Changes in the Enzyme Pattern of the Mammary Gland of the Lactating Rat after Hypophysectomy and Weaning

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1. The enzymes glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase, UDP-glucose pyrophosphorylase, phosphofructokinase, ATP-citrate lyase and acetyl-CoA carboxylase have been assayed in rat mammary glands in various stages of involution after hypophysectomy and weaning. 2. After hypophysectomy all seven enzymes decline in activity over a 12–16 hr. period but the extent of the decline varies, with acetyl-CoA carboxylase becoming almost totally inactive, ATP-citrate lyase and phosphofructokinase showing a large decrease, and the remaining enzymes a less marked decline. 3. Within 24 hr. of removing the litter a change in the pattern of enzyme activity is found very similar to that after hypophysectomy. 4. The significance of these results is discussed in relation to the endocrine control of mammary gland metabolism and the mechanisms of involution.

The mammary gland develops morphologically and biochemically during pregnancy and lactation under endocrine control (for reviews see Folley, 1961; Jacobsohn, 1961) and changes in various enzymes during the course of the pregnancy-lactation-involution cycle in the rat have been reported. These enzymes include arginase and alkaline phosphatase (Folley & Greenbaum, 1947), glutamate dehydrogenase and glutamate-aspartate transaminase (Greenbaum & Greenwood, 1954), succinate dehydrogenase (Greenbaum & Slater, 1957), glucose 6-phosphate and 6-phosphogluconate dehydrogenase (McLean, 1958; Willmer, 1960), UDP-glucose pyrophosphorylase and phosphoglucomutase (Malpress, 1961) and acetyl-CoA carboxylase and ATP-citrate lyase (Howanitz & Levy, 1965). Baldwin & Milligan (1966) have studied a range of 20 enzymes during pregnancy and lactation. Though there are some individual variations, all these enzymes show a similar pattern of activity, this rising during pregnancy or after parturition and falling abruptly after weaning.

The role of hormones in maintaining the metabolic pattern and enzyme content of the mammary gland has been studied by observing the changes after adrenalectomy and hypophysectomy of the lactating rat. Adrenalectomy (Willmer, 1960; Greenbaum & Darby, 1964) leads to a decrease in glucose utilization by the Embden-Meyerhof and pentose phosphate pathways and halts the steady rise in the dehydrogenases of the latter pathway,

which normally persists throughout lactation. Hypophysectomy, on the other hand, produces rapid changes in gland metabolism and an increase in lactate production by slices is detectable within 4 hr. (Bradley & Cowie, 1956).

The work reported in this paper was designed to study the changes occurring in the lactating rat after hypophysectomy in more detail by assaying the activities of a number of metabolically important enzymes in the mammary gland. By comparing the rate and magnitude of these changes during the 24 hr. post-operational period it was hoped to determine whether enzymic changes occurred sufficiently quickly to account for the rapid decline in milk production and whether there were any marked differences in the responses of different enzymes. As various surgical procedures other than hypophysectomy have been shown to produce no detectable change in the metabolism of the glands of lactating rats within 24 hr. (Bradley & Cowie, 1956) it was decided to dispense with sham-operated controls and to use normally lactating rats as a basis of comparison.

Hypophysectomy leads to an engorgement of the rat mammary gland with milk. This is because the posterior pituitary releases oxytocin, which is essential for the functioning of the milk-ejection reflex, and this reflex fails before the complete cessation of milk synthesis and secretion. To study the possible role of this engorgement in any changes after hypophysectomy the effects of oxytocin-replace-

ment therapy were observed. As milk secretion in the rat falls to a very low amount within 8 hr. of hypophysectomy (Bradley & Cowie, 1956), a single injection of oxytocin towards the end of this period allows the litter to remove the accumulated milk and subsequent re-engorgement does not occur.

Weaning has also been shown to lead to a fall in the activities of most mammary gland enzymes, though the activities of some enzymes associated with lysosome-like particles (Greenbaum, Slater & Wang, 1960), for example cathepsin and β -glucuronidase, increase during involution (Greenbaum & Greenwood, 1954). It was considered of interest to compare the changes after weaning (artificially induced by removing the litter during full lactation) with those after hypophysectomy to see whether involution produced by these two different mechanisms involved similar changes in enzyme activities.

The choice of enzymes was dictated by the importance of their metabolic role and the availability of a reliable assay method. All the enzymes chosen occur, as far as is known, only in the particle-free fraction of the cell homogenate. Five are concerned with the metabolism of glucose 6-phosphate, the starting point of the Embden-Meyerhof and pentose phosphate pathways and of lactose synthesis, and the other two with fatty acid synthesis. The seven enzymes assayed were:

1. Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49) and phosphogluconate dehydrogenase [6-phospho-D-gluconate-NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.44], enzymes of the pentose phosphate shunt, the importance of which in metabolism of the rat mammary gland is well established (McLean, 1958; Abraham & Chaikoff, 1959).

2. Phosphoglucomutase (D-glucose 1,6-diphosphate-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) and UDP-glucose pyrophosphorylase (UTP- α -D-glucose 1-phosphate uridylyltransferase, EC 2.7.7.9), the enzymes catalysing the first two stages in the synthesis of the galactose moiety of lactose from glucose 6-phosphate.

3. Phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11). This enzyme catalyses what is probably the rate-limiting reaction of the Embden-Meyerhof pathway under most metabolic conditions (Passonneau & Lowry, 1964).

4. ATP-citrate lyase [former trivial name citrate-cleavage enzyme, ATP-citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8] and acetyl-CoA carboxylase [acetyl-CoA-CO₂ ligase (ADP), EC 6.4.1.2], the enzymes catalysing the first two stages in the synthesis of fatty acids from citrate. The involvement of

ATP-citrate lyase in fatty acid synthesis in the rat mammary gland has been shown by Spencer, Corman & Lowenstein (1964).

MATERIALS AND METHODS

Animals and experimental treatments. Primiparous albino rats were used and fed *ad libitum* throughout. All animals, whether subject to surgery beforehand or not, were killed on the ninth, tenth or eleventh days of lactation. Two groups of rats were employed.

Expt. 1. Litter sizes were below eight and were not reduced. Rats were hypophysectomized by the parapharyngeal route and returned to their litters until they were killed 4, 8, 12, 16 or 24 hr. after hypophysectomy. Normally lactating rats acted as controls.

Expt. 2. Litter sizes were reduced to eight immediately after birth. Rats were hypophysectomized by the parapharyngeal route and returned to their litters until they were killed 24 hr. after hypophysectomy. Some of the hypophysectomized rats were injected intraperitoneally with 1 i.u. of oxytocin 5 hr. after hypophysectomy and the young held to the teats to ensure suckling. The litters were weighed before and after suckling to ascertain that milk had been obtained. The effects of weaning were studied in other rats of this group by removing the litter and killing the mother 12 or 24 hr. later. Normally lactating rats acted as controls.

Preparation of particle-free fraction. The rats were killed by a blow on the head. In the first experiment as much mammary tissue as possible was removed but in the second only the inguinal-abdominal glands were taken. The glands were placed in cold mannitol-sucrose-EDTA medium (0.25 M-mannitol, 0.027 M-sucrose, 1 mM-EDTA, pH 7.4), minced with scissors and squeezed in cheesecloth to remove as much milk as possible. Homogenization was carried out in a Potter-Elvehjem homogenizer in the first experiment and a Folley & Watson (1948) homogenizer in the second. The homogenate was filtered through cheesecloth and centrifuged in a Spinco model L ultracentrifuge for 6×10^6 g-min. The clear phase between the pellet and the cloudy surface layer was removed and frozen at -12° .

Enzyme assays. ATP-citrate lyase, phosphofructokinase and acetyl-CoA carboxylase were assayed within 3 days of the preparation of the supernatant; re-freezing, which caused some inactivation, was avoided. The other enzymes were completely stable when frozen.

Glucose 6-phosphate dehydrogenase was assayed in principle as described by Glock & McLean (1953). Medium: 0.2 mM-NADP⁺, 0.5 mM-glucose 6-phosphate, 0.075 M-tris, pH 7.4, total volume 1 ml. The reaction was started by the addition of glucose 6-phosphate and the initial rate of change of extinction at 340 m μ recorded.

As 6-phosphogluconate dehydrogenase is also present in the particle-free fraction it was essential to check the yield of NADPH/mole of glucose 6-phosphate oxidized. This was done by stopping the reaction at various time-intervals with HClO₄ and assaying for glucose 6-phosphate and NADP⁺ (Hohurst, 1963). The results indicated that 1 mole of NADP⁺ was reduced/mole of glucose 6-phosphate oxidized, i.e. during the initial period further oxidation of 6-phosphogluconate was not significant. In particle-free fractions from normally lactating rats NADP⁺ reduction in the absence

of substrate was undetectable but after weaning or hypophysectomy a considerable endogenous reduction took place. This endogenous reduction rate became constant after 1–2 min. and was subtracted from the initial reduction rate produced by the addition of substrate.

6-Phosphogluconate dehydrogenase was assayed in principle as described by Glock & McLean (1953). Medium: 0.2 mM-NADP⁺, 1 mM-6-phosphogluconate, 5 mM-MgCl₂, 0.065 M-tris, pH 9, total volume 1 ml. The reaction was started by the addition of 6-phosphogluconate and the initial rate of change of extinction at 340 m μ recorded. A yield of 1 mole of NADPH/mole of 6-phosphogluconate oxidized was assumed. The endogenous reduction rate was subtracted as described for the assay of glucose 6-phosphate dehydrogenase.

Phosphoglucomutase was assayed by using glucose 1-phosphate as substrate and allowing the glucose 6-phosphate produced to be oxidized by the glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase present in the particle-free fraction with an accompanying reduction of NADP⁺. The rate of change of extinction at 340 m μ was recorded. Medium: 0.2 mM-NADP, 0.5 mM-glucose 1-phosphate, 2 mM-MgCl₂, 0.07 M-tris, pH 7.4, volume 1 ml. As this is a coupled assay the rate increases during the initial period after the addition of substrate, becoming constant after about 1 min. To determine the stoichiometry the reaction was stopped with HClO₄ at various time-intervals and the reactants were assayed (Hohurst, 1963). The ratio was found to be 2 moles of NADP⁺ utilized/mole of glucose 1-phosphate, and the two-step nature of the oxidation was confirmed by the negligible accumulation of 6-phosphogluconate. As the ratio of activities of the three enzymes concerned remains essentially constant in the various involuted states studied the rate of NADPH production was halved to give the rate of utilization of substrate.

Phosphofructokinase was assayed by a procedure based on that of Mansour (1963), in which the product is cleaved to triose phosphates by aldolase and the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate coupled to NADH oxidation. The mammary gland particle-free fractions contained the relevant coupling enzymes in excess, so no further enzyme additions were required. Ammonium chloride was added to activate the enzyme (Underwood & Newsholme, 1965). In the absence of NH₄Cl the apparent specific activity of the particle-free fraction was a function of the volume added to the reaction medium. Medium: 0.1 mM-NADH, 0.2 mM-ATP, 4 mM-fructose 6-phosphate, 10 mM-NH₄Cl, 1 mM-MgCl₂, 40 mM-tris, pH 8.2, total volume 1 ml. With the medium complete except for ATP, addition of particle-free fraction produced a slow constant oxidation which was subtracted from the more rapid rate which followed the addition of ATP. The presence of an excess of the coupling enzymes was tested for by adding fructose 1,6-diphosphate, when a much more rapid oxidation of NADH ensued. The stoichiometry of the method was checked by comparing the results of the above assay with those obtained by using the same medium minus NADH, stopping the reaction with HClO₄ and assaying for fructose diphosphate and triose phosphate (Bücher & Hohurst, 1963). The two assays agreed to within better than 10%, assuming 2 moles of NADH oxidized/mole of fructose 6-phosphate phosphorylated.

ATP-citrate lyase was assayed by the method of Srere

(1959). The particle-free fractions contained a large excess of malate dehydrogenase, the indicator enzyme in this assay, so no further enzyme additions were required. The reaction was shown to be dependent on the presence of all three substrates, ATP, CoA and citrate.

UDP-glucose pyrophosphorylase was assayed by the method of Shatton, Gruenstein, Shay & Weinhouse (1965), in which the further oxidation of the product, UDP-glucose, by its dehydrogenase is coupled to NAD⁺ reduction with a yield of 2 moles of NADH/mole of UDP-glucose oxidized. The reaction was started by the addition of UDP-glucose dehydrogenase, no reaction occurring in its absence. The rate of reaction was proportional to the volume of supernatant added only at low concentrations, i.e. when the rate was in the range 0–0.5 μ mole/min./ml. of reaction medium. To increase the sensitivity of the assay the reaction was carried out in an E.I.L. model 27A direct-reading fluorimeter adapted for recording small fluorescence changes, essentially as described by Estabrook & Maitra (1962). The circuit incorporated a Pye 11370 amplifier and a Record 1mA, 1500 Ω recorder. The fluorimeter was calibrated between each enzyme assay by adding a known amount of NADH. To ensure that the limits of proportionality had not been exceeded the reaction was carried out with at least two different volumes of particle-free fraction.

Acetyl-CoA carboxylase was assayed by a procedure based on that of Waite & Wakil (1962), in which the incorporation of KH¹⁴CO₃ into malonyl-CoA is followed. In assays in the first experiment the medium contained 40 mM-tris-maleate, pH 6.5, 50 mM-sodium citrate, pH 6.5, 2 mM-MnCl₂, 2 mM-ATP, 0.1 mM-acetyl-CoA, 32 mM-KHCO₃ (containing 5 μ C of Na₂¹⁴CO₃ of specific activity 26.8 μ C/ μ mole), total volume 0.5 ml. The reaction was started by the addition of particle-free fraction and stopped after 10 min. with 0.2 ml. of 0.5 M-HClO₄. After centrifugation a known volume of supernatant was transferred to a 1½ in. aluminium planchet and dried in a stream of air with gentle heating. The incorporated activity was counted with an IDL 663 scintillation counter and 1700 scaler. Self-absorption was corrected for by reference to a standard curve prepared by counting a range of volumes of the normal reaction medium: the correction did not amount to more than 20% of the uncorrected count. An acetyl-CoA-free blank and two or three different volumes of particle-free fraction were run together for each assay. The amount of CO₂ incorporation was calculated from the known molarity of the KHCO₃ and the proportion of added counts recovered. To check the validity of the assay in this particular system a HClO₄ extract was made strongly alkaline and heated in a boiling-water bath for 30 min. to ensure the hydrolysis of CoA esters. After adding carrier malonate the organic acids were extracted and chromatographed as described by Jones & Gutfreund (1963). The paper strip was divided into segments and each segment extracted with 50% (v/v) ethanol, and the extracts were plated and counted as described above. All the activity recovered was associated with the malonate spot and accounted for more than 90% of the total incorporated activity determined by direct counting. The assay was modified in the second experiment by preincubating the enzyme in the presence of citrate as described by Howanitz & Levy (1965). The procedure was the same as that described above, except that the particle-free fraction was incubated 15 min. at 25° in the presence of citrate and tris-maleate and then added to the other

constituents of the medium to start the reaction. This modification accounts for the higher values recorded for acetyl-CoA carboxylase in Expt. 2.

All enzyme assays were carried out at 25° except that of UDP-glucose pyrophosphorylase, which was performed in a fluorimeter without temperature control at 15–19°. Spectrophotometric assays were carried out in a Beckman DB spectrophotometer.

Assay of DNA. DNA was assayed by the method of Burton (1956), as modified by Munford (1963), with herring-sperm DNA as standard.

Materials. Sugar phosphates and coenzymes were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), acetyl-CoA and UDP-glucose dehydrogenase from Sigma Chemicals Co. Ltd. (London), herring-sperm DNA from Koch-Light Laboratories Ltd. and oxytocin from Parke, Davis and Co. as Pitocin. Na₂¹⁴CO₃ was supplied by The Radiochemical Centre, Amersham, Bucks.

RESULTS

Time-course of the changes in enzyme activities after hypophysectomy. In mammary glands at different stages of involution enzyme activities cannot be compared on the basis of dry or wet weights or total protein because of the accumulation of varying amounts of milk of uncertain composition. However, Tucker & Reece (1962) have claimed that the amount of DNA per nucleus remains constant through the lactation cycle in the rat, so the expression of enzyme activities on the basis of DNA should give an estimate of the amount of enzyme per cell. It is probable that the DNA content of glands involuting after removal of the litter is elevated owing to a leucocyte invasion (Slater, 1962), but after only 24 hr. this effect should not be large. All activities in this paper are expressed per mg. of DNA, which was estimated in the gland homogenate after filtering through cheesecloth to remove intractable elastic connective tissue.

The results of Expt. 1 are shown in Figs. 1(a)–1(g), which illustrate the activities of the seven enzymes at different time-intervals after hypophysectomy. All the activities are lower after 24 hr. but there are large quantitative differences in behaviour. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucosutase and UDP-glucose pyrophosphorylase decline to about 50% of their control values during the first 12 hr., at which the activity remains relatively stable during the second 12 hr. period. There is a rise during the first 4 hr., especially marked with UDP-glucose pyrophosphorylase, but because of the size of the standard error this cannot be shown to be significant. With ATP-citrate lyase, phosphofructokinase and acetyl-CoA carboxylase the decreases in activity are more marked. ATP-citrate

lyase and phosphofructokinase fall to 15% of their control activities within 16 hr. of hypophysectomy and acetyl-CoA carboxylase declines rapidly to an activity near the limits of the assay method. For all the enzymes, activity remains relatively constant between 16 and 24 hr. and the differences between the control and pooled 16 and 24 hr. activities are significant (Fisher's *t* test, $P < 0.025$).

Role of gland engorgement in the enzyme changes after hypophysectomy. The results of the experiment in which hypophysectomized rats were treated with oxytocin 5 hr. after operation to allow the removal of accumulated milk are shown in Table 1. When these rats were killed 24 hr. after hypophysectomy the glands were free of any signs of milk engorgement, in contrast with the glands of similarly hypophysectomized rats which had not received oxytocin, which were distended and exuded large quantities of milk when minced. The activities of the mammary gland enzymes of the normally lactating control rats used in Expt. 2 show some variations from those used in Expt. 1 and the relative changes after hypophysectomy are larger. However, the enzymes show the same pattern of activity as in the first experiment, with glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucosutase and UDP-glucose pyrophosphorylase decreasing to 25% of their initial value, ATP-citrate lyase and phosphofructokinase to 7% and acetyl-CoA carboxylase to less than 1%. The differences produced by oxytocin treatment are very slight, none being significant, though there is a tendency for activities to be higher in the treated animals. However, it is apparent that gland engorgement is not a major factor in producing the enzyme changes after hypophysectomy.

Changes in activities of mammary gland enzymes after weaning. The effects of removing the litter on the activities of the mammary gland enzymes of the mother are shown in the fourth and fifth lines of Table 1. All activities have declined after 12 hr. and, after 24 hr., in all cases except UDP-glucose pyrophosphorylase and acetyl-CoA carboxylase, they are lower than the comparable values after hypophysectomy. For glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and phosphoglucosutase the differences between the activities after hypophysectomy and after weaning are significant (Fisher's *t* test, $P < 0.05$). The pattern of change after weaning is very similar to that after hypophysectomy with acetyl-CoA carboxylase showing the largest decline, ATP-citrate lyase and phosphofructokinase an intermediate one, and UDP-glucose pyrophosphorylase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and phosphoglucosutase the smallest decline.

Fig. 1. Activities of some enzymes of mammary gland at various times after hypophysectomy. Rats killed at zero time were normally lactating controls. Vertical lines represent the s.e.m. of the enzyme activities and the numbers in parentheses are the numbers of rats in each group. Further details are given in the Materials and Methods section. (a) Glucose 6-phosphate dehydrogenase; (b) 6-phosphogluconate dehydrogenase; (c) phosphoglucomutase; (d) UDP-glucose pyrophosphorylase; (e) phosphofructokinase; (f) ATP-citrate lyase; (g) acetyl-CoA carboxylase.

Table 1. Activities of some enzymes of the rat mammary gland after hypophysectomy or removal of the litter

Enzyme activities are expressed as μ moles of substrate utilized/min./mg. of gland DNA $\pm$ s.e.m. The numbers in parentheses are the activities expressed as a percentage of the activity in the normal lactating rat. Details of the assays and treatment of the rats are given in the Materials and Methods section.

Status of rats	No. in group	Enzyme activities						
		ATP-citrate lyase	Phospho-fructo-kinase	UDP-glucose pyrophosphorylase	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase	Phosphoglucomutase	Acetyl-CoA carboxylase
Tenth day of lactation	9	1.45 $\pm$ 0.25 (100)	0.86 $\pm$ 0.13 (100)	13.4 $\pm$ 1.3 (100)	23.3 $\pm$ 1.7 (100)	3.73 $\pm$ 0.61 (100)	1.40 $\pm$ 0.18 (100)	0.56 $\pm$ 0.07 (100)
24 hr. after hypophysectomy	7	0.104 $\pm$ 0.033 (7)	0.061 $\pm$ 0.016 (7)	4.55 $\pm$ 0.6 (34)	5.92 $\pm$ 0.78 (25)	0.794 $\pm$ 0.098 (21)	0.334 $\pm$ 0.043 (24)	0.008 $\pm$ 0.005 (1)
24 hr. after hypophysectomy; oxytocin-treated	6	0.120 $\pm$ 0.047 (8)	0.064 $\pm$ 0.018 (7)	5.0 $\pm$ 0.45 (37)	6.43 $\pm$ 0.56 (28)	1.02 $\pm$ 0.09 (27)	0.453 $\pm$ 0.053 (32)	0.002 $\pm$ 0.001 (0)
12 hr. after removal of litter	5	0.901 $\pm$ 0.062 (62)	0.288 $\pm$ 0.075 (33)	5.45 $\pm$ 0.75 (41)	13.1 $\pm$ 1.4 (56)	1.68 $\pm$ 0.21 (45)	0.800 $\pm$ 0.165 (57)	0.121 $\pm$ 0.033 (22)
24 hr. after removal of litter	5	0.050 $\pm$ 0.011 (3)	0.025 $\pm$ 0.002 (3)	5.4 $\pm$ 1.55 (40)	3.69 $\pm$ 0.40 (16)	0.354 $\pm$ 0.052 (9)	0.174 $\pm$ 0.053 (12)	0.004 $\pm$ 0.001 (1)

DISCUSSION

It is assumed that the results reported above arise from changes in the enzyme composition of the epithelial secretory cells and not from changes in the proportions of different types of tissues. This assumption can be justified by the rapidity of the changes, the preponderance of epithelial tissue in the fully lactating gland (Reese & Eversole, 1964) and the failure of electron-microscopic studies to detect any specialization of function in the secretory cells (Bargmann & Knoop, 1959; Wellings, DeOrme & Pitelka, 1960).

Any assessment of the effects of changes in enzyme activities on the metabolism of a tissue *in vivo* must be tentative, but it is a reasonable postulate that to have an immediate effect such changes must involve enzymes catalysing rate-limiting reactions. The metabolism of glucose 6-phosphate in the rat mammary gland will be considered.

1. The pentose phosphate shunt accounts for about half the glucose carbon atoms incorporated into acetyl-CoA (Abraham & Chaikoff, 1959; McLean, 1964). However, McLean (1960, 1964) has shown that the flux along this pathway is limited not by the enzymes of the pathway but by the rate of re-oxidation of NADPH, which in turn is largely dependent on the rate of fatty acid synthesis. According to Howanitz & Levy (1965), the rate-limiting enzyme of fatty acid synthesis in the rat mammary gland, as in other tissues, is acetyl-CoA carboxylase. Thus the rapid decline in acetyl-CoA carboxylase activity after hypophysectomy and weaning will not only prevent fatty acid synthesis but will inhibit the activity of the pentose phosphate shunt.

2. The Embden-Meyerhof pathway accounts for the remainder of acetyl-CoA synthesis from glucose. The control of this pathway has not been studied in detail in the mammary gland but in a variety of rat tissues which have been examined, the step catalysed by phosphofructokinase has been shown to be rate-limiting (Passonneau & Lowry, 1964; Bücher & Rüssmann, 1964; Williamson, 1965). Assuming this applies in the mammary gland the large decrease of phosphofructokinase activity during involution will reduce the potential flux through this pathway. Because of the complex control of this enzyme the changes in the flux *in vivo* will depend on the relative concentration of activators and inhibitors. Wang (1960) reported large decreases in the ratios ATP/AMP and ADP/AMP in the gland after weaning and these changes will tend to increase phosphofructokinase activity by reducing inhibition by ATP (Mansour, 1963). However, the decline in enzyme activity is probably large enough to produce some decrease of flux *in vivo*.

3. The other major glucose 6-phosphate-utilizing pathway is that concerned with lactose synthesis, the control of which has not been elucidated. As both phosphoglucomutase and UDP-glucose pyrophosphorylase possess significant activity after milk synthesis has ceased they do not presumably play a rate-limiting role.

Thus the changes in enzyme activities after hypophysectomy and weaning should stop fatty acid synthesis and cause a large reduction in the formation of acetyl-CoA from glucose 6-phosphate. Assuming that fuels other than glucose are not important this will lead to reduced citric acid-cycle activity and a reduced availability of ATP for synthetic processes. Of course, other rate-limiting enzymes, not studied here, may also be important in producing a decline in synthetic activity. In the absence of glycogen reserves in the mammary gland (Folley & French, 1949), hexokinase (glucose-ATP phosphotransferase) plays a key role in glucose metabolism but as the activity is still about 40% of its peak value 4 days after weaning (McLean, 1958; Shatton *et al.* 1965), a critical function in the involutory process is not suggested.

These predictions, based on changes in enzyme activity, are compatible with findings of other workers with gland slices. Weaning leads to a reduction in glucose utilization (M. L. McNaught, unpublished work quoted by Folley, 1961) and a decline in the R.Q. (Folley & French, 1949), which may be interpreted as resulting from a decline in fatty acid synthesis. Similar changes follow hypophysectomy (Bradley & Cowie, 1956). The increase in lactic acid production noted by these workers within 4 hr. of hypophysectomy is not explicable in terms of the enzyme changes reported here but may reflect a shift in the oxidation state of the nicotinamide nucleotides after a decrease in fatty acid synthesis.

The close resemblance between the changes after hypophysectomy and weaning suggests that these two methods of producing gland involution must at some level exert a common action. Two hypotheses can be offered to explain this.

1. The primary cause of involution is a general reduction in the synthetic activity of the secretory cells, which could be produced in various ways, and the subsequent changes in enzyme patterns are a function of the properties of the individual enzymes.

2. The primary cause of involution is a change in the pattern of enzyme synthesis which in turn leads to a change in cell metabolism and a reduction in synthetic activity. Hypophysectomy and weaning must in some way exert a common effect on enzyme synthesis.

Considering the first hypothesis, it is known that different enzymes in a tissue may have widely differing rates of turnover (Berlin & Schimke,

1965), so that a change in the rate of protein synthesis causes enzyme activities to vary at different speeds and a temporary change in the ratios of activity is produced which reverts to normal when a new steady state is achieved. However, the changes in the mammary gland after hypophysectomy do not seem to be of this type as the changed pattern of enzyme activity persists during the period 12–24 hr. after hypophysectomy when activities are almost constant, and the changes, though of widely differing magnitudes, all occur during the initial 12–16 hr. period. Thus the second hypothesis seems to fit the observations better.

The hormonal control of enzyme synthesis has been demonstrated in many tissues (for review see Karlson, 1965). For the mammary gland the evidence indicates that prolactin is the hormone most closely concerned with maintaining the enzyme pattern. Prolactin plus corticoids can initiate lactation in a suitably prepared rat in the absence of the pituitary (Lyons, Li & Johnson, 1958; Abraham, Cady & Chaikoff, 1960), and prolactin can initiate secretion in mammary gland explants from pregnant mice in the presence of insulin and aldosterone (Rivera, 1964). A lack of corticoids is unlikely to cause short-term changes after hypophysectomy, as adrenalectomy during lactation produces comparatively slow changes (Bradley & Cowie, 1956; Greenbaum & Darby, 1964; Willmer & Foster, 1965). As the accumulation of milk does not play a role in the changes after hypophysectomy, a lack of prolactin leading to a change in the pattern of protein synthesis seems the most reasonable hypothesis to explain these changes.

The changes that follow weaning cannot simply be ascribed to a decline in prolactin secretion, though according to current theories of the control of this secretion this may well occur (Folley, 1961; Cowie & Tindal, 1964; Meites & Nicoll, 1966). This is because unilateral involution, produced by preventing milk removal from the glands on one side of the rat, leads to changes very similar to those produced by removal of the litter (M. L. McNaught, unpublished work quoted by Folley, 1961; McLean, 1964; Spencer & Lowenstein, 1966). Unilateral involution cannot be under simple endocrine control as the glands still suckled continue to function normally. Thus some local factor associated with the cessation of milk removal can cause changes in the gland metabolism; this factor, combined with a lack of prolactin, may produce the changes seen after the removal of the litter. How this combined action can lead to changes in enzyme activities similar to those after hypophysectomy is a question that cannot be answered without further investigation.

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