The Sensitivity of Acetyl-Coenzyme A Carboxylase to Citrate Stimulation in a Homogenate of Rat Liver Containing Subcellular Particles

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1. Although citrate is known to activate purified preparations of acetyl-CoA carboxylase, it had no stimulatory effect on the incorporation of [^{14}C]acetate into long-chain fatty acids in a whole homogenate of rat liver ($S_{0.7}$) under conditions in which the activity of acetyl-CoA carboxylase was rate-limiting for fatty acid synthesis. 2. The rate of incorporation of acetyl carbon into fatty acids was estimated in $S_{0.7}$ preparations incubated with [^{14}C]acetate, by measuring the specific radioactivity of the acetyl carbon of acetyl-CoA and the incorporation of ^{14}C into fatty acids. These estimates were compared with estimates of acetyl-CoA carboxylase activity in the $S_{0.7}$ preparation obtained by direct assay in conditions in which the enzyme was in the fully activated state. 3. In the absence of citrate, incorporation of acetyl carbon into fatty acids was about 75% of the value expected if the acetyl-CoA carboxylase in the $S_{0.7}$ preparation were in the fully activated state. 4. Incorporation of acetyl carbon into fatty acids in the $S_{0.7}$ preparation was stimulated by citrate, but the effect was many times less than the stimulation of [^{14}C]acetate incorporation by citrate in particle-free preparations. 5. When the mitochondria and microsomes were removed from the $S_{0.7}$ preparation, [^{14}C]acetate incorporation into fatty acids fell to a negligible value and the preparation became highly sensitive to stimulation by citrate. 6. It is suggested that in the presence of mitochondria and microsomes, and in the intact liver cell, the degree of activation of acetyl-CoA carboxylase is such that citrate activation may not be of physiological significance.

Citrate and isocitrate stimulate the incorporation of acetate into long-chain fatty acids by particle-free preparations of liver (Brady & Gurin, 1952) and lactating mammary gland (Abraham, Matthes & Chaikoff, 1961). The effect of citrate on acetate incorporation into fatty acids is due to stimulation of the carboxylation of acetyl-CoA (Waite, 1962; Matsuhashi, Matsuhashi, Numa & Lynen, 1962; Martin & Vagelos, 1962), probably by allosteric activation of acetyl-CoA carboxylase (EC 6.4.1.2) (Vagelos, Alberts & Martin, 1963). Since the carboxylation of acetyl-CoA is the rate-limiting step in fatty acid synthesis from acetate in cell-free preparations of liver (Numa, Matsuhashi & Lynen, 1961) it has been suggested that allosteric modification of the activity of acetyl-CoA carboxylase by citrate is an important factor in the regulation of fatty acid synthesis in the intact cell. We have described a cell-free preparation of rat liver ($S_{0.7}$) that synthesizes long-chain fatty acids from acetate via the malonyl-CoA pathway, and that resembles an intact liver cell in having no exogenous cofactors and in deriving its supply of ATP from phosphorylating mitochondria (Iliffe & Myant, 1964). Since

citrate did not stimulate [^{14}C]acetate incorporation into fatty acids in this preparation, we suggested that its stimulatory effect on fatty acid synthesis in particle-free preparations may not occur under physiological conditions. In this paper we describe experiments in which we have tried to measure the degree of activation of acetyl-CoA carboxylase in $S_{0.7}$ preparations in the absence and in the presence of citrate. Our results suggest that the sensitivity of acetyl-CoA carboxylase to citrate stimulation is altered by the presence of subcellular particles. A brief account of this work has been published (Iliffe & Myant, 1969).

METHODS

Preparation of homogenates. The rats used were albino males weighing about 200 g. They were fed *ad libitum* on M.R.C. diet no. 18 (Bruce & Parkes, 1946). The $S_{0.7}$ preparation of rat liver, containing the mitochondria, microsomes and cell sap, was prepared as described previously (Iliffe & Myant, 1964), with a homogenizing fluid of the following composition: sucrose, 0.25 M; nicotinamide, 5 mM; EDTA, 2 mM. To obtain the $S_{8.5}$

preparation, the $S_{0.7}$ preparation was centrifuged at 8500g for 20 min to remove the mitochondria, and the supernatant, containing microsomes and the soluble cell sap, was decanted. To obtain the S_{100} preparation, the $S_{8.5}$ preparation was centrifuged at 100000g for 1 h and the clear supernatant between the microsomal pellet and the fatty layer at the top of the tube was removed with a fine pipette. For the preparation of microsomes, the whole supernatant from the 100000g centrifugation was decanted, the above homogenizing fluid was added in a volume equal to one-quarter of the volume of the $S_{8.5}$ supernatant, and the microsomal pellet was suspended by means of a close-fitting Teflon plunger driven at 100 rev./min for 2 min. The suspension of microsomes was centrifuged again at 100000g for 1 h and the supernatant was discarded. The washed pellet was resuspended in a volume of homogenizing fluid such that 0.2 ml contained the microsomes from 2 ml of $S_{8.5}$ supernatant. All these operations were carried out at 2°C.

Conditions of incubation. The standard incubation mixture (pH 7.4) used for the $S_{0.7}$, $S_{8.5}$ and S_{100} preparations contained 2 ml of preparation prepared from 400 mg of fresh liver, with tris-HCl buffer (45 mM), KCl (40 mM), K_2HPO_4 (5 mM), potassium acetate (10 mM), GSH (5 mM) and sodium [^{14}C]acetate (0.01 mM, 2 μ Ci/flask). Cofactors, including metal ions and HCO_3^- , were added to the incubation mixtures in concentrations found to be optimum for [^{14}C]acetate incorporation into fatty acids in each of the three fractions (see the Results section). In all experiments the final volume of the incubation mixture was 4.5 ml. Each flask contained 37–42 mg of $S_{0.7}$ protein or 31–36 mg of $S_{8.5}$ protein or 23–30 mg of S_{100} protein. All incubations were carried out in paired flasks at 37°C with air as the gas phase.

Enzyme assays. The activity of acetyl-CoA synthetase (EC 6.2.1.1) was assayed by the method of Lipmann & Tuttle (1945), as modified by Kornacker & Lowenstein (1965). Each sample was assayed at two protein concentrations in the linear range of the curve relating amount of hydroxamate formed to mg of protein in the sample. The activity of acetyl-CoA carboxylase was assayed by measuring the acetyl-CoA-dependent incorporation of [^{14}C]bicarbonate into acid-stable products in the presence of citrate by the method of Martin & Vagelos (1962), as modified by Smith, Easter & Dils (1966). The activity of fatty acid synthetase was assayed by measuring the incorporation of [^{14}C]malonyl-CoA into fatty acids by the method of Lynen (1962).

Measurement of the specific radioactivity of acetyl-CoA. The acetyl group of acetyl-CoA was trapped by adding to the incubation mixture *p*-aminobenzoic acid and arylamine acetyltransferase (EC 2.3.1.5) prepared from pigeon liver by the method of Tabor, Mehler & Stadtman (1953). The specific radioactivity of the *p*-acetamidobenzoic acid was determined after it had been isolated from a sample of the incubation mixture by extraction with 3-methylbutan-1-ol followed by t.l.c. Duplicate 1 ml samples, taken from each flask at the end of the incubation, were transferred to 2 ml of 5% (w/v) trichloroacetic acid. The precipitate was sedimented by centrifugation and washed once by resuspension in 1 ml of 5% (w/v) trichloroacetic acid. The combined supernatants were made up to 5 ml with water and extracted twice with 15 ml of 3-methylbutan-1-ol. The combined upper phases were washed with

10 ml of water and evaporated to about 0.05 ml. The samples were then submitted to t.l.c. on Kieselgel H plates with propionic acid–benzene–water (36:62.5:1.5, by vol.) as solvent. Markers of *p*-aminobenzoic acid and *p*-acetamidobenzoic acid were run on adjacent lanes. After chromatography, the sample lanes were covered with aluminium foil. The plates were sprayed with 2M-HCl and dried in an oven for 20 min at 100°C. They were then sprayed with 0.2% (w/v) ninhydrin in ethanol and returned to the oven. In about 20 min pink bands appeared corresponding to *p*-aminobenzoic acid (R_F 0.54) and *p*-acetamidobenzoic acid (R_F 0.36). The aluminium foil was removed from the lane containing the sample and the area adjacent to the *p*-acetamidobenzoic acid marker was scraped from the plate. The Kieselgel powder was eluted twice with 30 ml of ethanol. After centrifugation of the eluates, the combined supernatants were evaporated to dryness and made up to 4 ml with ethanol. A 1 ml sample was taken for assay of radioactivity. The remaining 3 ml was evaporated to dryness, resuspended in 10 ml of water, hydrolysed with HCl and assayed for *p*-aminobenzoic acid by the method of Bratton & Marshall (1939), by using a curve prepared from standard solutions of *p*-aminobenzoic acid. When samples from incubations to which no *p*-aminobenzoic acid had been added were taken through the above procedure, the region on the thin-layer chromatogram adjacent to the *p*-acetamidobenzoic acid marker gave no colour when assayed for *p*-aminobenzoic acid and contained negligible radioactivity. The total amount of *p*-acetamidobenzoic acid formed during the incubation was estimated as the difference between the amounts of *p*-aminobenzoic acid present in a sample before and after acid hydrolysis. Each value given for the specific radioactivity of acetyl-CoA is the mean of four determinations, these being duplicate determinations from paired flasks. The maximum variation from the mean was 7%.

The methods used for extracting the fatty acids from the incubation mixture and for measuring the concentration of ATP were those described previously (Iliffe & Myant, 1964). Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. Citrate in the incubation mixture was usually assayed by the colorimetric method of Taylor (1953). In some experiments, citrate was also assayed enzymically with citrate lyase (EC 4.1.3.6) (Moellering & Gruber, 1966). The enzyme method gave values which agreed to within 15% of those obtained by the colorimetric assay.

Materials. Sodium [^{14}C]acetate, sodium [^{14}C]malonate, $NaH^{14}CO_3$ and [^{14}C]citric acid were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The [^{14}C]citric acid was prepared from $K^{14}CN$ and bis(chloromethyl)glycolic acid by the method of Rothchild & Fields (1952). The citrate prepared by this method contains equal proportions of 1- ^{14}C - and 5- ^{14}C -labelled molecules, with a small proportion of doubly labelled molecules. [^{14}C]Malonyl-CoA was prepared by the method of Trams & Brady (1960). ATP, GSH, CoA, $NADP^+$, acetyl-CoA, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. *p*-Acetamidobenzoic acid was prepared from *p*-aminobenzoic acid by a standard method for the acetylation of aromatic amines.

RESULTS

Before investigation of the effect of citrate on fatty acid synthesis in liver homogenates, it was necessary to establish conditions in which the activity of acetyl-CoA carboxylase was rate-limiting for fatty acid synthesis.

Enzyme activities in the $S_{0.7}$, $S_{8.5}$ and S_{100} preparations. Measurement of the activities of the three enzymes required for fatty acid synthesis from acetate by the malonyl-CoA pathway showed that the activity of acetyl-CoA carboxylase, assayed under optimum conditions in the presence of citrate, was less than that of fatty acid synthetase and of acetyl-CoA synthetase in the $S_{0.7}$, $S_{8.5}$ and S_{100} preparations (Table 1). The activity of acetyl-CoA carboxylase would therefore be expected to be rate-limiting for fatty acid synthesis from acetate in the three cases, provided that conditions are optimum for all three enzymes.

Optimum cofactor concentrations. Optimum concentrations of cofactors required for fatty acid synthesis were found by measuring the incorporation of [14 C]acetate into fatty acids in the presence of various concentrations of cofactors, with no added citrate and with added citrate (20mM).

The results obtained with the $S_{0.7}$ preparation are shown in Fig. 1. The optimum concentration of ATP was about 2mM without added citrate and about 1mM in the presence of citrate. Measurement of the ATP concentration at the end of the incubation showed that the initial value was maintained by phosphorylation throughout the incubation in the presence of added citrate. In the absence of added citrate the ATP concentration fell significantly when the initial value was 5 or 8mM. The NADP⁺ curve showed a maximum at a concentration of about 0.5mM in the absence of added citrate, and at a slightly higher concentration in the presence of citrate. Bicarbonate, when tested over the concentration range 0–15mM, had only a slight stimulatory effect on [14 C]acetate incorporation with and without added citrate. The optimum con-

centration of CoA was about 0.05mM in the absence of added citrate. In the presence of added citrate, [14 C]acetate incorporation approached a maximum value when the CoA concentration was 0.2mM. No requirement for Mn²⁺ could be demonstrated in the $S_{0.7}$ preparation, either in the presence or in the absence of added citrate. The optimum concentration of Mg²⁺ in the absence of added citrate was between 3 and 5mM. The addition of 20mM-citrate increased the requirement for Mg²⁺, the optimum concentration rising to 15–20mM.

The results obtained with the $S_{8.5}$ and S_{100} preparations are shown in Figs. 2 and 3.

In the $S_{8.5}$ preparation the concentration of ATP fell significantly during the incubation, presumably owing to the absence of phosphorylating mitochondria and the presence of adenosine triphosphatase activity. The optimum concentration of added ATP (about 12mM) was therefore much higher than that in the $S_{0.7}$ preparation. The optimum concentrations of NADP⁺, bicarbonate and CoA were broadly similar to those found for the $S_{0.7}$ preparation. Mn²⁺ had a marked stimulatory effect on [14 C]acetate incorporation in the $S_{8.5}$ preparation, the effect approaching a maximum in the presence of 3mM-Mn²⁺ with and without added citrate. The optimum concentration of Mg²⁺ was 10mM in the absence of added citrate, and was not less than 20mM in its presence.

In the S_{100} preparation incorporation of [14 C]-acetate into fatty acids was negligible in the absence of added citrate. In the presence of 20mM-citrate, the optimum concentration of ATP was about 8mM. The ATP concentration fell during the incubation, but the fall was less marked than in the $S_{8.5}$ preparation. The optimum concentrations of NADP⁺, bicarbonate, CoA and Mn²⁺ were similar to those found for the $S_{8.5}$ preparation, but the optimum concentration of Mg²⁺ in the presence of citrate was 10mM.

Time-course of [14 C]acetate incorporation. In the presence of cofactors, at concentrations found in the above experiments to be optimum, the time-course

Table 1. Activity of acetyl-CoA synthetase, acetyl-CoA carboxylase and fatty acid synthetase in $S_{0.7}$, $S_{8.5}$ and S_{100} preparations of rat liver

See the Methods section for methods used. Each preparation was made from the pooled livers of two rats. The numbers of preparations assayed are given in parentheses.

	Activity (nmol of substrate converted/h per mg of protein)		
	$S_{0.7}$	$S_{8.5}$	S_{100}
Acetyl-CoA synthetase	236 ± 14 (3)	289 ± 20 (3)	323 ± 16 (3)
Acetyl-CoA carboxylase	85 ± 5.6 (4)	82.1 ± 7.6 (4)	110 ± 4.9 (4)
Fatty acid synthetase	216 ± 54 (3)	351 (1)	441 (1)

Fig. 1. Effect of various concentrations of cofactors on the incorporation of $[^{14}\text{C}]$ acetate into fatty acids in the $S_{0.7}$ preparation with ($\circ$) and without ($\bullet$) added citrate (20 mM). ATP concentrations at the end of the incubation with ($\square$) and without ($\blacksquare$) citrate are shown in (a). The incubations were carried out in the standard incubation mixture supplemented with potassium succinate (15 mM), ATP (2 mM) and the following additions: (a) NADP⁺ (1 mM), glucose 6-phosphate (5 mM), KHCO₃ (10 mM), CoA (0.2 mM with, and 0.05 mM without, added citrate) and MgCl₂ (15 mM with, and 4 mM without, added citrate); (b) glucose 6-phosphate (5 mM) and MgCl₂ (15 mM with, and 4 mM without, added citrate); (c) NADP⁺ (1.5 mM), glucose 6-phosphate (5 mM), CoA (0.2 mM with, and 0.05 mM without, added citrate) and MgCl₂ (15 mM with, and 4 mM without, added citrate); (d) NADP⁺ (1.5 mM), glucose 6-phosphate (5 mM) and MgCl₂ (15 mM with, and 4 mM without, added citrate); (e) NADP⁺ (1.5 mM), glucose 6-phosphate (5 mM) and MgCl₂ (15 mM with, and 4 mM without, added citrate). The concentration of the particular cofactor under investigation was varied over the range shown. The $[^{14}\text{C}]$ acetate was added at the beginning of the incubation, and the samples were incubated for 3 h. Other experimental details are given in the Methods section.

of incorporation of $[^{14}\text{C}]$ acetate into fatty acids was similar with the $S_{0.7}$, $S_{8.5}$ and S_{100} preparations. Both in the presence and in the absence of added citrate, there was a lag phase lasting 20–40 min in all three preparations, followed by a linear phase beginning about 1 h after zero time and lasting about 1 h. When the $[^{14}\text{C}]$ acetate was added to the

incubation mixture after it had been preincubated for 80 min at 37°C, the time-course of incorporation into fatty acids was linear from zero time for at least 40 min (Fig. 4).

Effect of citrate on $[^{14}\text{C}]$ acetate incorporation. As shown in Fig. 4, 20 mM-citrate stimulated $[^{14}\text{C}]$ acetate incorporation into fatty acids by the $S_{8.5}$ and

Fig. 2. Effect of various concentrations of cofactors on the incorporation of $[^{14}\text{C}]$ acetate into fatty acids in the $S_{8.5}$ preparation with (○) and without (●) added citrate (20 mM). ATP concentrations at the end of incubation with (□) and without (■) citrate are shown in (a). The incubations were carried out in the standard incubation mixture supplemented with ATP (12 mM) and the following additions: (a), (b), (c), (d) and (e) NADP^+ (1 mM), glucose 6-phosphate (5 mM), KHCO_3 (10 mM), CoA (0.2 mM with, and 0.05 mM without, added citrate), MgCl_2 (20 mM with, and 10 mM without, added citrate) and Mn^{2+} (3 mM); (f) NADP^+ (0.5 mM), glucose 6-phosphate (0.5 mM), CoA (0.05 mM) and KHCO_3 (5 mM). The concentration of the particular cofactor under investigation was varied over the ranges shown. The $[^{14}\text{C}]$ acetate was added at the beginning of the incubation, and the samples were incubated for 3 h. Other experimental details are given in the Methods section.

S_{100} preparations, but had no stimulatory effect on the $S_{0.7}$ preparation. In order to investigate the effect of citrate in greater detail, $[^{14}\text{C}]$ acetate incorporation was measured in the presence of various concentrations of citrate. For each preparation the incubation mixture was supplemented with cofactors at optimum concentrations. The results of these experiments are shown in Fig. 5.

With the $S_{0.7}$ preparation the addition of citrate at all concentrations tested between 2 and 20 mM diminished $[^{14}\text{C}]$ acetate incorporation. Although some of the added citrate was metabolized, the citrate concentration at the end of incubation was above 10 mM when the initial concentrations were 15 and 20 mM.

Citrate stimulated $[^{14}\text{C}]$ acetate incorporation into

fatty acids by the $S_{8.5}$ and S_{100} preparations at all concentrations tested up to 30 mM. With both preparations the maximal effect occurred when the concentration of added citrate was 20 mM. The maximal stimulatory effect in the $S_{8.5}$ preparation was threefold and in the S_{100} preparation was more than 50-fold.

Effect of citrate on the acetyl-CoA pool. The absence of a stimulatory effect of citrate on $[^{14}\text{C}]$ acetate incorporation into fatty acids in the $S_{0.7}$ preparation raised the possibility that some of the added citrate was converted into acetyl-CoA, and that this masked a stimulation of fatty acid synthesis by lowering the specific radioactivity of the acetyl-CoA pool.

To test this possibility, the specific radioactivity

Fig. 3. Effect of various concentrations of cofactors on the incorporation of $[^{14}\text{C}]$ acetate into fatty acids in the S_{100} preparation with ($\circ$) and without ($\bullet$) added citrate (20 mM). ATP concentrations at the end of incubation with ($\square$) and without ($\blacksquare$) citrate are shown in (a). The incubation mixtures were the same as those used for the $S_{8.5}$ preparation (see Fig. 2), except that the standard concentration of ATP was 8 mM. The concentration of the particular cofactor under investigation was varied over the range shown. The $[^{14}\text{C}]$ acetate was added at the beginning of the incubation, and the samples were incubated for 2 h. Other experimental details are given in the Methods section.

of the acetyl group of acetyl-CoA was measured in the $S_{0.7}$ preparation incubated with $[1,5-^{14}\text{C}]$ citrate; in these experiments potassium acetate and $[^{14}\text{C}]$ acetate were omitted from the incubation mixture. A representative fraction of the acetyl carbon of acetyl-CoA, formed during the incubation, was trapped as *p*-acetamidobenzoic acid by adding *p*-aminobenzoic acid and arylamine acetyltransferase at the beginning of the incubation. The results are shown in Table 2. Radioactivity was present in the acetyl carbon at all citrate concentrations tested. In the presence of 10 mM-citrate the specific radioactivity of the acetyl carbon averaged 51% of that of the $[1,5-^{14}\text{C}]$ citrate in the two experiments. Since the enzymic cleavage of citrate is stereospecific, only C-1 and C-2 giving rise to acetyl-CoA (Spencer & Lowenstein, 1962), the specific radioactivity of the acetyl-CoA formed from

$[1,5-^{14}\text{C}]$ citrate (expressed in $\mu\text{Ci/nmol}$) would be half that of the $[^{14}\text{C}]$ citrate. The observed confirmation of this therefore suggests that in the absence of added acetate the acetyl-CoA pool in the $S_{0.7}$ preparation is derived entirely from the added citrate and that there is no contribution from endogenous sources.

The finding that citrate gives rise to acetyl-CoA in the $S_{0.7}$ preparation showed that fatty acid synthesis could not be estimated from the incorporation of $[^{14}\text{C}]$ acetate into fatty acids in the presence of citrate, unless the specific radioactivity of the acetyl-CoA pool was measured at the same time. We therefore measured the specific radioactivity of the acetyl-CoA pool and the rate of incorporation of ^{14}C into fatty acids in three $S_{0.7}$ preparations in which $[^{14}\text{C}]$ acetate was used as precursor. From these measurements it was

Fig. 4. Time-course of incorporation of [^{14}C]acetate into fatty acids in the $S_{0.7}$ ($\circ$ and $\bullet$), $S_{8.5}$ (Δ and $\blacktriangle$) and S_{100} ($\square$ and $\blacksquare$) preparations, incubated under optimum conditions, with ($\circ$, Δ and $\square$) and without ($\bullet$, $\blacktriangle$ and $\blacksquare$) added citrate (20mM). For the $S_{0.7}$ preparation the standard incubation mixture was supplemented with potassium succinate (15mM), ATP (2mM), NADP^+ (1mM), KHCO_3 (10mM), MgCl_2 (15mM with, and 4mM without, added citrate), glucose 6-phosphate (5mM) and CoA (0.2mM with and 0.05mM without, added citrate). For the $S_{8.5}$ preparation the standard incubation mixture was supplemented with ATP (12mM), NADP^+ (1mM), glucose 6-phosphate (5mM), KHCO_3 (10mM), CoA (0.2mM with, and 0.05mM without, added citrate), MgCl_2 (20mM with, and 10mM without, added citrate) and Mn^{2+} (3mM). For the S_{100} preparation the standard incubation mixture was the same as that used for the $S_{8.5}$ preparation, except that the concentration of ATP was 8mM. The incubation mixtures were pre-incubated for 80min at 37°C before addition of [^{14}C]acetate. Citrate was added at the beginning of the preincubation. Other experimental details are given in the Methods section.

possible to estimate the rate of conversion of acetyl carbon into fatty acids in the absence and in the presence of citrate. The results of these experiments are shown in Table 3. For each experiment two sets of flasks were prepared. In one set, containing no trapping system for acetyl carbon, incorporation of ^{14}C into fatty acids was measured. The specific radioactivity of the acetyl carbon of acetyl-CoA was measured in the other set, to which the trapping system was added at zero time. In all three experiments the specific radioactivity of the acetyl-CoA was lowered by the addition of 20mM-citrate to about 25% of that of the added [^{14}C]acetate.

In Expt. 1 the $S_{0.7}$ preparation incorporated acetyl carbon into fatty acids at a rate of 72nmol/h per mg of protein. This was stimulated 2.2-fold by citrate. The activity of acetyl-CoA carboxylase in this homogenate, assayed directly in the presence of

an optimum concentration of citrate (see the Methods section), was 95nmol/h per mg of protein. In Expt. 2, although the incorporation of acetyl carbon was lower than in Expt. 1, citrate stimulated incorporation to the same extent. Expt. 3 shows that the highest extent of incorporation of acetate was reached at a citrate concentration of 20mM.

Effect of subcellular particles on citrate stimulation of [^{14}C]acetate incorporation. The marked difference between the effect of citrate on incorporation of [^{14}C]acetate and acetyl carbon into fatty acids in the $S_{0.7}$ preparation and on that in the S_{100} preparation suggested that the particles in the former in some way diminish the sensitivity of acetyl-CoA carboxylase to stimulation by citrate. To test this possibility, the effect of citrate was investigated in $S_{0.7}$, $S_{8.5}$ and S_{100} preparations, and in the S_{100} preparation to which the microsomes were added back. In each preparation the incubation mixture was supplemented with cofactors at the concentrations required for maximal incorporation of [^{14}C]acetate into fatty acids. The results are shown in Table 4.

The rate of incorporation of [^{14}C]acetate into fatty acids in the $S_{8.5}$ preparation was about one-fifth that in the $S_{0.7}$ preparation and was stimulated about twofold by citrate. In the S_{100} preparation incorporation was negligible in the absence of citrate and was stimulated 50-fold by addition of citrate. When the microsomes were added back to the S_{100} preparation the rate of incorporation of [^{14}C]acetate was increased to a value comparable with that in the $S_{8.5}$ preparation and the sensitivity of the preparation to stimulation by citrate was much diminished. Although some of the added citrate was metabolized during the incubations, the final citrate concentration was 7.6mM in the $S_{0.7}$ preparation and was more than 12mM in the other three preparations.

DISCUSSION

Stimulation of fatty acid synthesis by acids of the citric acid cycle has been shown most clearly with particle-free systems or preparations of acetyl-CoA carboxylase of various degrees of purity. In some systems, such as the soluble fraction of rat mammary gland used by Abraham *et al.* (1961) and the rat liver supernatant of Numa *et al.* (1961), very little fatty acid synthesis takes place in the absence of added citrate or isocitrate, and the addition of these acids leads to an increase of more than 50-fold in the rate of incorporation of [^{14}C]acetate or [^{14}C]acetyl-CoA into fatty acids. With purified preparations of acetyl-CoA carboxylase the effect of acids of the citric acid cycle is even more striking. Both the partially purified preparation of rat liver acetyl-CoA carboxylase obtained by Lynen,

Fig. 5. Effect of various concentrations of citrate on the incorporation of [^{14}C]acetate into fatty acids ($\circ$) in the $S_{0.7}$ (a), $S_{8.5}$ (b) and S_{100} (c) preparations, each incubated under optimum conditions. The incubation mixtures were the same as those used for the experiments shown in Fig. 4. The incubation mixtures were pre-incubated for 80 min at 37°C before addition of [^{14}C]acetate and the amount of ^{14}C incorporated into fatty acids was measured at the end of a further period of incubation for 40 min. To achieve adequate concentrations of citrate in the $S_{0.7}$ preparation during the incorporation of [^{14}C]acetate, the citrate was added to the incubation mixture at the end of the preincubation. In the $S_{8.5}$ and S_{100} preparations the citrate was added at the beginning of the preincubation. The citrate concentration at the end of incubation ($\bullet$) was measured in the $S_{0.7}$ preparation.

Table 2. *Specific radioactivity of acetyl-CoA formed from [1,5- ^{14}C]citrate in the $S_{0.7}$ preparation*

The standard incubation mixture was supplemented with MgCl_2 (4 mM), potassium succinate (15 mM), ATP (2 mM), *p*-aminobenzoic acid (2 mM) and arylamine acetyltransferase (100 units). The potassium acetate and sodium [^{14}C]acetate were replaced by [1,5- ^{14}C]citrate at the concentrations shown.

Expt. no.	Concn. of [^{14}C]citrate (mM)	Sp. radioactivity (c.p.m./nmol)		100 $\times$ Acetyl-CoA/citrate sp.-radioactivity ratio
		[^{14}C]Citrate	Acetyl-CoA	
1	1	2350	295	12.6
	5	470	154	32.8
	10	235	111	47.2
2	10	45	25	55.5

Matsuhashi, Numa & Schweizer (1963) and the enzyme prepared from chicken liver by Gregolin, Ryder, Kleinschmidt, Warner & Lane (1966) have an absolute requirement for citrate or isocitrate. In agreement with this, [^{14}C]acetate incorporation into fatty acids in the S_{100} preparation was negligible in the absence of citrate and was stimulated 50-fold by 20 mM-citrate (Table 4.)

The present work shows that citrate does not stimulate the incorporation of [^{14}C]acetate into fatty acids in the $S_{0.7}$ preparation in conditions in which the activity of acetyl-CoA carboxylase would be expected to be rate-limiting for fatty acid synthesis. However, Bhaduri & Srere (1964) have pointed out that in preparations containing citrate-ATP lyase (EC 4.1.3.8) citrate would give rise to

acetyl-CoA and that this would lower the specific radioactivity of the [^{14}C]acetyl-CoA formed from [^{14}C]acetate. Fatty acid synthesis in the presence of citrate would therefore tend to be underestimated if it were calculated from the incorporation of [^{14}C]acetate into fatty acids. Our observations on the specific radioactivity of the acetyl-CoA pool in the $S_{0.7}$ preparation show that formation of unlabelled acetyl-CoA could only mask a twofold stimulation of fatty acid synthesis by citrate.

The degree of stimulation may, in fact, be even smaller than this, since incorporation of acetyl carbon into fatty acids in the $S_{0.7}$ preparation may have been overestimated in the presence of citrate. In Expts. 1 and 3 (Table 3) the rate of incorporation of acetyl carbon into fatty acids, measured in the

Table 3. *Effect of added citrate on the specific radioactivity of acetyl-CoA, and on the incorporation of [1-¹⁴C]-acetate into fatty acids, in the S_{0.7} preparation*

The incubation mixture used for the homogenate was the same as that described for the S_{0.7} preparation in Fig. 4. All flasks were preincubated for 80 min before addition of [¹⁴C]acetate and were then incubated for a further 40 min. (The specific radioactivities of the [¹⁴C]acetate used in Expts. 1, 2 and 3 were 393, 396 and 400 c.p.m./nmol respectively.) Citrate was added at the beginning of the preincubation. In the flasks in which acetyl carbon was trapped, *p*-aminobenzoic acid (2 mM) and arylamine acetyltransferase (100 units) were added at the end of the preincubation.

Expt. no.	Concn. of citrate (mM)		Sp. radioactivity of acetyl-CoA (c.p.m./nmol)	[¹⁴ C]Acetate incorporated (nmol/h per mg of protein)	Acetyl-CoA/acetate sp.-radioactivity ratio	Acetyl-CoA incorporated (nmol/h per mg of protein)
	Added	Final				
1*	0	0.2	417	72.2	1.06	72
	20	4.4	96	35.8	0.24	155
2	0	0.3	381	32	0.95	34
	20	6.4	99	21	0.25	84
3	12.5	1.7	116	26.7	0.29	92.1
	20.0	4.2	88	30.2	0.22	137
	27.5	13.1	62	17.4	0.16	107

* The activity of the acetyl-CoA carboxylase in the preparation used for this experiment was assayed directly as described in the Methods section, and was found to be 95 nmol/h per mg of protein.

Table 4. *Effect of citrate on [1-¹⁴C]acetate incorporation into fatty acids in the S_{0.7}, S_{8.5} and S₁₀₀ preparations*

All homogenates were made from the same pool of rat livers. The incubation mixtures were those used in the experiments described in Fig. 4. The S₁₀₀ preparation + microsomes were incubated in the mixture used for the S_{8.5} preparation. [¹⁴C]Acetate was added after preincubation for 80 min at 37°C and the incubations were continued for a further 40 min. Citrate was added at the beginning of the preincubation.

Preparation	Concn. of citrate (mM)		[¹⁴ C]Acetate incorporated (nmol/h per mg of protein)
	Added	Final	
S _{0.7}	0	<0.2	31
	20	7.6	20
S _{8.5}	0	<0.2	6
	20	12.7	11
S ₁₀₀	0	<0.2	0.2
	20	14.5	11
S ₁₀₀ + microsomes	0	0.7	9
	20	13.0	12

presence of optimum citrate concentration and under conditions where acetyl-CoA carboxylase activity is rate-limiting, was higher than the activity of acetyl-CoA carboxylase assayed directly in the presence of 10 mM-citrate (Table 1). Possibly citrate supplies some acetyl carbon that is available for *p*-aminobenzoic acid formation before it has mixed completely with the acetyl-CoA formed from

[¹⁴C]acetate. If so, the specific radioactivity of the acetyl-CoA pool from which fatty acids are synthesized may be higher than that of the acetyl carbon trapped by *p*-aminobenzoic acid.

In Expt. 1 (Table 3) the activity of acetyl-CoA carboxylase in the absence of citrate, estimated from the incorporation of acetyl carbon into fatty acid, was 75% of the activity measured directly in the same preparation in conditions in which the enzyme would be in the fully activated state, and was over 80% of the mean activity measured by direct assay in four other S_{0.7} preparations (Table 1). This suggests that in the S_{0.7} preparation acetyl-CoA carboxylase is largely in the activated form and hence is relatively insensitive to stimulation by citrate.

The results shown in Table 4 suggest that the activation of acetyl-CoA carboxylase in the S_{0.7} preparation, and hence its lack of sensitivity to citrate, is due to the presence of subcellular particles. The microsomes seem to be particularly important. When these are removed there is a marked increase in sensitivity to citrate and this is lost when washed microsomes are added back. In keeping with this, citrate has no effect (Masoro, Panagos, Cohen & Rapport, 1956; Miller & Cooper, 1959; Bortz & Lynen, 1964), or only a small effect (Foster & Bloom, 1963), on the incorporation of acetate into fatty acids in tissue slices.

Siekevitz (1962) has suggested that the properties of an enzyme may be influenced by its association with subcellular membranes. Possibly an association with the membranes of mitochondria and

microsomes favours the aggregation of acetyl-CoA carboxylase into the activated form. Foster & McWhorter (1969) have shown that acetyl-CoA carboxylase is stimulated by a phospholipid present in the membrane of the endoplasmic reticulum. They suggest that the enzyme becomes activated by attachment to the phospholipid component of the membrane. If this occurs under physiological conditions, the association between enzyme and membrane must be a loose one, since acetyl-CoA carboxylase is confined largely to the soluble fraction of a liver homogenate separated by centrifugation (Smith *et al.* 1966).

Another possibility is that the subcellular particles remove an inhibitor that inactivates acetyl-CoA carboxylase. Microsomes stimulate the incorporation of acetate into fatty acids in particle-free preparations of rat liver (Abraham, Matthes & Chaikoff, 1959; Fletcher & Myant, 1961). This effect seems to be due partly to the conversion of the CoA esters of long-chain fatty acids into triglycerides and other complex lipids (Lorch, Abraham & Chaikoff, 1963; Howard & Lowenstein, 1964) by microsomal enzymes. Palmitoyl-CoA inhibits acetyl-CoA carboxylase (Tubbs & Garland, 1963; Bortz & Lynen, 1963), and Numa, Bortz & Lynen (1965) have shown that the inhibition is reversed competitively by citrate. It is therefore possible that in the absence of microsomes the acetyl-CoA carboxylase in the S_{100} preparation is inactivated by the presence of fatty acyl-CoA and that the enzyme is activated when either microsomes or citrate are added to the preparation.

Whatever the underlying reason for our findings may be, they suggest that acetyl-CoA carboxylase is relatively insensitive to citrate in the intact liver cell. This raises the possibility that citrate does not participate in the physiological control of fatty acid synthesis by regulating the activity of acetyl-CoA carboxylase, despite the undoubted influence of citrate on this enzyme in the purified state. The physiological significance of citrate activation of acetyl-CoA carboxylase has, in any case, been questioned by Myant & Iliffe (1963), Srere (1965) and Lynen (1967) on the ground that the intracellular concentration of citrate is below that required for activation of the enzyme *in vitro* (Martin & Vagelos, 1962). Moreover, Denton & Halperin (1968) have shown that citrate concentration in adipose tissue is not related to fatty acid synthesis in the manner expected if fatty acid synthesis were regulated by citrate activation of acetyl-CoA carboxylase. It should also be noted that the activity of acetyl-CoA carboxylase was about 40% of that of fatty acid synthetase in the $S_{0.7}$ preparation. Ganguly (1960) and Numa *et al.* (1961), on the other hand, concluded that the activity of the carboxylase in rat liver was 20–80-fold less than that

of the synthetase. Chang, Seidman, Teebor & Lane (1967), who found that the activity of acetyl-CoA carboxylase was more than half that of fatty acid synthetase in mouse liver, suggested that the low values for carboxylase activity obtained by Ganguly (1960) and by Numa *et al.* (1961) were due to their using suboptimum assay conditions. The high activity of acetyl-CoA carboxylase found by Chang *et al.* (1967), and in the present work, suggests that the activity of fatty acid synthetase could become rate-limiting for fatty acid synthesis in some physiological conditions.

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