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Diurnal Variation and Response to Food Withdrawal of Lactose Synthesis in Lactating Rats

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1. The incorporation of radiolabelled plasma glucose into mammary lactose was used to measure the rate of lactose synthesis in lightly anaesthetized lactating rats. 2. Lactose synthesis showed a diurnal variation with a minimum at 18:00h. 3. Food withdrawal for 6h did not affect lactose synthesis in the early morning but greatly decreased it in the afternoon or evening. 4. Plasma glucose, milk sugars and total galactosyltransferase activity (EC 2.4.1.22) did not show the above changes. 5. Measurements of plasma insulin, which varies diurnally, and experiments with injected insulin suggested that variations of insulin within the physiological range do not account for the changes in lactose synthesis described.

Wishing to examine the effects of nutritional and hormonal change on the rate of lactose synthesis by the mammary glands of lactating rats, we found it necessary to develop a technique for measuring lactose synthesis *in vivo*. Although rates of lactose synthesis might be calculated from rates of whole-milk production and values of the lactose content of milk, in practice milk production in small animals is not readily assessed over a wide range of conditions. The most suitable methods available allow the milk to accumulate in the tissue during a period (4–12h) of separation of the mother from the young. Then, either the weight gain of the maternal mammary glands can be measured or else, following the return of the young and the draining of the glands of the accumulated milk, the amount of the latter may be measured from the weight gain of the young, or from the weight loss of the mother (Brody & Nisbet, 1938; Grosvenor & Turner, 1959; Hanwell & Linzell, 1972). Although these approaches seem to be sound, at least during the removal of the young for not more than 4h (Hanwell & Linzell, 1972), they do not enable measurements to be made over short time periods, at parturition or weaning, or at very low milk-production rates.

With these shortcomings in mind, we have developed a simple radioisotopic method of measuring the rate of lactose synthesis over 30 min in lightly anaesthetized rats. In this paper we describe the method, and show that the derived rates of whole-milk production accord well with values that have been reported by workers using other methods. We show further how the method may be employed to follow changes in the rate of lactose synthesis during the 24th, and after short-term withdrawal of food. Investigations of the possible role of insulin in these changes are described. Some of the results were briefly reported earlier (Carrick & Kuhn, 1977).

Methods and Materials

Primiparous rats of a Wistar-derived strain bred in this Department were used on days 13–15 of lactation. Animals were given food and water *ad lib.*, except where described, and were maintained under an artificial lighting schedule of 10h dark and 14h light. So as to standardize any effect that litter size and suckling may have upon rates of lactose synthesis, only rats with litters of 10–12 pups were used, and undisturbed suckling was allowed until 1 min before anaesthesia.

Measurement of the rate of lactose synthesis in vivo

Rats were individually anaesthetized with 2–3% (v/v) halothane in O_2 throughout the following procedure. The femoral vein was rapidly dissected clear of surrounding connective tissue and injected with 17nmol of [U-^{14}C]glucose (268mCi/mmol) in 0.3ml of 0.9% (w/v) NaCl. Blood samples were then collected at intervals from the tail vein into cooled heparinized tubes. The plasma was then obtained by centrifugation at about 3000*g*. The animal was killed 30min after injection and the inguinal mammary glands from one side were removed and stored frozen at −20°C until required.

Plasma glucose was isolated by the ion-exchange procedure of Katz *et al.* (1974). The plasma (50μl) applied to the column was eluted with 2–3ml of water. It was later found that the cation-exchange resin used in this method could be dispensed with. Samples of the eluate were taken for the enzymic

determination of glucose (Bergmeyer & Bernt, 1965) and further samples were taken for the determination of ^{14}C by liquid scintillation. In control experiments the specific radioactivity of the plasma glucose isolated in this manner agreed well with that of glucose purified by paper chromatography (Vernon & Walker, 1972). Fig. 1 shows the result of a typical experiment, out of over 50 that were carried out, where the specific radioactivity of the plasma glucose is seen to fall during the half hour after injection.

Frozen mammary tissue was powdered under liquid N_2 with a mortar and pestle, and the duplicate portions (about 1g wet wt.) were transferred to preweighed thick-walled centrifuge tubes containing 5ml of 5%(w/v) trichloroacetic acid. The tubes were then reweighed to obtain the exact weight of tissue powder, and the contents were thoroughly ground round with a glass rod. After the tubes had been centrifuged at about 3000*g* at room temperature, the acid supernatants were removed and the pellets were extracted again with 5ml of trichloroacetic acid in the same way. The successive acid extracts were pooled, and 1ml portions were each added to a tube containing 200mg of solid lactose. The samples were heated at 70°C for 30min to dissolve the carrier lactose and to hydrolyse any *N*-acetylneuraminyl-lactose (Kuhn, 1972) to lactose. Control experiments with *N*-acetylneuraminyl-lactose purified from rat milk by ion-exchange and paper chromatography (Kuhn, 1972) showed that these conditions resulted in the quantitative hydrolysis of this trisaccharide without any detectable loss of lactose. The [^{14}C]lactose present in these extracts was then isolated together with the carrier lactose by selective precipitation with ethanol/diethyl ether (3:1, v/v). A portion of the final material was taken for the determination of ^{14}C by liquid scintillation, and a further portion was assayed for lactose by the phenol/sulphuric acid method (Ashwell, 1966), so that the recovered radioactivity could be corrected for losses (about 40%) during purification. Details of the method have been given by Kuhn & White (1975).

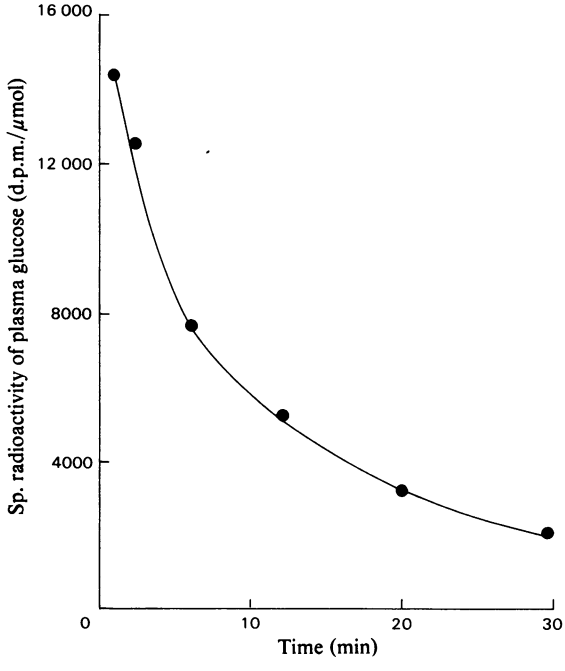

Fig. 1. *Change of specific radioactivity of plasma glucose over 30 min after injection of [U-^{14}C]glucose*
A typical result from a single rat is shown.

Calculation of the rate of lactose synthesis

From the changing specific radioactivity of plasma glucose, the amount of [^{14}C]lactose made in 30min, and the assumption that lactose is being synthesized at a constant rate L μmol/min, the value of L can be obtained from the following relationship:

amount of [^{14}C]lactose (d.p.m.)/wt. of tissue (g) = $\frac{1}{2}L \sum_{0}^{30\text{min}}$ specific radioactivity of plasma glucose (d.p.m./μmol)

using a simple graphical integration of the area under the specific-radioactivity–time curve. The fraction $\frac{1}{2}$ arises from the origin of 1mol of lactose from 2mol of glucose.

Assays and determinations

Assay of galactosyltransferase (EC 2.4.1.22). Mammary tissue was chopped (McIlwain & Buddle, 1953) and homogenized with 39 vol. of chilled 0.25M-sucrose with a Polytron PT 10 homogenizer at setting 2 for 1min. The total galactosyltransferase activity of the homogenate was then assayed under the conditions given by Kuhn & White (1977), which use *N*-acetylglucosamine as substrate and Triton X-100 to ensure disruption of the membranes.

Determination of plasma insulin and glucose. Rats were rapidly removed from their cages and decapitated, the trunk blood being then collected into chilled heparinized beakers. After centrifugation at 0–4°C at about 3000*g*, the plasma was separated and stored at −20°C until it was assayed. Insulin was measured by radioimmunoassay with a kit purchased from The Radiochemical Centre, Amersham, Bucks., U.K., but with a calibration curve prepared with pure rat insulin (Novo, Copenhagen, Denmark), and all samples were determined in duplicate. In experiments where pig insulin was administered, plasma insulin concentrations are expressed as rat insulin equivalents. Portions of plasma (0.02ml) were de-

proteinized and assayed enzymically for glucose (Bergmeyer & Bernt, 1965).

Determination of lactose and N-acetylneuraminyl-lactose in milk. Milk was collected from all the nipples of rats maintained under light diethyl ether anaesthesia after intraperitoneal administration of 1.0i.u. of oxytocin. Pooled samples were diluted 20-fold with water and portions (0.01ml) were assayed for lactose before and after incubation with neuraminidase (Kuhn, 1972). The difference between these two values was taken as being lactose originating from *N*-acetylneuraminyl-lactose.

Determination of mammary glycogen. Glycogen was extracted and hydrolysed to glucose (Pfleiderer, 1965), which was then assayed enzymically (Slein, 1965).

Isolation of mammary sugars

In the case of certain rats injected with [U-^{14}C]-glucose the mammary lactose was isolated and hydrolysed so that the specific radioactivities of the glucose and galactose moieties could be compared.

Frozen powdered mammary tissue (1g wet wt.) was extracted with 5% (w/v) trichloroacetic acid (5ml) and the acid extract was then shaken with six successive portions (10ml) of water-saturated diethyl ether to remove the trichloroacetic acid. The remaining aqueous phase was freeze-dried and redissolved in 0.2ml of water. The sugars were separated by descending chromatography on Whatman no. 4 paper with propan-1-ol/ethyl acetate/water (7:1:2, by vol.) for 24h at room temperature (Baar & Bull, 1953). After the paper had been dried in air, the region corresponding to standard lactose on an adjacent part of the paper was cut out and eluted with water. The lactose was hydrolysed with β-galactosidase (EC 3.2.1.23; 0.1 unit) in 50mM-Tris/HCl buffer, pH8.0, 20mM-$MgCl_2$ and bovine serum albumin (0.2mg/ml) overnight at room temperature, a trace of toluene being added to inhibit microbial activity. The glucose and galactose thus formed were then separated by descending chromatography on Whatman no. 3MM paper with pyridine/ethyl acetate/water (5:12:4, by vol.) for 36h at room temperature.

Lactose, glucose and galactose were determined enzymically by the methods of Reithel (1965), Slein (1965) and Kurz & Wallenfels (1974) respectively. Scintillation counting was carried out as previously described (Murphy *et al.*, 1973).

Enzymes were obtained from The Boehringer Corp. (London) Ltd., Lewes East Sussex, U.K., and from Sigma (London) Chemical Co., London S.W.6, U.K. All other reagents were of A.R. grade.

Results in this paper are expressed as mean values ± S.E.M., with the numbers of determinations in parentheses (S.E.M. values are denoted by error bars in Fig. 2).

Results

Measurement of the rate of lactose synthesis

It is well established that the flux along a biosynthetic pathway may be measured from the rate at which the end product acquires radioactivity from a precursor of known specific radioactivity. In the case of the intact lactating rat mammary gland, where the glucose and galactose moieties of lactose arise from free glucose and glucose 6-phosphate respectively, [U-^{14}C]glucose entering from the plasma appears to provide both sugars without isotopic dilution. Although homogenates can slowly hydrolyse glucose 6-phosphate, this activity is probably not due to a specific glucose 6-phosphatase and may not even be expressed in the intact tissue (Kuhn, 1968). This impression is supported by the finding that mannose alone does not permit lactose synthesis by slices of lactating rat mammary gland, although mannose is readily phosphorylated and oxidized, presumably by way of glucose 6-phosphate (Bartley *et al.*, 1966; Abraham *et al.*, 1961). Isotopic dilution of the glucose 6-phosphate pool of the tissue could possibly occur by the operation of gluconeogenesis, since lactating rat mammary tissue has been reported to contain some fructose bisphosphatase activity (Baird, 1969). However, lactating mammary tissue is generally believed not to carry out gluconeogenesis, being instead a major consumer of glucose (Davis & Bauman, 1974). Moreover, examination of the lactose isolated from the lactating mammary tissue of rats injected with [U-^{14}C]glucose revealed a specific radioactivity in the galactose moiety equal to 100 ± 10% (5) of that in the glucose moiety. Interestingly, Bartley *et al.* (1966) found a markedly lower specific radioactivity in the galactose moiety of lactose synthesized by slices of lactating rat mammary gland, although the ultimate source of these carbon atoms was not clear. In our experiments we could also rule out significant isotopic dilution by hexose phosphate arising from glycogen, of which we found only 0.32 ± 0.09 (13) μmol of glucose equivalent/g wet wt. of tissue.

Lactose itself is generally assumed to be the major end product of its biosynthetic pathway, but in fact substantial amounts are further converted into *N*-acetylneuraminyl-lactose at certain stages of lactation (Barra *et al.*, 1969; Carlson *et al.*, 1973; Kuhn, 1972). We therefore hydrolysed this to lactose before isolating the latter material for ^{14}C determination. No milk was removed from the gland by suckling during the 30min of measurement, although the pups were left undisturbed with the mother until the last minute. On the basis of experiments in the rat and goat (Murphy *et al.*, 1973; Kuhn & Linzell, 1970), loss of lactose by resorption into the bloodstream was assumed to be negligible.

Instead of trying to maintain the plasma glucose at

constant specific radioactivity we found it simpler to administer a single injection of [^{14}C]glucose and to measure the falling specific radioactivity thereafter. Fig. 1 shows a typical result, but the variation among animals nevertheless required that the procedure be applied to each one. For the purposes of calculation, each such curve was divided into 15 2min periods and the average specific radioactivity in each period, obtained from the graph, was taken as the effective specific radioactivity during those 2min. This afforded a simple way of integrating the specific radioactivity over the 30min of the experiment, the inaccuracy of which probably does not exceed the experimental errors of the method. Because the high specific radioactivity during the first few minutes after injection has a large influence on the final calculated rate of lactose synthesis, we were concerned to know how rapidly the injected glucose was distributed to the extracellular spaces of the mammary gland. The studies of Heath & Barton (1973) in male rats have shown that such distribution among extracellular spaces generally is largely complete within 30s. In two experiments we found that 1min after the injection of [^{14}C]glucose the specific radioactivity of mammary-gland glucose, essentially all of which is extracellular (Murphy *et al.*, 1973), was respectively 87 and 96% of that of the plasma glucose sampled at the same time. It was concluded that no important error was arising through undue delay in the mixing of plasma glucose with extracellular mammary glucose.

Actual rates of lactose synthesis

The mean rate of lactose synthesis *in vivo* throughout the 24h day was 7.29 ± 0.47 (32) μmol/h per g wet wt. of tissue. Taken together with the lactose and *N*-acetylneuraminyl-lactose content of rat milk at this stage of lactation (100μmol/ml; Kuhn, 1972) and a weight of 20g wet wt. of mammary tissue per rat, the daily whole-milk production is calculated to be about 35g/day. This compares with published values of 34g/day (Brody & Nisbet, 1938), 38g/day (Grosvenor & Turner, 1959) and 42g/day (Hanwell & Linzell, 1972). The mean rate of lactose synthesis *in vivo* between 08:00 and 12:00h was 9.2 ± 0.6 (13) μmol/h per g wet wt. of tissue, giving a calculated rate of milk synthesis of 7.4g/4h. The rate of milk formation measured over this period by the method of Hanwell & Linzell (1972) was 7.8 ± 0.9 (8) g/4h. The close agreement between these values appears to support the validity of the present method for the measurement of lactose synthesis *in vivo*.

Diurnal variation in the rate of lactose synthesis

Since an increasing number of metabolic parameters have been reported to exhibit diurnal fluctuations, we investigated this aspect of lactose synthesis before attempting to examine the effects of food withdrawal. Fig. 2 shows that the rate was high during the night and morning, but fell during the afternoon to reach about half that value by 18:00h. By contrast, the total galactosyltransferase activity of mammary tissue remained constant at a value that was 8–18-fold in excess of the actual rate of lactose synthesis *in vivo*. Plasma glucose concentrations did not vary significantly throughout the day, as has also been reported for non-lactating rats by Bellinger

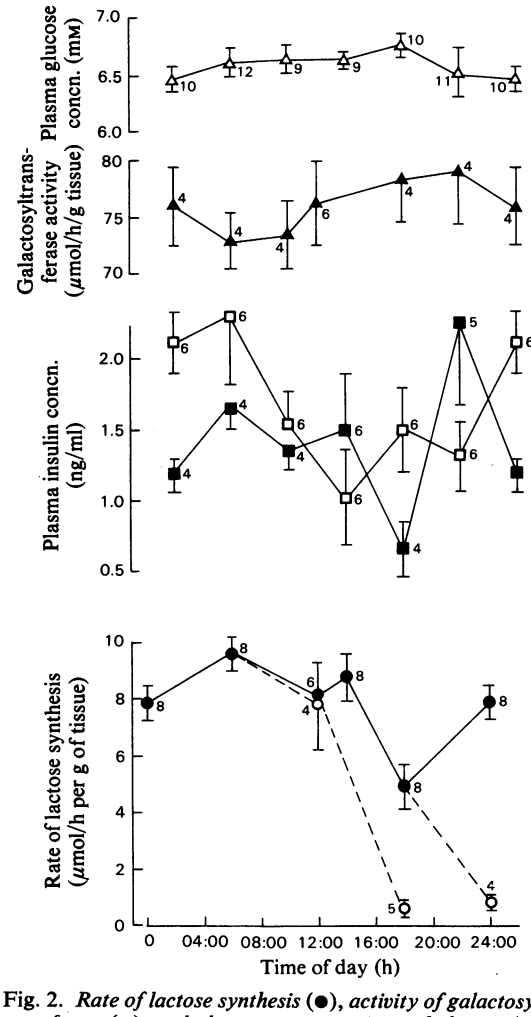

Fig. 2. *Rate of lactose synthesis* (●), *activity of galactosyltransferase* (▲), *and plasma concentrations of glucose* (△), *and insulin* (■, □) *in lactating rats during* 24*h*
The symbols ■ and □ refer to two series of rats sampled 4½ months apart. Rates of lactose synthesis after periods of food withdrawal are also given (○), with the number of separate animals shown by each value.

et al. (1975). The half-life of plasma glucose as calculated from the specific-radioactivity–time curves, averaged 10.5min, and did not alter significantly during the day.

Despite the diurnal variation in the rate of lactose synthesis, there was no discernible variation in the lactose content of the milk. The values at 12:00h and 18:00h were respectively 86.45 ± 4.92 (8) and 83.35 ± 3.65 (10) μmol/ml of whole milk. The molar ratio of *N*-acetylneuraminyl-lactose to lactose at these times was also unchanged at 0.13 and 0.15 respectively.

Plasma insulin concentrations exhibited a diurnal variation similar to that seen with lactose synthesis. Thus, in the two series of animals that we examined 4½ months apart, the concentration appeared to fall during daylight hours to reach a minimum value in the afternoon, although this minimum was reached at different times in the two series (Fig. 2). A similar diurnal variation in plasma insulin concentration was earlier reported for non-lactating rats (Kaul & Berdanier, 1972; Bellinger *et al.*, 1975).

Effects of short-term starvation

Since lactation is recognized to constitute a considerable drain on the body's reserves of carbohydrate, we examined the effect of short periods of food withdrawal. Removal of food at 06:00h did not affect the rate of lactose synthesis measured at 12:00h (Fig. 2). However, the withdrawal of food at 12:00h or at 18:00h resulted in very low rates of lactose synthesis 6h later. By contrast, these short periods of food withdrawal affected neither the galactosyltransferase activity of the tissue nor the plasma insulin concentrations for the expected time of day (results not shown).

Effects of insulin administration on lactose synthesis

We were unable to increase the rate of lactose synthesis by injecting 1i.u. of pig insulin subcutaneously. Rats injected at 14:00h and sampled at 18:00h had elevated plasma insulin concentrations of 5.32 ± 1.4 (4) ng/ml, but a mean rate of lactose synthesis of only 2.69 ± 0.25 (4) μmol/h per g wet wt. of tissue. Control rats injected with saline had a mean plasma insulin concentration of 1.10 ± 0.25 (3) ng/ml and a mean rate of lactose synthesis of 3.01 ± 0.17 (4) μmol/h per g wet wt. of tissue. Hypoglycaemia in the insulin-treated rats was prevented by the subcutaneous injection of 2.5ml of 2.5M-glucose.

Discussion

Although the radioisotopic method described above for measuring the rate of lactose synthesis *in vivo* appears not to have been used previously in mammary research, it is based on well-established approaches to the measurement of chemical flux in a complex system. The present situation appears to be favourable in so far as both the glucose and the galactose moieties of lactose can arise from plasma [U-^{14}C]glucose without isotopic dilution. The method, including estimation of lactose and *N*-acetylneuraminyl-lactose as it does, gives a value for the net chemical flux along the lactose-biosynthetic pathway.

The question arises whether the use of anaesthetized rats could lead to erroneous values for the rate of lactose synthesis. There is a tendency for plasma glucose concentrations to rise under halothane anaesthesia, and in the present work concentrations of 8mM were sometimes reached, compared with about 6.5mM in guillotined animals. This could affect the results only if the plasma glucose concentration itself affects the rate of lactose synthesis, for which we have no evidence. It is also possible that anaesthesia, and venous injection, cause the release of vasoconstrictor and vasodilator substances that affect the mammary blood flow (see discussion by Hanwell & Linzell, 1973). We have not attempted to assess these factors, which would in any case be difficult to control. However, the similarity of our calculated rates of milk formation with published values based on quite different techniques suggests that the present method is generally sound. It enables rates of lactose synthesis to be determined at times when the pups are not available, as during parturition (see, for example, Wilde & Kuhn, 1977) or weaning, or when the litter size is being varied. Especially, however, it enables rates to be measured over relatively short time periods, or when they are low, as in parts of the present work. One disadvantage remains, namely that repeated measurements cannot be made in single animals.

Application of the present technique reveals that the rate of lactose synthesis in rats fed *ad lib.* is not constant during the day, but falls at 18:00h to about 60% of maximum night-time values. Since there is no significant change in the lactose content of the milk, rates of whole-milk formation must change correspondingly. In a small study in which the gnawing sound of single feeding lactating rats (with litters) was recorded with aid of a microphone suspended over the food hoppers, we found that feeding proceeded in bursts of activity at about 30min intervals during the night. The intervals were longer, and the bursts of feeding shorter, during the daytime, unlike the pattern with non-lactating rats, which scarcely fed at all during the day (Kimura *et al.*, 1970; Bruckdorfer *et al.*, 1974). Thus the main increase in the rate of lactose synthesis between 18:00h and 24:00h coincided with the renewal of vigorous night-time feeding. The impression that lactose synthesis was closely linked with food intake, and hence with the very short-term nutritional status of the mother, was strengthened by the experiments involving food

feeding period, and shortly thereafter, the mother rat is in nutritional surplus and that lactose synthesis can withstand the withdrawal of food for a few hours. Later in the day, however, the nutritional status alters and becomes a limiting factor for lactose synthesis. The light daytime feeding of the animal prevents lactose synthesis from falling below 60% of maximal rates, but a few hours of complete food withdrawal causes it to drop rapidly to very low values.

It is already known that the plasma insulin concentrations undergo circadian variations in non-lactating rats. Similar variation was found in the two series of lactating rats studied here, with minima at 14:00h and 18:00h respectively, raising the possibility that plasma insulin might serve as a normal regulator of lactose synthesis. However, starvation from 18:00h to 24:00h appeared not to prevent the normal rise of plasma insulin concentration, whereas it almost abolished lactose synthesis. Further, we were unable to increase the rate of lactose synthesis by injecting insulin. These findings contrast with the insulin stimulation of [U-^{14}C]glucose incorporation into lactose shown for lactating rat mammary cells *in vitro* by Martin & Baldwin (1971). It must be concluded, therefore, that under normal physiological conditions changes in the concentration of plasma insulin do not serve to regulate lactose synthesis, at least over the short term. This does not exclude the possibility of insulin regulating other aspects of mammary metabolism, nor does it mean that over the long term or during pathological conditions insulin may not have profound effects on lactose synthesis.

Although changes in the rate of lactose synthesis appear related to short-term changes in nutritional status, it is by no means clear what factor(s) mediate this influence to the mammary gland. Since plasma insulin and glucose seem not to be indicated, it is open to speculation as to what other plasma hormones or metabolites may be involved. Longer periods of starvation (24h) reduce the apparent glucose uptake of lactating goat and rat mammary gland (Linzell, 1967; Robinson & Williamson, 1977*a*). While these observations may clearly relate to our recorded decreases in lactose synthesis, their causes are again obscure, although Robinson & Williamson (1977*b*) have examined the possible role of ketone bodies.

Whatever may be the plasma factor that informs the mammary gland of the nutritional status of the body, the question arises as to what change(s) within the secretory cell actually modulates the synthesis of lactose. In the uridine nucleotide cycle that supports the synthesis of lactose, it has recently been shown that galactosyltransferase constitutes the primary rate-limiting step (Kuhn & White, 1977). Yet the total assayable galactosyltransferase activity does not alter withdrawal. It thus appears that during the nocturnal significantly during any of the changes in rate of lactose synthesis documented here (Fig. 2). Such changes therefore reflect a varying efficiency in the operation of galactosyltransferase. In the present studies the efficiency varied over the range 6–13% in fed rats and fell to 0.8% in starved rats. The complexity of the lactose synthase reaction (Hill & Brew, 1975; Powell & Brew, 1976) and the problems of subcellular compartmentation (Kuhn & White, 1977) currently frustrate a satisfactory explanation of these efficiency changes.

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