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REVIEW

Carnitine Acetyltransferase: A Review of Its Biology, Enzymology, and Bioorganic Chemistry[1]

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The review begins with brief introductory remarks about the significance of carnitine. This is followed by a historical section on its discovery and function, ending with a listing of carnitine-dependent enzymes. Carnitine acetyltransferase then becomes the entire focus of the review. The ubiquity of the protein in tissues and organelles is emphasized in an initial section. A discussion of its enzymology follows, beginning with physical properties and kinetics and ending with substrate and inhibitor specificities. The review concludes with a discussion of proposed molecular mechanisms.

INTRODUCTION

Carnitine, the biological carrier molecule of fatty acids destined for transport into and oxidation by mitochondria (*1*), is required for efficient metabolism of long-chain fatty acids (*2–4*). Tissues with inadequate concentrations of carnitine exhibit severely impaired cellular energy metabolism (*5*). Until 1973, nutritionists assumed that individuals synthesize or ingest the required amount of carnitine, but later found that human carnitine deficiency can result from both nutritional deficiencies and disease (*6, 7*). Specific genetic disorders can result in primary muscle and systemic carnitine deficiency (*3–6*). Dietary carnitine is especially required in early infancy when the capacity for carnitine biosynthesis is limited (*6*) and for patients on hemodialysis (*5*). Newborns and adults undergoing extended parenteral nutrition often show low levels of carnitine in serum and tissue (*6*). Carnitine supplementation may be required to correct these deficiencies (*6*).

An abnormal balance between carnitine and acylcarnitine tissue levels has been noted in several disease states. Advanced cirrhosis of the liver is associated with subnormal plasma carnitine concentrations (*6*). Ischemia causes an increase in fatty acyl-CoA and -carnitine esters in myocardium, resulting in unfavorable metabolic effects (*8*). Carnitine administration, on the other hand, retards ischemic damage (*5, 8*).

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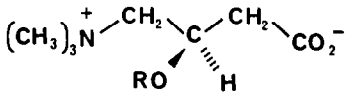


STRUCTURE **1.** R=H; carnitine.
STRUCTURE **2.** R=C(O)CH_3; acetylcarnitine.

DISCOVERY AND FUNCTION OF CARNITINE

Two groups of investigators independently discovered carnitine, **1,** in meat extracts 83 years ago (*9, 10*). Although a correct structure was proposed in 1907 (*11*), it was not proven until 1926 (*12, 13*). Interest in the metabolic significance of carnitine heightened after it was identified as the previously known growth factor, Vitamin B_T, of the meal worm (*Tenebrio molitor*) (*14*). Its ubiquity throughout the phyla and classes of the animal kingdom was subsequently demonstrated (*15*).

Carnitine is most concentrated in heart and skeletal muscles of vertebrates (*15, 16*), where it transports lipids into mitochondria (*17*). Unusually high concentrations also have been measured in the electric organ of the eel (*Torpedo occidentalis*) and the flight muscle of the fly (*Musca*) (*15*). Its presence (albeit in low concentrations) in plants (*13, 18*) suggests an even broader occurrence in all eucaryotes.

Carnitine's role in fatty acid metabolism was discovered in 1955, when Fritz (*19*) demonstrated that adding carnitine to liver slices and homogenates stimulated the oxidation of long-chain fatty acids. That same year, Friedman and Fraenkel (*20*) reported the reversible enzymatic acetylation of carnitine in the presence of coenzyme A, by crude pigeon-liver extracts (Eq. [1]), and identified the ester bond of acetylcarnitine as a high energy bond comparable with the thioester bond of acetylcoenzyme A (AcCoA):[3]

$$\text{AcCoA} + \text{carnitine} \rightleftharpoons \text{acetylcarnitine} + \text{CoA}. \qquad [1]$$

In 1962, Bremer (*17*) showed that isolated rat mitochondria from a variety of tissues catalyze the same reversible reaction (Eq. [1]). He concluded that acetylcarnitine, **2,** represents an active acetate that mitochondria efficiently utilize for oxidation, correctly surmising that because the inner mitochondrial membrane is impermeable to CoA and AcCoA, an acetylcarnitine-CoA-acetyltransferase exists. He also postulated that carnitine (as acetylcarnitine) transports acetyl groups across membranes and that because added carnitine stimulates palmitate oxidation, carnitine transports other acyl groups.

The large group-transfer potential of acetylcarnitine has been verified by thermodynamic studies using the acetyltransferase enzyme (*21, 22*) and by microcalorimetry (*23*). Table 1 compares the free energy of hydrolysis of acetylcarnitine to

[3] Abbreviations used: CAT, carnitine acetyltransferase; CPT, carnitine palmitoyltransferase; COT, carnitine octanoyltransferase; Cn, carnitine; ORD, optical rotary dispersion; HPC, hemipalmitoylcarnitinium; HAC, hemiacetylcarnitinium; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

TABLE 1

Comparison of Free Energies of Hydrolysis at pH 7.0

Ester	ΔG° (kcal/mol)			
	25°C	Ref.	38°C	Ref.
Acetylcarnitine	−7.90[a]	(*21*)	−8.20	(*22*)
Acetylcholine	−6.47	(*24*)	−6.99	(*22*)
Acetyl coenzyme A	−8.20	(*25*)	−8.54	(*22*)
ATP (terminal PO_4)	−7.30	(*24*)	−7.60	(*26*)

[a] Microcalorimetry suggests that this value is practically invariant with longer acyl chains (*23*).

that of other biologically significant esters. Acetylcarnitine has an even higher group transfer potential than either acetylcholine or the terminal phosphate of ATP. To date, no one has explained this phenomenon.

In investigating the stereospecificity of the enzyme-catalyzed acyltransferase, Bremer (*17*) found that mitochondria metabolized only one enantiomer of acetylcarnitine, from the D,L mixture (presumably the naturally occurring L-isomer). The L-isomer [also designated *l* or (−)] was subsequently shown, by synthesis from (*R*)-β-hydroxy-L-glutamic acid, to have the *R* absolute configuration (*27*).

CARNITINE SPECIFIC PROTEINS

The discovery that carnitine and its esters are involved in fatty acid metabolism has led to identification and characterization of the proteins involved. The eight proteins (or protein systems) specific to carnitine and its esters can be subdivided into five major groupings: First, a carnitine active-transport system is associated exclusively with the plasma membrane (*28–33*). Second, a carnitine–acylcarnitine translocase system is located on the inner mitochondrial membrane (*34–39*). Third, a long-chain carnitine ester hydrolase is found in liver and associated with microsomes (*40, 41*). Fourth, three unique carnitine acyltransferases are found in microsomes, peroxisomes, and mitochondria. Fifth, two degradative enzymes, carnitine dehydrogenase (EC 1.1.1.108) (*42*) and carnitine decarboxylase (EC 4.1.1.42) (*43*), are found in microbes and in insects and mammals, respectively. By comparing these enzymes and their inhibitors, we can identify the functional groups that account for molecular recognition of carnitine and acylcarnitines. In this review, we focus exclusively on carnitine acetyltransferase.

Carnitine acyltransferases. The discovery of carnitine's acetyl-carrying capacity (*17*) quickly led to the characterization of the protein, carnitine acetyltransferase (CAT,[3] EC 2.3.1.7), responsible for catalysis of acetyl transfer between carnitine and CoA (Eq. [1]) (*21, 44*). Fritz and Yue (*45*) had already demonstrated a more general acyl-carrying capacity of carnitine,

$$\text{Acyl-CoA} + \text{carnitine} \rightleftharpoons \text{acylcarnitine} + \text{CoA}, \qquad [2]$$

in heart muscle mitochondria and identified a unique long-chain transferase, carnitine palmitoyltransferase (CPT, EC 2.3.1.21). They based their conclusions on the finding that CAT does not catalyze the transfer of palmitoyl groups (*21*), whereas CPT shows maximal activity for this chain length. An enzyme specific for medium-chain (C_6–C_9) acylcarnitines, carnitine octanoyltransferase (COT, EC 2.3.1.–), was later postulated (*46, 47*), and its existence in mouse- and rat-liver peroxisomes subsequently established (*48–52*).

TISSUE AND ORGANELLAR DISTRIBUTION

Distribution in tissues. CAT is widely distributed in a variety of rat tissues, with the highest concentrations in heart and brown adipose (*16*). In addition, unusually high CAT activity is observed in testicular tissue (*16*). Rat caudal epididymal spermatozoa show the highest specific CAT activity of any tissue studied (*53*). Although the reason for CAT's presence in fatty tissues and the heart, which utilizes energy from fatty-acid metabolism, is easily understood, the function of the high concentration of CAT and its substrates in the male reproductive tract remains to be explained (*55*).

Pearson and Tubbs (*54*) suggest that CAT may buffer against rapid changes in the tissue content of AcCoA. This suggestion is based on an equilibrating ratio of CAT's substrates in rat tissues despite large variations in steady-state concentrations. They propose that CAT regulates the formation of an acetylcarnitine pool in the cystolic compartment. This pool is directly accessible to the mitochondria via the carnitine–acylcarnitine translocase and ensures a reserve of acyl energy for maintaining sperm motility. Recent studies of ram and bovine spermatozoa (*55, 56*) support these ideas.

Although CAT and carnitine are found in nerve tissue, their involvement in neurotransmission has not been substantiated (*57, 58*). Carnitine, choline, and their esters are structurally similar, but they perform different functions. Compartmentalization and the high selectivity of the carnitine and choline enzymes contribute to this difference. Whereas choline actyltransferase is found primarily in the plasma membrane, CAT is located in mitochondria and, unlike choline acetyltransferase, is uniformly distributed throughout the nervous system (*57*). Some evidence indicates that only the (*S*)-isomer of carnitine can effectively exhibit cholinergic, muscarinic activity (*59*). (*R*)-Acetylcarnitine is not a substrate for choline esterase (*60*), and choline is not an effective substrate for CAT (*61*).

Intracellular distribution. Although CAT is found primarily in mitochondria (*55, 62–65*), it occurs in other organelles. CAT has been isolated from chemically induced peroxisomes in the livers of rat (*63*) and mouse (*51*) as well as in yeast cells (*66*). In rat-liver peroxisomes, CAT is found in the soluble matrix (*48*). On the other hand, CAT from rat-liver microsomes (derived from endoplasmic reticulum) appears tightly associated with the microsomal membrane (*48*). Besides these organelles, CAT is present in pea chloroplasts (*67*).

How does CAT function in organelles other than mitochondria? Ueda *et al.* (*66*) postulate an acetylcarnitine shuttle between peroxisomes and mitochondria. Un-

der conditions of excess fatty acid metabolism, β-oxidation of acyl-CoAs located in peroxisomes leads to formation of acetylcarnitine, which is available to mitochondria. Conversely, McLaren *et al.* (*67*) suggest that CAT in the chloroplast enables acetylcarnitine originating in the mitochondria to be imported into the chloroplast.

The occurrence of CAT in different tissues and cellular organelles raises an intriguing question: Are these enzymes the same protein or isoenzymes? In mitochondria, *inner* (located on the matrix side of the inner membrane) and *outer* (located on the cytoplasmic side) pools of CAT have been postulated (*68*). Together these enzymes regulate the transfer of acetyl groups between cytoplasmic and matrix CoA (*69–71*). In nearly all studies to date, the purified CAT from different tissues (*56*) and from different organelles (*48*) is indistinguishable. Edwards *et al.* (72) have isolated mitochondrial protein from various tissues and uniformly find separate enzyme activity as soluble and membrane-associated forms. These forms have similar kinetic properties and are freely interconvertible, suggesting the existence of a single CAT. An exception may be peroxisomal and mitochondrial CAT from yeast, whose purified enzymes are easily separated by DEAE-Sephacel chromatography and may be distinct isoenzymes with similar kinetic properties (*66*).

ENZYMOLOGY

The best-characterized CATs, which have been purified from the various tissues and organelles shown in Table 2, are the pigeon-breast muscle CAT (commercially available) and rat-liver CAT. In general, CATs from different sources have similar properties and phylogenies.[4]

Molecular weights range from 51,000 to 75,000. Distinct subunits have been isolated from rat-liver mitochondria in at least two instances (*62, 63*), suggesting an $\alpha\beta$ composition. Miyazawa *et al.* (*63*) have demonstrated, however, that this *dimeric* form has kinetic properties nearly identical to those of a *monomeric* form isolated from the cell homogenate. They conclude that the method of purifying CAT from rat-liver mitochondria inadvertently cleaves the enzyme into two closely associated, but different, catalytically active subunits. (A specific protease responsible for this cleavage could not be identified.) Miyazawa *et al.* (*77*) have since demonstrated, by *in vitro* translation experiments, that rat-liver CAT is synthesized as a larger precursor with a molecular weight of 69,000, which subsequently loses a 1500 molecular weight fragment.

CAT shows a relatively broad optimal pH range, peaking at about 7.8 (Table 2). A slightly higher isoelectric point is observed, but some preparations give a minor band at about p*I* 5. The high and low p*I* bands are associated with soluble and membrane-bound forms, respectively, which are freely interconvertible and have similar kinetic properties (*48, 72*).

[4] When species differences exist they are noted; otherwise CAT refers generally to any of the enzymes in Table 2.

TABLE 2

Comparison of Purified Carnitine Acetyltransferases

Organism	Tissue–organelle	Molecular weight	Optimal pH range (pH opt)	Isoelectric point
Pigeon	Breast-muscle homogenate	55,000[a] (*44, 73*)	7.3–8.0 (7.8) (*74*)	7.9 (*48*)
		51,000[a] (*48*)		8.0[b] (*72*)
		75,000[c] (*62*)	Broad (*48*) (7.2–7.8)	
		58,000[d] (*75*)		
Pig	Heart		7.1–8.6 (7.8) (*21*)	
Mouse	Liver peroxisomes	60,000[a] (*51*)	(8.5) (*51*)	6.8 (*51*)
Yeast	Peroxisomes	60,000, 70,000[c] (*66*)	(8.0) (*66*)	
	Mitochondria	60,000, 70,000[c] (*66*)	(8.0) (*66*)	
	Cell free extract	60,000[c] (*76*)	Broad (7.2) (*76*)	
Rat	Liver mitochondria	56,000[a] (*62*)	Broad (8.0) (*63*)	
		61,900[a] (*63*)		
		34,000, 25,000[a] (*62*)		
		36,500, 27,000[c] (*63*)		
	Liver homogenate	66,100[a] (*63*)	Broad (8.0) (*63*)	
		67,500[c] (*63*)		
	Liver peroxisomes	59,000[a] (*48*)	Broad (*48*) (7.2–8.0)	8.3 (*48*)
	Liver microsomes	59,000[a] (*48*)	Broad (*48*) (7.2–8.0)	8.3 (*48*) (5.3)[e]
Bovine	Heart	61,000[c] (*56*)	7.5–8.2 (7.8) (*56*)	8.1 (*56*)
				8.1 (*72*) (5.2)[e]
	Spermatozoa	62,000[c] (*56*)	7.5–8.2 (7.8) (*56*)	8.2 (*56*)

[a] Gel filtration.
[b] A minor band at ca. p*I* 5 was observed in crude samples.
[c] Sodium dodecyl sulfate gel electrophoresis.
[d] Sedimentation–equilibrium.
[e] A minor band at ca. p*I* 5 was observed.

Acyl-group selectivity. Table 3 shows the acyl-group selectivity of CATs from several sources. The forward (from acyl-CoA) and reverse (from acylcarnitine) reactions are quite similar. Yeast CAT has exceptional selectivity for acetyl, whereas for most of the isolated CATs, C_3 or C_4 rather than C_2 (acetyl) is the optimal substrate. Chain lengths greater than C_4 show diminished activity. Neither palmitoylcarnitine (C_{16}) nor palmitoyl-CoA is a substrate, but the latter is a potent inhibitor (see Effect of chain length).

Reaction parameters. For the pigeon enzyme, Chase (*73*) finds a turnover number of 23,000 per minute for the forward reaction (pH 7.8 and 30°C). The reverse reaction shows a turnover number of 29,000 per minute; thus, the overall reaction (Eq. [1]) is readily reversible, with an equilibrium constant of 1.71 (*21, 79*). An Arrhenius plot for the forward reaction catalyzed by rat-liver mitochondrial CAT reveals an activation energy of 11.4 kcal/mol (*62*). The optimal reaction temperature for the rat enzyme is 37°C, yet thermal inactivation begins at 40°C (*62*). A crude preparation of CAT from human platelets has shown even more thermosensitivity (*80*).

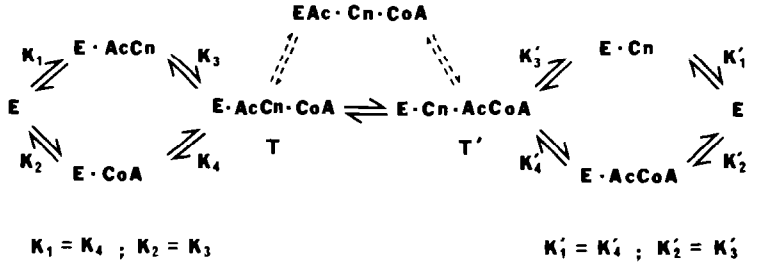
TABLE 3

Acyl-Group Selectivity of Carnitine Acetyltransferase Measured as Percentage of Velocity of the Acetyl Reaction

Organism	Tissue–organelle	Acyl-chain length												
		C_2[a]	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{12}	C_{14}	C_{16}	(Ref.)
Pigeon	Breast muscle homogenate	100[b]	77	41	18	13	11	8	5.5	4	<0.1	0	0	(*78*)
Pig	Heart	100[c]	123	100	37	23		13		3			0	(*21*)
Mouse	Liver peroxisomes	100[c,d]	121	132	47	26		24		16	1	0	0	(*51*)
		100[d,e]	141	115		17		25		17	7	3	0	(*51*)
Yeast	Peroxisomes	100[c]	9.4	0.12	0	0				0			0	(*66*)
	Mitochondria	100[c]	15	0.26	0	0				0			0	(*66*)
Rat	Liver mitochondria[f]	100[c,d]		124		15		7		2	0	0	0	(*63*)
		100[d,e]		117		28		17		6	2	0	0	(*63*)
	Liver homogenate[f]	100[c,d]		114		30		16		5	2	0	0	(*63*)
		100[d,e]		104		33		24		9	3	<1	0	(*63*)
	Liver													
	Peroxisomes	100[c]	110	82		14		3		0	0	0	0	(*48*)
	Microsomes	100[c]	112	75		17		3		0	0	0	0	(*48*)
Bovine	Heart	100[c,d]	147	114				26					0	(*56*)
	Spermatozoa	100[c,d]	144	121	52			24					0	(*56*)

[a] The reaction velocity for acetyl (C_2) is taken as 100%.
[b] Values are based on V'_{max} for the forward reaction.
[c] Initial velocity from forward reaction (acyl-CoAs).
[d] Graphical interpolation from the reference given.
[e] Initial velocity from reverse reaction (acyl-Cns).
[f] Enzyme from mitochondria was isolated in a dimeric form; the homogenate gave a single monomer (see text).

Kinetic enzymatic mechanism. Because the catalyzed reaction is reversible, product inhibition is readily apparent. For pigeon CAT, Chase and Tubbs (*79*) have determined that the reaction follows a random-order equilibrium (random Bi–Bi) mechanism. In this mechanism, Michaelis constants (K_m's) approximate true dissociation constants (K_s's), and binding of one substrate has little or no effect on binding of the second (i.e., $K_m \sim K_s$). Scheme I depicts Chase and Tubb's proposed mechanism of action for CAT (*79*). The observed kinetics are consistent with a rate-determining interconversion of the ternary complexes, T and T′ (i.e., acyl transfer is rate limiting). An acyl-enzyme intermediate cannot be excluded, but must arise via the ternary-enzyme complexes. The velocity in a



SCHEME I. Chase and Tubbs proposed random Bi–Bi mechanism for pigeon CAT (*79*). For clarity, free substrates and products are omitted in the equilibria.

random Bi–Bi system is described by Eq. [3], where K_A and K_B are K_m's for substrates A and B, respectively; Eq. [4] applies under conditions of competitive inhibition, where substance I competes with substrate A:

$$v = \frac{V_{max}}{\left(1 + \frac{K_A}{[A]}\right)\left(1 + \frac{K_B}{[B]}\right)} \quad [3]$$

$$v = \frac{V_{max}}{\left(1 + \frac{K_A}{[A]} + \frac{K_A[I]}{K_I[A]}\right)\left(1 + \frac{K_B}{[B]}\right)}. \quad [4]$$

Chase and Tubbs (*79*) observed competitive inhibition between (*R*)-carnitine and (*R*)-acetylcarnitine as well as between CoA and AcCoA, indicating separate recognition sites for the respective pairs. They also observed competitive inhibition of AcCoA with (*R*)-acetylcarnitine, results that indicate overlap of the acetyl groups and a common acetyl-recognition site.

Huckle and Tamblyn (*56*) have reported somewhat different kinetics for the bovine enzyme. Product inhibition appears mixed because one substrate's binding *depends* upon the second's concentration. As seen in Table 4, K_m's are less than K_s, indicating that binding of one substrate is enhanced in the presence of the second. For this enzyme, K_m's represent binding only in the presence of bound cosubstrate, as for example with carnitine:

$$E \cdot AcCoA \cdot Cn \overset{K_m}{\rightleftharpoons} E \cdot AcCoA + Cn,$$

where E represents CAT, and Cn represents carnitine. As usual, K_s describes binding of a substrate to free enzyme:

$$E \cdot Cn \overset{K_s}{\rightleftharpoons} E + Cn.$$

K_i's in Table 4 are defined as follows, where the pertinent equilibrium for carnitine is used as an example:

$$E \cdot CoA \cdot Cn \overset{K_i}{\rightleftharpoons} E \cdot CoA + Cn.$$

TABLE 4

Kinetic Constants of Bovine Carnitine Acetyltransferase: Data from Huckle and Tamblyn (*56*)

Enzyme source	Substrate	K_m (μM)	K_s (μM)	K_i (μM)
Spermatozoa	(*R*)-Cn	120	270	310
	(*R*)-AcCn	360	520	980
	AcCoA	12	27	52
	CoA	9.0	13	15
Heart	(*R*)-Cn	120	270	250
	(*R*)-AcCn	300	740	810
	AcCoA	20	43	47
	CoA	9.6	23	21

TABLE 5

Comparison of Michaelis Constants for Substrates of Purified Carnitine Acetyltransferases

Organism	Tissue–organelle	K_m (μM)				Ref.
		(*R*)-AcCn	CoA	(*R*)-Cn	AcCoA	
Pigeon	Breast-muscle homogenate	350	37	120	34	(*78, 79*)
Pig	Heart	310		310	41	(*61*)
Mouse	Liver peroxisomes	700	180	86	15.3	(*51*)
Yeast	Peroxisomes	417	304	719	42	(*66*)
	Mitochondria	639	257	622	36	(*66*)
	Cell free extract			3300[a]	130	(*76*)
Rat	Liver mitochondria	280		720[a]	30	(*62*)
		660	32			(*63*)
	Liver homogenate	390	28			(*63*)
	Liver peroxisomes			143	69	(*48*)
	Liver microsomes			150	69	(*48*)
Bovine	Heart	300[b]	9.6[b]	120[b]	20[b]	(*56*)
	Spermatoza	360[b]	9.0[b]	120[b]	12[b]	(*56*)

[a] Value for (*RS*)-carnitine.

[b] Substrate binding to the enzyme depends upon the concentration of the second substrate.

This latter form of binding is the least favorable for the bovine enzyme. For instance, although two acetylated substrates can bind simultaneously, each interferes with binding of the other.

Table 5 shows K_m's for purified CATs obtained from several sources. All CATs bind CoA (or AcCoA) more tightly than they bind carnitine (or acetylcarnitine), probably because of the greater molecular surface area of CoA. Compared to the other enzymes, both mouse and yeast CATs show markedly different K_m's for CoA and AcCoA. The lower value for AcCoA may result from a reorganization of the enzyme that is contingent upon a filled acetyl site or perhaps from a tight acetyl-recognition site.

Enzyme ORD spectrum. Tipton and Chase (*81*) have studied the effect of substrates on the optical rotatory dispersion (ORD) spectrum of pigeon CAT. (*R*)-Carnitine or (*R*)-acetylcarnitine causes a large perturbation in the spectrum, but neither CoA nor AcCoA, alone or in the presence of carnitine or acetylcarnitine, causes a change. The direction of change in the reduced mean residue rotation at 233 nm, $[M']_{233}$, suggests that binding of carnitine or acetylcarnitine causes the enzyme to unfold. Tipton and Chase (*81*) have determined K_s's for (*R*)-carnitine, (*S*)-carnitine, and (*R*)-acetylcarnitine by plotting the change in $[M']_{233}$ with degree of enzyme saturation. These values, shown in Table 6, closely agree with the kinetic constants and therefore support the random Bi–Bi mechanism.

In addition, the data show that (*S*)-carnitine binds as well as (*R*)-carnitine to pigeon CAT. As we previously proposed (*82*), this indiscriminate binding between (*R*)- and (*S*)-carnitine by pigeon CAT indicates a two-point recognition of carni-

TABLE 6

Comparison of Kinetic and Dissociation Constants for Carnitine Acetyltransferase from Pigeon-Breast Muscle

Substrate	Kinetic		Dissociation[a]	
	K_m or K_i (μM)	Ref.	K_s (μM)	Ref.
(*R*)-Carnitine	120	(*78*)	115	(*81*)
(*S*)-Carnitine	173[c]	(*79*)	106	(*81*)
(*R*)-Acetylcarnitine	350	(*78*)	312	(*81*)
(*R*)-Carnitine/CoA[b]			108	(*81*)

[a] Determined from change in ORD upon addition of variable quantities of substrate (*81*).

[b] Carnitine in the presence of 250 μM CoA.

[c] The K_i for (*S*)-Cn may deviate from K_s because of the absence of an enzymatic standardization procedure for this substance (*81*).

tine, involving the trimethylammonio and carboxylato groups. The AcCoA recognition site is the third locus required for chiral recognition (Fig. 1).

Inhibition (S)-isomers. Our model of the active site (Fig. 1) accounts for the inhibition by (*S*)-carnitine and (*S*)-acetylcarnitine of CATs from several sources. As Table 7 shows, pigeon CAT indiscriminately binds both enantiomers of carnitine or acetylcarnitine; yet the (*S*)-isomers do not undergo acetyl transfer. In contrast, the pig, bovine, and mouse enzymes discriminate substantially between (*S*)- and (*R*)-carnitine. These enzymes have a more restricted two-point recognition site or a three-point recognition site. The (*S*)-isomer competitively inhibits all CATs with respect to the (*R*)-isomer.

pH dependence. Chase (*74*) has studied the pH dependence of pigeon CAT over the pH range 6.0 to 9.0. The enzyme loses activity in solutions with a pH outside this range. He finds a constant V'_{max} for the reverse reaction over the range 6.0 to 8.8. Hence, the pH-activity curve reflects only changes in CAT's affinity for its

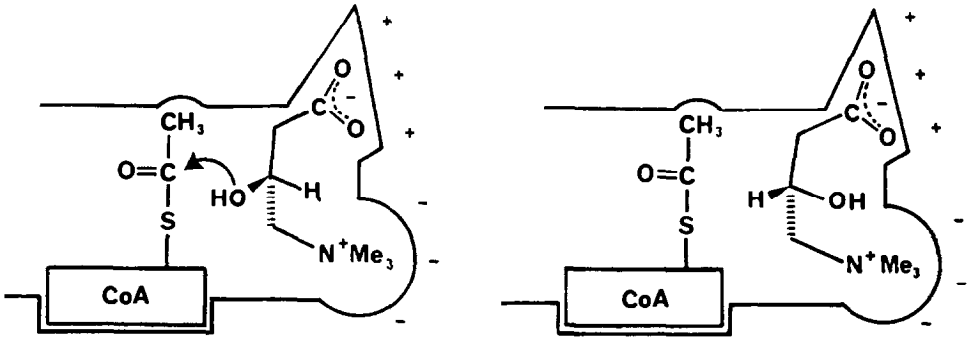


FIG. 1. Mode of chiral recognition in pigeon CAT.

TABLE 7

Competitive Inhibition of Carnitine Acetyltransferase by (*S*)-Carnitine and (*S*)-Acetylcarnitine

Inhibitor	K_i (μM)	Competitive with	K_m (μM) for (*R*)-substrate	Enzyme source	Ref.
(*S*)-Carnitine	1100	(*R*)-Cn	120	Bovine heart or spermatozoa	(*56*)
	2300	(*R*)-Cn	310	Pig heart	(*61*)
	1000	(*R*)-Cn	86	Mouse-liver peroxisomes	(*51*)
	173	(*R*)-Cn[a]	120	Pigeon breast	(*79*)
	106	[b]	120	Pigeon breast	(*81*)
(*S*)-Acetylcarnitine	256	(*R*)-AcCn[a]	350	Pigeon breast	(*79*)

[a] The authors reported apparent uncompetitive inhibition for (*S*)-carnitine with (*RS*)-carnitine and (*S*)-acetylcarnitine with (*RS*)-acetylcarnitine. The results, however, fit well to a competitive model, Eq. [4] (*73*).

[b] True dissociation constant measured by enzyme ORD.

substrates and not in its chemical catalysis. Chase concludes that no ionizing group on the enzyme with pK_a between 6.1 and 8.3 participates in the catalytic step, but does not rule out the possibility that ionizable groups outside of this range participate.

Chase implicates the following groups in the binding of substrates: (i) A group with pK_a 6.4, active in the basic form, is unequivocally identified as the 3′-phosphate of AcCoA. The lack of this group in acetyl-3′-dephospho-CoA results in a 300-fold reduction in binding efficiency without appreciably changing V'_{max} (Table 8). (ii) An unidentified ionizable group on the enzyme with an apparent pK_a of 7.85 becomes more basic (pK_a 8.25) upon binding of AcCoA or CoA. (iii) A group on the enzyme, active in its basic form, with pK_a of 7.2, (an imidazolyl or an α-ammonium group) is required for optimal binding of carnitine or acetylcarnitine.

Inhibition of dications. Sulfhydryl ligating dications such as Zn^{2+} and Hg^{2+} are inhibitors of CAT from bovine (*56*) and pig (*61*) heart, respectively (Table 9). For example, Zn^{2+} inhibits the bovine enzyme 50% (forward reaction) at a concentration of only 1–2 μM.[5] Only slight effects are noted for Ca^{2+} and Mg^{2+} on the same enzyme (*56*). Farrell *et al.* (*51*) have shown that Ca^{2+}, Mg^{2+}, and Mn^{2+} do not inhibit mouse CAT, whereas Zn^{2+} (50 μM) inhibits the *reverse* reaction. Complexation with the sulfhydryl of CoA cannot be excluded. Unlike avian and mammalian CATs, yeast CAT is sensitive to both Ca^{2+} and Mg^{2+} (*66*).

Sulfhydryl-specific reagents. CAT is sensitive to a wide spectrum of organic sulfhydryl-specific reagents. Table 8 lists two sulfhydryl-reactive compounds (methoxycarbonyl-CoA disulfide and CoA disulfide-*S*,*S*-dioxide), which are CoA analogs, and Table 9 shows several other reagents that have been successfully employed on CAT from various sources. The former are active-site specific and therefore suicide substrate inhibitors, and the latter are general reagents. All inactivate CAT, typically by interaction (or reaction) with one essential sulfhydryl

[5] This value is about 100 times the concentration of enzyme under typical assaying conditions.

TABLE 8

The Action of Coenzyme A Analogs on Pigeon-Breast-Muscle Carnitine Acetyltransferase

CoA Derivative		K_i or K_m (μM)	Mode of inhibition	Ref.
Name	Structure			
Chloroacetyl-CoA	CoAS—C(O)CH_2Cl	[a,b]	Form. of *S*-carboxymethyl-CoA Cn ester	(*75*)
Bromoacetyl-CoA	CoAS—C(O)CH_2Br	[a]	Form. of *S*-carboxymethyl-CoA Cn ester	(*75*)
Methoxycarbonyl-CoA disulfide	CoAS—S—C(O)OCH_3	[a]	Modified enzyme enz-SS-CoA	(*83*)
CoA disulfide-*S*,*S*-dioxide	CoAS$(O)_2$—S—CoA	[a]	Modified enzyme enz-SS-CoA	(*84*)
Desulfo-CoA	CoA—CH_3	23	Competitive with AcCoA	(*85*)
S-Dimethylarsino-CoA	CoAS—As$(CH_3)_2$	41	Competitive with AcCoA	(*86*)
S-Acetonyl-CoA	CoAS—CH_2C(O)CH_3	152	Competitive with AcCoA	(*87*)
Palmitoyl-CoA	CoAS—C(O)$C_{15}H_{31}$	0.43[c]	Competitive with AcCoA and (*R*)-Cn	(*78*)
Acryloyl-CoA	CoAS—C(O) 	47	Alternative substrate V'_{max} 9% of AcCoA[d]	(*88*)
4-Pentenoyl-CoA	CoAS—C(O) 	35	Alternative substrate V'_{max} 22% of AcCoA[d]	(*88*)
Sorboyl-CoA	CoAS—C(O) 	33	Alternative substrate V'_{max} <1% of AcCoA	(*78*)
Cyclopropanecarbonyl-CoA	CoAS—C(O) 	30	Alternative substrate V'_{max} 2% of AcCoA[d]	(*88*)
Cyclobutanecarbonyl-CoA	CoAS—C(O) 	31	Alternative substrate V'_{mx} 7% of AcCoA[d]	(*88*)
Acetyl-3′-dephospho-CoA		1300	Alternative substrate V'_{max} 97% of AcCoA	(*74*)

[a] Suicide substrate inhibitor.
[b] The apparent K_s, 113 μM, indicates that binding of (*R*)-Cn is limiting.
[c] K_s, palmitoyl-CoA is not a substrate; initial binding of palmitoyl-CoA is indicated by the kinetics.
[d] V'_{max}'s, originally reported for butyryl (C_4) = 100% (*88*), are corrected by 41% based on the results of Chase (*74*) for AcCoA = 100%.

residue in or near the active site (*83*, *84*). Methoxycarbonyl-CoA disulfide reacts with a nucleophilic sulfhydryl to give methanol, carbonyl sulfide, and a covalent enzyme disulfide derivative (*83*):

$$\text{Enz-SH} + \text{CoAS-SC(O)OCH}_3 \rightarrow \text{Enz-SSCoA} + \text{CH}_3\text{OH} + \text{COS}. \quad [5]$$

CoA disulfide-*S*,*S*-dioxide yields the same enzyme derivative but splits off CoA sulfenic acid as by-product (*84*):

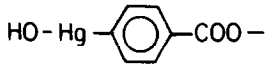
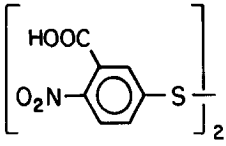
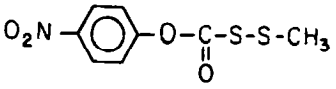
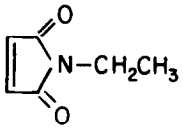
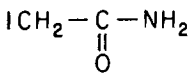
$$\text{Enz-SH} + \text{CoAS-S(O}_2\text{)-CoA} \rightarrow \text{Enz-SSCoA} + \text{CoA-SO}_2\text{H}. \quad [6]$$

CoA analogs react with pigeon CAT in both site-specific and non-site-specific fashions. Thus, either compound, when added in stoichiometric amounts, modifies a single sulfhydryl with concomitant loss of about 80% enzyme activity. When an excess of reagent is employed, however, the reaction kinetics for inactivation are biphasic. A fast bimolecular reaction with one specific active-site sulfhydryl precedes a slower reaction involving other sulfhydryls of the protein (*83*, *84*). Extrapolation to zero activity suggests a total of ten[6] modifiable sulfhydryl groups

[6] The complete titration of pigeon CAT with the non-site-specific reagent DTNB indicates 11 modifiable sulfhydryl groups (*83*). Amino acid analysis of the rat-liver enzyme reveals only six cysteine residues (*63*).

TABLE 9

Sulfhydryl-Specific Inhibitors of Carnitine Acetyltransferase

Name	Structure	Enzyme source	Ref.
Zinc chloride	$ZnCl_2$	Bovine heart	(*56*)
Mercuric chloride	$HgCl_2$	Pig heart	(*61*)
p-Hydroxymercuribenzoate		Pig heart Rat liver Yeast[a]	(*61*) (*62*) (*66*)
5,5′-Dithiobis(2-nitrobenzoic acid)		Pig heart Mouse liver Bovine heart Pigeon breast	(*21*) (*51*) (*56*) (*83*)
p-Nitrophenoxycarbonyl methyl disulfide		Pigeon breast	(*83*)
N-Ethylmaleimide		Pig heart Rat liver	(*61*) (*62*)
Iodoacetamide		Pig heart Rat liver Pigeon breast	(*61*) (*62*) (*89*)

[a] The authors report the use of *p*-chloromecuribenzoic acid, which can be converted to *p*-hydroxymercuribenzoic acid by ligand exchange in water.

(*83*). Because the rate of modification of the first sulfhydryl group is only an order of magnitude faster than that of the others, the active-site sulfhydryl is not excessively nucleophilic (*83*). For example, *S*-dimethylarsino-CoA (Table 8), a reagent selective for free sulfhydryl groups (Eq. [7]), does not react with CAT (*86*):

$$\text{Enz-SH} + \text{CoAS-As(CH}_3)_2 \rightarrow \text{Enz-S-As(CH}_3)_2 + \text{CoA}. \qquad [7]$$

Substrates partially protect CAT against inactivation from most sulfhydryl reagents, presumably by blocking these reagents' attack at the active site (*51, 61, 83*). For instance, pigeon CAT is protected from the action of methoxycarbonyl-CoA disulfide by (*RS*)-acetylcarnitine and AcCoA but not by (*R*)-carnitine, implying that the active-site sulfhydryl is close to the acetyl recognition site (*83*).

When the inhibitor forms enzyme–inhibitor disulfide linkages (for instance, DTNB), CAT can often be reactivated by adding disulfide-exchange reagents such as dithiothreitol (*84*) (Cleland's reagent), dithioerythritol (*83*), thiocholine (*83*), and mercaptoethanol (*61, 62*).

CoA analogs. Table 8 lists several other CoA and AcCoA analogs that react with pigeon CAT. Bromoacetyl- and chloroacetyl-CoA are suicide substrates that

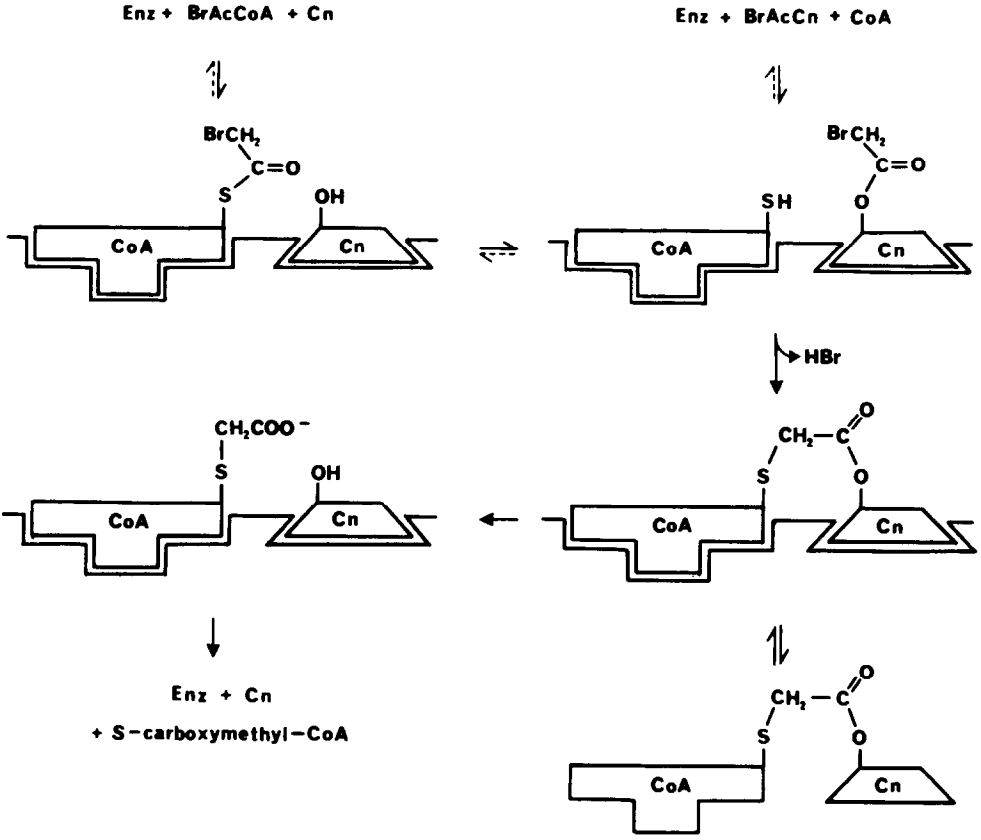
react with carnitine to form an enzyme-bound bisubstrate inhibitor. The modes of action of these compounds and of bromoacetylcarnitine, which reacts analogously, are discussed together below (see Suicide substrate inhibitors). Acetyl-3′-dephospho-CoA is an alternative substrate which binds poorly to CAT, but once bound reacts with a V'_{max} 97% that of AcCoA. Clearly the 3′-phospho site is a recognition point, but the 3′-phosphate is not essential for chemical steps. Unsaturated esters such as acryloyl-, 4-pentenoyl-, and sorboyl-CoA bind as tightly to CAT as their saturated analogs, propanoyl- (37 μM), pentanoyl- (33 μM), and hexanoyl-CoA (33 μM) (*74*). 4-Pentenoyl-CoA shows relative activity similar to that of pentanoyl-CoA base on V'_{max} (22% vs 18%), but both acryloyl- and sorboyl-CoA exhibit diminished activities compared to their saturated counterparts (9% vs 77% and 1% vs 13%, respectively). The velocity difference does not result from an inductive effect because an α,β-unsaturated carbonyl is more electrophilic than a saturated carbonyl. We speculate that an α,β-unsaturated carbonyl might undergo reversible conjugate addition, which would compete with acyl transfer. Cyclopropanecarbonyl- and cyclobutanecarbonyl-CoA bind to CAT as tightly as does AcCoA, but show markedly reduced activities based on V'_{max}. (Cyclopropanecarbonyl- and cyclobutanecarbonylcarnitine bind less tightly than (*R*)-acetylcarnitine—975 and 437 μM vs 350 μM.) Palmitoyl-CoA is not a substrate of CAT, but binds quite tightly (K_i = 0.43 μM), about two orders of magnitude better than AcCoA, with which it competes. Palmitoyl-CoA also competes with (*R*)-carnitine, an inhibition observed with other long-chain acyl-CoAs, (see discussion in the following section). Desulfo-, *S*-dimethylarsino-, and *S*-acetonyl-CoA (Table 8) are purely competitive inhibitors, which is surprising because the latter two have electrophilic moieties that react with sulfhydryls. The lack of reactivity of these compounds with CAT suggests that no sulfhydryl on the enzyme is involved in the acetyl transfer between carnitine and CoA (Scheme I).

Effect of chain length. Palmitoyl-CoA inhibits CAT purified from bovine (*56*), rat (*62*), and pigeon (*78*) sources. It competes with both AcCoA (K_i = 18.6 μM) and (*RS*)-carnitine (K_i = 8.0 μM) in rat-liver CAT. For bovine CAT, it is competitive with (*R*)-carnitine (K_i = 1.6 μM) and is noncompetitive with respect to AcCoA, showing mixed inhibition (K_i, slope = 1.4 μM; K_i, intercept = 4.2 μM). These latter values and that observed for pigeon CAT (K_i = 0.43 μM, competitive for free enzyme with either substrate) are below the critical micelle concentration (4 μM) of palmitoyl-CoA determined in similar buffers (*90*). Therefore, specific interactions with the enzyme rather than sequestration of substrates or enzyme cause the observed inhibition.

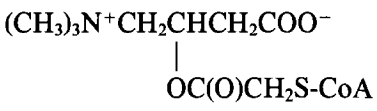
Chase (*78*) varies acyl-CoA-chain length and finds that the K_m for (*R*)-carnitine is nearly invariant (136 ± 29 μM) over the range C_2 to C_{10} and the K_m's for acyl-CoAs are invariant over the range C_2 to C_7 (38 ± 6 μM). Nevertheless, V'_{max} decreases 10-fold over this latter range. As their chain length increases from C_8 to C_{16}, acyl-CoAs bind more tightly to free enzyme (K_m's drop rapidly). The $\Delta G°$ of binding changes linearly with each additional methylene, −0.31 kcal/mol per CH_2, beyond C_8. Chase (*78*) concludes that CAT contains a hydrophobic region that interacts with the side chain of palmitoyl-CoA and thereby hinders the binding of carnitine. Mittal and Kurup (*62*) reach the same conclusions for rat-liver CAT.

Chase (*78*) proposes an inhibition mechanism involving carnitine and hydrocarbon recognition sites. Competition between the sites is regulated by conformational changes in the protein (*81*). Because palmitoylcarnitine is not an inhibitor or substrate (*78*), binding at the carnitine site disrupts the remote hydrocarbon recognition site. Correspondingly, a filled hydrocarbon site may either hinder access to the carnitine site or prevent the enzyme's conformational changes that are required for proper carnitine binding. Because palmitate ion only moderately inhibits pigeon CAT [K_i = 325 μM, competitive with respect to (*R*)-carnitine] (*78*) and is not an inhibitor of rat-liver CAT (*62*), a filled CoA site is required for the overall inhibition mechanism. The physiological relevance of this inhibition by acyl-CoAs is presently unknown.

Suicide substrate inhibitors. One of the earliest examples of a bisubstrate analog (*92*) (i.e., a single compound that resembles two separate substrates with the same relative juxtaposition as the ternary complex) is *S*-carboxymethyl-CoA-(*R*)-carnitine ester (**3**), discovered by Chase and Tubbs (*75, 91*). Bromoacetyl-CoA or bromoacetyl-(*R*)-carnitine react to form **3**, as shown in Scheme II. Byers (*92*)



SCHEME II. Chase and Tubbs' proposed inhibition of CAT by bromoacetyl derivatives of CoA and (*R*)-carnitine (*75*). Cn, (*R*)-Carnitine; BrAcCn, (*R*)-bromoacetylcarnitine; BrAcCoA, bromoacetyl-CoA.



STRUCTURE 3

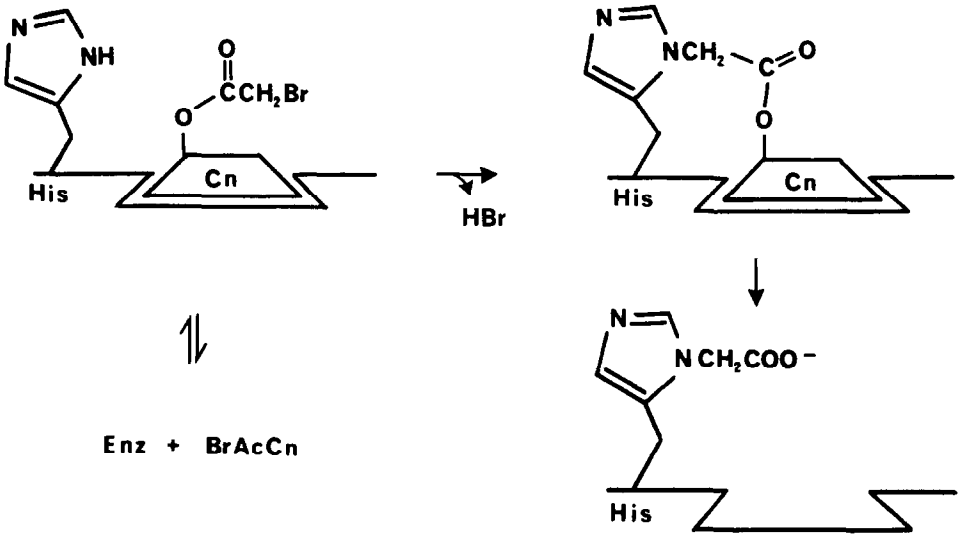
points out that bisubstrate analogs may have a lower K_i than the product of the K_s's of the two substrates. Compound **3** binds to the enzyme with an estimated K_i less than the product of the individual binding constants [K_m(Cn)·K_m(CoA) = 0.012 μM] (*75*). Bisubstrate formation is probably accompanied by a conformational change in the EI complex that enhances binding (*75*). Slow hydrolysis (half-life about 14.8 days) of the bisubstrate occurs in pH 7 buffer at 4°C and leads to recovery of enzyme activity and formation of *S*-carboxymethyl-CoA (*75*). Chase and Tubbs (*75*) find that chloroacetyl-CoA reacts the same as bromoacetyl-CoA but that (*S*)-bromoacetylcarnitine shows only minor activity, due perhaps to a trace impurity of the (*R*)-isomer.

When CAT is incubated with **3** in pH 7.0 buffer, inactivation peaks at 86% after 26 h. A large kinetic barrier to forming (or dissociating) the EI complex may result because the enzyme must undergo a substantial conformational change. The formation of enzyme-bound **3** from bromoacetyl substrates confirms the existence of the ternary complexes shown in Scheme I.

Chase and Tubbs (*89*) find that when CoA is absent, (*R*)-bromoacetylcarnitine irreversibly inhibits pigeon CAT. The bromoester alkylates a specific active-site histidine, as depicted in Scheme III. Slow hydrolysis (over a period of hours) of this adduct yields inactive alkylated enzyme. Isolation of *N*-3-carboxymethylhistidine after complete acid hydrolysis of the protein verifies the mechanism. A catalytic histidine (or a blocked active site) is implied because the alkylated enzyme is inactive. This histidine may be the group with a pK_a of 7.2 that was identified in the pH-activity study (*74*). Chase and Tubbs (*89*) suggest this histidine is a catalyst in the molecular mechanism; if so, it cannot be the pK_a 7.2 residue, which is involved only in substrate binding.

Competitive carnitine analogs. Studies of carnitine analogs with CAT have detailed the criteria for molecular recognition at its active site. Table 10 lists compounds that have been examined, mostly on CAT from pig heart (*61*). The only inhibitor that binds more tightly than the natural substrate is acetylaminocarnitine (acetoxy of acetylcarnitine replaced by acetamido). It is the best competitive inhibitor reported to date, binding 13-fold more tightly than (*R*)-acetylcarnitine to pigeon CAT and 6-fold more tightly to the rat enzyme (*93*). Because amides have a lower group-transfer potential than esters, acetyl transfer from acetylaminocarnitine does not occur.

Deoxynorcarnitine and γ-butyrobetaine are inactive with pig-heart CAT because, they lack the essential hydroxyl group. Their relatively high K_i's [factors of 4 and 8, respectively, larger than the K_m of (*R*)-carnitine] result partly from the loss of favorable interactions between the protein and hydroxy of carnitine. Similarly, (*S*)-carnitine has a K_i for pig-heart CAT exactly equal to that of γ-butyrobe-



SCHEME III. Mechanism proposed by Chase and Tubbs (*89*) for inhibition of CAT by (*R*)-bromoacetylcarnitine in the absence of CoA.

taine (Table 7). γ-Butyrobetaine inhibits pigeon CAT (*95*) with a K_i similar to that for the pig enzyme. Because pigeon CAT binds both (*S*)- and (*R*)-carnitine 10-fold better than γ-butyrobetaine, CAT's recognition of chirality is distinct from its recognition of trimethylammonium and carboxylate. When pig CAT binds substrates, it distinguishes chirality as well as carboxylate and trimethylammonium.

Choline and (*RS*)-acetyl-β-methylcholine are extremely poor inhibitors, showing K_i's much greater than the K_m of (*R*)-carnitine. This weak binding implies

TABLE 10

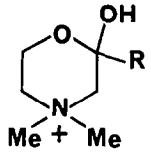
Compounds Tested as Potential Inhibitors of Carnitine Acetyltransferase

Name	Structure	K_i (μM)	Mode of inhibition	Enzyme source	Ref.
(*R*)-Bromoacetylcarnitine			Suicide substrate[a]	Pigeon breast	(*75, 89*)
(*S*)-Bromoacetylcarnitine			Unreactive	Pigeon breast	(*75, 89*)
(*RS*)-Acetylaminocarnitine		24	Comp. with (*R*)-AcCn	Pigeon breast	(*93*)
		130	Comp. with (*R*)-AcCn	Rat liver	(*93*)
Deoxynorcarnitine	$(CH_3)_2N(CH_2)_3COOH$	1200	Comp. with (*RS*)-Cn	Pig heart	(*61*)
γ-Butyrobetaine	$(CH_3)_3N^+(CH_2)_3COOH$	2300	Comp. with (*RS*)-Cn	Pig heart	(*61*)
Choline	$(CH_3)_3N^+(CH_2)_2OH$	27000	Comp. with (*RS*)-Cn	Pig heart	(*61*)
(*RS*)-Acetyl-β-methylcholine		71000	Comp. with (*RS*)-Cn	Pig heart	(*61*)
(*RS*)-β-Hydroxybutyric acid		[b]		Pig heart	(*61*)
(*trans*)-Crotonyl betaine	$(CH_3)_3N^+CH_2CH{=}CHCOO^-$		Inactive	Pigeon breast	(*94*)
(*RS*)-Carnitine nitrile	$(CH_3)_3N^+CH_2CH(OH)CH_2CN$	71000	Comp. with (*RS*)-Cn	Pig heart	(*61*)
(*RS*)-Norcarnitol	$(CH_3)_2NCH_2CH(OH)CH_2OH$	[b]		Pig heart	(*61*)
(*RS*)-Norcarnitine methyl ester	$(CH_3)_2NCH_2CH(OH)CH_2COOCH_3$	[b]		Pig heart	(*61*)
(*RS*)-Norcarnitinamide	$(CH_3)_2NCH_2CH(OH)CH_2CONH_2$	[c]		Pig heart	(*61*)

[a] Consult text for a complete description.
[b] Not an inhibitor up to 2000 μM concentration.
[c] Not an inhibitor up to 4000 μM concentration.

TABLE 11

Alternative Substrates of Carnitine Acetyltransferase

Name	Structure	K_m (μM)	Percentage relative activity[a]	Enzyme source	Ref.
(*RS*)-Thiocarnitine	$(CH_3)_3N^+CH_2CH(SR)CH_2COO^-$ R=H	170	3.3	Pigeon breast	(*97, 98*)
(*RS*)-*S*-Acetylthiocarnitine	R= —C(O)CH_3	140	100	Pigeon breast	(*97, 98*)
(*R*)-Thiocarnitine		190	4.5	Pigeon breast	(*98*)
(*R*)-*S*-Acetylthiocarnitine		100	95	Pigeon breast	(*98*)
(*RS*)-Aminocarnitine	$(CH_3)_3N^+CH_2CH(NH_2)CH_2COO^-$	3,800	7.5[b]	Pigeon breast	(*93*)
		4,050[c]		Pigeon breast	(*93*)
(*RS*)-Norcarnitine	$(CH_3)_2HN^+CH_2CH(OH)CH_2COO^-$	3,200	~26[d]	Pig heart	(*61*)
(*RS*)-γ-Amino-β-hydroxybutyrate (GABOB)	$H_3N^+CH_2CH(OH)CH_2COO^-$	40,000	<1[e]	Pig heart	(*61*)
	(*RS*)-$R_1R_2R_3N^+CH_2CH(OH)CH_2COO^-$	300–2,000[f]	100–<9[f]	Pigeon breast	(*99*)
				Rat spermatozoa	(*99*)
(*RS*)-Sulfocarnitine	$(CH_3)_3N^+CH_2CH(OH)CH_2SO_3^-$	25,000	<10[b]	Pigeon breast	(*95, 100, 101*)
(*R*)-Sulfocarnitine			0	Pigeon breast	(*100, 101*)
Hemicholiniums	R	X	3,000–4,000[g]	4–8[b,h]	Pigeon breast	(*94*)
	H	H			
	CH_3	OCH_3			
	CF_3	$NHC(O)CH_3$			
		NH_3+			
		Cl			
		NO_2			
		CN			

[a] V'_{max} of (*R*)-Cn and (*R*)-AcCn or (*RS*)-Cn and (*RS*)-AcCn are taken as 100%.

[b] Relative activity compared to V'_{max} of (*R*)-Cn.

[c] K_i (μM) for competition with (*R*)-Cn.

[d] Initial velocities interpreted from double-reciprocal plots. V'_{max} is equal to that obtained for (*RS*)-Cn. Ratio of K_m's gives 19% activity.

[e] Determined from ratio of K_m's, (*R*)-Cn/(*RS*)-GABOB.

[f] A range of values occurrs for increasing bulk and rigidity of nitrogen substituents (consult reference for specific structures).

[g] K_i's.

[h] A narrow range of activity is observed, extending from 4.1 for R=CF_3 to 8.0 for R=H.

CAT's recognition of the carboxy of carnitine. (*RS*)-β-Hydroxybutyric acid is also a poor inhibitor, implying trimethylammonium recognition. Both groups are necessary but not sufficient for recognition by CAT. (*trans*)-Crotonylbetaine, a compound that models the *extended* conformation (*96*) of carnitine, is inactive, which indicates that distance between and possibly orientation of these groups affect recognition.

The remaining compounds in Table 10 are carnitine analogs with modifications to the carboxyl group. The inactivity of these compounds indicates that the carboxyl group of carnitine is crucial for activity (see Molecular mechanisms of catalysis).

Alternative substrates. Table 11 lists alternative substrates of CAT, which are all carnitine analogs. (*R*)-Thiocarnitine binds nearly as well as (*R*)-carnitine but has 4.5% relative activity. (*R*)-*S*-Acetylthiocarnitine binds about 3.5 times more tightly than (*R*)-acetylcarnitine and is 95% as active. The high group-transfer potential observed for acetylthiocarnitine may cause this difference in activity. An equilibrium constant of 0.22, at pH 7.0 and 30°C, has been measured for Eq. [8] (*98*):

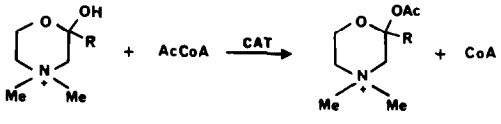
$$\text{AcCoA} + \text{thiocarnitine} \rightleftharpoons \text{acetylthiocarnitine} + \text{CoA}. \quad [8]$$

(*RS*)-Aminocarnitine binds about 13-fold less tightly to pigeon CAT than does (*R*)-carnitine and exhibits only 7.5% the activity (based upon V'_{max}). The weak binding and low enzymatic reactivity cannot be attributed to protonation of the 3-amino group, because its pK_a is quite low (6.37) compared to the buffer pH (7.6) (*93*). Instead, Jenkins and Griffith (*93*) suggest incorrect H bonding to an active-site base and misalignment of the amino group for acetyl transfer. For acetylation of (*RS*)-aminocarnitine by AcCoA, equilibrium favors acetylaminocarnitine; the racemate (3.3 mM) is acetylated 50% [presumably all available (*R*)-isomer] by AcCoA (6 mM) incubated in the presence of enzyme (*93*).

The results obtained for norcarnitine and GABOB (Table 11) further support CAT's recognition of trimethylammonium. Norcarnitine has the same V'_{max} as carnitine, but because it binds less well, its initial velocity is only 26% that for carnitine. Binding is reduced even more when all methyl groups are removed (e.g., GABOB). Because the amino groups on GABOB and norcarnitine are protonated at pH 7.8, the size of the ammonium ion affects binding more than the charge. When alkyl groups on nitrogen increase in size and rigidity, K_m increases and V'_{max} decreases (*99*). The increase in K_m is explained by a steric effect, but the decrease in V'_{max} implies a more complex mechanism, possibly involving slight disruption of the enzyme's catalytic machinery. How CAT selectively recognizes trimethylammonium groups is presently unknown.

Racemic sulfocarnitine is a minor substrate of the pigeon CAT [$V'_{max} < 10\%$ of (*R*)-carnitine rate], and (*R*)-sulfocarnitine is completely inactive because of incorrect stereochemistry. The apparent K_m of (*RS*)-sulfocarnitine (25,000 μM) is over 200-fold greater than that for (*R*)-carnitine with CAT (120 μM). The low activity observed for sulfocarnitine suggests that CAT's recognition of the carboxyl group is highly specific.

The racemic hemicholiniums are weak competitive inhibitors of CAT, with respect to carnitine. These compounds exhibit K_i's in the range of 3000–4000 μM for the pigeon CAT and bind to enzyme about 10-fold less tightly than (*R*)-acetylcarnitine (K_m = 350 μM). When these compounds are employed as substrates, at 10 mM concentration, enzyme-catalyzed acetyl transfer from AcCoA is observed,



but at a much slower rate than with (*R*)-carnitine. Relative V'_{max}'s [(*R*)-carnitine = 100 μmol min^{-1} mg^{-1}] are in the range 4.1 (R=CF_3) to 8.0 (R=H).

Conformationally rigid cyclic analogs. Table 12 shows several cyclic analogs of carnitine and acetylcarnitine that we have prepared and assayed. A predominant chair conformation of the morpholinium ring fixes the N–C–C–O torsion angle in a gauche conformation. We have previously postulated that CAT recognizes only the gauche conformation (*82, 96*). The data for 4,4-dimethylmorpholinium and

TABLE 12

Effects of Cyclic Carnitine and Acetylcarnitine Analogs on Pigeon Carnitine Acetyltransferase

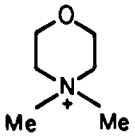
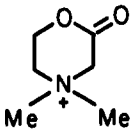
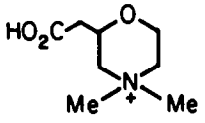
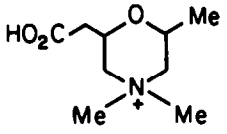
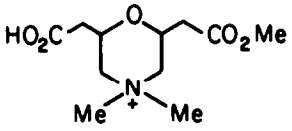
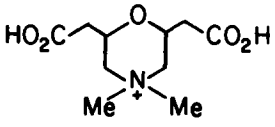
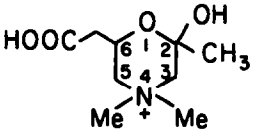
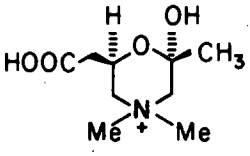
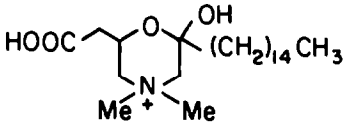
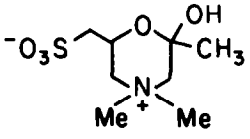
Compound	Structure	K_i (μM)	Mode of inhibition	Ref.
4,4-Dimethylmorpholinium			Inactive	(*102*)
4,4-Dimethyl-2-oxomorpholinium			Inactive	(*102*) (*103*)
(*RS*)-2-Carboxymethyl-4,4-dimethylmorpholinium		1000	Comp. with (*R*)-Cn	(*104*)
(2*R*, 6*S*:2*S*, 6*R*)-2-Carboxymethyl-4,4,6-trimethylmorpholinium		1080	Comp. with (*R*)-Cn	(*104*)
(2*R*, 6*S*:2*S*, 6*R*)-6-Carboxylatomethyl-2-methoxycarbonylmethyl-4,4-dimethylmorpholinium		8600	Comp. with (*R*)-Cn	(*104*)
(*meso*)-2,6-Bis(carboxymethyl)-4,4-dimethylmorpholinium		530	Comp. with (*R*)-Cn	(*104*)
(2*R*, 6*S*:2*S*, 6*R*)-Hemiacetylcarnitinium[a]		890 212.0[b]	Comp. with (*R*)-Cn	(*105*) (*102*)
(2*S*, 6*R*)-Hemiacetylcarnitinium		59.5[b]	Comp. with (*R*)-Cn	(*102*)

TABLE 12—*Continued*

Compound	Structure	K_i (μM)	Mode of inhibition	Ref.
(2*R*, 6*S*:2*S*, 6*R*)-Hemipalmitoylcarnitinium			Inactive	(*94*)
(2*R*, 6*S*:2*S*, 6*R*)-Hemiacetylsulfocarnitinium			Inactive	(*102*)

[a] By analogy with the hemicholiniums this compound is given the trivial name hemiacetylcarnitinium, and the class of compounds is referred to as hemiacylcarnitiniums.

[b] Racemic- and (2*S*, 6*R*)-HAC were assayed simultaneously using a single commercially available enzyme preparation. The K_m for (*R*)-carnitine under identical conditions is 105 μM vs 120 μM from the literature (Table 5).

4,4-dimethyl-2-oxomorpholinium indicate that this ring system alone is insufficient for enzyme recognition. Both dimethylammonium and carboxyl groups are necessary for adequate recognition, as the binding data for other compounds in Table 12 show.

Racemic hemiacetylcarnitinium (HAC), a structural isomer of acetylcarnitine, is a competitive inhibitor with respect to (*R*)-carnitine in both purified pigeon (K_i = 890 μM) and crude rat-liver (K_i = 4720 μM) CAT (*105*). Its K_i for the rat enzyme is about an order of magnitude higher than the K_m's for carnitine and acetylcarnitine (Table 5). A recent determination of its K_i (*102*) and that of (2*S*, 6*R*)-HAC gives values for pigeon CAT similar to the K_m for either (*R*)-carnitine (K_m = 120 μM) or (*R*)-acetylcarnitine (K_m = 350 μM).

The inhibitory activity of HAC is explained by its structural similarity to the tetrahedral intermediate proposed in the acetyl transfer between CoASH and acetylcarnitine (Fig. 2) (*82, 105*). Thus, HAC is a reaction intermediate analog (*92*).

Hemipalmitoylcarnitinium (HPC) is not an inhibitor of CAT, apparently because of a chain-length effect as observed with long-chain esters of carnitine.

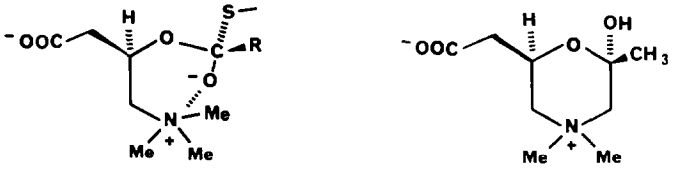


FIG. 2. Structural relationship between the proposed tetrahedral intermediate in the acetyl transfer reaction (shown on the left) and the structure of (2*S*, 6*R*)-hemiacetylcarnitinium (shown on the right).

The 2-carboxymethylmorpholiniums, shown in Table 12, are competitive inhibitors of pigeon CAT, with respect to (*R*)-carnitine (*104*). Of this series, (*meso*)-2,6-bis(carboxymethyl)-4,4-dimethylmorpholinium binds most strongly. This prochiral bilaterally symmetric molecule, a "Siameso" inhibitor, always presents the correct configuration for binding [i.e., a configuration equivalent to that of (*R*)-carnitine]. When the K_i's of 2-carboxymethyl-4,4-dimethylmorpholinium and 2-carboxymethyl-4,4,6-trimethylmorpholinium are reduced by half, they correspond with the K_i of the Siameso inhibitor, indicating that only one enantiomer of the racemates is active. We suggest that these inhibitors are models of the tetrahedral intermediate because pigeon-breast CAT recognizes their chirality. This CAT does not recognize chirality of carnitine or acetylcarnitine in binding, only in reacting with acetyl-CoA or CoA. These similar K_i's may indicate that for this class of inhibitors CAT does not distinguish hydro, methyl, or carboxylatomethyl at the acyl recognition site. The least effective inhibitor is the ester, 6-carboxylatomethyl-2-methoxycarbonylmethyl-4,4-dimethylmorpholinium. Assuming that only one enantiomer of the ester is active, the 8-fold reduction in binding compared to the Siameso inhibitor is significant. This difference may result from the increased size of the ester or from its decreased polarity compared to that of the carboxylate.

Binding improves with a hydroxyl at C_6 as seen when K_i's of racemic- and (2*S*, 6*R*)-HAC and K_i's of racemic 2-carboxymethyl-4,4-dimethylmorpholinium and racemic 2-carboxymethyl-4,4,6-trimethylmorpholinium are compared. The improved binding suggests hydrogen bonding or some other polar interaction of CAT with the hydroxy. The K_i of (2*S*, 6*R*)-HAC is nearly 3.6-fold lower than its racemate, instead of the expected 2-fold difference.

HAC is a better inhibitor than the analogous hemicholinium (R=CH_3, Table 11), another indication of the importance of the carboxylate recognition site. Similarly, the inactivity of the sulfo analog of HAC, hemiacetylsulfocarnitinium, shows that a seemingly minor change from carboxylate to sulfonate (as with carnitine and sulfocarnitine) disrupts interaction at what must be an extremely selective recognition site.

BIOORGANIC CHEMISTRY

Molecular mechanisms of catalysis. Fritz *et al.* have proposed a molecular mechanism (*21*) (Fig. 3a), based on forming a reactive enzyme-bound cyclic anhydride intermediate. In their mechanism the carboxylate does not affect binding, or once bound is free to rotate into position for reaction. Their mechanism, proposed before the random-order equilibrium kinetics were reported, does not properly apportion the decrease in activity between effects on K_m (binding) and effects on V'_{max} (chemical catalysis). For instance, with carnitinenitrile, if K_m and K_i represent true binding constants (as in the random-order equilibrium kinetics mechanism), then greater than 99% of the decreased CAT activity can be accounted for by binding alone (as measured by ratio of K_s). Correspondingly, for (*RS*)-norcarnitine, which shows a V'_{max} equal to that observed for (*RS*)-carnitine, the ratio

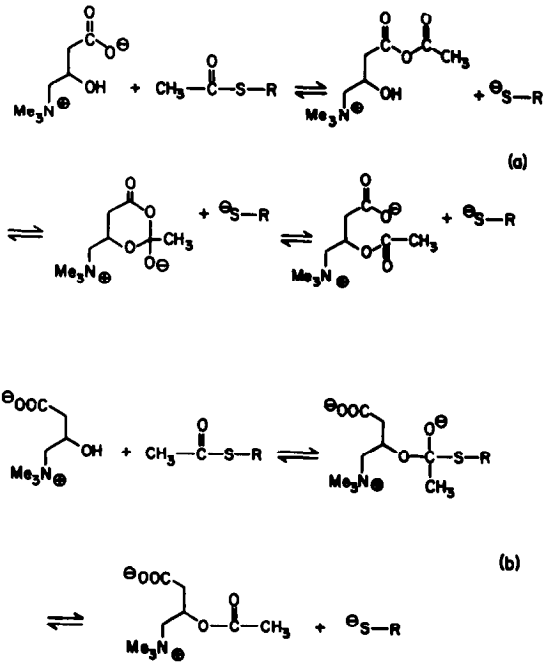
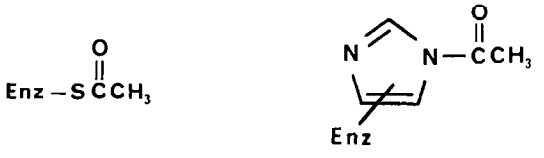


FIG. 3. (a) Mechanism of acetyl transfer proposed by Fritz *et al.* (*21*). (b) Mechanism of transfer proposed by Gandour *et al.* (*82*). Proton transfer steps are not included.

of binding constants predicts 19% activity, a value that agrees well with the percentage of relative activity determined from initial velocities (~26%, Table 11). Thus, most evidence indicates that carboxy is needed for binding more than catalysis.

In a second mechanism, proposed by Gandour *et al.* (*82*), a tetrahedral intermediate mediates *O*- to *S*-acetyl transfer (Fig. 3b). This mechanism requires the juxtaposition of the CoA and carnitine recognition sites. As the bisubstrate inhibitor studies of Chase and Tubbs (*75*) show, the sulfhydryl of CoA is close to the acetyl site, which in turn is adjacent to the carnitine hydroxyl site.

The kinetics do not preclude a third mechanism, in which a reactive acyl-enzyme intermediate forms. This mechanism is less likely, because there is no evidence of a strong nucleophile in the active site. A cysteine sulfhydryl and a histidine imidazole have been identified as residents of the active site, and either can act as a nucleophile in the formation of acyl intermediate **4** or **5**. Enzymes

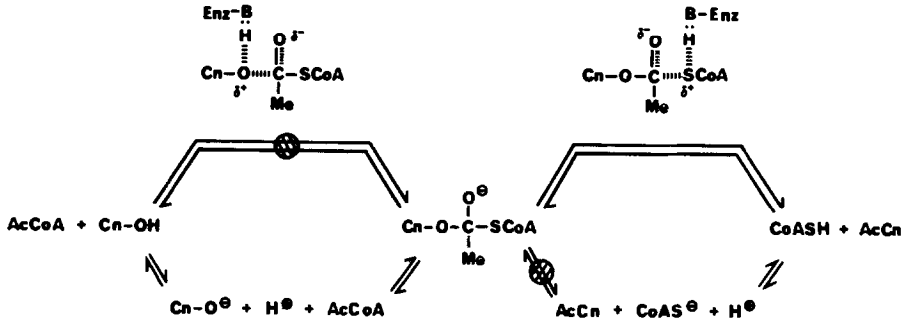


STRUCTURES **4** AND **5**

using CoA and acyl-enzyme intermediates usually exhibit either ordered sequential kinetics [e.g., choline acetyltransferase (*106*)] or ping-pong kinetics [e.g., arylamine acetyltransferase (*107*)]. Both form intermediates like **4.** Moreover, the sulfhydryl of CAT is only moderately nucleophilic as shown by its reactivity with several active-site-directed inhibitors (*75, 83, 84, 86*). The histidine is nucleophilic toward bromoacetylcarnitine when CoA is absent, but otherwise is inferior to the sulfhydryl of CoA (*75, 89*).

An active-site imidazole is probably a general base catalyst. All three proposed mechanisms require proton-transfer steps. A proton is lost at the hydroxyl of carnitine and sulfhydryl of CoA. If an active-site imidazole serves as a base, then either of two constraints must apply: First, in accordance with the pH dependence observed by Chase (*74*), the pK_a of this group must be less than 6.1 or greater than 8.3; or second, proton transfers to imidazole must occur only in non-rate-limiting steps.

From Hupe and Jencks' (*108*) studies on model reactions of acyl transfer between sulfur and oxygen nucleophiles, we draw further conclusions about our proposed molecular mechanism of CAT. Because carnitine oxyanion, with an estimated $pK_a \sim 13.9$ as in choline (*109*), is a poorer leaving group than CoA thioanion, $pK_a = 9.6$ (*109*), partitioning of the tetrahedral intermediate favors sulfur expulsion. If the mechanism follows the two lower pathways of Scheme IV, then formation of acetylcarnitine is favored. The principle of microscopic reversibility states that the forward and reverse reactions must have the same rate-limiting transition state, attack (forward) or expulsion (reverse) of oxyanion. At the optimal reaction pH (7.8), oxyanion concentration is small. If the lower left pathway is followed, then ionization of the hydroxy contributes substantially to the barrier for the forward reaction. This pathway can be avoided by general-base catalysis of hydroxy attack on the thio ester. (The reverse reaction is general-acid-catalyzed oxygen expulsion from the tetrahedral intermediate.) Conversely, the thioanion concentration (about 10%) at pH 7.8 implies that the lower right pathway of Scheme IV is more probable than the upper right pathway, general-acid catalysis of sulfur expulsion (forward) or general-base catalysis of sulfhydryl attack (reverse). Consequently, we suggest that the mechanism follows the upper



SCHEME IV. Mechanistic pathways for acyl transfer between carnitine and CoA. ⊗, Possible rate-limiting step.

left and lower right pathways. Catalysis increases the rate for oxygen expulsion and hydroxy attack, but by how much? The answer to the question about which step is rate limiting awaits more detailed kinetic studies.

SUMMARY

Although minor species variations exist for CAT, several generalizations concerning the mechanistic pathway of catalysis are nonetheless apparent. The binding of carnitine and acetylcarnitine probably requires recognition of both carboxylato and quaternary ammonium portions of the molecule and may involve recognition of charge, H-bonding capacity, end-to-end distance (C_1 . . . N^+) and relative orientation. Binding of carnitine occurs by an induced-fit mechanism, the Koshland model (*110*), rather than a simple complementary lock-and-key mechanism, the Fischer model (*111*). Hence, carnitine binding induces a major conformational change in the enzyme that results in organization of the reaction complex. The same conformational change may disrupt a remote hydrocarbon recognition site, which is responsible for inhibition by long-chain acyl-CoAs (C_8–C_{16}). A regulatory role might involve a selection mechanism for short-chain acyl-CoAs. Binding of CoA, or AcCoA, occurs in a lock-and-key fashion, whether or not bound carnitine is present. The 3′-phosphate of AcCoA affects binding to the enzyme. The kinetic mechanism fits a random-order equilibrium model where kinetic binding constants closely approach the true binding constants, and the rate-limiting step is acetyl exchange (i.e., interconversion of ternary enzyme–reactant and enzyme–product complexes). The presence of an acetyl-enzyme intermediate cannot be excluded by kinetics alone, but is not supported by other evidence. The molecular mechanism of acetyl transfer is probably addition–elimination with general base catalysis of hydroxy attack on a thioester in the forward reaction and general acid catalysis of oxygen expulsion in the reverse.

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