

The Substrate Specificity of Carnitine Acetyltransferase

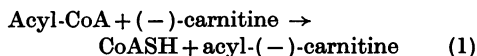
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(Received 16 December 1966)

1. A study of the acyl group specificity of the carnitine acetyltransferase reaction [acyl-($-$)carnitine + CoASH \rightleftharpoons ($-$)carnitine + acyl-CoA] has been made with the enzyme from pigeon breast muscle. Acyl groups containing up to 10 carbon atoms are transferred and detailed kinetic investigations with a range of acyl-CoA and acylcarnitine substrates are reported. 2. Acyl-CoA derivatives with 12 or more carbon atoms in the acyl group are potent reversible inhibitors of carnitine acetyltransferase, competing with acetyl-CoA. Lauroyl- and myristoyl-CoA show a mixed inhibition with respect to ($-$)carnitine, but palmitoyl-CoA competes strictly with this substrate also. Palmitoyl-DL-carnitine shows none of these effects. 3. Ammonium palmitate inhibits the enzyme competitively with respect to ($-$)carnitine and non-competitively with respect to acetyl-CoA. 4. It is suggested that a hydrophobic site exists on the carnitine acetyltransferase molecule. The hydrocarbon chain of an acyl-CoA derivative containing eight or more carbon atoms in the acyl group may interact with this, which results in enhanced acyl-CoA binding. Competition occurs between ligands bound to this hydrophobic site and the carnitine binding site. 5. The possible physiological significance of long-chain acyl-CoA inhibition of this enzyme is discussed.

Fritz, Schultz & Srere (1963) have reported that preparations of carnitine acetyltransferase [acetyl-CoA-($-$)carnitine *O*-acetyltransferase, EC 2.3.1.7] from pig heart catalyse the reversible transfer between CoA and ($-$)carnitine of *n*-acyl groups containing from two to 10 carbon atoms. For the reaction



the rate with propionyl-CoA was about 125% of that with acetyl-CoA; thereafter, rates decreased with increasing acyl group chain length. These measurements were only made, however, at a fixed concentration of both acyl-CoA and ($-$)carnitine. It was thus not clear whether the observed variations in reaction rate reflected changes in the affinity of the enzyme for the various acyl-CoA derivatives, in the catalytic rate constant for the transfer of different acyl groups, or in both of these factors. Subsequently, while Fritz *et al.* (1963) had found that palmitoyl-CoA was not a substrate for carnitine acetyltransferase, observations made in this Laboratory by Dr D. J. Pearson suggested that this compound was, in fact, a potent inhibitor of the enzyme.

The present work describes kinetic studies with a number of acyl-CoA and acylcarnitine derivatives as substrates of carnitine acetyltransferase, together

with the characterization of the inhibition by long-chain acyl-CoA. It was hoped that the determination of kinetic constants for a range of substrates of this enzyme might provide confirmatory evidence for the reaction mechanism proposed by Chase & Tubbs (1966). A brief report of this work has appeared (Chase, 1966).

MATERIALS

Enzymes. Pigeon breast muscle carnitine acetyltransferase was prepared as reported by Chase, Pearson & Tubbs (1965) and recrystallized twice.

Citrate synthase (EC 4.1.3.7) was made from pig heart according to Srere & Kosicki (1961).

Acyl-CoA synthetase (EC 6.2.1.2) was a gift from Dr P. K. Tubbs.

L-Malate dehydrogenase (EC 1.1.1.37) was obtained from Boehringer Corp. (London) Ltd., London, W. 5.

Chemicals. CoASH, NAD, NADH₂ and ATP were the products of Boehringer Corp. (London) Ltd. ($-$)Carnitine hydrochloride was isolated from Difco beef extract (Difco Laboratories, Detroit, Mich., U.S.A.) by the method of Friedman, Macfarlane, Bhattacharyya & Fraenkel (1960) and recrystallized from acetic acid-acetone (Chase & Tubbs, 1966). Palmitoyl-DL-carnitine hydrochloride was a gift from Dr D. J. Pearson.

n-Pentanoic acid, *n*-decanoic acid and lauric acid were the Puriss grade of Koch-Light Laboratories Ltd., Colnbrook, Bucks. *n*-Hexanoic acid, *n*-heptanoic acid, *n*-octanoic acid, and myristic acid were the Puriss grade of Fluka A.-G.,

Chemische Fabrik, Buchs SG, Switzerland. Palmitic acid was the Specially Pure grade of British Drug Houses Ltd., Poole, Dorset. Sorbic acid (Eastman Kodak Co., Rochester, N.Y., U.S.A.) was recrystallized twice from water. Sorboyl chloride and decanoyl chloride, prepared by treating the respective free acids with oxalyl chloride, were kindly given by Dr P. K. Tubbs. Oxaloacetic acid was from Koch-Light Laboratories Ltd.

5,5'-Dithiobis-(2-nitrobenzoic acid) was obtained from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Pyridine, tetrahydrofuran and ethyl chloroformate, all from Hopkin and Williams Ltd., Chadwell Heath, Essex, were freshly redistilled before use. Tris (Trizma Base; Sigma Chemical Co., St Louis, Mo., U.S.A.) was neutralized with HCl. Other reagents were of analytical grade and glass-distilled water was used throughout.

METHODS

Preparation of acyl-CoA derivatives. (1) Acetyl-, propionyl- and butyryl-CoA were prepared by treating CoASH with the corresponding acid anhydride (Simon & Shemin, 1953). Sorboyl-CoA was made from CoASH and sorboyl chloride (Seubert, 1960).

(2) Other acyl-CoA derivatives were obtained by causing CoASH to react with mixed anhydrides of the fatty acid concerned and ethyl hydrogen carbonate. These were prepared from the free acids and ethyl chloroformate as described by Stadtman (1957), although the subsequent procedure for the acylation of CoASH was modified as follows.

CoASH (25 mg., about 25 μ moles) is dissolved in 1.0 ml. of *m*-KHC₃ solution and treated with 0.125 ml. of neutral *m*-potassium thioglycollate (125 μ moles) to ensure complete reduction of the CoA. The solution, kept at room temperature, is adjusted to pH 7-8 if necessary and N₂ is bubbled through for a few minutes. This removes dissolved O₂ and continued gassing also ensures efficient mixing during the subsequent addition of 150 μ moles of mixed anhydride, the amount theoretically needed to acylate all the thiol present. Small further additions of anhydride are made, if required, until the solution gives a negative test for thiol with the nitroprusside reagent (Stadtman, 1957). Mixed anhydrides of *n*-octanoic acid and its higher homologues were found to be too insoluble in water to react satisfactorily in this system, a problem which may be overcome by adding 0.5 ml. of tetrahydrofuran to the CoASH solution before treating it with the acylating agent.

The pH of the nitroprusside negative reaction mixture is adjusted to about 2 with HCl and any tetrahydrofuran present is removed by passing N₂ through the solution at 35°. At this stage, the precise technique required for the removal of impurities, mainly free carboxylic acid and acylthioglycolic acid, from the acidified acyl-CoA preparations depends somewhat on the chain length of the acyl group in question. Thus, solutions of decanoyl-CoA and shorter-chain acyl-CoA derivatives, which are largely or completely soluble at this pH, should be extracted six to eight times with portions (5 ml.) of ether. The aqueous acyl-CoA solution is then brought to pH 4-6 by the addition of drops of *m*-KHC₃, residual ether is removed in a stream of N₂ at room temperature and the solution stored frozen. Lauroyl-CoA and longer-chain acyl-CoA derivatives are insoluble in acid solution. They may be purified by centrifuging, washing the precipitate twice with ether and redissolving the acyl-CoA by the dropwise addition of KHC₃, to give a solution of about pH 6. Any remaining undissolved material is removed by centrifugation and the cycle of acidification, extraction and neutralization is repeated twice. The product is stored either frozen in solution at pH 4-6 or as a solid in a vacuum desiccator at -15°; 50-90% yields of acyl-CoA derivatives are obtained by this procedure.

Preparation of acyl(-)-carnitine derivatives. Acetyl(-)-carnitine hydrochloride was made by treating (-)-carnitine hydrochloride in solution in acetic acid with acetyl chloride (Fraenkel & Friedman, 1957); butyryl(-)-carnitine hydrochloride was prepared similarly from carnitine dissolved in butyric acid and butyryl chloride. Both products were recrystallized twice from acetic acid-acetone (Chase & Tubbs, 1966).

Decanoyl(-)-carnitine hydrochloride, made from (-)-carnitine hydrochloride and decanoyl chloride by the method of Bremer (1962b), was recrystallized as above.

Purity and assay of substrates. All enzymic assays were performed on a Beckman DK-2 recording spectrophotometer. Solutions of the various substrates of carnitine acetyltransferase were prepared as described by Chase & Tubbs (1966) and assayed on the day of an experiment.

CoASH, (-)-carnitine and acetyl(-)-carnitine solutions were estimated as described by Chase & Tubbs (1966). Acetyl-CoA was specifically assayed by the procedure of Chase (1967).

Propionyl-, butyryl-, pentanoyl-, hexanoyl-, sorboyl-, heptanoyl-, octanoyl-, nonanoyl- and decanoyl-CoA were determined by measuring thiol release with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of carnitine acetyltransferase and an excess of carnitine. The system comprised: tris, pH 7.8, 100 mM; 5,5'-dithiobis-(2-nitrobenzoic acid), 0.5 mM; DL-carnitine, 2.5 mM; water to 2.0 ml. Free thiol in the preparation was calculated from the increase in extinction at 412 m μ on the addition of 10-50 m μ moles of acyl-CoA. The subsequent addition of about 10 μ g. of carnitine acetyltransferase gave a further increment equivalent to the acyl-CoA added, the increase in molar extinction at 412 m μ for the release of 5-thiol-2-nitrobenzoate being taken as 13.6 $\times 10^3$ cm.⁻¹ (Ellman, 1959). In no case was the free thiol content of any acyl-CoA derivative in excess of 2%. Measurements of acetyl-CoA present as an impurity in propionyl- and butyryl-CoA preparations were made by using the specific citrate synthase assay for acetyl-CoA (Chase, 1967) and showed a possible maximum contamination of 0.5%.

Lauroyl-, myristoyl- and palmitoyl-CoA solutions were estimated from their extinction at 232 and 260 m μ . Pure long-chain acyl-CoA derivatives have ϵ_{232} 9.4 $\times 10^3$ cm.⁻¹ and ϵ_{260} 16.4 $\times 10^3$ cm.⁻¹, i.e. $\epsilon_{260}/\epsilon_{232}$ 1.73 (Seubert, 1960). For the samples used here, this ratio was found to be 1.74 for lauroyl-CoA, 1.79 for myristoyl-CoA and 1.73 for palmitoyl-CoA, suggesting that no significant u.v.-absorbing impurity was present. None of these materials contained any detectable free thiol.

The determination of medium-chain-length acylcarnitine derivatives was based on reaction (2), which has an equilibrium



constant 0.6 (Fritz *et al.* 1963; J. F. A. Chase, unpublished work). In the presence of a large molar excess of

CoASH, a small amount of acylcarnitine should be almost quantitatively converted into acyl-CoA and carnitine, a reaction which may be followed at 232 m μ as the thio ester bond is formed. In practice, the amount of CoASH initially present is limited by the extinction of this material at 232 m μ and a correction has to be made for the acylcarnitine remaining at equilibrium, from the known values of the equilibrium constant and the concentrations of the other three reactants. As an example, the addition of 5 μ l. of 10 mM-acyl(-)-carnitine solution to a system containing tris, pH 7.8, 100 mM, CoASH, 0.5 mM, carnitine acetyltransferase, 100 μ g., and water to 2.0 ml. gives an increase in extinction at 232 m μ of 0.086 in a 10 mm. light-path. Taking $\Delta\epsilon_{232}$ for the acylation of CoASH to be 4.5×10^3 cm.⁻¹ (Stadtman, 1957), the acylcarnitine still present at equilibrium would be equivalent to a further extinction change of 0.027. Small changes such as these may be accurately measured on the Beckman DK-2 fitted with a scale-expansion device (Chase & Tubbs, 1966), but uncertainties in the value of the equilibrium constant may involve errors of up to one-third in the calculation of the correction factor. Estimates of the concentration of acylcarnitine solutions and of Michaelis constants based upon them may thus be in error by about 10%. Assays of the butyryl- and decanoyl(-)-carnitine preparations for free (-)-carnitine indicated less than 0.5% and 4% contamination respectively.

Palmitoyl-DL-carnitine hydrochloride solutions were prepared by dissolving a known weight of material in water at 50°.

Kinetic measurements. Initial reaction rates were followed spectrophotometrically at 232 m μ as described by Chase &

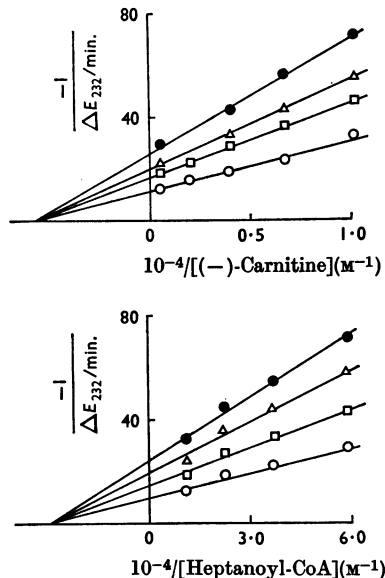


Fig. 1. Effect of varying the second substrate concentration on reciprocal plots for (-)-carnitine and heptanoyl-CoA. (a) For (-)-carnitine at different heptanoyl-CoA concentrations: 86 μ M (○), 43 μ M (□), 26 μ M (Δ) and 17 μ M (●). (b) For heptanoyl-CoA at different (-)-carnitine concentrations: 1260 μ M (○), 258 μ M (□), 155 μ M (Δ) and 103 μ M (●).

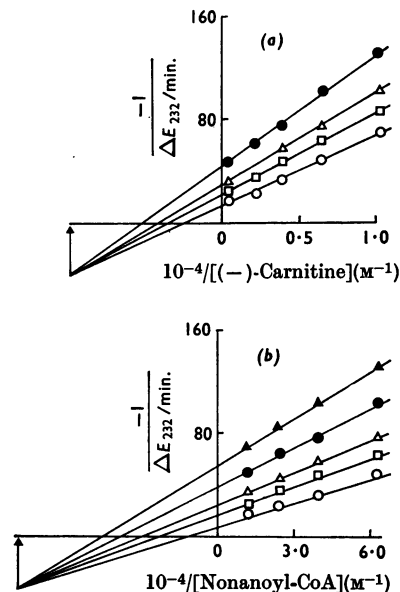


Fig. 2. Effect of varying the second substrate concentration on reciprocal plots for (-)-carnitine and nonanoyl-CoA. (a) For (-)-carnitine at different nonanoyl-CoA concentrations: 84 μ M (○), 42 μ M (□), 25 μ M (Δ) and 16 μ M (●). (b) For nonanoyl-CoA at different (-)-carnitine concentrations: 1290 μ M (○), 516 μ M (□), 258 μ M (Δ), 155 μ M (●) and 103 μ M (▲).

Tubbs (1966). All experiments were conducted at pH 7.8 and 30°, with an assay system identical with that described in the earlier paper, except that larger amounts of carnitine acetyltransferase, up to 2.5 μ g. per assay, were required when studying transfer of the longer-chain acyl groups.

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

RESULTS

Measurements of kinetic constants for the reaction between (-)-carnitine and a range of acyl-CoA derivatives. Michaelis constants were obtained for acyl-CoA derivatives of chain lengths between propionyl- and decanoyl- at a number of different (-)-carnitine concentrations and vice versa (Figs. 1 and 2). Values of V'_{max} , the maximum velocity of this reaction at infinite concentration of both substrates, were derived from these data by making secondary plots of intercept on the ordinate against the reciprocal of the second substrate concentration (Florini & Vestling, 1957). These values were related to V'_{max} with acetyl-CoA as a substrate by determining the latter in a similar manner on

Table 1. *Kinetic constants for the reaction between (-)-carnitine and a range of acyl-CoA derivatives*

Michaelis constants were obtained from double-reciprocal plots (e.g. Figs. 1 and 2). Values of V'_{\max} were calculated from secondary plots of these data (Florini & Vestling, 1957), the value for the reaction with acetyl-CoA as substrate being taken arbitrarily as 100. Inhibitor constants (K_I and K_{II}) were derived from experiments such as those shown in Figs. 3 and 4 as described in the text (see the Discussion section).

Acyl-CoA used	K_s for (-)-carnitine (μM)	K_s for acyl-CoA (μM)	K_I for acyl-CoA (μM)	K_{II} for acyl-CoA (μM)	V'_{\max}
Acetyl	120	34	—	—	100
Propionyl	104	37	—	—	77
Butyryl	159	44	—	—	41
Pentanoyl	154	33	—	—	18
Hexanoyl	172	33	—	—	13
Sorboyl	153	33	—	—	0.84
Heptanoyl	174	36	—	—	11
Octanoyl	131	32	—	—	8
Nonanoyl	114	15	—	—	5.5
Decanoyl	100	11	8.8*	12*	4
Lauroyl	—	—	3.4	10	<0.1
Myristoyl	—	—	1.5	4.2	0
Palmitoyl	—	—	0.43	—	0

* Results obtained with decanoyl-CoA as an inhibitor of the reaction between acetyl-CoA and (-)-carnitine as for lauroyl-CoA (Fig. 3). The contribution of the decanoyl-CoA, a poor substrate, to the observed initial rates was ignored.

the same day and with the same enzyme solution.

Two distinct types of result were seen. When acyl-CoA derivatives between propionyl- and heptanoyl-CoA, including sorboyl-CoA, were the substrates, K_m values for both the acyl-CoA and (-)-carnitine were independent of the concentration of each other (Fig. 1). This is also the pattern seen with acetyl-CoA (Chase & Tubbs, 1966). The K_m of carnitine acetyltransferase for each of these acyl-CoA substrates was found to be experimentally identical and the K_m for (-)-carnitine was independent of the acyl-CoA used (Table 1). V'_{\max} was found, in general, to decrease with increasing chain length of the group transferred, although for sorboyl-CoA it was an order of magnitude less than the value for the corresponding saturated derivative, hexanoyl-CoA (Table 1).

Kinetic data obtained with octanoyl-, nonanoyl- and decanoyl-CoA as substrates follow a rather different pattern. In each case, the K_m of the enzyme for (-)-carnitine rises with increasing acyl-CoA concentration and vice versa (Fig. 2). V'_{\max} diminishes further with increased acyl-group chain length (Table 1).

Inhibition of the enzyme by long-chain acyl-CoA. In agreement with the results of Fritz *et al.* (1963), palmitoyl-CoA was found not to be a substrate for carnitine acetyltransferase. The same applies to myristoyl-CoA, although, when very large amounts of enzyme were used, a small rate of reaction between lauroyl-CoA and (-)-carnitine was apparently observed. This was, however, less than 0.1% of the rate expected with acetyl-CoA under the same conditions and may have been due to contam-

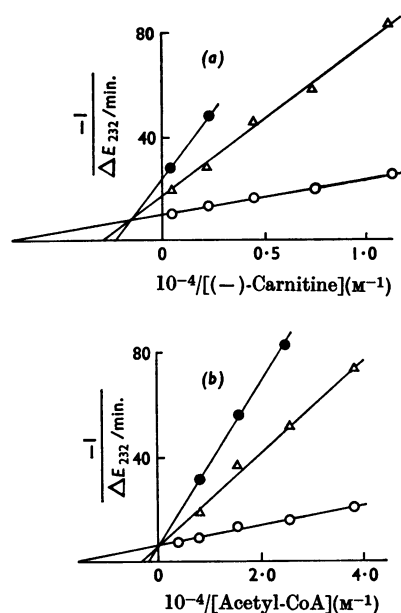


Fig. 3. Inhibition of the reaction between acetyl-CoA and (-)-carnitine by lauroyl-CoA. (a) With respect to (-)-carnitine at constant acetyl-CoA ($87 \mu\text{M}$). (b) With respect to acetyl-CoA at constant (-)-carnitine ($228 \mu\text{M}$). Lauroyl-CoA concentrations: 0 (\circ), $1.7 \mu\text{M}$ (Δ) and $3.4 \mu\text{M}$ (\bullet).

ination of the lauroyl-CoA with traces of shorter-chain substrates.

The three long-chain acyl-CoA derivatives were found to inhibit the enzyme reversibly, competing with acetyl-CoA (Figs. 3b and 4b). Lauroyl- and

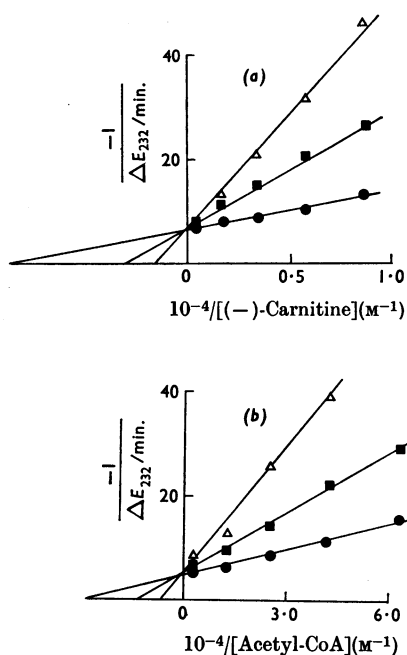


Fig. 4. Inhibition of the reaction between acetyl-CoA and (-)-carnitine by palmitoyl-CoA. (a) With respect to (-)-carnitine at constant acetyl-CoA ($39 \mu\text{M}$). (b) With respect to acetyl-CoA at constant (-)-carnitine ($250 \mu\text{M}$). Palmitoyl-CoA concentration: 0 (\bullet), $0.27 \mu\text{M}$ (\blacksquare) and $0.54 \mu\text{M}$ (Δ).

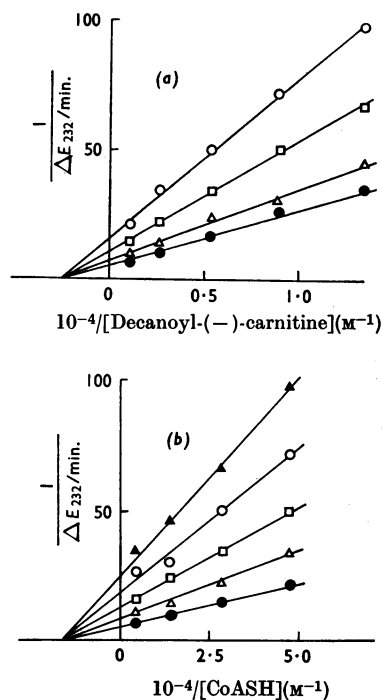


Fig. 5. Effect of varying the second substrate concentration on reciprocal plots for decanoyl(-)-carnitine and CoASH. (a) For decanoyl(-)-carnitine at different CoASH concentrations: $213 \mu\text{M}$ (\bullet), $71 \mu\text{M}$ (Δ), $36 \mu\text{M}$ (\square) and $21 \mu\text{M}$ (\circ). (b) For CoASH at different decanoyl(-)-carnitine concentrations: $750 \mu\text{M}$ (\bullet), $300 \mu\text{M}$ (Δ), $150 \mu\text{M}$ (\square), $90 \mu\text{M}$ (\circ) and $60 \mu\text{M}$ (\blacktriangle).

Table 2. Kinetic constants for the reaction between CoASH and a range of acyl(-)-carnitine derivatives

Michaelis constants were obtained from double-reciprocal plots (e.g. Fig. 6). Values of V_{max} were calculated from secondary plots of these data as described in the text, the value for the reaction with acetyl(-)-carnitine as substrate being taken arbitrarily as 100.

Acyl- (-)-carnitine used	K_m for acyl- (-)-carnitine (μM)	K_m for CoASH (μM)	V_{max}
Acetyl	350	37	100
Butyryl	455	45	100
Decanoyl	374	56	9

myristoyl-CoA exhibit a mixed inhibition with respect to (-)-carnitine (Fig. 3a), and palmitoyl-CoA is a strict competitor for this substrate (Fig. 4a).

Measurement of kinetic constants for the reaction between acyl(-)-carnitine derivatives and CoASH. Michaelis constants for butyryl- and decanoyl(-)-carnitine were found to be independent of the CoASH concentration used and vice versa (Fig. 5), the pattern being similar to that seen with acetyl-carnitine (Chase & Tubbs, 1966). V_{max} for the enzyme acting on each of the acylcarnitine sub-

strates was calculated as before. These results, together with the comparable figures for the reaction between acetyl(-)-carnitine and CoASH obtained with the same enzyme preparation, are shown in Table 2. K_m values for both CoASH and the acyl-carnitine are more or less independent of the length of the acyl group being transferred, in contrast with the findings above for the acyl-CoA series.

Palmitoyl-DL-carnitine is not a substrate for carnitine acetyltransferase. No inhibition of the enzyme was observed in the presence of this compound at a concentration of $100 \mu\text{M}$.

Inhibition of carnitine acetyltransferase by ammonium palmitate. Fig. 6 illustrates the reversible inhibition of the enzyme by 250 and $500 \mu\text{M}$ -ammonium palmitate. This inhibition is strictly non-competitive with respect to acetyl-CoA, but is competitive towards (-)-carnitine and K_i for the enzyme-inhibitor complex may be calculated as $325 \mu\text{M}$ (see the Discussion section). Ammonium ions at this concentration have no detectable effect on the activity of the enzyme.

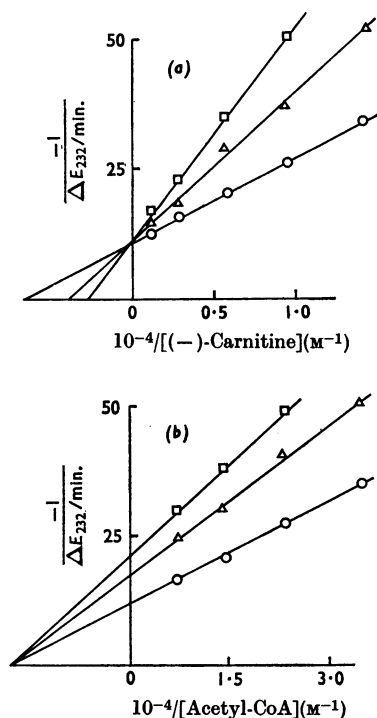


Fig. 6. Inhibition of the reaction between acetyl-CoA and (-)-carnitine by ammonium palmitate. (a) With respect to (-)-carnitine at constant acetyl-CoA ($70 \mu\text{M}$). (b) With respect to acetyl-CoA at constant (-)-carnitine ($193 \mu\text{M}$). Ammonium palmitate concentrations: 0 (○), $250 \mu\text{M}$ (Δ) and $500 \mu\text{M}$ (□).

DISCUSSION

Chase & Tubbs (1966) proposed that the kinetics of the acetyl group transfer between CoA and (-)-carnitine catalysed by carnitine acetyltransferase could be explained if the mechanism of action of the enzyme involves four binary and two ternary enzyme-substrate complexes in equilibrium with free enzyme and substrates. The rate-limiting step of this random-equilibrium mechanism is the interconversion of the ternary complexes, that is the acetyl group transfer itself. Two independent substrate-binding sites exist on the enzyme, one specific for carnitine or acetylcarnitine, the other for CoASH or acetyl-CoA, and binding of substrate to one site has no effect on the affinity of the enzyme for the other substrate.

Alberty (1953) has showed that the initial rate kinetics of an enzyme-catalysed reaction proceeding by such a mechanism could be described by equation (3).

$$v_0 = \frac{V_{\max.}}{\left(1 + \frac{K_m^1}{S_1}\right) \left(1 + \frac{K_m^2}{S_2}\right)} \quad (3)$$

Here K_m^1 and K_m^2 , the Michaelis constants for substrates S_1 and S_2 , approximate to the values of the equilibrium dissociation constants for the ES_1 and ES_2 complexes respectively. A characteristic of such a mechanism is that the catalytic rate constant is, by definition, absent from the combinations of rate constants that make up the K_m terms. In contrast, rate equations describing mechanisms involving the ordered addition of substrates to the enzyme, which were excluded with carnitine acetyltransferase by the effects of product inhibition (Chase & Tubbs, 1966), include the catalytic rate constant in the expressions for Michaelis constants (Alberty, 1953). Thus, for an enzyme working by an ordered addition mechanism, changes in the experimental conditions which alter $V_{\max.}$ should cause a parallel change in the K_m terms, although the reverse will not necessarily hold. In this paper and the preceding one a number of experiments are described in which $V_{\max.}$ and K_m values for the carnitine acetyltransferase system have been independently altered by several orders of magnitude. Such results will be interpreted as further evidence in favour of the application of the random-equilibrium mechanism to this system.

From the results of Fig. 1 and Table 1, it is seen that the Michaelis constants for acyl-CoA substrates from acetyl- to heptanoyl-CoA are experimentally identical ($38 \pm 6 \mu\text{M}$), whereas $V'_{\max.}$ for the catalysed reaction decreases about 10-fold over this range of substrates. Furthermore, carnitine acetyltransferase binds sorboyl-CoA with K_m $33 \mu\text{M}$, which is similar to the value found for the other short-chain acyl-CoA derivatives, but $V'_{\max.}$ with this substrate is only 0.84% of that with acetyl-CoA. Thus $V'_{\max.}$ is altered on changing from acetyl- to sorboyl-CoA by more than 100-fold whereas K_m is unaffected. As argued above, this is compatible with a random-equilibrium mechanism for carnitine acetyltransferase. By analogy with the earlier results for acetyl-CoA (Chase & Tubbs, 1966), K_m values for all the short-chain acyl-CoA substrates may be taken to represent dissociation constants (K_s values) for the respective ES complexes.

The pattern obtained with medium-chain-length (octanoyl-, nonanoyl- and decanoyl-) acyl-CoA substrates (Fig. 2) appears to show that binding of these derivatives to the enzyme lowers its affinity for (-)-carnitine and vice versa. Frieden (1957) has shown that, for a random-equilibrium mechanism giving rise to reciprocal plots of the type seen in Fig. 2, the lines obtained at various concentrations of the second substrate will intersect at a distance $-1/K_s$ along the abscissa. Hence, K_s values for both the acyl-CoA and (-)-carnitine may be determined in the three cases (Table 1). K_s for (-)-carnitine is independent of the nature of the acyl-CoA and is experimentally indistinguishable

from the values of this parameter obtained with short-chain acyl-CoA derivatives, but K_i for the acyl-CoA is now falling with increasing length of the acyl group.

This last trend is continued with the long-chain acyl-CoA inhibitors (Table 1). For an enzyme catalysing the reactions $S_1 + S_2 \rightarrow$ products by a random-equilibrium mechanism, in which K_m values for each substrate are independent of the concentration of the other substrate, the form taken by the initial rate equation in the presence of I , a competitive inhibitor for S_2 , is shown in equation (4),

$$v_0 = \frac{V_{\max}}{1 + \frac{K_m^1}{[S_1]} + \frac{K_m^2}{[S_2]} \left(1 + \frac{[I]}{K_{ii}}\right) + \frac{K_m^1 K_m^2}{[S_1][S_2]} \left(1 + \frac{[I]}{K_i}\right)} \quad (4)$$

where

$$K_i = \frac{[E][I]}{[EI]} \quad \text{and} \quad K_{ii} = \frac{[ES_1][I]}{[ES_1I]}$$

When $K_i = K_{ii}$, the inhibitor combines as easily with the ES_1 complex as with the free enzyme and non-competitive inhibition is observed with respect to S_1 . Inhibition of carnitine acetyltransferase by palmitate is of this type and the value of K_i for this inhibitor ($325 \mu\text{M}$) was derived by fitting the data of Fig. 6 to equation (4) and assuming the above equality.

If $K_i < K_{ii}$, a mixed competitive-non-competitive inhibition will be seen and, in the limit, when $K_{ii} = \infty$, the inhibitor cannot combine with ES_1 , giving competitive inhibition towards both substrates. Thus the results of Figs. 3 and 4 indicate that lauroyl- and myristoyl-CoA combine less well with the enzyme-carnitine complex than with free enzyme, whereas palmitoyl-CoA may only bind to the free enzyme. The K_i for palmitoyl-CoA shown in Table 1 was therefore calculated from the data of Fig. 4 according to equation (4) by assuming that $K_{ii} = \infty$; K_i and K_{ii} for the other acyl-CoA inhibitors were calculated from the abscissa intercepts (Fig. 3b) and the vertical height above the abscissa at which the mixed inhibition lines meet (Fig. 3a), also according to eqn. (4).

The observed interaction between acyl-CoA and (-)-carnitine binding for acyl groups containing eight or more carbon atoms provides a marked contrast with the independent binding characteristic of the short-chain substrates. This could be a reflexion of a different mechanism of action of the transferase when acting on the two sets of substrates but a more plausible explanation is apparent when the K_i values for the various acyl-CoA derivatives are expressed in the form shown in Fig. 7. Here pK_i ($pK_i = -\log K_i$; $K_i = K_m$ or K_i as applicable) is

plotted against the length of the acyl-CoA side chain. The horizontal portion of the graph represents the invariant value of K_m for short-chain acyl-CoA, but the subsequent rise between octanoyl- and palmitoyl-CoA shows that for these derivatives there is an equal increment in pK_i for each additional $-\text{CH}_2-$ in the acyl group, equivalent to a ΔG^0 of binding -0.31 kcal./mole ($\Delta G^0 = -RT \ln K_i$).

It is suggested that the carnitine acetyltransferase molecule contains a hydrophobic region with which an acyl-CoA hydrocarbon side chain containing eight or more carbon atoms may interact, enhancing acyl-CoA binding. The presence of a hydrocarbon chain on this site must hinder binding of (-)-carnitine to the carnitine-binding site and vice versa. The experiments described here do not, in themselves, justify the proposition that separate sites exist on the enzyme for carnitine and hydrocarbon chains, as the results would be equally compatible with a single site capable of binding carnitine or hydrocarbon in a mutually exclusive fashion. On the other hand, carnitine is a very hydrophilic molecule, soluble only in strongly polar solvents. It is thus hard to imagine a single enzyme site having affinity for both the carnitine molecule and a completely non-polar entity such as a hydrocarbon chain and it would seem more likely that separate carnitine and hydrophobic sites are present, the competition observed between ligands attached to the two sites being mediated by a conformational change in the enzyme protein.

If the above analysis of the inhibition of carnitine acetyltransferase by long-chain acyl-CoA were correct, it seemed likely that other molecules containing long aliphatic chains, such as fatty acids, might also inhibit the enzyme. Fig. 6 shows that

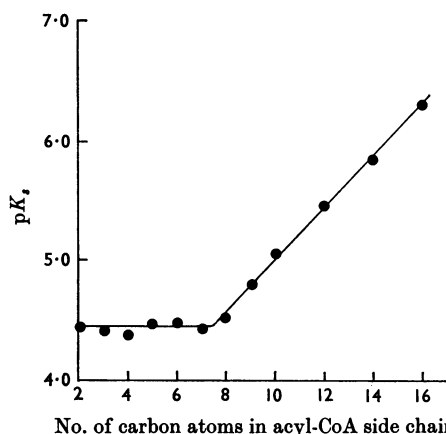


Fig. 7. Variation of pK_i ($-\log K_i$; $K_i = K_m$ or K_i as applicable) for a number of acyl-CoA substrates and inhibitors of carnitine acetyltransferase with the number of carbon atoms in the side-chain acyl group.

palmitate competes with respect to (-)-carnitine and inhibits non-competitively towards acetyl-CoA. Binding of a hydrocarbon chain to the hydrophobic site on the enzyme thus appears to be quite independent of the CoA-binding site. As with the acyl-CoA derivatives, palmitate inhibition is completely reversible.

The occurrence of hydrophobic bonds between proteins and small molecules is well established. Webb (1963, p. 300) has calculated the expected free-energy change on bringing together two hydrocarbon chains, initially surrounded by water, to a position in which the parallel chains are separated only by their van der Waals radii as -0.36 to -0.95 kcal./mole/ CH_2 group. This free energy is derived partly from the van der Waals forces between the components of the two chains and partly from the entropy change as water molecules, displaced from a hydrophobic environment, revert to a less ordered state in free solution. Interactions between a hydrocarbon chain and a hydrophobic region on a protein may reasonably be expected to be of the same order of magnitude as hydrocarbon-hydrocarbon interactions, but this will depend somewhat on the closeness of the 'fit' achieved between the two non-polar entities. Data collected by Webb (1963, p. 300) show a free-energy change of -0.55 kcal./mole/ CH_2 group for the binding of a series of aliphatic sulphates to serum albumin, whereas the extra binding to cholinesterase of

molecules containing a $(\text{C}_2\text{H}_5)_3\text{N}^+$ group in place of a $(\text{CH}_3)_3\text{N}^+$ group suggests an interaction of -0.34 kcal./mole/ CH_2 group (Webb, 1963, p. 288). Recently, the inhibition of pepsin by aliphatic alcohols (Tang, 1965) has been interpreted in terms of hydrophobic bonding. These alcohols inhibit competitively towards peptide substrates; the free-energy change on inhibitor binding increases linearly with hydrocarbon chain length, ΔG^0 -0.56 kcal./mole/ CH_2 group being calculated. In view of the analogous examples quoted, the observed increment in binding with increasing chain length of acyl-CoA derivatives is evidently compatible with the occurrence of hydrophobic interactions between the carnitine acetyltransferase molecule and the hydrocarbon chain on the substrate.

No evidence has been found that acyl groups attached to carnitine can be bound to the proposed hydrophobic state. Table 2 shows that K_m for CoASH and acylcarnitine substrates to be more or less independent of the nature of the acyl group involved. A tenfold diminution of V_{max} is seen with decanoyl(-)-carnitine compared with the values found with acetyl- or butyryl(-)-carnitine. As argued before, this favours the random-equilibrium mechanism for the carnitine acetyltransferase

reaction. The failure of $100\ \mu\text{M}$ -palmitoyl-DL-carnitine to inhibit the enzyme is in line with similar observations by Norum (1963) for enzyme preparations from various rat tissues. Presumably, as the binding of ligands to the carnitine and hydrophobic sites appears to be mutually exclusive, no very potent inhibition analogous to that seen with long-chain acyl-CoA and arising from the co-operative effect of binding to two sites at once would be expected. An inhibition by the hydrocarbon chain or the carnitine moiety alone would probably have K_i of the order of $300\ \mu\text{M}$, by analogy with the K_i for palmitate inhibition and the K_m values for acylcarnitine substrates. This might not have been detected at the palmitoylcarnitine concentration used, which was about as high as the limited water solubility of this compound will permit.

Finally, the physiological significance, if any, of the inhibition of carnitine acetyltransferase by long-chain acyl-CoA must be considered. Such inhibition has been made the basis of a number of possible regulatory mechanisms for several enzymes which are more or less directly involved in lipid metabolism. These include citrate synthase (Tubbs, 1963; Wieland & Weiss, 1963), acetyl-CoA carboxylase (Lynen, Matsushashi, Numa & Schweizer, 1963) and glucose 6-phosphate dehydrogenase (Eger-Neufeldt, Teinzer, Weiss & Wieland, 1965; Taketa & Pogell, 1966). The significance of such findings has recently been questioned by two groups of workers. Srere & Senkin (1964) found that preincubation of citrate synthase with palmitoyl-CoA gave rise to an enzyme form which could not be reactivated by the addition of the substrate oxaloacetate, even though this gave substantial protection when added before the inhibitor. Srere (1965) regarded this result as showing a 'non-specific' inactivation of the enzyme due to the detergent properties of the acyl-CoA. It is not clear exactly what is meant by 'non-specific' in this context as specific protection by a substrate does occur and there is, presumably, no reason why the irreversible inactivation of an enzyme should not result from a specific interaction with an effector. Indeed, the demonstration by Wieland, Weiss & Eger-Neufeldt (1964) that stearoyl-3'-dephospho-CoA does not inhibit citrate synthase suggests a very specific requirement for the CoA moiety of such inhibitors to be intact. Taketa & Pogell (1966), on the other hand, studied the effects of palmitoyl-CoA on a range of enzymes unconnected with lipid metabolism and found a quite marked inhibition of several of them, notably glutamate dehydrogenase and malate dehydrogenase, by low concentrations of this compound. Much of the inhibition observed was irreversible and substrates of the various enzymes afforded no protection. These authors

argued that such results are incompatible with a role for long-chain acyl-CoA as a metabolic regulator because its effects appear to be un-specific.

Bearing these considerations in mind, it is at least clear that the inhibition of carnitine acetyltransferase by long-chain acyl-CoA is of a classical reversible competitive type. The degree of inhibition observed is independent of time and no irreversible inactivation of the enzyme has been found. In fact, on one occasion, the incubation of a sample of carnitine acetyltransferase with $8\mu\text{M}$ -palmitoyl-CoA for 24 hr. at 0° caused no loss of activity (J. F. A. Chase, unpublished work), a result which contrasts sharply with those for citrate synthase, acetyl-CoA carboxylase and glucose 6-phosphate dehydrogenase, all of which show time-dependent inactivation. It should also be noticed that palmitoyl-CoA is a much more potent inhibitor of carnitine acetyltransferase than either palmitate or palmitoylcarnitine, molecules which might be expected to show comparable non-specific detergent effects, so that such inhibition would seem to be a specific effect by Srere's (1965) criteria.

Even if the inhibition of carnitine acetyltransferase by long-chain acyl-CoA may be taken to be specific, the consideration of the possible physiological importance of this effect is complicated by the present imprecise understanding of the primary metabolic role of this enzyme. It is tempting to postulate that its function includes the transport of acetyl-CoA out of mitochondria to the site of fatty acid synthesis (Bremer, 1962a; Bressler & Katz, 1965). If this were so, inhibition of the transferase by long-chain acyl-CoA might constitute a mechanism for feed-back control by the product of fatty acid synthesis upon the transport of precursors to the synthetic site. It is, however, far from certain that carnitine acetyltransferase has any major role in fat synthesis (Fritz, 1965) and, indeed, the tissue distribution of the enzyme (Beenackers & Klingenberg, 1964) may be taken as evidence to the contrary. The activity of the transferase in rat liver is only about 1% of that found in heart, a tissue in which little lipid synthesis takes place. Furthermore, Lowenstein and his group (Kornacker & Lowenstein, 1965; Kornacker & Ball, 1965) have found the main source of extra-mitochondrial acetyl-CoA in rat liver to be citrate, via the citrate-cleavage enzyme. It remains, however, possible that carnitine acetyltransferase is more important in this function in the livers of animals other than rat. Sauer & Erfle (1966) found the content of this enzyme in ox, sheep and guinea-pig livers to be 10–20 times that in rat, and Hardwick & Lowenstein (1966) reported very low activity of the citrate-cleavage enzyme in guinea-pig liver.

This work was carried out during the tenure of a Research Studentship from the Science Research Council. Thanks are also due to Dr P. K. Tubbs for much helpful discussion.

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