Rat Mammary-Gland Acetyl-Coenzyme A Carboxylase

INTERACTION WITH MILK FATTY ACIDS

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1. Highly purified rat mammary-gland acetyl-CoA carboxylase was inhibited by milk obtained from rats 12h after their young were weaned. 2. All the inhibitory activity was found in the particulate fraction (R_{105}) obtained on centrifuging the milk. It could be extracted from milk fraction R_{105} with acetone and identified as a complex mixture of non-esterified fatty acids, present in high concentration (nearly 10mM) in the milk. 3. Inhibition of acetyl-CoA carboxylase was observed at low concentrations (0.2–20 μ M) of several of these fatty acids when fresh fully active enzyme was used. Enzyme that had been partly inactivated by aging, or by storing in the absence of citrate, was stimulated by low concentrations but inhibited by high concentrations of fatty acids. 4. Various experiments suggested that fatty acids produce irreversible inactivation of acetyl-CoA carboxylase. 5. The effects of palmitoyl-CoA on mammary-gland acetyl-CoA carboxylase were found to resemble those of fatty acids, except that palmitoyl-CoA was effective at lower concentration. 6. The effect of milk fraction R_{105} was tested on six other enzymes previously shown to decline to various extents after weaning. Although several of these enzymes were affected by unfractionated milk fraction R_{105} , none was significantly inhibited by the acetone extract or by low concentrations of lauric acid. 7. The findings are consistent, both qualitatively and quantitatively, with a regulatory mechanism whereby milk fatty acids shut off fatty acid synthesis in the mammary gland after weaning by inhibiting acetyl-CoA carboxylase.

Some years ago we noted the marked correlation between the rapid cessation of fatty acid synthesis, measured in rat mammary-gland extracts, and the accumulation of milk engorging the glands during the first few hours after the weaning of the young. This effect could be produced in extracts prepared from mammary glands that the young were prevented from suckling, whereas extracts from the contralateral suckled glands remained fully active (Levy, 1964). Reasoning that an inhibitor might be present in the milk, we added rat milk to extracts prepared from suckled rat mammary glands and observed strong inhibition of fatty acid synthesis. Further experiments demonstrated that mammary acetyl-CoA carboxylase [acetyl-CoA- CO_2 ligase (ADP), EC 6.4.1.2] was markedly inhibited by non-esterified fatty acids associated with the particulate fractions of milk (Levy, 1963*b*, 1964). Since the acetyl-CoA carboxylase reaction may be the rate-limiting step of fatty acid synthesis

(Ganguly, 1960; Numa, Matsushashi & Lynen, 1961; Korchak & Masoro 1962; Martin & Vagelos, 1962; Howanitz & Levy, 1965) the foregoing results suggested that mammary-gland fatty acid synthesis was regulated through feedback inhibition by fatty acids. Substantiation of this proposal required the isolation and characterization of the inhibitor from milk, and an investigation of the interactions between inhibitor and highly purified enzyme. Recently we reported the isolation of highly purified rat mammary-gland acetyl-CoA carboxylase (Miller & Levy, 1969). In the present paper we describe the isolation and characterization of the milk inhibitor and its interaction with the enzyme. (It should be noted that the terms 'inhibitor' and 'inhibition' are used in a general sense. They are not to be construed as implying reversible inhibition, susceptible to the usual kinetic analyses; as will become evident in the Discussion section, it is unlikely that the effects described in this paper conform to such a mechanism.)

A preliminary report of this work has been given (Miller & Levy, 1968).

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EXPERIMENTAL

Assay of purified acetyl-CoA carboxylase. The procedures for assaying acetyl-CoA carboxylase were described by Miller & Levy (1969). Unless noted otherwise, these assays were conducted by using assay B (Miller & Levy, 1969), which provides optimum conditions, except that bovine serum albumin was omitted. In the description of the assay procedure that follows, numbers in parentheses refer to concentration in the final assay mixture (0.5 ml); concentrations of those components present during the 15 min activation period, when the volume was 0.4 ml, were correspondingly higher. Imidazole-HCl, pH 7.5 (60 mM), potassium citrate (30 mM), MgCl₂ (24 mM), 2-mercaptoethanol (0.3 mM) and enzyme were incubated for 15 min at 37°C in a volume of 0.4 ml to activate the enzyme. The assay was then started by the addition of the following: acetyl-CoA (0.63 mM), neutralized ATP (7.2 mM) and NaH¹⁴CO₃ (22.4 mM, 4.5 μCi/ml). The volume was 0.5 ml and the pH was 7.5. The reaction was allowed to proceed for 10 min, and the procedures previously described to stop the reaction and to assay for [¹⁴C]-malonyl-CoA (Miller & Levy, 1969) were applied. When the effects of fatty acids or other compounds on acetyl-CoA carboxylase were tested they were included in the 15 min activation period.

The experiments were performed with enzyme either from step 6 or from step 7 (Table 2 in Miller & Levy, 1969). These enzyme preparations are referred to as 'pre-sucrose gradient' and 'purified' respectively throughout this paper; their specific activities, measured under optimum conditions (i.e. by using assay B including bovine serum albumin), were 2.5 and 5.5 μmol/min per mg of protein respectively.

Assay of enzymes in crude homogenate. Acetyl-CoA carboxylase and six other enzymes were assayed in crude 23000g mammary-gland supernatant fractions, prepared essentially as described for Step 1 in the purification of mammary acetyl-CoA carboxylase by Miller & Levy (1969). For most experiments the glands were homogenized in a buffer of the following composition: 35 mM-potassium phosphate, pH 7.5, 20% (w/v) glycerol, 1 mM-EDTA and 7 mM-2-mercaptoethanol. Homogenates in which acetyl-CoA carboxylase was to be assayed were prepared in Buffer 2 (see under 'Materials' below). All homogenates were stored frozen in small volumes and thawed when needed. Acetyl-CoA carboxylase was assayed as described for the purified enzyme above. The other enzymes were assayed by modifications of spectrophotometric procedures (Jones, 1967). These assays involved measuring extinction changes at 340 nm due to the production or utilization of NADH or NADPH. Since the principal objective of these assays was to test the effect of milk fraction R₁₀₅, which was too turbid to allow direct measurement of extinction changes, the procedure employed was as follows. Assays were first conducted directly in the spectrophotometer in the absence of fraction R₁₀₅ to ascertain appropriate conditions. These direct assays were also employed to test the effects of laurate and of fraction R₁₀₅ extract. Discontinuous assays were then conducted under conditions that ensured that the rate was linear over the assay period. Reaction mixtures were prepared in tubes as for the direct assays, with and without fraction R₁₀₅. At appropriate times they were plunged into

boiling water for 30 s to stop the reaction and immediately placed into ice. After the tubes were cool, NaOH was added to 0.15 M and the samples were centrifuged at 25000g for 15 min. The extinction of the clear supernatant was read at 340 nm in a Zeiss spectrophotometer and the reaction rates were determined from the differences in extinction of mixtures allowed to react for different times. All assays were conducted in duplicate. Rates calculated from these assays were 80–110% of those observed in the continuous assays except that with phosphofructokinase they were approx. 60% of the rates measured directly. This might be related to the alkali-lability of NAD⁺ (Kaplan, Colowick & Barnes, 1951).

ATP citrate lyase [ATP-citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8] was assayed essentially by the procedure of Srere (1959). The malate dehydrogenase required in this coupled assay was present in large excess in the mammary-gland homogenate. The reaction mixture contained 0.05 M-tris chloride, pH 7.4, 2.5 mM-MgCl₂, 0.02 M-potassium citrate, 0.01 M-2-mercaptoethanol, 0.1 mM-NADH, 3.3 mM-ATP, enzyme and 0.187 mM-CoA, which was used to start the reaction. In the discontinuous assay the reaction was allowed to proceed for 1 min and 7 min. Phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) was assayed by the procedure described by Jones (1967), which is based on the method of Mansour (1963). The ATP concentration was raised to 1.0 mM, as 0.2 mM-ATP employed by Jones (1967) gave lower rates, presumably because of contaminating adenosine triphosphatase. It was found that the mammary-gland extracts contained excess of glycerol 3-phosphate dehydrogenase, but it was necessary to add aldolase to ensure that phosphofructokinase was rate-limiting. The reaction mixture contained 0.04 M-tris chloride, pH 8.2, 1.0 mM-MgCl₂, 0.01 M-NH₄Cl, 4.0 mM-fructose 6-phosphate, 0.1 mM-NADH, 1.2 units of aldolase, enzyme and 1.0 mM-ATP, which was used to start the reaction. The discontinuous assay was allowed to proceed for 3½ and 8 min. Phosphoglucumutase (D-glucose 1,6-diphosphate-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) was assayed essentially as described by Jones (1967). The endogenous glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49), present in large excess, was used as coupling enzyme. The reaction mixture contained 0.069 M-tris chloride, pH 7.4, 2.1 mM-MgCl₂, 0.205 mM-NADP⁺, enzyme and 0.75 mM-glucose 1-phosphate, which initiated the reaction. The discontinuous assay was conducted from 2 to 9 min. Glucose 6-phosphate dehydrogenase was assayed as described by Levy (1963a), and 6-phosphogluconate dehydrogenase [6-phospho-D-gluconate-NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.44] was assayed essentially by the method of Glock & McLean (1953). The assay mixture for glucose 6-phosphate dehydrogenase contained 0.066 M-tris acetate, pH 8.6, 0.83 mM-NADP⁺, enzyme and 5.0 mM-glucose 6-phosphate, which was used to initiate the reaction. The discontinuous assay was conducted from 1 to 6 min. For 6-phosphogluconate dehydrogenase assays the conditions were the same except that 0.209 mM-NADP⁺ was used and the reaction was initiated with 6-phosphogluconate (final concentration 1.5 mM). UDP-glucose pyrophosphorylase (UDP-α-D-glucose 1-phosphate uridylyltransferase, EC 2.7.7.9) was assayed by the

method of Shatton, Gruenstein, Shay & Weinhouse (1965). It was necessary to use low concentrations of this enzyme to ensure linear dependence of velocity on enzyme concentration (see Table 9). The reaction mixture contained 0.1M-glycine adjusted to pH 9.2 with NaOH, 3.0mM-MgCl₂, 3.0mM-NAD⁺, 1.25mM-UTP, 7.5mM-glucose 1-phosphate and enzyme; the reaction was started with 250 units of UDP-glucose dehydrogenase. The discontinuous assay was conducted for 1 and 12 min. In this assay the addition of base after the heat inactivation was omitted, since it caused high readings at 340nm.

In all the discontinuous assays the enzyme was incubated with the reaction components (including fraction R₁₀₅ when added) before the reaction was initiated. This incubation period was 7 min for glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and UDP-glucose pyrophosphorylase, and 10 to 15 min for the other enzymes. Protein concentrations in the 23000g mammary-gland supernatant fractions were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Preparation of methyl esters of fatty acids. The acetone extract of milk fraction R₁₀₅ (see below) was evaporated to dryness at 0°C under N₂. Boron trifluoride (14%, w/v) in methanol (5ml) was added, the mixture was refluxed for 2–5 min on a steam bath and then transferred to a separating funnel containing 30ml of light petroleum (b.p. 30–60°C). To this was added 20ml of water and the mixture was shaken vigorously. The light-petroleum layer was filtered through Whatman no. 1 filter paper, that had been washed by refluxing with light petroleum, into a test tube with a Teflon-lined screw cap. The sample was evaporated to dryness at 0°C under N₂, redissolved in acetone and stored at 0°C under N₂ until needed.

Gas-liquid chromatography. The methyl esters prepared from the milk fraction R₁₀₅ were separated in a Perkin-Elmer model 801 gas-liquid chromatograph on a 6ft glass column of 0.076in internal diameter containing 8% by weight of butanediol succinate polyester on a support of acid-washed Chromasorb DMCS (mesh size 80–100). Purified N₂ was the carrier gas. A dual hydrogen flame-ionization detector was used, and the instrument was connected to a Leeds and Northrup Speedomax G recorder. The temperature of the column was programmed to increase generally from 60 to 100°C at the beginning of a run until a temperature of 180 or 190°C was attained. The injector and detector were generally set at 300 and 225°C respectively. The methyl esters prepared from the milk fraction R₁₀₅ were identified by comparing their retention times with those of standard fatty acid methyl esters chromatographed under identical conditions. Except where noted otherwise, identification was based on results from at least two runs employing different mixtures of standards. Estimates of the relative amounts of the various components in the samples prepared from milk fraction R₁₀₅ were made by measuring the areas of the recorded peaks by triangulation. These values were corrected to allow for the differential response of the flame-ionization detector to methyl esters of fatty acids of various chain lengths (Sternberg, Gallaway & Jones, 1962; Karmen, 1963).

Determination of Ca²⁺. The concentration of Ca²⁺ in milk fraction R₁₀₅ and extracted fraction R₁₀₅ was determined by using a Perkin-Elmer model 303 atomic absorption spectrophotometer.

Materials. The compositions of the two buffers used in the experiments with the purified acetyl-CoA carboxylase, and referred to as Buffer 1 and Buffer 2 respectively, were as follows: both contained 2-mercaptoethanol (7mM), EDTA (1.0mM for Buffer 1, 0.1mM for Buffer 2), glycerol (20%, v/v), imidazole-HCl (50mM), and both had a pH of 6.5; Buffer 2 also contained 20mM-potassium citrate. Palmitoyl-CoA was obtained from P. L. Biochemicals Inc. (Milwaukee, Wis., U.S.A.), [U-¹⁴C]glucose was from New England Nuclear Corp. (Boston, Mass., U.S.A.), 0.1% 2',7'-dichlorofluorescein was from Brinkmann Instruments Inc. (Westbury, N.Y., U.S.A.), boron trifluoride in methanol, standard qualitative and quantitative mixtures of fatty acid methyl esters and high-purity fatty acids were from Applied Science Laboratory Inc. (State College, Pa., U.S.A.), purified gases (N₂, H₂ and air) were from The Matheson Co. Inc. (New York, N.Y., U.S.A.), Nembutal was from Abbott Laboratories (North Chicago, Ill., U.S.A.) and Pitocin was from Parke, Davis and Co. (Detroit, Mich., U.S.A.). The following reagents were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.): ATP, UTP, CoA, NAD⁺, NADP⁺, NADH, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, 6-phosphogluconate, aldolase and UDP-glucose dehydrogenase. Glycine was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Calcium caseinate was purchased from Mann Research Laboratories (New York, N.Y., U.S.A.) and prepared as a 2% (w/v) suspension by brief treatment with an ultrasonic probe. The sources for other reagents and for the rats used were given previously (Miller & Levy, 1969). All other chemicals were reagent grade and all organic solvents and water were glass-distilled. Acetone was purified by refluxing with successive quantities of KMnO₄ until the violet colour persisted. It was dried over K₂CO₃, filtered and then fractionally distilled. Fatty acids were dissolved as their sodium or potassium salts by adding a slight excess of 1M-KOH (or 1M-NaOH) to an aqueous suspension and heating to 80–90°C. The solutions were stored at 4°C and rewarmed to 80–90°C before use.

RESULTS

Isolation and identification of inhibitor from milk

Milk fractions. Lactating Sprague-Dawley-derived rats, 15–19 days *post partum*, were separated from their young. Approx. 12h later they were anaesthetized by intraperitoneal injection of 2ml of Nembutal diluted tenfold in 0.9% sodium chloride (final concentration, 5mg/ml); subsequently 1ml of Pitocin, diluted tenfold in 0.9% sodium chloride (final concentration 1 unit/ml), was injected intraperitoneally. They were then milked with the aid of a specially constructed device which permitted collection of the milk at 0°C and under nitrogen. Generally 1.5–5ml of milk could be collected from one rat.

The milk was centrifuged for 4h at 105000g at 4°C in a Spinco model L ultracentrifuge (SW 39 rotor). Three distinct fractions were obtained: milk fat, a clear milk supernatant fraction (S₁₀₅)

and a heterogeneous sediment, the milk residue (fraction R_{105}). These fractions were carefully separated, and the milk fat and milk fraction R_{105} were each washed by resuspending in water and centrifuging for 1 h at 30000g at 4°C in an RC-2B Sorvall centrifuge. The milk fat and milk fraction R_{105} were then resuspended in water to a volume equal to that of the milk from which they were derived. Thus the concentrations of components in all the milk fractions were the same as they were in the unfractionated milk. Milk fraction S_{105} was also centrifuged at 30000g and 4°C for 1 h to free it of any residual fat or sediment (higher centrifugal force was required to sediment the fraction R_{105} into a tightly packed precipitate from whole milk than when it was resuspended in water).

It is evident from the results in Table 1 that milk inhibits acetyl-CoA carboxylase, and that the inhibitory activity is located in fraction R_{105} . Both milk fat and milk fraction S_{105} enhance the activity of the enzyme. In separate experiments it was demonstrated that the stimulatory effects of these milk fractions was totally abolished in the presence of fraction R_{105} . For example, in one experiment addition of 0.05 ml of milk fraction S_{105} gave 128% activity of the control, 0.05 ml of milk fraction R_{105} gave 32% activity, and adding 0.05 ml of each gave 30% activity. Neither the fat nor fraction S_{105} catalysed incorporation of [14 C]bicarbonate in the absence of enzyme.

The inhibition by milk or milk fraction R_{105} was not due to quenching of the counts in the liquid-scintillation spectrometer. This was demonstrated by performing assays in the usual manner, but using unlabelled bicarbonate and adding [14 C]glucose. The addition of 0.05, 0.10 or 0.15 ml of milk or milk fraction R_{105} to the assay gave no decrease in the counts recorded for [14 C]glucose.

Extraction of inhibitor. A procedure was developed whereby the inhibitor could be quantitatively extracted with acetone, identified and the effects of the extract on the enzyme tested. The aqueous suspension of milk fraction R_{105} (8 ml) was centrifuged for 1 h at 30000g at 4°C. The residue was extracted ten times with 5 ml volumes of acetone with the aid of a glass homogenizer, and the acetone extracts were combined. The extracted residue was washed ten times with water by homogenization and centrifugation, and then resuspended in water to 8 ml. This fraction R_{105} residue ($R_{105}R$) was tested for inhibitory activity. The results show (Table 2) that all inhibitory activity was extracted from fraction R_{105} .

The combined acetone extract was evaporated under nitrogen at 0°C until the volume was 4 ml. It was then divided into two 2.0 ml portions, one of which served for identification procedures and the other was used to test for the inhibitory activity.

Table 1. *Interaction of milk and milk fractions with acetyl-CoA carboxylase*

Purified enzyme, diluted in Buffer 2, was used for this experiment. Pre-sucrose gradient enzyme gave essentially the same results.

Addition	Volume (ml)	HCO ₃ ⁻ incorporated	
		(μmol/min per mg of protein)	(%)
None		2.82	[100]
Milk	0.05	1.05	38
	0.10	0.62	22
	0.15	0.44	15
Milk fat	0.05	4.90	173
	0.10	4.90	173
	0.15	4.58	162
Fraction S_{105}	0.05	5.30	188
	0.10	3.19	114
	0.15	2.30	82
Fraction R_{105}	0.05	1.01	36
	0.10	0.75	27
	0.15	0.24	12

Table 2. *Interaction of fractions $R_{105}R$ and $R_{105}E$ with acetyl-CoA carboxylase*

Pre-sucrose gradient enzyme diluted in Buffer 2 was used; similar results were obtained with purified enzyme.

Addition	Volume (ml)	HCO ₃ ⁻ incorporated	
		(μmol/min per mg of protein)	(%)
None		1.22	[100]
Fraction $R_{105}R$	0.05	1.25	102
	0.10	1.21	98
	0.15	1.17	95
Fraction $R_{105}E^*$	0.025	0.97	79
	0.05	0.57	46
	0.075	0.30	24
	0.10	0.27	21
	0.15	0.18	14

* Concentrations of fatty acids for the five additions, based on dry-weight determinations (see the text), were 0.98, 1.95, 2.93, 3.90 and 5.86 mM respectively.

Except for the final step, these two 2.0 ml portions were then treated identically, as follows. The extracts were further concentrated until all the acetone was removed and only an aqueous residue remained. Water and 0.1 M-hydrochloric acid were added until the pH was 2 and the volume 10 ml. This solution was extracted three times with an equal volume of light petroleum (b.p. 30–60°C) and then three times with an equal volume of diethyl ether. These extracts were combined, dried with anhydrous sodium sulphate and then transferred to pre-weighed tubes with Teflon-lined screw caps. Each extract was evaporated to dryness at 0°C under nitrogen and the tubes were reweighed. The net weights of

the two residues were 7.5 and 9.0mg respectively. One of the samples was dissolved in 0.5ml of acetone and used for identification procedures (see below). To the other sample, water and 0.1M-potassium hydroxide were added until the volume was 2.0ml and the pH was 7.0. The sample was heated to 80°C with gentle swirling until most of the material had dissolved; a small residue resisted solubilization and remained as a very fine suspension. This fraction R_{105} extract ($R_{105}E$) was tested for inhibitory activity (Table 2). The results demonstrate that the inhibitory activity was extracted by the acetone, and recovered in the alkaline fraction R_{105} extract. It should be noted that, whereas the concentration of fraction $R_{105}R$ was the same as that of fraction R_{105} , that of fraction $R_{105}E$ was twice as great.

Identification and quantification of inhibitor. Samples of the acetone solution of the fraction R_{105} extract were spotted on Eastman Chromogram sheets impregnated with silica gel (Distillation Products Industries, Rochester, N.Y., U.S.A.) and developed with a solvent system (Marinetti, 1965) consisting of *n*-heptane-di-isobutyl ketone-acetic acid (85:15:1, by vol.). Ascending chromatography was carried out in an Eastman developing apparatus for 45 min at room temperature. The chromatograms were dried for 2h, sprayed with 2',7'-dichlorofluorescein and examined with u.v. light. A single spot with R_F 0.87 was observed. A mixture of fatty acids chromatographed on the same sheet migrated with R_F 0.85. Marinetti (1965) showed that in this system phospholipids and monoglycerides do not migrate, diglycerides travel with a low R_F , and triglycerides and free fatty acids migrate well, but are clearly separated.

These results from the t.l.c. suggested that the fraction R_{105} extract consisted of fatty acids. For further identification the extract was treated with boron trifluoride in methanol and the products were subjected to g.l.c. as described in the Experimental section. Various samples were run under different temperature-programming conditions, and appropriate standard mixtures were run under identical conditions. The results from these runs are assembled in Table 3. Of the 30 distinct peaks that could be discerned in the mixtures, 16 could be tentatively identified by the close correspondence (in at least two runs) between their retention times and those of standards. Thirteen peaks could not be identified, but only five of these were present in more than trace amounts, and only two were major components. For one other major component identification was especially tenuous. It was assigned as $C_{4:0}$ acid ester because it had a retention time exactly one-half of that for $C_{6:0}$ acid ester, and since $C_{8:0}$ acid ester and $C_{10:0}$ acid ester gave retention times that were exactly twice and four times respectively that for $C_{6:0}$ acid ester, the assignment seemed reasonable.

Table 3. *Identification and quantification of fatty acids in rat milk*

Component	Composition (%, by wt.)	Component	Composition (%, by wt.)
$C_{4:0}$ *	11	$C_{17:0}$	Trace (<1)
$C_{6:0}$	1.5	$C_{18:0}$	3.1
$C_{8:0}$	2.2	$C_{18:1}$	8.7
$C_{10:0}$	8.3	$C_{18:2}$	6.0
$C_{11:0}$	Trace (<1)	$C_{18:3}$	Trace (<1)
$C_{12:0}$	6.3	$C_{22:0}$	Trace (<1)
$C_{13:0}$	Trace (<1)	U-1†	2.3
$C_{14:0}$	5.7	U-2†	14
$C_{15:0}$	Trace (<1)	U-3†	1.3
$C_{16:0}$	12	U-4†	1.3
$C_{16:1}$	3.0	U-5†	7.0

* Identification is only tentative (see the text).

† Unidentified: these peaks had retention times as follows: U-1, between $C_{6:0}$ and $C_{8:0}$; U-2, between $C_{8:0}$ and $C_{10:0}$; U-3, between $C_{10:0}$ and $C_{11:0}$; U-4, between $C_{11:0}$ and $C_{12:0}$; U-5, greater than $C_{22:0}$.

The values for percentage composition in Table 3, calculated as described in the Experimental section, account for 93.7% of the total fatty acids. The balance consists of 13 components present in quantities of less than 1% each. By using those components comprising 1% or more, we calculated the concentration of non-esterified fatty acids in milk as follows. On the basis of their retention times, and for purposes of this calculation only, we assumed Unknowns 1-5 (Table 3) were $C_{7:0}$ acid, $C_{9:0}$ acid, $C_{10:1}$ acid, $C_{11:1}$ acid and $C_{23:0}$ acid respectively. We could then calculate an average molecular weight of 211 for the non-esterified fatty acids. This compares reasonably well with an average fatty acid chain length of 14.2 found by Smith, Watts & Dils (1968) for rat milk triglycerides. From the dry weights of the extracts (see above) it was calculated that the milk fraction R_{105} , and therefore the original milk, contained 2.06mg of non-esterified fatty acids/ml. From the average molecular weight of 211, this gives a concentration of 9.77mM-non-esterified fatty acids in rat milk and in the inhibitory fraction R_{105} .

Portions of the aqueous solution of the extract equal to those used in the enzyme assays were tested for thiol esters by using the procedure of Stadtman (1957). No thiol esters were detected; however, with palmitoyl-CoA under the same conditions we would only have detected an amount corresponding to a concentration of 0.4mM in milk or milk fraction R_{105} .

Interaction of fatty acids with acetyl-CoA carboxylase

To examine the mechanism of inhibition of acetyl-CoA carboxylase by the milk inhibitor, we chose five representative fatty acids: octanoic ($C_{8:0}$) acid,

Table 4. *Effect of laurate on acetyl-CoA carboxylase*

Expt. 1 used pre-sucrose gradient enzyme diluted in Buffer 2. Expt. 2 used purified enzyme diluted in Buffer 2. Expt. 3 used pre-sucrose gradient enzyme diluted in Buffer 1.

Concn. of laurate (μM)	Expt. 1 HCO_3^- -incorporated		Expt. 2 HCO_3^- -incorporated		Expt. 3 HCO_3^- -incorporated	
	$(\mu\text{mol/min per mg of protein})$		$(\mu\text{mol/min per mg of protein})$		$(\mu\text{mol/min per mg of protein})$	
		(%)		(%)		(%)
0	1.54	[100]	2.48	[100]	0.26	[100]
0.02	1.38	90	2.20	88	—	—
0.2	1.27	83	1.82	73	—	—
2.0	1.24	80	1.62	65	0.35	134
20	1.01	65	1.42	57	0.30	117
60	—	—	—	—	0.24	94
100	0.61	40	0.31	12	0.23	89
300	0.05	3	0.08	3	0.02	8

Table 5. *Effect of aging on interaction between acetyl-CoA carboxylase and laurate*

All tubes contained pre-sucrose gradient enzyme diluted in Buffer 2.

Age of enzyme (days)	HCO_3^- -incorporated in the absence of laurate (A) $(\mu\text{mol/min per mg of protein})$	HCO_3^- -incorporated with $20\mu\text{M}$ -laurate (B) $(\mu\text{mol/min per mg of protein})$	$100 \times B/A$
0	1.39	0.86	61
1	1.27	1.14	89
2	1.12	1.17	104

decanoic ($\text{C}_{10:0}$) acid, lauric ($\text{C}_{12:0}$) acid, myristic ($\text{C}_{14:0}$) acid and palmitic ($\text{C}_{16:0}$) acid. All fatty acids were used as their sodium or potassium salts (see the Experimental section).

It became apparent early during this investigation that the interaction between fatty acids and the enzyme was critically dependent on the environment and age of the enzyme, and on the time of incubation of the enzyme with the fatty acids; it was also dependent on the chain length of the fatty acids. These effects are illustrated by the following results.

Enzyme stored in the presence of citrate (in Buffer 2) at -20°C was thawed, diluted in Buffer 2 and incubated with various fatty acids, by using assay B. Under these conditions low concentrations of all the fatty acids inhibited acetyl-CoA carboxylase. Laurate and myristate appeared to be the most effective inhibitors. Table 4 illustrates typical results obtained with laurate with both purified and pre-sucrose gradient enzyme (Expts. 1 and 2).

A completely different picture emerged when such experiments were performed with enzyme dialysed against Buffer 1 (containing no citrate), and diluted in the same buffer for assay (but assayed, as usual, in the presence of citrate). Under these circumstances, as noted previously (Miller & Levy,

1969), the activity of the enzyme was considerably decreased. At low concentrations ($2\text{--}20\mu\text{M}$), all the fatty acids gave significant stimulation of activity. Typical results for laurate are shown in Table 4 (Expt. 3). Myristate gave very similar results, but the other fatty acids failed to inhibit even at $300\mu\text{M}$.

Another factor that determined the response of the enzyme to fatty acids was aging. As shown in Table 5, freshly thawed enzyme stored and diluted in buffer containing citrate was sensitive to fatty acids, but if the enzyme was allowed to stand at 4°C it lost this sensitivity while undergoing a moderate loss of catalytic activity. Tests conducted after the enzyme had been kept at 4°C for 3 weeks showed little further change. In some cases we observed that aged enzyme, like enzyme stored in the absence of citrate, became susceptible to activation by fatty acids.

Numa, Ringelmann & Lynen (1965) observed that the mechanism of inhibition of partially purified rat liver acetyl-CoA carboxylase by palmitoyl-CoA was non-competitive with respect to acetyl-CoA and competitive with respect to citrate. Our results of similar studies with the mammary-gland enzyme demonstrate that laurate behaves like a non-competitive inhibitor with respect

Fig. 1. Effect of laurate on acetyl-CoA carboxylase at various concentrations of citrate. ○, Without laurate; ●, with 100 μM-laurate. All assay tubes contained purified enzyme (0.20 μg of protein) diluted in Buffer 2. Citrate was varied as specified on the abscissa. The results are expressed as total nmol of [¹⁴C]bicarbonate incorporated in 10 min.

Fig. 2. Double-reciprocal plot of variation in velocity (v) with citrate concentration, in the absence (○) and presence (●) of 100 μM-laurate. Data from Fig. 1 were used.

to acetyl-CoA. However, laurate is not a competitive inhibitor with respect to citrate, although at low citrate concentration the mechanism resembles competitive inhibition (Figs. 1 and 2).

We examined the possibility that the inhibition of acetyl-CoA carboxylase by fatty acids was due to time-dependent irreversible inactivation of the enzyme. Enzyme was incubated with and without laurate for various times before initiating the

Fig. 3. Effect of varying the preincubation time on the activity of acetyl-CoA carboxylase in the presence and absence of laurate. With fresh enzyme: ○, without laurate; ●, with 100 μM-laurate. With aged enzyme: △, without laurate; ▲, with 100 μM-laurate. All tubes contained pre-sucrose gradient enzyme (2.1 μg of protein) diluted in Buffer 2. The solution containing substrates was added after the specified preincubation times, as noted on the abscissa. The aged enzyme was the same preparation used for the experiment shown in Table 5, aged now for 3 weeks at 4°C. The results are expressed as total nmol of [¹⁴C]bicarbonate incorporated in 10 min.

reaction with substrates in the normal manner (Fig. 3). With the usual 15 min activation laurate appeared to inhibit fresh enzyme. However, this apparent inhibition did not take place if insufficient time was allowed for the laurate and enzyme to interact before the reaction was initiated. In fact, if the reaction was initiated immediately, an apparent slight stimulation by laurate was seen. This experiment suggests that the interaction between enzyme and fatty acids is time-dependent. Fig. 3 also illustrates an experiment with aged enzyme, demonstrating its diminished sensitivity to fatty acid inhibition.

It is also evident from Fig. 3 that enzyme stored in the presence of citrate did not require incubation with citrate to activate it: in fact, a slight progressive decline in activity with time is seen. Nevertheless it is clear that inhibition by fatty acids did require an incubation period. Other experiments showed that after the 15 min incubation no further changes in the degree of laurate inhibition of fresh enzyme took place during the assay.

The results in Tables 6 and 7 suggest that bovine serum albumin can prevent fatty acid inhibition if included in the incubation with enzyme, but that it cannot reverse it (at the concentration tested) if added with the substrates after enzyme and laurate

had been incubated for 15 min. These findings suggest that the incubation between fatty acids and acetyl-CoA carboxylase is irreversible.

Interaction of palmitoyl-CoA with acetyl-CoA carboxylase

The effects of palmitoyl-CoA (Table 8) are strikingly similar to those of laurate (Table 4) except that they occur at a much lower concentration. Like fatty acids, palmitoyl-CoA stimulated acetyl-CoA carboxylase stored in the absence of citrate, but inhibited the enzyme stored in citrate, the purified enzyme being more sensitive to inhibition. Fig. 4 illustrates the effect of varying the citrate concentrations on the effect of palmitoyl-CoA, and is similar to the results with laurate in Fig. 1 and with palmitoyl-CoA with the rat liver enzyme (Numa *et al.* 1965). Results included in Table 7 suggest that palmitoyl-CoA, like laurate, may cause irreversible inhibition of mammary-gland acetyl-CoA carboxylase.

Physiological significance: effects on other mammary-gland enzymes.

To assess the physiological significance of the effects of milk fraction R₁₀₅ and milk fatty acids on mammary-gland acetyl-CoA carboxylase, six

other mammary-gland enzymes were examined. The enzymes chosen are known to play important roles in mammary-gland metabolism and were shown by Jones (1967) to decline in activity to various extents after either hypophysectomy or weaning.

The enzymes tested were ATP citrate lyase, phosphofructokinase, phosphoglucosmutase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and UDP-glucose pyrophosphorylase. Acetyl-CoA carboxylase was also examined in these experiments. The enzymes were assayed in crude 23000g supernatant fractions, prepared as described in the Experimental section. Milk fractions R₁₀₅, R₁₀₅E and R₁₀₅R were prepared as described above, except that only five acetone extractions and five water washings were performed.

The results of these experiments are summarized in Table 9. Both ATP citrate lyase and phosphofructokinase are inhibited by milk fraction R₁₀₅ to an even greater extent than acetyl-CoA carboxylase. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are not significantly affected by fraction R₁₀₅, whereas phosphoglucosmutase and UDP-glucose pyrophosphorylase are markedly stimulated. For ATP citrate lyase and phosphofructokinase additional experiments demonstrated that the effect of fraction R₁₀₅ was not on one of the enzymes required in the coupled assay.

To test whether these effects of fraction R₁₀₅ were caused by fatty acids, fraction R₁₀₅ was extracted with acetone, as described above, and both fraction R₁₀₅E and fraction R₁₀₅R were tested for their effect on the enzymes. Table 9 shows that with acetyl-CoA carboxylase most of the inhibitory activity was extracted with acetone. The results with ATP citrate lyase and phosphofructokinase, in contrast, show that very little inhibitory activity was extracted and that fraction R₁₀₅R was still strongly inhibitory. None of the other enzymes tested was inhibited by fraction

Table 6. *Effect of bovine serum albumin, present during the activation period and assay, on laurate inhibition of acetyl-CoA carboxylase*

All tubes contained pre-sucrose gradient enzyme diluted in Buffer 2.

Concn. of bovine serum albumin (mg/ml)	No laurate HCO ₃ ⁻ incorporated (μmol/min per mg of protein)	100 μM-Laurate HCO ₃ ⁻ incorporated (μmol/min per mg of protein)
0	1.64	0.54
0.2	2.62	2.02
0.4	2.84	2.78
0.6	2.97	3.02

Table 7. *Effect of bovine serum albumin, present during the assay only, on inhibition of acetyl-CoA carboxylase by laurate and palmitoyl-CoA*

All tubes contained pre-sucrose gradient enzyme diluted in Buffer 2. Bovine serum albumin was absent during the activation period and was added, where indicated, with the substrates.

Addition	No bovine serum albumin HCO ₃ ⁻ incorporated		Bovine serum albumin (0.206 mg/ml) HCO ₃ ⁻ incorporated	
	(μmol/min per mg of protein)	(%)	(μmol/min per mg of protein)	(%)
None	1.47	[100]	1.77	[100]
10 μM-Laurate	1.38	94	1.54	87
100 μM-Laurate	0.86	58	0.95	54
0.1 μM-Palmitoyl-CoA	1.10	75	1.25	71

Table 8. *Effect of palmitoyl-CoA on acetyl-CoA carboxylase*

Expt. 1 used pre-sucrose gradient enzyme diluted in Buffer 2. Expt. 2 used purified enzyme diluted in Buffer 2. Expt. 3 used pre-sucrose gradient enzyme diluted in Buffer 1.

Concn. of palmitoyl-CoA (μM)	Expt. 1 HCO_3^- -incorporated		Expt. 2 HCO_3^- -incorporated		Expt. 3 HCO_3^- -incorporated	
	($\mu\text{mol}/\text{min}$ per mg of protein)	(%)	($\mu\text{mol}/\text{min}$ per mg of protein)	(%)	($\mu\text{mol}/\text{min}$ per mg of protein)	(%)
0	1.50	[100]	2.92	[100]	0.38	[100]
0.02	1.26	86	2.77	95	0.57	148
0.08	1.11	75	1.69	58	0.52	134
0.2	1.03	69	0.94	32	0.47	121
0.8	0.72	48	0.51	18	0.32	84
2.0	0.16	11	0.07	2	0.16	42
8.0	0.00	0	0.00	0	—	—

Fig. 4. Effect of palmitoyl-CoA on acetyl-CoA carboxylase at various concentrations of citrate. ○, Without palmitoyl-CoA; ●, with 0.2 μM palmitoyl-CoA. All tubes contained pre-sucrose gradient enzyme (1.25 μg of protein) diluted in Buffer 2. Citrate was varied as specified on the abscissa. The results are expressed as total nmol of [^{14}C]bicarbonate incorporated in 10 min.

R_{105}E . These results suggest that, of the enzymes tested, acetyl-CoA carboxylase is most sensitive to the fatty acids in the acetone extract of milk fraction R_{105} . Further support for this idea was provided by the demonstration (Table 9) that acetyl-CoA carboxylase is much more sensitive to laurate inhibition than are any of the other enzymes examined.

During these studies it was found that the response of acetyl-CoA carboxylase in crude homogenates to the milk fractions is sensitive to the age of the

enzyme. Freshly prepared enzyme was inhibited by fraction R_{105}E , but on aging this sensitivity was lost and sometimes stimulation was observed. This behaviour resembles the response of the purified enzyme to fatty acids, noted above. At the same time the enzyme becomes more sensitive to inhibition by fraction R_{105}R . Since fractions R_{105} and R_{105}R contain Ca^{2+} (see below) and since Mg^{2+} activates mammary-gland acetyl-CoA carboxylase (Miller & Levy, 1969), it may be that Ca^{2+} antagonizes Mg^{2+} activation and that this effect is enhanced on aging. Experiments to test this idea were not performed.

We have not investigated in detail the nature of the other effectors present in fraction R_{105} , but evidently they are not extracted by acetone. The fact that the three ATP-linked enzymes tested were inhibited by fraction R_{105} suggested at first that possibly fraction R_{105} contained an adenosine triphosphatase. However, substitution of an ATP-generating system for ATP did not affect the results and tests for adenosine triphosphatase were negative. Further, it appears unlikely that the adenosine triphosphatase could survive the vigorous acetone extraction procedure and remain fully active in fraction R_{105}R . An alternative explanation is that the Ca^{2+} of the calcium caseinate, which probably forms a major component of milk fraction R_{105} , inhibits ATP citrate lyase and phosphofructokinase (and acetyl-CoA carboxylase). The concentrations of Ca^{2+} in fractions R_{105} and R_{105}R were found to be 48 and 30 mM respectively, by atomic absorption spectrophotometry. Thus the concentrations of Ca^{2+} in the assays would be 4.8 and 3.0 mM in the presence of fractions R_{105} and R_{105}R respectively. The effects of calcium caseinate and calcium chloride were tested on several enzymes under our assay conditions. It was found that a suspension of 0.4% calcium caseinate gave 83% inhibition of ATP citrate lyase, 78% inhibition of

Table 9. *Effects of milk fraction R₁₀₅, acetone extract fraction of R₁₀₅ and laurate on various mammary-gland enzymes*

The values are expressed as percentage activities of controls. The control activities varied somewhat with different enzyme preparations; the values for the experiments cited in this table are as follows. For acetyl-CoA carboxylase, 520 c.p.m. For the other enzymes the activities are expressed in change in E_{340}/min : ATP citrate lyase, 0.0224; phosphofructokinase, 0.0266; phosphoglucomutase, 0.0241; glucose 6-phosphate dehydrogenase, 0.0487; 6-phosphogluconate dehydrogenase, 0.0370; UDP-glucose pyrophosphorylase, 0.0152. The volume of the assay solution was 0.5 ml for acetyl-CoA carboxylase and 1.0 ml for all others. The protein concentrations during the assay were as follows ($\mu\text{g}/\text{ml}$): acetyl-CoA carboxylase, 54; ATP citrate lyase, phosphofructokinase and phosphoglucomutase, 190; glucose 6-phosphate dehydrogenase, 19; 6-phosphogluconate dehydrogenase, 63; UDP-glucose pyrophosphorylase, 9.5. The amount of fraction R₁₀₅ added was 0.1 of the total reaction mixture volume for all assays, i.e. 0.05 ml for acetyl-CoA carboxylase and 0.1 ml for all others. Corresponding volumes of fractions R₁₀₅E and R₁₀₅R were used. The latter was washed to remove acetone and redissolved as described in the Experimental section.

Addition	Acetyl-CoA carboxylase	ATP citrate lyase	Phospho- fructokinase	Phospho- glucomutase	Glucose 6-phosphate dehydrogenase	6-Phospho- gluconate dehydrogenase	UDP-glucose pyro- phosphorylase
Control	[100]	[100]	[100]	[100]	[100]	[100]	[100]
Fraction R ₁₀₅	65	19	17	150	114	113	215
Fraction R ₁₀₅ R	85	36	20	122	—	—	157
Fraction R ₁₀₅ E	72.5	85	87	96	102	97	88
Laurate*	52 (0.10)	100 (0.10)	90 (0.10)	96 (0.10)	88 (0.10)	106 (0.10)	87 (0.10)
	9 (0.30)	86 (0.44)	82 (0.38)	84 (0.44)	91 (0.38)	102 (0.44)	65 (0.35)

* Concentrations (mM) in parentheses.

phosphofructokinase, but only 55% inhibition of acetyl-CoA carboxylase and 24% inhibition of phosphoglucomutase. Similarly 1.0 mM-calcium chloride gave 67% inhibition of ATP citrate lyase, 75% inhibition of phosphofructokinase, but only 25% inhibition of acetyl-CoA carboxylase and it did not inhibit phosphoglucomutase.

These experiments demonstrate that the unfractionated milk fraction R₁₀₅ contains components that inhibit several enzymes. Of the enzymes that we tested, only acetyl-CoA carboxylase appears to be significantly sensitive to the fatty acids in fraction R₁₀₅. Although Ca²⁺ inhibits acetyl-CoA carboxylase, both ATP citrate lyase and phosphofructokinase appear to be more sensitive to Ca²⁺ inhibition. The findings are thus in accord with the idea that unesterified fatty acids in fraction R₁₀₅ inhibit acetyl-CoA carboxylase, and that the inhibition of ATP citrate lyase and phosphofructokinase may be due to Ca²⁺ in fraction R₁₀₅. The stimulations of UDP-glucose pyrophosphorylase and phosphoglucomutase have not been further examined.

Effect of fraction R₁₀₅ prepared from milk obtained from lactating rats

The rat milk used for all the experiments discussed so far was obtained from rats whose young had been removed approx. 12 h before milking. It seemed possible that the presence of inhibitor in

such milk might result from processes associated with the early stages of mammary-gland regression. To test this possibility milk was obtained from two rats whose young were still suckling as they were taken to be anaesthetized ('suckling') and from two rats whose young were removed 12 h previously ('weaned'). Milk fraction R₁₀₅ was prepared from both samples and its effect noted on the activity of pre-sucrose gradient acetyl-CoA carboxylase. The percentage activities on adding 0.05, 0.10 and 0.15 ml of 'suckling' fraction R₁₀₅ were 55, 42 and 28; corresponding volumes of 'weaned' fraction R₁₀₅ gave 64%, 38% and 28% activities. Fraction R₁₀₅ from both milk samples was extracted with acetone, and methyl esters were prepared as described above. When these methyl esters were subjected to g.l.c. the patterns from both samples appeared essentially identical. These findings suggest that the acetyl-CoA carboxylase inhibitor is a normal component of the milk rather than a product resulting from physiological changes in the mammary gland after weaning.

DISCUSSION

To our knowledge, no one has previously analysed non-esterified fatty acids in rat milk. Several reports give information on the fatty acid composition of rat milk (Beare, Gregory, Smith & Campbell, 1961; Beare, 1961; Rees, Shuck & Ackerman, 1966; Glass, Troolin & Jennes, 1967;

Smith *et al.* 1968), but these either include, or are specifically restricted to, fatty acids from triglycerides. Our results show that the composition of non-esterified fatty acids appears to be identical in milk collected immediately after the young stop suckling and milk removed from the glands 12h later. Extensive enzymic hydrolysis of milk fat therefore does not seem to occur within the gland during the early stages of mammary-gland regression. Because of the procedures used, it is unlikely that the fatty acids in milk were derived from esters or thiol esters during collection, storage or subsequent manipulations.

Although non-esterified fatty acids were shown to be present in high concentration in rat milk (approx. 10mM) and acyl-CoA thiol esters were not found, our test for the latter was not sufficiently sensitive to detect quantities that might cause significant inhibition of mammary-gland acetyl CoA carboxylase. Experiments showed that palmitoyl-CoA was a potent inhibitor and that it behaved in every respect like laurate except that it was effective at lower concentrations. Our studies, although limited, are thus in agreement with the view that the effects of long-chain fatty acids and their acyl-CoA thiol esters upon various enzymes are mediated via the same mechanism (Pande & Mead, 1968). Rat liver acetyl-CoA carboxylase is also inhibited by long-chain fatty acids (Korchak & Masoro, 1964; Numa *et al.* 1965) and, more effectively, by long-chain acyl-CoA thiol esters (Bortz & Lynen, 1963; Numa *et al.* 1965).

Numa *et al.* (1965) reported that the inhibition of rat liver acetyl-CoA carboxylase by long-chain acyl-CoA thiol esters was competitive with respect to citrate and non-competitive with respect to acetyl-CoA. Our results show that the effect of laurate on mammary-gland acetyl-CoA carboxylase appears to be associated with an antagonistic effect on citrate activation (Fig. 2), which cannot, however, be ascribed to classical competitive inhibition, and that laurate behaved like a non-competitive inhibitor with respect to acetyl-CoA. It should be noted, however, that irreversible inactivation, after proceeding for a fixed time, gives kinetic results resembling non-competitive inhibition. In fact, our evidence (Fig. 3, Tables 6 and 7) supports just such a mechanism for the interaction of laurate with mammary-gland acetyl-CoA carboxylase. Support for irreversible inactivation is also provided by experiments (A. L. Miller, unpublished work) showing that the effects of fatty acids on acetyl-CoA carboxylase were not significantly different whether the substrates, cofactors and activator were present at optimum concentrations (assay B), or at concentrations giving approximately half-optimum rates. If fatty acids inhibit reversibly it would be expected that their effect

would be markedly enhanced under the latter conditions.

The stimulation of acetyl-CoA carboxylase by isolated milk fat and fraction S₁₀₅ may be due to phospholipids, known to be present in mammalian milk (Parsons & Patton, 1967; Morrison, 1968) and shown to stimulate acetyl-CoA carboxylase from the mammary gland (Miller & Levy, 1969) and liver (Foster & McWhorter, 1969). Like phospholipids (Miller & Levy, 1969) the milk fat and fraction S₁₀₅ stimulated enzyme stored either with or without citrate. The finding that stimulation is not related to the amount of added milk fat or milk fraction S₁₀₅ may be due to the fact that low concentrations of phospholipids are sufficient to produce a maximum response, and that inhibitors are also present in these fractions.

Stimulation by fatty acids and palmitoyl-CoA, in contrast to stimulation by milk fat or milk fraction S₁₀₅, was never observed with fully active enzyme but only when the activity had been partly lost after aging or storage in the absence of citrate. We have not investigated the nature of this aging process further, but we noted previously that the activity of this enzyme is very sensitive to its environment (Miller & Levy, 1969). Proper precautions should be taken therefore to ensure that aging does not interfere with any investigation of the interaction of various ligands with acetyl-CoA carboxylase from the mammary gland, or possibly from other sources. It should be pointed out that neither whole milk nor milk fraction R₁₀₅ stimulated acetyl-CoA carboxylase under any conditions. Perhaps other components present, such as phospholipids, insured that the enzyme was in its fully active form, although we have no evidence for this. Thus with either freshly prepared enzyme or with natural inhibitor only inhibition could be observed. The mechanism of the stimulatory effect of fatty acids and palmitoyl-CoA is not yet understood, but it seems clear, in any case, that it is the inhibitory effect of the fatty acids (and fatty acyl-CoA) which must be examined for its possible physiological significance.

Several investigators, most notably Pande & Mead (1968), have suggested that enzyme inhibition by fatty acids and acyl-CoA thiol esters is due to non-specific detergent effects and therefore has no regulatory significance. Pande & Mead (1968) also pointed out that often the most inhibitory fatty acids are only minor constituents in mammalian systems, and that many cellular components bind fatty acids and acyl-CoA thiol esters, thus probably preventing these compounds from ever reaching effective inhibitory concentrations *in vivo*. Although inhibitory effects of fatty acids must be considered cautiously in the light of such arguments, the results of the present investigation suggest that

the interaction between mammary gland acetyl-CoA carboxylase and milk fatty acids represents a rather special situation. The arguments supporting the physiological significance of this interaction may be summarized as follows.

First, the concentration of non-esterified fatty acids in rat milk, nearly 10mM, is considerably higher than in tissues (Rees *et al.* 1966). We showed previously (Levy, 1964) that 12h after the young were weaned, approximately 50% of the weight of the excised rat mammary glands could be attributed to retained milk. During this time the synthesis of fatty acids, measured in mammary-gland extracts, decreased by 75% (Levy, 1964). Assuming a uniform distribution of milk fatty acids within the gland, the concentration of non-esterified fatty acids would thus be about 5mM. This is nearly 20 times the concentration shown, in the present study, to cause over 90% inhibition of either crude or purified acetyl-CoA carboxylase. We have not examined the mechanism whereby the particle-bound fatty acids in milk gain access to the intracellular acetyl-CoA carboxylase. Relevant to this question may be electron micrograph studies by Hollman (Hollman, 1966; Hollman & Verley, 1967), which show resorption of milk particles accompanying morphological changes during mammary-gland regression after weaning.

Second, the non-esterified fatty acids in rat milk are tightly bound to a particulate component, resisting solubilization upon repeated washings, and yet they cause strong inhibition under our assay conditions. We do not know whether the fatty acids are released during the assay, but evidently binding of fatty acids to other components does not necessarily prevent them from inhibiting acetyl-CoA carboxylase.

Third, in the case of the mammary gland, those fatty acids found to be the most effective inhibitors do not constitute a minor fraction of the fatty acids synthesized. Thus laurate and myristate together comprise 12% of the non-esterified fatty acids in rat milk; when decanoate and palmitate (which were found to be nearly as inhibitory) are included this value becomes 30%. Laurate and myristate constitute about one-fourth, and, with decanoate and palmitate, over two-thirds of the fatty acids of rat milk fat (Glass *et al.* 1967).

Finally, we have presented evidence that milk fatty acids may specifically affect mammary-gland acetyl-CoA carboxylase. The effects of milk fractions R₁₀₅, R₁₀₅R and R₁₀₅E and laurate on seven mammary enzymes (including acetyl-CoA carboxylase) were examined. The enzymes chosen were those for which Jones (1967) had demonstrated that either hypophysectomy or weaning produced a similar pattern of change. Jones ruled out diminished prolactin secretion as the common factor

underlying these changes by demonstrating that the same changes could be produced unilaterally by preventing milk removal from the mammary glands on only one side of the rat (Jones, 1967). We used the same technique previously to show that fatty acid synthesis is inhibited only in unsuckled mammary glands, thus supporting our suggestion that an inhibitor is present in the retained milk (Levy, 1964). Jones (1967) explained his results by the presence of some 'local factor' in the unsuckled gland. More recent data from his laboratory (Jones, 1968) also support our suggestion (Levy, 1964) that milk accumulation in the unsuckled gland directly affects the accompanying decline in fatty acid synthesis. The experiments presented in the present report strongly suggest that the 'local factor' for acetyl-CoA carboxylase is non-esterified fatty acids in the milk. Of the seven enzymes tested, acetyl-CoA carboxylase is the only one for which it could be clearly shown that inhibition by fraction R₁₀₅ from milk was due to an acetone-extractable component. The fact that the acetone extract contains a high concentration of fatty acids, together with the direct demonstration that, of the enzymes tested, acetyl-CoA carboxylase was by far the most sensitive to laurate inhibition, affords strong support for the unique physiological role of the milk fatty acids in regulating mammary-gland acetyl-CoA carboxylase.

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REFERENCES

- Beare, J. L. (1961). *Can. J. Biochem. Physiol.* **39**, 1855.
- Beare, J. L., Gregory, E. R. W., Smith, D. M. & Campbell, J. A. (1961). *Can. J. Biochem. Physiol.* **39**, 195.
- Bortz, W. M. & Lynen, F. (1963). *Biochem. Z.* **337**, 505.
- Foster, D. W. & McWhorter, W. P. (1969). *J. biol. Chem.* **244**, 260.
- Ganguly, J. (1960). *Biochim. biophys. Acta*, **40**, 110.
- Glass, R. L., Troolin, H. A. & Jennes, R. (1967). *Comp. Biochem. Physiol.* **22**, 415.
- Glock, G. E. & McLean, P. (1953). *Biochem. J.* **55**, 400.
- Hollman, K. H. (1966). *Z. Zellforsch.* **69**, 395.
- Hollman, K. H. & Verley, J. M. (1967). *Z. Zellforsch.* **82**, 222.
- Howanitz, P. J. & Levy, H. R. (1965). *Biochim. biophys. Acta*, **106**, 430.
- Jones, E. A. (1967). *Biochem. J.* **103**, 420.
- Jones, E. A. (1968). *Biochim. biophys. Acta*, **177**, 158.
- Kaplan, N. O., Colowick, S. P. & Barnes, C. C. (1951). *J. biol. Chem.* **191**, 461.
- Karmen, A. (1963). *Science, N.Y.*, **142**, 163.

- Korchak, H. M. & Masoro, E. J. (1962). *Biochim. biophys. Acta*, **58**, 354.
- Korchak, H. M. & Masoro, E. J. (1964). *Biochim. biophys. Acta*, **84**, 750.
- Levy, H. R. (1963a). *J. biol. Chem.* **238**, 775.
- Levy, H. R. (1963b). *Biochem. biophys. Res. Commun.* **13**, 267.
- Levy, H. R. (1964). *Biochim. biophys. Acta*, **84**, 229.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. N. (1951). *J. biol. Chem.* **193**, 265.
- Mansour, T. E. (1963). *J. biol. Chem.* **238**, 2285.
- Marinetti, G. V. (1965). *J. Lipid Res.* **6**, 315.
- Martin, D. B. & Vagelos, P. R. (1962). *J. biol. Chem.* **237**, 1787.
- Miller, A. L. & Levy, H. R. (1968). *Abstr. Pap. Am. chem. Soc., 156th Meet., Atlantic City, N.J.*, Biol. Abstr. no. 77.
- Miller, A. L. & Levy, H. R. (1969). *J. biol. Chem.* **244**, 2334.
- Morrison, W. R. (1968). *Lipids*, **3**, 101.
- Numa, S., Matsushashi, M. & Lynen, F. (1961). *Biochem. Z.* **334**, 203.
- Numa, S., Ringelmann, E. & Lynen, F. (1965). *Biochem. Z.* **343**, 243.
- Pande, S. V. & Mead, J. F. (1968). *J. biol. Chem.* **243**, 6180.
- Parsons, J. G. & Patton, S. (1967). *J. Lipid Res.* **8**, 696.
- Rees, E. D., Shuck, A. E. & Ackerman, H. (1966). *J. Lipid Res.* **7**, 396.
- Shatton, J. B., Gruenstein, M., Shay, M. & Weinhouse, S. (1965). *J. biol. Chem.* **240**, 22.
- Smith, S., Watts, R. & Dils, R. (1968). *J. Lipid Res.* **9**, 52.
- Srere, P. A. (1959). *J. biol. Chem.* **234**, 2544.
- Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. **3**, p. 938. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Sternberg, J. C., Gallaway, W. S. & Jones, D. T. S. (1962). In *Gas Chromatography*, p. 231. Ed. by Brennen, N., Calles, J. E. & Weiss, M. D. New York: Academic Press Inc.