Biochem. J. (1967) **105**, 225
Printed in Great Britain

Regulation of Triglyceride Synthesis in the Parturient Guinea-Pig Mammary Gland

By N. J. KUHN*
Department of Biochemistry, University of Oxford

(*Received* 17 *January* 1967)

1. The specific activity of the enzyme palmitoyl-CoA–L-glycerol 3-phosphate palmitoyltransferase (EC 2.3.1.15) in the mammary tissue of guinea pigs has been shown to increase 37-fold at parturition. 2. Increases also occur in tissue concentrations of glycerol 3-phosphate, CoA and free fatty acid, but not in that of acid-insoluble CoA. 3. The isolation and fatty acid composition of plasma triglyceride and of mammary-tissue free fatty acid, diglyceride and triglyceride are described. 4. The findings are discussed in relation to the regulation of milk fat synthesis.

In the preceding paper (Kuhn, 1967) evidence was provided for the presence of palmitoyl-CoA–L-glycerol 3-phosphate palmitoyltransferase in lactating guinea-pig mammary glands. The probable role of this enzyme in triglyceride synthesis was demonstrated by showing the reaction product *in vitro* to be phosphatidic acid, which, in the same preparation, was further metabolized to glycerides under suitable conditions.

The present paper adduces support for the association of this enzyme with the biosynthesis of milk fat by showing a rapid increase in its activity in the tissue *post partum*. Observations on the concentrations of metabolites in the tissue before and after parturition are given, together with measurements of the fatty acid composition of mammary-gland triglyceride, diglyceride, free fatty acids and plasma triglyceride. The interpretation of the findings in relation to the regulation of lactogenesis is discussed.

METHODS

Transferase assay. 'Mitochondrial+microsomal' fractions (MM fractions) were prepared as previously described (Kuhn, 1967) and assayed by the method of Kornberg & Pricer (1953), which measures the incorporation of L-[^{14}C]-glycerol 3-phosphate into an acid-insoluble ethanol-soluble form. Reaction mixtures contained: MM fraction (0·02ml., equivalent to 0·02g. of tissue); sodium phosphate buffer, pH6·5 (20μmoles); serum albumin (2·5mg.); cysteine, pH7 (1·0μmole); palmitoyl-CoA (approx. 127mμmoles); L-glycerol 3-phosphate (1·57μmoles, $2{\cdot}32\times10^6$counts/min.). The final volume was 0·2ml. Reactions were started by the addition of enzyme and carried out at 37°. Each tissue preparation was assayed at 1min. intervals over the first 5min. and showed linear incorporation of label over this period.

Since the initial palmitoyl-CoA concentrations (0·59–0·73mM) were already such as to partially inhibit the transferase, the measured activities have been corrected to obtain the activities at optimum concentrations of this substrate. The required correction factor was 1·65, on the basis of the curve shown in the preceding paper (Kuhn, 1967, Fig. 7). The correction did not alter the pattern of results.

Tissue extractions. Guinea pigs were anaesthetized by intraperitoneal injection with 15–20mg. of Nembutal [60mg./ml. in 10% (v/v) ethanol–20% (v/v) propylene glycol, diluted fivefold with 0·9% NaCl]. Mammary glands were dissected free of surrounding fatty and connective tissue, care being taken to leave the blood supply intact and unoccluded, and to see that the animals were respiring well throughout. The glands were removed by rapid freezing *in situ* (Hohorst, Kreutz & Bücher, 1959). The frozen tissue was powdered in a mortar and extracted with 10vol. of 5% (w/v) $HClO_4$ at 0° by further grinding in the mortar. The slurry was centrifuged for 30min. in the cold at 78000*g* in a Spinco model L preparative ultracentrifuge, and the clear supernatant withdrawn and neutralized with solid $KHCO_3$ to pH6·7. The extract remaining after removal of the precipitated $KClO_4$ was analysed for glycerol, glycerol 3-phosphate and CoA+acetyl-CoA.

The tissue residue was re-extracted with acid, the extract being discarded. The residue was frozen and divided into two, roughly equal, weighed portions for subsequent determination of free fatty acids and long-chain acyl-CoA.

Metabolite determinations. (*a*) CoA+acetyl-CoA. These were determined together by the arsenolysis method of Stadtman (1955). To avoid erroneously low values due to the presence of inhibitory substances (probably high salt concentrations), each extract was assayed in the following manner. To four separate tubes, each containing an identical portion of the extract (about 0·3ml.), were added 0, 1, 2 and 3mμmoles of standard CoA respectively. The arsenolysis of acetyl phosphate, catalysed by the total CoA present, was then measured over a 1 or 1½hr. reaction period at 30° and compared with that in a blank tube containing everything but CoA (either as standard or extract). By plotting graphically the extent of arsenolysis in each tube against standard CoA added, a straight line is obtained that,

* Present address: Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge.

when produced to cut the abscissa, gives the amount of CoA present in the extract sample. This device amounts to comparing the unknown with the standard CoA under identical conditions, and gives results proportional to the amount of extract used.

(*b*) Long-chain acyl-CoA. The tissue residue, after two $HClO_4$ extractions, still contains CoA that becomes acid-soluble only after alkaline hydrolysis. The only known CoA derivatives with these properties are those of long-chain fatty acids, and it has been shown by Wieland, Löffler, Weiss & Neufeldt (1960) and Tubbs & Garland (1964) that the acid-insoluble CoA of rat liver is all of this nature. Until this identification has been made in mammary gland, it is assumed to hold true for this tissue also. The concentration was determined by CoA assay after alkaline hydrolysis for 45min. at pH 10·5 at room temperature (Bortz & Lynen, 1963).

(*c*) Glycerol and glycerol 3-phosphate. These were successively determined on the same sample by the method of Wieland (1963). Where necessary, the tissue extract was concentrated by freeze-drying before assaying the glycerol 3-phosphate.

(*d*) Free fatty acids. These were extracted from a portion of the tissue residue by the method of Dole & Meinertz (1960). In some cases, the final heptane extract was washed with 0·05% H_2SO_4 (Trout, Estes, & Friedberg, 1960) to remove contaminating titratable substances that might be present. Only with tissue extracts from pregnant animals did this make a significant difference. Free fatty acids were titrated with 0·01 N-NaOH as described by Salaman & Robinson (1961).

(*e*) Ester determinations were by the method of Stern & Shapiro (1953).

Isolation of lipid fractions. Twenty lactating mammary glands (105g. of tissue) were removed by the method of rapid freezing and homogenized directly in 2l. of chloroform–methanol (2:1, v/v) in a Waring Blendor. The extraction mixture was stood in the dark for 9½ hr. at room temperature and then filtered. The clear filtrate was evaporated to dryness in a rotary-film evaporator at 30–40° and the residue evacuated with an oil pump for 1½ hr. This yielded 8·8g. of yellow oil, which was diluted to 50ml. with chloroform. Then 40g. of Mallinckrodt A.R. silicic acid (100 mesh), activated overnight at 115°, was suspended in chloroform and poured into a glass column. After being washed for 1 day with chloroform, the column was loaded with 25ml. of lipid extract, and eluted successively with chloroform (850ml.) followed by 5% (600ml.), 10% (700ml.), 15% (1200ml.) and 20% (700ml.) methanol in chloroform (v/v). Fractions eluted by methanol in chloroform contained phosphatides, and were concentrated and examined by thin-layer chromatography for the presence of phosphatidic acid. None was detected. Although two spots appeared close to the position of reference phosphatidic acid, they did not release glycerol 3-phosphate after elution and mild alkaline hydrolysis (Dawson, 1960).

The chloroform eluate, containing the free fatty acids and neutral lipids, was evaporated to dryness, redissolved in *n*-hexane and fractionated on a column of Florisil as described by Carroll (1961). From these fractions, triglyceride and diglyceride were further purified by thin-layer chromatography, under the conditions already described (Kuhn, 1967), and were further characterized by analysis, which gave theoretical glycerol/ester ratios. Free fatty acids, eluted fron the Florisil, were identified by thin-layer chromatography. Glycerides were saponified and the fatty acids esterified with diazomethane in ether before examination by gas–liquid chromatography.

Qualitative identification was performed by gas–liquid chromatography on two different columns [Apiezon L high-vacuum grease (10%, w/w) and polyethylene glycol adipate (25%, w/w) on 100–200-mesh Celite] by comparing retention times of major peaks with those of pure standard methyl esters. Their carbon numbers were plotted graphically against the logarithm of their retention times (Woodford & van Gent, 1960), giving a straight line. The carbon numbers of other peaks were derived from the graph, by using their measured retention times. Identification of the unknown fatty acids was performed by a comparison of their derived carbon numbers with values given for known fatty acids by Enser & Bartley (1962).

Fatty acids were determined quantitatively by measuring their peak areas (Pecsok, 1959). Trials with an artificial equimolar mixture of pure palmitic acid, stearic acid, oleic acid and linoleic acid methyl esters showed a variation of ±9% about the mean.

Materials. L-[^{14}C]Glycerol 3-phosphate, diglyceride and phosphatidic acid were prepared as described previously (Kuhn, 1967). Phospholipase D, glycerokinase, glycerol 3-phosphate dehydrogenase, phosphotransacetylase, CoA, ATP and NAD were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Acetyl phosphate was synthesized by the method of Stadtman (1957). Palmitoyl-CoA was prepared by the method of Seubert (1959). Pure fatty acids were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and Calbiochem, Los Angeles, Calif., U.S.A. Cardiolipin and sphingomyelin were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., ethanolamine phosphatide was from Mann Research Laboratories Inc., New York, N.Y., U.S.A., and bovine serum albumin (Cohn fraction V) was from Armour Pharmaceutical Co., Eastbourne, Sussex. Thin-layer chromatography was performed on apparatus from Desaga, Heidelberg, Germany. A.R. chloroform, methanol and ether and redistilled reagent-grade hexane were used for column chromatography.

RESULTS

Transferase assays. Assays were performed on mammary tissue taken from guinea pigs either just before parturition (estimated within 1 day of term) or at known times thereafter. The results given in Fig. 1 show a 37-fold increase of specific activity during the 2–3 days *post partum*. It is clear that the enzyme increase has begun by parturition, though the measurements do not show whether any increase has occurred *pre-partum* or not. A mean activity of 0·74 μmole/min./g. fresh wt. at 37° is found at 2–3 days *post partum* and, since the total tissue fresh weight also increases about 2½-fold over this period, there is approximately a 100-fold increase of total enzyme activity.

Measurements were also made on two animals that, after 3 days of lactation, were withdrawn from their litters for a further 2 days. By this time the

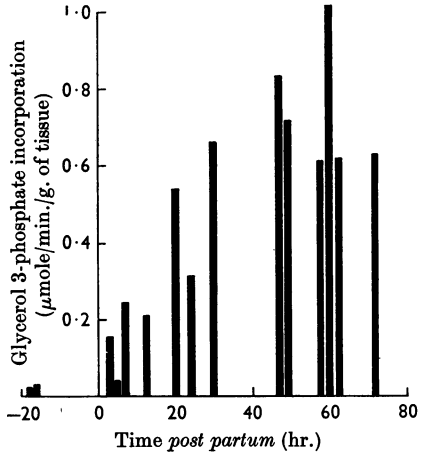

Fig. 1. Variation in specific activity of mammary-gland transferase before and after parturition.

glands were extremely swollen with milk, which had also penetrated into the surrounding tissue. The true weights of these glands were taken as 10·9g., this being the mean of the weights in the group of normal animals at 2–3 days *post partum*. The transferase specific activities in these glands were 0·35 and 0·32μmole of glycerol 3-phosphate incorporated/min./g. fresh wt., which is about half the mean value at 2–3 days of lactation.

Tissue metabolite concentrations. L-Glycerol 3-phosphate, glycerol, long-chain acyl-CoA, CoA + acetyl-CoA and free fatty acids were determined in the mammary glands of lactating guinea pigs during the first week *post partum*, and in those of animals just before parturition. In addition, the effects of anoxia and of tying off one nipple to prevent suckling were noted.

Table 1 compares the mean values obtained from lactating and from pregnant animals. Glycerol 3-phosphate concentrations rise nearly threefold at parturition, though the concentration is still low by comparison with values published for rat liver (Hohorst *et al.* 1959; Bortz & Lynen, 1963; Tzur, Tal & Shapiro, 1964). However, the techniques of handling the tissue described in the last two references may have involved anoxia, which can lead to rapid increases in tissue glycerol 3-phosphate (see below). Though the increase in glycerol concentration is not statistically significant, a significant rise in the concentration of this metabolite has been found in rat mammary gland at the time of parturition (N. J. Kuhn, unpublished work). There is essentially no change in the concentration of acid-insoluble CoA, a surprising finding in view of the twofold increases of both CoA + acetyl-CoA and free fatty acids. Two perchloric acid extracts, assayed before and after alkaline hydrolysis, showed no increase in CoA + acetyl-CoA content. It is therefore concluded that essentially all the tissue CoA is accounted for by CoA, acetyl-CoA and acid-insoluble CoA (assumed to be long-chain acyl-CoA).

In a separate experiment (the results of which are not given in detail) acetyl-CoA was concentrated from the pooled perchloric acid extracts of the mammary glands from five lactating guinea pigs, by the procedure of Wieland *et al.* (1960), and determined by the method of Ochoa, Stern & Schneider (1951). A tissue concentration of 5mμmoles/g. fresh wt. was measured, after correction for the percentage recovery as determined on extracts to which known amounts of acetyl-CoA had been added. Acetyl-CoA therefore accounts for less than 10% of the measured 'CoA + acetyl-CoA'.

It is noteworthy that, of the total CoA (free + esterified) measured in these glands, as much as 63% (pregnant) and 42% (lactating) was in the form of the long-chain acyl ester, stressing the importance of this ester in mammary metabolism. The absolute concentration of long-chain acyl-CoA compares with that reported for normal rat liver [15mμmoles/g. (Bortz & Lynen, 1963); 52mμmoles/g. (Tubbs & Garland, 1964)]. The values for CoA + acetyl-CoA, which are similar to the value for CoA in normal rat liver (47mμmoles/g.; Garland, 1964), also compare favourably with those of Ringler, Becker & Nelson (1954) in guinea-pig mammary gland, and with those of Lauryssens, Peeters & Donck (1956) in rat mammary gland. The latter authors showed an increase from 33mμmoles/g. fresh wt. at parturition to 95mμmoles/g. fresh wt. at 15 days of lactation.

Very high concentrations of free fatty acids were found in the present experiments. There is unlikely to be any gross contamination by plasma free fatty acids, since the concentration of these in the plasma of various species is only about 1μmole/ml. (Fredrickson & Gordon, 1958). It is possible that some phosphatide degradation has occurred during the perchloric acid treatment, but no experiments were done to examine this possibility.

Further determinations were performed on animals of which one nipple had been tied 13–15hr. beforehand. Since each gland has only a single nipple, this procedure stops milk withdrawal from that gland, leaving the opposite one to be suckled. Table 2 compares metabolite concentrations in the two glands of each of five animals. The mean values for the tied glands are given, and may be compared with the mean values for normal glands in Table 1. There is no consistent change in the concentration of any metabolite.

Experiments were carried out on three lactating animals, in each of which the one gland was removed under 'normal' oxygenated conditions, the other

Table 1. *Metabolite concentrations in mammary tissue of lactating and late-pregnant guinea pigs*

Values are expressed per g. fresh wt. of tissue, and are given as means ± S.E.M. with the numbers of animals used in parentheses.

Physiological state	Glycerol 3-phosphate (mμmoles/g.)	Glycerol (mμmoles/g.)	Long-chain acyl-CoA (mμmoles/g.)	CoA+ acetyl-CoA (mμmoles/g.)	Free fatty acids (μmoles/g.)
Lactating	89±11 (11)	260±52 (9)	44±6 (10)	62±7 (10)	4·62±0·13 (9)
Pregnant	32±7 (4)	186±10 (5)	52±12 (6)	31±3 (6)	2·38±0·41 (6)

Table 2. *Metabolite concentrations in mammary glands with tied (a) and untied (b) nipples*

Mean values ± S.E.M. for glands with tied nipples are given. Values are expressed per g. fresh wt. of tissue.

Guinea pig	Glycerol 3-phosphate (mμmoles/g.)	Glycerol (mμmoles/g.)	Long-chain acyl-CoA (mμmoles/g.)	CoA+ acetyl-CoA (mμmoles/g.)	Free fatty acids (μmoles/g.)
1 (*a*)	74	446	22	56	3·62
(*b*)	148	440	30	81	4·90
2 (*a*)	66	608	28	68	5·87
(*b*)	106	580	26	32	5·27
3 (*a*)	64	371	57	77	3·20
(*b*)	114	310	67	56	4·28
4 (*a*)	147	186	63	92	4·78
(*b*)	121	103	70	94	4·68
5 (*a*)	173	198	49	53	2·73
(*b*)	116	170	45	58	3·79
Mean (*a*) values	105±21	362±71	44±7	69±6	4·04±0·51

Table 3. *Metabolite concentrations in normal and anoxic mammary glands of lactating guinea pigs*

Values are expressed per g. fresh wt. of tissue.

Guinea pig	State of gland	Period of anoxia (min.)	Glycerol 3-phosphate (mμmoles/g.)	Glycerol (mμmoles/g.)	Long-chain acyl-CoA (mμmoles/g.)	CoA+ acetyl-CoA (mμmoles/g.)	Free fatty acids (μmoles/g.)
1	Anoxic	2	212	172	64	—	4·17
	Normal	—	35	104	70	—	4·63
2	Anoxic	4	237	—	24	53	6·30
	Normal	—	28	—	26	55	5·10
3	Anoxic	6	299	314	44	74	4·52
	Normal	—	87	308	51	94	4·69

being removed after short period of anoxia caused by clamping the vascular supply. Table 3, which shows the metabolite concentrations in these tissues, reveals a large and rapid increase in glycerol 3-phosphate. This resembles the findings of Hohorst *et al.* (1959) with respect to lactate in anoxic rat liver. In particular, it should be noted that no change occurred in the long-chain acyl-CoA concentrations.

Fatty acid composition of glycerides and free fatty acids. Triglyceride, diglyceride and free fatty acid fractions were isolated from lactating tissue, as well as plasma triglyceride from the same animals, as described in the Methods section, and analysed for their component long-chain fatty acids. The percentage fatty acid composition for each lipid fraction is shown in Table 4. The present study is concerned only with major fatty acids, and the following conclusions may be drawn:

(1) Palmitate, oleate and linoleate together

Table 4. *Fatty acid composition of plasma triglyceride and mammary-gland free fatty acids and glycerides*

	Fatty acid composition (%, by wt.)					
		Mammary gland				
Fatty acid	Plasma triglyceride	Free fatty acids	Diglyceride	Triglyceride	Diglyceride *a*	Diglyceride *b*
Lauric acid	—	0·3	—	—	—	—
Myristic acid	0·9	0·9	1·4	1·3	0·7	0·3
$C_{15:1}$ acid	1·1	0·3	0·5	0·3	0·2	0·8
Palmitic acid	22·6	23·7	24·6	21·3	13·4	40·7
Palmitoleic acid	2·0	1·7	3·8	2·3	1·6	5·4
$C_{17:2}$ acid*	1·7	0·6	1·7	0·7	0·9	1·4
Stearic acid	3·6	7·6	3·8	3·9	3·1	4·6
Oleic acid	23·8	26·4	27·8	29·2	36·9	30·4
Linoleic acid	40·7	24·7	33·5	36·6	37·6	5·3
Unknown	4·2	3·8	3·0	4·3	4·2	8·5
$C_{20:1}$ acid*	—	0·9	—	1·3	1·0	—
Unknown	—	9·1	—	—	—	—

* Tentatively identified.

make up 75% of all the fatty acids in each lipid fraction.

(2) Stearate is the next most common identified fatty acid.

(3) The percentages of palmitate and oleate are essentially constant throughout the fractions. In relation to other fractions, the percentage of linoleate is low in the free fatty acid fraction and rather high in the plasma triglyceride.

(4) The picture obtained is generally consistent with the idea that free fatty acid, diglyceride and triglyceride bear precursor–product relationships to each other, and that plasma triglyceride is a major source of the long-chain fatty acids in mammary gland.

The diglyceride isolated from the tissue extract on Florisil columns was partially resolved into three fractions, which revealed quite different fatty acid compositions. The fastest and slowest of these are represented in Table 5 as 'diglyceride *a*' and 'diglyceride *b*' respectively. The faster fraction contained less than 18% of saturated fatty acids, and the slowest contained only 5·3% of linoleate. It is clear that the diglyceride fraction comprises molecules of very different composition.

DISCUSSION

Since guinea pigs are born at a relatively late stage of foetal development, their milk demand is high immediately after birth but continues for only 1–2 weeks, by which time the young have weaned themselves. This explains the pattern of milk yield found by Nelson, Kaye, Moore, Williams & Herrington (1951) where a rapid increase to a peak at 2 days *post partum* is followed by a relatively rapid decline. In the face of these requirements, a rapid increase of transferase activity during the first days *post partum* may be seen as demonstrating its role in milk triglyceride synthesis. A similar pattern of increase was earlier shown for clearing-factor lipase (McBride & Korn, 1963; Robinson, 1963), which is believed to cause the uptake of plasma triglyceride by the lactating mammary gland. The mean specific activity of the transferase at 2–3 days *post partum* was 0·74 μmole of glycerol 3-phosphate incorporated/min./g. fresh wt. Since, from the results of Nelson *et al.* (1951) on the milk yield and fat content at the peak of lactation, the guinea-pig mammary glands must esterify about 0·019 μmole of glycerol 3-phosphate/min./g. fresh wt., it is clear that abundant transferase exists to carry this out. However, it must be remembered that these transferase activities refer to optimum concentrations of glycerol 3-phosphate and palmitoyl-CoA. These substrates are almost certainly present at sub-optimum concentrations *in vivo*, as is shown by the data in Table 1.

In the present study, possible factors that may regulate triglyceride formation have been examined. Other workers have studied this mainly in other tissues. For adipose tissue, glucose availability is generally considered a major regulating factor (Fredrickson & Gordon, 1958; Bally, Cahill, Leboeuf & Renold, 1960; Randle, Hales, Garland & Newsholme, 1963) through its capacity to provide glycerol 3-phosphate. In liver, where phosphatide synthesis is also of importance, the situation is less clear. High concentrations of free fatty acids and long-chain acyl-CoA are associated with low rate of lipogenesis (Bortz & Lynen, 1963; Tubbs & Garland,

1964) and triglyceride release (Otway & Robinson, 1967) during starvation. Contradictory values are available for concentrations of glycerol 3-phosphate (Bortz & Lynen, 1963; Tzur *et al.* 1964; J. M. Lowenstein, unpublished work). The small contribution of phosphatide to milk fat (Rhodes & Lea, 1958/9) and the sudden onset of milk fat synthesis at parturition, together with the low rate of fatty acid oxidation (Annison, Fazakerley, Linzell & Nichols, 1965), make the mammary gland a suitable tissue for examining the regulation of glyceride synthesis.

The transferase studied here is a branch-point enzyme catalysing an irreversible step. Phosphatidic acid and diglyceride, the two successive reaction products, do not accumulate in the tissue despite large quantities of triglyceride. The measured activity of the transferase *in vitro* is sufficient for the known rate of milk fat synthesis, and the pattern of activity increase after parturition closely resembles the pattern of milk yield. The tissue concentrations of the substrates glycerol 3-phosphate and long-chain acyl-CoA do not fall when lactation begins, suggesting that the enzymes concerned in their formation are at least as active as the transferase. This is supported by the rapid rise of tissue glycerol 3-phosphate concentration that is induced by anoxia (Table 3), and in rat mammary gland the dehydrogenase is present in great excess, both before and after parturition (Baldwin & Milligan, 1966; N. J. Kuhn, unpublished work). These facts are consistent with the role of the transferase as a rate-limiting enzyme on the pathway of milk fat synthesis, though they do not prove it.

Concentrations of long-chain acyl-CoA do not change at parturition, despite the increases in the concentrations of free fatty acids and CoA shown here, and the rise in ATP concentration shown for the mammary tissue of the rat (Wang & Greenbaum, 1960) and cow (R. A. Freedland, unpublished work quoted by Hansen & Carlson, 1961). Either this is a fortuitous similarity between two steady-state levels of metabolism, or else unknown factors work to maintain constant concentrations. The latter possibility is supported by the finding that the increased concentrations of glycerol 3-phosphate caused by anoxia do not produce a fall in long-chain acyl-CoA concentration, despite the very small pool size. The possibility that long-chain acyl-CoA might regulate the activity of the transferase through inhibition at high concentrations has been considered previously (Kuhn, 1967).

The mean concentration of glycerol 3-phosphate in the lactating tissue (89 mμmoles/g. fresh wt.) is well below the K_m (2·7 mM) measured previously (Kuhn, 1967), so that this substrate is almost certainly not saturating the transferase. Hence the threefold rise in glycerol 3-phosphate concentration at parturition could, by itself, account for a part of the increased rate of esterification.

Glycerol 3-phosphate probably originates from two sources in this tissue. In other species isotopic-tracer experiments (Kleiber *et al.* 1955; Popják, Hunter & French, 1953) and the demonstration of glycerol 3-phosphate dehydrogenase (Baldwin & Milligan, 1966) have indicated that glycerol 3-phosphate may be derived from the Embden–Meyerhof pathway via dihydroxyacetone phosphate. The recent demonstration of glycerokinase (McBride & Korn, 1964*a*) has shown that it may also be derived from glycerol, of which the present study has demonstrated appreciable tissue concentrations. Glycerol could appear either by the action of clearing-factor lipase on plasma triglyceride (McBride & Korn, 1963; Robinson, 1963; Barry, Bartley, Linzell & Robinson, 1963) or through the action of unknown lipases on mammary-tissue fat. The reversible nature of the reaction catalysed by glycerol 3-phosphate dehydrogenase and the irreversible nature of that catalysed by glycerokinase suggest that, whereas the latter enzyme may lead to net synthesis of glycerol 3-phosphate, the former may enable its absolute tissue concentration to be determined by the concentration of dihydroxyacetone phosphate and the $NAD^+/NADH$ concentration ratio in the cell sap.

It is therefore possible to regard the great increase of triglyceride synthesis at parturition as primarily the result of increased transferase activity, whereas the concentration of glycerol 3-phosphate may act as a finer control to relate rates of triglyceride synthesis with the metabolic activity of carbohydrate degradation.

Current evidence suggests that plasma triglyceride is a major source of the long-chain fatty acids of milk fat (Barry *et al.* 1963; McBride & Korn, 1964*b*), and the evidence provided in the preceding paper (Kuhn, 1967) suggests that the biosynthetic pathway involves free fatty acids, long-chain acyl-CoA, phosphatidic acid and diglyceride as successive intermediates. One might therefore expect those substances to carry a similar complement of fatty acids. Although reported analyses of these intermediates in rat liver (Hübscher & Clark, 1960; Bortz & Lynen, 1963; Connellan & Masters, 1965) reveal almost no overlap of composition, liver has a more complicated metabolism, and some of these substances may not be acting there purely as biosynthetic intermediates. In mammary gland, where fatty acid oxidation is relatively unimportant (Annison *et al.* 1965), the role of such intermediates is more easily defined. In the present study, long-chain acyl-CoA was not successfully isolated, and phosphatidic acid could not even be qualitatively detected (cf. Hölzl, 1965). The other intermediates were analysed, however, and showed reasonable

uniformity of fatty acid composition, with palmitate, oleate and linoleate as the major fatty acids of each. This is consistent with the proposed biosynthetic pathway. On the other hand, it must be pointed out that mammary-gland fat is not necessarily identical with milk fat, and the heterogeneity of the cell population in this tissue complicates interpretation of results.

I am grateful to Dr D. S. Robinson for the hospitality of his Laboratory and for his supervision during the work described in this and the preceding paper. I also thank Mr J. H. D. Kent and his staff for providing lactating guinea pigs, and the Medical Research Council for their financial support through a research scholarship. Gas–liquid chromatography was kindly performed by Mr A. Renshaw.

REFERENCES

Annison, E. F., Fazakerley, S., Linzell, J. L. & Nichols, B. W. (1965). *Biochem. J.* **94**, 21P.

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

Bally, P. R., Cahill, G. F., Leboeuf, B. & Renold, A. E. (1960). *J. biol. Chem.* **235**, 333.

Barry, J. M., Bartley, W., Linzell, J. L. & Robinson, D. S. (1963). *Biochem. J.* **89**, 6.

Bortz, W. & Lynen, F. (1963). *Biochem. Z.* **339**, 77.

Carroll, K. K. (1961). *J. Lipid Res.* **2**, 135.

Connellan, J. H. & Masters, C. J. (1965). *Biochem. J.* **94**, 81.

Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.

Dole, V. P. & Meinertz, M. (1960). *J. biol. Chem.* **235**, 2595.

Enser, M. & Bartley, W. (1962). *Biochem. J.* **85**, 607.

Fredrickson, D. S. & Gordon, R. S. (1958). *Physiol. Rev.* **38**, 585.

Garland, P. B. (1964). *Biochem. J.* **92**, 10C.

Hansen, R. G. & Carlson, D. M. (1961). In *Milk: The Mammary Gland and Its Secretion*, vol. 1, p. 371. Ed. by Kon, S. K. & Cowie, A. T. New York: Academic Press Inc.

Hohorst, H. J., Kreutz, F. M. & Bücher, Th. (1959). *Biochem. Z.* **332**, 18.

Hölzl, J. (1965). *Biochem. Z.* **341**, 168.

Hübscher, G. & Clark, B. (1960). *Biochim. biophys. Acta*, **41**, 45.

Kleiber, M., Black, A. L., Brown, M. A., Baxter, C. F., Luick, J. R. & Stadtman, F. H. (1955). *Biochim. biophys. Acta*, **17**, 252.

Kornberg, A. & Pricer, W. E. (1953). *J. biol. Chem.* **204**, 345.

Kuhn, N. J. (1967). *Biochem. J.* **105**, 213.

Lauryssens, M., Peeters, G. & Donck, A. (1956). *Ann. Endocr., Paris*, **17**, 543.

McBride, O. W. & Korn, E. D. (1963). *J. Lipid Res.* **4**, 17.

McBride, O. W. & Korn, E. D. (1964*a*). *J. Lipid Res.* **5**, 442.

McBride, O. W. & Korn, E. D. (1964*b*). *J. Lipid Res.* **5**, 459.

Nelson, W. L., Kaye, A., Moore, M., Williams, H. H. & Herrington, B. L. (1951). *J. Nutr.* **44**, 585.

Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.

Otway, S. & Robinson, D. S. (1967). *J. Physiol.* **190**, 321.

Pecsok, R. L. (1959). In *Principles and Practice of Gas Chromatography*, p. 135. Ed. by Pecsok, R. L. London: Chapman and Hall, Ltd.

Popják, G., Hunter, G. D. & French, T. H. (1953). *Biochem. J.* **54**, 238.

Randle, P. J., Hales, C. N., Garland, P. B. & Newsholme, E. A. (1963). *Lancet*, i, 785.

Rhodes, D. N. & Lea, C. H. (1958/9). *J. Dairy Res.* **25/26**, 60.

Ringler, I., Becker, N. & Nelson, W. L. (1954). *Arch. Biochem. Biophys.* **52**, 348.

Robinson, D. S. (1963). *J. Lipid Res.* **4**, 21.

Salaman, M. & Robinson, D. S. (1961). In *The Enzymes of Lipid Metabolism*, p. 218. Ed. by Desnuelle, P. Oxford: Pergamon Press Ltd.

Seubert, W. (1959). *Biochem. Prep.* **7**, 80.

Stadtman, E. R. (1955). In *Methods in Enzymology*, vol. 1, p. 596. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, p. 229. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Stern, I. & Shapiro, B. (1953). *J. clin. Path.* **6**, 158.

Trout, D. L., Estes, E. H. & Friedberg, S. J. (1960). *J. Lipid Res.* **1**, 199.

Tubbs, P. K. & Garland, P. B. (1964). *Biochem. J.* **93**, 550.

Tzur, R., Tal, E. & Shapiro, B. (1964). *Biochim. biophys. Acta*, **84**, 18.

Wang, D. V. & Greenbaum, A. L. (1960). *Biochem. J.* **77**, 552.

Wieland, O. (1963). In *Methods of Enzymatic Analysis*, p. 211. Ed. by Bergmeyer, H. U. London: Academic Press (Inc.) Ltd.

Wieland, O., Löffler, G., Weiss, L. & Neufeldt, I. (1960). *Biochem. Z.* **333**, 10.

Woodford, F. P. & van Gent, C. M. (1960). *J. Lipid Res.* **1**, 188.