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Some Kinetic Studies on the Mechanism of Action of Carnitine Acetyltransferase

By J. F. A. CHASE and P. K. TUBBS
Department of Biochemistry, University of Cambridge

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1. Michaelis constants for substrates of carnitine acetyltransferase have been shown to be independent of the concentration of second substrate present. This applies to the forward reaction between acetyl-L-carnitine and CoASH, and to the back reaction between L-carnitine and acetyl-CoA. 2. Product inhibition of both forward and back reactions has been studied. Evidence has been obtained for independent binding sites for L-carnitine and CoASH. Acetyl groups attached to either substrate occupy overlapping positions in space when the substrates are bound to the enzyme. 3. Possible reaction mechanisms involving the ordered addition of substrates have been excluded by determining kinetic constants in the presence and absence of added product. 4. D-Carnitine and acetyl-D-carnitine have been shown to inhibit competitively with respect to L-carnitine and acetyl-L-carnitine. 5. It is concluded that the mechanism of action of carnitine acetyltransferase involves four binary and two or more ternary enzyme complexes in rapid equilibrium with free substrates, the interconversion of the ternary complexes being the rate-limiting step. The possible intermediate formation of an acetyl-enzyme cannot be excluded, but this could only arise from a ternary complex.

Carnitine acetyltransferase (acetyl-CoA–carnitine *O*-acetyltransferase, EC 2.3.1.7) catalyses the reversible reaction:

$$\text{O-Acetyl-L-carnitine} + \text{CoASH} \rightleftharpoons \text{L-carnitine} + \text{acetyl-CoA}$$

which has an equilibrium constant of 0·6 (Fritz, Schultz & Srere, 1963). The partially purified enzyme from pig heart is sensitive to reagents for thiol groups, and the presence of a substrate, acetyl-CoA, protects against such inhibition (Fritz & Schultz, 1965). As a result, it has been suggested that the mechanism of action of this enzyme involves the transfer of an acetyl group from acetyl-CoA or acetylcarnitine to a thiol group on the enzyme to form an acetyl-enzyme thio ester as an intermediate, and releasing the deacylated substrate. The acetyl-enzyme would then react with either carnitine or CoASH, thus catalysing an acetyl transfer or an exchange reaction (Fritz, 1963).

A kinetic study has been made of the catalysis by carnitine acetyltransferase of both forward and back reactions, and of the effects of product inhibition. It was hoped that this would provide evidence as to the mechanism of action of the enzyme, and in particular would show whether the mechanism postulated above is applicable. A preliminary report of part of this work has appeared (Chase, 1965).

MATERIALS

Enzymes. Pigeon-breast-muscle carnitine acetyltransferase was prepared and recrystallized twice to a specific activity of 100–120 as reported by Chase, Pearson & Tubbs (1965).

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

Acyl-CoA synthetase (EC 6.2.1.2) was a gift from Dr D. J. Pearson.

L-Malate dehydrogenase (EC 1.1.1.37) was obtained from Boehringer Corporation (London) Ltd.

Chemicals. CoASH was obtained from Boehringer Corporation (London) Ltd. Acetyl-CoA was prepared from CoASH by treatment with acetic anhydride (Simon & Shemin, 1953).

DL-Carnitine hydrochloride was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. L-Carnitine hydrochloride was isolated from Difco beef extract (Difco Laboratories, Detroit, Mich., U.S.A.) by the method of Friedman, Macfarlane, Bhattacharyya & Fraenkel (1960). *O*-Acetyl-DL-carnitine hydrochloride and *O*-acetyl-L-carnitine hydrochloride were prepared from the corresponding carnitine hydrochlorides by the method of Fraenkel & Friedman (1957), and recrystallized twice from butanol.

NAD, $NADH_2$ and ATP were obtained from Boehringer

Corporation (London) Ltd., and L-malic acid was from Koch–Light Laboratories Ltd. Sorbic acid (Eastman Kodak Co., Rochester, N.Y., U.S.A.) was recrystallized twice from water. 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. As far as possible, all other reagents were of analytical grade, and glass-distilled water was used throughout.

The pH of solutions was measured with a Radiometer type 22 meter fitted with a type B glass electrode (Radiometer, Copenhagen, Denmark).

METHODS

Purity and assay of substrates. All assays were carried out in a Beckman DK–2 recording spectrophotometer.

L-Carnitine hydrochloride was recrystallized twice. A 1g. portion was dissolved in 20ml. of warm acetic acid. The solution was cooled, 6 vol. of acetone was added and crystals were allowed to form overnight. These were dried over P_2O_5 in a desiccator. The yield was 0·75g. Twice-recrystallized L-carnitine was 98·5% pure by enzymic assay (see below). Thin-layer chromatography on Silica G (Macherey, Nagel and Co., 516 Duren, Germany), with as solvent chloroform–methanol–aq. ammonia (sp.gr. 0·88)–water (50:40:3:9, by vol.), followed by development with I_2 vapour, showed only a single spot (Friedberg & Bressler, 1965). L-Carnitine was assayed by either of two enzymic methods. In each case, the CoASH released in the presence of carnitine acetyltransferase and an excess of acetyl-CoA was measured, either by coupling to sorbate in the presence of acyl-CoA synthetase and ATP, when EDTA and GSH were omitted from the assay which was otherwise similar to that of Pearson & Tubbs (1964), or by using 5,5′-dithiobis-(2-nitrobenzoic acid) to detect thiol release (Fritz *et al.* 1963). The increase in molar extinction at 300mμ for the formation of sorboyl-CoA was taken as 23×10^3cm.$^{-1}$ (P. K. Tubbs, unpublished work) and that for the release of 5-thio-2-nitrobenzoate as $13{\cdot}6\times10^3$cm.$^{-1}$ at 412mμ (Ellman, 1959).

Acetyl-L-carnitine was assayed enzymically by the coupled assay of Fritz *et al.* (1963) by using carnitine acetyltransferase, citrate synthase and malate dehydrogenase:

$$\text{Acetylcarnitine} + \text{CoASH} \rightleftharpoons \text{carnitine} + \text{acetyl-CoA}$$
$$\text{Acetyl-CoA} + \text{oxaloacetate} \rightarrow \text{citrate} + \text{CoA}$$
$$\text{Malate} + \text{NAD} \rightleftharpoons \text{oxaloacetate} + \text{NADH}_2$$

As pointed out by Pearson (1965), this assay is only stoicheiometric if the equilibrium concentration of oxaloacetate in the absence of acetyl-CoA is negligible compared with the acetyl-CoA to be assayed, or in this case produced from acetylcarnitine. To achieve this, the initial malate concentration was decreased to 7·5mM, and $NADH_2$ (50μM) was added to the assay mixture, under which conditions the production of 1equiv. of acetyl-CoA gives rise to 1equiv. of $NADH_2$ with an accuracy greater than 99%. Acetyl-L-carnitine was found to be 99% and acetyl-DL-carnitine 95% pure on a weight basis. Assays of the two compounds for free L-carnitine indicated less than 0·1% and 0·8% contamination respectively. Thin-layer chromatography of both compounds (as above) showed a spot clearly resolved from free carnitine markers, and a faint trace of free carnitine in the DL-preparation.

CoASH was assayed by coupling to sorbate in the presence of ATP and acyl-CoA synthetase (Wakil & Hübscher, 1960).

Acetyl-CoA was assayed by measuring thiol release in the presence of an excess of carnitine, carnitine acetyltransferase and 5,5′-dithiobis-(2-nitrobenzoic acid). The assay system comprised: tris, pH7·8, 100mM; 5,5′-dithiobis-(2-nitrobenzoic acid), 0·5mM; L-carnitine, 0·5mM; and water to 2ml. The extinction change at 412mμ on the addition of 10–50mμmoles of acetyl-CoA was a measure of free thiol and the further increase on the addition of about 10μg. of carnitine acetyltransferase corresponded to the acetyl-CoA added. Free thiol was 0·5–1% of the acetyl-CoA in all samples used.

Kinetics of carnitine acetyltransferase. Solutions of all substrates were assayed on the day of an experiment. CoASH was dissolved in water, and acetyl-CoA was prepared in solution and kept at pH4–6. The hydrochlorides of carnitine and acetylcarnitine were brought to about pH6 with phosphate buffer. Solutions of carnitine and acetyl-CoA were stable frozen, but acetylcarnitine and CoASH were dissolved as required.

Carnitine acetyltransferase was kept as a solution containing 2–8 units/ml. (Chase *et al.* 1965) in 0·1M-phosphate buffer, pH7·5. Such very dilute solutions lost only 10–12% of their activity in 2 months at 4°.

The assay system comprised: tris, pH7·8, 100mM; 10μl. of carnitine acetyltransferase (0·18–0·72μg. of protein); substrates and water to 2·0ml. Volumes of substrates were delivered from 100μl. Hamilton graduated syringes (Hamilton Co. Inc., Whitties, Calif., U.S.A.).

Reaction rates were followed in a Beckman DK–2 recording spectrophotometer, the molar extinction change at 232mμ for the acetylation of CoASH being taken as $4{\cdot}5\times10^3$cm.$^{-1}$ (Stadtman, 1957). Temperature was controlled by passing water from a constant-temperature bath through the cell housing and a thermometer in the housing registered 30±0·3° throughout. In general, all assay components except carnitine or acetylcarnitine were mixed in the cuvette, and allowed to warm up in the cell housing for 1min. The reaction was started with carnitine or acetylcarnitine. Identical rates were obtained if the reaction was initiated with enzyme.

The spectrophotometer was fitted with a supplementary recorder that enabled any extinction range of 0·1 to be chosen to give full-scale deflexion. This device enabled rates of extinction change down to 0·005/min. to be measured accurately. In turn, this permitted the use of less enzyme, ensuring that the initial reaction rate was maintained for a measurable length of time. Progress curves were found in practice to be linear for 20–120sec.

Neither carnitine nor acetylcarnitine had any appreciable extinction at 232mμ, and the alkaline hydrolysis of acetylcarnitine showed no change at that wavelength when followed spectrophotometrically.

Expression of results. Michaelis constants were determined by expressing initial reaction velocities at various substrate concentrations as double-reciprocal plots (Lineweaver & Burk, 1934), and extrapolating the resulting lines to cut the abscissa. Lines were fitted to the experimental points by eye.

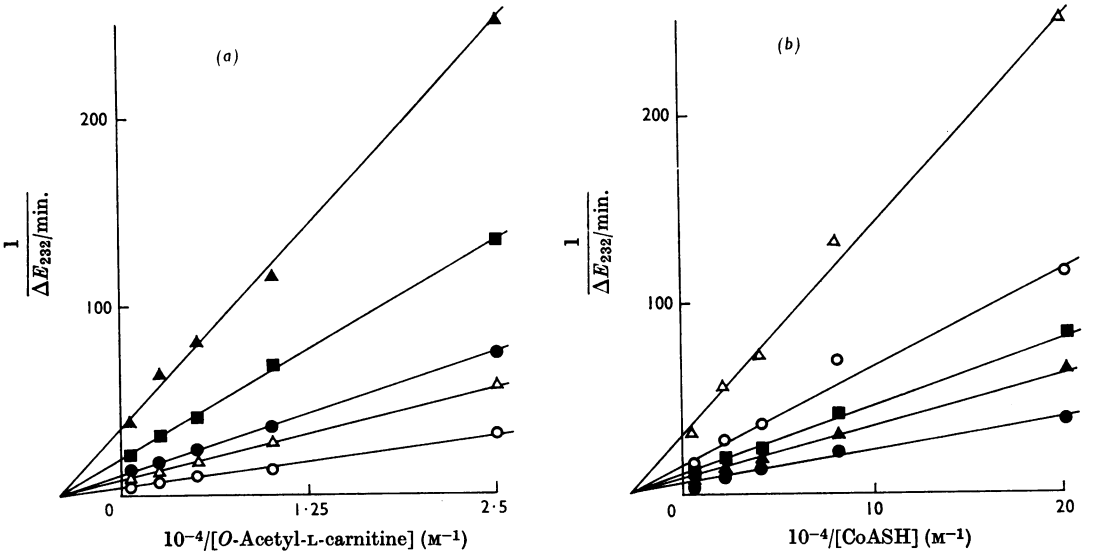


Fig. 1. Effect of varying the second substrate concentration on reciprocal plots for *O*-acetyl-L-carnitine and CoASH. (*a*) For acetylcarnitine at different CoASH concentrations: 270 μM (○), 53 μM (△), 27 μM (●), 14 μM (■) and 5·5 μM (▲). (*b*) For CoASH at different acetylcarnitine concentrations: 2000 μM (●), 400 μM (▲), 200 μM (■), 100 μM (○) and 40 μM (△). The amount of enzyme used was 0·18 μg./assay.

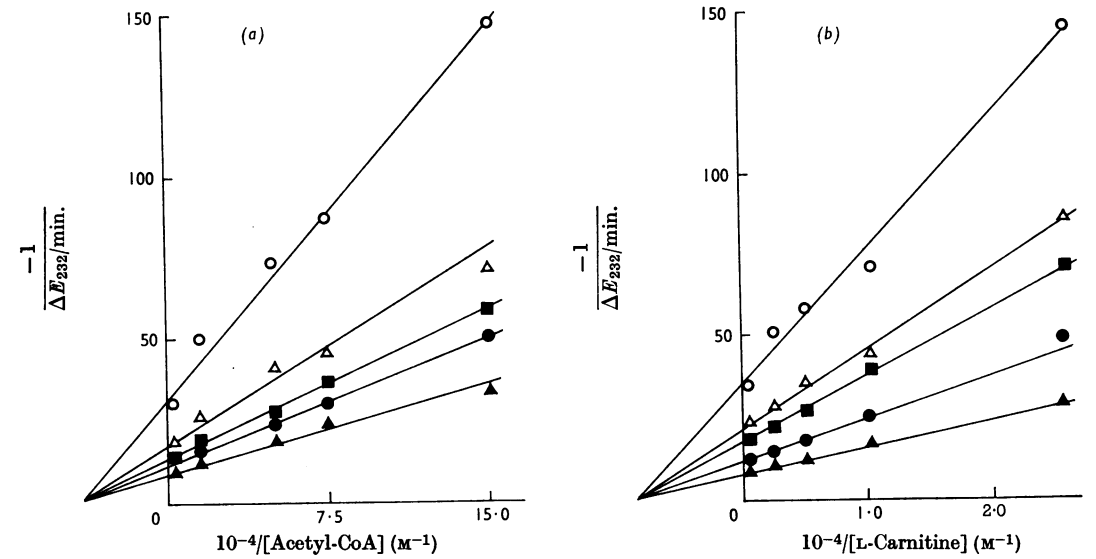


Fig. 2. Effect of varying the second substrate concentration on reciprocal plots for L-carnitine and acetyl-CoA. (*a*) For acetyl-CoA at different L-carnitine concentrations: 1900 μM (▲), 380 μM (●), 190 μM (■), 95 μM (△) and 38 μM (○). (*b*) For carnitine at different acetyl-CoA concentrations: 169 μM (▲), 34 μM (●), 20 μM (■), 13·5 μM (△) and 6·8 μM (○). The amount of enzyme used was 0·18 μg./assay.

RESULTS

Michaelis constants for acetyl-L-carnitine were measured in the presence of a range of CoASH concentrations, and vice versa (Fig. 1). Similar determinations were made for the back reaction between L-carnitine and acetyl-CoA (Fig. 2). In every case, it was found that the K_m for one substrate was independent of the concentration of the second substrate. All reciprocal plots were linear

over the range of substrate concentrations studied. From the reciprocal plots, secondary plots of intercept on the ordinate against second substrate concentration, and slope against second substrate concentration, were made, and values of kinetic constants were obtained by the procedure of Dalziel (1957) for a rate equation of the form:

$$\frac{[E]}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}$$

(Tables 1 and 2).

Product inhibition of forward and back reactions. Reciprocal plots for acetyl-L-carnitine and CoASH were obtained in the presence of both products, L-carnitine and acetyl-CoA, separately (Figs. 3 and 4). Similar determinations were made for the back reaction in the presence of its products (Figs. 5 and 6). The nature of the inhibition effects observed may be summarized as follows: (*a*) acetyl-CoA competes with CoASH and acetylcarnitine; (*b*) acetylcarnitine competes with carnitine and with acetyl-CoA; (*c*) CoASH competes only with acetyl-CoA; (*d*) carnitine competes only with acetylcarnitine; (*e*) CoASH and carnitine inhibit non-competitively with respect to each other.

Determination of kinetic constants in the presence of product. Reciprocal plots for L-carnitine and acetyl-CoA at a number of second substrate concentrations were obtained as above, and also in the

Table 1. *K_m values for substrates, and K_i values for* D-*carnitine and acetyl-*D-*carnitine*

Values for K_m were obtained from the intercepts on the abscissae of the reciprocal plots in Figs. 1 and 2, and from three other similar experiments. The results given are average values.

Substrate	K_m (μM)	Inhibitor	K_i (μM)
L-Carnitine	120	D-Carnitine	173
Acetyl-L-carnitine	350	Acetyl-D-carnitine	256
CoASH	37		
Acetyl-CoA	34		

Table 2. *Kinetic constants for forward and back reactions*

Secondary plots of the data in Figs. 1 and 2 were prepared according to Dalziel (1957), as described in the text. S_1, Acetyl-L-carnitine; S_2, CoASH; primed coefficients apply to the back reaction, when S_1' is L-carnitine and S_2' is acetyl-CoA. As the molecular weight of the enzyme is not accurately known, ϕ_0 and ϕ_0' are expressed in arbitrary units. ϕ_1, ϕ_2, ϕ_1' and ϕ_2' are in moles, and ϕ_{12} and ϕ_{12}' in moles^2.

$$\phi_0 = 4{\cdot}9 \quad ; \quad \phi_0' = 6{\cdot}2$$
$$\phi_1 = 1{\cdot}1 \times 10^{-3} \quad ; \quad \phi_1' = 8{\cdot}2 \times 10^{-4}$$
$$\phi_2 = 1{\cdot}95 \times 10^{-4} ; \quad \phi_2' = 1{\cdot}95 \times 10^{-4}$$
$$\phi_{12} = 4{\cdot}24 \times 10^{-8} ; \quad \phi_{12}' = 2{\cdot}48 \times 10^{-8}$$
$$\frac{\phi_1\phi_2}{\phi_{12}} = 5{\cdot}05 \quad ; \quad \frac{\phi_1'\phi_2'}{\phi_{12}'} = 6{\cdot}3$$
$$\frac{\phi_0\phi_1'\phi_2'}{\phi_0'\phi_1\phi_2} = K_{eq.} = 0{\cdot}585$$

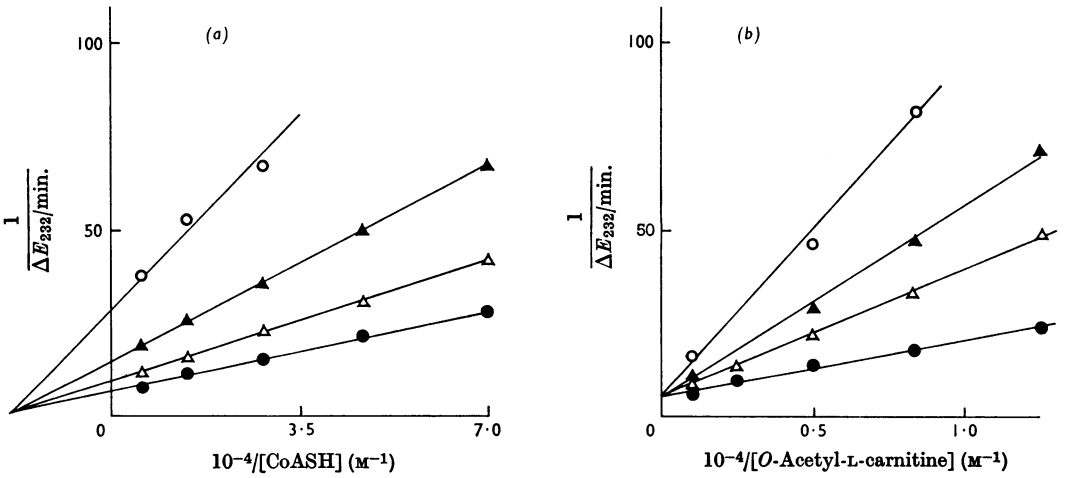


Fig. 3. Inhibition of forward reaction by L-carnitine. (*a*) Reciprocal plots for CoASH with constant acetyl-L-carnitine (200 μM) at different L-carnitine concentrations: none (●), 225 μM (△), 450 μM (▲) and 1125 μM (○). (*b*) Reciprocal plots for acetylcarnitine with constant CoASH (35 μM) at different L-carnitine concentrations: none (●), 225 μM (△), 450 μM (▲) and 1125 μM (○). The amount of enzyme used was 0·66 μg./assay.

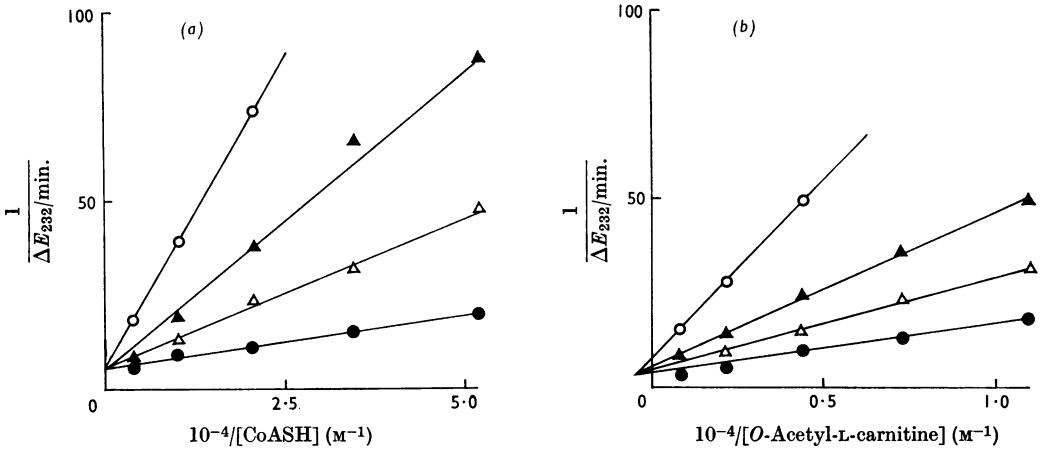


Fig. 4. Inhibition of the forward reaction by acetyl-CoA. (*a*) Reciprocal plots for CoASH with constant acetyl-L-carnitine (220μM) at different acetyl-CoA concentrations: none (●), 40μM (△), 80μM (▲) and 200μM (○). (*b*) Reciprocal plots for acetylcarnitine with constant CoASH (47·5μM) at different acetyl-CoA concentrations: none (●), 40μM (△), 80μM (▲) and 200μM (○). The amount of enzyme used was 0·60μg./assay.

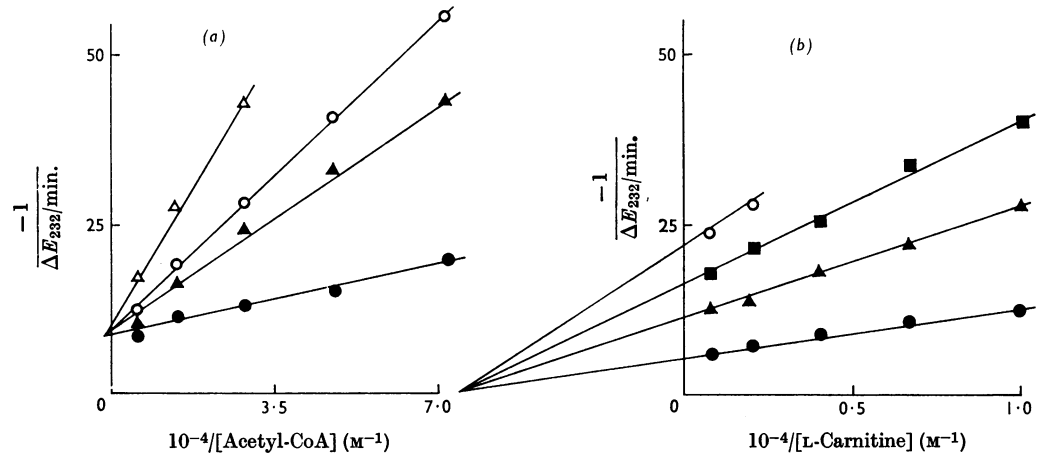


Fig. 5. Inhibition of the back reaction by CoASH. (*a*) Reciprocal plots for acetyl-CoA with constant L-carnitine (250μM) at different CoASH concentrations: none (●), 42μM (▲), 84μM (○) and 210μM (△). (*b*) Reciprocal plots for L-carnitine with constant acetyl-CoA (35μM) at different CoASH concentrations: none (●), 42μM (▲), 84μM (■) and 210μM (○). The amount of enzyme used was 0·39μg./assay.

presence of 500μM-acetyl-L-carnitine and 90μM-CoASH separately. Values of kinetic constants in the three cases were calculated from secondary plots, and the results are shown in Table 3.

Inhibition by D-*carnitine and acetyl-*D-*carnitine.* Reciprocal plots were obtained for L-carnitine and DL-carnitine at a fixed acetyl-CoA concentration, and for acetyl-L-carnitine and acetyl-DL-carnitine at a fixed CoASH concentration (Fig. 7). It was found in both cases that the presence of the D-isomer was inhibitory, and lowered $V_{max.}$ and increased K_m for the L-isomer by the same factor.

DISCUSSION

Dalziel (1957) considered possible mechanisms of two-substrate enzymic reactions and pointed out that for a mechanism of the type shown in Scheme 1,

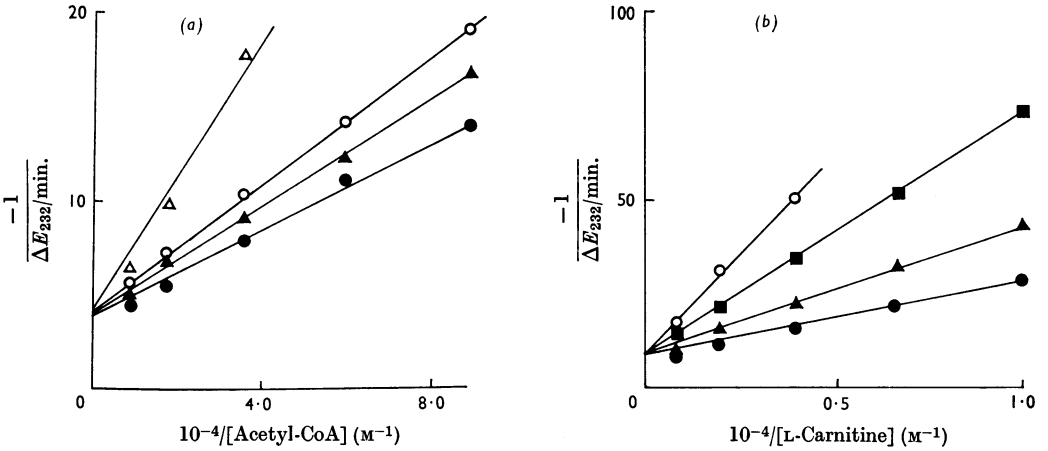


Fig. 6. Inhibition of the back reaction by *O*-acetyl-L-carnitine. (*a*) Reciprocal plots for acetyl-CoA with constant L-carnitine (208 μM) at different acetylcarnitine concentrations: none (●), 217 μM (▲), 434 μM (○) and 1080 μM (△). (*b*) Reciprocal plots for L-carnitine with constant acetyl-CoA (48 μM) at different acetylcarnitine concentrations: none (●), 222 μM (▲), 445 μM (■) and 1110 μM (○). The amounts of enzyme used were 0·60 μg./assay in (*a*) and 0·39 μg./assay in (*b*).

Table 3. *Effect of added products on the kinetic constants for the back reaction*

Kinetic constants were evaluated as in Table 2 for the reaction between L-carnitine and acetyl-CoA in the absence of product, and with the addition of 500 μM-acetyl-L-carnitine or 90 μM-CoASH. Δ_0' is the ratio of ϕ_0' measured in the presence of inhibitor to ϕ_0' in the absence of inhibitor, and Δ_1', Δ_2' and Δ_{12}' are defined similarly. K_i for an inhibitor = [inhibitor]/(Δ_{12}' − 1).

Constant	No inhibitor	500 μM-Acetylcarnitine added	90 μM-CoASH added
ϕ_0'	6·0	5·9	8·2
ϕ_1'	$1·6\times10^{-3}$	$2·4\times10^{-3}$	$2·0\times10^{-3}$
ϕ_2'	$4·2\times10^{-4}$	$5·5\times10^{-4}$	$15·6\times10^{-4}$
ϕ_{12}'	$1·2\times10^{-7}$	$3·0\times10^{-7}$	$4·8\times10^{-7}$
Δ_0'	—	0·98	1·36
Δ_1'	—	1·50	1·25
Δ_2'	—	1·31	3·72
Δ_{12}'	—	2·50	4·00
K_i		333 μM	30 μM

in which four binary and two or more ternary complexes are in equilibrium with substrates and in which the rate-determining step involves the interconversion of ternary complexes, certain relationships exist between kinetic constants. Reciprocal plots are linear, and the Haldane relationship:

$$K_{eq} = \frac{\phi_0\phi_1'\phi_2'}{\phi_0'\phi_1\phi_2}$$

will hold where the primed coefficients apply to the back reaction, as first shown by Alberty (1953). In addition, this reaction mechanism [type I(*b*) in Dalziel's (1957) classification] exhibits a diagnostic relationship between kinetic coefficients in that:

$$\phi_0 = \phi_1\phi_2/\phi_{12} \text{ and } \phi_0' = \phi_1'\phi_2'/\phi_{12}'$$

The data of Figs. 1 and 2 and Table 2 are in good agreement with these requirements, assuming a value of 0·6 for the equilibrium constant as measured at pH 7·0 and 35° by Fritz *et al.* (1963) and found not to be significantly different at pH 7·8 and 30° (J. F. A. Chase, unpublished work). It is tentatively suggested therefore that catalysis by carnitine acetyltransferase proceeds by a mechanism of the type shown in Scheme 1. If this is so, measured K_m values do represent true dissociation constants for the substrates in question. Such a mechanism cannot rule out the intermediate formation of an acetyl-enzyme, but this could only arise from a ternary complex (Scheme 1), and the postulated scheme of Fritz (1963) involving an acetyl-enzyme free of associated substrates cannot apply.

The product-inhibition experiments of Figs. 3–6 are compatible with the proposed mechanism. They suggest the existence of two independent substrate-binding sites, one for carnitine or acetylcarnitine and the other for CoASH or acetyl-CoA. It is envisaged that acetyl groups attached to either substrate occupy overlapping positions in space when on the enzyme. Thus the acetylated reaction

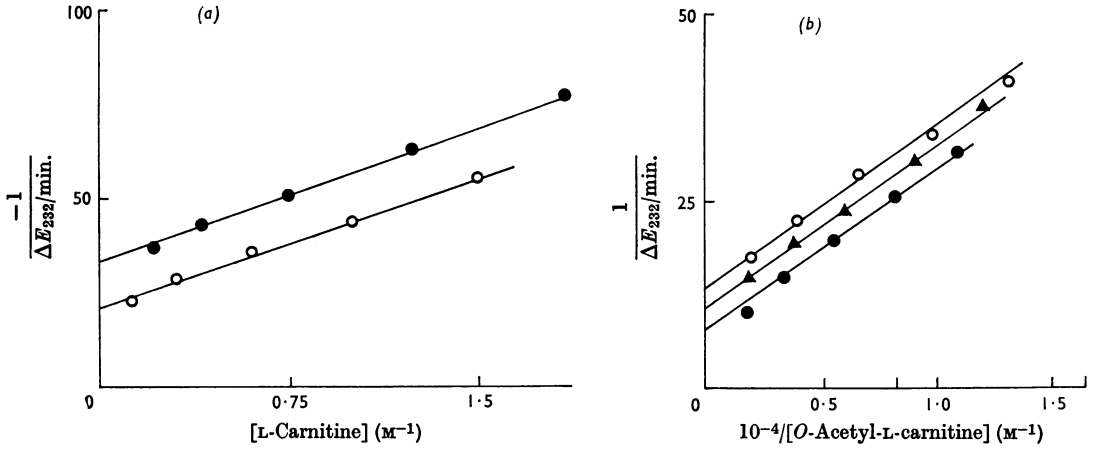
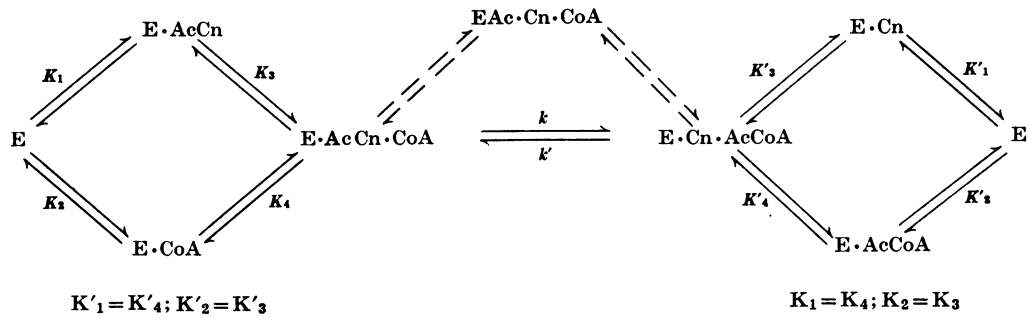


Fig. 7. Inhibition by D-carnitine and acetyl-D-carnitine. (*a*) Reciprocal plots for L-carnitine (○) and DL-carnitine (●) at constant acetyl-CoA (73 μM). (*b*) Reciprocal plots for acetyl-L-carnitine (●), acetyl-L-carnitine + acetyl-D-carnitine (1:0·45) (▲) and acetyl-DL-carnitine (○) at constant CoASH (87 μM).



Scheme 1. Proposed scheme for the mechanism of action of carnitine acetyltransferase. Cn, L-carnitine; AcCn, acetyl-L-carnitine; AcCoA, acetyl-CoA (all bound to enzyme E); EAc, acetyl-enzyme. In the interests of clarity, free substrates are omitted. K_1–K_4 and K'_1–K'_4 represent the dissociation constants of the enzyme complexes shown. k and k' are the velocity constants of the forward and back reactions respectively.

product will compete with both its non-acetylated analogue and the acetylated form of the other substrate, whereas a non-acetylated product will only compete with its acetylated analogue. Non-competitive inhibition between carnitine and CoASH will result if two separate binding sites exist, the binding of substrate to one having no effect on the affinity of the enzyme for the other substrate. A similar scheme has been proposed for pyruvate kinase (Reynard, Hass, Jacobsen & Boyer, 1961). The similarity between the K_m values for carnitine and acetylcarnitine on the one hand and CoASH and acetyl-CoA on the other (Table 1) suggests that little if any binding occurs between the enzyme and acetyl groups associated with either substrate.

The observation that the data of Figs. 1 and 2 and Table 2 are compatible with the mechanism of Scheme 1 cannot completely exclude all other mechanisms. In cases such as this, in which the reaction catalysed has an equilibrium constant close to 1, the Haldane relationship is of little use in excluding alternative mechanisms, for which higher powers of $V_{max.}$ and $V'_{max.}$ appear in the Haldane relationships given by Alberty (1953). Further, the Dalziel relationship:

$$\phi_0 = \phi_1\phi_2/\phi_{12}$$

can apply to other mechanisms if certain restrictions are placed on these.

Thus for a two-substrate reaction with a com-

pulsory order of substrate binding and a single ternary complex:

$$E + S_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1$$

$$ES_1 + S_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E(XY) \underset{k'_3}{\overset{k'_4}{\rightleftharpoons}} EP_1 + P_2$$

$$EP_1 \underset{k'_1}{\overset{k'_2}{\rightleftharpoons}} E + P_1$$

If $k_4 \gg k_2$, $k'_4 \gg k'_2$ and $1/k_2 = 1/k'_2$ (i.e. $\phi_0 = \phi'_0$), the same relation holds [mechanism type II (ii) in Dalziel's (1957) classification]. The Theorell–Chance mechanism (Theorell & Chance, 1951), which is a limiting case of the above scheme in which no ternary complex is kinetically significant, could also apply if $\phi_0 = \phi'_0$. In fact, for carnitine acetyltransferase, ϕ_0 does not equal ϕ'_0 (Table 2), but the difference is too small to rule out satisfactorily the alternative schemes cited, in view of the possible errors that may arise in terms derived from secondary plots. Alberty (1958) has shown that the effects of product inhibition may be used to distinguish between the mechanism of Scheme 1 and other possible mechanisms. He derived an expression showing the type of inhibition expected for the addition of product to an enzyme-catalysed reaction of the type:

$$S_1 + S_2 \rightleftharpoons P_1 + P_2$$

proceeding by a mechanism of the type shown in Scheme 1. This was of the form:

$$\frac{[E]}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}\left(1 + \frac{[P]}{K_p}\right) \quad (1)$$

where [P] represents the concentration of either product P_1 or P_2 which is added to the original reaction mixture, and K_p is the appropriate dissociation constant for the enzyme–product complex formed. The form of the inhibition equation is thus the same, whichever product is added. In fact, inhibition by product will only be of this form if the added product, e.g. P_1, can only combine with free enzyme E. If, as must often happen in practice, P_1 may also combine with the binary complex ES_2, a different type of inhibition is expected, described by the equation:

$$\frac{[E]}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]}\left(1 + \frac{[P_1]}{K_{p_1}}\right) + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}\left(1 + \frac{[P_1]}{K_{p_1}}\right) \quad (2)$$

For carnitine acetyltransferase, the product-inhibition studies shown in Figs. 3 and 5 suggest that CoASH, a non-acetylated substrate, when added as a product inhibitor of the back reaction, will compete with acetyl-CoA both for free enzyme and for the enzyme–carnitine binary complex. Table 3 shows that, in the presence of added CoASH, ϕ'_0 and ϕ'_1 are not significantly altered, whereas ϕ'_2 and ϕ'_{12} are raised by about the same factor in accordance with eqn. (2). However, when product inhibition by an acetylated substrate, e.g. acetylcarnitine, is studied, the inhibitor is only able to react with free enzyme as the presence of two acetylated substrates on the enzyme is sterically hindered (Figs. 4 and 6). Inhibition by acetylcarnitine may thus be expected to follow eqn. (1), in the same way as the inhibition of pyruvate kinase by a phosphorylated product (Reynard *et al.* 1961). The results shown in Table 3 largely bear this out, the ϕ'_{12} term being altered much more than the other constants. The slight effect on ϕ'_1 may be due to the presence of one acetylated substrate on the enzyme only incompletely excluding binding of the acetylated form of the other substrate, as is in fact suggested in Figs. 4(*b*) and 6(*a*), where the inhibition by acetyl-CoA with respect to acetylcarnitine and vice versa is not perfectly competitive. ϕ'_0 and ϕ'_2 are not significantly altered in the presence of acetylcarnitine, since the errors involved in determining the ratio of two kinetic constants may well approach 30% (Baker & Mahler, 1962).

Product inhibition according to eqn. (1) is indicative of the reaction mechanism shown in Scheme 1, and is not compatible with the ordered addition mechanisms considered, for which effects on the ϕ_{12} term should always be paralleled by effects on one of the other terms (Alberty, 1958). When equilibrium conditions apply to all substrate-binding steps, the K_i for a substrate acting as a product inhibitor should be equal to the K_m when it acts as a substrate, as both represent the dissociation constant of the enzyme–substrate complex. Tables 1 and 3 show that, for acetyl-L-carnitine and CoASH, K_m and K_i measurements are in good agreement. From all the results discussed above, it seems reasonably certain that carnitine acetyltransferase acts by a mechanism of the type shown in Scheme 1.

Fritz & Schultz (1965) found that carnitine acetyltransferase from pig heart is inhibited by D-carnitine, which competes with L-carnitine. They also reported that comparison of reciprocal plots for DL-carnitine with those for L-carnitine showed an increased K_m for L-carnitine and an unaltered $V_{max.}$ for the racemic mixture. This does not agree with the results of Fig. 7, in which the presence of a fixed ratio of D- to L-carnitine produces a parallel decrease in $V_{max.}$ and K_m. Alberty (1953) showed

that the mechanism of Scheme 1 may be described by the rate equation:

$$v = \frac{V_{max.}}{(1+K_A/[A])(1+K_B/[B])}$$

From the work of Tubbs (1962) and Dalziel (1962, 1963) it may be deduced that the form of inhibition expected when an enzyme following this mechanism acts on a substrate A containing a fixed molar ratio of an inhibitor I, which competes with A and not with B, is given by the equation:

$$v = \frac{V_{max.}}{\left\{1+K_A\left(\frac{1}{[A]}+\frac{R}{K_i}\right)\right\}\left(1+\frac{K_B}{[B]}\right)}$$

or

$$\frac{1}{v} = \left\{\frac{1}{V_{max.}}+\frac{K_A}{V_{max.}}\left(\frac{1}{[A]}+\frac{R}{K_i}\right)\right\}\left(1+\frac{K_B}{[B]}\right)$$

where $[I]=R[A]$. $V_{max.}$ and K_A will both be decreased by the factor $1/(1+RK_m/K_i)$, leaving the slope of the reciprocal plot unaltered compared with the uninhibited reaction. K_i values for D-carnitine and acetyl-D-carnitine may thus be obtained from the data of Fig. 7, as shown in Table 1. It appears that the K_m for the L-form of each compound is approximately equal to the K_i for the D-form, suggesting that the binding of carnitine and acetylcarnitine to the enzyme does not involve the asymmetry of the molecule, and providing further evidence that K_m measurements for L-carnitine and acetyl-L-carnitine represent true dissociation constants.

The kinetic studies of Fritz & Schultz (1965) gave results differing in one further respect from those reported here. They found acetyl-L-carnitine to inhibit non-competitively with respect to L-carnitine, whereas the results shown in Fig. 6(*b*) show competitive inhibition. Unless this represents a true difference between the pig-heart and pigeon-breast-muscle enzymes, it is suggested that the use of different assays in the two cases may be responsible. In this paper, progress of the reaction is measured directly, whereas Fritz & Schultz (1965) measured the rate of thiol release from acetyl-CoA with 5,5′-dithiobis-(2-nitrobenzoic acid), which is present in the assay system throughout.

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