Conditions for the Self-Catalysed Inactivation of Carnitine Acetyltransferase

A NOVEL FORM OF ENZYME INHIBITION

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1. Carnitine acetyltransferase is very rapidly inhibited in the presence of bromoacetyl-(−)-carnitine plus CoA or of bromoacetyl-CoA plus (−)-carnitine.
2. Under appropriate conditions, the enzyme may be titrated with either bromoacetyl substrate analogue; in each case about 1 mole of inhibitor is required to inactivate completely 1 mole of enzyme of molecular weight $58\,000 \pm 3000$.
3. Inhibition by bromoacetyl-CoA plus (−)-carnitine results in the formation of an inactive enzyme species, containing stoichiometric amounts of bound adenine nucleotide and (−)-carnitine in a form that is not removed by gel filtration. This is shown to be *S*-carboxymethyl-CoA (−)-carnitine ester.
4. The inhibited enzyme recovers activity slowly on prolonged standing at 4°.
5. Incubation with *S*-carboxymethyl-CoA (−)-carnitine ester causes a slow inhibition of carnitine acetyltransferase.
6. The formation of bound *S*-carboxymethyl-CoA (−)-carnitine ester by the enzyme is discussed. Presumably the resulting inhibition reflects binding of the ester to both the CoA- and carnitine-binding sites on the enzyme and its consequent very slow dissociation. These observations confirm that carnitine acetyltransferase can form ternary enzyme-substrate complexes; this also appears to be the case with carnitine palmitoyltransferase and choline acetyltransferase.

The enzyme carnitine acetyltransferase [acetyl-CoA-(−)-carnitine *O*-acetyltransferase, EC 2.3.1.7] is widespread and active in animal tissues (Beenakkers & Klingenberg, 1964), but as yet no precise physiological role can be ascribed to it (Fritz, 1965). It might be expected, however, that a study of the metabolic disturbances caused by specific inhibitors of the enzyme would yield information relevant to this problem.

The effects of bromoacetyl derivatives of CoA and carnitine on isolated preparations of carnitine acetyltransferase have been investigated. It was hoped that such substrate analogues, after being specifically bound to the enzyme, would alkylate susceptible amino acid residues at or near the active centre. Similar active-site-directed inhibitors have been designed for a number of enzymes (Baker, 1967). Although it appears that bromoacetyl-(−)-carnitine at least may be used to inhibit the enzyme irreversibly and probably in the manner envisaged above, it has also become clear that under certain conditions either analogue can cause a much more rapid inhibition, which is slowly reversible. It is the somewhat novel nature of this rapid inhibition that is reported here.

Preliminary reports of part of this work have been published (Chase & Tubbs, 1966b, 1967).

MATERIALS

Enzymes. Pigeon breast muscle carnitine acetyltransferase was obtained from Boehringer Corp. (London) Ltd. (London, W. 5) and recrystallized twice to a specific activity of 120–130 units/mg. (Chase, Pearson & Tubbs, 1965).

Citrate synthase (EC 4.1.3.7) was prepared from pig heart by the method of Srere & Kosicki (1961).

Chemicals. CoA was bought from Boehringer Corp. (London) Ltd.; desulpho-CoA was prepared from it by the method of Chase, Middleton & Tubbs (1966). Chloroacetyl chloride, bromoacetyl bromide, bromoacetic acid, thiophenol and β -alanine were the reagent grade of British Drug Houses Ltd. (Poole, Dorset). *S*-Carboxymethyl-2-mercaptoethylamine was prepared from 2-mercaptoethylamine hydrochloride (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) by the method of De Marco, Riva & Dupre (1964) and recrystallized twice. (+)-, (−)- and (±)-Carnitine hydrochlorides were obtained from Koch-Light Laboratories Ltd.; samples of the two optically active isomers from British Drug Houses Ltd. were also used. (±)-[Me-¹⁴C]-Carnitine hydrochloride, nominally 11.65 μ C/ μ mole, was a gift from Dr P. B. Garland. Before use it was chromatographed on a column of Zeo-Karb 225

(SRC16) cation-exchange resin (British Drug Houses Ltd.) by the procedure of Friedman, McFarlane, Bhattacharyya & Fraenkel (1960). An emergent peak with 95% of the radioactivity of the starting material proved to contain all the carnitine. This material was pooled and stored frozen.

5,5'-Dithiobis-(2-nitrobenzoic acid) was obtained from the Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.). Tris (Trizma base; Sigma Chemical Co., St Louis, Mo., U.S.A.) was neutralized with HCl. Phosphate buffers were prepared from KH_2PO_4 and KOH.

Other reagents were of analytical grade and glass-distilled water was used throughout.

METHODS

Preparation of chloroacetyl-CoA and bromoacetyl-CoA. In the early stages of this work these compounds were prepared by treating CoA solutions with the appropriate haloacetyl halide (Seubert, 1960). However, as this procedure gives rather poor yield (20–30%) of impure haloacetyl-CoA, a milder technique has been developed based on transhaloacetylation between thiophenol and CoA.

(i) Preparation of *S*-bromoacetylthiophenol. A 2.0 ml. portion (19.2 m-moles) of thiophenol was added to 2.4 ml. (26.4 m-moles) of freshly redistilled bromoacetyl bromide in a Thunberg tube. HBr was removed as formed by means of a water pump. After about 60 min. at room temperature no further gas was evolved and saturated KHCO_3 solution was added to bring the pH of the mixture to about 8. This precipitated bromoacetylthiophenol as colourless crystals. The mixture was extracted twice with ether, which dissolved the crystals, and the aqueous supernatant was washed twice with this solvent. The ethereal solution was washed three times with dilute aqueous KHCO_3 to remove traces of bromoacetic acid and dried with Na_2SO_4 . Removal of the ether in a stream of N_2 left a pale-yellow solid, which was recrystallized from aqueous ethanol. A yield of 2.7 g. (57% of theory) of colourless bromoacetylthiophenol (m.p. $36.3\text{--}37.3^\circ$, uncorr.) was obtained. On storage at 4° *in vacuo* in the dark, bromoacetylthiophenol develops a yellow colour, and it was freshly recrystallized before use.

(ii) Bromoacetylation of CoA. A 0.5 g. portion (2.0 m-moles) of bromoacetylthiophenol was dissolved in 3 ml. of acetone–0.1 M- KHCO_3 (5:2, v/v) and mixed with 20 mg. (approx. 20 μmoles) of CoA dissolved in the same solvent. This system was usually monophasic; if it was not, drops of acetone or KHCO_3 were added as required. After 45 min. at room temperature, the solution was brought to pH 1–2 with conc. HCl and the acetone removed with a stream of N_2 . The aqueous slurry that remained was extracted ten times with ether to remove thiophenol and acylthiophenol, and the last traces of ether were evaporated with N_2 as before.

The pH of the solution was adjusted to 3.5–4.5 with KHCO_3 . About 75% of the CoA originally present was recovered as bromoacetyl-CoA, which may be stored frozen with only a low rate of decomposition (less than 5%/week).

Assay of haloacetyl-CoA derivatives. Both chloroacetyl-CoA and bromoacetyl-CoA are substrates for citrate synthase. Solutions of these derivatives may therefore be assayed in the system described for acetyl-CoA (Chase, 1967a), although the time taken for the reaction to reach equilibrium must be considerably extended and larger amounts (100–200 $\mu\text{g.}$) of citrate synthase used.

Preparation and assay of bromoacetylcarnitine. The bromoacetyl derivatives of (+), (–) and ($\pm$)-carnitine were prepared by treating the appropriate carnitine hydrochloride, dissolved in bromoacetic acid, with bromoacetyl bromide. The procedure was otherwise identical with that described by Fraenkel & Friedman (1957) for acetylcarnitine. Bromoacetylcarnitine was recrystallized twice from acetic acid–acetone (Chase & Tubbs, 1966a) and stored in a desiccator away from light.

No specific assay for bromoacetylcarnitine is available, as this compound strongly inhibits carnitine acetyltransferase. Two indirect methods have therefore been used. In the first, bromoacetylcarnitine was hydrolysed by incubation at room temperature for 10 min. in the presence of a 0.1 M concentration excess of KOH. After neutralization the (–)-carnitine released was determined enzymically (Pearson & Tubbs, 1964). This procedure can only be used with derivatives of (–)- or ($\pm$)-carnitine. Bromoacetyl esters of all carnitine enantiomers were also estimated chemically by a hydroxamate method (Friedman & Fraenkel, 1955). The first method measures both free and alkali-labile carnitine in the sample, whereas the second measures all esters of carnitine, so that the reported concentrations of bromoacetylcarnitine are maximum values.

Substrates of carnitine acetyltransferase. Solutions of CoA, acetyl-CoA, (–)-carnitine and acetyl-(–)-carnitine were prepared, stored and assayed as described by Chase & Tubbs (1966a).

Measurements of carnitine acetyltransferase activity. Except where specific conditions are mentioned, carnitine acetyltransferase activities were compared by adding enzyme samples to a system containing tris-HCl buffer, pH 7.8 (100 mm), acetyl-CoA (0.1 mm) and (–)-carnitine hydrochloride (1.25 mm) in a final volume of 2.0 ml. The initial rate of decrease of E_{232} , due to the deacetylation of CoA, was observed by using a Beckman DK-2 recording spectrophotometer with the cell housing maintained at $30 \pm 0.3^\circ$.

Molecular-weight determinations. A single determination of the molecular weight of carnitine acetyltransferase was made by using the Archibald technique as modified by Ehrenberg (1957). Observations on the meniscus at the air/solvent interface were carried out on a solution containing 9.85 mg. of protein/ml. in 0.1 M-phosphate buffer, pH 7.5, by using a Spinco model E analytical ultracentrifuge at 7950 rev./min. and 17° . The subsequent artificial-boundary experiment was conducted at 16000 rev./min. Calculated values for the molecular weight after 48, 64 and 80 min. were 59300, 58900 and 57500 respectively. A value of 0.74 was taken for the partial specific volume of the enzyme. This was obtained from the amino acid composition (J. F. A. Chase, unpublished work) by the procedure of McMeekin & Marshall (1952).

Two experiments were performed by using the 'short-column' sedimentation-equilibrium technique of Yphantis (1960). With 4.5 mg. of protein/ml. dissolved in 0.03 M-phosphate buffer, pH 7.8, containing NaCl (0.1 M), these gave values for the molecular weight of 61120 and 60580.

A previous estimate of the molecular weight of carnitine acetyltransferase, obtained by gel filtration (Andrews, 1964), was 55000 (Chase *et al.* 1965). Taking this result with those reported above, the molecular weight of the enzyme has here been assumed to be 58000 ± 3000 .

Protein concentration. Carnitine acetyltransferase solu-

tions were estimated spectrophotometrically, by taking $E_{1\text{cm}}^{1\%}$ to be 8.25 at 280m μ . This value was obtained by submitting enzyme samples to micro-Kjeldahl digestion and determining their nitrogen content (as ammonia) with ninhydrin. It was then assumed that the protein contained 16% of nitrogen (Bailey, 1962, p. 300).

Amino acid analyses. Acid hydrolysates were analysed by using a Technicon AutoAnalyzer. Known samples of β -alanine and *S*-carboxymethyl-2-mercaptoethylamine were used to determine the elution positions and ninhydrin colour yields of these compounds. Under the usual conditions suggested by the manufacturers of the AutoAnalyzer for the estimation of amino acids, β -alanine was eluted 23–26 min. before *S*-carboxymethyl-2-mercaptoethylamine, which was itself eluted 40–44 min. before ammonia.

Radioactivity measurements. Samples (100 μ l.) of solutions containing [^{14}C]carnitine dissolved in 0.1M-phosphate buffer, pH 7.0, were plated on to planchets, dried *in vacuo* and counted with a Geiger-Müller tube fitted with a Panax type D558 scaler.

RESULTS

Establishment of experimental conditions for the rapid inhibition of carnitine acetyltransferase by bromoacetylcarnitine. Fig. 1(a) shows the effect of adding 100 μ M-bromoacetyl-($\pm$)-carnitine to a reaction system containing carnitine acetyltransferase and its substrates, acetyl-CoA and (–)-carnitine. Complete inhibition of the enzyme is observed within 1 min.; a second sample of enzyme added to the same system, which now contains some free CoA, is inhibited even more rapidly. The experiments shown in Figs. 1(b)–1(d) establish that,

for rapid inhibition of the enzyme to occur, it must be exposed simultaneously to CoA and bromoacetylcarnitine. Neither acetyl-CoA nor desulpho-CoA will substitute for CoA in promoting inhibition.

In experiments similar to those of Fig. 1, bromoacetyl-(+)-carnitine was found to be very much less inhibitory than the (–)-derivative. Thus, even at a concentration of 2mM, only a very slow inhibition was observed and this may well have been due to trace (about 1%) contamination with the natural isomer of the (+)-carnitine used in the synthesis of bromoacetyl-(+)-carnitine.

Establishment of experimental conditions for the rapid inhibition of carnitine acetyltransferase by haloacetyl-CoA. In Fig. 2(a) it is shown that the addition of 0.4 μ M-bromoacetyl-CoA to the reaction mixture used above caused virtually instant abolition of carnitine acetyltransferase activity. Even 0.02 μ M-inhibitor stopped the reaction in about 1 min. (Fig. 2b). Fig. 2(c)–2(e) establish that for rapid inhibition to occur the enzyme must be simultaneously exposed to bromoacetyl-CoA and (–)-carnitine. (+)-Carnitine, the unnatural isomer, did not promote inhibition by bromoacetyl-CoA (Fig. 2f).

Enhancement of chloroacetyl-CoA inhibition by (–)-carnitine. Under the conditions of Fig. 2, chloroacetyl-CoA inhibited in the same way as bromoacetyl-CoA. Loss of enzyme activity was, however, less rapid and this made it easier to investigate the quantitative effects of (–)-carnitine on the rate of inhibition. Fig. 3(a) shows that no

Fig. 1. Inhibition of carnitine acetyltransferase by bromoacetyl-($\pm$)-carnitine. Progress curves are shown for the reaction between acetyl-CoA and (–)-carnitine as followed at 232m μ . (a) Acetyl-CoA, 55 μ M; (–)-carnitine, 1.25mM; tris-HCl buffer, pH 7.7, 0.1M. The reaction was started at A by the addition of enzyme. At B bromoacetyl-($\pm$)-carnitine was added (final concn. 100 μ M). A second addition of enzyme was made at A'. The broken line shows the progress of the uninhibited reaction. (b) Enzyme preincubated with 100 μ M-bromoacetyl-($\pm$)-carnitine for 2 min. in buffer at 30°. The reaction was started at C by adding acetyl-CoA and (–)-carnitine (final concentrations as before). A second addition of enzyme was made at A'. (c) Enzyme preincubated as in (b) with 100 μ M-bromoacetyl-($\pm$)-carnitine, but plus 50 μ M-CoA. Additions at C and A' were as in (b). (d) Enzyme preincubated as in (c), but with 55 μ M-desulpho-CoA in place of CoA. Acetyl-CoA and (–)-carnitine were added at C as in (b) and (c).

Fig. 2. Inhibition of carnitine acetyltransferase by bromoacetyl-CoA. Progress curves are shown as in Fig. 1. (a) Acetyl-CoA, $55 \mu\text{M}$; (—)-carnitine, 1.4 mM ; tris-HCl buffer, pH 7.7, 0.1 M . The reaction was started at A by the addition of enzyme ($0.58 \mu\text{g}$). At B bromoacetyl-CoA was added (final concn. $0.35 \mu\text{M}$). The broken line shows the progress of the uninhibited reaction. (b) Reaction started at A as in (a). At C bromoacetyl-CoA was added (final concn. 17 nM). A second addition of enzyme was made at A'. (c) Enzyme ($0.58 \mu\text{g}$) preincubated for 2 min. in buffer at 30° with 17 nM -bromoacetyl-CoA. The reaction was started at D by adding acetyl-CoA and (—)-carnitine (final concentrations as before). (d) Enzyme preincubated as in (c) with 17 nM -bromoacetyl-CoA plus $55 \mu\text{M}$ -acetyl-CoA. The reaction was started at E by adding (—)-carnitine. (e) Enzyme preincubated as in (c) with 17 nM -bromoacetyl-CoA plus 1.4 mM (—)-carnitine. The reaction was started at F with acetyl-CoA. (f) Enzyme preincubated as in (e) with 1.25 mM (+)-carnitine in place of (—)-carnitine. The reaction was started at D with acetyl-CoA and (—)-carnitine.

Fig. 3. Effect of (—)-carnitine concentration on inhibition by chloroacetyl-CoA. (a) In a series of experiments carnitine acetyltransferase was mixed with (—)-carnitine, at the indicated concentration, in 100 mM -tris-HCl buffer, pH 7.8, at 30° . Chloroacetyl-CoA (final concn. $0.38 \mu\text{M}$) was added at zero time and, after various intervals, acetyl-CoA and (—)-carnitine were added simultaneously (final concns. $100 \mu\text{M}$ and $900 \mu\text{M}$ respectively) to start the transferase reaction. Rates were linear for 30–60 sec., showing effective protection by acetyl-CoA against further inhibition. Initial (—)-carnitine concentrations: ●, zero; △, $36 \mu\text{M}$; □, $72 \mu\text{M}$; ▲, $180 \mu\text{M}$; ○, $900 \mu\text{M}$. (b) Reciprocal of the pseudo-first-order inactivation rates (in arbitrary units) observed at the various (—)-carnitine concentrations in (a) plotted against the reciprocal of the corresponding (—)-carnitine concentrations.

inhibition occurred in the absence of (—)-carnitine; in its presence the progressive inhibition obeyed pseudo-first-order kinetics. The apparent first-order rate constant for the development of inhibition depends on the (—)-carnitine concentration in

a manner that suggests that it is an enzyme-carnitine complex with K_i $1.13 \times 10^{-4} \text{ M}$, and not the free enzyme itself, that is susceptible to attack by chloroacetyl-CoA (Fig. 3b). A value of $1.20 \times 10^{-4} \text{ M}$ has been reported for the dissociation

Table 1. *Protection by desulpho-CoA against bromoacetyl-CoA inhibition of carnitine acetyltransferase*

Two incubation mixtures were set up containing: tris-HCl buffer, pH 7.7, 100 mM; (–)-carnitine, 1 μ M; desulpho-CoA, 0 or 112 μ M; carnitine acetyltransferase, 0.81 μ M; water to final volume 0.98 ml. At zero time 0.02 ml. of bromoacetyl-CoA solution was added (final concn. 1.25 μ M) to each mixture. Samples (0.05 ml.) were removed for assay after 0, 20, 120 and 600 sec. The percentages of the original enzyme activity remaining in each sample are shown.

Concn. of desulpho-CoA (μ M)	Time ...	Activity remaining (%)		
		20 sec.	120 sec.	600 sec.
0		17.5	8	4
112		46.5	34.5	21

Fig. 4. Titration with bromoacetyl-(–)-carnitine. To each of two tubes kept at room temperature was added 0.2 ml. of carnitine acetyltransferase (1.25 nmole) dissolved in 0.1 M-phosphate buffer, pH 7.0, and 10 μ l. (100 nmole) of CoA. Samples (2 μ l.) were removed for enzymic assay and then 1 μ l. (0.129 nmole) of bromoacetyl-(–)-carnitine was added to one tube and 1 μ l. of water to the control. After 5 min. the enzyme activity of each tube was determined as before. Further additions of inhibitor and water were made in the same way. ●, Titration with inhibitors; ○, control. Enzymic rates are expressed in arbitrary units.

constant of the enzyme-carnitine complex formed during the catalytic activity of carnitine acetyltransferase (Chase & Tubbs, 1966a). The close similarity between these values strongly suggests that the same enzyme-carnitine complex is involved in catalysis and in the promotion of haloacetyl-CoA inhibition.

Protection by desulpho-CoA against inhibition by bromoacetyl-CoA. As shown in Table 1, the CoA analogue desulpho-CoA, which is bound to carnitine acetyltransferase similarly to other CoA derivatives (Chase *et al.* 1966), affords considerable protection against inhibition by bromoacetyl-CoA.

Titration of carnitine acetyltransferase with bromoacetyl-(–)-carnitine. Fig. 4 shows the effect of successive additions, each of 0.129 nmole, of bromoacetyl-(–)-carnitine on the activity of carnitine acetyltransferase (1.25 nmole) in the presence of excess of CoA. Until at least 85% of the

Fig. 5. Titration with bromoacetyl-CoA. Samples of carnitine acetyltransferase (0–0.052 nmole) were incubated at 30° in tris-HCl buffer, pH 7.8 (100 mM), EDTA (0.25 mM), (–)-carnitine (0.25 mM) and bromoacetyl-CoA in a final volume of 1.9 ml. After 10 min. residual enzyme activity was measured by the addition of 0.05 ml. of 100 mM-acetyl-(±)-carnitine and 0.05 ml. of 10 mM-CoA. Bromoacetyl-CoA added: ●, none; △, 0.0148 nmole; ○, 0.0296 nmole.

enzyme's activity is lost, the degree of inhibition observed is proportional to the amount of inhibitor added. By extrapolation it may be calculated that 1 mole of enzyme is inactivated by 0.97 mole of inhibitor.

Titration of carnitine acetyltransferase with bromoacetyl-CoA. Fig. 5 summarizes the results of an experiment in which the residual activities of carnitine acetyltransferase solutions were measured after incubation with bromoacetyl-CoA in the presence of excess of (–)-carnitine. The molar proportion of enzyme to inhibitor was varied from about 0.25:1 to 2:1, and 1 mole of bromoacetyl-CoA in this system completely inactivated up to 1.17 moles of enzyme. Further additions of enzyme above the amount required to titrate the inhibitor had undiminished activity.

The results shown in Figs. 4 and 5 suggest that the enzyme has only one active centre/molecule.

Recovery of activity by inhibited enzyme. Carnitine acetyltransferase, after inhibition with bromoacetyl-CoA plus (–)-carnitine and the subsequent

Fig. 6. Recovery of activity by inhibited enzyme. A 40mg. sample (0.69 μ mole) of carnitine acetyltransferase in 0.1M-phosphate buffer, pH 7.15, was treated with 1.05 μ -moles of (–)-carnitine and 0.93 μ mole of bromoacetyl-CoA in a final volume of 3.0ml. After 5 min. at room temperature residual enzyme activity was less than 1%. Excess of inhibitor was then removed from the enzyme by passage through a column of Sephadex G-25 as described for the experiment shown in Fig. 7. The eluted protein in a volume of 24ml. was stored at 4°, and 1 μ l. samples were assayed for carnitine acetyltransferase activity over a period of about 6 weeks. Control samples of enzyme showed less than 5% loss of activity after a corresponding time in the refrigerator.

removal of excess of inhibitor by gel filtration, showed a slow progressive recovery of activity on standing at 4° (Fig. 6). The rate of this process followed pseudo-first-order kinetics with a half-time of about 14.8 days. After 37 days 85% of the original activity was present; thereafter reactivation took place more slowly and never exceeded 89% (51 days).

Enzyme that had been inhibited with bromoacetylcarnitine plus CoA (Fig. 1) also regained activity (5–10% of the uninhibited value) on dialysis overnight against 0.1M-phosphate buffer, pH 7.0, at 4°.

The finding that the inhibition of carnitine acetyltransferase by bromoacetyl-CoA plus carnitine, or by bromoacetylcarnitine plus CoA, was reversible on prolonged incubation was unexpected, as these inhibitors had been designed to act irreversibly by alkylating groups on the protein. Consideration had therefore to be given to possible alternative mechanisms for their action. The striking quantitative similarity in the enzyme's requirement for (–)-carnitine, and not (+)-carnitine, both for the inhibition by haloacetyl-CoA derivatives (Figs. 2 and 3) and for its normal catalytic function, suggested that the processes of inhibition and catalysis have features in common. Further, it appears likely from the results of Table 1 that bromoacetyl-CoA binds to the enzyme at the

Scheme 1. Proposed scheme for the inhibition of carnitine acetyltransferase by the bromoacetyl derivatives of CoA and (–)-carnitine. Abbreviations: Cn, (–)-carnitine; BrAcCn, bromoacetyl-(–)-carnitine; BrAcCoA, bromoacetyl-CoA; CoA·S·CH₂·CO·O·Cn, S-carboxymethyl-CoA (–)-carnitine ester.

same site as other CoA derivatives (Chase & Tubbs, 1966a). A hypothesis developed from these observations is illustrated in Scheme 1. It is assumed that, after independent binding of bromoacetyl-CoA and (–)-carnitine to the usual enzyme sites for CoA and carnitine derivatives (Scheme 1a), transfer of the bromoacetyl group from CoA to carnitine takes place by the normal catalytic mechanism (Scheme 1b). This is not unreasonable as the enzyme has a broad specificity for the transfer of short-chain acyl groups (Chase, 1967b). At this stage, the newly formed enzyme-bound bromoacetyl-(–)-carnitine is involved in an alkylation reaction, not of a group on the enzyme, but rather of the terminal thiol group of the other substrate, CoA (Scheme 1c). The resulting adduct, S-carboxymethyl-CoA (–)-carnitine ester, having affinity for both the CoA- and carnitine-binding sites on the enzyme, might be expected to dissociate extremely slowly. Inhibition of carnitine acetyltransferase by bromoacetylcarnitine plus CoA would presumably follow an abbreviated scheme without a preliminary transbromoacetylation (Scheme 1b–c).

Such a sequence of events is compatible with the experiments described in Figs. 4 and 5 on the stoichiometry of inhibition, and the further work described here was designed to establish that the above hypothesis is correct.

Large-scale preparation of inhibited enzyme. Carnitine acetyltransferase (0.86 μ mole) in 3.5ml. of 0.1M-phosphate buffer, pH 7.0 (14.2mg. of protein/ml.), was mixed with 250 μ l. (2.08 μ moles) of 8.3 mM-($\pm$)-[¹⁴C]carnitine hydrochloride (specific radioactivity 3.6 $\times$ 10⁵ counts/min./ μ mole). Then 200 μ l. (1.12 μ moles) of 5.6 mM-bromoacetyl-CoA

Fig. 7. Elution profile from Sephadex G-25 of carnitine acetyltransferase treated with bromoacetyl-CoA plus $(\pm)$ - $[^{14}\text{C}]$ carnitine. $\bullet$, E_{260} (10mm. light-path); Δ , radioactivity.

Fig. 8. Altered absorption spectrum of the inhibited enzyme. Curve A ($\bullet$), spectrum of $7.2\mu\text{M}$ inhibited carnitine acetyltransferase obtained by diluting fraction 12 of the Sephadex G-25 eluate shown in Fig. 7 with 0.1M -phosphate buffer, pH 7.1. Extinction measurements over a 10mm. light-path were made by using a Unicam SP.500 spectrophotometer. The protein concentration of the inhibited enzyme solution was obtained by the microbiuret method (Bailey, 1962, p. 294), with untreated carnitine acetyltransferase as a standard. Curve B (Δ), corresponding spectrum of $7.2\mu\text{M}$ native enzyme. Curve C ($\circ$), difference spectrum obtained by subtracting curve B from curve A.

was added and, after 5min. at room temperature, less than 0.3% of the original enzyme activity was detectable.

The entire reaction mixture (3.95ml.) was applied

to a column (2cm. $\times$ 35cm.) of Sephadex G-25 equilibrated with 0.1M -phosphate buffer, pH 7.0, and was eluted with the same buffer; 26-drop fractions were collected automatically. Fig. 7 shows the elution pattern of protein and unchanged bromoacetyl-CoA as determined from the E_{260} value of each fraction. Samples (100 μl .) were also taken for radioactivity determinations to show the fate of the carnitine label.

Absorption spectrum of the inhibited enzyme. A difference spectrum (curve C in Fig. 8) may be calculated by comparing the spectrum of inhibited carnitine acetyltransferase (curve A in Fig. 8) with that of the native enzyme (curve B in Fig. 8). It has a peak at $260\text{m}\mu$, which could be caused by an adenine nucleotide residue bound to the inhibited enzyme.

Radioactivity associated with the inhibited enzyme. Fractions 11–15 of the eluate from Sephadex G-25 (Fig. 7) were pooled and found to contain 41.3mg. of protein ($0.71\mu\text{mole}$). Assay of this enzyme, immediately after gel filtration, showed it to have 18% of the specific activity of the original carnitine acetyltransferase. The radioactivity associated with this protein was equivalent to that from $0.43\mu\text{mole}$ of the $(-)$ -carnitine used to promote inhibition. Thus, if it is assumed that no radioactivity was bound to reactivated enzyme, each mole of inhibited enzyme contained 0.74mole of a $(-)$ -carnitine derivative.

Release of radioactivity from the inhibited enzyme. Preliminary experiments showed that neither precipitation of the enzyme with ammonium sulphate nor its adsorption on calcium phosphate gel and subsequent elution promoted release of radioactivity from the protein or the return of its catalytic function. However, irreversible denaturation of the enzyme with trichloroacetic acid followed by passage through a Millipore filter left more than 90% of the radioactivity in the filtrate.

To 9.5ml. of inhibited enzyme (39mg., $0.67\mu\text{mole}$) from the experiment shown in Fig. 7 was added an equal volume of cold 5% (w/v) trichloroacetic acid. Denatured protein was removed by centrifugation and washed once with cold 2.5% trichloroacetic acid. The volume of the combined supernatant and washings was reduced to 4.7ml. by rotary evaporation (bath temperature 30°) and trichloroacetic acid was removed by extracting six times with ether.

The sample was diluted to 50ml. with water and applied to a column (1.5cm. $\times$ 21cm.) of DEAE-cellulose equilibrated with 3mM-hydrochloric acid. Elution was performed with a salt gradient, the mixing vessel originally holding 100ml. of 3mM-hydrochloric acid, and the reservoir 100ml. of 3mM-hydrochloric acid containing 0.25M -potassium chloride (Moffatt & Khorana, 1961); 26-drop

fractions were collected automatically. The E_{260} value of each fraction was measured and 25 μ l. samples were taken for radioactivity determinations (Fig. 9). A single sharp peak of 260 $m\mu$ -absorbing material emerged coincidentally with all the radioactivity of the sample. Fractions 45–48, which comprised this peak, were pooled for analysis.

Analysis of the pooled material: identification of S-carboxymethyl-CoA (–)-carnitine ester. (i) Ultra-violet-absorption spectrum. This was measured both at pH 2.7 and at pH 7.0. In acid there was an extinction maximum at 257 $m\mu$, a minimum at 230.5 $m\mu$ and the E_{280}/E_{260} ratio was 0.202. In

neutral solution the maximum was at 260 $m\mu$, the minimum at 228 $m\mu$ and the E_{280}/E_{260} ratio was 0.136. These values agree well with published data for adenine nucleotides (Beaven, Holiday & Johnson, 1955). Assuming the molar extinction coefficient for adenine nucleotides in acid solution to be $14.2 \times 10^3 \text{ cm}^{-1}$ at 260 $m\mu$ (Beaven *et al.* 1955), the total yield of nucleotide was calculated to be 0.33 μ mole.

(ii) Alkali-labile (–)-carnitine content of the nucleotide. The radioactivity associated with the nucleotide was that present in 0.366 μ mole of the [^{14}C]carnitine used to promote inhibition of the original enzyme.

Three samples of nucleotide were adjusted to 0.1 M concentration excess of potassium hydroxide and left at room temperature for 20 min. They were then neutralized with hydrochloric acid and assayed for (–)-carnitine (Chase & Tubbs, 1966a), together with another nucleotide sample that had not been exposed to alkali. It was found (Table 2) that each mole of nucleotide contained about 1 mole of (–)-carnitine and that at least 95% of this carnitine was released only on alkaline hydrolysis.

(iii) Acid hydrolysis of the nucleotide. A sample containing 0.205 μ mole of nucleotide was evaporated to dryness in a rotary evaporator, dissolved in 1 ml. of 6 M-hydrochloric acid and hydrolysed in a sealed evacuated tube for 18 hr. at 105°. The hydrolysate, after evaporation to dryness, was dissolved in 1 ml. of 0.1 M-hydrochloric acid and applied to a Technicon AutoAnalyzer. It proved to contain stoichiometric amounts of β -alanine and S-carboxymethyl-2-mercaptoethylamine (Table 2).

This analysis strongly suggests that the nucleotide formed during the inhibition of carnitine acetyltransferase by bromoacetyl-CoA plus (–)-carnitine is indeed S-carboxymethyl-CoA (–)-carnitine

Fig. 9. Chromatography on DEAE-cellulose of the radioactive nucleotide released from inhibited carnitine acetyltransferase by acid denaturation. ●, E_{260} (10 mm. light-path); Δ, radioactivity.

Table 2. *Analysis of S-carboxymethyl-CoA (–)-carnitine ester*

The values for the amount of ester taken for analysis are based on E_{260} (see the text).

Amount of ester taken for analysis (nmoles)	Component estimated	Amount of component found	
		(nmoles)	(moles/mole of ester taken)
18.3*	(–)-Carnitine	0.7	0.04
18.3†	(–)-Carnitine	15.9	0.87
21.1†	(–)-Carnitine	22.4	1.06
36.6†	(–)-Carnitine	32.5	0.89
0.92‡	(–)-Carnitine	1.02	1.11
211	β -Alanine§	181	0.86
211	S-Carboxymethyl-2-mercaptoethylamine§	191	0.91

* (–)-Carnitine estimated without alkaline hydrolysis of the ester.

† (–)-Carnitine estimated after alkaline hydrolysis of the ester.

‡ Radioactivity measurement.

§ Estimated after acid hydrolysis of the ester by using the Technicon AutoAnalyzer.

Fig. 10. Time-course (●) of the inhibition of carnitine acetyltransferase ($6.8\mu\text{M}$) by *S*-carboxymethyl-CoA (—)-carnitine ester ($15\mu\text{M}$). Δ, Control from which the ester was omitted.

ester, as predicted in Scheme 1. Its spectrum is that of an adenine nucleotide and it contains (—)-carnitine in a form with the alkali-lability expected of acid-soluble carnitine *O*-esters (Pearson, Chase & Tubbs, 1969). The finding of β -alanine and *S*-carboxymethyl-2-mercaptoethylamine is crucial in that it shows the CoA moiety of the nucleotide to be intact and also requires that the process of inhibition involves an enzyme-catalysed trans-bromoacetylation (Scheme 1c), since alkylation of CoA must have occurred. Although ($\pm$)-[^{14}C]-carnitine had to be used to prepare the inhibited enzyme, the close agreement between the values of the carnitine content of the nucleotide estimated from its radioactivity and by the stereospecific enzyme assay after alkaline hydrolysis (Table 2) indicate that only the laevorotatory isomer can have been incorporated into the nucleotide, again providing support for Scheme 1.

Inhibition of carnitine acetyltransferase by isolated *S*-carboxymethyl-CoA (—)-carnitine ester. An incubation mixture containing $6.8\mu\text{M}$ -carnitine acetyltransferase, $15\mu\text{M}$ -*S*-carboxymethyl-CoA (—)-carnitine ester (isolated from inhibited enzyme as described above) and 25mM -phosphate buffer, pH 7.0, was kept at room temperature. Samples were removed at intervals and assayed for enzyme activity. A very slow loss of activity was observed (Fig. 10), the degree of inhibition never exceeding 80%, even after 26 hr. A control from which the nucleotide was omitted showed no loss of activity over this period.

DISCUSSION

The results described here demonstrate that the rapid inhibition of carnitine acetyltransferase by bromoacetyl derivatives of CoA and (—)-carnitine

may be represented by Scheme 1. Certain aspects of the inhibition, however, require further comment.

First, the alkylation of CoA by bromoacetyl-(—)-carnitine, which takes place within the enzyme-substrate ternary complex, is an extraordinarily fast reaction. Bromoacetylcarnitine is not in itself an unusually powerful alkylating agent. Thus, in an attempt to prepare *S*-carboxymethyl-CoA (—)-carnitine ester synthetically, a mixture containing 15mM -CoA and 140mM -bromoacetyl-(—)-carnitine was kept at room temperature at pH 8–9; complete reaction, as judged by the mixture becoming nitroprusside-negative, was achieved only after 60–90 min. In contrast, in Fig. 2(a), at a maximum concentration of enzyme-bound bromoacetyl-CoA and (—)-carnitine of 5mM , complete inhibition takes less than 5 sec. This short time is sufficient for both the alkylation and for the essential preliminary transbromoacetylation (Scheme 1a–b); in fact the latter must determine the rate at which inhibition develops, as the stoichiometry of the reaction of carnitine acetyltransferase with bromoacetyl-CoA (Fig. 5) requires that virtually none of the bromoacetylcarnitine formed as an intermediate (Scheme 1b) is released from the enzyme before taking part in the alkylation of CoA. Presumably the enzyme's ability to accelerate the alkylation reaction results solely from its capacity to bind the reactants in close proximity and in a favourable orientation (Koshland, 1960), rather than from any specific chemical catalysis.

An explanation must also be sought as to why the enzyme is so powerfully inhibited by *S*-carboxymethyl-CoA (—)-carnitine ester formed *in situ*. This adduct of the two substrates is imagined as forming a 'bridge' between the independent CoA- and carnitine-binding sites, previously proposed to exist on this enzyme on kinetic grounds (Chase & Tubbs, 1966b; Chase, 1967a,b). On a naive interpretation, it might be expected that this inhibitor would be bound to the enzyme with a free energy of binding roughly equal to the sum of the binding energies of CoA and carnitine derivatives. Thus, by taking the product of the dissociation constants for short-chain acyl-CoA derivatives and for acetyl-(—)-carnitine [$34\mu\text{M}$ (Chase, 1967b) and $350\mu\text{M}$ (Chase & Tubbs, 1966a), respectively], a value of $1.2 \times 10^{-8}\text{M}$ might be expected for the K_s of the adduct. However, the equilibrium formation of an enzyme-inhibitor complex with such a dissociation constant cannot explain the observed results. For example, in the experiment shown in Fig. 4, in which the enzyme was assayed at a concentration of 6.25nM , virtually no inhibition would have been observed when less than stoichiometric amounts of bromoacetylcarnitine had been added. It is, of course, possible that the dissociation constant deduced above for the adduct might be much too

high, for simple multiplication of the component dissociation constants may not be appropriate. For metal complexes it is well known that the free energy of binding of a multidentate ligand (here the adduct) is greater than the sum of the free energies of binding of its individual parts; this is due to slow dissociation of the complex rather than to its accelerated formation (Martell & Calvin, 1952). Such a chelate effect could presumably also contribute to enzyme-inhibitor (or, indeed, to enzyme-substrate) interactions. Another possibility is that adduct formation is accompanied by a conformational change in the enzyme-inhibitor complex, which enhances binding.

A number of cases are known of protein-small molecule complexes with very low dissociation constants. Although in principle such constants give no indication of the absolute rates of formation and breakdown of complexes, in practice low values are usually caused by slow breakdown. For example, the pseudo-first-order release of FAD from D-amino acid oxidase has a half-time of 133 sec., K_s for coenzyme binding being 2.5×10^{-7} M (Dixon & Kleppe, 1965); aminopterin, which inhibits folate reductase with K_s about 10^{-10} M, dissociates from the enzyme-inhibitor complex with a half-time of 5.4×10^5 sec. (Werkheiser, 1961); and in the extreme case of the combination of biotin with avidin, K_s 10^{-15} M is accompanied by a half-time for dissociation of 1.1×10^7 sec. (Green, 1963).

Fig. 6 shows that carnitine acetyltransferase recovers from inhibition by bound adduct with a half-time of 14.8 days. Since this virtually complete recovery occurs in comparatively concentrated solution ($28 \mu\text{M}$) it cannot be due to the slow dissociation of an inhibitor of high affinity. Fig. 10 shows that, as expected, added *S*-carboxymethyl-CoA (-)-carnitine ester inhibits the enzyme. However, for a non-covalent interaction, the process is unusually slow and contrasts strongly with the virtually instant inhibition observed when the adduct is formed *in situ*. Other examples of sluggish combination between enzymes and complex small molecules are known, e.g. the slow reactivation of yeast apo-pyruvate decarboxylase by thiamine pyrophosphate (Morey & Juni, 1968). Possibly the favoured configuration of the adduct in free solution is unsuitable for binding; it is well known that NAD and FAD, which have structural similarities to CoA derivatives, have preferred conformations (Kaplan, 1960; Beinert, 1960). Equally the structure of the protein may be such as to hinder sterically both binding and desorption of the adduct.

The exceedingly potent inhibition of carnitine acetyltransferase by *S*-carboxymethyl-CoA (-)-carnitine ester, when formed *in situ*, is evidently a consequence, not of a very small dissociation con-

stant, but rather of the very low rate of adduct release, i.e. it is a kinetic and not an equilibrium effect. Possible explanations of this slow dissociation have been discussed above. It is probable that the recovery from inhibition (Fig. 6) is largely governed by the hydrolysis of *S*-carboxymethyl-CoA (-)-carnitine ester either *in situ* or after release (Scheme 1c-f). The equilibrium constant for the reversible binding of the adduct by the enzyme has not been measured. Indeed, for reasons already discussed above, it would not be directly relevant to the observed inhibition effects.

The ability of the enzyme to promote the formation of *S*-carboxymethyl-CoA (-)-carnitine ester directly demonstrates that it can bind CoA and carnitine simultaneously, confirming the existence of a ternary complex previously inferred from kinetic evidence (Chase & Tubbs, 1966a). Indeed, the isolated inhibited enzyme may be regarded as a 'frozen' ternary enzyme-substrate complex. Since carnitine palmitoyltransferase (EC 2.3.1.-) is powerfully inhibited by 2-bromolauroyl-CoA (or 2-bromostearoyl-CoA) in a (-)-carnitine-dependent manner (Tubbs & Chase, 1967), its action too presumably involves the simultaneous binding of both substrates. Such also appears to be the case with choline acetyltransferase (EC 2.3.1.6), for the inhibition of this enzyme by bromoacetyl-CoA is choline-dependent (Chase & Tubbs, 1966b), although such a conclusion is at variance with the kinetic studies of Schuberth (1966).

Finally, it may be noted that carnitine acetyl- and palmitoyl-transferases can be inhibited in intact mitochondria by the mechanism described in this paper. This fact has been used to demonstrate (Tubbs & Chase, 1966, 1967) that each of these enzymes exists in two mitochondrial pools, only one of which is inactivated by externally added bromoacyl-CoA plus (-)-carnitine. The other pool is, however, inhibited by bromoacylcarnitine plus intramitochondrial CoA.

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