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THE ROLE OF MICROSOMES IN FATTY ACID SYNTHESIS FROM ACETATE BY CELL-FREE PREPARATIONS OF RAT LIVER AND MAMMARY GLAND

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SUMMARY

1. The conversion of acetate to fatty acids by particle-free supernatant and microsomal fractions of rat livers and of lactating and non-lactating rat-mammary glands was studied.

2. Stimulation of fatty acid synthesis by addition of microsomes to the supernatant fraction was related to the amounts of microsomal protein and ATP added to the incubation mixtures. Excessive amounts of either one depressed the level of synthesis, which could be reversed by addition of larger amounts of the other.

3. Microsomal stimulation cannot be explained solely by the presence of microsomal ATPase since the highest level of synthesis was observed at a critical ratio of microsomal protein to ATP concentration. This suggests that microsomal stimulation is related to an ATP-dependent process.

4. The newly synthesized fatty acids were firmly bound to the incubated protein. Upon re-isolation of microsomes at the end of incubation, it was found that 80 % of the newly synthesized fatty acids was bound to these particles. However, microsomal stimulation cannot be explained solely on the basis of a binding phenomenon.

5. The possibility that microsomal stimulation of fatty acid synthesis from acetate or acetyl-CoA is due to an effect on acetyl-CoA carboxylase present in the supernatant fraction is discussed.

INTRODUCTION

It is now well established that fatty acid synthesis from acetate via the malonyl-CoA pathway[1-7] occurs in the particle-free supernatant fraction obtained from a variety of tissues. Several observations[5,7-12] indicate, however, that addition of microsomes to a properly fortified medium containing the particle-free supernatant fraction modifies the extent of fatty acid synthesis taking place in this incubation mixture. The effect obtained depends upon the amount of microsomes added: small amounts augment the

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synthesis, but additions beyond a certain level result in decreased formation of fatty acids[9,12,13].

The present report deals with the relation of ATP to the action of microsomes on fatty acid synthesis. The binding of the newly synthesized fatty acids to protein of the supernatant fraction and to cytoplasmic particles was also investigated. Two tissues were studied: livers of rats and mammary glands of lactating and non-lactating rats.

EXPERIMENTAL

Animals and their treatment

Livers were excised from normal, Long-Evans rats weighing 200–300 g, that had been fed an adequate stock diet (Diablo Labration). Mammary glands were excised from lactating (10–15 days postpartum) and non-lactating rats. The former had suckled at least five pups at the time they were killed; the latter had lactated for 21 days and were killed two days after removal of the pups, by which time lactation was arrested. The lactating and non-lactating animals were fed a specially prepared diet consisting of 67.5 % whole wheat, 15 % casein, 7.5 % whole milk powder, 6.75% vegetable oil, 1.5 % calcium carbonate, 1.0 % fish oil concentrate (containing 3000 USP units of vitamin A and 400 chick units of vitamin D per g) and 0.75 % potassium chloride with 150 mg of potassium iodide/280 lbs. of diet.

Preparation of tissue homogenate fractions and incubation procedures

Liver experiments. The livers were first minced and then homogenized with 3 volumes of an isotonic (0.25 M) sucrose solution in a Potter–Elvehjem homogenizer, the teflon pestle of which had a tolerance of 0.5 mm. The nuclei and cellular debris were removed by centrifugation at 1000 × g* for 10–15 min. Mitochondria were separated by centrifugation at 8700 × g for 10 min in the Spinco model L ultracentrifuge. The microsomes** were sedimented by centrifugation at 78000 × g for 45 min, the fatty surface layer was discarded, and the clear supernatant fraction was removed with a pipet. The mitochondrial and microsomal pellets were separately rehomogenized in isotonic sucrose solution, and each was resedimented, the former at 8700 × g for 10 min, the latter at 78000 × g for 45 min. The isolated, washed particles were separately resuspended by homogenization in the isotonic sucrose solution, and the volume of each suspension was adjusted to 1 ml/2 g of original tissue homogenized. All of these procedures were carried out in a cold room maintained at 2–4°.

Unless otherwise specified, 1.5 ml of the particle-free, supernatant fraction (20–30 mg protein), either alone, or with 0.1 ml of the microsomal suspension (4–6 mg protein) obtained from the same liver, were incubated, in stoppered tubes, for 2 h at 37°, with 2 ml of incubation medium. The medium contained 240 μmoles of glycylglycine–KOH buffer (pH 7.5), 10 μmoles of $KHCO_3$, 70 μmoles of $MgCl_2$, 1 μmole $MnCl_2$, 60 μmoles of GSH (K^+ salt), 48 μmoles of ATP (K^+ salt), 1.0 μmole of TPN, 0.1 μmole of CoASH, 75 μmoles of potassium citrate, and 6.0 μmoles of potassium [1-^{14}C]acetate. The total volume was 3.6 ml, and air was the gas phase[5].

* All centrifugal values refer to the average force in the center of the tube.

** Electron microscopic study of the microsomal pellet showed that no intact mitochondria were present in this fraction, and revealed a typical microsomal picture. As jugded by the absence of mitochondrial-bound enzymes in the supernatant fraction[9,14], apparently little or no destruction of mitochondria occurred.

Mammary-gland experiments: Mammary-tissue slices 0.4 mm thick were prepared with a McILWAIN–BUDDLE[15] tissue chopper, and washed at least 4 times with isotonic sucrose solution to remove as much of the preformed milk as possible. The slices were next finely minced with scissors, and then homogenized. The homogenate was separated into mitochondria, microsomes, and a particle-free supernatant fraction, as described above.

1.5 ml of the particle-free supernatant fraction (12–18 mg protein) were incubated in stoppered tubes for 2 h, at 37°, with 2 ml of medium containing (unless otherwise specified) 255 μmoles of glycylglycine–KOH buffer (pH 7.2), 10 μmoles of $KHCO_3$, 70 μmoles of $MgCl_2$, 1 μmole of $MnCl_2$, 60 μmoles of GSH (K^+ salt), 10 μmoles of ATP (K^+ salt), 1.0 μmole of TPN, 0.1 μmole of CoASH, 50 μmoles of potassium citrate, and 6.0 μmoles of potassium [1-^{14}C]acetate. The total volume was 3.5 ml, and air was the gas phase[6].

Analytical procedures

The contents of the incubation tube were first saponified with KOH and then acidified with HCl. The fatty acids were isolated from the mixture by two extractions with *n*-hexane. This procedure completely extracts the fatty acids of chain lengths higher than heptanoic, and no acetic acid can be found in these hexane extracts[6]. The hexane extracts were assayed for ^{14}C activity, in a Packard automatic tri-carb liquid scintillation spectrometer to within ± 3 % as described[6].

Protein was determined by the biuret method described by GORNALL *et al.*[16], and inorganic phosphate by the method of FISKE AND SUBBAROW[17].

RESULTS

ATP requirement for microsomal stimulation

Liver: Fig. 1 shows the effect of adding varying amounts of ATP on fatty acid synthesis by the particle-free supernatant fraction and by the system composed of the supernatant fraction plus microsomes. In the former, highest fatty acid synthesis was observed in the presence of 12 μmoles of ATP/3.7 ml of incubation volume. In the latter, synthesis of fatty acids continued to rise with increasing ATP concentrations.

Lactating mammary gland: In the experiments in which only the particle-free supernatant fraction was used, highest fatty acid synthesis was observed in the presence of 10 μmoles of added ATP/3.6 ml of medium (Fig. 2). When greater amounts of ATP were added the incorporation of acetate carbon into fatty acids fell off. This effect of the larger amounts of ATP was overcome by the addition of microsomes obtained from the same gland; in those experiments the ratio of supernatant to microsomal protein was about 8 to 1 (Fig. 2).

The amount of synthesis induced by addition of microsomes to the particle-free supernatant fractions in the presence of 48 μmoles of ATP (Fig. 2) exceeded by far the highest level of fatty acid synthesis observed in the experiments with the particle-free supernatant fraction alone.

It should be noted that the synthesis of fatty acids per milligram supernatant protein was several fold higher in the experiments with lactating rat-mammary-gland fractions than in those with liver fractions (compare Figs. 1 and 2).

The addition of lactating mammary-gland microsomes to the particle-free liver-

supernatant fractions resulted in a pronounced increase in the conversion of acetate carbon to fatty acids (compare Figs. 1 and 3). Indeed, in this respect, the lactating mammary-gland particles were superior to liver microsomes. For example, when 7.4 mg of lactating mammary-gland microsomal protein were added to 29.5 mg of particle-free liver supernatant fraction (Curve A, Fig. 3), synthesis of fatty acids

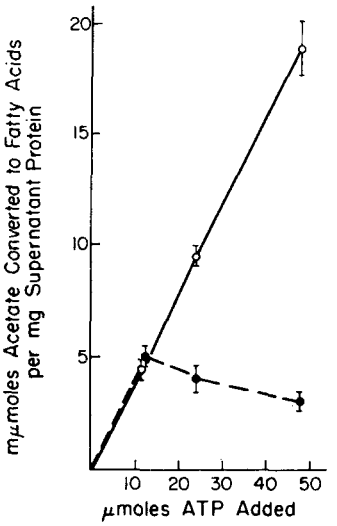

Fig. 1. Effect of ATP on fatty acid synthesis in normal rat-liver fractions. ●—●, the results obtained in experiments with supernatant fractions alone; O—O, those with the system composed of microsomes plus supernatant fraction. The particle-free supernatant fraction contained 29.5 mg of protein, and the microsomes 6.0 mg of protein per incubation volume of 3.6 ml. Each value is the mean of the results obtained in three experiments, and the vertical lines are the standard errors of the means.

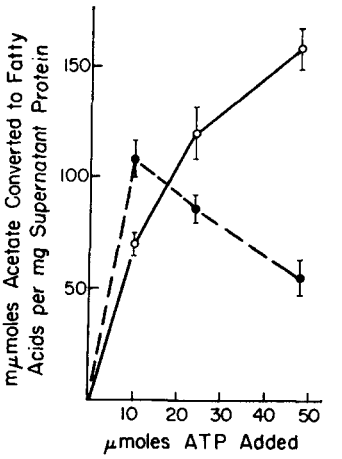

Fig. 2. Effect of ATP on fatty acid synthesis in lactating mammary-gland fractions. ●—●, results obtained in experiments with the supernatant fraction alone; O—O, those in which microsomes plus the supernatant fraction (both obtained from the same gland) were used. The particle-free supernatant fraction contained 14.8 mg of protein, and the microsomes 1.8 mg of protein per incubation. Each value is the mean, and the vertical line its standard error, of results obtained in three experiments.

from acetate amounted to 70 mμmoles/mg supernatant protein in the presence of 48 μmoles of added ATP, whereas when 6.0 mg of liver microsomal protein were added to the same amount of liver supernatant protein, the synthesis of fatty acids amounted to only 19 mμmoles/mg supernatant protein at this same ATP level* (Fig. 1).

The results of experiments recorded in Fig. 3 demonstrate the delicate balance that exists between the supernatant-to-microsomal protein ratio on the one hand and ATP concentration on the other. The lower the ratio of supernatant protein to microsomal protein (Curve A, 4/1; Curve B, 8/1; curve C, 16/1), the higher were the amounts of ATP required to obtain optimal fatty acid synthesis. Thus, when the ratio was 4/1 (Curve A), 48 μmoles of ATP were required to produce optimal synthesis, whereas when the ratio was 16/1 (Curve C), 36 μmoles of ATP produced optimal synthesis. Optimal fatty acid synthesis from acetate did not result when the super-

* It should be noted in this connection that, when larger amounts of liver microsomes are added under these conditions, fatty acid synthesis from acetate decreases[9,12,13].

natant-to-microsomal protein ratio was high at any ATP concentration (compare Curves A and C).

Non-lactating mammary glands: The addition of microsomes from non-lactating mammary-gland homogenates to particle-free supernatant fractions prepared from livers or lactating mammary glands failed to stimulate conversion of acetate to fatty

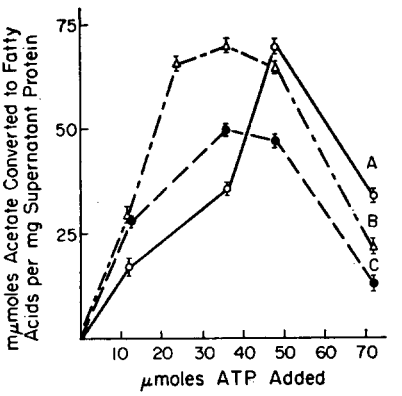

Fig. 3. Effect of ATP on fatty acid synthesis in incubation mixtures containing liver supernatant fractions and lactating mammary-gland microsomes. Each incubation mixture contained 29.5 mg of liver supernatant protein and varying amounts of mammary-gland microsomal protein: 7.4 mg, Curve A; 3.7 mg, Curve B; 1.8 mg, Curve C. Each point represents the mean, and the vertical line its standard error, of three experiments with different rats.

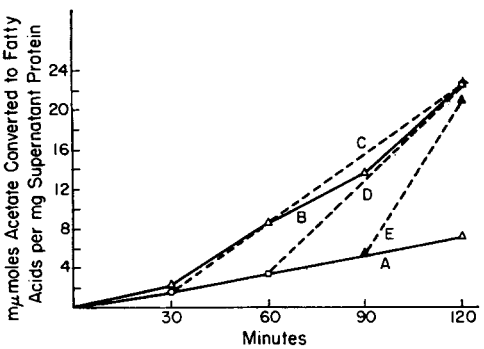

Fig. 4. Effect on fatty acid synthesis of adding rat-liver microsomes to the particle-free liver-supernatant fractions at different intervals during the incubation. To 0.5 ml of liver medium (p. 358) were added: 0.4 ml of supernatant fraction (8.8 mg protein) and, as indicated, 0.05 ml of a microsomal suspension (1.3 mg protein). Each point is the mean of two closely agreeing values obtained in two separate experiments with different rats. Curve A, no microsomes added. Microsomes added at 0 time (curve B), 30 min (Curve C), 60 min (Curve D), 90 min (Curve E) after the start of incubation.

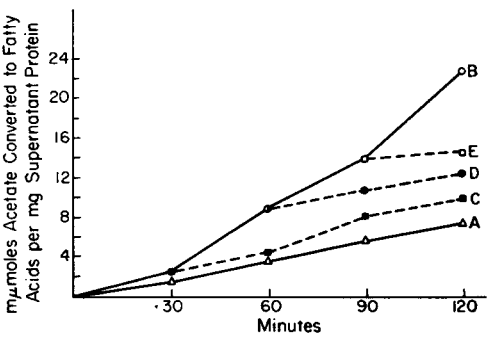

Fig. 5. Effect of removal of microsomes at different incubation times on fatty acid synthesis by the reaction mixture containing supernatant plus microsomes, both obtained from the same liver. See Fig. 4 for experimental details. Supernatant fraction plus added microsomes was incubated for 30, 60, or 90 min before the microsomes were removed ultracentrifugally at 0° as described in text. The reaction mixture, devoid of microsomes, was then reincubated for 90, 60, or 30 min. Each point on the curves is the mean of two closely agreeing values from two separate experiments with different rats. Curve A shows results of the experiment in which supernatant alone was incubated, and Curve B that in which supernatant plus microsomes were present in the reaction mixture throughout the entire 2-h incubation period. The results of the experiments in which microsomes were removed after 30 min are shown in Curve C; those in which they were removed after 60 min in Curve D; and after 90 min in Curve E.

acids at any ATP concentration. Indeed, even in the presence of 48 μmoles of ATP, in experiments with liver-supernatant fractions, as well as in the presence of 10 μmoles of ATP, in experiments with lactating gland-supernatant fractions, the addition of non-lactating gland microsomes strongly inhibited fatty acid synthesis.

The effect of addition and removal of liver microsomes at various time intervals during incubation on synthesis of fatty acids by liver supernatant fractions

The results of experiments in which liver microsomes were added to reaction mixtures containing liver-supernatant fractions at various intervals during the incubation are shown in Fig. 4. The reactions were allowed to proceed for a total of 2 h. The rate of fatty acid synthesis was increased in all experiments by the addition of microsomes. In the experiment in which microsomes were added at the start of the incubation period, a lag of about 30 min occurred before a rise in the rate of fatty acid synthesis was observed.

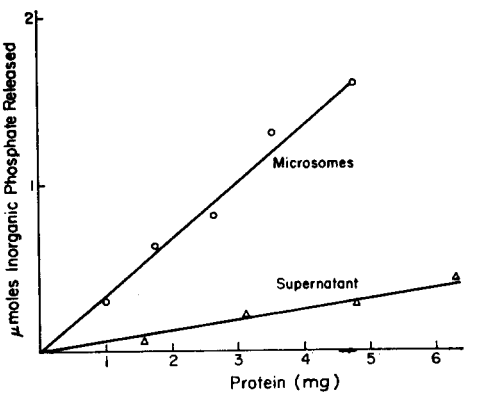

Fig. 6. Relation of ATPase activity to protein content of supernatant and microsomal fraction. Particle-free supernatant or microsomal fractions were incubated with 35 μmoles of glycylglycine–KOH buffer (pH 7.5), 20 μmoles $MgCl_2$ and 5 μmoles ATP (Na^+ salt) for 5 min at 37° in a final volume of 1.0 ml, with air as the gas phase. The reactions were stopped with 1.0 ml of 5% perchloric acid, and inorganic phosphate was determined[17] on duplicate aliquots of the protein-free filtrates. Each point is the average of the two closely agreeing values.

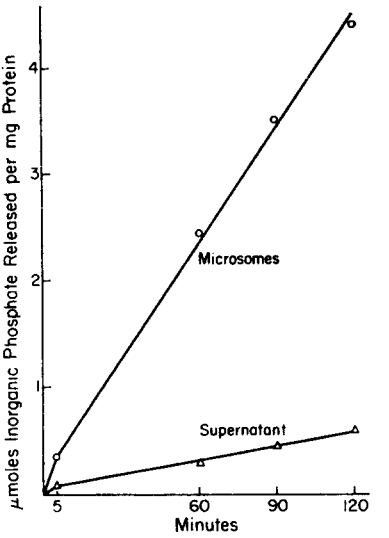

Fig. 7. Time dependence of normal rat-liver supernatant fraction and normal rat-liver microsomal ATPase activity. Particle-free supernatant fraction (3.14 mg protein) or microsomes (2.63 mg protein) were incubated, and inorganic phosphate was determined as described in Fig. 6.

The effect of removal of microsomes from the reaction mixtures at various times of incubation is shown in Fig. 5. When microsomes were removed, they were analysed for [^{14}C]fatty acids, and the values were added to those obtained from the supernatant fractions. Regardless of when the particles were removed the result was a reduction in the rate of fatty acid synthesis.

ATPase activity of liver supernatant and of liver and lactating mammary-gland microsomal fractions

Fig. 6 shows a typical linear response between the amounts of inorganic phosphate released from ATP and amounts of liver supernatant or liver-microsomal protein added to the incubation medium. The specific activity* of the microsomal ATPase was more than 5 times that of the particle-free supernatant ATPase.

In Fig. 7 the release of inorganic phosphate from ATP per milligram protein of liver supernatant or liver microsomal fractions was plotted against time. The reactions proceeded linearly from 5 min to 2 h. In these experiments, ATPase activity of the microsomes was about 7 times higher than that of the supernatant fraction. For example, after 2 h of incubation, the microsomes released about 4.4 μmoles of inorganic phosphate from ATP whereas the supernatant fraction released about 0.6 μmoles per mg protein.

TABLE I

CATION-DEPENDENT ADENOSINE TRIPHOSPHATASE ACTIVITY IN RAT LIVER AND LACTATING MAMMARY-GLAND HOMOGENATE FRACTIONS

1–2 mg of protein were incubated with 320 μmoles of triethanolamine buffer (pH 7.4), 6 μmoles of ATP, 12 μequiv. of Na^+, and either 8 μequiv. of Mg^{2+} or 20 μequiv. of K^+ in a final volume of 2.0 ml for 20 min at 30° with air as the gas phase. Average values of the results obtained in 4–6 experiments, and standard errors, are given below. Each experiment was carried out with liver fractions obtained from a single rat, and the results are expressed as μmoles inorganic phosphate released[17] from ATP/mg protein/h.

No. of Expts.	*Cations added*	*Inorganic phosphate per milligram protein released*		
		Liver		*Lactating mammary gland*
		Supernatant fraction	*Microsomes*	*Microsomes*
4	Na^+	0.004 ± 0.002	0.21 ± 0.06	0.33 ± 0.12
6	Na^+ and Mg^{2+}	0.200 ± 0.012	3.04 ± 0.18	4.73 ± 0.42
6	Na^+ and Ca^{2+}	0.094 ± 0.017	2.10 ± 0.14	3.60 ± 0.08
4	Na^+ and K^+	0.010 ± 0.004	0.34 ± 0.12	0.51 ± 0.16

The results shown in Table I demonstrate that both liver fractions have highest ATPase activities in the presence of sodium and magnesium ions. These results also illustrate the central role played by Mg^{2+} in the cation-dependent ATPase of rat-liver supernatant and of liver and lactating mammary-gland microsomal fractions. Magnesium ion can be partially replaced by calcium ion. In the absence of these bivalent ions, ATPase activity is very low. Under the incubation conditions recorded in Table I, the amount of inorganic phosphate released from ATP per milligram liver-microsomal protein was about 15 times greater than that released per milligram liver-supernatant protein.

Nature of the fatty acid binding

In experiments with the supernatant-plus-microsome system of rat liver, it was observed, immediately after the incubation mixture had been acidified, that the amount of [^{14}C]fatty acids extracted with hexane was only 5–10 % of that extracted

* Specific activity of ATPase refers to the amount of inorganic phosphate released from ATP per milligram of supernatant or microsomal protein per unit time.

with hexane after the mixture was subjected to prolonged alkaline saponification and subsequent acidification. Of the [^{14}C]fatty acids extracted from the acidified mixture before saponification, only 5 % was neutral lipid which was not adsorbed on Amberlite-IRA 400 (OH^-) resin. Practically no [^{14}C]cholesterol (digitonin-precipitable sterols[18]) and very little other non-saponifiable ^{14}C activity (less than 0.1 % of the total [^{14}C] fatty acid fraction) were found in any of the fractions.

In the experiments with lactating mammary gland, particle-free supernatant fractions, about 40–50 % of the total [^{14}C]fatty acids appeared in the hexane extract of the acidified mixture before saponification, all of which was adsorbed on Amberlite-IRA 400 (OH^-) resin. No [^{14}C]cholesterol or other [^{14}C]-labeled non-saponifiable lipids were synthesized from [1-^{14}C]acetate by this system.

When the enzymic reactions were stopped, either by treatment with trichloroacetic acid or by boiling, a large portion of the [^{14}C]fatty acids synthesized by both the composite liver system (supernatant-plus-microsomes) and the supernatant alone,

TABLE II

BINDING OF FATTY ACIDS FORMED FROM [1-^{14}C]ACETATE TO PROTEIN

The liver system contained 25.5 mg of supernatant protein and 5.1 mg of microsomal protein. The lactating mammary-gland system contained 17.9 mg of supernatant protein. The [1-^{14}C]acetate incubated had an activity of $1.86 \cdot 10^6$ counts/min. One μmole of each pyridine nucleotide indicated below was added to the incubation medium. For other incubation conditions and experimental details see text.

System incubated	*Pyridine nucleotides added*	*Treatment after incubation*	*Total [^{14}C]-fatty acids recovered (counts/min)*	*Percent of total [^{14}C]fatty acids isolated from:*		
				Protein fraction	*Deproteinized supernatant fraction*	
					Acid extract	*After saponification*
Liver	DPN + TPN	TCA*	281 750	91.1	4.2	4.7
Liver	DPN	TCA	116 550	71.0	10.1	18.9
Liver	DPN + TPN	Boiled	258 650	90.6	6.0	3.4
Liver	DPN	Boiled	78 750	62.7	26.5	10.8
Lactating mammary gland	DPN + TPN	TCA*	789 450	78.1	18.9	3.0
Lactating mammary gland	DPN	TCA	196 000	62.0	29.9	8.1
Lactating mammary gland	DPN + TPN	Boiled	980 200	58.2	33.4	8.5
Lactating mammary gland	DPN	Boiled	174 800	38.4	50.1	11.5

* Trichloroacetic acid added to a final concentration of 5 %

prepared from the lactating rat-mammary gland, was associated with the protein precipitate. Table II shows the results obtained with rat liver and lactating mammary-gland-homogenate fractions. Striking differences were observed in the amounts of [^{14}C]fatty acids bound to the protein fraction. When fatty acid synthesis was measured under optimum conditions (*i.e.*, in the presence of DPN and TPN[5]), the ^{14}C activities in the fatty acids bound to protein amounted to about 90 % of the total ^{14}C incorporated in the case of liver. When TPN was omitted from the incubation mixtures, the

proportion of fatty acid ^{14}C activity bound to the protein fraction was decreased. Experiments with the particle-free supernatant fractions prepared from lactating rat-mammary-gland homogenates revealed that a similar binding of [^{14}C]fatty acid to protein had occurred (Table II).

The possibility that microsomes act as a specific acceptor for the synthesized fatty acids was next considered. For this purpose the enzymic reactions were arrested after 2 h of incubation by cooling the mixtures to 0°, and the microsomes were separated from the supernatant fractions by high-speed centrifugation (78000 × *g* for 45 min in the refrigerated ultracentrifuge). The supernatant protein was then precipitated by immersion in boiling water, and separated from the soluble fraction by centrifugation. Each fraction, the microsomes, the supernatant protein precipitate, and the deproteinized supernatant, was then separately saponified with KOH at 90° under reflux overnight. The ^{14}C activity in the hexane extracts of each fraction, after acidification with HCl, showed that almost all of the fatty acids synthesized from [1-^{14}C]acetate were bound to the microsomes (Table III) in the experiments with liver and lactating rat-mammary-gland microsomes. In experiments performed with non-lactating rat-mammary-gland microsomes and with particle-free supernatant fractions from liver, more ^{14}C activity was bound to the supernatant protein than was associated with the non-lactating rat-mammary-gland microsomes (Table III).

TABLE III

BINDING OF FATTY ACIDS FORMED FROM [1-^{14}C]ACETATE TO MICROSOMES

For incubation conditions and experimental details see text. The [1-^{14}C]acetate incubated contained $9.0 \cdot 10^5$ counts/min.

Composite system incubated		*Total [^{14}C]-fatty acids recovered (counts/min)*	*Percent of total [^{14}C] fatty acids isolated from:*		
Supernatant prepared from:	*Microsomes prepared from:*		*Supernatant fraction*		*Microsomes*
			Soluble fraction	*Protein fraction*	
Liver (35.7 mg protein)	Liver (7.8 mg protein)	550 720	1.7	13.1	85.2
Liver (35.7 mg protein)	Non-lactating mammary gland (7.4 mg protein)	210 030	12.4	50.0	37.6
Lactating mammary gland (16.5 mg protein)	Lactating mammary gland (2.1 mg protein)	556 840	1.7	7.1	91.2

These findings raised the question of whether microsomes function as an acceptor for fatty acid synthesized by the supernatant fractions. When the supernatant fraction plus microsomes both prepared from the same liver, was incubated, about 80 % of the newly synthesized fatty acids was associated with the microsomal fraction (Expt. 1, Table IV). When isolated mitochondria were added to this composite system, the amount of fatty acid synthesis was about the same, but in this case most of the [^{14}C]fatty acids were recovered in the mitochondrial fraction; the amount of [^{14}C]fatty acid recovered in the supernatant fraction was unchanged. In the experiments with mitochondria, the [^{14}C]fatty acids bound to these particles represented by far the major portion of the [^{14}C]fatty acids synthesized.

In Expt. 2 (Table IV), the ability of cytoplasmic particles (microsomes and mitochondria) to bind newly preformed, synthesized fatty acids was studied. Here also, most of the [^{14}C]fatty acids synthesized by the supernatant fraction were associated with mitochondria and microsomes. Mitochondria bound more [^{14}C]fatty acids in the presence of microsomes than in their absence. In addition, these experiments show that, in the presence of mitochondria, microsomes bind a smaller portion of the total [^{14}C]fatty acids than they do in the absence of the larger particles. Thus, even though microsomes will bind synthesized [^{14}C]fatty acids, the stimulation of fatty acid synthesis from [1-^{14}C]acetate by addition of microsomes to the supernatant fraction seems not to depend solely upon binding capacity since mitochondria, particles which do not stimulate fatty acid synthesis, also bind [^{14}C]fatty acids to a similar extent.

TABLE IV

LOCALIZATION OF [^{14}C]FATTY ACIDS SYNTHESIZED FROM [1-^{14}C]ACETATE BY RAT-LIVER SUPERNATANT FRACTIONS, MICROSOMES AND MITOCHONDRIA

The supernatant fractions, microsomes, and mitochondria contained 26.4, 5.1, and 5.0 mg of protein, respectively. The [1-^{14}C]acetate incubated contained $9.9 \cdot 10^6$ counts/min. For additional incubation conditions and experimental details see text. In Expt. 1, supernatant fraction plus added particles was incubated for 2 h. In Expt. 2, the indicated particles were added to the reaction mixtures containing only the supernatant fraction 5 min before the end of the 2-h incubation period.

Expt.	*Liver fractions incubated*	*Total [^{14}C]-fatty acids recovered (counts/min)*	*Percent of total [^{14}C]fatty acids isolated from:*		
			Supernatant fraction	*Microsomes*	*Mitochondria*
1	Supernatant + microsomes	252 435	19	81	
	Supernatant + mitochondria	100 080	55		45
	Supernatant + microsomes + mitochondria	262 010	18	20	62
2	Supernatant + microsomes	65 225	42	58	
	Supernatant + mitochondria	64 990	78		22
	Supernatant + microsomes + mitochondria	68 225	27	32	41

DISCUSSION

It is apparent from the results presented here that the stimulation of fatty acid synthesis observed in the presence of microsomes depends upon a delicate balance between ATP concentrations and amounts of microsomes added. Some of the discrepancies noted in the literature might be explained on this basis. With addition of excessive amounts of ATP or with amounts of microsomal protein exceeding one fifth of the supernatant protein, a pronounced inhibitory effect on conversion of acetate to fatty acids was observed.

The ATPase in microsomes might explain the high ATP requirement for fatty acid synthesis in the presence of these particles. The finding that the specific activity of the ATPase of the microsomal fraction was higher than that of the supernatant fraction offers a reasonable explanation for the composite liver system's requiring a higher ATP concentration than did the supernatant fraction alone, for optimum fatty

acid synthesis. For example, in the presence of magnesium ion, when 25 mg of supernatant protein alone were incubated for 2 h, about 10 μmoles of ATP would be consumed by its ATPase activity and, when 5 mg of microsomal protein were used, about 30 μmoles of ATP would be hydrolyzed by the microsomal ATPase (see Table I). The composite system would then consume about 40 μmoles of ATP. This explains why, with a supernatant protein-to-microsomal protein ratio of 25 to 5 mg, the optimal ATP level is high (48 μmoles) and with the supernatant alone it is low (12 μmoles). But ATPase activity alone cannot account for the stimulatory effect of microsomes on fatty acid synthesis because: (a) the addition of small amounts of lactating rat-mammary-gland microsomes to liver supernatants resulted in greater stimulation of fatty acid synthesis than that observed by addition of optimal amounts of liver microsomes at all ATP concentrations studied, even though liver and lactating mammary-gland microsomes showed similar ATPase activities. (b) The addition of microsomes to liver or lactating mammary-gland-supernatant fractions, in the presence of high ATP levels, yielded a significantly higher ^{14}C incorporation into fatty acids than did supernatant fractions alone at their optimal ATP concentrations. Although the inhibition of fatty acid synthesis brought about by excessive amounts of microsomes results from their ATPase activity, in addition to other factors*, their stimulating ability apparently cannot be ascribed to release of an ATP inhibition of fatty acid synthesis by the supernatant fraction.

The stimulation of fatty acid synthesis by microsomes might be due to a reaction involving ATP and carried out either by the microsomes themselves or by an interaction between the microsomes and the supernatant fraction. This reaction cannot be the one catalyzed by acetokinase, as the microsomes do not contain detectable amounts of this enzyme[12]. Additional evidence supporting this latter point comes from experiments showing that the stimulating of [^{14}C]fatty acid synthesis from [1-^{14}C]-acetate and from [1-^{14}C]acetyl-CoA by microsomes was identical[12,19]. The enhancement of fatty acid synthesis from acetate or acetyl-CoA induced by addition of microsomes to the particle-free supernatant fractions might, however, be ascribed to a specific effect on the enzyme concerned with malonyl-CoA synthesis from acetyl-CoA. This latter reaction can be performed by a biotin-dependent enzyme which transfers HCO_3^- or CO_2 to acetyl-CoA at pH 6.0–7.0 as described by WAITE AND WAKIL[21]. This acetyl-CoA carboxylase activity, according to these workers, is present in the particle-free supernatant fraction. Under the conditions of the experiments reported here, *i.e.*, at pH 7.5, evidence for the transfer of free HCO_3^- to acetyl-CoA by the microsomes alone could not be demonstrated, In experiments reported elsewhere[12,19] it was shown that [1-^{14}C]acetyl-CoA was not converted to fatty acids in the presence of ATP, citrate, and TPNH by the microsomes alone, whereas a large amount of the ^{14}C added as [2-^{14}C]malonyl-CoA was introduced into fatty acids in this system containing only microsomal protein**.

Evidence presented elsewhere suggests that a rate-limiting step in the synthesis of fatty acids from acetate or acetyl-CoA by particle-free supernatant fractions of

* Liver-microsomal protein solubilized by either ultrasonic vibration or desoxycholate treatment of the particles can be precipitated with ammonium sulfate. The fraction of solubilized protein precipitated between 0 and 50% saturated ammonium sulfate will inhibit fatty acid synthesis from acetate in the rat-liver-supernatant system[20].

** With malonyl-CoA as substrate the specific activities of the fatty acid-synthesizing enzymes present in the supernatant fraction are 4–5 times greater than those of the microsomes[19].

liver, mammary gland, and yeast at a pH above 7 is a carboxylation reaction yielding malonyl-CoA[12,19,22-24]. Thus, a specific interaction between microsomes and supernatant enzymes might be required for optimal carboxylation of acetyl-CoA under our experimental conditions.

The addition of palmitate, in the form of the sodium salt or as the albumin complex, will inhibit fatty acid synthesis from acetate by liver systems[13,25]. It is therefore possible that another function of the microsomes in our composite system is to bind fatty acids as they are formed and remove them from the soluble phase of the system. However, mitochondria also have this binding ability even though they do not stimulate fatty acid synthesis.

It is difficult to decide from the results presented here whether [^{14}C]fatty acids synthesized by the composite liver system were actually formed in the microsomes or whether they arose solely via enzymic reactions in the supernatant fraction and were then bound to the protein of the microsomal particles.

We suggested earlier that the presence of the α,β-unsaturated acyl-CoA reductase, an enzyme first described by LANGDON[26,27] and later by SEUBERT *et al.*[28], in the microsomes[29] indicates that microsomal protein is involved in certain reactions concerned with fatty acid synthesis. Since our earlier experiments were carried out with a microsomal fraction solubilized with cholate, it is possible that the enzymic activity measured in those experiments represented a part of a protein complex similar to an electrophoretically pure protein isolated by LYNEN[4], which contains the multiplicity of enzyme activities required for fatty acid synthesis. LYNEN has suggested that the condensation occurs with enzyme-bound reactants rather than with the CoA derivatives[4]. It seems reasonable to assume that this reductase, released from the enzyme complex, could function with crotonyl-S-CoA instead of crotonyl-S-enzyme as substrate, or that a transacylase is involved. The observation that this TPNH-dependent reductase does not occur in the particle-free supernatant fractions of liver homogenates in any detectable amounts has been confirmed by GIBSON *et al.*[30].

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