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IN VITRO RESPONSE OF ATPase ACTIVITIES IN TISSUE SUBCELLULAR PARTICLE PREPARATIONS TO A SERIES OF MONO-UNSATURATED C_{18} FATTY ACIDS*

ROBERT B. KOCH
Department of Biochemistry, Mississippi State University, Mississippi State, MS 39762, U.S.A.

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Abstract—The positions of the double bond and the *cis/trans* configurations of six mono-unsaturated C_{18} fatty acids (FA) showed selectivity for inhibition and stimulation of ATPase activities of tissue homogenate fractions. The 13,000 *g* and 100,000 *g* sediments (fractions B and C respectively) of tissue homogenates were used as sources of Na^+–K^+ ATPase and of oligomycin-sensitive (OS) and -insensitive (OIS) Mg^{2+} ATPase activities. Tissue sources included bovine brain and rat brain, kidney, heart and liver. *Cis* mono-unsaturated C_{18} FA caused an apparent uncoupling of mitochondrial coupling factor F_1 (Mg^{2+} ATPase). This was indicated by the loss of oligomycin and 1,1,1-trichloro-2,2 bis (*p*-chlorophenyl) ethane (DDT) sensitivity in the presence of 50 μM FA (12-$C_{18:1}$). Thus, a precipitous decrease in OS-Mg^{2+} ATPase activity of mitochondria was accompanied by an equally steep increase in OIS-Mg^{2+} ATPase activity. This was especially apparent in a heart tissue preparation. The uncoupling action of the FA was not observed with the *trans* mono-unsaturated C_{18} FA. Na^+–K^+ ATPase activity from a bovine brain nerve ending particle (B) fraction was also sensitive to 12-$C_{18:1}$ FA. However, inhibitory responses of Na^+–K^+ ATPase activity were different for OS-Mg^{2+} ATPase activity. The latter (OS) was not sensitive to the *trans* 12-$C_{18:1}$, while the former (Na^+–K^+) was sensitive to both *cis* and *trans* forms of 12-$C_{18:1}$ and inhibition appeared to be dependent on the position of the double bond.

Long chain fatty acids (FA)†, especially those that are mono-unsaturated, have been shown to inhibit Na^+–K^+ ATPase activity [1]. It was reported [1] that the mono-unsaturated C_{16} and C_{18} FA (palmitoleate and oleate) were much more effective as inhibitors of Na^+–K^+ ATPase than were the corresponding saturated FA. Earlier studies had shown that FA caused both stimulation and inhibition of mitochondrial metabolism. Pressman and Lardy [2] reported stimulation of latent ATPase activity of mitochondria by saturated and unsaturated FA. Lehninger and Remmert [3] found that isooctane extracts of liver mitochondria contained a factor with properties of long chain FA that caused uncoupling of oxidative phosphorylation. The ATP–$^{32}P_i$ exchange reaction of mitochondrial preparations has also been shown to be sensitive to FA [4, 5].

The present studies were conducted on a series of *cis/trans* isomers of highly purified mono-unsaturated C_{18} FA differing in the position of the single double bond. This series of FA was used to investigate the importance of these structural differences to their inhibitory effects on ATPase activities of several different tissues.

MATERIALS AND METHODS

Bovine brain was obtained from cows freshly slaughtered at the Bryan Brothers Meat Packing Co., West Point, MS. Special chemicals (ATP, PK-LDH, NADH, etc.) were obtained from the Sigma Chemical Co., St. Louis, MO. Analytical grade chemicals were obtained from the Fisher Scientific Co., Pittsburgh, PA. Rat brain, kidney, liver and heart tissues were obtained from a Sprague–Dawley-derived strain of white rats. Tissues were removed immediately after stunning the rats with a sharp blow to the base of the head and were homogenized in 0.32 M sucrose, 10 mM imidazole·Cl (pH 7.5) and 1 mM EDTA solution (1/10, w/v). Homogenization and fractionation were as previously described [6]. The 13,000 *g* pellets, designated fraction B, contained mitochondria and nerve ending particles (brain only) and the 100,000 *g* pellets, designated fraction C, contained primarily microsomal membranes. The pellets were resuspended in homogenization solution (buffered sucrose–EDTA), were frozen as concentrates in liquid nitrogen, and were stored at −20°. Just before use, the concentrates were diluted with sucrose solution to give 25–50 μg protein per 50 μl solution.

F_o and F_1 coupling factors were isolated from mitochondria [18]. ATPase activities were assayed by the continuous method of Pullman *et al.* [7] as described by Koch and Gilliland [8]. Absorbance changes were measured at 340 nm using a Gilford

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† Abbreviations: FA, fatty acids; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; F_1, water-soluble Mg^{2+} ATPase; OS, oligomycin sensitive; OIS, oligomycin insensitive; and Ouab-Ins, ouabain-insensitive.

2400 recording spectrophotometer with the temperature controlled at 37°. Different types of ATPase activity were distinguished by the use of ouabain (cardiac glycoside specific inhibitor of Na^+–K^+ ATPase) and oligomycin (antibiotic specific inhibitor for mitochondrial Mg^{2+} ATPase).

Mono-unsaturated FA were obtained from the Hormel Institute of the University of Minnesota, Austin, MN, as solutions containing approximately 20 mM FA in 47.5% ethanol, 30 mM NaOH. Six different samples were used: *cis* and *trans* isomers of 18 carbon straight-chain FA with a double bond at position C_{12}, C_9 or C_6 from the carboxyl carbon (e.g. 9-$C_{18:1}$ *cis*, oleic acid; 9-$C_{18:1}$ *trans*, elaidic acid). FA additions to a 1-ml rapidly stirring reaction mixture were made with a 1-μl Hamilton syringe, using appropriate aliquots of each solution of FA to give the desired concentration. The order of additions to the reaction mixture was important for maximal and reproducible inhibition of enzyme activities. The procedure used was: (1) 0–1 μl FA was added to 0.9 ml of reaction mixture, then 50 μl of tissue fraction was added and the mixture was stirred and held at 37° for 2 min; (2) 50 μl substrate (4.5 mM ATP and 0.5 mM PEP) was added, and the mixture was stirred and held at 37° for 3 min; and (3) the decrease in absorbance at 340 nm was recorded for 5–10 min.

Proteins were determined by the method of Lowry *et al.* [9] using bovine serum albumin (BSA) as the standard.

RESULTS

Responses of rat heart Mg^{2+} *ATPase activities*

Effect of 12-$C_{18:1}$ *cis FA on rat heart OS- and OIS-*Mg^{2+} *ATPase activities.* Figure 1 shows the time course of the response of rat heart ATPase activity to the presence or absence of 50 μM 12-$C_{18\ 1}$ *cis* FA. Rat heart OS-Mg^{2+} ATPase activity (curves 1 minus 2) was almost completely inhibited by 5 μM DDT (curves 3 minus 4). Curves 5–8 were obtained in the presence of 50 μM FA and showed a complete loss of OS-Mg^{2+} ATPase activity. Curves 7 and 8 also contained 5 μM DDT and also showed loss of sensitivity to DDT.

Effects of increasing the FA (12-$C_{18.1}$ cis *or* trans) *concentration on OS- and OIS-*Mg^{2+} *ATPase activities from rat heart subcellular particle fractions B and C.* Figure 2 shows that *cis* FA caused concentration dependent changes in ATPase activities. For the heart B fraction, with 25 μM FA a slight increase in OS activity, and a slight decrease in OIS activity, occurred. However, with 50 μM *cis* FA a complete loss of OS activity, and a concomitant large increase in OIS activity, were observed; with a 100 μM concentration of *cis* FA the OIS activity was inhibited to about 50% of the activity with the 50 μM concentration of *cis* FA. The results of similar tests using *trans* FA showed a slight stimulation of OS activity with a 25 μM concentration, but little or no further effect was observed with increasing *trans* FA con-

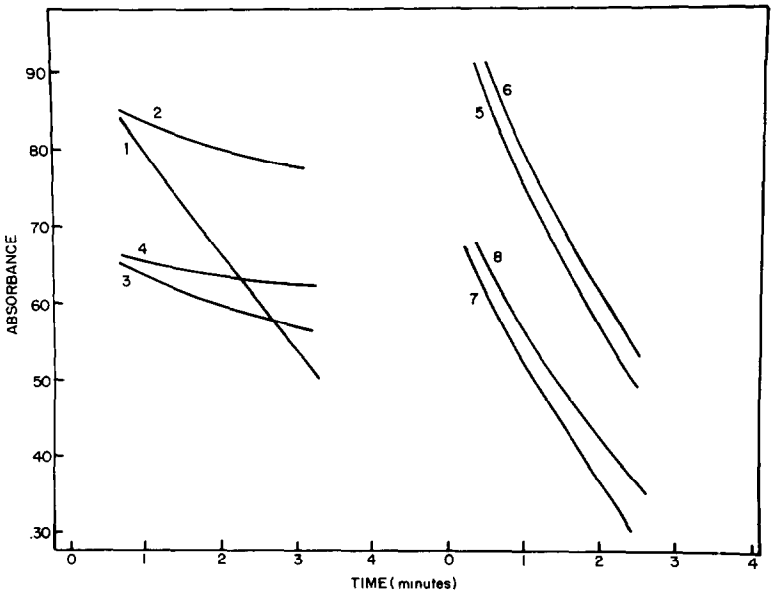

Fig. 1. Time courses (tracings of recordings of absorbance at 340 nm) of ATPase activities in the absence and presence of DDT and of 12-$C_{18.1}$ *cis* FA. Odd numbered curves indicate that the reaction mixtures contained ouabain as a specific inhibitor of Na^+–K^+ ATPase activity. See Materials and Methods for reaction conditions. Even numbered curves indicate that the reaction mixtures contained ouabain + oligomycin as inhibitors of Na^+–K^+, and mitochondrial Mg^{2+}, ATPase activities. Odd numbered curves depict total Mg^{2+} ATPase activities; even numbered curves depict OIS-Mg^{2+} ATPase activity. OS-Mg^{2+} ATPase activity equaled total Mg^{2+} ATPase minus OIS-Mg^{2+} ATPase activities. Key: Curves numbered 1 and 2 are controls; 3 and 4, plus 5 μM DDT; 5 and 6, plus 50 μM 12-$C_{18\ 1}$ *cis*; 7 and 8, plus 5 μM DDT and 50 μM 12-$C_{18:1}$ *cis*. Specific activities for individual tracings were: (1) 102.0, (2) 18.8, (3) 207.8, (4) 10.0, (5) 137.4, (6) 137.4, (7) 122.9, and (8) 108.4 μmoles Pi·(mg protein)$^{-1}$·hr^{-1}.

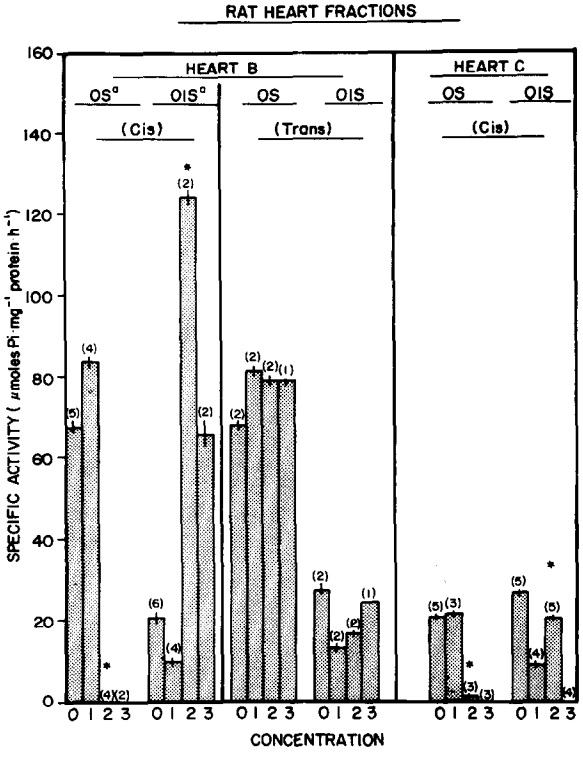

Fig. 2. Effects of 12-$C_{18:1}$ *cis* and *trans* FA on OS- and OIS-Mg^{2+} ATPase activities from rat heart homogenate fractions B and C. Abscissa numbers 0, 1, 2 and 3 indicate 0, 25, 50 and 100 μM fatty acid respectively. Key: (a) OS = oligomycin sensitive and OIS = oligomycin insensitive Mg^{2+} ATPase activities. (*) FA concentration at which opposite responses occurred for OS and OIS activities. The number of determinations and S.E.M. are indicated at the top of each bar. See Materials and Methods for reaction conditions.

centration for either OS or OIS activity. The microsomal (C) fraction from rat heart showed a pattern in response to increasing *cis* FA concentrations of OS and OIS activities, similar to those shown by heart fraction B. The much lower OIS activity in heart fraction C was totally inhibited by 100 μM *cis* FA.

A similar investigation was conducted on a B fraction from rat heart using 49 μM 6-$C_{18:1}$ *cis* FA in place of 50 μM 12-$C_{18:1}$ *cis* FA with the same (e.g. OS activity was completely inhibited and OIS activity was stimulated about 10-fold) results (data not presented). This comparison showed that the position of the double bond in the $C_{18:1}$ *cis* FA was not the cause of the changes in the responses of the OS- and OIS-Mg^{2+} ATPase activities.

Responses of rat liver, brain and kidney ATPase activities

Effects of 12:$C_{18:1}$ cis *FA concentration on OS- and OIS-Mg^{2+} ATPase activities from rat liver subcellular particle fractions B and C.* Figure 3 shows that the OS-Mg^{2+} ATPase activity of liver fraction B was stimulated significantly by 25 μM FA. This enzyme activity was only partially inhibited at 50 μM FA and some activity remained even in the presence of 100 μM FA. Also, stimulation of OIS-Mg^{2+} ATPase from fraction B was higher at 100 μM FA

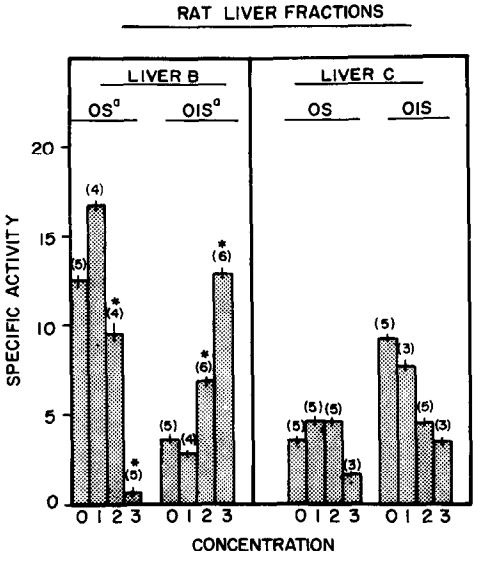

Fig. 3. Effect of 12-$C_{18:1}$ *cis* FA on OS- and OIS-Mg^{2+} ATPase activities from rat liver homogenate fractions B and C. Abscissa numbers 0, 1, 2, and 3 indicate 0, 25, 50 and 100 μM FA respectively. Key: (a) OS = oligomycin sensitive and OIS = oligomycin insensitive Mg^{2+} ATPase activities. (*) FA concentrations at which opposite responses occurred for OS and OIS activities. The number of determinations and S.E.M. are indicated at the top of each bar. See Materials and Methods for reaction conditions.

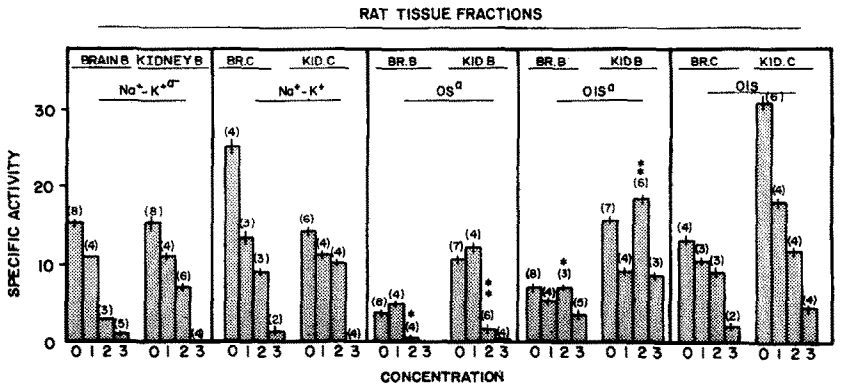

Fig. 4. Effect of 12-$C_{18:1}$ *cis* FA on Na^+–K^+, OS-, and OIS-Mg^{2+} ATPase activities from rat brain and kidney homogenate fractions B and C. Abscissa numbers 0, 1, 2 and 3 indicate 0, 25, 50 and 100 μM FA respectively. Key: (a) Na^+–K^+ = Na^+–K^+ ATPase activity; OS = oligomycin sensitive and OIS = oligomycin insensitive Mg^{2+} ATPase activities. (*) and (**) FA concentrations at which opposite responses occurred for OS and OIS activities. The number of determinations and S.E.M. are indicated at the top of each bar. See Materials and Methods for reaction conditions.

than at 50 μM. OS- and OIS-Mg^{2+} ATPase activities of liver fraction C showed quite different response patterns to increased FA concentration compared to fraction B activities. OS-Mg^{2+} ATPase activity in fraction C was quite low (as would be expected for a microsomal fraction) and only showed inhibition by 100 μM FA. OIS-Mg^{2+} ATPase (fraction C) showed a slow but steady decrease in activity with increase in FA.

Effects of 12-$C_{18:1}$ cis *FA concentration on* Na^+–K^+ *OS-, and OIS-*Mg^{2+} *ATPase activities from rat brain and kidney subcellular particle fractions B and C.* The Na^+–K^+ ATPase activities, in relatively high proportions in rat brain and kidney B and C fractions, showed similar inhibitory response patterns with increasing concentrations of 12-$C_{18:1}$ *cis* FA (Fig. 4). Little or no OS-Mg^{2+} ATPase activity was present in brain and kidney C fractions. The OIS activity was much higher in the kidney C fraction than in the brain C fraction. However, both C fractions showed similar patterns of inhibition of enzyme activity with increasing concentrations of FA. As was observed for OIS activities in brain and kidney B fractions, the OIS activities in the C fractions were not completely inhibited at 100 μM FA.

Responses of bovine brain ATPase activities

Effects of cis/trans *configuration and of the position of the double bond in mono-unsaturated FA on* Na^+–K^+ *and Ouab-Ins-*Mg^{2+} *ATPase activities.* Figure 5 shows the effects of the *cis* and *trans* isomers of three mono-unsaturated 18 carbon FA, with the double bond at carbon atom 12, 9, or 6, on the activity of a nerve ending particle (B) fraction from a bovine brain homogenate. The bovine brain Na^+–K^+ ATPase activity was strongly inhibited by both the *cis* and *trans* isomers of the three mono-unsaturated C_{18} FA. At the lowest concentration tested (15–16 μM), there was a difference in sensitivity of the Na^+–K^+ ATPase to the three *cis* FA. The order of inhibition of Na^+–K^+ ATPase was $\Delta^6 > \Delta^9 > \Delta^{12}$. However, at 30–32 and 60–64 μM concentrations there was little difference in the amount of inhibition by the six FA. The total Mg^{2+} ATPase activities (Ouab-Ins) were only slightly inhibited at all levels of fatty acid concentration tested.

DISCUSSION

The results presented in this paper show that under *in vitro* conditions the position and *cis/trans* configuration of the double bond in mono-unsaturated C_{18} FA have modulating effects on ATPase activities,

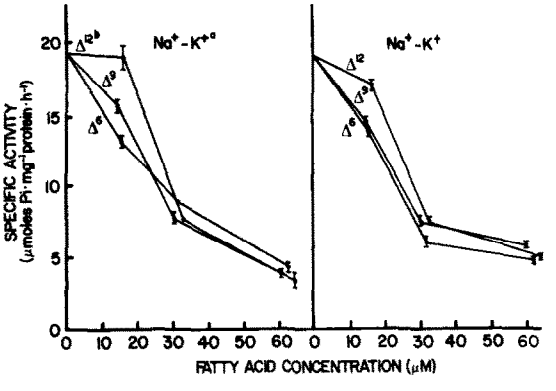

Fig. 5. Effects of $C_{18:1}$ *cis* and *trans* FA on Ouab-Ins, and Na^+–K^+, ATPase activities from bovine brain fraction B. Each point is the average of a minimum of four determinations, and the error limits for S.E.M. are indicated. At 15–16 μM, the differences in inhibition by the *cis*-fatty acids (left side) were highly significant. According to Scheffe's procedure, all three means were different at less than the 0.0005 confidence level [H. Scheffé, *Biometrika* **40**, 87 (1953)]. According to Duncan's procedure, the inhibition by Δ_6 *trans*-fatty acid (right side) was different from the other two fatty acids at less than the 0.05 confidence level; inhibitions by the other two fatty acids were not different. Key: (a) Na^+–K^+ = sodium–potassium stimulated Mg^{2+} ATPase activity. (b) Δ^{12}, Δ^9 and Δ^6 indicate the positions of the double bond in the different fatty acids.

depending on the type and the source of the activity. As seen in Figs. 2 and 3, the increase in Mg^{2+} ATPase activity with 25 μM 12-$C_{18:1}$ *cis* FA was due to stimulation of OS-Mg^{2+} ATPase activity, whereas with 50 μM FA the further increase in Mg^{2+} ATPase activity was oligomycin insensitive. The large increase in OIS activity with a concurrent, complete loss of OS activity at 50 μM FA (Fig. 2) was indicative of uncoupling of F_1 ATPase. The FA-induced Mg^{2+} ATPase activity was insensitive to oligomycin and DDT (Fig. 1) as are purified F_1 preparations from beef heart [7] and cockroach coxal muscle [10, 11].

Studies similar to those conducted on rat heart (fractions B and C) were conducted on rat liver, brain and kidney preparations. The results from heart and liver preparations (Figs. 2 and 3) were qualitatively the same except that liver OS-Mg^{2+} ATPase (Fig. 3) was less sensitive to the FA, as indicated by a lack of complete inhibition of OS activities and stimulation of OIS activities with 100 μM FA. Also, brain and kidney C fractions, which would be expected to lack OS-Mg^{2+} ATPase activity, showed only inhibition of OIS-Mg^{2+} ATPase activity with increase in FA concentration. However, except that brain and kidney preparations were lower in specific activities, the patterns of response for OS and OIS activities (Fig. 4) were very similar to those observed for the heart preparations (Fig. 2).

Na^+–K^+ ATPase activities from rat brain and kidney B and C fractions showed concentration-dependent inhibition responses to increases in FA (12-$C_{18:1}$ *cis*) concentration (Fig. 4). Bovine brain fraction B was used for a more detailed study of the effect of position and *cis/trans* configuration of mono-unsaturated C_{18} FA. Inhibitory responses to *cis* or *trans* FA were essentially similar. However, at 15–16 μM FA the position of the double bond had significantly different inhibitory effects on Na^+–K^+ ATPase activity (Fig. 5). Inhibition of Na^+–K^+ ATPase by 50 μM oleate (9-$C_{18:1}$ *cis*) was about 70% for bovine brain, in close agreement with results reported by Ahmed and Thomas [1] for a microsomal rat brain preparation (66% inhibition). Karli *et al.* [12] observed 55% inhibition of sarcolemma Na^+–K^+ ATPase at 100 μM myristate, while Ahmed and Thomas [1] reported 41% inhibition with 50 μM myristate for the enzyme from rat brain. The length of the saturated hydrocarbon chain is also important for inhibition of Na^+–K^+ ATPase activity, since stearic acid has little or no effect [1, 13]. Skou [13] also reported complete inhibition of rabbit brain microsomal Na^+–K^+ ATPase activity with 0.5 mM oleic acid.

Three reports which appeared during the final preparation of this manuscript add further evidence of the importance of phospholipoprotein complex formation. Reactivation of a yeast Mg^{2+} ATPase preparation after lipid depletion by solubilization with lysolecithin and purification by density gradient centrifugation was accomplished by mixing the enzyme with preformed lipid micelles or vesicles [13]. Lecithins of varying FA chain length and unsaturation reactivated the enzyme to different extents [14]. The inhibition of anti-immunoglobulin cap formation by *cis*, but not by saturated or *trans* FA, reported by Klausner *et al.* [15], was found to be associated with effects of FA on ATP levels in cells [16]. They showed that 70 μM linoleic acid caused uncoupling of oxidative phosphorylation as effectively as did 2.5 μg/ml oligomycin. The above concentration of linoleic acid is very similar to the concentration of oleic or linoleic acids (50 μM) that caused apparent "uncoupling" of F_1 from the mitochondrial (B) fraction of heart, liver and kidney preparations in the present study.

The results of the present and the above cited studies could have been due to disruption or perturbation of the secondary bonding forces of the phospholipoprotein complex in the native membrane structures. "Fine tuning" of lipoprotein complexes of membrane bound enzymes may be accomplished by intimate association of the unsaturated FA alkyl chain with the nonpolar groups of amino acids in the integral membrane proteins. Such perturbations have been observed (the results of a long-term study in my laboratory) as result of the action of odorous chemicals on Na^+–K^+ ATPase from homogenates of olfactory epithelial tissue and have been proposed to be associated with the mechanism for initiation of odor sensing [8, 17].

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