Citrate and the Conversion of Carbohydrate into Fat

THE REGULATION OF FATTY ACID SYNTHESIS BY RAT LIVER EXTRACTS

BY MARIE FANG AND JOHN M. LOWENSTEIN
*Graduate Department of Biochemistry, Brandeis University,
Waltham, Mass. 02154, U.S.A.*

(Received 31 March 1967)

1. The rate of fatty acid synthesis by particle-free extracts prepared from rat liver is increased greatly if the enzyme system is first activated with citrate. 2. The extent of the activation depends on the citrate concentration and on the time of activation in an interdependent manner. 3. Citrate activation is strongly dependent on temperature. 4. Tricarballoylate can replace citrate as an activator, but its presence in the assay inhibits fatty acid synthesis. 5. Mg^{2+} ions can replace citrate in the activation but not in the complete reaction system. 6. ATP prevents the activating effect of citrate and Mg^{2+} ions. 7. The rate of fatty acid synthesis is increased by palmitoyl-DL-carnitine. This type of activation, additional to that caused by citrate, is rapid and does not depend on prior incubation. 8. Inhibition of fatty acid synthesis by palmitoyl-CoA can be prevented by palmitoyl-DL-carnitine or by increasing the concentration of protein.

Acetyl-CoA carboxylase from adipose tissue of rat is activated by incubation with citrate before the assay of the enzyme (Martin & Vagelos, 1962). In applying this finding to a study of the complete pathway of fatty acid synthesis we found that incubation of liver extracts with citrate or Mg^{2+} ions or both leads to a large increase in the rate of fatty acid synthesis. Such activation is prevented almost completely by physiological concentrations of ATP.

The rate of fatty acid synthesis by particle-free extracts of rat liver is stimulated considerably by the addition of microsomes and glycerol 3-phosphate. This effect may be due in part to the removal of palmitoyl-CoA or a near biochemical relative of palmitoyl-CoA by esterification with glycerol 3-phosphate (Howard & Lowenstein, 1965). When we attempted to reproduce the inhibitory effect of palmitoyl-CoA by the use of palmitoylcarnitine, coenzyme A and carnitine palmitoyltransferase, we observed a stimulation of fatty acid synthesis instead of the expected inhibition. (C. F. Howard & J. M. Lowenstein, unpublished work.) The present paper shows that this stimulation is caused by palmitoylcarnitine.

METHODS

Male rats were obtained from Charles River Breeding Laboratories, Wilmington, Mass., U.S.A. They were maintained on a stock diet of Wayne Lab-Blox (Allied Mills

Inc., Chicago, Ill., U.S.A.). Rats that had been starved for 2 days were re-fed with a diet high in glucose for 3 or 4 days (Kornacker & Lowenstein, 1965). The rats were killed by decapitation. The livers were excised quickly, placed in ice-cold 0.25 M-sucrose, washed several times with this medium, and then homogenized in 3 vol. of 0.25 M-sucrose with a Philpot homogenizer (Philpot & Stanier, 1956). The homogenate was centrifuged in a Spinco model L preparative ultracentrifuge (head no. 40) at 105000g at 0° for 30 min. The clear supernatant was withdrawn with a drawn-out dropper, care being taken not to disturb the particulate and fat layers. The resulting solution is referred to as high-speed supernatant. Other methods were as described by Howard & Lowenstein (1965). Fatty acid esters of DL-carnitine were a gift from Dr G. Deltour, Labaz, Brussels, Belgium.

RESULTS

Effect of citrate on fatty acid synthesis. The rate of fatty acid synthesis by high-speed supernatant prepared from rat liver was increased twofold by incubating the supernatant with 50 mM-glycylglycine-sodium hydroxide buffer, pH 7.5, and 30 mM-GSH at 37° for 20 min. before the complete reaction was started. Addition of 20 mM-sodium citrate to the same activation medium resulted in an 11-fold increase compared with the non-activated control (Fig. 1). The ratio of the rates of fatty acid synthesis obtained when the enzyme extract was activated with or without citrate was initially about 9.5. The ratio fell with time, perhaps

Fig. 1. Activation of fatty acid synthesis by citrate. High-speed supernatant was prepared from the liver of a rat that was starved for 48 hr. and re-fed with a diet high in glucose for 4 days. The supernatant either was used without activation (○), or was first activated at 37° for 20 min. in a solution containing 50 mM-glycylglycine-NaOH buffer, pH 7.5, 30 mM-GSH and 10 mg. of protein/ml. in the presence (●) or absence (×) of 20 mM-sodium citrate. The assay mixture contained 20 mM-sodium [1,5-¹⁴C]citrate (10⁴ counts/min./μmole), 50 mM-glycylglycine-NaOH buffer, pH 7.5, 30 mM-GSH, 10 mM-MgCl₂, 0.5 mM-MnCl₂, 0.26 mM-CoA, 0.68 mM-NADP, 5 mM-glucose 6-phosphate, 5 mM-ATP, 25 mM-NaHCO₃, 15 mM-creatine phosphate, 10 μg. of creatine phosphokinase, approx. 0.1 M-sucrose and high-speed supernatant (5.0 mg. of protein). Assays were carried out for various times at pH 7.5-7.6 in a final volume of 1.0 ml. at 37°. The reaction was stopped by the addition of 2 ml. of 5 N-NaOH. The remainder of the procedure was as described by Spencer & Lowenstein (1962). The broken line shows the citrate incorporation expected for the curve (×) if the activating effect of citrate in the complete assay medium were the same as in the activation medium.

because of greater end-product inhibition in the reaction mixture that was activated with citrate. Nevertheless, the ratio of rates was still 5 and 3 after 30 and 60 min. respectively in the complete assay system. If citrate had had the same activating effect during the assay as it did during the activation before the assay, then citrate incorporation would have followed the course shown by the broken line in Fig. 1. This line was plotted by taking tangents from the upper curve in Fig. 2(a) at various activation times. The actual results show that citrate caused virtually no activation during the assay (Fig. 1). This finding suggested that there was a substance present during the assay that prevented the activating effect of citrate, and that this substance was not present during the activation. It is shown below that this substance is ATP.

Fig. 2. Effect of various citrate concentrations on the activation of fatty acid synthesis as a function of time. Activation mixtures were as in Fig. 1, except that the following concentrations (mM) of sodium citrate were used: ●, 20; ○, 10; ▲, 4; △, 2; ■, 1; □, 0.5; ×, 0. The assays were as described in Fig. 1, for 30 min.

The extent of the activation depended on the concentration of citrate and on the time of activation in an interdependent manner. For example, the effect of 20 mM-citrate for 10 min. was the same as that of 10 mM-citrate for 20 min., or of 4 mM-citrate for 30 min. (Fig. 2a). Similarly the effectiveness of 4 mM-citrate for 20 min. was the same as that of 0.5 mM-citrate for 100 min. (Fig. 2b).

The effect of varying the temperature of the activation is shown in Fig. 3. Temperatures of 30°

Fig. 3. Effect of temperature on the activation of fatty acid synthesis. Filled-in symbols indicate that the activation mixtures were as in Fig. 1. Open symbols indicate that the activation mixtures were the same except that citrate was omitted. The activations were at 37° (● and ○), 30° (■ and □), 22° (◆ and ◇) and 0° (▲ and △), for the times shown. The assays were carried out as in Fig. 1, for 30 min.

or below were very much less effective than a temperature of 37°. Very little activation by citrate occurred at 0°, as observed by Martin & Vagelos (1962) and Matsuhashi, Matsuhashi & Lynen (1964) with acetyl-CoA carboxylase. Incubation of the high-speed supernatant at 0° in the presence or absence of citrate for 30 min. did not reduce the rate of fatty acid synthesis to values below those of the controls. This shows that there was no loss of activity due to cold-inactivation of acetyl-CoA carboxylase under the conditions of our experiments. Numa & Ringelmann (1965) have reported that the purified, activated enzyme is inactivated almost completely when stored at 0° for 30 min.

The rate of fatty acid synthesis depended not only on the concentration of citrate used for activation, but also on the concentration of citrate in the assay. This is demonstrated by the experiment shown in Fig. 4. High-speed supernatant was activated with 4 mM-citrate and was then assayed at various concentrations of citrate. The optimum rate of fatty acid synthesis was attained with 16–20 mM-citrate. The concentration required for one-half of the maximum rate was about 8 mM. As has already been stated, the activation of the enzyme that can be produced by preincubation with citrate did not occur under the conditions of the assay (Fig. 1). The results in Fig. 4 show that high concentrations of citrate must nevertheless be present to obtain maximum rates in the assay.

Fig. 4. Effect of citrate concentration on the assay of fatty acid synthesis. The activation mixtures contained 50 mM-glycylglycine-NaOH buffer, pH 7.5, 30 mM-GSH, 4 mM-sodium citrate and 6 mg. of protein/ml. Activation was at 37° for 60 min. The assay conditions were as in Fig. 1 for 30 min., except that the concentrations of citrate were as indicated and the concentration of protein was 3 mg./ml.

This suggests that citrate plays different roles during the activation and during the complete reaction sequence. The K_m for citrate of citrate-cleavage enzyme (ATP citrate lyase, EC 4.1.3.8) is 0.1–0.3 mM (M. Fang & J. M. Lowenstein, unpublished work), so the high concentration of citrate needed for fatty acid synthesis (Fig. 4) is clearly not necessary to saturate citrate-cleavage enzyme.

Effect of tricarballoylate. Prior incubation of the enzyme system with tricarballoylate results in a strong activation of fatty acid synthesis. To make comparisons easier all the assays in these experiments (Fig. 5) were adjusted to contain 20 mM-tricarballoylate. Maximum activation was obtained with about 30 mM-tricarballoylate. The presence of tricarballoylate in the assay medium lowered the rate of citrate incorporation into fatty acid (Fig. 6). For example, the inhibition was 40% with 20 mM-tricarballoylate. The maximum activation obtained with tricarballoylate alone in the experiment shown in Fig. 5 was 87 mμmoles/mg. of protein/hr. The corrected optimum rate for activation by tricarballoylate was therefore $87 \times 100/60 = 145$ mμmoles/mg. of protein/hr. This is in reasonable agreement with the optimum obtained in the presence of 4 mM-citrate, namely 130 mμmoles/mg. of protein/hr. Lynen, Matsuhashi, Numa & Schweizer (1963) stated that tricarballoylate does not activate acetyl-CoA carboxylase even though it increases the rate of sedimentation of the enzyme

Fig. 5. Effect of tricarballoylate on the activation of fatty acid synthesis. The activation mixtures contained 50mm-glycylglycine-NaOH buffer, pH 7.5, 30mm-GSH, 6mg. of protein/ml., and sodium tricarballoylate at the concentrations shown. Activation was in the presence (●) and absence (×) of 4mm-sodium citrate at 37° for 20 min. The assay conditions were as described in Fig. 1, except that 20mm-tricarballoylate was present in all tubes and the concentration of protein was 3mg./ml. The assays were carried out for 30 min.

Fig. 6. Effect of tricarballoylate on the assay of fatty acid synthesis. Activation was as in Fig. 4. The assay conditions were as described in Fig. 1, except that 50mm-glycylglycine-NaOH buffer was replaced by 50mm-tris-HCl buffer, pH 7.5; the concentrations of citrate were either 4mm (●) or 20mm (●); tricarballoylate was present in the concentrations shown. A control was run in which the enzyme system was not activated by prior incubation, but in which 20mm-citrate was present in the assay (○).

in the ultracentrifuge. The activity of citrate-cleavage enzyme, as assayed by the hydroxamate method over a concentration range 1–20mm-citrate, was not inhibited by 20mm-tricarballoylate.

Effect of protein concentration. The effect of protein concentration on the rate of fatty acid synthesis is shown in Fig. 7. A deviation from linearity was observed at low protein concentrations. This was most marked for the non-activated enzyme or the enzyme activated in the absence of citrate. Activation of acetyl-CoA carboxylase is accompanied by aggregation (Vagelos, Alberts & Martin, 1963). The dependence of the aggregation phenomenon on the protein concentration may account for the deviations from linearity shown in Fig. 7.

Effect of Mg^{2+} ions. The activation of fatty acid synthesis by prior incubation of the enzyme system with Mg^{2+} ions was discovered when various controls were being run in a study of the inhibitory effects of ATP. This activation by Mg^{2+} ions occurred in the presence and absence of citrate. However, the effects of Mg^{2+} ions and citrate were not additive (Fig. 8). The activation attained with magnesium chloride alone at concentrations greater than 13mm somewhat exceeded the activation attained with 20mm-citrate alone.

Fig. 7. Effect of protein concentration on the rate of fatty acid synthesis. The high-speed supernatant was used either without activation (○), or was activated in a mixture containing 50mm-glycylglycine-NaOH buffer, pH 7.5, 30mm-GSH, in the presence (●) or absence (×) of 20mm-sodium citrate. The activation was carried out at 37° for 30 min. The assay conditions were as described in Fig. 1; the assay time was 15 min. The abscissa shows the protein concentrations in the assay; the activation was conducted at twice these concentrations.

Fig. 8. Effect of MgCl_2 on the activation of fatty acid synthesis. High-speed supernatant (6mg./ml.) was activated with 50mm-glycylglycine- NaOH buffer, pH 7.5, 30mm-GSH, 20mm-sodium citrate, and ATP as indicated, in the presence (●) and absence (Δ) of 20mm-sodium citrate, at 37° for 20 min. The assays were carried out for 30 min. as described in Fig. 1 except that the MgCl_2 concentration was 20mm.

Effect of ATP. The activation of fatty acid synthesis by citrate or by citrate and Mg^{2+} ions is inhibited by ATP. For example, the activating effect of 20mm-citrate was inhibited 50% by 1mm-ATP. The presence of 10mm-magnesium chloride did not reverse the inhibition, but appeared to enhance it somewhat (Fig. 9).

ATP was much more effective as an inhibitor when it was present during the activation than when it was present only during the complete reaction. The rate of fatty acid synthesis was optimum at about 2mm-ATP. It decreased at higher ATP concentrations, being 50% of maximum at 10mm-ATP. The effects of ATP were similar regardless of whether the complete reaction was run with or without prior activation (Fig. 10).

Effect of acylcarnitines. Palmitoyl-DL-carnitine stimulated fatty acid synthesis under a variety of different conditions (Fig. 11). When the enzyme system was first activated by incubation with citrate, 100μm-palmitoyl-DL-carnitine stimulated the rate about twofold. When the activation was carried out in the presence of both citrate and ATP, there was a sixfold stimulation by 100μm-palmitoyl-DL-carnitine, even though the maximum activity attained under these conditions was only about one-half of that attained when ATP was absent from the activation. This shows that the presence of ATP does not prevent the activation by palmitoyl-DL-carnitine. Even larger relative

Fig. 9. Effect of ATP on the activation of fatty acid synthesis. High-speed supernatant (6mg./ml.) was activated with 50mm-glycylglycine- NaOH buffer, pH 7.5, 30mm-GSH, 20mm-sodium citrate, and ATP as indicated, in the presence (Δ) and absence (●) of 10mm- MgCl_2 . Tubes incubated in the absence of citrate and MgCl_2 served as controls (x). The activation was carried out at 37° for 20 min. The assay conditions were as described in Fig. 1, except that the concentration of ATP in all tubes was adjusted to 10 mm, and the assay time was 30 min.

Fig. 10. Effect of ATP on the assay of fatty acid synthesis. High-speed supernatant (6mg./ml.) was activated with 50mm-glycylglycine- NaOH buffer, pH 7.5, and 30mm-GSH in the presence (●) and absence (x) of 20mm-sodium citrate at 37° for 20 min. The assay conditions were as in Fig. 1 except that the concentrations of ATP were as indicated, and the assay time was 30 min.

effects of palmitoyl-DL-carnitine were observed when the activation was carried out in the presence of ATP and the absence of citrate, or in the absence of both (Fig. 11). In each case the optimum

Fig. 11. Effect of palmitoyl-DL-carnitine on fatty acid synthesis. The activation mixture contained 50mm-glycylglycine-NaOH buffer, pH 7.5, 30mm-GSH, 5mg. of protein/ml., and 20mm-sodium citrate (●), or 20mm-sodium citrate and 5mm-ATP (Δ) or 5mm-ATP (●), or neither citrate nor ATP (x). Activation was at 37° for 30min. The assays were carried out as described in Fig. 1, with an assay time of 30min., except that palmitoyl-DL-carnitine was added at the indicated concentrations.

Fig. 12. Effect of palmitoyl-DL-carnitine on the assay of fatty acid synthesis. Results shown with filled-in symbols were obtained with high-speed supernatant (6mg./ml.) that was activated with 50mm-glycylglycine-NaOH buffer, pH 7.5, 30mm-GSH and 20mm-sodium citrate at 37° for 30min. Results shown with open symbols were obtained with high-speed supernatant that was not activated before assay. The conditions for the assay were as described in Fig. 1, with an assay time of 30min. Palmitoyl-DL-carnitine (final concn. 100 μ M) (▲ and Δ), or an equivalent volume of water (● and ○) was added to the assay medium at time 'A'.

Table 1. Effect of palmitoyl-DL-carnitine and palmitoyl-CoA on the rate of fatty acid synthesis

Reaction conditions were as described in Fig. 1, except that the protein concentration in the assay was 3mg./ml., and the assays were for 30min. Where indicated, the reaction mixture also contained palmitoyl-DL-carnitine and palmitoyl-CoA. In experiment (a) the high-speed supernatant was not activated before the assay; in (b) it was activated for 30min. at 37° with 50mm-glycylglycine-NaOH buffer, 30mm-GSH and 20mm-sodium citrate, pH 7.5.

	Additions to assay mixture		Citrate incorporation (mμmoles/mg. of protein/hr.)
	Palmitoyl-DL-carnitine (μM)	Palmitoyl-CoA (μM)	
(a) Not activated	—	—	8.2
	50	—	109
	150	—	192
	—	50	7.1
	—	150	3.5
	50	50	57
	50	150	4.9
	150	50	133
	150	150	6.9
(b) Activated	—	—	165
	150	—	453
	—	150	75
	150	150	237

concentration of palmitoyl-DL-carnitine was about 100 μ M, and the concentration for one-half of maximum stimulation was about 15 μ M.

Although the relative activation by palmitoyl-DL-carnitine was much greater when the enzyme system was not first activated than when it was activated with citrate, the actual increase in fatty acid synthesis observed over a period of 30 or 40min. was about the same in both cases (Fig. 12). A comparison of palmitoyl-DL-carnitine and stearoyl-DL-carnitine showed that these have about the same stimulating effects, whereas octanoyl-DL-carnitine and unesterified carnitine had no effect on the rate of fatty acid synthesis. The stimulating effect of palmitoyl-DL-carnitine has also been reported by Fritz & Hsu (1966).

Palmitoyl-CoA inhibits fatty acid synthesis *in vitro* (Porter & Long, 1958; Bortz & Lynen, 1963a,b; Robinson, Brady & Bradley, 1963; Tubbs & Garland, 1964; Howard & Lowenstein, 1965). We have tested the effect of palmitoyl-DL-carnitine on this inhibition. Table 1 shows the effect of the palmitoyl esters when the high-speed supernatant was not activated before assay. At a protein concentration of 3mg./ml., 50 μ M- and 150 μ M-

Fig. 13. Effects of palmitoyl-DL-carnitine and palmitoyl-CoA on fatty acid synthesis as a function of time. Activation was as in Fig. 12. The assay conditions were as described in Fig. 1, except that the following additions were made to the assay in a volume of 0.1 ml., at time 'A': ▲, 100 μ M-palmitoyl-DL-carnitine; ●, 100 μ M-palmitoyl-CoA; Δ, 100 μ M-palmitoyl-CoA plus 100 μ M-palmitoyl-DL-carnitine; ●, none.

Fig. 14. Effects of palmitoyl-CoA and palmitoyl-DL-carnitine on fatty acid synthesis as a function of protein concentration. Activation of high-speed supernatant protein at the concentrations indicated was as in Fig. 12. The assays were carried out under the conditions described in Fig. 1 for 30 min. The following additions were made at the start of the assay: ●, none; ▲, 200 μ M-palmitoyl-DL-carnitine; ●, 200 μ M-palmitoyl-CoA.

palmitoyl-CoA inhibited fatty acid synthesis by 15% and 60% respectively, whereas 50 μ M- and 150 μ M-palmitoyl-DL-carnitine stimulated the syn-

thesis 12- and 25-fold respectively. Addition of palmitoyl-DL-carnitine to the system inhibited by palmitoyl-CoA resulted in a reversal of the inhibition. Table 1 also shows the effect of the palmitoyl esters when the high-speed supernatant was first activated with citrate. Palmitoyl-CoA was more effective as an inhibitor, and palmitoyl-DL-carnitine more effective as an activator, when the high-speed supernatant had not been activated by prior incubation with citrate. The effects of palmitoyl-DL-carnitine and palmitoyl-CoA on fatty acid synthesis as a function of time are shown in Fig. 13. The activation observed with palmitoyl-DL-carnitine alone and the reversal of the palmitoyl-CoA inhibition by palmitoylcarnitine follow similar time-courses.

Palmitoyl-CoA was a very effective inhibitor of fatty acid synthesis at low protein concentrations, but its effectiveness diminished rapidly as the protein concentration was increased. In the presence of 200 μ M-palmitoyl-CoA the inhibition was almost complete with 2 mg. of protein/ml., but it was only 23% with 6 mg. of protein/ml. The stimulating effect of palmitoyl-DL-carnitine (200 μ M), on the other hand, diminished little with increasing protein concentrations, being about twofold over the entire range used in the experiment shown in Fig. 14.

DISCUSSION

In the synthesis of fatty acids by mammalian enzyme systems, citrate acts both as a source of carbon (Spencer & Lowenstein, 1962; Bhaduri & Srere, 1963; Spencer, Corman & Lowenstein, 1964) and as an activator of acetyl-CoA carboxylase (Martin & Vagelos, 1962; Kallen & Lowenstein, 1962; Waite & Wakil, 1962; Matsushashi *et al.* 1964). The present paper shows that prior incubation of particle-free liver extracts with citrate leads to an increased rate of fatty acid synthesis by such extracts (Fig. 1). Although this effect might be predicted from the known behaviour of acetyl-CoA carboxylase from rat liver (Numa, Ringelmann & Lynen, 1965b; Numa & Ringelmann, 1965; Numa, Ringelmann & Riedel, 1966; and references quoted above), prior activation of extracts with citrate does not appear to have been used previously in studies of the whole pathway of fatty acid synthesis. Earlier measurements of rates of fatty acid synthesis *in vitro* were therefore too low, in many cases by a factor of as much as 5–10. It should be noted that acetyl-CoA carboxylase from chicken liver apparently does not require activation by prior incubation with citrate or Mg^{2+} ions (Gregolin, Ryder, Kleinschmidt, Warner & Lane, 1966).

The data in Fig. 2 show that activation of the enzyme preparation with a low concentration of

citrate for a prolonged period can give the same effect as activation with a high concentration of citrate for a short period. This interdependence between the citrate concentration and the time of incubation makes it difficult at present to assign a physiologically meaningful value for the citrate concentration that gives one-half maximum activation of fatty acid synthesis. In most previous experiments involving measurements of the rate of fatty acid synthesis *in vitro*, relatively high concentrations of citrate, such as 10mM or greater, were used (Spencer & Lowenstein, 1962; Spencer *et al.* 1964; Bhaduri & Srere, 1963). The citrate content of rat liver is about 0.3 $\mu\text{mole/g.}$ fresh wt., which corresponds to an intracellular concentration of about 0.6mM (Spencer & Lowenstein, 1967). At such low concentrations of citrate virtually no fatty acid synthesis *in vitro* was observed in previous work. Fig. 2 shows that under the proper conditions of activation such low concentrations of citrate could in fact elicit high rates of fatty acid synthesis. Tricarballoylate, an analogue of citrate, can replace citrate as activator, although high concentrations of this substance in the assay were inhibitory (Figs. 5 and 6).

Fatty acid synthesis can also be activated by prior incubation with Mg^{2+} ions (Fig. 8). There is no evidence of changes in the Mg^{2+} content of liver under conditions that lead to major changes in the rate of fatty acid synthesis. However, it is conceivable that a control mechanism based on changes in the intracellular distribution of Mg^{2+} ions operates in the liver.

Activation of the enzyme system by citrate was inhibited strongly by ATP, and the inhibition was not affected by the presence of high concentrations of Mg^{2+} ions (Fig. 9). Other experiments showed that ATP also effectively inhibits activation by Mg^{2+} ions in the absence of citrate. ATP exerts a lesser effect in the complete assay system. The optimum rate was obtained with about 2mM-ATP, and 10mM-ATP inhibited the rate by about 50%. The activity of a number of enzymes is regulated by ATP. For example, ATP inhibits phosphofructokinase, for which it is also a substrate (Lardy & Parks, 1956; Passonneau & Lowry, 1963), and activates adenylate deaminase (Mendicino & Muntz, 1958; Setlow & Lowenstein, 1967). ATP inhibition of citrate synthase (Hathaway & Atkinson, 1965; Shepherd & Garland, 1966) is abolished *in vitro* by low concentrations of Mg^{2+} ions (Kosicki & Lee, 1966). The tissue content of Mg^{2+} ions is generally much greater than that of ATP. Considerable caution must therefore be exercised in assessing the physiological significance of the effects of unchelated ATP. Difficulties of interpretation also are encountered for the results shown in Figs. 9 and 10. Here ATP strongly inhibits the

activation (even in the presence of Mg^{2+} ions) but it does not inhibit the complete reaction except at very high concentrations.

Palmitoyl-DL-carnitine stimulates the rate of fatty acid synthesis (Table 1), an effect that has also been reported by Fritz & Hsu (1966). The highest rate of fatty acid synthesis was achieved by adding palmitoyl-DL-carnitine to high-speed supernatant previously activated with citrate. The greatest relative stimulation by palmitoyl-DL-carnitine was observed with enzyme extract that had not been activated by incubation with citrate or magnesium. The activation by palmitoyl-DL-carnitine was rapid, whereas the activation by citrate or Mg^{2+} ions required prolonged incubation. Figs. 11 and 12 also show that the two stimulating effects were approximately additive. These results all suggest that palmitoyl-DL-carnitine acts in a manner different from citrate and ATP.

Fritz & Hsu (1966) reported optimum rates of fatty acid synthesis, in the presence of palmitoyl-DL-carnitine, of 1–40 $\mu\text{moles/mg.}$ of protein/hr., whereas those reported in the present paper are 450–800 $\mu\text{moles/mg.}$ /hr. for preparations activated with citrate, and 190–400 $\mu\text{moles/mg.}$ /hr. for preparations not activated with citrate (Table 1 and Fig. 11).

The concept that fatty acid synthesis is controlled through feed-back inhibition by palmitoyl-CoA (Porter & Long, 1958; Bortz & Lynen, 1963a,b; Robinson *et al.* 1963; Tubbs & Garland, 1963, 1964) is supported strongly by measurements of the long-chain acyl-CoA content of liver under various nutritional conditions. According to Tubbs & Garland (1964) the following amounts are found ($\mu\text{moles/g.}$ wet weight): control, 53; starved 110; starved and re-fed with fat, 135; starved and re-fed with sugar, 30; alloxan-diabetic, 92. Studies *in vitro* show that the inhibition of fatty acid synthesis by long-chain acyl-CoA is affected by at least five variables: (a) the concentration of palmitoyl-CoA (Numa, Bortz & Lynen, 1965a; Howard & Lowenstein, 1965); (b) the concentration of citrate (Numa & Ringelmann, 1965; Numa *et al.* 1965b); (c) the extent of prior activation with citrate (Table 1); (d) the concentration of protein (Fig. 14); (e) the concentration of palmitoylcarnitine (Table 1 and Fig. 13).

The inhibitory effect of palmitoyl-CoA (200 μM) is strong at low concentrations of protein, but diminishes rapidly as the protein concentration is increased, being only 23% at a protein concentration of 6mg./ml., and less at higher protein concentrations (Fig. 14). The concentration of the non-particulate protein of rat liver *in vivo* is about 103mg./g. wet weight (Spencer *et al.* 1964). This suggests that palmitoyl-CoA may not have any inhibitory effect on fatty acid synthesis at physio-

logical protein concentrations. Numa *et al.* (1965*b*) reported that the amount of palmitoyl-CoA needed for 50% inhibition of acetyl-CoA carboxylase depended strongly on the amount of bovine serum albumin that they used in their reaction mixtures, and assumed that the effectiveness of palmitoyl-CoA as an inhibitor of fatty acid synthesis would also depend on the protein concentration.

The inhibitory effect of palmitoyl-CoA is reversed by the addition of palmitoyl-DL-carnitine. The tissue content of long-chain acylcarnitine of heart and liver from rats under various nutritional conditions varies in a manner qualitatively similar to that of acyl-CoA (Pearson & Tubbs, 1964; Tubbs, Pearson & Chase, 1965; Böhmer, Norum & Bremer, 1966). Moreover, the stimulating effect of palmitoylcarnitine does not diminish with increasing protein concentrations. This strengthens the tentative conclusion reached above, namely that under physiological conditions fatty acyl-CoA is not a controlling factor in fatty acid synthesis. These arguments, however, do not take into account the possibility of compartmentation of metabolites. If fatty acyl-CoA were highly concentrated in the vicinity of the fatty acid-synthesizing system it might reach concentrations that would be inhibitory even at the high concentrations of protein that prevail in the cell.

REFERENCES

- Bhaduri, A. & Srere, P. A. (1963). *Biochim. biophys. Acta*, **70**, 221.
- Böhmer, T., Norum, K. R. & Bremer, J. (1966). *Biochim. biophys. Acta*, **125**, 244.
- Bortz, W. M. & Lynen, F. (1963*a*). *Biochem. Z.* **337**, 505.
- Bortz, W. M. & Lynen, F. (1963*b*). *Biochem. Z.* **339**, 77.
- Fritz, I. B. & Hsu, M. P. (1966). *Biochem. biophys. Res. Commun.* **22**, 737.
- Gregolin, C., Ryder, E., Kleinschmidt, A. K., Warner, R. C. & Lane, M. D. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 148.
- Hathaway, J. A. & Atkinson, D. E. (1965). *Biochem. biophys. Res. Commun.* **20**, 661.
- Howard, C. F., jun. & Lowenstein, J. M. (1965). *J. biol. Chem.* **240**, 4170.
- Kallen, R. & Lowenstein, J. M. (1962). *Arch. Biochem. Biophys.* **96**, 188.
- Kornacker, M. S. & Lowenstein, J. M. (1965). *Biochem. J.* **94**, 209.
- Kosicki, G. W. & Lee, L. P. K. (1966). *J. biol. Chem.* **241**, 3571.
- Lardy, H. A. & Parks, R. E., jun. (1956). In *Enzymes: Units of Biological Structure and Function*. Ed. by Gaebler, O. H. New York: Academic Press Inc.
- Lynen, F., Matsuhashi, M., Numa, S. & Schweizer, E. (1963). *Symp. biochem. Soc.* **24**, 43.
- Martin, D. B. & Vagelos, P. R. (1962). *J. biol. Chem.* **237**, 1787.
- Matsuhashi, M., Matsuhashi, S. & Lynen, F. (1964). *Biochem. Z.* **340**, 263.
- Mendicino, J. & Muntz, J. A. (1958). *J. biol. Chem.* **233**, 178.
- Numa, S., Bortz, W. M. & Lynen, F. (1965*a*). In *Advances in Enzyme Regulation*, vol. 3, p. 407. Ed. by Weber, G. New York: Pergamon Press Inc.
- Numa, S. & Ringelmann, E. (1965). *Biochem. Z.* **343**, 258.
- Numa, S., Ringelmann, E. & Lynen, F. (1965*b*). *Biochem. Z.* **343**, 243.
- Numa, S., Ringelmann, E. & Riedel, B. (1966). *Biochem. biophys. Res. Commun.* **24**, 750.
- Passonneau, J. V. & Lowry, O. H. (1963). *Biochem. biophys. Res. Commun.* **3**, 372.
- Pearson, D. J. & Tubbs, P. K. (1964). *Biochim. biophys. Acta*, **84**, 772.
- Philpot, J. St L. & Stanier, J. E. (1956). *Biochem. J.* **63**, 214.
- Porter, J. W. & Long, R. W. (1958). *J. biol. Chem.* **233**, 20.
- Robinson, J. D., Brady, R. O. & Bradley, R. M. (1963). *J. Lipid Res.* **4**, 144.
- Setlow, B. C. & Lowenstein, J. M. (1967). *J. biol. Chem.* **242**, 607.
- Shepherd, D. & Garland, P. B. (1966). *Biochem. biophys. Res. Commun.* **22**, 89.
- Spencer, A. F., Corman, L. & Lowenstein, J. M. (1964). *Biochem. J.* **93**, 378.
- Spencer, A. F. & Lowenstein, J. M. (1962). *J. biol. Chem.* **237**, 3640.
- Spencer, A. F. & Lowenstein, J. M. (1967). *Biochem. J.* **103**, 342.
- Tubbs, P. K. & Garland, P. B. (1963). *Biochem. J.* **89**, 25*P*.
- Tubbs, P. K. & Garland, P. B. (1964). *Biochem. J.* **93**, 550.
- Tubbs, P. K., Pearson, D. J. & Chase, J. F. A. (1965). In *Recent Research on Carnitine*, p. 117. Ed. by Wolf, G. Cambridge, Mass.: The M.I.T. Press.
- Vagelos, P. R., Alberts, A. W. & Martin, D. B. (1963). *J. biol. Chem.* **238**, 533.
- Waite, M. & Wakil, S. J. (1962). *J. biol. Chem.* **237**, 2750.