*Biochem. J.* (1969) **113**, 409
*Printed in Great Britain*



# Hormonal Induction and Regulation of Lactose Synthetase in Mouse Mammary Gland

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(*Received* 7 *January* 1969)

The augmentation of lactose synthetase activity during late pregnancy and lactation was measured by using both a tissue-culture assay and a cell-free assay. The results indicated at least a 100-fold augmentation in specific activity between late pregnancy and lactation. The cell-free assay indicated that the activities of both subunits of this enzyme had increased to 20–30% of the value during lactation by the last day of pregnancy. The tissue-culture assay, however, showed activities only 3–4% of the maximum at the time of parturition. This suggests that not all the enzyme present in the tissue before lactation commenced was active. Since at all stages of pregnancy and lactation the B subunit, α-lactalbumin (which is also a milk protein), was rate-limiting, it is suggested that the rate of lactose synthesis may be linked to the rate of milk-protein synthesis. Both subunits of lactose synthetase could be induced in tissue culture by the hormones insulin + hydrocortisone + prolactin. Of the three hormones, prolactin appeared to be the 'trigger' that induced the synthesis of these proteins if the tissue had been stimulated previously by insulin + hydrocortisone.

The induction of lactose synthesis in the adult mammary gland is an example of hormonal control of macromolecular synthesis and serves as a model for the study of cytodifferentiation.

Lactose is synthesized only in the mammary gland in mammals. Its synthesis is catalysed by the enzyme lactose synthetase (EC 2.4.1.22), which transfers galactose from UDP-galactose to glucose (Watkins & Hassid, 1962; Bartley, Abraham & Chaikoff, 1966). The substrate, UDP-galactose, is synthesized from either glucose or galactose, as illustrated in Scheme 1.

The discovery by Brodbeck & Ebner (1966*a*) that lactose synthetase is composed of two subunits, A and B (both of which are required for enzymic activity), raises the possibility that lactose synthetase activity in the mammary gland may be regulated by varying the concentration of either or both of the subunits. The present paper gives evidence that the specific activity of both subunits increases over 100-fold during late pregnancy and lactation. It also demonstrates that at all stages the activity of lactose synthetase is limited by the availability of the B subunit. The B subunit is identical with one of the common milk proteins, α-lactalbumin (Brodbeck, Denton, Tanahashi & Ebner, 1967). Thus the synthesis of the major carbohydrate of milk may be linked to the synthesis of milk proteins.

The present paper demonstrates that the augmentation of lactose synthetase activity is mediated by the same hormones that Juergens, Stockdale, Topper & Elias (1965) and Lockwood, Turkington & Topper (1966) have shown are necessary for the differentiation of this tissue.

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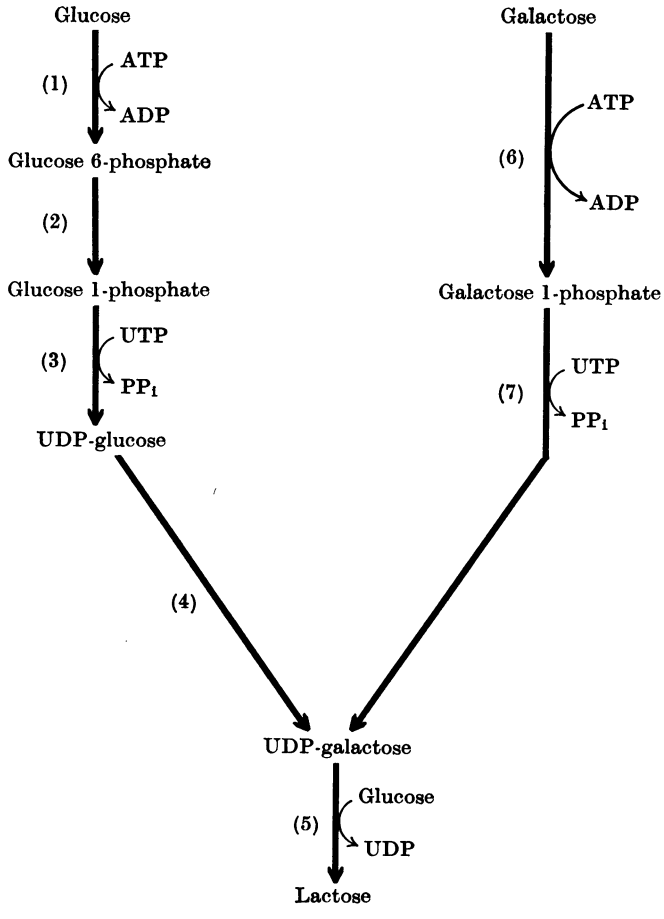
## MATERIALS AND METHODS

*Culture.* First-pregnancy (mid-to-late) BALB/C mice were killed by cervical dislocation and the anterior inguinal mammary glands were removed and placed in sterile Hanks salt solution. Lymph nodes and superficial connective tissues were discarded and the mammary glands were cut into pieces of wet wt. 0·5–1·0mg. These pieces of tissue float on the incubation medium because of their high content of fat cells. Medium 199 in Hanks salts (Grand Island Biological, Oakland, Calif., U.S.A.) was used with the addition of penicillin (50i.u./ml.), streptomycin (50μg./ml.), Fungizone (2·5μg./ml.), glutamine (292μg./ml.) and $NaHCO_3$ (375μg./ml.). Hormone stock solutions were made up as follows: insulin (Eli Lilly and Co., Indianapolis, Ind., U.S.A.) (2·5mg./ml. in 5mM-HCl), prolactin (National Institutes of Health, Bethesda, Md., U.S.A.) (2·5mg./ml. in 0·8% NaCl) and hydrocortisone alcohol (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) (8mg./ml. in ethanol). Each hormone was diluted to a final concentration of 5μg./ml. of culture medium before use. All culture media




Scheme 1. Pathways of lactose synthesis. Enzymes: (1) hexokinase (EC 2.7.1.1); (2) phosphoglucomutase (EC 2.7.5.1); (3) UDP-glucose pyrophosphorylase (EC 2.7.7.9); (4) UDP-glucose 4-epimerase (EC 5.1.3.2) or hexose 1-phosphate uridylyltransferase (EC 2.7.7.12); (5) lactose synthetase (EC 2.4.1.22); (6) galactokinase (EC 2.7.1.6); (7) galactose 1-phosphate uridylyltransferase (EC 2.7.7.10) or hexose 1-phosphate uridylyltransferase (EC 2.7.7.12.)

were sterilized by filtration through a 0·45 $\mu$m. Nalgene filter unit before being added to sterile plastic Petri dishes. Medium was changed at approx. 30 hr. intervals. Incubation was at 37°, with air and $CO_2$ adjusted to maintain the pH of the media between 7·0 and 7·5.

*Tissue-culture assay of lactose synthetase.* Mammary tissue was prepared as described above. The cultures consisted of five to ten pieces of tissue (0·5–1·0 mg. wet wt. each) in 1 ml. of medium 199 without hormones. Each culture was labelled with 0·5 $\mu$c (3·05 mc/m-mole) of [$^{14}$C]galactose (New England Nuclear Corp., Boston, Mass., U.S.A.) for up to 5 hr. Incorporation was linear for this period. The cultures were terminated by floating the plastic culture dishes on a 90° water bath for 10 min. The medium was removed and saved. The tissue and dish were washed once with 1 ml. of water and the wash was combined with the medium. The tissue was weighed and the weight corrected for the loss due to heating. The medium and washes were deproteinized and defatted by shaking with an equal volume of chloroform. The aqueous phase was removed and the chloroform layer washed with another 1 ml. of water. The media and washes were treated by shaking the sample with 200 mg. of AG 501 (X8; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) mixed ion-exchange resin (this process removes over 95% of the material absorbing at 260 and 280 nm.). The resin was then separated by a basket-centrifuge technique. The sample was freeze-dried and redissolved in 100 $\mu$l. of water. Three 3 $\mu$l. portions of sample and one 3 $\mu$l. portion of carrier galactose and lactose were spotted on Whatman no. 1 chromatography paper. Lactose was separated from galactose by using as solvent water–propan-2-ol (1:4, v/v). After 16–20 hr. of ascending chromatography, galactose had migrated 8–10 cm. The migration of lactose relative to galactose was $R_{Gal}$ 0·44.

The chromatograms were stained by dipping them into diphenylamine–aniline reagent (1g. of diphenylamine, 1ml. of aniline, 10ml. of phosphoric acid and 100ml. of acetone) and were then dried in an oven at 95–105° for 5–10min. The galactose and lactose spots were cut out and placed in scintillation vials with 0·2ml. of water followed by 10ml. of Bray's (1960) solution. The counting efficiency was 80%. The percentage of the total radioactivity in the lactose spot was calculated. That percentage was then multiplied by the input of [$^{14}$C]galactose (0·0285mg.) to give the amount (in milligrams) of lactose synthesized. This was then converted into nmoles. A 10mg. portion of lactating tissue converted about 20% of the radioactive galactose into lactose in 4hr.

Recovery of [$^{14}$C]lactose and [$^{14}$C]galactose by the isolation procedure was 80–90%. A major problem with the technique was that a small amount (0·3–0·5%) of the faster-migrating galactose always trailed into the lactose spot. Controls without tissue were therefore run through the complete isolation procedure to measure background lactose radioactivity. This high background made the assay relatively insensitive.

The product of the reaction was identified as lactose by chromatography in several solvent systems by paper chromatography and t.l.c., by both one- and two-dimensional techniques. The product was neutral since it was not lost during the deionization step. It had the property of binding to activated charcoal (Darco G60) and being released by ethanol (monosaccharides do not bind to Darco G60) (Whistler & Durso, 1950). It was also cleaved by $\beta$-galactosidase (EC 3.2.1.23) into a moiety that migrated like galactose.

*RNA and protein synthesis in mammary-gland cultures.* Cultured tissues were doubly labelled with a $^{14}$C-labelled amino acid mixture (New England Nuclear Corp.) and [$^{3}$H]uridine (48·5mc/mg.) (New England Nuclear Corp.). Incorporation was terminated by blotting the tissue, weighing it and then placing it in cold acetone. It was then washed twice with cold 5% trichloroacetic acid and twice with ethanol–ether (3:1, v/v). The acetone and the washes were pooled and extracted extensively with diethyl ether to remove the trichloroacetic acid, and then the radioactivity was measured in 10ml. of Bray's (1960) solution as an estimate of the soluble radioactivity (c.p.m.). Washed tissue was dissolved in 0·5ml. of NCS (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) at 55° in tightly capped scintillation vials. The insoluble radioactivity (c.p.m.) was determined by measuring the radioactivity of the dissolved tissue in 10ml. of toluene scintillation fluid [5g. of 2,5-diphenyloxazole and 0·3g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l.]. Corrected values for the radioactivity (d.p.m.) for both $^{14}$C and $^{3}$H were calculated by using a three-channel quench-correction technique. $^{14}$C counting efficiency was 35–55% and the $^{3}$H counting efficiency was 12–25% (Nuclear-Chicago Corp. mark I scintillation counter).

*Preparation of whole homogenates.* Anterior inguinal mammary glands of first-pregnancy BABL/C mice were excised, and lymph nodes and connective tissue removed (as for the culture above), cut into strips and washed in 10ml. of TM buffer (10mM-$MgCl_2$–20mM-tris–HCl buffer, pH7·5). This tissue (0·5–1·5g.) was homogenized in 10ml. of fresh TM buffer in a 30ml. motor-driven glass–Teflon homogenizer by using four strokes in about 30sec. at 4°. The crude homogenates were filtered through a Pasteur pipette containing a plug of glass wool to remove unbroken clumps of cells and connective tissues. Such extracts were used for enzyme determinations within 30min., since lactose synthetase in crude homogenates decays with a half-life of about 10hr. at 4° (Palmiter, 1969*a*). Homogenates of lactating tissue were passed through a column (1·5cm. × 25cm.) of Bio-Gel P6 equilibrated with TM buffer to remove all small molecules, as washing the tissue did not remove all the glucose.

Tissue-culture samples (10–30mg.) were washed in 10ml. of TM buffer and then homogenized in 1·5ml. of TM buffer in a 5ml. conical motor-driven glass–Teflon homogenizer by using four strokes in 30sec. at 4°.

*Enzyme and protein assays.* Lactose synthetase was assayed as described by Palmiter (1969*a*). In brief, a 150$\mu$l. reaction mixture containing 2$\mu$moles of tris–HCl buffer, pH7·5 at 4°, 1$\mu$mole of $MgCl_2$, 0·5$\mu$mole of $MnCl_2$, 0·15$\mu$mole of ATP, 3$\mu$moles of glucose and 32·3nmoles of UDP-[$^{14}$C]galactose (24000d.p.m.) (International Chemical and Nuclear Corp., City of Industry, Calif., U.S.A.) was incubated in a 400$\mu$l. plastic tube for up to 25min. at 37–38°. The non-ionic radioactive products were separated from the UDP-[$^{14}$C]galactose by anion-exchange chromatography. A description of the controls, which demonstrate that the major product of this assay is indeed lactose, and a description of the kinetics and properties of this enzyme were given by Palmiter (1969*a*).

*N*-Acetyl-lactosamine synthetase was assayed in the same way as described above, except that 0·45$\mu$mole of *N*-acetylglucosamine replaced the 3$\mu$moles of glucose. *N*-Acetylglucosamine at 3mM (final concentration) was chosen because at that concentration $\alpha$-lactalbumin does not affect *N*-acetyl-lactosamine synthetase activity (Brew, Vanaman & Hill, 1968).

Protein in homogenates was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), except that, if much fat was present, samples and standards were extracted with diethyl ether after the reaction and development of the colour.

*Partial purification of A and B subunits of lactose synthetase.* The A subunit was purified from lactating mammary glands of the mouse by the method of Palmiter (1969*a*). The A-subunit activity that was eluted from Sephadex G-100 with apparent mol.wt. 110000–130000 was used in these experiments. Commercial bovine $\alpha$-lactalbumin (Nutritional Biochemical Corp.) was purified as in the studies by Palmiter (1969*a*).

## RESULTS AND DISCUSSION

In a tissue that is not functional throughout the life of an animal a common mechanism for regulating the synthesis of small molecules required only at specific times is the modulation of the concentration of the enzyme(s) catalysing their synthesis. Such a mechanism is apparently used in the regulation of lactose synthesis by the mammary gland. This has been demonstrated by using both a tissue-culture and a cell-free assay of lactose synthetase activity.

*Tissue-culture assay of lactose synthetase activity.* Twarog & Larson (1964) and Bartley *et al.* (1966) showed that lactose synthesis in cultures of

mammary-gland tissue can be measured by labelling the cultures with [$^{14}$C]galactose, which is enzymically converted by the tissue into one of the substrates of lactose synthetase, UDP-galactose (see Scheme 1). Thus the formation of the product, [$^{14}$C]lactose, is a measure of lactose synthetase activity, assuming that the two enzymes involved in the synthesis of UDP-galactose from galactose (galactokinase and galactose 1-phosphate uridylyltransferase) are not limiting. (It was not possible to measure the activity of lactose synthetase directly with the tissue-culture assay since one of the substrates, glucose, was present at a concentration of 5mM in the media, thereby lowering the specific radioactivity of any labelled glucose added by several orders of magnitude, and the other substrate, UDP-galactose, was cleaved to UDP and galactose on entering the cells.) The rate of lactose formation by cultures of mammary-gland tissue isolated from mice during various stages of pregnancy and lactation is shown in Table 1. There was approximately a 100-fold rise in the rate of lactose synthesis between late pregnancy and early lactation. The tissue-culture assay has the advantage of measuring only the lactose synthetase that is active within the tissue. This is an important consideration, since one of the subunits of this two-subunit enzyme is secreted into the lumen of the gland along with the other milk proteins, thus making it probable that both subunits are not distributed identically within the cell (Brodbeck & Ebner, 1966*b*).

However, this assay has the disadvantage of being indirect. The cell-free assay, on the other hand, is direct, is more sensitive and permits the determination of the activity of both subunits of lactose synthetase.

*Determination of the activity of the A and B subunits of lactose synthetase by using the cell-free assay.* Brew *et al.* (1968) expanded the original observation by Watkins & Hassid (1962) that lactose synthetase transfers galactose from UDP-galactose, not only to glucose, but also to *N*-acetylglucosamine:

$$\text{UDP-galactose} + N\text{-acetylglucosamine} \xrightarrow[N\text{-Acetyl-lactosamine synthetase (lactose synthetase, A protein)}]{Mn^{2+}} N\text{-acetyl-lactosamine} + \text{UDP}$$

They showed that, whereas the synthesis of lactose requires both subunits, the synthesis of *N*-acetyl-lactosamine is catalysed by the A subunit alone. This result allows an easy assay for both subunits. The A subunit is assayed by using the *N*-acetyl-lactosamine synthetase assay (see the Materials and Methods section) in which radioactive galactose is transferred from UDP-galactose to *N*-acetyl-glucosamine; the B subunit is assayed by using the lactose synthetase assay (which is identical with the *N*-acetyl-lactosamine synthetase assay except that glucose replaces *N*-acetylglucosamine). However, the lactose synthetase assay is an accurate measure of B-subunit activity only when the A subunit is present in excess. Fig. 1 demonstrates that, with partially purified subunits (see the Materials and Methods section) under the assay conditions used, the activity of lactose synthetase was proportional to the B subunit concentration as long as the activity obtained in the lactose synthetase assay did not exceed three times the activity (in terms of $\mu$moles of galactose transferred/min./mg. of protein) measured with the *N*-acetyl-lactosamine synthetase assay. After that point, the A subunit became limiting. In whole homogenates of glands at various stages of pregnancy and lactation the activity of B subunit never exceeded the activity of A subunit by more than 20%; therefore the activity of both subunits could be measured in crude homogenates from any physiological state by adding, as substrate, glucose to one portion, *N*-acetylglucosamine to another and none to a control, along with the other reagents.

*Cell-free assay of lactose synthetase subunits from freshly excised mouse mammary-gland tissue during pregnancy and lactation.* Mammary-gland tissue was isolated as described in the Materials and Methods section and assayed for A- and B-subunit activity

Table 1. *Tissue-culture assay of lactose synthetase*

The rate of lactose synthetase activity in cultures of mammary tissue was assayed as described in the Materials and Methods section. Cultures were labelled with [$^{14}$C]-galactose for up to 5hr. The radioactive lactose formed was determined by paper chromatography of the defatted, deproteinized, deionized culture medium.

| Physiological stage | No. of cultures | Rate of lactose synthesis (ng./hr./mg. wet wt.) Mean | Range |
|---|---|---|---|
| Virgin | 3 | 1·3 | 0·7–1·7 |
| Pregnant, 3 days | 4 | 2·0 | 1·3–2·5 |
| Pregnant, 9 days | 4 | 1·2 | 0·6–2·0 |
| Pregnant, 16 days | 2 | 3·9 | 3·5–4·3 |
| Pregnant, 18 days | 4 | 4·8 | 3·2–6·2 |
| Pregnant, 19 days; 8hr. after birth, unsuckled | 4 | 33 | 29–36 |
| Lactating, 2–6 days | 4 | 142 | 124–160 |
| Lactating (12 days); pups weaned 30hr. earlier | 5 | 2·9 | 2·1–3·5 |

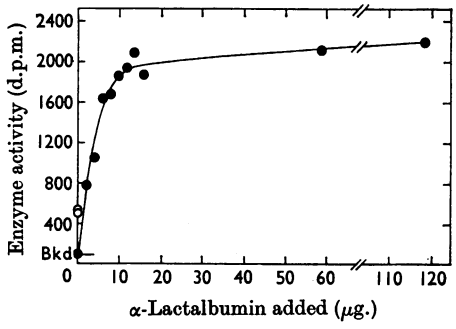



Fig. 1. Saturation of the A subunit of lactose synthetase with bovine α-lactalbumin (the B subunit). Partially purified α-lactalbumin (see the Materials and Methods section) was added to a constant quantity of partially purified A protein that could transfer 530 d.p.m. of radioactive galactose from UDP-galactose to 3 mM-*N*-acetylglucosamine in 10 min. Lactose synthetase activity was measured with 20 mM-glucose for 10 min. Maximum lactose synthetase activity was 4·8 times the *N*-acetyl-lactosamine synthetase activity. UDP-[$^{14}$C]galactose was present at 2·2 μM (24000 d.p.m.). Enzyme activity is expressed as radioactivity (d.p.m.) of product formed/10 min.; 100 d.p.m. of product equals 2 pmoles. $Mn^{2+}$ was present at 3·3 mM and ATP at 1 mM. Bkd, background radioactivity observed without enzyme. ●, Lactose synthetase activity; ○, A-subunit activity (*N*-acetyl-lactosamine synthetase).

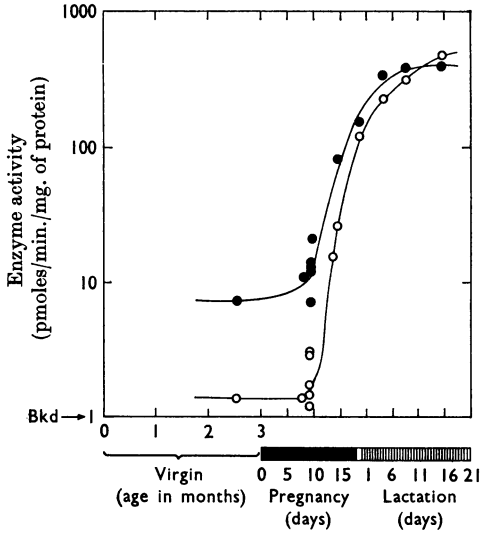



Fig. 2. Activity of the A and B subunits of lactose synthetase in crude homogenates from the mammary gland of the mouse during various stages of pregnancy and lactation. The A-subunit activity was determined by using the standard assay (see the Materials and Methods section) with 3 mM-*N*-acetylglucosamine, and the B-subunit activity in another portion of the same homogenate was assayed by using 20 mM-glucose. UDP-[$^{14}$C]galactose was present at 2·2 μM (24000 d.p.m.). $Mn^{2+}$ was present at 3·3 mM and ATP at 1 mM. Bkd, average UDP-galactose hydrolysis without added *N*-acetylglucosamine or glucose. ●, A-subunit activity; ○, B-subunit activity.

within 1 hr. Fig. 2 illustrates the rise in lactose synthetase activity that occurs after about day 10 of pregnancy. Initially the A-subunit activity was about five times the B-subunit activity. During late pregnancy and early lactation the activity of both subunits rose to about the same value.

By using the cell-free assay (Fig. 2) the following can be concluded about mouse lactose synthetase activity during pregnancy and lactation. First, there is approximately a 500-fold increase in the specific activity of this enzyme during late pregnancy and early lactation. (This may be an underestimate, since lactating tissue contains large amounts of milk protein in the alveoli, which complicates expression of enzyme activities in terms of total protein.) Secondly, 20–30% of the rise in lactose synthetase activity occurs before parturition, so that the gland is already prepared for synthesis of large amounts of lactose at the time of birth. This result contrasts with the induction of lactose synthetase (B subunit) at the time of parturition as reported by Turkington, Brew, Vanaman & Hill (1968) for the mouse and with that reported by Kuhn (1968) for the rat. The explanation for this discrepancy may lie in the fact that lactose synthetase (B subunit) activity measured *in vitro* is sensitive to the method of breaking the cells (Palmiter, 1969*a*; Coffey & Reithel, 1968*a*). Interestingly, the A subunit is not affected by the method of breaking the cells. It has been suggested that the loss of B-subunit activity is due to dissociation of the two subunits rather than to a destruction of B subunit (Palmiter, 1969*a*). Thus under suboptimum isolation conditions, if the AB complex were disrupted, the induction of B-subunit activity measured with the lactose synthetase assay would appear to lag behind the induction of A-subunit activity. The upturn in lactose synthetase activity before parturition argues against a specific hormonal stimulus for induction of this enzyme at this time. Thirdly, there is a definite, low, lactose synthetase activity even in virgin animals.

Comparison of the two assay systems reveals a smaller and delayed augmentation of lactose synthetase in the tissue-culture assay. There are several possible explanations for this result. It may be that in the tissue-culture assay the two enzymes that catalyse the synthesis of UDP-galactose from galactose are rate-limiting until the time of parturition. Or this result may indicate that some of the enzyme assayed after disruption of the cells is not active in whole cells, possibly owing to compartmentalization of lactose synthetase into secretory vesicles (Coffey & Reithel, 1968*b*).

The mechanisms by which the differentiated mammary gland regulates the rate of lactose synthesis so that the concentration of lactose in the milk is relatively constant throughout most of the lactation period are poorly understood. The results presented in Figs. 1 and 2 suggest one regulatory mechanism. Fig. 1 demonstrates that, as long as the B-subunit activity is less than three times as great as the A-subunit activity, the A subunit is present in excess. In Fig. 2 it is apparent that at all stages in the life cycle of the mouse the B-subunit activity never exceeds the A-subunit activity by more than 20%. These results suggest that at all times α-lactalbumin is the rate-limiting subunit of lactose synthetase. This conclusion is supported by experiments in which purified A subunit or B subunit was added to crude homogenates. The addition of A subunit had no effect on lactose synthetase activity, whereas the addition of the limiting subunit always enhanced the activity. Kuhn's (1968) findings suggest that α-lactalbumin is limiting in the rat also, since its addition to cell-free extracts always enhanced lactose synthetase activity. In view of the findings that A subunit is predominantly bound to the microsomes (Brodbeck & Ebner, 1966*b*; Coffey & Reithel, 1968*b*) and is always present in excess and that α-lactalbumin is secreted into the lumen of the gland along with other milk proteins, it is tempting to suggest that the rate of lactose synthesis may be linked to the rate of milk-protein synthesis. Thus a model is envisaged in which newly synthesized α-lactalbumin may first be used as part of the lactose synthetase enzyme, and then be packaged into protein-containing vesicles (Sekhri, Pitelka & DeOme, 1967; Kurosumi, Kobayashi & Baba, 1968; Coffey & Reithel, 1968*b*) and secreted into the alveoli. Some A subunit is also secreted into the milk (Babad & Hassid, 1966; Brew *et al.* 1968).

Other potential forms of regulation include feedback inhibition and enzyme repression. Feedback inhibition by lactose on lactose synthetase is apparently not involved in the mouse, since concentrations of lactose up to 5% inhibit lactose synthesis in the cell-free assay by no more than 10% (Palmiter, 1969*b*). The possibility of repression of the synthesis of one or both of the lactose synthetase subunits during hormonal induction of the enzyme *in vitro* has also been examined. Lactose (3%) in the culture medium had little effect on the extent of the induction of either subunit (Palmiter, 1969*b*).

*Hormonal induction of both lactose synthetase subunits.* The differentiation of mammary-gland epithelial cells of the mouse is thought to be the result of hormone action, since both the adrenal cortex and the pituitary are necessary for the functional development of the mammary gland (Nandi & Bern, 1961). The role of hormones in the induction of lactose synthetase was investigated by adding purified hormones to cultures consisting of pieces of mammary-gland tissue from mid-pregnancy in completely defined media. It

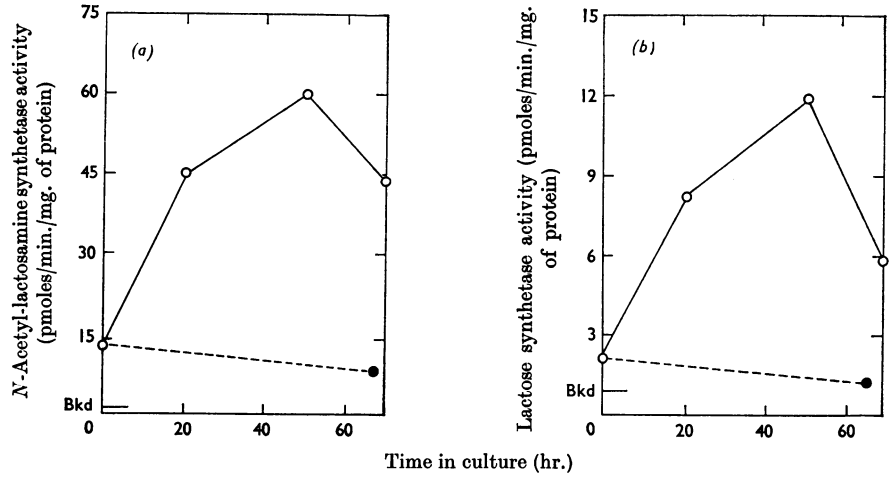



Fig. 3. Time-course of the induction of the A subunit (*a*) and B subunit (*b*) of lactose synthetase. For each set of A-subunit and B-subunit activities, 35 pieces of mammary-gland tissues from 9-days-pregnant mice were cultured in 10 ml. of medium 199, homogenized and then assayed as in Fig. 2. Each point represents the rate of product formation determined from a six-point time-course covering 20 min. Protein was determined in quadruplicate. The indicated hormones were present at a final concentration of 5 μg./ml. each and were added at the start of culturing. Bkd has the same meaning as in Fig. 2. ○, Insulin + hydrocortisone + prolactin; ●, no hormones.

was found that the combination of hormones, insulin + hydrocortisone + prolactin, which is instrumental in the induction of the major milk proteins (Juergens *et al.* 1965; Lockwood *et al.* 1966), also stimulates the induction of both lactose synthetase subunits. These results are essentially in agreement with those of Turkington *et al.* (1968).

Addition of insulin + hydrocortisone + prolactin to the culture medium at the time the tissue is explanted resulted in about a four- to five-fold increase in A-subunit activity and an eight- to nine-fold increase in B-subunit activity after about 50hr. of incubation (Fig. 3); however, more units of A-subunit activity than of B-subunit activity were induced. In contrast, no enhancement of specific activity took place in the absence of all three hormones. Insulin alone, insulin + hydrocortisone and insulin + prolactin gave no more than a twofold enhancement of the activity of either subunit. The slight enhancement obtained with these hormonal combinations is probably due to the insulin-mediated increase in the number of epithelial cells during the first day in culture (Stockdale & Topper, 1966). These results indicate that both A and B subunits are maximally induced only in the presence of all three hormones, since none of the other possible combinations of the three hormones results in more than a slight enhancement of activity.

The hormonal requirements for and the time-course of the induction of the lactose synthetase subunits presented here are in general agreement with those for other milk proteins (Lockwood *et al.* 1966). The data available suggest that the induction of the two subunits is temporally linked. An assessment of the extent of induction of the two subunits is more difficult. In terms of percentage increase in activity there was always a larger augmentation of B-subunit activity than of A-subunit activity, principally because the B subunit initially had very low activity; but in terms of units of activity induced in the parallel assays, more units of A-subunit activity were induced than of B-subunit activity. (However, owing to differences in the substrate concentrations of glucose and *N*-acetylglucosamine in the two assays and to possible differences in the kinetics of the two reactions used in measuring the A-subunit and B-subunit activities, it is not possible to equate the activity units of the two reactions to protein concentrations.)

*Role of prolactin in the induction of milk proteins and lactose synthetase* in vitro. Stockdale & Topper (1966) showed that insulin is necessary for cell division. If cell division occurs in the presence of hydrocortisone, prolactin can then stimulate the cells to synthesize the milk proteins casein, $\alpha$-lactalbumin and $\beta$-lactoglobulin (Lockwood, Stockdale & Topper, 1967; Turkington, Lockwood & Topper, 1967).

To isolate the effect of prolactin from those of insulin and hydrocortisone, mammary-gland tissue was incubated first in the presence of insulin and hydrocortisone for 24–34hr. (the period during

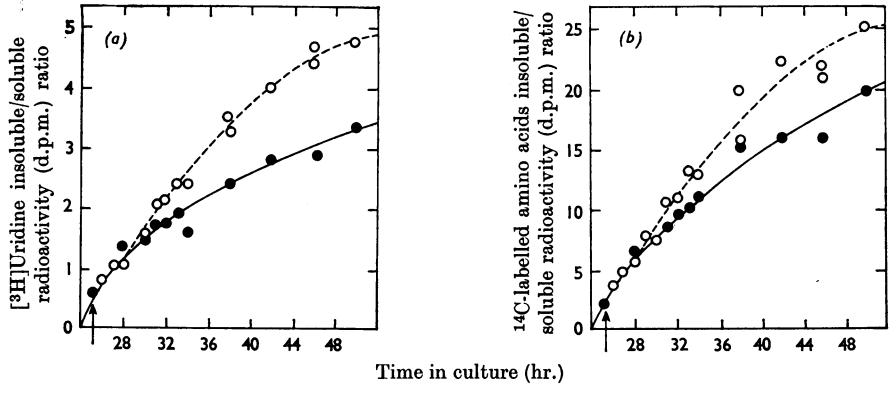



Fig. 4. Effect of prolactin on incorporation of uridine (*a*) and amino acid (*b*) into trichloroacetic acid-insoluble material. Ten pieces of mammary-gland tissue from 13–14-days-pregnant mice were incubated in 1ml. of medium 199 with insulin + hydrocortisone (●) for 24hr. before the addition of labelled compound. Then 0·1$\mu$c of $^{14}$C-labelled amino acids and 1·0$\mu$c of [$^3$H]uridine were added to each culture at 24hr. At 25hr. (shown by arrows) 5$\mu$g. of prolactin was added to some of the cultures (○). The results are plotted as the ratio of trichloroacetic acid-insoluble to trichloroacetic acid-soluble radioactivity (d.p.m.), to rule out effects of prolactin on the soluble pools and to minimize errors in determining the fresh weight of the tissue. The medium was changed 3hr. before the addition of labelled compounds. Maximum [$^3$H]uridine incorporation into trichloroacetic acid-insoluble material was 119000d.p.m.; maximum $^{14}$C-labelled amino acid incorporation into trichloroacetic acid-insoluble material was 31000d.p.m.

which most of the cell division takes place; Stockdale & Topper, 1966), and then prolactin was added. Fig. 4 illustrates the effect of this procedure on protein and RNA synthesis. To rule out permeability changes in response to prolactin and to avoid errors in determining the wet weight of the tissue, the results are presented as the ratio of trichloroacetic acid-insoluble to trichloroacetic acid-soluble radioactivity (d.p.m.). Prolactin had no effect on the soluble [$^3$H]uridine radioactivity (d.p.m.)/mg. of tissue, but increased soluble $^{14}$C-labelled amino acid radioactivity (d.p.m.)/mg. of tissue by about 20%. As shown in Fig. 4, after about 5hr. there was a noticeable change in the rate of synthesis of both RNA and protein in the cultures. This increased rate of protein synthesis was most likely to be due to an augmentation of differentiated-product synthesis (mainly casein) (Turkington *et al.* 1967).

Part of Fig. 5 (the black circles) illustrates the same type of experiment as described above, except that the A and B subunits of lactose synthetase were assayed. Mammary-gland tissue was incubated in the presence of insulin+hydrocortisone for 34hr. During this time there was a small increase in the activity of both subunits. Then, after the addition of prolactin, there was an additional increase in both A- and B-subunit activities. Approx. 80% of the maximal enzymic activity of both subunits was induced by the addition of prolactin to insulin+hydrocortisone-stimulated cells. In this experiment both activities reached maximal values 16hr. after the addition of prolactin.

Fig. 5 also illustrates the effect of adding hydrocortisone to tissue that had been stimulated with insulin+prolactin for 34hr. (white circles). The extent of induction, especially of the B-subunit activity, was lowered by this procedure. These results are in agreement with the proposal by Stockdale & Topper (1966) and Lockwood *et al.* (1967) that hydrocortisone acts during the period

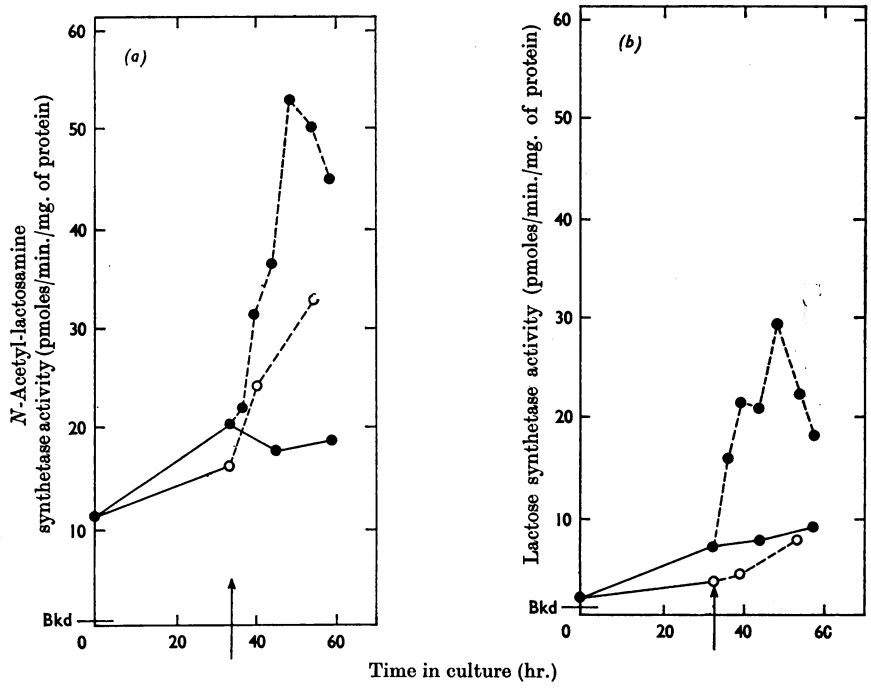



Fig. 5. Induction of the A subunit (*a*) and the B subunit (*b*) of lactose synthetase by generating the complete insulin+hydrocortisone+prolactine combination in two ways: (1) insulin+hydrocortisone (●——●) for 34hr. followed by the addition of prolactin (shown by the arrow); the result is indicated by ●-----●; (2) insulin+prolactin for 34hr. (●——○) followed by the addition of hydrocortisone; the result is indicated by ○---○. Control (●——●) was in the presence of insulin+hydrocortisone for the entire period. The final concentration of all hormones was 5$\mu$g./ml. The medium was changed at 29hr. For each set of A-subunit and B-subunit activities, 25 pieces of mammary-gland tissue from 9-days-pregnant mice were cultured in 10ml. of medium 199. Enzyme activity and protein were assayed as in Fig. 3. Bkd has the same meaning as in Fig. 2.

of cell division (the first 24–36hr. of culture) and that prolactin is not necessary at this time.

The experimental results of Fig. 5 demonstrate that the major portion of the rise in activity of both subunits is dependent on prolactin. Although the mechanisms of the action of prolactin on mammary-gland tissue are yet to be elucidated, several independent findings are pertinent. When added to insulin + hydrocortisone-stimulated cells, prolactin augments the synthesis of both RNA and protein (Fig. 4). Turkington (1968) has shown that, if actinomycin D is added concomitantly with prolactin to insulin + hydrocortisone-stimulated cells, there is no stimulation of α-lactalbumin synthesis. Both of these findings are consistent with the idea that synthesis of the subunits is evoked by prolactin.

In conclusion, it is apparent that, in this tissue, whether the milk carbohydrate, lactose, is synthesized or not depends primarily on the concentration of the terminal enzyme in the lactose pathway, lactose synthetase. The specific activities of the other enzymes involved in this pathway do not change markedly during pregnancy and lactation. Heitzman (1967) showed that the specific activities of UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase increase only tenfold from mid-pregnancy to mid-lactation compared with the 500-fold increase in lactose synthetase activity. Further, the augmentation of lactose synthetase activity appears to be under the control of hormones (Figs. 3 and 5, and Turkington *et al.* 1968).

These results are part of a Thesis offered in partial fulfilment of the requirements for the Ph.D. degree of Stanford University. I am grateful to my major professor, Dr Norman Wessells, for his sustained interest and encouragement in this study and to Dr R. Schimke, Dr F. Stockdale and the many graduate students who critically read this manuscript. This work was supported by U.S. Public Health Service Grants GM-08719, GM-30044 and by Grant IN-32 from the American Cancer Society.

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