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Co-ordinate Changes in Enzymes of Fatty Acid Synthesis, Activation and Esterification in Rabbit Mammary Gland during Pregnancy and Lactation

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1. The activities of fatty acid synthetase, acyl-CoA synthetase, glycerol phosphate acyltransferase and phosphatidate phosphatase were measured in the mammary glands of rabbits from day 16 of pregnancy to day 15 *post partum*. 2. There were significant correlations between the increases in activities of these enzymes during this period. This was the case whether the activities were expressed per mg of homogenate protein, per g wet wt. of tissue or per total wet weight of the whole glands. The only exception was the lack of correlation between the activities of fatty acid synthetase and of phosphatidate phosphatase per g wet wt. of tissue. 3. These co-ordinate increases are discussed in relation to the changes which occur in fatty acid metabolism in the mammary gland during pregnancy and lactation.

The mammary gland undergoes differentiation during pregnancy so as to produce milk at parturition. Triacylglycerol, which is a major component of milk, is synthesized from fatty acids derived from the blood and from fatty acids synthesized within the gland itself. The activities of the enzymes of fatty acid biosynthesis dramatically increase in a co-ordinated manner in the gland during the period of pregnancy to lactation (Gul & Dils, 1969; Hartman & Jones, 1970; Gumaa *et al.*, 1973; Mellenberger & Bauman, 1974).

The present study was designed to investigate whether similar increases occur in the activities of enzymes responsible for glycerolipid synthesis in mammary gland and whether these enzymes behave as a co-ordinated group. If they do not respond in this way, it may be possible to identify those enzymes which have a regulatory function from those which are more constitutive in nature.

The enzyme activities investigated during pregnancy and lactation were acyl-CoA synthetase (EC 6.2.1.3), glycerol 3-phosphate acyltransferase (EC 2.3.1.15) and phosphatidate phosphatase (EC 3.1.3.4). The activity of the fatty acid synthetase system was also measured to provide a comparison with enzymes of fatty acid biosynthesis. The enzyme activities were measured in the nuclei-free homogenate of rabbit mammary gland, since fatty acid activation and esterification may take place in both the mitochondria and endoplasmic reticulum, and phosphatidate phosphatase can be largely recovered in the particle-free supernatant fraction of tissue homogenates.

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Materials and Methods

Animals

Pregnant and lactating New Zealand White rabbits were supplied by the University of Nottingham Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K. The virgin animals were at least 6 months old and pregnancy was timed from the day of mating. The gestation period for these rabbits was 31–32 days. They were fed *ad libitum* with rabbit pellets, which were supplied by F. E. Stevens Ltd., Trent Corn Mills, Shardlow, Derby, U.K. The rabbits were killed by cervical dislocation. Each experiment was done with mammary tissue obtained from a single rabbit.

Materials

The chemicals and the enzymes used were purchased or prepared as described previously (Sánchez *et al.*, 1973; Brindley & Bowley, 1975; Lloyd-Davies & Brindley, 1975). Acetyl-CoA was a gift from Dr. B. K. Speake of this Department. Malonyl-CoA and NADPH were obrained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Preparation of nuclei-free homogenate from mammary gland

As soon as the rabbits were killed, the mammary glands were quickly dissected away from muscle, adipose and connective tissues. Excess of milk was removed from the lobulo-alveolar tissue by repeated washing with ice-cold 0.25M-sucrose which had been adjusted to pH7.4 with $KHCO_3$. The tissue was finely chopped with scissors and then homogenized in this medium (approx. 4ml/g wet wt.) by using a Teflon pestle in a stainless-steel vessel. The homogenate was filtered through nylon gauze and then centrifuged for 10min at 4°C and 800*g* ($r_{max.}$ 10.7cm) to yield a nuclei-free homogenate. The freshly prepared homogenate was used to study enzyme activities.

Enzyme assays

The activity of each enzyme was determined at several protein concentrations under optimum assay conditions. This ensured that the reaction rate was proportional to the enzyme concentration. The assays were usually done in duplicate.

(*a*) *Palmitoyl-CoA synthetase.* This was assayed by two different methods, each of which was based on those described by Brandes *et al.* (1973). In the first assay, which measures total palmitoyl-CoA synthetase activity, palmitoyl-CoA was generated from palmitate, CoA, ATP and Mg^{2+}. In the second assay, palmitoyl-AMP was used as the direct precursor of palmitoyl-CoA. The second assay only measures that portion of the palmitoyl-CoA synthetase activity which is able to use palmitoyl-AMP as substrate.

(i) Palmitoyl-CoA synthesis from palmitate. The activity was measured by following the disappearance of CoA from the assay system in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid). Each assay contained in a volume of 0.6ml:125mM-Tris buffer, adjusted to pH7.4 with HCl, 5mM-NaF, 1.67mM-EDTA, 1mg of fatty acid-poor bovine serum albumin, 5mM-ATP, 16.7mM-$MgCl_2$, 0.8mM-potassium palmitate and 208μM-CoA. The reaction was started by adding up to 500μg of protein from nuclei-free mammary homogenates from 16-day-pregnant rabbits, or up to 30μg of protein from nuclei-free mammary homogenates from lactating rabbits. The mixtures were incubated for 10min at 37°C. The reaction was stopped with 0.2ml of 15% (w/v) trichloroacetic acid. The mixture was shaken, cooled in ice and the sediment removed by centrifugation in a bench centrifuge. Samples (0.2ml) of the clear supernatant were added to 0.8ml of a mixture which consisted of 3vol. of 0.2M-potassium phosphate buffer, pH7.4, and 1vol. of a solution of 0.54μM-KOH in 0.4M-potassium phosphate buffer, pH6.8 (Brandes *et al.*, 1973). Then 50μl of 10mM-5,5′-dithiobis-(2-nitrobenzoic acid) in 0.2M-potassium phosphate buffer, pH7.4, was added and the A_{413} of the solution was measured immediately. The results were calculated by using $\varepsilon_{413} = 13600$litre·mol^{-1}·cm^{-1} for CoA (Means & Feeney, 1971).

(ii) Palmitoyl-CoA synthesis from palmitoyl-AMP. The assay contained in a volume of 0.6ml: 125mM-Tris buffer adjusted to pH7.4 with HCl, 5mM-NaF, 1.67mM-EDTA, 1mg of fatty acid-poor bovine serum albumin, 20mM-$MgCl_2$, 1mM-palmitoyl-AMP and 333μM-CoA. The reaction was started by adding up to 500μg of protein from nuclei-free mammary homogenates from 16-day-pregnant rabbits, or up to 80μg of protein from nuclei-free mammary homogenates from lactating rabbits. The mixture was incubated for 10min at 37°C. The reaction was stopped with 0.2ml of 15% (w/v) trichloroacetic acid. The concentration of CoA in the supernatant was determined as described above for assay (i).

(*b*) *Glycerol 3-phosphate palmitoyltransferase.* This was assayed by measuring the incorporation of *sn*-[1,3-^{3}H]glycerol 3-phosphate into lipid in a system in which palmitoyl-CoA was generated from palmitoylcarnitine. Each assay contained in a volume of 0.25ml: 25mM-Tris buffer, adjusted to pH7.4 with HCl, 5mM-dithiothreitol, 100μM-CoA, 0.4mM-palmitoyl-(−)-carnitine, 1.5mg of fatty acid-poor bovine serum albumin, an excess (approx. 200μg of protein) of carnitine palmitoyltransferase (EC 2.3.1.21) and 4mM-*sn*-[1,3-^{3}H]glycerol 3-phosphate (0.25μCi/μmol). The reaction was started with up to 500μg of protein from nuclei-free mammary homogenates from 16-day-pregnant rabbits, or up to 50μg of protein from nuclei-free mammary homogenates from lactating rabbits. The mixture was incubated for 6min at 37°C. The reaction was stopped with 1.88ml of chloroform/methanol (1:2, v/v) and lipids were extracted by the method of Hajra *et al.* (1968) by using the washing procedure of Brindley & Bowley (1975). In a number of experiments, the lipid extract was analysed by t.l.c. as described by Brindley & Bowley (1975). About 70% of the radioactivity was incorporated into phosphatidate and 30% was incorporated into lysophosphatidate. Only trace amounts of radioactive diacylglycerol could be detected.

(*c*) *Phosphatidate phosphatase.* The assay system used was essentially that described by Brindley & Bowley (1975). Each assay contained, in a final volume of 0.25ml:20mM-potassium phosphate buffer, pH6.8, 1.25mg of fatty acid-poor bovine serum albumin, 560μg of microsomal protein containing [^{3}H]phosphatidate (Brindley & Bowley, 1975). EDTA (0.5mM) was added to adjust the concentration of Mg^{2+} bound to phosphatidate to that required for optimum reaction rates. The mixtures were incubated for 15min at 37°C.

(*d*) *Fatty acid synthetase.* This was assayed spectrophotometrically as described by Speake *et al.* (1975).

Determination of radioactivity and of protein concentrations

These have been described previously (Sánchez *et al.*, 1973).

Statistical procedures

The results obtained were analyzed statistically as described by Bradford-Hill (1967).

Results and Discussion

Table 1 shows the activities of the enzymes expressed per mg of nuclei-free homogenate protein and per g wet wt. of mammary gland. These values have not been corrected for retained milk or for mammary fluid, since previous work (Gul & Dils, 1969) showed that anomalous results can be obtained if the lactose content of the milk or retained fluid is used for this correction. On the basis of the lactose content, the calculated weight of fluid retained in pregnant-rabbit mammary glands exceeded the total wet weight of the gland. Kuhn & Lowenstein (1967) found no correlation between the presence of lactose and of visible milk in the mammary glands of parturient rats.

Enzyme activities could also have been related to DNA to provide an estimate of the activities per cell. However, this does not necessarily reflect the activities in those cells which are synthesizing milk lipids, since there is a variety of cell types in the tissue used for this study, and these change in proportion during development. In addition, Sod-Moriah & Schmidt (1968) found that the DNA content of the nuclei of epithelial cells from rabbit mammary gland varies between animals, between glands from the same animal and between areas within the same gland. The DNA content of the nuclei appeared to be high at times of intense secretory activity and low at times of secretory quiescence. However, the total DNA/g wet wt. of rabbit mammary gland does not change significantly throughout pregnancy (Hartman & Jones, 1970). At the beginning of lactation, this content increases up to 1.2-fold (B. Gul & R. Dils, unpublished work; D. E. Bauman, personal communication) or up to twofold (Hartman & Jones, 1970). The DNA content then remains constant until day 15 of lactation (Hartman & Jones, 1970). Gul & Dils (1969) studied a number of enzymes involved in supplying cofactors and substrates for fatty acid biosynthesis in rabbit mammary gland and found a correlation when these enzyme activities were expressed relative to DNA and to protein. It was therefore decided that there was no advantage in expressing the results relative to DNA.

The activities of acyl-CoA synthetase and of glycerol phosphate acyltransferase were measured by using palmitate as the acyl donor. The latter substrate was chosen because the rates of glycerolipid synthesis from glycerol phosphate in lactating-rabbit mammary gland were essentially similar when fatty acyl-CoA esters were generated from acylcarnitines of chain lengths $C_{10:0}$–$C_{16:0}$. However, a preferential esterification of palmitate was observed in this tissue when acyl-CoA esters were produced by the endogenous acyl-CoA synthetase (Breach & Dils, 1975).

The activities of the enzymes of glycerolipid synthesis and of the fatty acid synthetase system increased progressively through the period of pregnancy to day 15 of lactation (Table 1). The patterns of the changes were essentially similar for the two different ways of expressing the enzyme activities. The most dramatic effect was seen in the increases in the activity of the fatty acid synthetase system. These are consistent with the results reported by Mellenberger & Bauman (1974) and by Speake *et al.* (1976) for the fatty acid synthetase system in this tissue. Of the enzymes shown in Table 1, the increases in the activity of phosphatidate phosphatase were the least marked. It is noteworthy that the specific activity of the total palmitoyl-CoA synthetase was approx. 10 times that of glycerol phosphate acyltransferase, phosphatidate phosphatase, and the fatty acid synthetase system (based on nmol of fatty acid products). This need not imply, however, that such a difference in enzymic rates occurs in the mammary gland *in vivo*.

The results in Table 1 and also the activities of the enzymes expressed relative to the total wet weight of mammary-gland tissue (not shown) were examined by linear-regression analysis. This established that there are significant temporal correlations between the increases in the activities of the enzymes, whether expressed per mg of homogenate protein, per g wet wt. of tissue or per total gland (r in the range 0.65–0.93; $P<0.05$–0.001). The exception was a lack of significant correlation between the activities of phosphatidate phosphatase and of fatty acid synthetase when expressed per g wet wt. of tissue ($r=0.51$; $P>0.1$). Nevertheless this correlation was significant when the activities of these two enzymes were expressed per mg of homogenate protein ($r=0.82$; $P<0.01$), and all of the enzyme activities shown in Table 1 were significantly correlated with the weight of the mammary tissue (r in the range 0.61–0.93; $P<0.05$–0.001). This demonstrates that as the weight of the mammary gland increases so do the specific activities of the fatty acid synthetase system and of the enzymes of glycerolipid synthesis.

The rate of palmitoyl-CoA synthesis was measured either from palmitate plus ATP, or from palmitoyl-AMP. Brandes *et al.* (1973) reported that these two procedures do not determine the same activity in liver and that these activities show different responses to dietary change and to diabetes. The explanation offered for this was that either there are

Table 1. *Activities of enzymes of fatty acid synthesis, activation and esterification in rabbit mammary gland*

Enzyme activities were measured as described in the Materials and Methods section. They are given as nmol of substrate transformed/min per mg of nuclei-free homogenate protein or per g wet wt. of tissue. The activity of fatty acid synthetase is expressed in terms of nmol of NADPH oxidized/min. n.d., Not detectable.

	Palmitoyl-CoA synthesis from palmitate		Palmitoyl-CoA synthesis from palmitoyl-AMP		Glycerol 3-phosphate palmitoyltransferase		Phosphatidate phosphatase		Fatty acid synthetase		Total wet weight of glands (g)
Time	Per mg of homogenate protein	Per g wet wt. of tissue	Per mg of homogenate protein	Per g wet wt. of tissue	Per mg of homogenate protein	Per g wet wt. of tissue	Per mg of homogenate protein	Per g wet wt. of tissue	Per mg of homogenate protein	Per g wet wt. of tissue	
Pregnancy											
Day 16	13	130	n.d.	n.d.	1.1	11	2.4	24	5.9	60	24
22	11	170	n.d.	n.d.	2.7	42	1.4	22	2.6	40	23
29	27	530	7.7	150	2.8	55	2.3	45	15	290	40
31	31	580	17	310	4.4	81	3.7	65	8.7	160	54
Post partum											
Day 1	150	1180	20	160	11	91	6.1	48	140	1070	95
2	93	1090	27	320	10	120	5.6	65	180	2090	102
3	130	1110	25	210	15	130	5.5	46	150	650	133
3	110	1250	23	270	7	86	5.0	59	130	750	139
15	90	950	43	440	15	150	4.4	45	230	2900	142
15	140	2100	42	620	18	270	5.7	85	160	2310	138
15	190	2840	36	540	16	250	4.1	60	280	3420	115

at least two palmitoyl-CoA synthetases, or that the enzyme can undergo a conformational change which makes it impossible for one form of the protein to accept palmitoyl-AMP as a substrate. As stated above, the two different assays give results with mammary gland which correlated significantly with each other. The present results therefore give no indication that such a situation exists in rabbit mammary gland.

There are a number of other differences in the relationships between the enzymes of fatty acid metabolism in mammary gland and in liver. In mitochondria (Sánchez *et al.*, 1973) and microsomal fractions (Lloyd-Davies & Brindley, 1973, 1975) of rat liver there was no correlation between the activity of palmitoyl-CoA synthetase and the rate of glycerol phosphate esterification with palmitate. However, the activities correlated in rabbit mammary gland (Table 1) as they did in the small-intestinal mucosa of adult (Brindley, 1973) and of foetal and neonatal (Short *et al.*, 1975) guinea pigs. In rat liver there is no direct correlation between the activities of glycerol phosphate acyltransferase and of the fatty acid synthetase system, and it was suggested that the former may be a constitutive enzyme of the endoplasmic reticulum in this tissue (Wiegand *et al.*, 1974). The activities of glycerol phosphate esterification and of the fatty acid synthetase system correlate in rabbit mammary gland (Table 1) and it appears that a similar relationship occurs in rat adipose tissue (Dodds *et al.*, 1976). In general, where there are changes in the flux of fatty acids to triacylglycerol synthesis in rat liver there are relatively minor changes in the activities of fatty acyl-CoA synthetase and of glycerol phosphate acyltransferase. The largest effects are observed with phosphatidate phosphatase (Mangiapane *et al.*, 1973; Lamb & Fallon, 1974). However, the changes in specific activity of phosphatidate phosphatase observed in Table 1 were the least marked of all the enzymes studied.

These differences are probably accounted for by differences in metabolism in these organs. In the small intestine, lactating mammary gland and adipose tissue, the major route of fatty acid metabolism is directed to the synthesis of triacylglycerols. Therefore one might expect a close relationship to exist between the activities of enzymes of fatty acid biosynthesis, fatty acid activation and of the subsequent esterification of fatty acids to produce triacylglycerol. In liver, other routes of fatty acid metabolism are relatively more important with respect to the rate of triacylglycerol synthesis. The control of fatty acid metabolism is therefore probably different in liver.

The relationship between fatty acid synthesis and glycerolipid synthesis changes in the mammary gland during its development. From day 12 to day 18 of pregnancy, rabbit mammary gland synthesizes mainly long-chain fatty acids. After day 18 there is a striking increase in the rate of fatty acid synthesis, and the fatty acid products consist mainly of $C_{8:0}$ and $C_{10:0}$ acids, which are characteristic of rabbit milk (Strong & Dils, 1972; Mellenberger & Bauman, 1974). Between days 16 and 30 of pregnancy the proportion of acetate incorporated into triacylglycerol *in vitro* increases from about 48 to 90%; there are corresponding decreases in the proportions incorporated into diacylglycerol and phospholipid (Strong & Dils, 1972). Some of the phospholipid synthesized is required for membrane formation in the developing mammary gland. The relative contributions to this process of fatty acids derived from the blood or from synthesis within the gland are not known. Neither is it certain to what extent the changes in the activities of the enzymes of glycerolipid synthesis observed in Table 1 are involved with cell proliferation rather than milk-fat synthesis.

The increases in the rate of fatty acid biosynthesis which are observed in the mammary gland during pregnancy through to lactation are paralleled by increases in the activities of enzymes which supply cofactors and substrates for this process (Gul & Dils, 1969; Hartman & Jones, 1970; Gumaa *et al.*, 1973; Mellenberger & Bauman, 1974). The enzymes include ATP citrate lyase (EC 4.1.3.8), acetyl-CoA synthetase (EC 6.2.1.1), acetyl-CoA carboxylase (EC 6.4.1.2), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44). A close temporal relationship was demonstrated (Mellenberger & Bauman, 1974) between the rate of fatty acid synthesis measured in rabbit mammary gland *in vitro* and the activities of ATP citrate lyase ($P<0.01$) and of acetyl-CoA carboxylase ($P<0.01$). The increases in activities of glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) and $NADP^+$–isocitrate dehydrogenase (EC 1.1.1.42) were smaller than those of the other enzymes mentioned, and the activity of $NADP^+$–malate dehydrogenase (EC 1.1.1.40) was negligible at all the periods tested (Mellenberger & Bauman, 1974). The uptake of fatty acids from the blood is also increased during this period, and this is facilitated by increases in the activity of lipoprotein lipase (EC 3.1.1.34) (Scow *et al.*, 1973).

We can say that the increased supply of fatty acids from the blood and from synthesis *de novo* in the mammary gland is accompanied by co-ordinate increases in the activities of enzymes responsible for fatty acid activation and esterification. The factors which control the co-ordinated increase in this group of enzymes, which are metabolically related, are not fully understood. However, the increased rate of triacylglycerol synthesis that occurs in rabbit mammary gland between pregnancy and lactation (Strong & Dils, 1972) can be mimicked by culturing mammary

explants from mid-pregnant rabbits with insulin, corticosterone and prolactin (Strong *et al.*, 1972; Forsyth *et al.*, 1972).

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