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FATTY ACID SYNTHESIS BY COMPLEX SYSTEMS

THE POSSIBILITY OF REGULATION BY MICROSOMES

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SUMMARY

1. Fatty acid synthesis from malonyl-CoA catalyzed by rat-liver microsomal protein does not seem to be due to contamination with enzymes from the particle-free supernatant or mitochondrial fractions.

2. Optimum conditions for the conversion of malonyl-CoA to fatty acids by rat-liver microsomes with respect to substrate concentration, protein concentration, and incubation time were established. The enzyme systems concerned with fatty acid synthesis from malonyl-CoA in the particle-free supernatant fraction were 4–5 times more active than those in the microsomes.

3. Preincubation of the microsomes caused a release of malonyl-CoA decarboxylase (EC 4.1.1.9) but not of fatty acid synthetase. This resulted in a greater conversion of the available malonyl-CoA to fatty acids.

4. The products of synthesis in the supernatant system were free fatty acids bound to protein, whereas the products of the microsomal system were predominantly complex lipids (phospholipid).

5. The predominant fatty acid synthesized from malonyl-CoA by the supernatant fraction was palmitate, and that synthesized by the microsomes was stearate.

6. In the system composed of the supernatant fraction plus microsomes, the fatty acids synthesized were complex lipids (phospholipids), and the pattern of fatty acid synthesis resembled that found in the liver slice.

7. The mechanism of the stimulatory effect of microsomes on fatty acid synthesis by the supernatant fraction is discussed and tentatively localized at the level of acetyl-CoA carboxylation. The stimulation may be due to a release of feedback inhibition.

INTRODUCTION

Studies on fatty acid synthesis at the subcellular level have been confined mainly to the particle-free supernatant fractions obtained from homogenates of various mammalian tissues and to purified preparations derived from these supernatant fractions[1–8]. In this laboratory we have been concerned with reconstructing non-

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cellular systems capable of converting acetate to fatty acids under conditions approaching the physiological state. An outcome of our studies was the demonstration that the addition of a specific amount of microsomes to the particle-free supernatant fraction obtained from rat-liver homogenates resulted in a pronounced increase in the rate of fatty acid synthesis from acetate[9-11] or acetyl-CoA[12,13]. This microsomal stimulation has been confirmed by FLETCHER AND MYANT, in a rat-liver system[14], and by DILS AND POPJAK, in a mammary-gland system[6]. Furthermore, these particles, isolated by classical techniques, are by themselves capable of synthesizing fatty acids from malonyl-CoA[12,13,15]. The present study is concerned with (a) the locus and mechanism of the microsomal-stimulating effect, and (b) the chain-length of the fatty acids synthesized and the form in which they appear at the end of the incubation period.

EXPERIMENTAL PROCEDURES

The rats used in this study were of the Long-Evans strain. They weighed from 250 to 350 g and, unless otherwise stated, had been raised on an adequate stock diet (Diablo Labration). They were killed by a blow on the head, and their livers were rapidly excised and placed in a chilled sucrose solution. A portion of each liver was sliced[16], and the slices were incubated. The rest of the liver was then minced and homogenized with 3 vol. of 0.25 M sucrose solution as previously described[8]. Unless otherwise specified, nuclei and cellular debris (800 $\times$ g* for 15 min), mitochondria (8700 $\times$ g for 15 min), microsomes, and the particle-free supernatant fraction (100 000 $\times$ g for 45 min) were separated centrifugally as described in ref. 8.

Mammary glands were excised from lactating rats that had suckled at least 6 pups for 18–20 days following parturition. The glands were sliced, washed in isotonic sucrose solution to remove much of the preformed milk, minced, and homogenized with 3 vol. of 0.25 M sucrose solution in a manner similar to that described in ref. 8. The high-speed, particle-free, supernatant fraction obtained centrifugally as described in ref. 8 was then carefully fractionated with solid $(NH_4)_2SO_4$**. The fraction of protein precipitating between 0 and 29 % saturation was collected by centrifugation (10000 $\times$ g for 15 min), dissolved in 0.15 M glycylglycine buffer (pH 7.2), and used as a source of acetyl-CoA carboxylase (EC 6.4.1.2). As judged by spectrophotometric assay and conversion of [1,3-$^{14}C_2$]malonyl-CoA to ^{14}C-labelled fatty acids[17,***], this fraction, although it contained substantial acetyl-CoA carboxylase activity, showed no measurable fatty acid synthetase§ activity. All preparative procedures for liver and mammary glands were carried out in a cold room maintained at 2–4°.

Homogenate fractions were incubated in vessels that were mechanically agitated in a water bath set at 37°. The composition of the incubation medium, which varied with the homogenate preparation used, is given in the legends to figures and in tables,

* This refers to the gravitational force in the center of the tube.

** The ammonium sulfate concentration was calculated from the formula[37] $x = \frac{51.47\ (S_2 - S_1)}{1 - 0.27\ S_2}$

where x = the weight in grams $(NH_4)_2SO_4$ to be added to 100 ml of solution; S_1 = original fractional saturation and S_2 = final fractional saturation.

*** Since only one of the isotopically labelled carbons of [1,3-$^{14}C_2$]malonyl-CoA is converted to fatty acids or CO_2[1,19-21], an appropriate correction was applied in calculating the yields of these products.

§ The enzymes responsible for the conversion of malonyl-CoA to long-chain fatty acids are referred to as fatty acid synthetase.

as are the final volumes and incubation times. When fatty acid synthesis was studied, the incubation vessel was a stoppered test tube[15]; when conversion of a substrate to CO_2 was measured, the vessel was a specially designed flask provided with a center well and closed with a gas-tight rubber serum cap[18].

At the end of the incubation period, total lipids were extracted from the entire incubation mixture with 10 vol. of a mixture of chloroform–methanol (2:1, v/v) under reflux*. The extraction was repeated 5 times, and the extracts were pooled, and evaporated to a small volume under N_2. The residue lipid material was then washed with water and finally dissolved in petroleum ether (b.p. 30–60°). The clarified petroleum ether solution was applied to a micro silicic acid column, and the chromatogram was developed as described by LIS, TINOCO AND OKEY[22]. Each fraction was evaporated under N_2 and redissolved in hexane. Aliquots were assayed for ^{14}C activity, to within $\pm 3\%$, in a Packard automatic Tri-carb liquid scintillation spectrometer. The phospholipid fraction was dissolved in methanol and assayed as described above. The correction for quenching of ^{14}C activity due to methanol was determined by use of an internal standard. Aliquots of the cholesterol, mono- and diglyceride, and free fatty acid fractions were further separated into their individual components on a Florisil column[23], and assayed for ^{14}C activity as given above.

The ^{14}C-labelled fatty acids were isolated in *n*-hexane, after saponification and acidification of the entire contents of the incubation vessel, and assayed for ^{14}C activity, to within $\pm 3\%$, in the Packard automatic Tri-carb liquid scintillation spectrometer[8]. The method for quantitative recovery of $^{14}CO_2$ trapped with 1 M hyamine in methanol, and its assay, has been presented in ref. 15. The methods for the gas-chromatographic analysis of the fatty acids and the determination of their ^{14}C-content have also been described in ref. 8.

Protein was determined by the biuret method described by GORNALL, BARDAWILL AND DAVID[24].

SUBSTRATES AND COFACTORS

[1-^{14}C]Acetate was prepared from $^{14}CO_2$ and methylmagnesium iodide by the Grignard reaction, and isolated as the potassium salt[25]. It was converted to [1-^{14}C]acetic anhydride by reaction with *p*-toluene sulfonic acid[26], and then to [1-^{14}C]acetyl-CoA by the procedure outlined by STADTMAN[27]. The [1-^{14}C]acetyl-CoA was finally obtained as the pure, lyophilized, dry substance by chromatography on Whatman No. 3 MM paper with a 1:1 mixture of 0.1 M sodium acetate buffer (pH 4.5) and ethanol (95 %) as the developing solvent[27].

[1,3-$^{14}C_2$]diethylmalonate was synthesized by the method described in ref. 28. It was saponified, and the [1,3-$^{14}C_2$]malonic acid obtained was purified by sublimation *in vacuo*. This latter procedure insured a chromatographically pure product. The [1,3-$^{14}C_2$]malonic acid was converted to *S*-[1,3-$^{14}C_2$]malonyl-*N*-caprylcysteamine and then to [1,3-$^{14}C_2$]malonyl-CoA by the procedure of LYNEN[17], and finally isolated chromatographically pure by means of the solvent mixture mentioned above.

* Two types of experiments showed that complete extraction of the ^{14}C-labelled lipids was effected by this procedure. Saponification, acidification and *n*-hexane extraction of the protein residue after chloroform–methanol extraction of the incubation contents yielded no ^{14}C activity. In duplicate experiments the values for ^{14}C activity in the fatty acids obtained after saponification of the entire contents of the incubation vessel were the same as those for the total lipid extract.

CoA, TPN and ATP were purchased from Pabst Laboratories; disodium glucose 6-phosphate and purified glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from California Corporation for Biochemical Research; *dl*-isocitric lactone (allo-free) and isocitrate dehydrogenase (EC 1.1.1.42) from Sigma Chemical Company; glycylglycine from Nutritional Biochemical Corporation; and reduced glutathione from Schwarz Laboratories.

RESULTS

Characterization of the fatty acid-synthesizing system isolated from rat liver

In order to be reasonably certain that the microsomal pellet isolated from rat-liver homogenates was not contaminated with protein from the supernatant fraction or with mitochondria, the following experiments were performed. After removal of mitochondria, the pellet isolated by centrifugation of the supernatant fraction at 100000 × *g* for 45 min was resuspended in isotonic sucrose solution and used either ((a)) directly ((unwashed))*; or ((b)) after resedimentation at 100000 × *g* for 45 min ((washed)); or ((c)) after being spun at 115000 × *g* for 115 min (a speed higher than that required for removal of liver mitochondria), the minute residue that formed being removed, and being resedimented at 100000 × *g* for 45 min. The preparation of these fractions is shown in Fig. 1. The extent to which the ^{14}C of [1,3-$^{14}C_2$]malonyl-CoA was

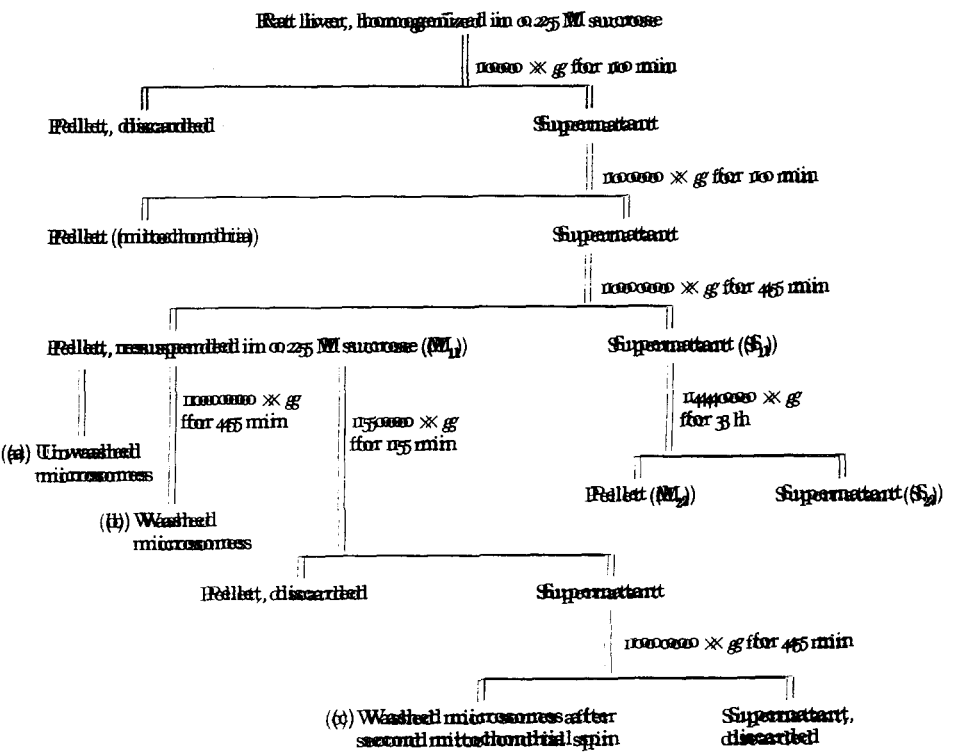


Fig. 1. Centrifugal fractionation of rat-liver homogenates: washed and unwashed microsomes.

*Electron microscopic studies of this pellet failed to reveal the presence of intact mitochondria.

TABLE I

EFFECT OF WASHING MICROSOMES ON THEIR ABILITY TO CONVERT MALONYL-CoA TO FATTY ACIDS

Unless otherwise specified, the standard incubation conditions for microsomes were 24 μmoles of glycylglycine buffer (pH 7.5), 1 μmole of $KHCO_3$, 7 μmoles of $MgCl_2$, 0.1 μmole of $MnCl_2$, 6 μmoles of reduced glutathione, 4.8 μmoles of ATP, 0.05 μmole of TPN, 8.4 μmoles of *dl*-isocitrate, 20 mμmoles of acetyl-CoA, 100 mμmoles of [1,3-$^{14}C_2$]malonyl-CoA ($3.4 \cdot 10^6$ counts/min), and enough isocitric dehydrogenase to make 160 mμmoles of TPNH per minute were incubated for 30 min at 37° with 1.5 mg of microsomal protein in a total volume of 0.4 ml with air as gas phase. Each value is the average of results and its standard error, from experiments with 6 different rat livers, and represents mμmoles of malonyl-CoA converted to fatty acids per mg protein per 30 min. See RESULTS section and Fig. 1 for preparation of microsomal fractions.

System	*^{14}C-labelled fatty acids*
Unwashed microsomes	3.2 ± 0.2
Washed microsomes	3.1 ± 0.3
Washed microsomes after a second mitochondrial spin	2.9 ± 0.1

converted to fatty acids per mg protein by these three differently prepared microsomes was the same (Table I).

If the particle-free supernatant solution (S_1) isolated after the removal of the microsomal pellet at 100000 × *g* (M_1) is centrifuged at 144000 × *g* for 3 h, a very small, clear red pellet (M_2) can be isolated and easily separated from its supernatant (S_2) (see Fig. 1 for flow diagram). For example, when 14 g of rat liver were homogenized with 35 ml of 0.25 M sucrose, the protein yield was 846 mg in S_1, 609 mg in M_1, 770 mg in S_2, and 55.8 mg in M_2. The ability of the M_1 and M_2 fractions to stimulate fatty acid synthesis from acetate by the S_1 and S_2 fractions was studied, and the results are presented in Table II. As previously noted[9-13], the addition of this M_1 fraction increased fatty acid synthesis by the S_1 fraction 4-fold; it also stimulated this conversion by the S_2 fraction. The M_2 fraction, however, did not stimulate either supernatant fraction. It should be noted that fatty acid synthesis by the S_2 fraction in the presence of M_2 was about the same as that observed with the S_1 fraction alone. For example, the S_1 fraction (10.5 mg protein) converted 3.5 mμmoles of acetate to fatty acids per mg supernatant protein, whereas the $S_2 + M_2$ fractions (containing 10.9 mg + 11.0 mg of protein, or twice as much as the S_1 fraction) converted 7.0 mμmoles of acetate to fatty acids. Apparently the fraction sedimented by centrifugation at 144000 × *g* for 3 h contains some—but not all—of the supernatant fatty acid-synthesizing activity. A somewhat similar finding has also been reported by WAKIL[20] with a particle-free fraction of avian liver.

It was of interest to determine whether protein was released into the supernatant fraction from microsomes during their isolation or incubation. Two types of protein were studied: (a) that containing malonyl-CoA decarboxylase (EC 4.1.1.9) activity, and (b) that concerned with conversion of malonyl-CoA to fatty acids (fatty acid synthetase). A liver homogenate prepared in the usual manner (see METHODS) was first cleared of unbroken cells and cellular debris by centrifugation at 1000 × *g* for 10 min, and the supernatant fraction so obtained was then freed of mitochondria and microsomes by centrifugation at 100000 × *g* for 45 min. The resulting particle-free supernatant fraction is designated S_1. The cytoplasmic particles (mitochondria and

TABLE II

EFFECT OF 100000 × *g* PELLET (M_1) AND 144000 × *g* PELLET (M_2) ON THE SYNTHESIS OF FATTY ACIDS FROM ACETATE BY PARTICLE-FREE SUPERNATANT FRACTIONS

Unless otherwise specified, the standard incubation conditions were: 60 μmoles of glycylglycine buffer (pH 7.5), 2.5 μmoles of $KHCO_3$, 17.5 μmoles of $MgCl_2$, 0.25 μmole of $MnCl_2$, 15 μmoles of reduced glutathione, 12 μmoles of ATP, 0.13 μmole of TPN, 0.25 μmole of CoA-SH, 1.25 μmoles of potassium [1-^{14}C]acetate ($3.24 \cdot 10^5$ counts/min), and 18.8 μmoles of potassium citrate were incubated with the homogenate fractions recorded below in a final volume of 1.0 ml for 2 h at 37°; gas phase, air. The values are given as mμmoles of acetate converted to fatty acids per mg supernatant protein (S_1 or S_2) per 2 h. See text and Fig. 1 for additional experimental details and preparation of the homogenate fractions.

Supernatant S_1 *(mg)*	*Protein* S_2 *(mg)*	*Pellet* M_1 *(mg)*	*Protein* M_2 *(mg)*	^{14}*C-labelled fatty acids*
10.5				3.5
10.5		1.0		10.0
10.5		2.1		13.5
10.5		10.5		7.0
10.5			1.1	4.7
10.5			2.2	3.6
10.5			11.0	3.4
	10.9			4.1
	10.9	1.0		7.0
	10.9	2.1		10.7
	10.9	10.5		8.3
	10.9		1.1	4.3
	10.9		2.2	6.3
	10.9		11.0	7.0

microsomes) were resuspended in isotonic sucrose solution and separated, by centrifugation at 15000 × *g* for 10 min, into mitochondria which were discarded, and into microsomes (100000 × *g* for 45 min, M_1). The supernatant solution from this final microsomal spin was called S_M (see Fig. 2).

The microsomal pellet (M_1) obtained from 20 g of liver was resuspended in 12 ml of a mixture containing 1500 μmoles of glycylglycine buffer (pH 7.5) 60 μmoles of

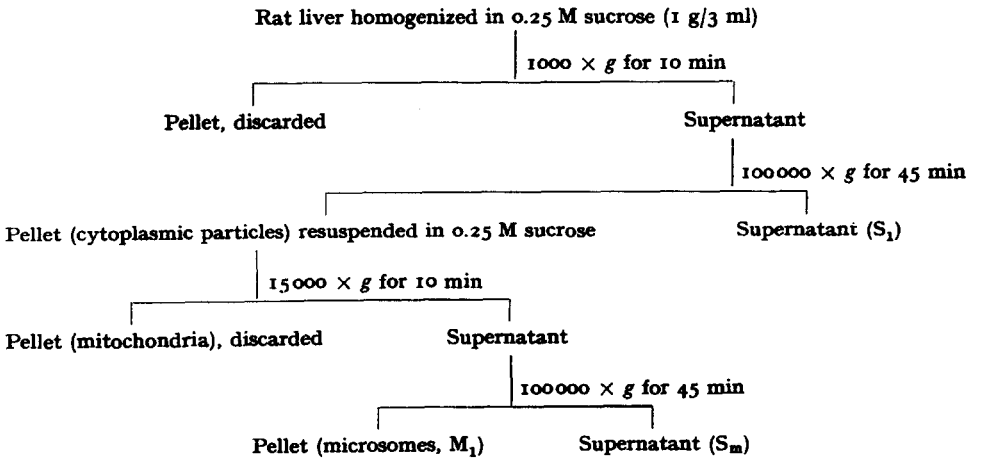


Fig. 2. Centrifugal fractionation of rat-liver homogenates: microsome preparation for preincubation studies.

reduced glutathione, and 290 μmoles of ATP (final volume 13.7 ml), and incubated in air at 37° for 5, 60 and 120 min. At the end of each incubation period, 4.2 ml of the suspension were withdrawn, cooled in an ice bath, and immediately centrifuged at 100000 × *g* for 45 min at 0°. This served to sediment the preincubated microsomes, which were designated M:5 (5 min), M:60 (60 min), and M:120 (120 min). The corresponding supernatants are called S:5, S:60, and S:120.

Each of the preincubated microsomal pellets was resuspended in isotonic sucrose (1.0 ml for M:5, 1.9 ml for M:60, and 2.0 ml for M:120). 3 mg of M:5, 3 mg of M:60 and 1.1 mg of M:120 were incubated in air for 30 min at 37° with 60 μmoles of glycylglycine (pH 7.5), 2.5 μmoles of $KHCO_3$, 17.5 μmoles of $MgCl_2$, 0.25 μmoles of $MnCl_2$, 15 μmoles of reduced glutathione, 12 μmoles of ATP, 0.13 μmole of TPN, 10 μmoles of glucose 6-phosphate, 100 mμmoles of acetyl-CoA, 500 mμmoles of [1,3-$^{14}C_2$]malonyl-CoA ($1 \cdot 10^5$ counts/min), and enough purified glucose-6-phosphate dehydrogenase to produce 0.7 μmole of TPNH per min, all in a final volume of 0.7 ml. To each 4 ml of the S:5, S:60 and S:120 fractions were added 72 μmoles of ATP, 0.75 μmole of TPN, 30 μmoles of glucose 6-phosphate, 100 mμmoles of acetyl-CoA, 500 mμmoles of [1, 3-$^{14}C_2$]malonyl-CoA ($1 \cdot 10^5$ counts/min), and enough purified glucose-6-phosphate dehydrogenase to produce 0.7 μmole of TPNH per min, making a final volume of 4.2 ml. These were incubated under the same conditions as were the microsomal fractions. In addition, as controls, S_1, S_M and M_1, were each incubated alone with the standard cofactors.

TABLE III

RELEASE OF MICROSOMAL PROTEIN BY PREINCUBATION

See Table I for incubation conditions for S_1, S_M and M_1. For experimental details and incubation conditions for S:5, S:60, S:120, M:5, M:60, and M:120, see RESULTS section

System	Protein incubated (mg)	mμmoles of [1,3-$^{14}C_2$]malonyl-CoA per mg protein per 30 min converted to:		CO_2 (fatty acids)
		CO_2	fatty acids	
S_1	1.0	31.7	11.4	2.8
S_M	1.5	21.0	3.6	5.8
M_1	1.5	13.5	2.2	6.1
S:5		35.6	0	
S:60		40.8	0	
S:120		75.0	0	
M:5	3.0	5.2	2.0	2.6
M:60	3.0	7.3	3.1	2.4
M:120	1.1	20.8	8.9	2.3

At the end of the incubation periods, all mixtures were analysed for $^{14}CO_2$ and ^{14}C-labelled fatty acids. The results presented in Table III show that the strong decarboxylase activity in the microsomes—but not the fatty acid-synthesizing activity—can be partially removed by preincubation of these particles. Furthermore, it is interesting to note that, after removal of some of the decarboxylase activity (by preincubation), the microsomes are now capable of converting malonyl-CoA more efficiently to fatty acids per mg protein.

The malonyl-CoA decarboxylase activity of liver microsomes was much higher than that of liver supernatant fractions (Table IV). A fraction obtained from lactating

TABLE IV

MALONYL-CoA DECARBOXYLASE OF SUPERNATANT FRACTIONS AND OF MICROSOMES

The incubation mixture consisted of 24 μmoles of glycylglycine buffer (pH 7.5), 1 μmole of $KHCO_3$, 7 μmoles of $MgCl_2$, 0.1 μmole of $MnCl_2$, 6 μmoles of reduced glutathione, 4.8 μmoles of ATP, 0.05 μmole of TPN, 20 mμmoles of acetyl-CoA, 100 mμmoles of [1,3-$^{14}C_2$]malonyl-CoA ($3.4 \cdot 10^4$ counts/min), and either (a) 6.3 μmoles of glucose 6-phosphate or (b) 10 μmoles of dl-isocitrate for 3.8 mg of liver supernatant fraction; or (a) 6.3 μmoles of glucose 6-phosphate and enough glucose-6-phosphate dehydrogenase to yield 240 mμmoles of TPNH per min or (b) 10 μmoles of dl-isocitrate and enough isocitric dehydrogenase to produce 210 mμmoles of TPNH per min for 1.6 mg of liver microsomal protein. The mixtures were incubated at 37° in a total volume of 0.4 ml with air as gas phase for the time indicated below. Each value recorded below is the average of two results obtained with each liver fraction.

Incubation time (time)	Ratio of $^{14}CO_2$: ^{14}C-labelled fatty acids from [1,3-$^{14}C_2$]malonyl-CoA by:			
	supernatant fraction in the presence of:		microsomes in the presence of:	
	glucose 6-phosphate	dl-isocitrate	glucose 6-phosphate	dl-isocitrate
15	1.7	1.7	2.4	2.3
30	1.5	1.6	2.4	2.8
120	1.4	1.5	3.8	4.0

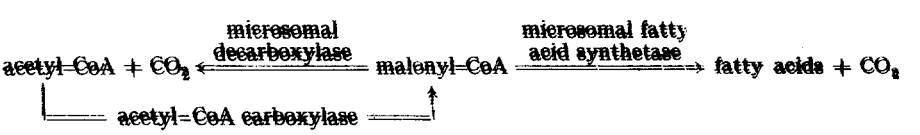
TABLE V

SYNTHESIS OF FATTY ACID FROM [1,3-$^{14}C_2$]MALONYL-CoA BY RAT-LIVER MICROSOMES IN PRESENCE AND ABSENCE OF ADDED CARBOXYLASE OBTAINED FROM LACTATING RAT MAMMARY GLANDS

See Table I and Fig. 1 for experimental details and incubation conditions. The acetyl-CoA carboxylase was isolated from homogenate fractions prepared from the mammary gland of a lactating rat as described in text. Each value is the average of results from two experiments with separate, 60 % glucose-fed rat-liver microsomal fractions, and represents mμmoles of malonyl-CoA converted to fatty acids per mg microsomal protein per 30 min.

Microsomal protein (mg)	Carboxylase protein (mg)	^{14}C-labelled fatty acids
0	0.31	0
1.5	0	10.8
1.5	0.31	25.1

rat mammary gland supernatant fractions, rich in acetyl-CoA carboxylase activity but devoid of fatty acid synthetase activity (Table V), increased the conversion of malonyl-CoA to fatty acids by the liver microsomes. This indicates that decarboxylation of malonyl-CoA by microsomes limits the amounts of malonyl-CoA converted to fatty acids. When carboxylase was added, this inhibition was overcome, and more malonyl-CoA was converted to fatty acids, probably by the following mechanism:



Chain length of fatty acids synthesized in the presence of the supernatant fraction alone, supernatant plus added microsomes, microsomes alone, and slices, prepared from rat liver

The carbon length of the fatty acids synthesized by these four systems was investigated by gas-chromatographic methods, and the results are shown in Table VI. In the supernatant system, the predominant fatty acid synthesized from acetate, acetyl-CoA and malonyl-CoA was palmitate, with about equal amounts of myristate coming from acetate and acetyl-CoA and smaller amounts from substrate malonyl-CoA. The synthesis of stearate, under these conditions, from any of the ^{14}C-labelled precursors used as substrates was very low. However, in the composite system (supernatant plus added microsomes), the amounts of stearate synthesized were increased.

TABLE VI

TYPES OF FATTY ACIDS SYNTHESIZED FROM [1-^{14}C]ACETATE, [1-^{14}C]ACETYL-CoA, AND [1,3-$^{14}C_2$]MALONYL-CoA BY RAT-LIVER HOMOGENATE FRACTIONS

See Table II for experimental conditions when supernatant or supernatant plus microsomes were incubated with [1-^{14}C]acetate. The incubation conditions for the experiments with [1,3-$^{14}C_2$]-malonyl-CoA are given in Tables I and IV. In the experiments with [1-^{14}C]acetyl-CoA, the incubation conditions were the same as those with [1-^{14}C]acetate except that CoA was omitted and the amount of acetyl-CoA was 0.3 μmole. In the experiments with slices, 250 mg (0.4 mm thick) were incubated with 2.0 ml of Krebs–Henseleit bicarbonate buffer[38] (pH 7.4) containing 2.5 μmoles of [1-^{14}C]acetate ($3 \cdot 10^6$ counts/min) for 2 h at 37° with 95% O_2 and 5% CO_2 as gas phase. The fatty acids were isolated after saponification of the slices in a manner similar to that for the homogenate fractions.

*Carbon chain length****	*Percent of total fatty acids synthesized by liver*							
	Supernatant fractions from:			*Supernatant fraction plus added microsomes from*:*			*Microsomal fraction from:*	*Slice from:*
	*[1-^{14}C]-acetate (5)***	*[1-^{14}C]-acetyl-CoA (3)***	*[1,3-$^{14}C_2$]-malonyl-CoA (6)***	*[1-^{14}C]-acetate (5)***	*[1-^{14}C]-acetyl-CoA (3)***	*[1,3-$^{14}C_2$]-malonyl-CoA (10)***	*[1,3-$^{14}C_2$]-malonyl-CoA (4)***	*[1-^{14}C]-acetate (4)***
6–12	1.1	1.9	1.3	1.3	0.5	0.8	3.3	0.2
12	5.7	4.2	1.4	2.6	1.0	0.7	1.9	0.2
12–14	1.2	1.4	0.6	0.6	0.4	0.3	1.7	0.2
14	38.5	24.6	8.5	20.3	20.9	6.2	1.5	5.2
14–16	2.5	2.0	4.4	1.6	1.3	1.2	1.5	5.2
16	27.6	28.1	56.9	31.8	44.5	45.3	11.6	53.3
16–18	1.8	4.5	2.2	2.3	1.9	5.0	3.4	0.9
18 + 18:1	1.5	2.8	3.4	12.3	11.0	13.6	35.6	31.9
18:2	1.1	1.4	1.5	1.2	0.8	2.3	1.1	0.3
18:2–20	2.9	4.2	3.3	2.0	1.0	3.6	3.4	0.3
20	3.1	1.3	3.1	1.1	1.0	2.8	4.5	0.7
20–20:4	4.2	6.9	3.3	7.3	3.3	4.3	6.5	0.5
20:4	2.3	3.3	3.5	2.0	1.8	2.1	2.6	1.9
20:4–22		3.1	1.8	3.7	1.3	1.4	2.4	0.3
22	4.7	4.2	1.8	5.5	2.7	3.7	7.6	1.2
above	7.0	7.1	3.5	4.3	6.6	6.8	12.3	3.2
Total	100.2	100.0	100.5	99.9	100.0	100.0	100.0	100.5

* The ratio of supernatant protein to microsomal protein was 5 to 1.

** The figure in parentheses is the number of experiments with different rat livers.

*** The figure after the colon represents the number of double bonds in the fatty acid (18:1 is oleic acid).

In contrast to what was observed with the supernatant, the microsomal fraction made little myristate, a much lower amount of palmitate, and a great deal more stearate*.

The last column in Table VI shows the results obtained in experiments with slices. It is of interest to note that the pattern of chain lengths of the synthesized fatty acids from acetate by the slice more closely resembles that observed with the composite system than that with the supernatant fraction alone.

Since the microsomes produced mainly stearate from malonyl-CoA, and since the addition of microsomes to the supernatant produced a shift in the pattern of the fatty acids synthesized, it became of interest to see if the addition of more microsomes would alter the distribution of ^{14}C in the fatty acids of longer chain lengths. The results of such experiments are shown in Table VII. The addition of larger amounts of microsomes shifted the synthesis in favor of stearate.

TABLE VII

THE EFFECT OF MICROSOMES ON CHAIN LENGTH OF FATTY ACIDS SYNTHESIZED FROM [1-^{14}C]ACETATE

See Table II for experimental details and incubation conditions. Particle-free supernatant fraction S_1 contained 10.5 mg protein, the microsomal fraction M_1, when added, contained 2.1 mg protein (5:1 protein ratio) and 5.1 mg protein (2:1 protein ratio). Each value is the average of two separate, closely agreeing determinations.

Carbon chain length	*% of total fatty acids synthesized from [1-^{14}C]acetate by:*		
	supernatant alone	*supernatant plus microsomes, protein ratio of 5:1*	*supernatant plus microsomes, protein ratio of 2:1*
14	33.5	19.1	6.3
16	27.6	27.3	9.8
18	1.5	5.3	39.8

Table II presents evidence indicating that the microsomal fraction (M_1), isolated from liver homogenates as a pellet, by centrifugation at 100000 $\times$ *g* for 45 min (after the nuclear fraction, cellular debris and mitochondria were removed), was distinctly different from another pellet (M_2) isolated from the supernatant fraction S_1 by a longer spin at a greater gravitional force. In view of the different fatty acids synthesized from acetate by the supernatant fractions S_1 and the supernatant S_1 plus microsomal fraction M_1, we investigated the chain lengths of the fatty acids synthesized from acetate by various recombinations of the two types of supernatant fractions and two types of pellets. Table VIII shows the results of gas-chromatographic analysis of the fatty acid methyl esters obtained with these fractions. Only systems that contained microsomal fractions produced significant amounts of stearate. This type of evidence further supports our conclusion that the activities of the M_1 and M_2 pellets differ, and suggests that the microsomal stimulation is restricted to the particles sedimented at 100000 $\times$ *g* for 45 min after removal of mitochondria.

* Preliminary degradative studies on these isolated fatty acids indicate that, at least in part, *de novo* synthesis occurred.

TABLE VIII

CHAIN LENGTH OF FATTY ACIDS SYNTHESIZED FROM [1-^{14}C]ACETATE BY A COMBINATION OF 2 TYPES OF SUPERNATANT FRACTIONS, S_1 AND S_2, AND 2 TYPES OF PELLETS, M_1 AND M_2

See Fig. 1 for preparation of liver homogenate fractions and Table II for experimental details and incubation conditions. 10.5 mg of S_1, 3.1 mg of M_1, 10.9 mg of S_2, and 2.2 mg of M_2 protein were added as indicated below.

Carbon chain length	*% of total fatty acids synthesized from [1-^{14}C]acetate by:*			
	$S_1 + M_1$	$S_2 + M_1$	$S_1 + M_2$	$S_2 + M_2$
14	19.6	23.7	28.9	23.4
16	36.5	37.4	25.0	15.7
18	8.9	9.9	2.0	1.2

Types of lipids synthesized by the supernatant fraction alone, supernatant plus added microsomes, microsomes alone, and slices prepared from rat liver

Table IX shows the types of lipids synthesized by the different enzyme systems under investigation. The supernatant fraction predominantly catalysed the formation of free fatty acids, while the microsomal fraction produced phosphorus-containing lipids (phosphatide acids, phospholipids, etc.). When they were combined (composite system) again, the pattern shifted from that observed with the supernatant fraction alone to that found with the microsomal system alone. It should be noted that this shift in the pattern of the synthesized lipids is towards that produced by slices.

TABLE IX

TYPE OF LIPIDS SYNTHESIZED BY THE LIVER SUPERNATANT FRACTION, SUPERNATANT FRACTION PLUS MICROSOMES, MICROSOMES ALONE, AND BY THE SLICE

Results are presented as the average percentage of the total lipids isolated from each fraction.

System	*^{14}C-labeled substrate*	*No. of expts.*	*Cholesterol ester*	*Triglyceride*	*Cholesterol; mono- and diglycerides, and free fatty acids*	*P-containing lipids*
Supernatant alone	[1-^{14}C]Acetate	3	0.8	3.8	90.1*	5.3
Supernatant plus microsomes	[1-^{14}C]Acetate	3	1.6	11.1	13.4	73.9
Microsomes alone	[1,3-$^{14}C_2$]Malonyl-CoA	2	6.4	5.8	2.9	84.9
Slice	[1-^{14}C]Acetate	4	2.3	18.5	40.5**	38.7

* This fraction was applied to a Florisil column, and separated into its individual components[22]. It was composed of 0% hydrocarbon, 0.3% triglyceride, 0.3% cholesterol, 4.6% diglyceride, 0.6% monoglyceride and 94.2% free fatty acids.

** This fraction was composed mainly of ^{14}C-labelled mono- and diglycerides. Only 1–2% of the total ^{14}C-activity was precipitated as the digitonide[36] and almost no ^{14}C-activity was adsorbed on Amberlite IR-4B ion-exchange resin.

Factors influencing the fatty acid synthesis by rat-liver microsomes

Effect of incubation time. Fig. 3 shows a comparison of fatty acid synthesis from malonyl-CoA, as a function of time, by the supernatant fraction and by the microsomal fraction. With both fractions, the conversion proceeded linearly during the

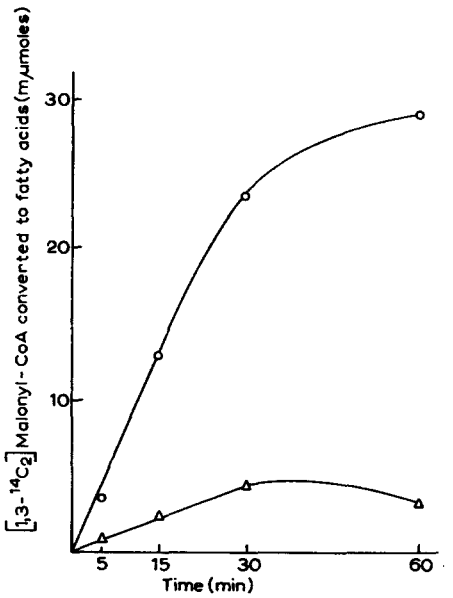


Fig. 3. Effect of incubation time on incorporation of the ^{14}C of [1,3-$^{14}C_2$]malonyl-CoA into fatty acids by rat-liver supernatant (1.2 mg protein) and microsomal (1.2 protein) fractions. Glucose 6-phosphate was used as TPNH-generating substrate. For experimental details see Table IV. Each value is the average of two experiments with different rat-liver fractions. O—O, supernatant fraction; Δ—Δ, microsomes.

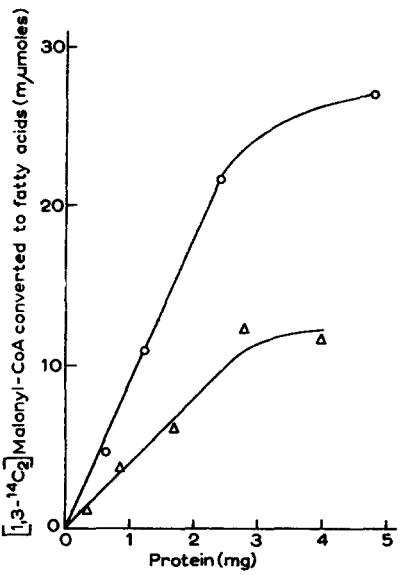


Fig. 4. Effect of protein concentration on incorporation of the ^{14}C of [1,3-$^{14}C_2$]malonyl-CoA into fatty acids by rat-liver supernatant and microsomal fractions. 24 μmoles of glycylglycine buffer (pH 7.5), 1 μmole of $KHCO_3$, 7 μmoles of $MgCl_2$, 0.1 μmole of $MnCl_2$, 6 μmoles of reduced glutathione, 4.8 μmoles of ATP, 0.1 μmole of TPN, 30 mμmoles of acetyl-CoA, 150 mμmoles of [1,3-$^{14}C_2$]malonyl-CoA ($0.47 \cdot 10^5$ counts/min), 2 μmoles of glucose 6-phosphate, and 0.5 μg of purified glucose-6-phosphate dehydrogenase (enough to produce 0.4 μmole of TPNH per min) were incubated with either (a) the supernatant fraction for 15 min, or (b) the microsomal fraction for 30 min at 37° with air gas phase, in a total volume of 0.4 ml. Each value is the average of two experiments with different rat-liver fractions. O—O, supernatant fraction; Δ—Δ, microsomes.

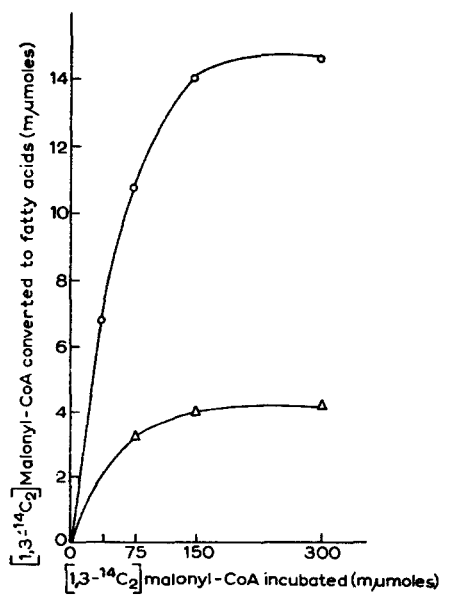


Fig. 5. Effect of malonyl-CoA concentration on its conversion to fatty acids by rat-liver supernatant and microsomal fractions. 1 mg of supernatant fraction protein, or 1.5 mg of microsomal fraction protein, were incubated for 15 min under the conditions recorded in Fig. 4, and with the amounts of [1,3-$^{14}C_2$]malonyl-CoA indicated above. At each point studied, the amount of acetyl-CoA added was one-fifth of the added malonyl-CoA. O—O, supernatant fraction; Δ—Δ, microsomes.

first 30 min. It should be noted that the activity (mμmoles of malonyl-CoA converted to fatty acids per mg protein) of the supernatant fraction is about 6 times greater than that of the microsomal fraction.

Effect of protein concentration. Fatty acid synthesis from malonyl-CoA by the supernatant fraction and by the microsomal fraction was compared as a function of protein concentration (Fig. 4). Under these specific incubation conditions, the reaction in the supernatant fraction proceeded linearly for 15 min, up to 2.5 mg of protein; the reaction in the microsomes proceeded linearly for 30 min, up to 2.5 mg of protein.

Effect of malonyl-CoA concentration. Fig. 5 shows that, when the malonyl-CoA concentration was varied from 37 mμmoles to 300 mμmoles per 0.4 ml of incubation volume, the enzymes present in the supernatant and in the microsomal fractions responsible for converting malonyl-CoA to fatty acids are saturated with substrate at about the 150-mμmole level. At each concentration studied, the amount of unlabelled acetyl-CoA added was one-fifth that of the malonyl-CoA.

Malonyl-CoA decarboxylase activities of the particle-free supernatant fraction and of the microsomal fraction

The decarboxylase activity of the microsomal fraction was 2–3 times higher than that of the supernatant fraction (Table IV). In addition, it should be noted that the ratio of $^{14}CO_2$ to ^{14}C-labelled fatty acids produced from [1,3-$^{14}C_2$]malonyl-CoA by the supernatant fraction was only slightly higher than the theoretical value of 1 which would be expected as a result of fatty acid synthesis from this precursor. This suggests that this fraction contains but a slight amount of decarboxylase. However, as shown in Table III, the decarboxylase activity present in the microsomes can be released into the supernatant. Thus, it is of importance to clear the supernatant of these particles as rapidly as possible to keep the supernatant relatively free of this enzyme.

DISCUSSION

The mitochondria isolated by WAKIL *et al.*[30,31] from avian-liver homogenates are capable of converting acetyl-CoA to fatty acids —predominantly stearate. These workers have presented evidence for *de novo* synthesis by these particles. Since our microsomal system, which was devoid of intact mitochondria, was also capable of synthesizing this C_{18}-acid as the principle product, a more detailed study of these particles became our primary concern. In previous communications it was shown that malonyl-CoA, but not acetate nor acetyl-CoA, can serve as an efficient precursor of the fatty acids synthesized by the rat-liver microsomes[12,13]. The mitochondria, on the other hand, can utilize acetyl-CoA as substrate[29–31]. The present report shows that microsomes will convert malonyl-CoA to complex lipids (phospholipids) rather than to acyl-CoA derivatives of free fatty acids. Evidence presented elsewhere[10] concerning the very low oxidative capacity of the microsomes suggests that it is very unlikely that these small particles are contaminated with mitochondria or mitochondrial sub-units. Thus, from our evidence, it may reasonably be concluded that the microsomes represent a locus for fatty acid synthesis.

LYNEN and his coworkers[32] have isolated a fatty acid-synthesizing system from yeast which converts malonyl-CoA to fatty acids (fatty acid synthetase) and behaves

as a single protein of high "molecular weight" containing a multiplicity of enzymic activities. In view of this finding, we might be tempted to conclude that microsomal stimulation is due to contamination of those particles by a synthetase complex in the particle-free supernatant fraction, such as that described by LYNEN. But the following considerations rule out this possibility: (1) the pellet obtained after prolonged high-speed centrifugation of the particle-free supernatant fraction did not exhibit the stimulatory properties of microsomes; (2) the addition of microsomes to the particle-free supernatant fraction stimulated fatty acid synthesis from acetate and acetyl-CoA, but not from malonyl-CoA[12,13,15]; (3) it is well established that the carboxylation of acetyl-CoA is the rate-limiting step in the synthesis of fatty acids from acetate and acetyl-CoA[17,33,34]. Thus, if the stimulatory effect of microsomes was due solely to their ability to convert malonyl-CoA to fatty acids, we should expect the stimulation to occur only when malonyl-CoA was used as substrate.

Our finding of a fatty acid synthetase in the microsomal fraction raises the question of localization of fatty acid-synthesizing systems in the mammalian cell. The evidence presented here indicates no ready leakage of fatty acid synthetase activity from these particles into the supernatant fraction. That the microsomes can release protein into the supernatant, however, is shown by the appearance of additional malonyl-CoA decarboxylase activity after a preincubation period. Under all conditions studied, the specific activity of the crude supernatant synthetase was 5–6 times greater than that of the crude microsomal synthetase. By decreasing the microsomal content of malonyl-CoA decarboxylase, the apparent specific activity of the microsomal synthetase was increased, probably because of competition between fatty acid synthetase and decarboxylase for the substrate malonyl-CoA.

The observed shift in carbon chain length of the fatty acids synthesized as well as in the synthesis of complex lipids to a pattern similar to that found with slices justifies the assumption that the activity of the composite system resembles more closely that of the physiological state than does the activity of the supernatant system alone. The addition of mitochondria to the supernatant or to the supernatant plus microsomal system invariably resulted in a decrease in the ^{14}C-labelled fatty acid yields from [^{14}C]acetate.

Reports from this laboratory have shown[10,35] that, when the composite system is used, after an initial lag-phase, the microsomes cause an increase in the reaction rate as well as in the final level of product. This observation, together with the fact that the microsomal stimulation is observed only with acetate and acetyl-CoA but not when malonyl-CoA serves as substrate, appears to localize the microsomal action to the carboxylase step. This effect of rat-liver microsomes upon fatty acid synthesis in the supernatant fraction can be imitated by the addition of purified acetyl-CoA carboxylase obtained from lactating rat mammary gland homogenates (S. ABRAHAM, E. LORCH AND I. L. CHAIKOFF, unpublished observations).

It is conceivable that microsomes enhance fatty acid synthesis by stimulating or by removing an inhibitor of acetyl-CoA carboxylase. It is possible that one of the reaction products (acyl-CoA, fatty acid, protein-bound fatty acid, or fatty acid ester) is an inhibitor of this enzyme (feedback inhibition) and that the further conversion of these intermediates by microsomal enzymes results in a non-inhibitory complex lipid. Another possibility, the release from microsomes of an activator of the carboxylase enzyme, cannot be ruled out at present.

In view of their high content of malonyl-CoA decarboxylase and their ability to stimulate fatty acid synthesis from acetate and acetyl-CoA, the interesting possibility that microsomes function as an important regulatory system for fatty acid synthesis within the cell should be kept in mind.

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