J. theor. Biol. (1980) **82,** 559–572

Conformations of Carnitine and Acetylcarnitine and the Relationship to Mitochondrial Transport of Fatty Acids

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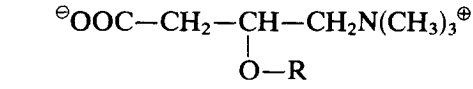
(*Received* 1 *June* 1979)

The conformations of carnitine and acetylcarnitine by EHT and CNDO/2 molecular orbital calculations show that carnitine has two low energy conformers. One of these is an extended conformer corresponding to a charge separated species, while the other is a folded conformer having a charged onium head and carboxylate anion in close proximity forming an internal ionic bond. These results suggest that the folded conformation is responsible for the active transport of acetyl- and acyl-carnitine by enzymes which transfer acyl groups into the mitochondria for subsequent fatty acid oxidation.

1. Introduction

(A) BIOCHEMISTRY OF CARNITINE

L-(−)-Carnitine [3-(R)-hydroxy-4-trimethylaminobutyric acid; (R)-carnitine, **I**] is widely distributed in the tissues of animals, plants and microorganisms. It is found in highest concentrations in the muscles of vertebrates and invertebrates. The principal effect of carnitine is due to its ability to stimulate fatty acid oxidation thereby increasing oxygen consumption. It does this without itself being depleted in the process. Heart muscle shows the greatest response to these effects (Fritz, 1961), and there is growing acknowledgement of the role fatty acid oxidation may play in the development of myocardial ischemia and cardiac arrhythmias (Oliver, 1976; Hillis & Braunwald, 1977).



I = Carnitine R = —H
II = Acetylcarnitine R = —$COCH_3$
III = Palmitylcarnitine R = —$CO(CH_2)_{14}CH_3$

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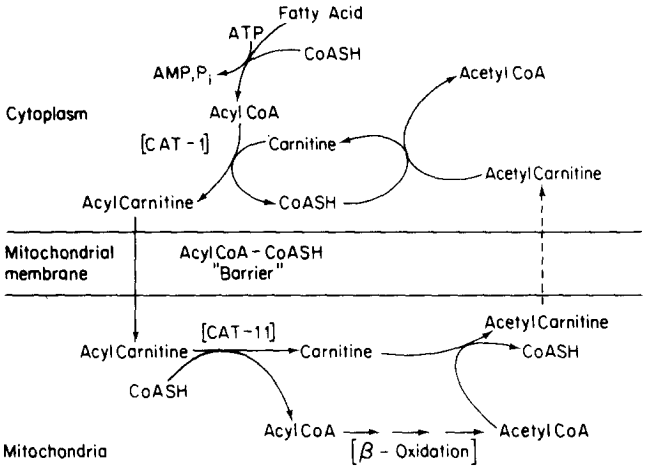


FIG. 1. Sequence of steps leading to the transport of fatty acids into the mitochondria and β-oxidation.

It is well established that carnitine provides a shuttle mechanism whereby fatty acids are transported into the mitochondria. Figure 1 illustrates the sequence of events leading to mitochondrial oxidation of fatty acids. The first step occurs in the cytoplasm and involves the activation of fatty acids to acyl CoA derivatives. However, the inner mitochondrial membrane is impermeable to coenzyme A and to acyl CoA thioesters. This is depicted as the acyl CoA-CoASH "barrier" (Snyder, 1977). Two transferase systems are believed to exist for the purpose of carrying acyl groups into the mitochondria. These systems are carnitine acyltransferase I (CAT-I) located within the outer mitochondrial membrane, and carnitine acyltransferase II (CAT-II) located on the inner mitochondrial membrane. Within the outer mitochondrial membrane CAT-II catalyzes the transfer of the acyl group from acyl CoA to carnitine. CAT-II is believed to be responsible for the vectorial transport of acylcarnitine to the inner membrane where the reverse process occurs and carnitine and acyl CoA are released into the mitochondrial matrix. The mitochondrial acyl CoA can then undergo β-oxidation.

Pande (1975) has offered evidence for a translocase enzyme system, carnitine acylcarnitine translocase, which he proposes as providing a mechanism for transporting the fatty acyl group from the cytoplasm into the mitochondria. His modified scheme is based on evidence for facilitated diffusion, and is shown in Fig. 2.

An intramitochondrial enzyme, carnitine acetyltransferase, has been isolated which catalyzed the conversion of acetyl CoA to acetylcarnitine

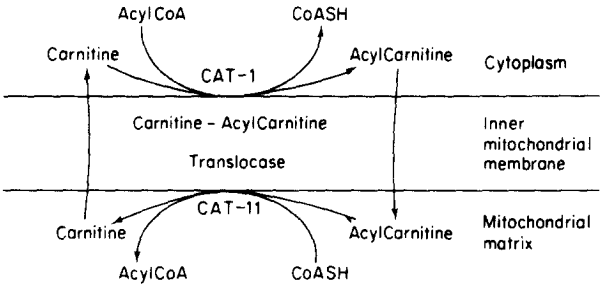


FIG. 2. Proposed scheme for fatty acid transport invoking a carnitine-acylcarnitine translocase system.

(Fritz, 1963). The acetylcarnitine thus formed may be transported to the cytoplasm where reconversion to acetyl CoA can occur. During times of high carbohydrate utilization, the principal route for formation of cytoplasmic acetyl CoA is via emigration of mitochondrial citrate. It is tempting to speculate that during periods of high fatty acid utilization, or in those tissues which predominantly use fatty acids as energy substrates, the route for formation of cytoplasmic acetyl CoA is via the carnitine acetyltransferase mechanism. To date, there have been no reports of the affinity of acetylcarnitine for the carnitine acylcarnitine translocase. Acetylcarnitine has also been proposed to act as a "buffer" to maintain constant levels of acetyl CoA (Pearson & Tubbs, 1967). This would be analogous to the role creatine prosphate plays in buffering changes in ATP levels in muscle.

Acylcarnitine is the ester formed between the fatty acyl group and the β-hydroxy group of carnitine. Since the transport system appears to be specific for the acylated derivatives of carnitine it seems likely that this substitution affects the affinity for transport. At physiological pH, the carboxylate group is ionized, effectively forming a zwitterion for both carnitine, **I**, and acylcarnitine, **III**. The substitution at the β-hydroxy position also introduces a certain amount of steric bulk in the center of the molecule. It is of interest, therefore, to examine the influence of β-substitution on the orientation of carnitine to see if there is a correlation between structural conformation and mitochondrial transport. It is also of interest to examine the influence of the acyl group on the electronic interaction between the two charged functional groups.

(B) CONFORMATION STUDIES

There are three principal means by which the preferred conformation of a molecule may be studied. Two of these are experimental approaches: X-ray

crystallography and nuclear magnetic resonance (NMR) spectroscopy. The third is a theoretical approach using semiempirical quantum chemical methods. One advantage of the experimental approaches is that they represent actual states of the molecule. However, X-ray crystallography requires solid state conditions and the conformations which are determined are influenced by the intermolecular forces of crystal packing. Conformations determined by X-ray crystallography are, therefore, a distortion of what occurs under biological conditions. NMR studies of molecular conformation, normally proton magnetic resonance (PMR), are also usually not possible under normal physiological conditions. Either a nonaqueous solvent is required, or conformations are determined in D_2O to diminish the large absorption band given by H_2O. Quantum chemical theoretical methods are often utilized and, qualitatively, the preferred conformations determined by these methods are quite similar to those obtained experimentally. Quantitatively, some semiempirical methods, such as the extended Hückel theory (EHT), exaggerate the differences between conformational states. The quantum chemical calculations are normally performed on single molecules in the ideal vapor state, i.e. no intermolecular influences. In recent studies, attempts to simulate the influence of small molecules, such as H_2O, on conformation have yielded comparable results to those obtained on the isolated molecule (Langlet *et al.*, 1977).

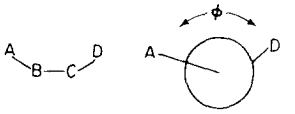
In this paper, data from X-ray crystallographic studies of carnitine (Tomita *et al.*, 1974) and acetylcarnitine (Sass & Werness, 1973) are compared to results from theoretical quantum chemical studies.

In comparing the differences in conformation between carnitine and β-substituted derivatives, acetylcarnitine, **II**, was selected as a surrogate structure for the long chain acyl derivatives. It was believed that any changes in conformation seen with the acetyl derivative would be magnified with a bulkier substituent such as a palmityl group, **III**.

2. Comparisons of Results between Experimental and Theoretical Studies

The numbering scheme for carnitine and acetylcarnitine and the torsional angles considered are given in Fig. 3. The conformation problem concerns the steric and electronic effects of the C^1-carboxylate anion, the N^5-quaternary ammonium cation, and the β-hydroxy vs. β-ester group in determining the rotational and conformational properties of the carnitine molecule. A torsional angle, ϕ(A–B–C–D), between the bonded atoms A–B–C–D represents the angle between the planes of ABC and BCD. Viewed from the direction of A, positive rotations of ϕ are clockwise and negative rotations counter-clockwise, the BCD plane rotating with respect

to the ABC plane. The value $\phi = 0°$ corresponds to the *cis*-planar arrangement of the bonds AB and CD.



The theoretical studies to determine the conformation of (R)-carnitine and (R)-acetylcarnitine were carried out using the EHT method (Hoffman, 1963) and the CNDO/2 method (Pople, Santry & Segal, 1965; Pople & Segal, 1965). Although EHT usually exaggerates the energy barriers to rotation, it does correctly predict the preferred rotational conformer. The CNDO/2 calculations utilize a self-consistent field (SCF) technique to estimate the electron interactions, thus, less reliance is placed on empirical parameters for calculations of electronic properties. Compared to EHT, CNDO/2 gives more realistic values for charge densities, electron populations, and estimations of energy barriers to rotation. The CNDO/2 method is used to verify the EHT calculations of preferred conformations, and permits the determination of the effect of the electrostatic interaction between the quaternary ammonium group and the carboxylate anion.

The problem is analogous to that encountered by Kier in his study on chlolinergic agents (Kier, 1967). To simplify his calculations on acetylcholine Kier segmented the molecule into two parts and determined the

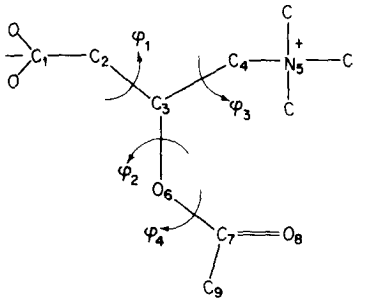


FIG. 3. Molecular structure of acetylcarnitine showing the numbering scheme and notation of torsional angles used for carnitine and acetylcarnitine.

Torsional angle	Notation
C1–C2–C3–O6	ϕ_1
C2–C3–O6–H (or C7)	ϕ_2
C2–C3–C4–N5	ϕ_3
C3–O6–C7–O8	ϕ_4

preferred conformation of each segment which are dependent only on vicinal groups. This approach was used in the present study with both (R)-carnitine and (R)-acetylcarnitine.

The carnitine molecule was segmented to include the three torsional angles: ϕ_1(Cl–C2–C3–O6), ϕ_2(C2–C3–O6–H6), ϕ_3(C_2–C3–C4–N5). The calculated lowest energy conformer of (R)-carnitine is with ϕ_1(C1–C2–C3–O6) = 60°, ϕ_2(C2–C3–O6–H6) = 60°, ϕ_3(C2–C3–C4–N5) = 150°. A comparison of these values with those obtained by X-ray crystallography (Tomita *et al.*, 1974) is given in Table 1.

TABLE I

Comparison of X-ray and EHT-calculated torsional angles for (R)-carnitine

Dihedral angle	X-ray data†	EHT calculation
ϕ_1(C1–C2–C3–O6)	73·4°	60°
ϕ_2(C2–C3–O6–H6)		60°
ϕ_3(C2–C3–C4–N5)	174·8°	150°

† Tomita *et al.* (1974).

The principal torsional angles that determine the overall conformation of (R)-carnitine are ϕ_1 and ϕ_3. As determined by X-ray crystallography, the value of $\phi_1 = 73{\cdot}4°$ (Tomita *et al.*, 1974) is in close agreement with the theoretically derived valued, $\phi_1 = 60°$. The value of ϕ_3 from X-ray data is 174·8° as compared to the theoretically derived value of 150°. These are in fair agreement. An examination of the energy diagram determined for the torsional angle, ϕ_3, (Fig. 4), shows the energy difference between $\phi_3 = 180°$ and $\phi_3 = 150°$ to be 13 kcal mol^{-1} as estimated by EHT. This is probably an exaggerated barrier, and there is a relatively low total energy barrier to rotation in this region, i.e. the molecule possesses "flexibility". It seems reasonable that crystal packing forces may stabilize (R)-carnitine in the conformation in which $\phi_3 = 174{\cdot}8°$.

The theoretical data show that the preferred conformation for the unsubstituted (R)-carnitine is an extended form. In this conformation the onium nitrogen group is functionally separated from the carboxylate anion (Fig. 5).

For (R)-acetylcarnitine, the same partitioning procedure was followed. In this case, however, the acetyl group offers an additional complication since the additional bulky group at the β-position affects the orientations about the torsional angles ϕ_1(C1–C2–C3–O6) and ϕ_3(C2–C3–C4–N5). In addition, the preferred conformation about an additional torsional angle,

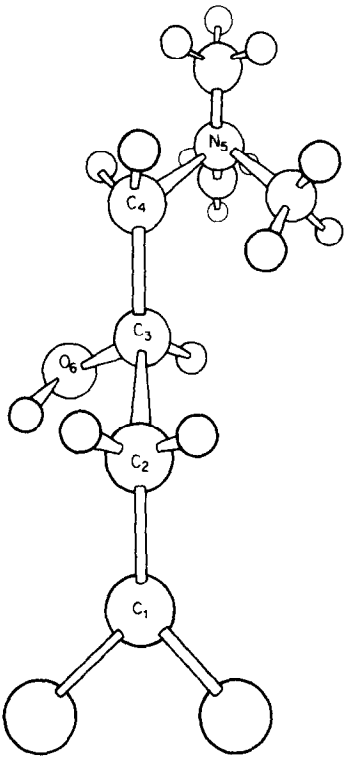


FIG. 4. Preferred conformation of (R)-carnitine is an "extended" molecule where the quaternary nitrogen, N5, is separated from the carboxylate anion, C1.

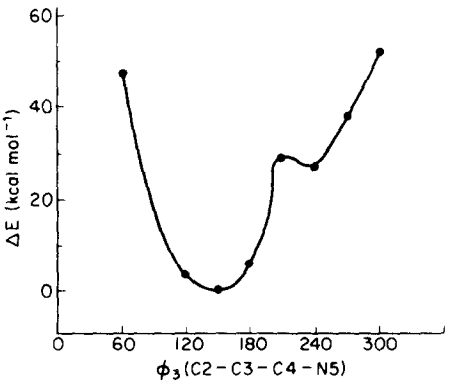


FIG. 5. Energy diagram showing energy change (kcal mol^{-1}) as a function of changes in the torsional angle, ϕ_3, in (R)-carnitine.

ϕ_3(C3–O6–C7–O8), must be calculated before the remaining torsional angles are determined (Fig. 3).

The EHT calculations for (R)-acetylcarnitine show two energetically favorable conformations. One of these corresponds to an "extended" conformation similar to that obtained with (R)-carnitine. The torsional angle values for this "extended" form are as follows: ϕ_1(C1–C2–C3–O6) = 300°; ϕ_2(C2–C3–O6–C7) = 240°; ϕ_3(C2–C3–C4–N5) = 120°; ϕ_4(C3–O6–C7–O8) = 300°. The other predicted favorable conformation is "folded" differing from the "extended" in that ϕ_1(C1–C2–C3–O6) = 180°. These two conformations are illustrated in Fig. 6. The "folded" conformer refers to the molecule having the onium group and the carboxylate group in close

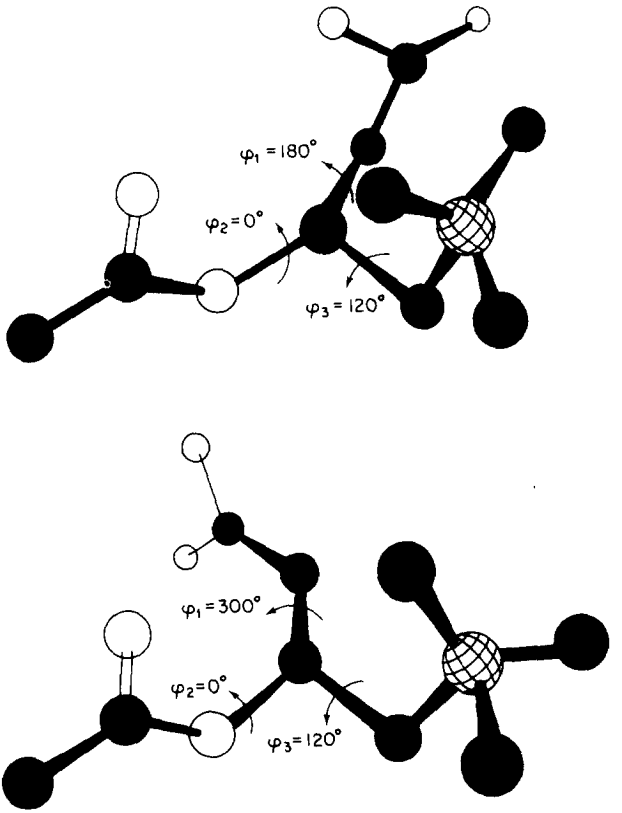


FIG. 6. Preferged conformations of (R)-acetylcarnitine. (a) "Folded" conformer where the quaternary nitrogen interacts with the carboxylate anion: ϕ_1(C1–C2–C3–O6) = 180°; ϕ_2(C2–C3–O6–C7) = 0°; ϕ_3(C2–C3–C4–N5) = 120°. (b) "Extended" conformer where the quaternary nitrogen and carboxylate anion are spread apart: ϕ_1(C1–C2–C3–O6) = 300°; ϕ_2(C2–C3–O6–C7) = 0°; ϕ_3(C2–C3–C4–N5) = 120°.

proximity whereas the "extended" conformer is the molecule having these charged groups separated.

The X-ray data of Sass & Werness (1973) on acetylcarnitine indicate a "folded" conformation in the crystal. The values as interpreted from their data are: $\phi_1 \sim 190°$, $\phi_2 = 284{\cdot}5°$ and $\phi_3 = 152°$. Calculations using the CNDO/2 method also show the preferred conformer of acetylcarnitine to be the "folded" conformer. Table 2 shows the comparison of the torsional angles found by the three methods.

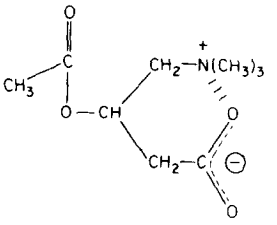
TABLE 2

Comparison of X-ray, EHT-calculated and CNDO/2-calculated torsional angles for (R)-acetylcarnitine

Dihedral angle	X-ray data†	EHT-calculation	CNDO/2-calculation
ϕ_1(C1–C2–C3–O6)	193°†	180°; 300°	180°
ϕ_2(C2–C3–O6–C7)	284·5°	240°	240°
ϕ_3(C2–C3–C4–N5)	152·1°	120°	120°
ϕ_4(C3–O6–C7–O8)	5·3°	300°	300°

† Sass & Werness (1973).

The theoretical molecular orbital calculations show that acetylcarnitine may exist in two, nearly equal conformations. One of these is a charged-separated, "extended" form; the other is a charge-interacting, "folded" form. The CNDO/2 calculations show that when the positively charged quaternary nitrogen group is close to the negatively charged carboxylate group, changes in the atomic charges of the involved atoms occur. There is a net electrostatic attraction between the onium head and carboxylate anion that is best represented by a "quasi" six-membered ring, **IV**:



IV

3. Conformations of Carnitine vs. Acetylcarnitine and FFA Oxidation

The rate-limiting step in fatty acid oxidation is believed to be the migration of the fatty acyl group from the cytoplasm to the mitochondria

(Zuurendonk & Tager, 1974; Challoner & Steinberg, 1966). Two carnitine palmityl transferases, carnitine acyl transferase I and II, and a translocase, carnitine acyl translocase, are proposed as playing important roles in this transport process. Though the transport kinetics and structural requirements have been characterized, the enzymes themselves have not been isolated and appear to be dependent on the intact mitochondrial membrane. A third transferase, carnitine acetyltransferase, has been isolated from mitochondria (Fritz & Schultz, 1966), and its properties have been characterized. Based on this experimental information and our conformational analysis, we believe the conformations of carnitine and acylcarnitine play important roles in the activities of these various enzymes.

Carnatine acetyltransferase (2.3.1.7.) catalyzes the reversible reaction:

$$\text{(R)-Carnitine} + \text{acetylCoA} \rightleftharpoons \text{CoASH} + \text{(R)} = \text{acetylcarnitine} \qquad (1)$$

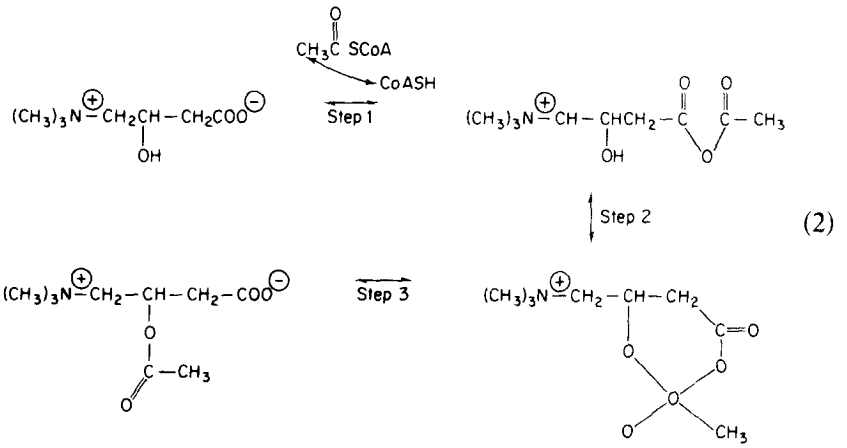
Carnitine acetyltransferase also catalyzes the transfer of propionyl and butyryl groups, but not longer chained groups (Fritz, Schultz & Srere, 1963). Branched chained alkyl groups, such as valyl, leucyl and isoleucyl, are also transferred (Solberg & Bremer, 1970). (S)-Acetylcarnitine, (S)-carnitine and deoxycarnitine are competitive inhibitors of this reaction. It appears that, structurally, the enzyme requires the "extended" conformation of carnitine for the forward reaction. Based on our results with carnitine, deoxycarnitine (α-trimethylaminobutyrate) most likely favors an extended conformation and therefore, could "fit" the enzyme active site. However, it lacks the necessary β-hydroxy group to which the acetyl group would transfer making it a competitive inhibitor. (S)-Carnitine is the mirror image of (R)-carnitine, and its lowest energy conformation is the extended form (Reed, Murray & Roche, 1979). (S)-Carnitine could also fit the enzyme active site, but since its β-hydroxy group is not oriented correctly, i.e. opposite the natural R-isomer, it may not be able to accept an acetyl group.

The role of (S)-acetylcarnitine as a competitive inhibitor of carnitine acetyltransferase may be viewed in two ways. The preferred conformation for both isomers of acetylcarnitine is the folded conformation. If we assume that the last step in the acetyl transfer is release of the "folded" conformation, then the (S)-isomer cannot undergo the reverse reaction in equation (1), i.e. transfer of the acetyl group, since the acetyl group is incorrectly positioned. It can, however, occupy the active site for an indefinite length of time, becoming a competitive inhibitor. Alternatively, (S)-acetylcarnitine may be exerting its effect on the first step of the reaction. Though the "folded" conformation is preferred. It is energetically feasible for the extended conformer to exist. If the extended conformer interacts at the active site, the β-position, in addition to being in the wrong configura-

tion, is already acetylated. Therefore, the active site is occupied, cannot be acetylated, cannot undergo the reverse reaction and, thus, the enzyme is inhibited.

(R)-Acetylcarnitine acts as a non-competitive inhibitor of carnitine acetyltransferase. A likely explanation for this observation is the presence of an allosteric site on the enzyme, distinct from the catalytic or active site, which when occupied modifies the rate of enzymatic activity through a feedback control mechanism. If (R)-acetylcarnitine exists in the "quasi" ring conformation, **IV**, a three point arrangement of the quaternary nitrogen, carboxylate anion and acyl carbonyl oxygen may be envisioned. The allosteric site may require this arrangement of charged groups for proper recognition of specific binding substances giving the allosteric site stereoselective properties. On the other hand, (S)-acetylcarnitine cannot interact with this allosteric site since in its quasi-ring conformation the carbonyl oxygen is not positioned correctly for recognition.

Fritz (1963) has suggested the following scheme for the transfer of an acetyl group from acetylCoA to carnitine:



(2)

In the lowest energy conformation of (R)-carnitine the carboxylate group and hydroxy group are in close proximity to one another, suggesting the possibility of a hydrogen bonding interaction. The "quasi"-ring conformation of (R)-acetylcarnitine, **IV**, would maintain a large distance between the carboxylate group and the acetylated oxygen. It seems plausible that following the formation of the anhydride in Step 1, the close proximity of the hydroxy group facilitates the esterification reaction occurring in Step 2. Once transfer of the acetyl group has been achieved, Step 3, the carboxylate

group is free to swing around and interact with the quaternary nitrogen, and it is this folded conformation which is released from the enzyme. In this model, the quaternary nitrogen assumes the role of a primary binding group to a complimentary group on the enzyme. This interaction is compromised when the carboxylate anion is free to interact strongly with the positively charged nitrogen. Supporting this hypothesis is the observation that high concentrations of choline derivatives having free onium nitrogens inhibit carnitine acetyltransferase (Fritz & Schultz, 1965).

Carnitine palmityltransferase catalyzes the transfer of the long-chain acyl group from palmityl CoA to carnitine:

$$\text{Palmityl CoA} + \text{carnitine} \longleftrightarrow \text{palmitylcarnitine} + \text{CoASH} \qquad (3)$$

A β-hydroxy group, carboxylate and trimethyl ammonium group are required for a molecule to be a substrate of carnitine palmityltransferase. The same mechanism proposed for carnitine acetyltransferase is assumed [equation (2)]. That is, the palmityl group transfers from coenzyme A to the carboxylate of carnitine to form the anhydride. Esterification of the β-hydroxy group follows via the intermediate at Step 2 [equation (2)]. Once (R)-palmitylcarnitine is formed, the "folded" conformation is preferred, and palmitylcarnitine is transferred vectorally into the mitochondria.

Pande (1975), Ramsey & Tubbs (1975) and Pande & Parvin (1976) have proposed a carnitine : acylcarnitine translocase enzyme which catalyzes the exchange diffusion of extramitochondrial acylcarnitine for intramitochondrial carnitine. There are a number of questionable points raised by Pande's description of the proposed translocase (Pande, 1975). Apparently, the carboxylate and trimethylammonium groups of carnitine are essential for activity. The β-hydroxy group does not appear to be important since deoxycarnitine facilitated the rapid exchange of carnitine across the membrane (Pande & Parvin, 1976). However, Pande & Parvin (1976) also state that the translocase is stereoselective since the R-isomers of carnitine and acylcarnitine are preferred over the S-isomers. It is difficult to reconcile his assignment of stereoselectivity, implying a three point interaction, with the high activity of deoxycarnitine which has only a two point interaction.

Pande & Parvin (1976) also propose that a sulfhydryl group on the translocase enzyme is involved in substrate binding. Their reason for this proposal is that competitive inhibition of carnitine : acylcarnitine exchange was noted upon addition of mersalyl. There is some difficulty in accepting this explanation for two reasons. First are the unanswered questions of how carnitine is "bound", and why it should have an affinity for sulfhydryl groups? Second, the mode of action of mersalyl, an organic mercurial, is to form a covalent bond between the mercury atom and a sulfhydryl group.

Thus, if mersalyl were acting in its usual manner one would expect to observe noncompetitive not competitive inhibition.

The acylcarnitine : carnitine translocase enzyme has not been isolated. The measure of its enzyme characteristics are based upon effects on the exchange of extramitochondrial carnitine for intramitochondrial carnitine in intact mitochondrial preparations. The major evidence for the existence of a translocase enzyme is based on the differential effects of mersalyl, β-hydroxy-α-aminobutyrate and α-aminobutyrate on carnitine : carnitine exchange, versus their effects on the transferase enzymes. However, one usually notes differences in properties for isolated or partially isolated acyltransferases as opposed to acyltransferases in intact mitochondria. For example, (S)-palmitylcarnitine does not inhibit partially purified palmityltransferase, but does inhibit mitochondrial bound palmityltransferase (Fritz, Schultz & Srere, 1963). The evidence for the existence of a translocase enzyme is, therefore, inconclusive.

In summary, it is proposed from this study that in the carnitine acetyl- and acyltransferase systems, the "extended" conformation of carnitine interacts with the enzyme. Employing the mechanism of Fritz (1963), the acyl group of acyl CoA is transferred to the carboxylate group of carnitine to give an anhydride. Following this, the acyl group is transferred from the anhydride to the β-hydroxy group to give the acylcarnitine ester. This is followed by formation of the "folded" conformer and the quasi-ring structure, **IV**, and vectoral transfer into the mitochondria. Within the mitochondrial matrix the reverse reaction occurs.

In this instance, CAT-II may require initial contact with the "folded" conformer of acylcarnitine followed by transfer of the acetyl group to CoASH, and in the final step, release of the "extended" conformer of carnitine.

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