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Lactogenesis in the Rat

METABOLISM OF URIDINE DIPHOSPHATE GALACTOSE BY MAMMARY GLAND

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1. Lactose synthetase activity in the rat mammary gland increases during the last day of pregnancy from an essentially zero value. There is a parallel increase of tissue lactose and of glucose 6-phosphate dehydrogenase activity. 2. Mammary-gland homogenates prepared both before and after parturition hydrolyse the lactose precursors glucose 6-phosphate, glucose 1-phosphate, UDP-glucose, UDP-galactose and also maltose, but not lactose. 3. A role of lactose synthetase as the rate-limiting enzyme for lactose biosynthesis and the possible significance of the hydrolytic activities are discussed with respect to lactogenesis.

At the time of parturition the mammary glands of adult primiparous rats contain about 12 μmoles of lactose/g. fresh wt. of tissue, whereas none is present 24hr. previously (Kuhn & Lowenstein, 1967). Although this finding illustrates the sudden initiation of lactation, the magnitude and the timing of the increases that occur at this time in the activity of three enzymes on the pathway of lactose biosynthesis, namely phosphoglucomutase (EC 2.7.5.1), UDP-glucose pyrophosphorylase (EC 2.7.7.9) and UDP-glucose 4-epimerase (EC 5.1.3.2), do not appear sufficient to account for it. Further, all these enzymes are appreciably active in the tissue before parturition (Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967) at a time when lactose is apparently not being synthesized.

The present paper deals with the activity of lactose synthetase (uridine diphosphate D-galactose–D-glucose 1-D-galactosyltransferase, EC 2.4.1.22), which catalyses the reaction:

$$\text{UDP-D-galactose} + \text{D-glucose} \rightarrow \text{UDP} + \text{lactose}$$

The activity of this enzyme, which is the final enzyme on the pathway of lactose biosynthesis (Watkins & Hassid, 1962; Babad & Hassid, 1966), is shown to be virtually zero in homogenates of rat mammary tissue until just before parturition, and thereafter remains rate-limiting on the pathway. Observations are also presented on the ability of tissue homogenates to hydrolyse UDP-galactose and other hexose derivatives.

METHODS

Primiparous Sprague–Dawley rats were obtained from the Charles River Breeding Laboratories, Wilmington, Mass., U.S.A. For the measurement of enzyme activities over the period of parturition, 50 rats mated simultaneously over a 24hr. period were used. Of these, 30 were allowed to go to term and the exact time of parturition was noted for each (28 of these gave birth within a period of 8hr.). These rats were subsequently killed at known times thereafter. The mean time of parturition of these rats was used to establish the stage of pregnancy of the remaining 20 rats, which were killed before parturition.

Assay of lactose synthetase and UDP-galactose-hydrolysing activity. Incubation of mammary-gland homogenates with UDP-[^{14}C]galactose in the presence of glucose results in the release of radioactive free lactose and galactose as a result of lactose synthetase and UDP-galactose-hydrolysing activity respectively. The combined formation of these sugars was followed by a method adapted from Babad & Hassid (1966), with anion-exchange resin used to separate them from unchanged UDP-[^{14}C]galactose.

Tissue homogenates were prepared from chopped mammary gland in 4 vol. of ice-cold 0·25M-sucrose or 1·15% (w/v) KCl in a glass homogenizer with a motor-driven glass pestle. The standard assay mixture contained homogenate (10 μl.), $MnCl_2$ (0·665 μmole), glucose (none or 2 μmoles), UDP-[^{14}C]-galactose (0·025 μmole, 10000 counts/min.) and dimethylglutaric acid–NaOH buffer, pH 7·4 (2·5 μmoles), in a final volume of 50 μl. Each reaction was carried out in a Pyrex glass tube (internal dimensions 0·4cm. × 3·0cm.) at 37° for 10min. It was stopped by heating at 95° in a water bath for 100sec. or, on occasion, by chilling in ice. After dilution to 0·1ml. with water, the reaction mixture was transferred with a Pasteur pipette to a column containing approx. 0·5ml. of wet Dowex 1 (formate form; 100–200 mesh) resin in a cut-off Pasteur pipette (0·5cm. internal diam.). The reaction tube was rinsed out twice with 0·1ml. of water. After the reaction mixture and washings had sunk into the resin, the neutral sugars on the column were eluted with 1·0ml. of water into a glass scintillation vial, the UDP-galactose being retained on the column. The radioactivity of the eluate was determined in a refrigerated scintillation counter (Ansitron–Picker X-ray Corp., White Plains, N.Y., U.S.A.) in the presence of 20ml. of dioxan solution containing 7g. of 2,5-diphenyloxazole, 0·3g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 100g. of naphthalene/l.

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(essentially as described by Langham, Eversole, Hayes & Trujillo, 1956). Quenching of counting efficiency by the water was negligible.

In separate experiments it was found that [^{14}C]glucose was quantitatively recovered in this procedure. Control tubes were run in which reaction mixtures containing UDP-[^{14}C]galactose, but no enzyme, were applied to the resin and the amount of eluted radioactivity was determined. This represented about 1% of the radioactivity applied to the resin except at low concentrations of UDP-galactose (25 μM), where it rose to 10%. The appropriate correction was applied in all cases. Because such correction was relatively large when enzyme activity was measured at low concentrations of UDP-galactose, only an approximate value for the K_m of this substrate can be determined from the data in Fig. 2.

Lactose synthetase activity was calculated as the difference between the UDP-galactose-hydrolysing activity (glucose absent) and the combined UDP-galactose-hydrolysing and lactose synthetase activities (glucose present). Apart from the presence or absence of glucose, reaction mixtures were identical, except in the experiment described in Fig. 3, where the UDP-galactose-hydrolysing activity was measured only in the absence of added α-lactalbumin. In a separate experiment the UDP-galactose-hydrolysing activity in the presence of 0·3 mg. of α-lactalbumin/ml. was 86% of the activity in its absence. Therefore the measured stimulation of lactose synthetase by α-lactalbumin may be less than its true value.

Paper chromatography of sugars. Neutralized $HClO_4$ extracts of glands (Kuhn & Lowenstein, 1967) and reaction mixtures were deionized by passage through a column containing a bed of Dowex 50 (H^+ form) resin superimposed on a bed of Dowex 1 (CO_3^{2-} form) resin. The eluates were freeze-dried and dissolved in a convenient volume of water. Carrier sugars were added when necessary and the mixture was applied to Whatman no. 1 paper. The sugars were separated by ascending or descending chromatography with either the upper phase of ethyl acetate–pyridine–water (2:1:2, by vol.) (Jermyn & Isherwood, 1949), or propan-1-ol–ethyl acetate–water (7:1:2, by vol.) (Baar & Bull, 1953). The complete chromatograms were dried in air, and the sugars made visible by spraying the paper with 4% diphenylamine in ethanol–4% aniline in ethanol–syrupy phosphoric acid (5:5:1, by vol.), followed by heating for 5 min. at 80°. Radiochromatograms were scanned in a Tracerlab 4π scanner.

Determination of tissue sugars. Glucose was determined enzymically by the hexokinase method (Slein, 1963) or the glucose oxidase method (Huggett & Nixon, 1957) as indicated. Lactose was determined on unconcentrated extracts of mammary glands (experiment described in Fig. 3) by the method of Kuhn & Lowenstein (1967). Determination of lactose in a highly concentrated deionized tissue extract was performed after removal of over 90% of the glucose by the charcoal technique of Whistler & Durso (1950).

Hydrolysis of glucose derivatives. Release of glucose from a variety of glucose derivatives was measured enzymically. Each reaction mixture contained homogenate (0·2 ml.), glycylglycine–NaOH buffer, pH 7·4 (25 μmoles), glucose derivative (1·0 or 10 μmoles) and water in a final volume of 1·0 ml. After incubation at 37° for 30 min., the reaction mixture was deproteinized with $Ba(OH)_2$ and $ZnSO_4$ and assayed for glucose (Slein, 1963). Controls were run in the absence of the added glucose derivative, and for one derivative (maltose) correction was made for the presence of contaminating glucose.

Materials. UDP-galactose was purchased from Calbiochem, Los Angeles, Calif., U.S.A., and was homogeneous by paper chromatography in the solvent ethanol–M-ammonium acetate, pH 3·8 (2:1, v/v). UDP-[^{14}C]galactose was supplied by New England Nuclear Corp., Boston, Mass., U.S.A., and gave a single radioactive peak when chromatographed in the same system. Hexokinase (type III), glucose 6-phosphate dehydrogenase (type VI), glucose oxidase (type V), peroxidase and $NADP^+$ were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Purified bovine α-lactalbumin was generously provided by Dr F. M. Robbins and Dr M. J. Kronman of the U.S. Army Natick Laboratories, Natick, Mass., U.S.A.

RESULTS

Metabolism of UDP-galactose by mammary-gland homogenates

Preparations of lactating mammary gland metabolize UDP-[^{14}C]galactose in the presence of glucose to give radioactive free lactose and hexose (Watkins & Hassid, 1962; Brodbeck & Ebner, 1966*b*). The combined reactions can be conveniently followed by passing the incubation mixture over a bed of anion-exchange resin, which retains unchanged UDP-[^{14}C]galactose, but allows the un-ionized sugars to pass through. Table 1 shows the requirements of this system, when the homogenate was prepared from a rat mammary gland after about 2 weeks of lactation. In agreement with the findings of Watkins & Hassid (1962) on guinea-pig mammary gland, and of Babad & Hassid (1966) on a preparation from cow's milk, there is a requirement for glucose that is not replaceable by

Table 1. *Requirement for glucose and manganous chloride for the release of* ^{14}C*-labelled sugar from UDP-*[^{14}C]*galactose*

The complete reaction mixture contained sodium dimethylglutarate buffer, pH 7·4 (5 μmoles), glucose (2 μmoles), $MnCl_2$ (1·3 μmoles), UDP-[^{14}C]galactose (0·15 μmole, 30 000 counts/min.), tissue homogenate (equivalent to 5·4 mg. fresh wt. of tissue) and, where indicated, glucose 1-phosphate (2 μmoles) and $MgCl_2$ (1·5 μmoles). The final volume was 0·1 ml. Incubations were carried out at 37° for 15 min.

Reaction mixture	Sugar released (mμmoles)
Complete	23·8
Complete − glucose	3·2
Complete − glucose + glucose 1-phosphate	1·6
Complete − $MnCl_2$	11·3
Complete − $MnCl_2$ + $MgCl_2$	16·4

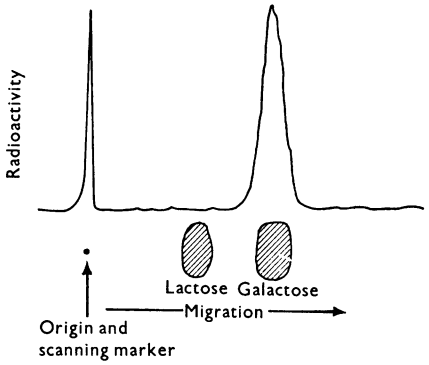

Fig. 1. Radiochromatogram showing galactose as the sole sugar released by the action of mammary-gland homogenates on UDP-[^{14}C]galactose in the absence of added glucose. The tissue homogenate (25μl.) was incubated under standard conditions, but for 40min.

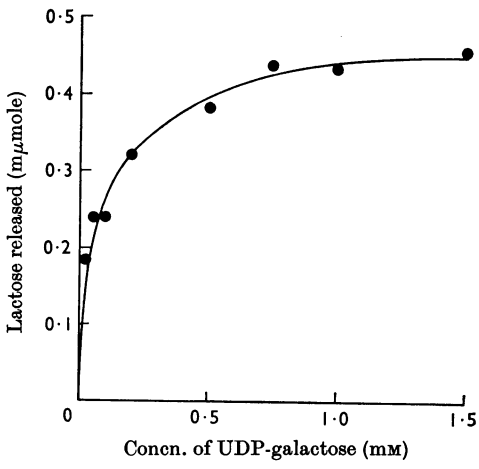

Fig. 2. Effect of UDP-galactose concentration on the amount of lactose formed. The homogenate was prepared from a rat 2–3 weeks after parturition, and was diluted 40-fold before use.

glucose 1-phosphate. Manganous chloride also stimulates the formation of sugar, and can be partially replaced by magnesium chloride. Identification of the free sugars as [^{14}C]lactose and [^{14}C]galactose was carried out in the present study by means of paper chromatography (results not shown), and no other radioactive sugar was detected.

When UDP-[^{14}C]galactose is incubated with preparations of lactating mammary gland in the absence of glucose, however, only [^{14}C]galactose is formed (Fig. 1). Further, the formation of this sugar is not significantly affected by the presence or absence of glucose. Thus in an experiment where the [^{14}C]galactose formed under standard conditions was separated from lactose by retention of the latter on charcoal (Whistler & Durso, 1950), it contained 3286counts/min. and 3278counts/min. after incubations in the absence and presence of 40mM-glucose respectively (each value a mean of three determinations). This formation of galactose presumably represents the action of one or more hydrolytic enzymes on UDP-galactose. This hydrolytic activity may therefore be simply assayed in crude homogenates of mammary gland by determining the amount of radioactive sugar formed from UDP-[^{14}C]galactose in the absence of glucose. The extra radioactive sugar formed in the presence of glucose represents the formation of lactose by lactose synthetase.

The UDP-galactose-hydrolysing activity does not require added metal ion, and is depressed by only 20% or less in the presence of 40% (v/v) methanol, ethanol, ethylene glycol or acetone.

Kinetics of lactose synthetase and UDP-galactose-hydrolysing activities. The amounts of lactose and of galactose formed increase linearly over an incubation period of 30min. and over a fivefold range of protein concentration (data not given in detail). The combined amount of these sugars was not allowed to exceed 25% of the added UDP-galactose. The Michaelis plot for lactose synthetase activity obtained by varying the concentration of UDP-galactose is given in Fig. 2. Although a reciprocal plot of the data does not give a straight line, possibly because values at low concentration are not very accurate (see the Methods section) the curve shows a half-maximum rate at about 0·06mM-UDP-galactose. A K_m value of 0·5mM has been reported for the enzyme of cow's milk (Babad & Hassid, 1966).

The effect of varying the UDP-galactose concentration on the UDP-galactose-hydrolysing activity has not given consistent results, possibly because one is here not dealing with a single enzyme. The affinity for the substrate is comparable with that of lactose synthetase, but the results do not coincide with a hyperbolic curve.

Appearance of lactose synthetase activity at parturition

Preparations of mammary gland taken from rats before parturition resemble those taken during lactation in acting on UDP-galactose to release a sugar, identified as galactose by paper chromatography. In contrast, however, there is no increase in the amount of radioactive sugar formed when glucose is added to the reaction mixture. These findings are shown in Fig. 3, where, in a study of the relative activities of lactose synthetase and of UDP-galactose hydrolysis, groups of five rats each

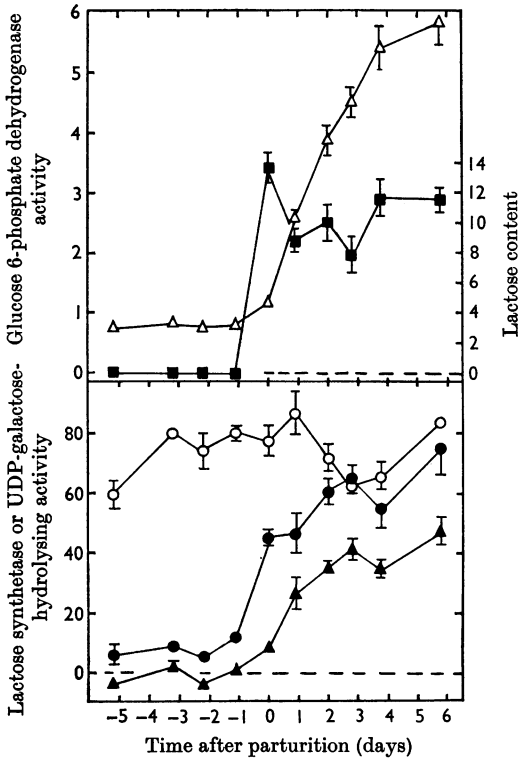

Fig. 3. Metabolic changes in mammary tissue during parturition: ▲, lactose synthetase activity (mμmoles of lactose formed/mg. of DNA/min.); ●, lactose synthetase activity assayed with added α-lactalbumin (0·3mg./ml.); ○, UDP-galactose-hydrolysing activity (mμmoles of galactose formed/mg. of DNA/min.); △, glucose 6-phosphate dehydrogenase activity (μmoles of NADHP formed/mg. of DNA/min.); ■, lactose content (μmoles/g. fresh wt.). Each point represents a mean value obtained from five rats. Vertical bars show S.E.M. values; where no bars are present, they lie within the area of the symbol.

were sampled at known times before, at and after parturition. The mammary tissue of each rat was analysed for lactose, DNA, lactose synthetase, UDP-galactose-hydrolysing activity and glucose 6-phosphate dehydrogenase. The results given in Fig. 3 show that the activity of lactose synthetase in the absence of added α-lactalbumin is essentially zero until about 24hr. before parturition, and increases from that time. α-Lactalbumin has been shown to be a component of the lactose synthetase of cow's milk (Brodbeck & Ebner, 1966*a*) and of mammary gland (Ebner, Denton & Brodbeck, 1966). When purified bovine α-lactalbumin was added to the assay medium, a greater activity of lactose synthetase was observed at all times, including a low, but definite, activity during the

Table 2. *Hydrolysis of hexose derivatives by homogenates of mammary gland from pregnant and lactating rats*

Each column contains the measurements made on tissue from a single rat, killed 3 days before (pregnant) or 8 days after (lactating) parturition. Details are given in the Methods section.

Hexose derivative	Activity (mμmoles of substrate hydrolysed/mg. of DNA/min.)			
	Pregnant		Lactating	
Glucose 6-phosphate (1mM)	40·8	56·3	18·4	21·4
Glucose 1-phosphate (1mM)	76·3	106	23·1	28·1
UDP-glucose (1mM)	43·6	61·0	9·2	9·5
Lactose (1mM)	0·0	1·3	0·0	0·0
Lactose (10mM)	2·7	4·3	1·4	1·0
Maltose (1mM)	33·2	34·2	21·5	22·7
Maltose (10mM)	36·0	—	21·0	25·5

days before parturition. There was maximum stimulation at the concentration used (0·3mg./ml.).

The results for glucose 6-phosphate dehydrogenase, which were closely comparable with those obtained by Kuhn & Lowenstein (1967), followed a similar pattern to that observed with lactose synthetase except that the enzyme shows appreciable activity before parturition. With the routine assay used in these experiments lactose was not detected until parturition, at which time it was present to the extent of 13·7 μmoles/g. fresh wt. of tissue.

UDP-galactose-hydrolysing activity does not show the same pattern of change. The activity is similar before and after parturition, and shows no relationship to the kinetics of lactose appearance. Its activity can be exceeded by that of lactose synthetase at later stages of lactation (e.g. in Table 1).

Hydrolysis of hexose derivatives. In view of their ability to hydrolyse UDP-galactose, homogenates of mammary gland were examined for their ability to hydrolyse other hexose derivatives. Table 2 shows that glands from both pregnant and lactating rats can hydrolyse glucose 6-phosphate, glucose 1-phosphate, UDP-glucose and maltose at rates comparable with that for UDP-galactose. Lactose, however, is virtually unaffected even at high concentrations.

Isolation of sugar from mammary glands of pregnant rats. The sugars present in mammary glands taken from rats 3 days before parturition were examined by enzymic and chromatographic means. The inguinal glands of five rats (total of 30·7g.) were extracted with 5% (w/v) perchloric acid, the combined neutralized extract was

deionized and concentrated 250-fold, and portions were chromatographed on paper with propan-1-ol–ethyl acetate–water (7:1:2, by vol.). The tissue extract was found to contain glucose and lactose, but no galactose. In addition there was an unidentified spot with R_{Glc} 1·5. Enzymic determination of glucose and of lactose in the pooled extract gave values of 0·65 and 0·091 μmole/g. fresh wt. of tissue respectively.

DISCUSSION

The application of a specific and quantitative method for the determination of tissue lactose, one of the major components of milk, has shown that the time of its appearance in the mammary gland at the onset of lactation is sharply defined. The mapping of tissue amounts of enzymes and metabolites concerned in lactose formation near the critical time of parturition (Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967) has shown a rather general increase in these amounts at, or close to, parturition. Up to the present time, however, all the biosynthetic machinery for the formation of lactose and other milk products that has been examined has proved to be present in the gland in appreciable quantities before parturition. This has opened up the question as to why lactose should be absent, or virtually so, during pregnancy. The negligible lactase activity found in this study makes it unlikely that the absence of lactose is due to its rapid destruction. Although the enzymes that are present before parturition might derive from cells other than those of the secretory alveoli, it has been shown that they probably do not derive from adipose cells, despite the fact that adipose tissue makes up 50% (w/w) of the mammary gland before parturition (Rees, 1960; Kuhn & Lowenstein, 1967).

Lactose synthetase is an enzyme exclusively concerned with the synthesis of lactose, which is in turn a substance unique to milk. Mammary cells not directly concerned with lactose synthesis should therefore not contribute to the measured enzymic activity of the whole tissue. The extremely low activity of this enzyme before parturition (Fig. 3) provides the first reasonable explanation for the virtual absence of lactose at this time. Fig. 3 shows that lactose synthetase begins to appear during the last day of pregnancy, simultaneous with the appearance of tissue lactose. The mean activity of lactose synthetase during this day, measured in the absence of added α-lactalbumin, is 12 mμmoles/mg. of DNA/min., which could account for the formation of 39·4 μmoles of lactose/g. fresh wt. during the day. Its activity is therefore sufficient to account for the 13·7 μmoles of lactose/g. fresh wt. that are actually found at the end of this day. A comparison of the activity of lactose synthetase with that of other enzymes on the pathway of lactose biosynthesis (Malpress, 1961; Shatton, Gruenstein, Shay & Weinhouse, 1965; Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967) indicates that it is rate-limiting. The mean enzyme activity on the sixth day of lactation is sufficient to account for the daily formation of about 36 g. of milk, taking the total weight of mammary tissue as 15 g., the DNA content as 2·6 mg./g. fresh wt. (Kuhn & Lowenstein, 1967) and the lactose content of milk as 2·8% (Cox & Mueller, 1937). This probably exceeds the actual milk yield at this time, since the data of Grosvenor & Turner (1959) give a daily yield of 38 g. on the fourteenth day of lactation.

Data on the activity of glucose 6-phosphate dehydrogenase before and after parturition are presented for comparison, this enzyme being used as a marker for the large group of enzymes that increase in activity at parturition (Baldwin & Milligan, 1966). The increase in activity, originally demonstrated by Glock & McLean (1954), shows the same timing as that for lactose synthetase, suggesting that these two enzymes may respond to a common stimulus.

Fig. 3 shows that the activity of lactose synthetase *in vitro* is increased in the presence of added bovine α-lactalbumin, as originally reported by Ebner *et al.* (1966), and that a small but definite activity is found in tissue from pregnant rats under these conditions. Although it is not clear whether the stimulated or unstimulated activity should be taken as characteristic of the tissue activity, the finding of traces of lactose in mammary gland before parturition (0·091 μmole/g. fresh wt.) implies that the activity of the synthetase at this time is not completely absent. Lactose has also been reported to be formed before parturition in the cow (Wheelock & Rook, 1966), and a slow incorporation of [^{32}P]-phosphate into casein has been found in the mammary gland of the pregnant mouse (Turkington, Juergens & Topper, 1965).

The presence of UDP-galactose-hydrolysing activity in mammary gland, first indicated by Watkins & Hassid (1962), raises the question of its physiological role. Several observations suggest that this hydrolytic activity is not very high in the intact tissue. First, although its measured activity greatly exceeds that of lactose synthetase in mammary homogenates of the parturient rat, the continuing formation of lactose in the intact tissue at this time suggests that an adequate pool of UDP-galactose is still being maintained. Secondly, the data in Table 2 show that several other hexose derivatives are hydrolysed at similar rates. These include all the other precursors of lactose, and it is improbable that material is thus drained away from every step in a major biosynthetic pathway.

Finally, no galactose was detected by the chromatography of highly concentrated extracts of tissue taken from rats a few days before parturition. Lactose was detected in these extracts, indicating the presence of some UDP-galactose, although the lactose synthetase activity is less than 10% that of the UDP-galactose hydrolysis at this time. It therefore seems likely that the activities of these hydrolytic enzymes are masked in the intact tissue, possibly by spatial separation from their substrates or through the presence of inhibitory substances. The latter explanation is supported by the ability of UTP to inhibit the hydrolysis of UDP-galactose by mammary gland preparations *in vitro* (Watkins & Hassid, 1962). The detailed metabolic pathways by which these precursors of lactose are degraded *in vitro* have not been examined in this study. The inability of mammary gland to hydrolyse lactose is striking, especially in view of the hydrolysis of maltose.

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REFERENCES

Baar, S. & Bull, J. P. (1953). *Nature, Lond.*, **172**, 414.

Babad, H. & Hassid, W. Z. (1966). *J. biol. Chem.* **241**, 2672.

Baldwin, R. L. & Milligan, L. P. (1966). *J. biol. Chem.* **241**, 2058.

Brodbeck, U. & Ebner, K. E. (1966*a*). *J. biol. Chem.* **241**, 762.

Brodbeck, U. & Ebner, K. E. (1966*b*). *J. biol. Chem.* **241**, 5526.

Cox, W. M. & Mueller, A. J. (1937). *J. Nutr.* **13**, 249.

Ebner, K. E., Denton, W. L. & Brodbeck, U. (1966). *Biochem. biophys. Res. Commun.* **24**, 232.

Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.

Grosvenor, C. E. & Turner, C. W. (1959). *Endocrinology*, **101**, 699.

Huggett, A. St G. & Nixon, D. A. (1957). *Biochem. J.* **66**, 12P.

Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.

Kuhn, N. J. & Lowenstein, J. M. (1967). *Biochem. J.* **105**, 995.

Langham, W. H., Eversole, W. J., Hayes, F. N. & Trujillo, T. T. (1956). *J. Lab. clin. Med.* **47**, 819.

Malpress, F. M. (1961). *Biochem. J.* **78**, 527.

Rees, E. D. (1960). *Amer. J. Physiol.* **199**, 1067.

Shatton, J. B., Gruenstein, M., Shay, H. & Weinhouse, S. (1965). *J. biol. Chem.* **240**, 22.

Slein, M. W. (1963). In *Methods of Enzymatic Analysis*, p. 117. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.

Turkington, R. W., Juergens, W. G. & Topper, Y. J. (1965). *Biochim. biophys. Acta*, **111**, 573.

Watkins, W. M. & Hassid, W. Z. (1962). *J. biol. Chem.* **237**, 1432.

Wheelock, J. V. & Rook, J. A. F. (1966). *J. Dairy Res.* **33**, 37.

Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.