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Specific Alkylation of a Histidine Residue in Carnitine Acetyltransferase by Bromoacetyl-L-Carnitine

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Incubation of carnitine acetyltransferase with low concentrations of bromoacetyl-L-carnitine causes a rapid and irreversible loss of enzyme activity; one mol of inhibitor can inactivate one mol of enzyme. Bromoacetyl-D-carnitine, iodoacetate or iodoacetamide are ineffective. L-Carnitine protects the transferase from bromoacetyl-L-carnitine. Investigation shows that the enzyme first reversibly binds bromoacetyl-L-carnitine with an affinity similar to that shown for the normal substrate acetyl-L-carnitine; this binding is followed by an alkylation reaction, forming the carnitine ester of a monocarboxymethyl-protein, which is catalytically inactive. The carnitine is released at an appreciable rate by spontaneous hydrolysis, and the resulting carboxymethyl-enzyme is also inactive. Total acid hydrolysis of enzyme after treatment with 2-[^{14}C]bromoacetyl-L-carnitine yields *N*-3-carboxy[^{14}C]methylhistidine as the only labelled amino acid. These findings, taken in conjunction with previous work, suggest that the single active centre of carnitine acetyltransferase contains a histidine residue.

Bromoacetyl-L-carnitine, a substrate analogue for carnitine acetyltransferase [acetyl-CoA–L-carnitine *O*-acetyltransferase, EC 2.3.1.7], causes rapid inactivation of the enzyme when CoA is also present (Chase & Tubbs, 1969). This inhibition involves binding of the analogue and CoA to the normal substrate-binding site, followed by alkylation of the CoA thiol group by the adjacent bromoacetyl group. The product of this reaction, enzyme-bound *S*-carboxymethyl-CoA–L-carnitine ester, has affinity for both the CoA and carnitine binding sites and is released only extremely slowly. Such inhibition is stoicheiometric, 1 mol of enzyme being completely inactivated by 1 mol of bromoacetyl-L-carnitine in the presence of excess of CoA, and is fully reversible on prolonged incubation as no covalent modification of the enzyme protein is involved.

In the present work, it is shown that bromoacetyl-L-carnitine also inhibits carnitine acetyltransferase in the absence of CoA. Under these conditions, irreversible inhibition follows specific binding of the inhibitor to the enzyme and results from the alkylation of a single histidine residue at or near the active centre.

MATERIALS

Carnitine acetyltransferase from pigeon breast muscle was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. and was recrystallized to a specific activity of 105–120 units/mg (Chase, Pearson & Tubbs, 1965).

D- and L-carnitine hydrochlorides were bought from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. L-Carnitine perchlorate was prepared from the hydrochloride by passage through an anion-exchange resin (De-Acidite FF-1P, SRA 71; Permutit Co. Ltd., London W.4, U.K.) in the hydroxide form and adjustment of the resulting solution of L-carnitine in the free base form to pH 2.0 with $HClO_4$. Water was removed by rotary evaporation and the solid L-carnitine perchlorate stored in a dessicator over P_2O_5. Acetyl-L-carnitine hydrochloride was made from L-carnitine hydrochloride by the method of Fraenkel & Friedman (1957). CoA was the product of Boehringer Corp. (London) Ltd.; acetyl-CoA was prepared from it by treatment with acetic anhydride (Simon & Shemin, 1953).

Iodoacetic acid and iodoacetamide, both from BDH Chemicals Ltd., Poole, Dorset, U.K., were recrystallized from hot water. Bromo[2-^{14}C]acetic acid, 46.6 mCi/mmol, was from The Radiochemical Centre, Amersham, Bucks., U.K. Bromoacetyl bromide and *S*-carboxymethylcysteine were laboratory-reagent grade, from BDH Chemicals Ltd. *N*-1- and *N*-3-carboxymethylhistidines were prepared as described by Crestfield, Stein & Moore (1963*a*), recrystallized from warm water–ethanol and were found to be electrophoretically homogeneous.

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 the highest available grade and glass-distilled water was used throughout.

METHODS

Measurements of carnitine acetyltransferase activity. These were performed as described by Chase & Tubbs (1969).

Substrates of carnitine acetyltransferase. Except where specific details are given in the text, solutions of CoA, acetyl-CoA, L-carnitine and acetyl-L-carnitine were prepared, stored and assayed as described by Chase & Tubbs (1966).

Radioactivity measurements. Samples (10–50 μl) of solutions containing [^{14}C]-labelled materials dissolved in water were plated on to planchets, dried *in vacuo* and counted with a Geiger–Müller tube fitted with a Panax type D558 scaler.

Paper electrophoresis. High-voltage paper electrophoresis was carried out as described by Perham (1967) with Whatman no. 1 paper. Buffers used were: pH 2.0, acetic acid–formic acid–water (4:1:45, by vol.); pH 3.5, pyridine–acetic acid–water (1:10:189, by vol.); pH 6.5, pyridine–acetic acid–water (25:1:225, by vol.). A potential gradient of 60 V/cm for 50 min was employed. Amino acids on the paper were detected with ninhydrin–collidine.

Total acid hydrolysis. Samples of protein (usually about 1 mg) were hydrolysed with constant-boiling HCl (1 ml) in sealed, evacuated tubes at 105°C for 24 h. The HCl was removed *in vacuo* over fresh NaOH pellets.

Preparation and assay of unlabelled bromoacetylcarnitine. Bromoacetyl esters of D- and L-carnitine were prepared by treating the appropriate carnitine hydrochloride, dissolved in bromoacetic acid, with bromoacetyl bromide, and were recrystallized twice (Chase & Tubbs, 1969). Solutions containing these esters were assayed by the alkaline hydroxamate procedure of Friedman & Fraenkel (1955), using enzymically determined acetylcarnitine as a standard. Bromoacetyl-L-carnitine made in this way contained no impurities detectable on t.l.c. as described in the following section.

Preparation of 2-[^{14}C]*bromoacetyl-L-carnitine.* Bromo-[2-^{14}C]acetic acid (10.7 μmol, i.e. 1.5 mg; 46.6 mCi/mmol) was mixed with 97.5 μmol (8.5 μl) of freshly redistilled bromoacetyl bromide and left in a stoppered tube in the dark for 24 h at room temperature to ensure incorporation of radioactivity into bromoacetyl bromide. L-Carnitine perchlorate (100 μmol, i.e. 26.4 mg), dissolved in 0.1 ml of dry redistilled acetonitrile, was then added to the labelled bromoacetyl bromide and the mixture left in the dark at room temperature. After 85 h, the acetonitrile was removed *in vacuo* and the residue dissolved in about 1 ml of water. This solution was extracted eight times with ether to remove bromoacetic acid, the residual ether was evaporated in a stream of N_2 and the preparation was stored frozen at −15°C. Small samples of the solution proved extremely inhibitory when added to a carnitine acetyltransferase assay system, suggesting that it contained at least some bromoacetyl-L-carnitine, and a yield of 53 μmol of ester was found by the hydroxamate method. As recrystallization of this small amount of material was impracticable, its purity was investigated by a t.l.c. procedure to measure the specific radioactivity of the bromoacetyl-L-carnitine. Glass plates were coated with a layer of silica gel G (E. Merck A.-G., Darmstadt, Germany) and washed once with methanol–acetone–conc. HCl (1:9:1, by vol.; Lindstedt & Lindstedt, 1965). To each of two plates were applied 5 and 10 μl spots of the crude bromoacetyl-L-carnitine solution, together with a third spot of carnitine as a marker. A sample (100 μl) of the same bromoacetyl-L-carnitine preparation was applied as a streak to a third, preparative, plate. The plates were developed with the above solvent mixture, after which one of the two spotted plates was exposed to I_2 vapour. This revealed that the bromoacetyl-L-carnitine preparation contained four clearly separated components with R_F values 0, 0.10, 0.47 and 0.60. The other spotted plate was treated with a spray of M-hydroxylamine hydrochloride dissolved in 1.75M-KOH, followed 10 min later by 0.37M-$FeCl_3$ in 3.8M-HCl (Friedman & Fraenkel, 1955). The third of the four spots shown by I_2 (R_F 0.47) coincided exactly with the carnitine marker. All the other components were hydroxamate-positive and were presumably carnitine esters. Autoradiography of one of the plates was performed overnight (Kodak Blue Brand film) and showed that only the second (R_F 0.10) and fourth (R_F 0.60) iodine spots were radioactive. By reference to these findings, two bands of silica gel, expected to contain the radioactive components, were scraped from the preparative plate. Each portion of gel powder was extracted with 1 ml of water for 5 min and the silica was removed by centrifugation. The supernatants were tested for bromoacetyl-L-carnitine by adding samples to a carnitine acetyltransferase assay system (Chase & Tubbs, 1969). The extract from the component with R_F 0.1 had no inhibitory effect, but 5 μl of that from the fastest-moving material rapidly inactivated the enzyme. Measurement of the radioactivity and acylcarnitine content of this latter solution established that the specific radioactivity of the bromoacetyl-L-carnitine in the crude preparation was 2.55×10^5 c.p.m./μmol. In experiments with radioactive bromoacetyl-L-carnitine as label for the active site of carnitine acetyltransferase, the unpurified inhibitor preparation was employed, since none of the contaminants was at all inhibitory. The nature of the hydroxamate-positive material remaining at and near the origin in the t.l.c. system was not investigated. It is, however, possible that the synthetic procedure used here might lead to the formation of carnitoylcarnitine and bromoacetylcarnitoylcarnitine.

RESULTS

Stereospecificity of the inhibition of carnitine acetyltransferase by bromoacetylcarnitine. Each of four samples of enzyme was treated with one of the following; bromoacetyl-D-carnitine, bromoacetyl-L-carnitine, iodoacetamide or iodoacetic acid. Table 1 shows the relative potency of these compounds as inhibitors of carnitine acetyltransferase. Bromoacetyl-L-carnitine inactivates the enzyme extremely rapidly. Bromoacetyl-D-carnitine, however, is much less effective, being only slightly more inhibitory than the general protein reagents iodoacetamide and iodoacetic acid, and even this activity may well be due to contamination with the L-isomer. The inhibition of carnitine acetyltransferase by bromoacetyl-L-carnitine added alone cannot be reversed by dialysis or gel filtration. This

Table 1. *Inactivation of carnitine acetyltransferase by some alkylating agents*

Enzyme samples in 0.1M-potassium phosphate buffer, pH 7.2, were incubated with various alkylating agents at 21°C. After the time-intervals shown, 5μl samples were removed for assay of residual carnitine acetyltransferase activity.

Inhibitor used	Final concn. (mM)	Incubation time (min)	Residual enzyme activity (%)
Bromoacetyl-D-carnitine	0.75	45	>95
	4.50	60	45
Bromoacetyl-L-carnitine	0.20	5	0*
Iodoacetamide	10.0	45	84
Iodoacetic acid	10.0	45	95

*After passage of the incubation mixture through Sephadex G-25 to remove excess of bromoacetyl-L-carnitine, which would interfere with the assay (Chase & Tubbs, 1969).

contrasts with the time-dependent return of activity observed in enzyme samples which have been treated with bromoacetyl-L-carnitine plus CoA (Chase & Tubbs, 1969).

Dependence upon inhibitor concentration of the rate of inactivation of carnitine acetyltransferase by bromoacetyl-L-carnitine. Since very low concentrations of bromoacetyl-L-carnitine cause rapid inhibition of carnitine acetyltransferase when CoA is also present (Chase & Tubbs, 1969), it is impossible to assay the residual activity of an enzyme–bromoacetyl-L-carnitine mixture until excess of inhibitor has been removed. The rate of inactivation of carnitine acetyltransferase at a particular bromoacetyl-L-carnitine concentration was therefore measured as follows. In each of four tubes was placed 0.8ml of an enzyme solution (3.15μM in 0.1M-potassium phosphate buffer, pH 7.2) at 0°C. At zero time, small volumes (less than 10μl) of bromoacetyl-L-carnitine solution were added to give final inhibitor concentrations of 57.5, 113, 192, or 1240μM. Samples (0.2ml) were removed from the incubation mixtures at various times and applied to a small column (1.1cm×6.1cm) of Sephadex G-25 (medium grade). This was equilibrated with 0.1M-phosphate buffer, pH 7.2, and maintained at 2–4°C in a cold-room. The first 2.6ml of eluate were discarded and the remaining 1.6ml kept for enzyme assay. Control experiments established that this fraction, which contained more than 95% of the enzyme eluted from the column, was entirely free of contaminating bromoacetyl-L-carnitine. The time taken between application of a sample to the column and the end of the emergence of eluted enzyme was 2±0.25min. In constructing a time-course for the inhibition of carnitine acetyltransferase at a given bromoacetyl-L-carnitine concentration, the effective periods of exposure of the enzyme to inhibitor were obtained by adding 1min (half of the period of gel-filtration) to the time at which each sample was applied to the Sephadex column. This attempt to allow for continuation of the inhibitory reaction on the column, before complete separation has been achieved, is arbitrary and makes estimation of the time each enzyme sample had been in contact with the inhibitor rather imprecise. The degree of uncertainty (at worst±1min) is clearly more important for the shorter periods of incubation.

Fig. 1(*a*) shows that, at each of the four inhibitor concentrations tried, the inactivation of carnitine acetyltransferase by bromoacetyl-L-carnitine followed pseudo-first-order kinetics. The relationship between inhibitor concentration and the pseudo-first-order rate constant for inhibition is shown as a double-reciprocal plot in Fig. 1(*b*). The results imply that the reversible formation of an enzyme–bromoacetyl-L-carnitine complex with a dissociation constant of 890μM precedes the step in which the enzyme becomes irreversibly inhibited.

Protection by L-carnitine against the inactivation of carnitine acetyltransferase by bromoacetyl-L-carnitine. The rate of inactivation of carnitine acetyltransferase by 260μM-bromoacetyl-L-carnitine at 0°C was measured as described above both in the presence and absence of L-carnitine. As shown in Fig. 2, both concentrations of L-carnitine tried gave significant protection against the inhibitor.

The alkali-labile L-carnitine content of carnitine acetyltransferase after inactivation with bromoacetyl-L-carnitine. The obvious mechanism by which bromoacetyl-L-carnitine might act as an irreversible inhibitor of carnitine acetyltransferase involves alkylation by the bromoacetyl group of some reactive nucleophile in the enzyme. If this is the case, the inhibited form of the enzyme should contain L-carnitine esterified to the carboxymethylated protein. Since carnitine esters are hydrolysed under mildly alkaline conditions, determination of the L-carnitine released by alkali might be expected to provide both confirmation of the above scheme

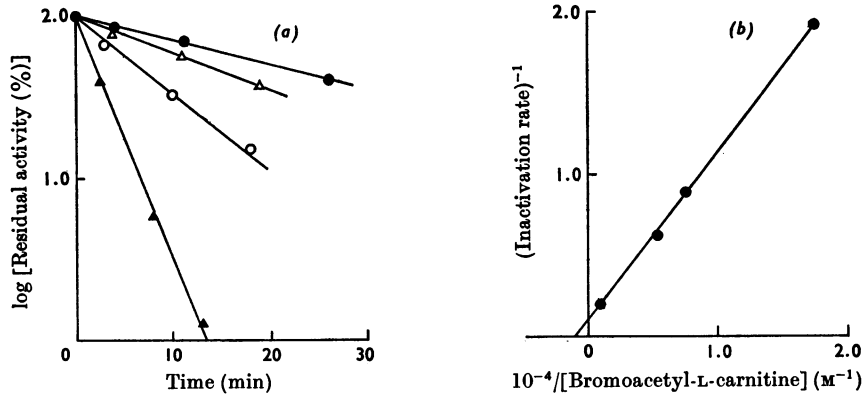

Fig. 1. Rate of inactivation of carnitine acetyltransferase by bromoacetyl-L-carnitine. (*a*) Carnitine acetyltransferase (3.15 μM in 0.1M-potassium phosphate buffer, pH 7.2) was treated at 0°C with bromoacetyl-L-carnitine at a final concentration of: ●, 57.5 μM; △, 113 μM; ○, 192 μM; ▲, 1240 μM. Samples removed from these incubation mixtures at intervals were passed through a column of Sephadex G-25 to remove inhibitor (see the text) and assayed for residual enzyme activity. (*b*) Reciprocals of the pseudo-first-order inactivation rates (in arbitrary units) observed at various inhibitor concentrations in (*a*) plotted against the reciprocals of the corresponding bromoacetyl-L-carnitine concentrations.

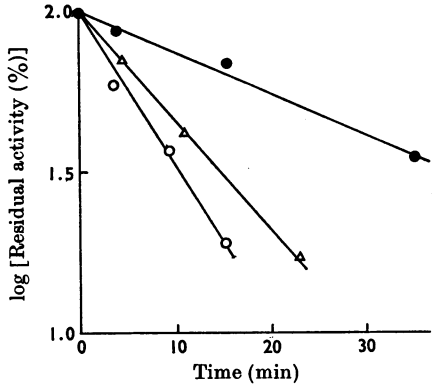

Fig. 2. Protection by L-carnitine against bromoacetyl-L-carnitine inhibition of carnitine acetyltransferase. The rate of inactivation of carnitine acetyltransferase by 260 μM-bromoacetyl-L-carnitine at 0°C was measured in the presence and absence of L-carnitine. Experimental details are as described in the text and in the legend for Fig. 1. L-Carnitine concentrations: ○, zero; △, 177 μM; ●, 875 μM.

for the inhibitory reaction and also a measure of its stoicheiometry. It was, therefore, surprising to find that when a sample of carnitine acetyltransferase, which had been treated with bromoacetyl-L-carnitine, separated from excess of inhibitor by gel-filtration and found to be totally inactive, was adjusted to about pH 13 with potassium hydroxide and kept at room temperature for 30 min, only 0.35 mol of L-carnitine was released/mol of enzyme [assuming mol. wt. 58000 (Chase & Tubbs, 1969)]. This could mean either that the proposed inhibitory mechanism is incorrect or that, after alkylation, the carboxymethyl-enzyme–carnitine ester linkage is slowly hydrolysed to leave a catalytically inert carboxymethyl-enzyme. The results of an experiment designed to investigate this are shown in Fig. 3. Enzyme samples were rapidly inactivated with a high concentration of bromoacetyl-L-carnitine. After various time-intervals, the protein in each sample was precipitated with perchloric acid, washed to remove unchanged inhibitor and treated with alkali under conditions known to hydrolyse carnitine esters (D. J. Pearson, personal communication). The amount of alkali-labile carnitine covalently bound to the enzyme at the time of addition of perchloric acid increased rapidly over the first 6 min of exposure of the enzyme to inhibitor. This presumably reflects the rate at which bromoacetyl-L-carnitine reacted irreversibly with carnitine acetyltransferase (Fig. 1), and reached a maximum of about 0.7 mol of carnitine/mol of enzyme. Thereafter, a decline in enzyme-bound carnitine was observed. It thus seems likely that, although the initial product of the inhibitory reaction is the carnitine ester of a carboxymethylated enzyme form, spontaneous hydrolysis of the ester bond finally gives rise to free carboxymethyl-enzyme, which is still inactive. The release of carnitine from the alkylated enzyme is unaffected by the presence of CoA. The experiment in Fig. 3 also suggests that 1 mol of bromo-

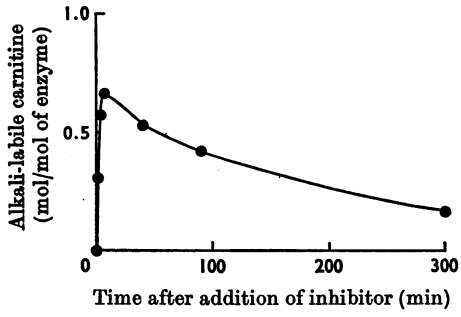

Fig. 3. The protein-bound alkali-labile L-carnitine content of carnitine acetyltransferase after treatment with bromoacetyl-L-carnitine. Samples (0.5ml), each containing 28nmol of enzyme in 12.5mM-potassium phosphate buffer, pH7.0, were treated with bromoacetyl-L-carnitine (final concn. 590μM) at 0°C. After various times, protein was precipitated by the addition of 2ml of ice-cold $HClO_4$ (2.5%, w/v) and centrifuged. The precipitate was drained, washed twice with cold perchloric acid to remove excess of inhibitor and dissolved in 0.5ml of 0.15M-KOH. This alkaline solution was kept at room temperature for 15min and neutralized by the addition of 0.37ml of 0.2M-HCl, 0.2ml of M-tris–HCl buffer, pH7.8, and 0.9ml of water. After centrifuging to remove the precipitate (denatured protein and $KClO_4$), as much of the supernatant as possible was transferred to a spectrophotometer cuvette (10mm light-path) and mixed with 10μl of 19.4mM-acetyl-CoA and 20μl of 10mM-5,5'-dithiobis-(2-nitrobenzoic acid). The increase in extinction that occurred on the further addition of 1μl of carnitine acetyltransferase (5mg of protein/ml) was a measure of the alkali-labile L-carnitine bound to the enzyme at the time of addition of $HClO_4$ (Marquis & Fritz, 1964). Control experiments showed that two washes of the protein precipitate were sufficient to remove unreacted bromoacetyl-L-carnitine and that alkaline hydrolysis for 15min liberated all the protein-bound carnitine.

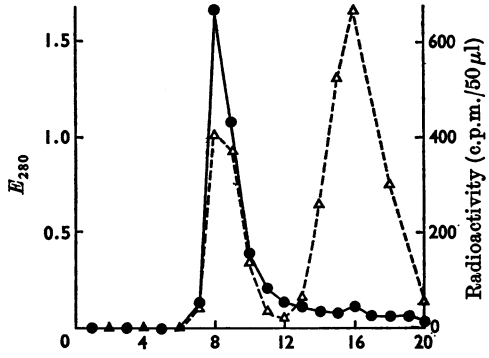

Fig. 4. Elution profile from Sephadex G-25 of carnitine acetyltransferase treated with 2-[^{14}C]bromoacetyl-L-carnitine. ●, E_{280} (10mm light-path); △, radioactivity (50μl samples from each fraction).

acetyl-L-carnitine can inactivate 1mol of carnitine acetyltransferase.

Stoicheiometry of the reaction between bromoacetyl-L-carnitine and carnitine acetyltransferase. Because of the apparent lability of the carboxymethyl-enzyme–carnitine ester bond, it was decided to obtain a better estimate of the stoicheiometry of the inhibitory reaction by using bromoacetyl-L-carnitine labelled with ^{14}C in the bromoacetyl group. 1.5ml of a carnitine acetyltransferase solution (8mg/ml; 0.207μmol) in 50mM-potassium phosphate buffer, pH7.1, was treated with 40μl (1.16μmol of ester) of bromo[2-^{14}C]acetyl-L-carnitine perchlorate (specific radioactivity 2.55×10^5c.p.m./μmol). After 40min at room temperature, the whole sample was applied to a column (2cm×14cm) of Sephadex G-25 (medium grade) equilibrated with glass-distilled water and fractions (26 drops) were collected. Fig. 4 shows the elution profile of protein and radioactivity from the column. The first peak of radioactivity to emerge coincided with the appearance of the protein, which was enzymically inactive. To determine the specific radioactivity of the labelled carboxymethyl-enzyme, the protein content of fractions 8–10 of the eluate was obtained by the Folin–Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) with native carnitine acetyltransferase as standard. Hence it was calculated that the number of carboxymethyl groups/mol of inhibited enzyme in fractions 8, 9 and 10 was 0.67, 0.90 and 0.95 respectively. This confirms the 1:1 stoicheiometry of the enzyme–inhibitor reaction and provides further evidence in favour of the suggestion of Chase & Tubbs (1969) that carnitine acetyltransferase has only one active site/molecule.

Identification of N-3-carboxymethylhistidine in a total acid hydrolysate of the inhibited enzyme. Fractions 8–10 from the Sephadex eluate (Fig. 4), which contained radioactive monocarboxymethylated carnitine acetyltransferase in water, were combined and freeze-dried. Samples containing about 1mg of protein were acid-hydrolysed, dried and subjected to paper electrophoresis at pH2.0, 3.5 or 6.5 together with appropriate amino acid markers. After autoradiography (12–20 days; Kodak Blue Brand film) and location of amino acids with ninhydrin, it was found that at each of the three pH values studied all the radioactivity was associated with a single band on the paper. Fig. 5, a diagram showing the electrophoretic mobilities of various amino acids at pH6.5, shows that the radioactive band

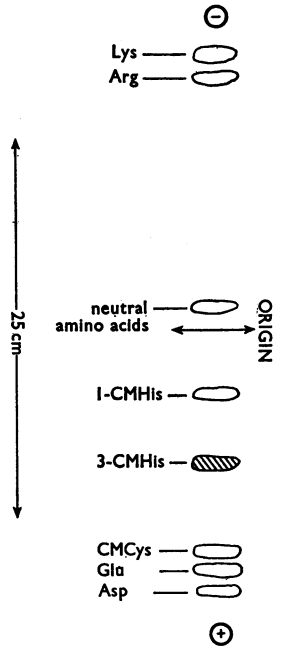

Fig. 5. Diagram showing the separation of a number of marker amino acids by one-dimensional paper electrophoresis at pH 6.5 and 60 V/cm for 50 min. The hatched area shows the location of radioactivity, determined radioautographically, after a total acid hydrolysate from carnitine acetyltransferase that had been inhibited with bromo[2-^{14}C]acetyl-L-carnitine was subjected to the same procedure. Abbreviations: CMCys, *S*-carboxymethylcysteine; 1-CMHis, *N*-1-carboxymethylhistidine; 3-CMHis, *N*-3-carboxymethylhistidine.

has the same mobility as *N*-3-carboxymethylhistidine and is clearly separated from *N*-1-carboxymethylhistidine. The radioactive amino acid also coincided with *N*-3-carboxymethylhistidine at pH 2.0 and 3.5, although under these conditions the two monocarboxymethylhistidine isomers were not conclusively separable. In each case, the labelled material was distinctly more cationic than *S*-carboxymethylcysteine.

DISCUSSION

The experiments described here establish that bromoacetyl-L-carnitine inactivates carnitine acetyltransferase in the manner expected of an active-site-directed inhibitor. Rigorous stereochemical requirements exist for rapid inhibition, as is clear from the ineffectiveness of bromoacetyl-D-carnitine and the insensitivity of this enzyme to non-specific alkylating agents (Table 1). Fig. 1 shows that the rate of inactivation is not proportional to bromoacetyl-L-carnitine concentration but tends instead to a limiting value. This indicates that the reaction between bromoacetyl-L-carnitine and carnitine acetyltransferase is a two-stage process, involving reversible binding of inhibitor to the enzyme followed by an irreversible alkylation. Two lines of evidence suggest that the preliminary reversible binding of the inhibitor is analogous to the normal binding of carnitine substrates and therefore occurs at the active centre of the enzyme. Firstly, the dissociation constant for the reversible enzyme–inhibitor complex as calculated from the data of Fig. 1 (890 μM) is comparable with the corresponding K_s value for the enzyme–acetyl-L-carnitine complex (720 μM at 30°C; Chase, 1967). In addition, the presence of L-carnitine slows the rate at which irreversible inhibition develops (Fig. 2), as expected if substrate and inhibitor are competing for a common binding site.

The radioactive amino acid derivative, present in a total acid hydrolysate of carnitine acetyltransferase after treatment with bromo[2-^{14}C]-acetyl-L-carnitine, showed identical electrophoretic mobility to *N*-3-carboxymethylhistidine at pH 2.0, 3.5 and 6.5. It was clearly separated from *S*-carboxymethylcysteine at each pH and from *N*-1-carboxymethylhistidine at pH 6.5. pK_a values for the imidazole ionization of monocarboxymethylhistidines have been reported to be 6.35 and 5.70 for the *N*-1 and *N*-3 derivatives respectively (Gurd, 1967), so that at pH 6.5 each of these compounds bears a net negative charge of less than one unit. This accounts for the electrophoretic mobility of the two carboxymethylhistidine isomers shown in Fig. 5, which is intermediate between that of electrophoretically neutral amino acids and those bearing a full net negative charge. None of the other monocarboxymethyl amino acids that could in principle have been formed in the reaction of enzyme and inhibitor would show this behaviour, as none of them contains an ionizing group with a pK_a near to 6.5. At this pH, any *N*-carboxymethyl derivative that might have resulted from alkylation of an *N*-terminal amino acid would have a net negative charge of one to two units, ϵ-*N*-carboxymethyllysine and *S*-carboxymethylmethionine would be electrophoretically neutral, and of the three breakdown products of *S*-carboxymethylmethionine formed during acid hydrolysis (Gurd, 1967), methionine and homocysteine have lost the carboxymethyl group and *S*-carboxymethylhomocysteine would carry a full net negative charge. The electrophoretic evidence therefore establishes unambiguously that the amino acid residue alkylated in the reaction between carnitine acetyltransferase and bromoacetyl-L-carnitine is histidine. Substitution of this residue occurs largely, and probably exclusively, at the *N*-3 position. Any

side reaction leading to alkylation of as little as 5% of the enzyme at the histidine *N*-1 position, or at some other residue, would have been detected by the radioautographic procedure used. Such specificity in the alkylation reaction taken in combination with the observed 1:1 stoicheiometry between enzyme and inhibitor (Figs. 3 and 4) suggests very strongly that there is a single unique histidine residue in the enzyme protein that is modified. It is conceivable that two histidine residues might be present near the inhibitor-binding site, alkylation of either of which would preclude reaction of the other. This latter situation would be analogous to the reaction of iodoacetate with ribonuclease, in which either histidine-12 or histidine-119, but not both, may be carboxymethylated (Crestfield, Stein & Moore, 1963*a*,*b*). It may be possible to resolve the two possibilities in the present case by determining the amino acid sequence or sequences around *N*-3-carboxymethylhistidine in the inhibited enzyme.

Many examples are now known of the specific inhibition of enzymes by active-site-directed inhibitors. In a number of cases, mostly involving hydrolytic enzymes, the nature of the amino acid residue modified in the inhibitory reaction has been elucidated and has frequently proved to be histidine. Thus, chymotrypsin is alkylated at the *N*-3 position of histidine-57 by reaction with 1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one (Schoellmann & Shaw, 1963; Stevenson & Smillie, 1965); *N*-3-substituted histidine residues also result from the treatment of trypsin with 7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one (Petra, Cohen & Shaw, 1965) and of subtilisin with 3-benzyloxycarbonylamido-1-bromo-4-phenylbutan-2-one (Shaw & Ruscica, 1968). Histidine residues alkylated in the *N*-1 position are also known. Husain & Lowe (1968) found that papain could be irreversibly inhibited by treatment with 1,3-dibromoacetone. This reacted first with the active centre residue cysteine-25 and then intramolecularly with the *N*-1 position of the spatially adjacent histidine-106. In every case quoted, the histidine residue that reacts with the inhibitor is also believed to play a direct role in the catalytic function of the enzyme concerned.

The action of bromoacetyl-L-carnitine as an active-site-directed inhibitor of carnitine acetyltransferase does not, of course, prove that the histidine alkylated in this process is part of the active centre of the enzyme. The inhibited enzyme derivative formed initially is a carboxymethyl-enzyme–L-carnitine ester (Fig. 3) in which, presumably, a covalently bound carnitine moiety is in permanent occupation of the binding site for this substrate. Such an enzyme form could hardly catalyse a reaction involving dissociable carnitine molecules, whether the alkylated residue was important for catalysis or not. However, Fig. 3 also shows that the carboxymethyl-enzyme–L-carnitine ester hydrolyses spontaneously at an appreciable rate, losing carnitine and leaving a monocarboxymethyl-enzyme, which is still catalytically inert. Although it is quite possible that such a carboxymethyl group might interfere both sterically and electrostatically with substrate binding or impose some catalytically unfavourable configuration on the enzyme protein, there is evidence to suggest that the susceptible histidine residue is of direct importance in the catalytic mechanism of carnitine acetyltransferase. Bromoacetyl-L-carnitine can give rise to inhibited forms of this enzyme in two distinct ways. As shown here, it may react with a histidine residue at or near the active site. If, however, CoA is also present when enzyme and inhibitor react, the bromoacetyl group preferentially alkylates the thiol group of enzyme-bound CoA (Chase & Tubbs, 1969). With such an indication that the histidine residue is in close juxtaposition to the CoA-binding site as well as that for carnitine, it is hard to imagine that its imidazole side chain could fail to influence the rate of the catalytic reaction; it is well known that imidazole can catalyse non-enzymic acyl-transfer reactions by a number of mechanisms (Jencks, 1969). In view of these considerations we suggest that the single active centre of carnitine acetyltransferase contains a histidine residue that is directly involved in the catalysis of the acyl transfer reactions carried out by this enzyme.

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