expressed as nmol/g wet wt. of tissue or nmol/ml of plasma and are means ± S.E.M. for at least five rats.

Time after ovariectomy (h) ...	0	19	23	30
Tissue metabolites				
Glucose	936 ± 76	1037 ± 71	1082 ± 48	921 ± 146
Glucose 6-phosphate	54.1 ± 7.8	54.9 ± 4.1	57.0 ± 10.7	59.6 ± 1.6
Glucose 1-phosphate	4.4 ± 0.6	3.2 ± 0.3	3.8 ± 0.6	4.9 ± 0.8
UDP-glucose	47.4 ± 9.0	56.0 ± 3.1	51.0 ± 2.2	63.4 ± 4.7
UDP-galactose	12.6 ± 1.0	—	—	8.8 ± 0.2
ATP	486 ± 50	526 ± 35	574 ± 141	536 ± 55
ADP	165 ± 30	191 ± 16	215 ± 22	163 ± 30
AMP	45 ± 2	65 ± 11	80 ± 7	65 ± 6
UTP + UDP	112 ± 7	130 ± 9	132 ± 14	122 ± 6
PP_i	6.7 ± 1.7	15.1 ± 16	11.0 ± 2.9	12.1 ± 2.1
P_i	1726 ± 359	1427 ± 127	2029 ± 361	2842 ± 441
Pyruvate	70.0 ± 3.1	58.9 ± 5.6	85.0 ± 11.0	51.2 ± 5.7
Lactate	1485 ± 364	1303 ± 36	2333 ± 509	1638 ± 176
Total water (mg/g)	383 ± 27	391 ± 15	419 ± 33	448 ± 18
Interstitial water (mg/g)	253 ± 25	183 ± 6	196 ± 12	190 ± 25
Plasma metabolites				
Glucose	4140 ± 152	4080 ± 19	4170 ± 59	4520 ± 403
P_i	1012 ± 89	1200 ± 86	1080 ± 72	920 ± 155

Table 2. *Calculated mean concentrations of tissue metabolites or of their ratios*

For details see the Methods and Materials section. Energy charge = [ATP] + ½[ADP]/[ATP] + [ADP] + [AMP].

Time after ovariectomy (h) ...	0	19	23	30
[ATP]/[ADP]	2.95	2.76	2.67	3.29
[ATP]/[AMP]	10.8	8.1	7.2	8.3
Energy charge	0.82	0.80	0.79	0.81
$[ATP][AMP]/[ADP]^2$	0.80	0.93	1.04	1.31
[Lactate]/[pyruvate]	21.2	22.2	27.4	32.0
[Glucose 1-phosphate]/[glucose 6-phosphate]	0.082	0.058	0.067	0.082
Tissue UTP (nmol/g wet wt.)	83.5	95.0	96.0	94.0
Tissue UDP (nmol/g wet wt.)	28.5	35.0	36.0	28.0
[UDP-glucose][PP_i]/[UTP][glucose 1-phosphate]	0.86	2.8	1.5	1.7
[UDP-galactose]/[UDP-glucose]	0.27	—	—	0.14
Retained milk water (g/g wet wt.)	0.001	0.013	0.027	0.156
Intracellular water (g/g wet wt.)	0.129	0.195	0.196	0.102

the [^{3}H]inulin contents of tissue and plasma. In some experiments [^{3}H]sucrose was used, with similar results. Milk water, from milk in the alveoli and ducts, was gauged from the amount of tissue lactose and the concentration of lactose and water in the first milk of parturient rats (Table 3). Lactose is one of the very few milk constituents that is not also a common constituent of tissue. The method assumes that the lactose concentration in the retained milk water is the same as that expressed from the nipples, and that all the tissue lactose is truly extracellular. However, the calculations below with respect to milk P_i and α-lactalbumin retained in the tissue show that these assumptions may not be wholly justified.

The calculated intracellular water ranges from 0.102 to 0.196g/g wet wt. of tissue (Table 2). The apparent large fall at 30h follows mainly from the large correction for milk water which, as discussed, is open to criticism. Therefore we prefer to take a mean value of 0.16g/g wet wt. of tissue.

Phosphate

At 0h the total tissue P_i, corrected for the contribution by interstitial-fluid P_i (Table 1), is 1.47 μmol/g wet wt., and the calculated intracellular concentration is 9.2mM. This value is similar to that for lactating goat udder (9.1mM; D. C. Hardwick, personal communication), but about 50% higher than in rat liver (Veech *et al.*, 1970). The rise in tissue P_i during lactogenesis is probably due to the accumulation of extracellular milk P_i (Table 3). However, the value at 30h (2.8 μmol/g wet wt.) is much less than the expected contribution from milk (8.6 μmol/g wet wt.). These findings imply that different milk components are not recruited simultaneously in the process of milk secretion, and underline the difficulty of measuring retained milk from the tissue content of any one milk component.

Adenine nucleotides

These were assayed partly as a means of assessing the integrity of the tissue at the time of freeze-clamping. This was important in view of the impossibility of exposing undisturbed mammary tissue for clamping. The overall tissue contents (Table 1) were similar to those found by Baldwin & Cheng (1969) and the calculated intracellular concentrations are similar to those found by Gumaa *et al.* (1971). However, the degree of phosphorylation as expressed in the ATP/ADP, ATP/AMP and energy-charge ratios (Table 2) are higher than those found by the above authors (Table 4), and compare favourably with similar values from other tissues, including those that are more readily freeze-clamped (Table 4). Energy-charge values are especially useful in a comparison of this sort, since they are less dependent on the mass-action ratio of the adenylate kinase reaction, the equilibrium of which depends on the free Mg^{2+} concentration (Rose, 1968) and may in any case not always be attained (Ballard, 1970). The data for the heart may be distorted by the presence of ATP bound to myosin. We conclude that the mammary tissue was removed in a good state of adenine nucleotide phosphorylation.

The mass-action ratio of the adenylate kinase reaction, $[ATP][AMP]/[ADP]^2$, increases from 0.80 to 1.31 during lactogenesis (Table 2). This increase is greater than can be accounted for by a change in the apparent equilibrium constant with Mg^{2+} (Rose, 1968). We suggest that at 0h, when little synthetic activity is occurring, the reaction is close to equilibrium in the presence of 0.1–0.2mM free Mg^{2+}, and becomes displaced from equilibrium as increased synthetic activity generates AMP by means of pyrophosphorylytic reactions such as the activation of amino acids and fatty acids.

Lactate and pyruvate

The lactate/pyruvate ratio may be used to indicate the redox status of tissue cytosol when lactate dehydrogenase is present in high activity (Krebs & Veech, 1969). This appears to obtain for parturient mammary tissue, where the activity of this enzyme (approx. 40 μmol/min per g wet wt.; Gul & Dils, 1969) is high relative to the rate of glucose uptake (approx. 0.05–0.5 μmol/min per g wet wt.; A. R.

Table 3. *Composition of initial milk*

Values are means ± S.E.M. with numbers of rats in parentheses. The density of these milk samples was about 1.0.

Lactose (μmol/ml)	33.1 ± 0.7 (5)
P_i (μmol/ml)	28.6 ± 19.4 (4)
α-Lactalbumin (mg/ml)	1.46 ± 0.07 (4)
Water (g/ml)	0.522 ± 0.021 (5)

Table 4. *Comparison of published values for adenine nucleotide ratios and lactate/pyruvate ratios for different tissues of the rat or mouse*

	[ATP]/[ADP]	[ATP]/[AMP]	Energy charge	[Lactate]/[pyruvate]	Reference
Rat mammary gland (pregnant)	1.6	4.0	0.66	25	Baldwin & Cheng (1969)
Rat mammary gland (pregnant)	1.9	2.7	0.67	48	Gumaa *et al.* (1971)
Rat mammary gland (lactating)	—	—	—	27	D. H. Williamson (personal communication)
Rat liver	3.2	18.8	0.75	7.2	Start & Newsholme (1968)
Rat liver	2.1	8.2	0.78	12.1	Brosnan *et al.* (1970)
Rat kidney	2.7	9.1	0.84	—	Nishiitsutsuji *et al.* (1967)
Mouse brain	2.7	11.2	0.81	—	Lowry *et al.* (1964)
Rat uterus	2.0	23.7	0.81	—	Oliver & Kellie (1970)
Rat heart	4.8	26	0.89	—	Newsholme & Randle (1964)
Rat adipose tissue	3.0	1.3	0.50	25.6	Saggerson & Greenbaum (1970)

Elkin & N. J. Kuhn, unpublished work). Our lactate/pyruvate ratios compare favourably with those of others for mammary tissue but are 2–4-fold higher than those in liver (Table 4). It is not clear whether this represents a true difference between these tissues, or whether the mammary gland was slightly anaerobic at the time of sampling. The free-NAD^+/free-NADH ratio in mammary cytosol is calculated from these data to be 525–282 during lactogenesis. As was observed by Baldwin & Cheng (1969) the couple appears to become more reduced during lactogenesis, possibly reflecting an influence of insulin (Martin & Baldwin, 1971).

Hexokinase metabolites

The constant content of tissue glucose during experimentally induced lactogenesis (Table 1) agrees with our previous finding (Kuhn & Lowenstein, 1967), but not with that of Baldwin & Cheng (1969), during normal lactogenesis at parturition. It follows from the constancy of plasma glucose concentrations (Table 1) and the calculation that extracellular glucose accounts almost entirely for the tissue glucose. The difference is too small and variable to permit a useful estimation of intracellular glucose concentration which is, however, probably not more than 1mM. Combining this value with other data in Table 1 gives a mass-action ratio [glucose 6-phosphate][ADP]/[glucose][ATP] = 0.01, clearly far removed from the equilibrium value of 1550, at pH7 (Robbins & Boyer, 1957).

Phosphoglucomutase metabolites

The mass-action ratio [glucose 1-phosphate]/[glucose 6-phosphate] is 0.06–0.08, close to the equilibrium value of 0.06 (Colowick & Sutherland, 1942; Najjar, 1948).

UDP-glucose pyrophosphorylase metabolites

UTP contents (Table 2) were calculated from the sum of UTP+UDP measured in the tissue, and the UTP/UDP ratio which was assumed to equal the measured ATP/ADP ratio. This assumption is supported by the presence of a very active UDP-kinase in mammary tissue (see below). The tissue contents of UDP-glucose are severalfold lower than those reported by Baldwin & Cheng (1969). The tissue contents of PP_i give a calculated intracellular concentration of about 70μM, similar to the value of 50μM for perfused rat heart (Jefferson *et al.*, 1971) but far lower than the very high values reported for liver (Flodgaard, 1970), which we have been unable to confirm.

From these tissue contents the mass-action ratio [UDP-glucose][PP_i]/[UTP][glucose 1-phosphate] = 0.86–2.8 (Table 2). In a redetermination of the equilibrium constant in which equilibrium was approached from both sides by using pure UDP-glucose pyrophosphorylase of calf liver at pH7.0, 37°C and $I = 0.25$, the value of 0.41–0.44 was obtained over the range of free Mg^{2+} concentration of 0.09–0.9mM, as maintained by a Mg^{2+}–citrate buffer. All four reactants were determined by enzymic–spectrophotometric methods.

The discrepancy of the mass-action ratio from the equilibrium constant is in the wrong direction to be due to disequilibrium, and may be ascribable to errors in the determination of low tissue contents of glucose 1-phosphate and PP_i or to problems of cell compartmentation.

UDP-glucose epimerase metabolites

The tissue contents of UDP-glucose and UDP-galactose show an apparent slight rise and fall respectively as lactogenesis proceeds. The mass-action ratio [UDP-galactose]/[UDP-glucose] = 0.27 and 0.14 at 0 and 30h respectively. These compare with an equilibrium value of 0.29–0.32 (Maxwell *et al.*, 1962; Wilson & Hogness, 1964), implying that the reaction is very close to equilibrium at 0h, but moves into slight disequilibrium as lactogenesis proceeds.

Enzyme assays

Table 5 shows the activities of several enzymes of the lactose-biosynthetic pathway, and of glucose 6-phosphate dehydrogenase, in high-speed supernatants of mammary homogenates prepared at 0 and 30h after ovariectomy. The activity of hexokinase is about one-sixth of that reported by Gumaa *et al.* (1971), who used the same assay. Although it exceeds the rate of glucose uptake *in vitro* by mammary tissue from pregnant rats (about 0.05μmol/min per g wet wt.; A. R. Elkin & N. J. Kuhn, unpublished work), it only just exceeds the estimated rate of lactose synthesis at 30h. Since only a small further activity occurs in particulate fractions of mammary homogenates (Gumaa *et al.*, 1971) it appears that, for unknown reasons, our measurements underestimate the true activity.

The activities of phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-glucose epimerase and glucose 6-phosphate dehydrogenase were generally similar to those reported previously (Glock & McLean, 1954; Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967). Only UDP-glucose pyrophosphorylase increased markedly in activity (5-fold) during the period studied, so that these enzymes form a pattern similar to that at normal lactogenesis during parturition (Kuhn & Lowenstein, 1967).

Table 5. *Activities of enzymes in pregnant-rat mammary tissue 0 and 30h after ovariectomy*

For details see the Methods and Materials section. Values are means±S.E.M.

	Enzyme activity (μmol/min per g wet wt. at 30°C)	
Time after ovariectomy (h)	... 0	30
Hexokinase	0.12±0.01 (6)	0.14±0.03 (5)
Phosphoglucomutase	0.58±0.05 (6)	0.77±0.09 (5)
Nucleoside diphosphokinase	5.3* (6)	7.3* (5)
UDP-glucose pyrophosphorylase	0.14±0.02 (6)	0.70±0.14 (5)
UDP-glucose epimerase	0.10±0.01 (6)	0.17±0.01 (5)
Glucose 6-phosphate dehydrogenase	0.67±0.06 (6)	0.82±0.11 (5)

* S.E.M. not available.

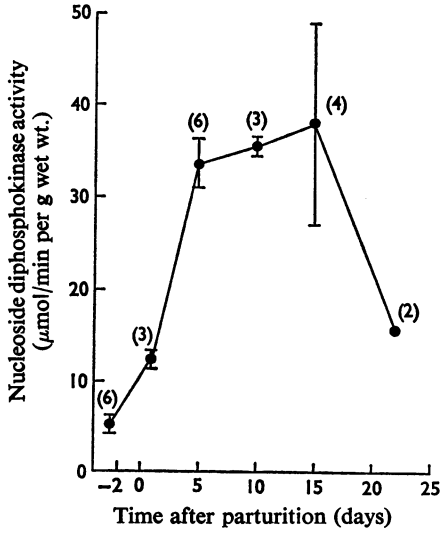


Fig. 3. *Increase in nucleoside diphosphokinase activity in mammary tissue during lactation*

Values are means±S.E.M. for the number of rats shown (except for day 22).

We observed very great nucleoside diphosphokinase activity, which has apparently not previously been studied in this tissue. It was assayed by an optical method in the direction of ATP formation (see the Methods and Materials section), where the optimum concentrations of reactants were UTP (2mM), ADP (0.1mM) and $MgCl_2$ (2mM). UTP, CTP and GTP (all at 1mM) gave relative rates of 100:44:8 respectively, and no attempt was made to establish whether more than one enzyme was involved. The formation of [^{32}P]UTP from [γ-^{32}P]-ATP (Glynn & Chappell, 1964) in the presence of UDP was demonstrated by paper chromatography (Krebs & Hems, 1953). Although the enzyme was studied mainly in high-speed supernatants of mammary homogenates, a roughly equivalent amount of nucleoside diphosphokinase activity was released from particulate fractions by freezing and thawing. The soluble enzyme activity changed little after ovariectomy but rose 7-fold during normal lactation (Fig. 3).

Lactose synthetase A protein showed significant activity in the late-pregnant rat, confirming previous observations in this species (Kuhn, 1968; McKenzie *et al.*, 1971) and in the mouse (Turkington *et al.*, 1968; Palmiter, 1969; McKenzie *et al.*, 1971; Jones, 1972). It rose 3.8-fold after ovariectomy (Fig. 4) to reach an activity of 0.15μmol/min per g wet wt. by 30h. This was largely prevented by progesterone administration at the time of ovariectomy.

α-Lactalbumin was present in both particulate and soluble fractions of homogenates, with the latter comprising about 90% of the total. Tissue contents remained extremely low for 13h after ovariectomy, and then rapidly increased some 28-fold by 30h (Fig. 5). These increases, which were visibly accelerating during part of the time, were largely prevented by progesterone administration at the time of ovariectomy. Similarly large increases have previously been noted during parturition in the rat and mouse (Turkington *et al.*, 1968; Palmiter, 1969; McKenzie *et al.*, 1971), although these measurements probably did not include the particulate material. Jones (1972) has suggested that the soluble α-lactalbumin of mammary homogenates may derive from material already secreted into the alveoli. By assuming that it occurs in the tissue in the same proportion to lactose as it does in first milk (Table 3), one could expect 438μg/g wet wt. of tissue, easily accounting for the 299μg/g wet wt. actually found at 30h and so supporting Jones' (1972) interpretation. We assume therefore that only the particulate α-lactalbumin participates in the lactose synthetase reaction, although it is

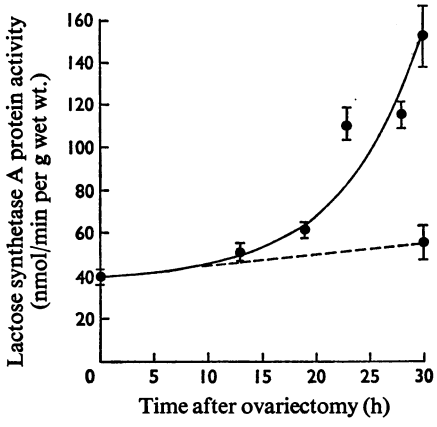


Fig. 4. *Increased tissue activity of lactose synthetase A protein after ovariectomy of pregnant rats*

The effect of progesterone administration at ovariectomy (----) is indicated. Values are means±S.E.M. for five or six rats.

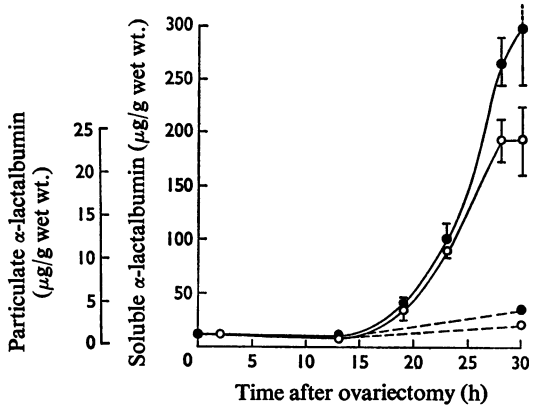


Fig. 5. *Increase in the soluble* (●) *and particulate* (○) *α-lactalbumin of mammary tissue from ovariectomized pregnant rats*

The effect of progesterone administration (----) at ovariectomy is indicated. Values are means ±S.E.M. for five rats.

possible that a portion of this was lost to the supernatant fraction during homogenization and isolation of the particles (see Jones, 1972).

Particulate lactose synthetase of mammary homogenates showed an affinity for glucose that was not changed by the addition of α-lactalbumin. Fig. 6 shows the results of six experiments in which the mean half-saturating concentration of glucose was 1.4mM.

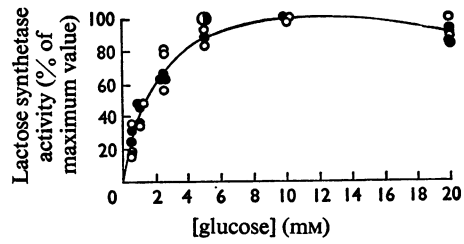


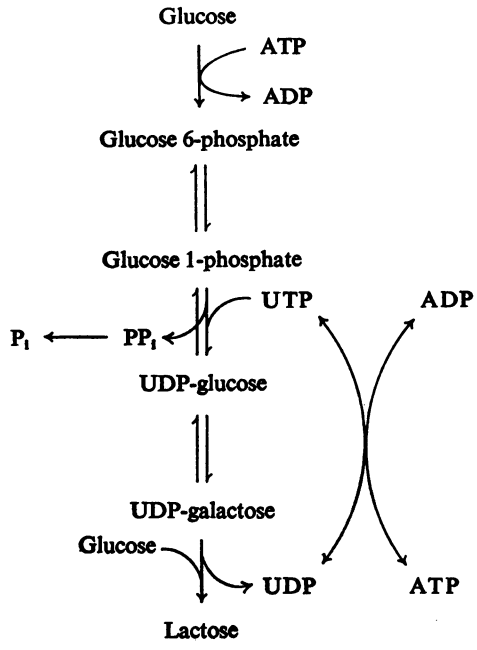
Fig. 6. *Effect of glucose concentration on the lactose synthetase activity of lactating rat mammary homogenates with* (○) *and without* (●) *added α-lactalbumin (2mg/ml)*

The data represent six experiments, and activities are expressed as percentages of the maximum activity found in each experiment.

Discussion

The information on metabolite contents and enzyme activities presented in this paper must be seen in relation to the approximately 126-fold increase in flux through the lactose-biosynthetic pathway during the period studied. Such an increase, which may be paralleled by similar increases in the fluxes through other biosynthetic pathways, has probably also taken place by the time of normal parturition in the rat.

Mammary tissue is not ideally suited for such analyses owing to the presence of tissue elements other than secretory epithelial cells. As has been discussed before, however (Rees, 1960; Kuhn & Lowenstein, 1967), the other main cell type is adipose which, because of its very low cytoplasmic content, probably contributes not more than 10% to the whole tissue metabolism and therefore, presumably, to the tissue enzymes and metabolites. Despite these drawbacks, and although we have made no attempt to account for subcellular compartmentation, the metabolite data indicate that the phosphoglucomutase, UDP-glucose pyrophosphorylase and UDP-glucose epimerase reactions are all very close to equilibrium whereas the hexokinase reaction and presumably the lactose synthetase reaction are far displaced from equilibrium. These conclusions are reinforced by the measured tissue activities of these enzymes which, relative to the activities of the pathways on which they lie, are high for those catalysing the proposed equilibrium reactions and low for hexokinase and lactose synthetase. In addition the very high activity of nucleoside diphosphokinase suggests that the UTP/UDP ratio is in close equilibrium with the ATP/ADP ratio. The pathway for lactose biosynthesis may therefore be depicted as in Scheme 1, where hexokinase and lactose synthetase catalyse rate-determining steps.



Scheme 1. *'Near-equilibrium' and 'disequilibrium' reactions on the pathway of lactose biosynthesis*

We find it of particular interest that UDP-glucose pyrophosphorylase appears to catalyse a reaction close to equilibrium. Discussions in the literature of this and of related enzymes have sometimes implied that the reaction is irreversible and far removed from equilibrium. The very low tissue contents of PP_i and the irreversible nature of its hydrolysis have been invoked in support (e.g. Stetten, 1960; Kornberg, 1962). It is presumably from this viewpoint that UDP-glucose pyrophosphorylase of calf liver (Albrecht *et al.*, 1966) and ADP-glucose pyrophosphorylase of *Escherichia coli* (Preiss *et al.*, 1965; Shen & Atkinson, 1970) have been studied with respect to their potential regulatory properties. Our data show, however, that mammary contents of PP_i are not lower than those of glucose 1-phosphate or UDP-galactose, and show a constancy in the face of large changes in the rate of PP_i generation indicative of a tightly regulated inorganic pyrophosphatase. The effective irreversibility of the pyrophosphorylase in this tissue stems not from any lack of PP_i but from the lack of alternative sources of UDP-glucose.

The tissue contents of reaction intermediates show a striking constancy during the large increase in flux through the pathway, implying an impressive homeostatic capacity of the tissue. This is due in part to the constant content of glucose 6-phosphate, control of which is poorly understood in mammary tissue, and partly to the constant energy charge of the adenine nucleotide system. Other authors have shown this constancy to extend even into full lactation (Baldwin & Cheng, 1969; Gumaa *et al.*, 1971).

It is clear that only the lactose synthetase components, A protein and α-lactalbumin, show changes in amount that might account for the increased flux, and the question arises as to whether they account for it completely. It appears that during pregnancy the activity of lactose synthetase is limited primarily by the extremely small amounts of α-lactalbumin. This is underlined by the 13h lag which precedes the increases of both lactose and α-lactalbumin after ovariectomy, and which is apparently not shown by the A protein. However, the overall 28-fold increase in particulate α-lactalbumin appears insufficient to explain the estimated 126-fold increase in the rate of lactose synthesis, and the 3.8-fold increase in A-protein activity is also likely to make a contribution. In the discussion that follows we accept the model, suggested mainly by Brew (1969), where soluble α-lactalbumin within the lumen of endoplasmic (possible Golgi) reticular vesicles interacts reversibly with membrane-bound A protein to form an enzymically active complex of 1:1 stoicheiometry in the manner shown by the purified, soluble, proteins of bovine milk (Klee & Klee, 1972):

$$A + B \rightleftharpoons AB$$

(B = α-lactalbumin). If it is assumed that rat A protein resembles that of the cow in having a molecular weight of 42000 and a specific activity of 14.1 μmol/min per mg (Trayer & Hill, 1971; Klee & Klee, 1972) and that rat α-lactalbumin resembles that of the cow and guinea pig in having a molecular weight of 15000 (Brew *et al.*, 1967; Brew & Campbell, 1967), then the particulate fraction of 1g of rat mammary gland at 0 and 30h after ovariectomy may be calculated to contain respectively 0.068 and 0.26nmol of A protein and 0.058 and 1.8nmol of α-lactalbumin. By comparing the estimated rates of lactose synthesis with the measured galactosyltransferase activities of the tissue A protein, one might expect the latter to be about 2 and 53% in the AB form at 0 and 30h respectively, although these values might be higher if the enzyme is not saturated with its substrates, as discussed below. It is seen that α-lactalbumin occurs in sufficient molar excess to permit this. In fact, the saturation of A protein by α-lactalbumin might change less than 26-fold if there were a simultaneous increase in the concentration of substrates. We have suggested 1mM as an upper limit to the intracellular concentration of glucose. This may be compared with the value of 1.4mM-glucose that was observed to give half-maximum activity of particulate rat mammary lactose synthetase (Fig. 6). The intracellular concentration of UDP-galactose calculated from data in

Tables 1 and 2 is 0.06–0.08mM, which compares with the half-saturating concentration of 0.06mM for the particulate enzyme (Kuhn, 1968). Thus it is likely that both substrates are non-saturating and that the enzyme activity is sensitive to changes in their concentrations.

From the above treatment of our experimental results, the rate-limiting step of the lactose-biosynthetic pathway is clearly the lactose synthetase reaction. Within this step α-lactalbumin is particularly rate-limiting, but A protein, UDP-galactose and possibly glucose are also partly rate-limiting once the α-lactalbumin has started to increase. Within the limits of accuracy of our data, changes in these factors are probably sufficient to explain the increased rate of lactose synthesis during lactogenesis. Insofar as progesterone withdrawal triggers lactogenesis its influence must be brought to bear primarily at this point on the pathway, though naturally it might also be responsible for the other, less critical, changes such as the increase of UDP-glucose pyrophosphorylase or the later increases of other enzymes. Our data also hint that UDP-glucose epimerase may begin to be partly rate-limiting at high flux rates along the pathway. In examining the role of α-lactalbumin during lactogenesis, Jones (1972) used an ingenious approach to determine its concentration within the membrane vesicles of mouse mammary homogenates. Unexpectedly, he found little change. We have preferred to measure quantities as opposed to concentrations of α-lactalbumin, since this avoids some of the assumptions inevitable in Jones' (1972) approach. However, both methods suffer from current ignorance of the exact nature of the A protein–α-lactalbumin interaction within these vesicles of the cell.

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