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Effect of Ionic Strength on the Activity of Carnitine Palmityltransferase I†

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ABSTRACT: Changes in ionic strength at a constant osmolarity produced increases in carnitine palmityltransferase I activity in intact mitochondria. This enzyme catalyzes the reaction: palmityl-CoA + carnitine ⇄ palmitylcarnitine. The linearity of the effect of ionic strength on palmitylcarnitine formation was present only at ionic strengths below 0.060 M. The use of the Brönsted–Bjerrum equation for the effect of ionic strength on ion–ion interactions yielded a positive slope of 3 that was pH sensitive. A decrease in pH lowered the slope of the equation, suggesting involvement of the negative charges 3 and 1 (Z_A,Z_B) in the effect of ionic strength on the reaction rate. Similar increases in ionic strength increased linearly the association of palmityl-CoA with mitochondria incubated in the presence of antimycin A. A positive slope of 3 was obtained when the Brönsted–Bjerrum equation was applied. A positive correlation between increases in palmitylcarnitine formation and palmityl-CoA bound to the mitochondria at different ion concentrations was observed. Carnitine palmityltransferase activity released with digitonin was not affected by changes in ionic strength. Addition of a mitochondrial outer membrane fraction to the released enzyme activity restored the ionic strength dependency. This effect was proportional to increased association of palmityl-CoA with the outer membrane fraction. The augmentation of carnitine palmityltransferase activity with increased ionic strength was postulated to be related to the increased concentration of palmityl-CoA in the vicinity of the enzyme active site.

The partial latency of carnitine palmityltransferase has been described (Yates and Garland, 1970). It was originally proposed by Fritz and Yue (1963) that the mitochondria contain two carnitine palmityltransferase activities (transferases I and II) that catalyze reactions 1 and 2. In unbroken mitochondria, type-I transferase is accessible to the inhibitor

$$\text{palmityl-CoA} + \text{carnitine} \rightleftharpoons \text{palmitylcarnitine} + \text{CoA}^{1} \quad (1)$$
(transferase I)

$$\text{palmitylcarnitine} + \text{CoA} \rightleftharpoons \text{carnitine} + \text{palmityl-CoA} \quad (2)$$
(transferase II)

2-bromostearyl-CoA, whereas the type-II ("latent") transferase is inaccessible (Yates and Garland, 1970). The type-I transferase ("external") is located on the external portion of the inner membrane of mitochondria, whereas the type-II transferase is bound within the inner mitochondrial membrane (Yates and Garland, 1970; Hoppel and Tomec, 1972; Brosnan *et al.*, 1973).

Brosnan and Fritz (1971) examined factors that might influence the expression of the "external" carnitine palmityltransferase. Changes in the ability of intact mitochondria to oxidize palmityl-CoA were shown to depend on the ionic strength of the assay medium. Under the appropriate conditions, the "latency" or hypothesized deficiency of fetal heart mitochondria for the external transferase (Wittels and Bressler, 1965; Warshaw and Terry, 1970) was expressed in intact mitochondria by increased ionic strength or by storage in ice (Brosnan and Fritz, 1971).

The apparent "masked" activity of the external enzyme, carnitine palmityltransferase I, in media of low ionic strengths was examined in the present study. Maximal expression of carnitine palmityltransferase I was seen to be directly related to increased association of palmityl-CoA with the mitochondrion.

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[1] Abbreviations used are: CoA, coenzyme A; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; μ, ionic strength; mosm, milliosmolar.

Experimental Procedure

Mitochondria were prepared from hearts from mongrel dogs as described by Sordahl *et al.* (1971) in a medium containing 0.18 M KCl, 10 mM EDTA, and 0.5% bovine serum albumin (fraction V, Sigma). The membrane integrity of heart mitochondria prepared in this medium has been described (Sordahl *et al.*, 1971). Mitochondrial protein was measured using the Biuret method (Layne, 1957).

The digitonin method for fractionation of mitochondria was used (Schnaitman and Greenawalt, 1968; Hoppel and Cooper, 1968). Ice-cold digitonin (100 mg/ml) was added to mitochondrial suspensions (50–70 mg/ml) in 0.25 M sucrose (pH 7.3). After 30 min at 0°, the mitochondria were diluted with 1.5 volumes of 0.25 M sucrose. The suspension was then centrifuged at 12,000*g* for 15 min. The supernatant was decanted and centrifuged at 100,000*g* for 60 min. The pellet from this centrifugation was described as outer membrane and was washed once and then resuspended in 0.25 M sucrose. Monoamine oxidase activity of the outer membrane pellet was assayed on a Gilford recording spectrophotometer (Gilford Instrument Laboratories) at 37° using 7.5 μmol of benzylamine as substrate and following absorbance of benzaldehyde at 250 nm (Tabor *et al.*, 1954). Fifteen minutes before assay, samples were activated with Lubrol (0.3 mg/mg of protein) for minimization of changes in optical density that are associated with mitochondrial swelling (Schnaitman and Greenawalt, 1968).

Following removal of the outer membrane pellet, the 100,000*g* supernatant was dialyzed against two changes of 10 volumes of 0.25 M sucrose–1 mM EDTA (pH 7.2) to remove the digitonin. This fraction contained the digitonin-releasable carnitine palmityltransferase activity as described by Hoppel and Tomec (1972). Protein content of the outer membrane and soluble fractions was determined by the Lowry procedure (Lowry *et al.*, 1951).

Carnitine palmityltransferase activity was measured by a modification of the original method of Bremer and Norum (1967), as described by Pande and Blanchaer (1970), with *dl*-[*methyl*-^{14}C]carnitine hydrochloride (ICN Chemical and Radioisotope Division), specific activity 1165–1220 dpm/nmol of L-carnitine or *dl*-[*methyl*-^{3}H]carnitine hydrochloride (Amersham/Searle), specific activity 5000 dpm/nmol of L-carnitine. Hepes,[1] at a final concentration of 2 mM, was added to maintain the pH at 7.0. Varying amounts of 0.5 M salt solutions were added to change the ionic strength. Osmolarity was kept constant at 290 mosm using 440:140 mM mannitol–sucrose. Enzyme activity was determined at 25° using 0.2–0.4 mg of mitochondrial protein in a final volume of 0.5 ml. The concentration of the radioactive product extracted in butanol, [^{14}C]palmitylcarnitine, or [^{3}H]palmitylcarnitine was estimated by scintillation counting of aliquots taken from the butanol phase. An external standard method for quench correction was employed in all experiments. The scintillation fluid for radioactive counting was composed of Beckman Bio-Solv Solubilizer BBS-3 and fluoralloy in toluene. Samples were counted in a Packard Tri-Carb spectrometer Model 574 and in a Beckman liquid scintillation counter (Model LS-200B).

Association of [1-^{14}C]palmityl-CoA (ICN Chemical and Radioisotope Division), specific activity 5500 dpm/nmol, with intact mitochondria was studied by the following procedure. Aliquots of mitochondria (0.2–0.3 mg) were preincubated at 18° for 15 sec in solutions of varying ionic strengths containing 0.225 μM antimycin A. Substrate binding was initiated by addition of [1-^{14}C]palmityl-CoA. After 30 sec, the solutions were transferred to a microfuge (Beckman–Spinco 152) and centrifuged. The pellets were dissolved in 0.05 ml of 2 N NaOH by heating. Aliquots for radioactivity and protein measurements were removed and determined. The same procedure was followed for binding of [1-^{14}C]palmityl-CoA to the outer membrane fraction except the centrifugation, which was performed in an L3-40 Beckman ultracentrifuge at 100,000*g* for 60 min.

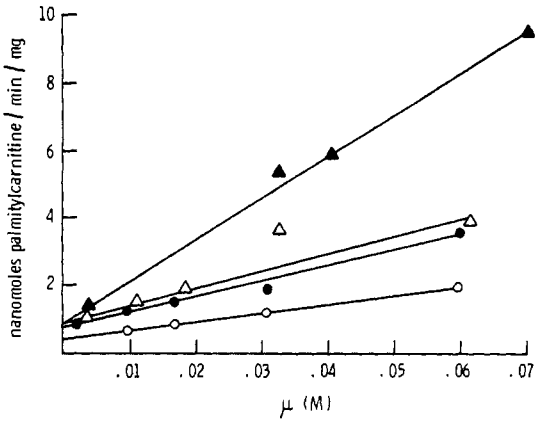

FIGURE 1: The effect of ionic strength (μ) on carnitine palmityltransferase activity in dog heart mitochondria. Carnitine palmityltransferase was assayed as described in Methods in a total volume of 0.5 ml. Mitochondrial protein (0.3–0.5 mg) was added to each reaction tube and the effect of changes in ionic strength on carnitine palmityltransferase activity was determined after incubation for 4 min at 25°. Osmolarity was kept constant at 290 mosm by addition of varying amounts of 440:140 mM mannitol–sucrose. Each determination was repeated on one to four other mitochondrial preparations: Tris-Cl, ▲; KCl, △; choline-Cl, ●; LiCl, ○.

The effect of varying salt (KCl) concentrations on the critical micelle concentration of palmityl-CoA was examined by using the procedure of Zahler *et al.* (1968) for determination of critical micelle concentration, with the following modifications. Rhodamine 6G (Allied Chemical Corp.) was prepared fresh at a final concentration of 0.5 μM in 2 mM Hepes buffer (pH 7.0) and in varying concentrations of KCl. Appropriate aliquots of 0.8 mM palmityl-CoA prepared in the same buffer were added and the absorption at 525 nm was determined.

Results

Using polarographic techniques, Brosnan and Fritz (1971) reported that the carnitine-stimulated oxidation of palmityl-CoA by adult and fetal heart mitochondria was increased by increased ionic strength of the assay medium. Because the addition of palmitylcarnitine increased total oxygen consumption in intact mitochondria *independent* of any change in ionic strength (Brosnan and Fritz, 1971), the involvement of carnitine palmityltransferase I was implicated in the effects described. Therefore, the activity of the transferase I in intact mitochondria was determined at different concentrations of added ions. The osmolarity was kept constant by varying volumes of 440:140 mM mannitol–sucrose added to the incubation vials. The results are shown in Figure 1. The addition of choline^{+}, Li^{+}, and K^{+} produced a 4.5- to 5-fold increase in carnitine palmityltransferase activity measured at 25°. The K_m values for these ions were 2, 5.5, and 4 mM, respectively. The addition of Tris^{+} (pH 7.4), produced an approximate 8-fold increase in enzyme activity when compared to a similar range of ion concentrations for Li^{+}, K^{+}, and choline^{+} (see below). Therefore, the activity of carnitine

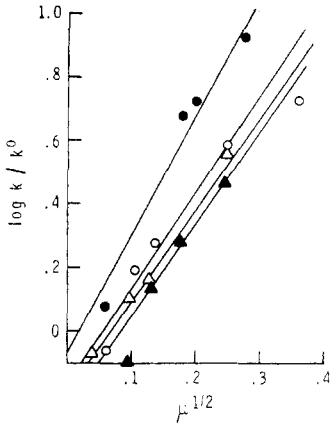

FIGURE 2: The effect of ionic strength on the rate of palmityl-carnitine formation. The rate of change of the log of the rate constant (k) (expressed as nmol of palmitylcarnitine/min per mg), where k^0 = the rate constant at infinite dilution, is plotted *vs.* the square root of the ionic strength ($\mu^{1/2}$). Carnitine palmityltransferase was measured in a total final volume of 0.5 ml (see Methods). Osmolarity was kept constant at 290 mosm. These data were derived from the experiments described in Figure 1: Tris-Cl, ●; KCl, ○; choline-Cl, △; LiCl, ▲.

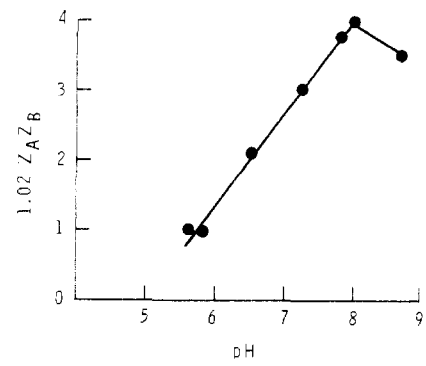

FIGURE 3: The effect of pH on the slope of the line when the rate of change of the log of the rate constant (nmol of palmitylcarnitine/min per mg) is plotted *vs.* the square root of the ionic strength. Carnitine palmityltransferase was measured in a total final volume of 0.5 ml (see Methods). Hepes buffer was added to the reaction medium to achieve the indicated pH. Ionic strength was varied to obtain the slopes at varying pH's by use of appropriate quantities of KCl. Osmolarity was held constant at 290 mosm. Experiments at each pH were repeated in duplicate.

palmityltransferase I exhibited a marked dependence on the salt concentration of the incubation medium. However, when the enzyme activity was measured at higher concentrations of ions (>60 mM), the rate of change of the formation of [^{14}C]-palmitylcarnitine was not consistently affected. Lesser increases in carnitine palmityltransferase I activity were noted along with either no effects or a negative effect at higher concentrations of ions. Therefore, since the effect was consistent only at $\mu < 0.06$ M (here, a linear response of the enzyme to μ was observed) and since the change in enzyme activity was nonspecific for the ion added (the case of Tris$^+$ will be reported below), the possibility of purely ionic interactions being responsible for the changes observed was considered. Here, we must assume that the effects on the enzyme molecule itself are independent of and secondary to the increases in ionic strength.

The effect of ionic strength on ion–ion interactions has been proposed by Brönsted (1922). The expression derived from the Brönsted–Bjerrum equation is as follows (Benson, 1960)

$$\text{in } H_2O \text{ at } 25^\circ\text{: } \log k/k^0 = 1.02 Z_A Z_B \mu^{1/2}$$

where k^0 = the rate constant at infinite dilution and $Z_A Z_B$ = charges on ions A and B. This equation predicts that at low ion concentrations, the rate constant for ion–ion interactions should vary logarithmically with the square root of the ionic strength. Reactions between ions of opposite charge, $Z_A Z_B < 0$, should slow down with increasing μ and reactions between ions of like sign should increase with increasing μ. The equation also predicts that reactions between ions and uncharged species should not be affected by changing μ.

A plot of this equation is shown in Figure 2. The log of the activity of carnitine palmityltransferase varied linearly with the square root of μ. For the ions Tris$^+$, K$^+$, choline$^+$, and Li$^+$, the values for $Z_A Z_B$ = 3.7, 3.04, 2.84, and 2.89, respectively. The positive slope indicated that ions of like charges (3 and 1) were interacting. The more positive slope obtained with addition of Tris$^+$ suggested a pH sensitivity of these slopes.

The pH of the different ionic solutions was varied with 2 mM Hepes. Figure 3 is a plot of the slopes ($1.02 Z_A Z_B$) obtained at the indicated pH's when μ was varied as before. A decrease in the slope, with a decrease in pH, suggested a role for negative charges in the interaction of ions. Moreover, an apparent maximum slope of 3.8 to 4 was obtained at pH 7.8–8.5 indicating the presence of a possible 4 negative charges. Since the pH optimum for carnitine palmityltransferase lies in a range of pH from 7 to 8.2 (Norum, 1964), the slopes at pH 7.4–8.5 were measured at higher total enzyme activities than were the slopes measured at lower pH. However, the logarithm of the rate constant still varied in a linear manner with increases in $\mu^{1/2}$ at pH's below 7.0.

TABLE I: Binding of [1-^{14}C]Palmityl-CoA to Mitochondria in Media of Varying Ionic Strengths.

KCl (mM)	[1-^{14}C]Palmityl-CoA (nmol/mg ± SEM)
0	14.05 ± 1.69
7	22.5 ± 0.9
15	28.3 ± 5.23
30	45.6 ± 1.19
60	71.8 ± 6.2

The net ionic charges on substrates involved in the carnitine palmityltransferase reaction at neutral pH were 1 positive charge in the case of carnitine [$(CH_3)_3N^+$–] and three negative charges in the case of the coenzyme A moiety of palmityl-CoA. The possibility was then considered that in intact mitochondria, ionic strength influenced the association of substrate, in particular, palmityl-CoA, with the mitochondrial membranes. The following results illustrate that this mechanism does indeed occur *in vitro*. While maintaining a constant osmolarity, increases in salt concentration increased the association of [1-^{14}C]palmityl-CoA with the mitochondria 5-fold over an ionic strength range of 0–60 mM (Table I). When these data were replotted according to the Brönsted–Bjerrum derivation, a positive slope of 3.1 was observed (Figure 4A). In addition, the graphic correlation between palmityl-CoA bound and carnitine palmityltransferase activity was linear (Figure 4B).

To determine the role of mitochondrial membranes in the increases in external transferase activity seen with increases in

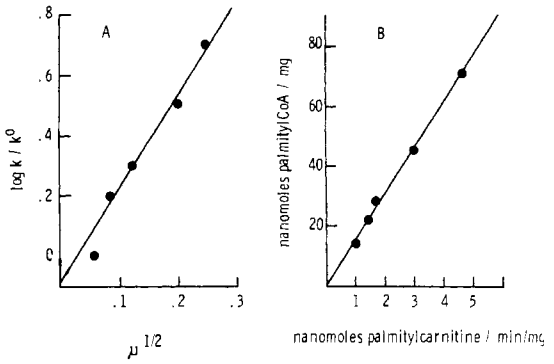

FIGURE 4: (A) The effect of ionic strength on the binding of palmityl-CoA to mitochondria. The effect of ionic strength on the binding of [1-^{14}C]palmityl-CoA to 0.2 mg of mitochondria was determined after incubation for 30 sec at 20°. Osmolarity of the reaction medium was kept constant at 290 mosm by addition of varying amounts of 440:140 mM mannitol–sucrose. KCl was added to vary the ionic strength. The data were plotted from the average of four separate experiments. (B) Association of palmityl-CoA with mitochondria and increased carnitine palmityltransferase activity. The binding of [1-^{14}C]palmityl-CoA to mitochondrial fractions at various ionic strengths was plotted *vs.* carnitine palmityltransferase activity at the same ionic strengths, *i.e.*, 3, 7, 15, 30, and 60 mM ion. Values on the ordinate are derived from Figure 1 (mean of four determinations) and values on the abscissa are derived from Table I (mean of four determinations).

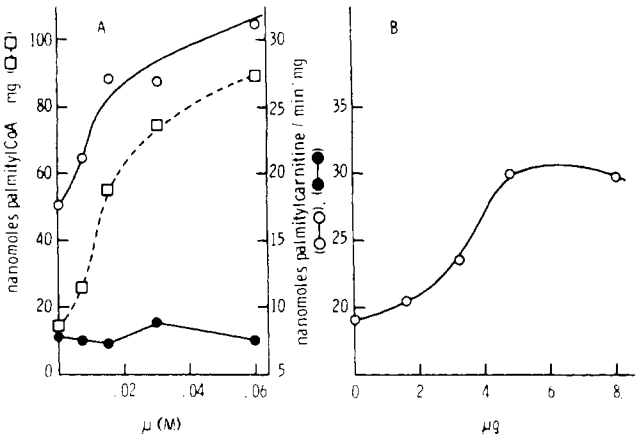

FIGURE 5: (A) The effect of ionic strength on palmityl-CoA binding to outer membrane and on the activity of the digitonin-released carnitine palmityltransferase in the absence and presence of outer membrane. For binding of [1-^{14}C]palmityl-CoA to outer membrane fractions, 0.2 mg of outer membrane was added to a final total volume of 0.5 ml of solution at varying ionic strengths and constant osmolarities. The membranes were spun down and radioactivity and protein concentration determined. Binding at each ionic strength was repeated in duplicate. Binding was expressed as nmol of palmityl-CoA/mg (□). Carnitine palmityltransferase was assayed as described in Methods in a total volume of 0.5 ml. Digitonin-released enzyme protein (22 μg) was added to each reaction tube and the effect of ionic strength on carnitine palmityltransferase activity was determined after incubation for 4 min at 25°. When outer membrane was added to the reaction tubes in 5-μg amounts, enzyme activity was simultaneously determined on "blanks" containing outer membrane alone. The results are representative of three separate determinations. Digitonin-released carnitine palmityltransferase activity was expressed as nmol of palmitylcarnitine/min per mg (●). Digitonin-released carnitine palmityltransferase activity assayed in the presence of 5 μg of outer membrane protein was expressed as nanomol of palmitylcarnitine/min per mg (○). (B) Stimulation of the digitonin-released carnitine palmityltransferase activity by outer membrane protein. Carnitine palmityltransferase was measured in a total final volume of 0.125 ml for 1 min at 30° at an ionic strength of 120 mM Tris (pH 7.6). Varying amounts of outer membrane protein were added to the incubation tube containing 22 μg of digitonin-released carnitine palmityltransferase. Blank tubes contained outer membrane protein alone, and these values were subtracted from the activities in the reaction tubes containing the digitonin-released enzyme. The results are representative of three separate determinations. Activity was expressed as nmol of palmitylcarnitine/min per mg of digitonin-released carnitine palmityltransferase protein.

ionic strength, mitochondria were treated with digitonin to release carnitine palmityltransferase activity associated with the external surface of the inner membrane (Hoppel and Tomec, 1972). Figure 5A demonstrates a lack of effect of ionic strength on the released enzyme activity. Because the outer membrane is not believed to contain endogenous carnitine palmityltransferase activity (Hoppel and Tomec, 1972; Brosnan *et al.*, 1973), and since mitochondrial membranes may provide binding sites for palmityl-CoA molecules (Vaartjes *et al.*, 1972) thereby affecting substrate availability to the enzyme, preparations of outer membrane were added back to the soluble carnitine palmityltransferase activity. Monoamine oxidase activities of these outer membrane suspensions averaged 148 nmol/min per mg and were comparable to those activities reported by Schnaitman and Greenawalt (1968) for their digitonin-treated preparation. The addition of varying amounts of outer membrane protein to the digitonin-released carnitine palmityltransferase activity significantly enhanced total enzyme activity (Figure 5B). Although contaminating amounts of transferase activity were present in the outer membrane fraction (less than $^1/_{20}$th of the total activity), these amounts were corrected for in the final expression of the data. When outer membrane was added to the released transferase preparation in varying concentrations of KCl, increases in ionic strength significantly enhanced enzyme activity (Figure 5A). With increases in ionic strength, the response of the carnitine palmityltransferase corresponded qualitatively to increases in palmityl-CoA binding to the outer membrane (Figure 5A).

No consistent change in critical micelle concentration of palmityl-CoA with increased salt concentration was observed in a range of μ from 0 to 10 mM KCl (cmc = 3–4 μM). These results were similar to those reported by Zahler *et al.* (1968) for palmityl-CoA at 3 and 30 mM K^+. At 60 mM KCl, the critical micelle concentration for palmityl-CoA decreased to 1.9 μM. It does not appear that an increase in the number of micelles formed over the ionic strength range tested could account for the increased absorption of palmityl-CoA molecules to the mitochondrial or outer membrane fractions.

Discussion

Recently, Hoppel and Tomec (1972) demonstrated dual localization of carnitine palmityltransferase in the mitochondria using digitonin fractionation procedures. Carnitine palmityltransferase I (or A) was loosely bound to the external surface of the inner membrane and digitonin treatment of the mitochondria released this enzyme activity. Tubbs and Chase (1967) had reported that DL-2-bromostearoyl-CoA was a potent inhibitor of the carnitine-dependent mitochondrial oxidation of palmityl-CoA, *i.e.*, of the transferase I, but not the oxidation of palmitylcarnitine. Since there is no reason to think that penetration of DL-2-bromostearoyl-CoA through the outer mitochondrial membrane to the site of the carnitine palmityltransferase I should be any different from that of the substrate, palmityl-CoA, the activity of the external transferase has been described as "nonlatent." It was inferred that

the location of the type I transferase was easily accessible to added carnitine, CoA, and their acyl esters. A factor that influences the response of carnitine palmityltransferase activity has been described in our studies. Ionic strength increases were shown to influence both the binding of palmityl-CoA to mitochondria and the activity of carnitine palmityltransferase, the latter phenomenon first described in intact mitochondria by Brosnan and Fritz (1971). These effects appeared to be directly related and dependent upon the presence of a membrane fraction in the reaction medium, since ionic strength had no effect on the digitonin-released carnitine palmityltransferase activity alone. Outer membrane fractions added back to the released transferase in media of different ionic strengths enhanced the total activity of the enzyme and restored the effect of ionic strength on increasing enzyme activity. These experiments suggest that the released enzyme may "seek out" a membrane in order to function optimally. Association of the soluble transferase with the membrane itself seems likely since the binding capacity of the membrane for palmityl-CoA molecules did not compete with the enzyme activity by making substrate less available to the active site. These experiments are not meant to suggest that the outer membrane is a physiological receptor for carnitine palmityltransferase, but rather that its function may be to act as a "reservoir" for palmityl-CoA molecules. A similar binding to the inner membrane may occur since palmityl-CoA has been demonstrated to inhibit the atractyloside-sensitive site for adenine nucleotide translocation (Pande and Blanchaer, 1971; Vaartjes *et al.*, 1972). Similar experiments with measurements of digitonin-releaseable enzyme activity recombined with the inner membrane were not pursued because of the high levels of the internal carnitine palmityltransferase present in sonic particle preparations of inner membrane (Brosnan *et al.*, 1973). In fact, the significant increases in external transferase activity with increased ionic strength appear to be unique to this form of the enzyme since the internal transferase (measured on sonicated particles from dog heart) responded only minimally to changes in ionic strength (unpublished data). The different degrees of association of the enzymes with the membrane, *i.e.*, loosely bound *vs.* tightly and internally bound, may be an important factor in the observed effect.

The mechanism of the effect of the ionic strength on the increase in palmityl-CoA association with the mitochondrial membranes was not elucidated. An increased dispersion of the palmityl-CoA molecules with increases in salt concentration is probably not an explanation for the results, since Corrin and Harkins (1947) have shown that amphipathic electrolytes with a low critical micelle concentration suffered a large depression of the critical concentration by the addition of salts. Experiments on the critical micelle concentration of palmityl-CoA in solutions of ionic strengths from 0 to 60 mM confirm the original data of Zahler *et al.* (1968), *i.e.*, that very little effect on critical micelle concentration was observed with this range of salt concentrations. The use of the Brönsted and Bjerrum equation has not previously been applied to a biological system or to such a complex series of reaction events.[2] However, considering the specificity of the equation for strictly ionic interactions and the prediction by the slope (as a function of $\mu^{1/2}$ and the logarithm of the rate constant) that negative charges of 3 and 1 were involved in the effect of ionic strength on carnitine palmityltransferase activity, a justification for its use may be made. In addition, the linearity seen in palmitylcarnitine formation is a reflection of the initial assumption that linearity in the slope is produced only through a reaction between ions. Finally, the linear response to increased ionic strength is present only in those ranges of ionic strength where the Debye–Hückel limiting law for the ionic activities can be properly applied (Benson, 1960). The reaction

$$[\text{palmityl-CoA}\cdot H_3]^0 \rightleftharpoons [\text{palmityl-CoA}\cdot H]^{3-} + 3H^+$$

is proposed to provide the ionic charge of -3 at pH 7.0. However, the origin of the charge of -1 (Z_B) in combination with -3 (Z_A) is not clear and may be attributable to a charge on a membrane lipid (Meisner *et al.*, 1972).

In summary, a direct correlation between increased palmityl-CoA binding to mitochondrial membranes and concomitant increases in the activity of the external carnitine palmityltransferase was made. The mechanism of the increased association of palmityl-CoA with mitochondria and with the outer membrane fraction is not clear but may be a function of the ionizable phosphate groups on the coenzyme A moiety of the substrate. The presence of a membrane component not only enhanced transferase activity but appeared to be essential for manifestation of the ionic strength effect on expression of enzyme activity. Finally, this study points to a role of the mitochondrial membranes in a possible control of fatty acid metabolism.

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Phospholipase Activity of Retina and Pigment Epithelium†

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ABSTRACT: The retina proper and pigment epithelium of the bovine eye showed significant phospholipase activity at pH 4.2–4.8 and relatively low activity above pH 8.1. Phospholipases A_1 and A_2 were demonstrated in lysosomal fractions from both structures at the lower pH range while microsomes contained the A_1 form and mitochondria the A_2 form at the alkaline pH. Substrate preference and ionic requirements varied widely within the subcellular fractions. Vitamin A aldehyde and vitamin A alcohol were without effect on phospholipase activity with added substrate but the two compounds differed in their effect on release of fatty acid from endogenous substrates. No significant, consistent phospholipase activity was detected in light-adapted rod outer segments.

Phospholipase activity of two positionally specific types has been reported present in a number of mammalian tissues (Waite, 1973; Franson *et al.*, 1971; Cooper and Webster, 1970; Blaschko *et al.*, 1967; de Haas *et al.*, 1971; Ottolenghi, 1964; Gatt, 1968). Enzymes hydrolyzing β-glycerophosphate or aryl sulfate and demonstrating phospholipase-like activity have been studied in preparations of retina and pigment epithelium (Eichner, 1958; Lessell and Kuwabara, 1964; Abraham *et al.*, 1969; Ishikawa and Yamada, 1970; Burden *et al.*, 1971; Marshall and Ansell, 1971; Magalhaes and Coimbra, 1972); however, the phospholipases of these tissues have not been differentiated nor characterized.

This paper reports on studies of phospholipase A_1 and A_2 activities of bovine retinal and pigment epithelial homogenates and subcellular organelles. A preliminary summary of this work has been previously presented (Swartz and Mitchell, 1973).

Materials and Methods

Bovine eyes, obtained from a local supplier, were placed in appropriate buffers at 0–4° upon removal and retina, pigment epithelium, and rod outer segments were taken immediately from nonfrozen, iced material. Rod outer segments were gently shaken off and treated according to Frank *et al.* (1973); lysosomes were isolated by the method of Sawant *et al.* (1964), and mitochondrial membrane fractions and smooth and rough endoplasmic reticulum were harvested following the sonication method of Sottocasa *et al.* (1967). Particles were sonicated for 1–4 min at 1.0–3.0 A using a 20-kHz Branson sonifier, Model 140, with an ice–salt bath to prevent heating of the particulate preparation. Nonsonicated particles were isolated using 0.25 M sucrose and conventional flotation methods with a Beckman Model L centrifuge and SW 50.1 rotor.

The following marker enzymes were used: monoamine oxidase for outer mitochondrial membrane (McCaman *et al.*, 1965), cytochrome oxidase for inner mitochondrial membrane (Smith, 1955), acid phosphatase for lysosomes (Gianetto and de Duve, 1955), and glucose-6-phosphatase (Swanson, 1955) and ribonuclease (de Duve *et al.*, 1955) for smooth and rough endoplasmic reticulum, respectively. Enzyme specific activities were calculated from the linear segment of plots of concentration of product formed *vs.* time.

Reaction mixtures contained 6 μmol of substrate emulsified in 0.1% Triton X-100, 0.01 M Tris-HCl or acetate buffer, whole homogenate yielding 5–7.5 mg of protein or subcellular fraction containing 0.1–1.0 mg of protein, and $CaCl_2$, EDTA, $MgCl_2$ (0–20 mM), or other additions to give a final volume of 2.0 ml. Alkaline and acid phospholipase activities were linear with time for 15–20 min with up to 60 μg of protein. Maximal hydrolysis (nonlinear activity), however, was noted at a protein concentration of 150–200 μg. Emulsification of substrates in Triton X-100 eliminated the lag period of hydrolysis occasionally noted under alkaline conditions when substrates were originally merely sonicated. Incubation was for 90 min at 37.5° in preliminary experiments to determine "total" hydrolysis and for 20 min at the same temperature in routine analyses. The hydrolyzed fatty acids did not inhibit the forward reaction during this incubation period as long as membrane material was present in the mixture. Emulsification of substrate in Triton X-100 was adopted as a routine procedure after it was found that sonication of substrates in

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