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MEMBRANE LIPID FATTY ACIDS AND REGULATION OF MEMBRANE-BOUND ENZYMES

ALLOSTERIC BEHAVIOUR OF ERYTHROCYTE Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase AND ACETYLCHOLINESTERASE FROM RATS FED DIFFERENT FAT-SUPPLEMENTED DIETS*

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SUMMARY

Studies were carried out to determine the Hill coefficients for the inhibition by F^- of the erythrocyte membrane-bound Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase and acetylcholinesterase from rats fed with seven different diets. Five groups were fed with different natural fats or oil supplements, one with a hydrogenated fat supplement and the other with fat-free diet. The responses of the red cell fatty acids to dietary fats were recorded. The values of n for the inhibition by F^- of the three enzymes revealed a particular and different behaviour in each group. Correlations between the fatty acid compositions of erythrocyte membranes and cooperativity of each enzyme were calculated. The results indicate that neither the essential fatty acid family nor the non-essential ones are particularly involved in the allosteric phenomena. The increase of the double bond index/saturation ratio of fatty acids, which is taken as indicative of membrane fluidity, was accompanied in an inverse manner by changes in allosteric transitions of the $(Na^+ + K^+)$-ATPase and acetylcholinesterase, whereas the Mg^{2+}-ATPase was not dependent on this ratio. Diminution of membrane fluidity, carried out by *in vitro* increase of its cholesterol content, yields confirmatory results of this regulatory mechanism since the value of n for acetylcholinesterase shifted as predicted.

These facts indicate that the membrane fluidity is a physiological regulator for the allosteric behaviour of the membrane-bound enzymes and that each enzyme exhibits a particular behaviour in this phenomenon.

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INTRODUCTION

The allosteric regulation of membrane-bound enzymes has been investigated by our laboratory. This regulation phenomenon was previously reported in erythrocyte membrane-bound ATPases from rats fed a fat-free diet[1,2]. In these animals, the fatty acids of the oleic acid family ($n-9$) are increased and the fatty acids of the linoleic acid family ($n-6$) are decreased[3].

The isolation of mutants of *Escherichia coli*[4] which require an unsaturated fatty acid for growth has made possible investigations into the presence of the oleic and linoleic acid families in the lipid composition and its effect on the allosteric behaviour of the membrane-bound ATPase[5].

In both systems, animal and bacterial, a decrease in the allosteric behaviour was observed when the ($n-9$) fatty acid content increased in the fatty acid composition of the membrane[1,2,5]. The assumption of a relationship between the "phenotypic allosteric desensitization phenomenon"[6] and ($n-9$) fatty acid content seems therefore plausible. However, we describe in this paper, that when the rats were fed a hydrogenated fat supplement in order to produce an essential fatty acids deficiency, the accumulation of ($n-9$) fatty acid was not accompanied by a decrease in the allosteric behaviour of the ATPases. In addition, even though low values of n were previously observed with rats fed a commercial diet[7], a high ($n-6$) fatty acid content is reported here for erythrocytes from this groups of animals.

Experiments are described which furnish further evidence for the role assumed by the unsaturated fatty acids in the regulation exerted by the membrane structure on the allosteric behaviour of bound enzymes. It is shown that changes in the fatty acid composition of the erythrocyte membrane lipids, induced by the nature of the dietary fat, are accompanied by marked changes in the allosteric inhibition by F^- of the ATPases (ATP phosphohydrolase, EC 3.6.1.3) and acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7). An inverse relationship between the double bond index/saturation ratio and the absolute values of n for the inhibition by F^- of $(Na^+ + K^+)$-ATPase was observed. The situation was the opposite for acetylcholinesterase since the allosteric transitions increased with the rise of the double bound index/saturation ratio. Mg^{2+}-ATPase presented no correlation between these parameters.

In addition, we offer biochemical evidence regarding the molecular basis of the interaction of membrane lipids and membrane enzymes through studies of the effect of cholesterol-dependent fluidity on the allosteric behaviour.

METHODS

Animals and diet

Male Sprague–Dawley rats were put on solid diets after weaning and used after 15 weeks. The composition of the basic diet without fat supplement has been published previously[2]. In order to produce a deficiency in essential fatty acids, the rats were fed with a fat-free diet and a diet with a hydrogenated fat (5%) supplement. In the fat-sufficient diets, 5% corn oil, olive oil, linseed oil or lard were used as fat supplements. One group of rats was fed a standard rat diet manufactured by Molinos, Rio de la Plata (Argentina).

Enzymatic preparation

Red cell ghosts were prepared by the method of Dodge *et al.*[8] with the addition of 1 mM EDTA in the 20 mosM sodium phosphate buffer (pH 7.4). They were washed twice with a solution of 1 mM cysteine in 20 mM imidazole buffer (pH 7.5), and then resuspended in the same buffer. The determinations of the ATPase activities were performed with freshly prepared cell ghost suspensions or with preparations which had been kept frozen in liquid nitrogen for not more than 1 week. Suspensions of freshly prepared red cell ghosts in 20 mosM phosphate buffer (without additional washes with imidazole buffer) or preparations which had been kept at 4 °C for not more than 3 days were used for determinations of acetylcholinesterase activity.

Solubilization and reaggregation of red cell ghost acetylcholinesterase

The solubilization of the acetylcholinesterase and the preparation of membrane-like material were carried out by the method previously described[6]. The membrane-like reconstituted material was loaded with cholesterol by adding it before dialysis.

Assays of enzyme activities

The reaction mixture for the determination of total-ATPase, which is the sum of the activities of the Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase, consisted of: 25 mM Tris–HCl buffer (pH 8.0); 2 mM $MgCl_2$; 1 mM ATP (Tris salt); 80 mM NaCl; 33 mM KCl, 40–80 μg of ghost protein, in a final volume of 2 ml. The Mg^{2+}-ATPase component of the total-ATPase was determined by omitting Na^+ and K^+ from the reaction mixture. The mixture was incubated at 37 °C for 30–60 min. The reaction was stopped by the addition of 0.3 ml 40% trichloroacetic acid, and after 5 min at 0 °C it was centrifuged. P_i liberated was estimated in the supernatant fluid by the method of Fiske and SubbaRow[9].

Acetylcholinesterase activity was determined in the following incubation mixture; 100 mM sodium phosphate buffer, (pH 8.0); 0,87 mM $MgCl_2$, 0.5 mM acetylthiocholine iodide, 20–40 μg ghost protein, final volume 3 ml. The thiocholine liberated was determined by the method of Ellman *et al.*[10]. The mixture was incubated at 30 °C for 30 min.

Inhibition by F^- was investigated by measuring the enzymatic activities in presence of a concentration of F^- from 0.3 to 2.5 mM. The values of n and K_i of the acetylcholinesterase, Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase were determined graphically by using the following equations:

$$\log(v/V_0 - v) = \log K_i - n \log(I) \text{ (refs 11 and 12) and}$$

$$\log\left(\frac{V_t - V_b}{v_t - v_b} - 1\right) = \log K_i + n_a \log(I) \text{ (ref. 2)}$$

since the inhibition by F^- approached 100% (not shown). In the latter equation, V_t and V_b are the maximal reaction velocities for the total- and Mg^{2+}-ATPase, respectively; v_t and v_b the reaction velocities for the total- and Mg^{2+}-ATPase, respectively; K_i, the Hill equation constant; n_a is the Hill coefficient and I is the F^- concentration. The % of $(Na^+ + K^+)$ activation in the ATPase system was calculated as the ratio $\times 100$ of the $(Na^+ + K^+)$-ATPase and the total ATPase activities.

TABLE I

FATTY ACID COMPOSITION (MOLAR PERCENTAGE) OF DIETARY FATS AND ERYTHROCYTE GHOSTS

The analyses were performed as described under Methods. The values given represent an average for three preparations with a S.E. of less than ±4% in each case.

Fatty acid	*Fat-free ghosts*	*Hydrogenated fat*		*Lard*		*Olive oil*		*Corn oil*		*Linseed oil*		*Standard diet*	
		Fat	*Ghosts*	*Fat*	*Ghosts*	*Fat*	*Ghosts*	*Fat*	*Ghosts*	*Fat*	*Ghosts*	*Fat*	*Ghosts*
16:0	28.4	30.3	31.5	26.9	31.0	14.9	29.5	14.3	28.6	9.1	31.2	22.3	27.5
16:1 (*n*−7)	2.9	—	3.1	3.1	1.7	1.5	5.7	0.1	4.8	0.8	1.4	2.7	2.4
18:0	13.1	57.2	13.9	11.5	16.1	2.8	13.9	2.5	12.6	4.9	16.5	8.6	15.0
18:1 (*n*−9)	16.5	—	17.6	42.9	14.9	64.8	14.1	33.3	10.8	20.5	12.8	27.1	10.0
18:2 (*n*−6)	2.6	—	2.6	10.6	6.5	14.9	5.6	47.3	10.9	13.7	7.3	34.3	10.8
18:3 (*n*−3)	—	—	—	0.5	—	0.6	—	2.2	—	51.0	0.5	2.4	—
20:1 (*n*−9)	0.5	—	0.4	0.8	0.4	—	0.2	—	0.2	—	—	—	0.2
20:3 (*n*−9)	10.5	—	8.9	—	0.8	—	0.8	—	0.1	—	0.1	—	0.1
20:3 (*n*−6)	—	—	—	—	0.8	—	0.5	—	0.4	—	0.5	—	0.1
20:4 (*n*−6)	18.2	—	14.6	—	20.8	—	22.6	—	23.9	—	12.4	—	22.7
20:5 (*n*−3)	—	—	—	—	—	—	0.2	—	0.1	—	5.6	—	0.9
22:3 (*n*−9)	1.1	—	0.8	—	—	—	—	—	—	—	—	—	—
22:4 (*n*−6)	0.1	—	—	—	1.8	—	1.8	—	2.1	—	—	—	1.5
22:5 (*n*−6)	0.8	—	0.9	—	0.9	—	0.7	—	0.7	—	—	—	0.5
22:5 (*n*−3)	0.2	—	0.3	—	0.6	—	0.6	—	1.0	—	4.8	—	2.5
22:6 (*n*−3)	1.0	—	1.4	—	1.0	—	1.0	—	1.4	—	3.4	—	2.9
24:0	1.3	—	1.3	—	0.8	—	0.9	—	1.1	—	2.0	—	1.6
24:1 (*n*−9)	2.8	—	2.5	—	1.8	—	1.8	—	1.4	—	1.6	—	1.2

Chemical analyses

The method of Lowry *et al.*[13] was used to determine the protein contents. The cholesterol determinations were performed by the method of Zlatkis *et al.*[14].

The analysis of ghost fatty acids were carried out on samples extracted according to the method of Folch *et al.*[15]. Lipids were transesterified with 14% boron trifluoride in methanol for 90 min in sealed ampoules. The methyl esters were purified by thin-layer chromatography and analyzed by gas–liquid chromatography. The antioxidant BHT (2,6, di-*tert*-butyl-*p*-cresol) was used throughout the procedure in order to avoid autoxidation of the highly unsaturated fatty acids[16]. As an aid in identification of fatty acid methyl esters of overlapping peaks on chromatograms, samples were fractioned according to their degree of unsaturation by thin-layer chromatography on $Ag(NH_2)_2NO_3$-impregnated silica gel before gas chromatographic running. Gas–liquid chromatography was performed with an F and M instrument Model 700 equipped with paired 6-ft columns of EGSS-X 10% on chromsorb W. AW DMCS (Applied Sciences Lab. Inc.) and dual flame-ionization detectors. The peaks were identified by comparison with standards (Sigma Chem. Co. and Applied Science Lab. Inc.). Quantitation was achieved by multiplication of the peak height by the width at half-height.

RESULTS

Effects of dietary fat on erythrocyte fatty acids

Variations in the nature of the dietary fat resulted in changes in the mixed fatty acid composition of rat erythrocytes[17]. The analytical fatty acid compositions of dietary fat supplements and erythrocyte membranes are presented in Table I. The fatty acid composition of red cells was remarkably reproducible in different animals of the same groups. These were essentialy the same as described by several authors[18,19].

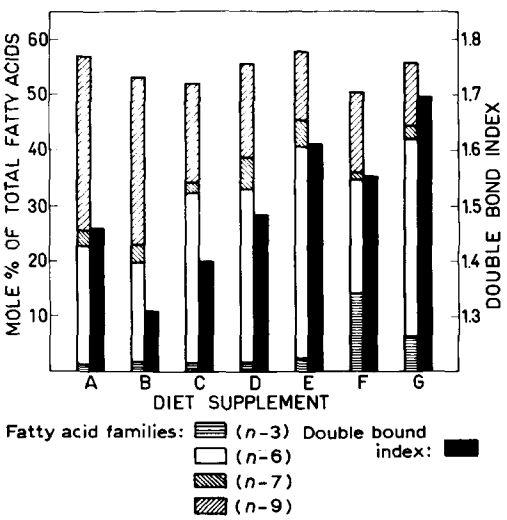

Fig. 1. Effect of dietary fat supplements on the content of unsaturated fatty acids and double bond index of erythrocyte lipids. Diet supplements: A, fat-free; B, hydrogenated fat; C, lard; D, olive oil; E, corn oil; F, linseed oil; and G, standard diet.

A marked distinction between the fatty acid families which can be synthesized endogenously and those which are dependent on a dietary source can be seen in Fig. 1. Evidence of a regulated synthesis and distribution of fatty acid, as described above, has been reported[3,17]. Although the rats were able to maintain fairly constant the proportion of unsaturated fatty acid in spite of the wide differences in the unsaturation of the dietary supplements, they were unable to maintain a constant number of total double bounds in the membrane (Fig. 1 and refs 20 and 21). No dietary influence was observed in the cholesterol and lipid phosphorus content (not shown).

Essential fatty acid deficiency and the allosteric inhibition by F^- of the ATPases

Similar and typical signs of essential fatty acid deficiency were found in rats fed the fat-free diet and the diet with a hydrogenated fat supplement. An increase of $(n-9)$ and a reduction of $(n-6)$ fatty acids were observed (Fig. 1). Holman[23] showed that when the ratio $20:3(n-9)/20:4\,(n-6)$ was higher than 0.4, the rats were deficient in essential fatty acids. This ratio was similar and higher than 0.4 in red cells from both groups of animals; (0.58 for fat-free and 0.61 for hydrogenated-fat fed rats). The kinetic parameters for F^- inhibition of the ATPase system of the two groups of essential fatty acid-deficient animals are presented in Table II. As can be observed, the values of n for the ATPases of animals fed a diet with a hydrogenated fat supplement are significantly higher ($P<0.001$) than those corresponding to animals fed a fat-free diet. The values of K_i were similar for the enzymes of the two groups (not shown). The % of $(Na^+ + K^+)$-activation showed no difference between both groups.

The values of n from rats fed a fat-free diet presented in Table II agreed with the values communicated in a previous work[2].

TABLE II

ACTIVATION BY $Na^+ + K^+$ AND n VALUES FOR THE INHIBITION BY F^- OF THE ATPases FROM RATS FED FAT-FREE AND HYDROGENATED FAT-SUPPLEMENTED DIET

The enzymatic assays and the calculation of the kinetic parameters were performed as described under Methods. (N), number of experiments. The results are expressed as the mean ± S.E.

Diet	(N)	*Kinetic parameter*		
		n*		% *Activation*
		Mg^{2+}-*ATPase*	$(Na^+ + K^+)$-*ATPase*	
Fat-free	8	-1.34 ± 0.06	-1.86 ± 0.13	57 ± 3
Hydrogenated fat-supplemented	8	-1.81 ± 0.06	-2.86 ± 0.19	45 ± 7

* The values for the two groups are significantly different ($P<0.001$).

Effects of dietary fat on the allosteric inhibition by F^- of the ATPases

The fact that rats fed the diet with a hydrogenated fat supplement show values of n similar to that reported for rats fed a fat-sufficient diet[2] indicates no apparent relation between the allosteric behaviour of ATPases and essential fatty acid deficiency.

The behaviour of membrane-bound ATPases, under conditions where the interactions between proteins and lipids might be altered by variations in the nature of the dietary fat-supplement, may provide a sensitive indicator of structured features in the allosteric changes. Walker and Kummerow[24] showed that when rats were fed diets differing in their fat-supplement, the essential fatty acid (*n*—6) content of the erythrocytes was related to the resistance of the red blood cells to haemolysis. Consequently, with these ideas we studied the relationship between allosteric modification of ATPases and lipid composition.

The Hill coefficient for the inhibition by F^- of the ATPases was determined in erythrocyte membranes obtained from five groups of rats fed with different fat-supplemented diet (Table III). The values of *n* for $(Na^+ + K^+)$-ATPase ranged from -2.0 for rats fed on a standard diet to -3.0 for those fed on the linseed oil diet. Significant differences between some groups are presented. The Mg^{2+}-ATPase of the rats fed on the diet with the linseed oil supplement and on the standard diet are significatively different in their allosteric behaviour from the other groups. The low *n* values found for rats fed the standard diet agreed with those previously observed[7]. The % of activation by $(Na^+ + K^+)$ was the same in all five cases. The values of K_i and the specific activities showed no differences between the groups (not shown).

TABLE III

ACTIVATION BY $(Na^+ + K^+)$ AND *n* VALUES FOR THE INHIBITION BY F^- OF THE ATPases FROM RATS FED DIFFERENT DIETS

The enzymatic assays and the calculation of the kinetic parameters were performed as described under Methods. (*N*), number of experiments. The results are expressed as the mean ± S.E.

Diet	(*N*)	*Kinetic parameter*		
		n		% *Activation*
		Mg^{2+}-*ATPase*	$(Na^+ + K^+)$-*ATPase*	
Standard	6	-1.31 ± 0.03*	-2.04 ± 0.15	45 ± 7
Supplemented with:				
Lard	6	-1.84 ± 0.10	-2.93 ± 0.16**	52 ± 3
Olive oil	7	-1.80 ± 0.10	-2.23 ± 0.10	52 ± 3
Corn oil	9	-1.87 ± 0.09	-2.13 ± 0.06	58 ± 3
Linseed oil	11	-1.51 ± 0.03*	-2.99 ± 0.10**	54 ± 2

* Significantly different from the other groups ($P < 0.005$).
** Significantly different from the other groups ($P < 0.001$).

Effects of dietary fat on the allosteric inhibition by F^- of the acetylcholinesterase

Studies similar to those described above were carried out with acetylcholinesterase. The values of $n = -1.0$ for the inhibition by F^- in rats fed a fat-free diet were previously reported[6]. As can be observed, the values of *n* ranged from -0.9 in the case of lard-supplemented rats to -1.6 in the case of corn oil-supplemented ones. Significative differences are presented between several groups (Table IV).

TABLE IV

HILL COEFFICIENT (n) VALUES FOR THE INHIBITION BY F^- OF ACETYLCHOLINESTERASE FROM RATS FED DIFFERENT DIETS

The enzymatic assays and the calculation of n were performed as described under Methods. (N), number of experiments. The results are expressed as the mean ± S.E. Values followed by the same letters are not significantly different ($P > 0.05$).

Diet	(N)	n
Standard	7	-1.44 ± 0.05^{a}
Fat-free	6	$-1.06 \pm 0.03^{c,d}$
Supplemented with:		
Hydrogenated fat	7	$-1.00 \pm 0.08^{c,d}$
Lard	7	-0.94 ± 0.03^{c}
Olive oil	6	-1.25 ± 0.05^{b}
Corn oil	6	-1.58 ± 0.06^{a}
Linseed oil	7	$-1.19 \pm 0.04^{b,d}$

Differing from that found with the ATPase system, the values of n for the F^- inhibition of erythrocyte acetylcholinesterase of rats grown on a hydrogenated fat diet were indistinguishable from those of animals fed a fat-free diet. But it is interesting to remark that low n values were not characteristic of erythrocytes containing low (n–6) fatty acid levels, as can be seen in the case of lard-fed rats (−0.9). Values of K_i and specific activities were similar in all seven cases (not shown).

Correlations between the allosteric behaviour of the enzymes and fatty acid composition

Several relations were calculated with the data presented in this work. The mole percentage of the ($n-9$) ($n-6$) ($n-3$) fatty acid families were plotted against the values of n for the ATPases and acetylcholinesterase. No significative values were found for the correlation coefficient in all cases. This discards the possibility that the allosteric modification of membrane-bound enzymes could be due to the direct action of some fatty acid family. However, inverse and specific effects of oleic and linoleic acid contents on the values of n of the acetylcholinesterase is illustrated in Fig. 2. The allosteric transitions of the ATPases presented no relationship with any fatty acids.

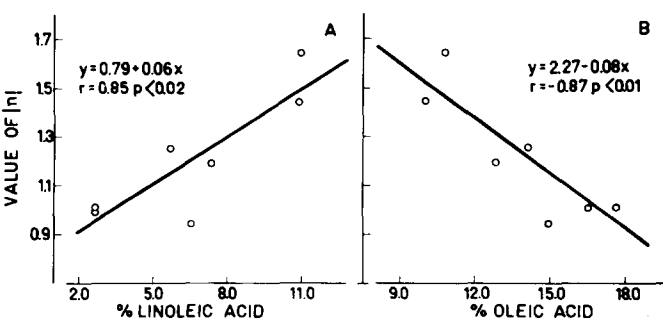

Fig. 2. Relationship between absolute values of n for inhibition by F^- of acetylcholinesterase and (A) linoleic acid, (B) oleic acid content of erythrocyte lipids.

The decrease of oleic acid was compensated by an increase in the content of linoleic acid (Table I), the sum of them being the same in all the diets examined. Other authors showed the same compensatory changes in unsaturated fatty acids[21,25]. This fact could be due to an equally important effect of oleic acid and linoleic acid on the physiological allosteric regulation by the cell membrane.

The plots of the unsaturated/saturated fatty acids ratio against the absolute values of *n* for ATPases and acetylcholinesterase are presented in Fig. 3A. The correlation coefficient was significant for $(Na^+ + K^+)$-ATPase but not for Mg^{2+}-ATPase and acetylcholinesterase.

Since the fluidity of a lipid may be more directly dependent upon the total number of double bonds present than upon the percentage of unsaturated fatty acids, the double bound index/saturated fatty acids ratio was calculated and plotted against the absolute values of *n* for the enzymes. As can be seen in Fig. 3B the animals with a higher double bond index/saturated ratio exhibit minor *n* values for $(Na^+ + K^+)$-ATPase. This relation is inverse for acetylcholinesterase. Mg^{2+}-ATPase presented no significant correlation.

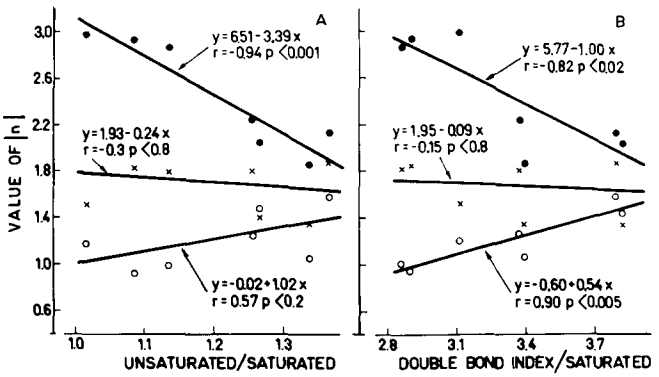

Fig. 3. Relationship between absolute values of *n* for inhibition by F⁻ of $(Na^+ + K^+)$-ATPase (●), Mg^{2+}-ATPase (×), and acetylcholinesterase (○) and the ratio (A) unsaturated/saturated and (B) double bond index/saturation of fatty acids from erythrocyte lipids.

Reconstitution of the membrane in the presence of cholesterol and the allosteric behaviour of acetylcholinesterase

Since the correlation seen above suggest that the $(Na^+ + K^+)$-ATPase and acetylcholinesterase are regulated by the fluidity of the lipid portion of the membrane, we have studied the effect of increasing its cholesterol content. The addition of cholesterol to phospholipid suspensions or to monolayers restricts the motion of the hydrocarbon chains[17]. The values of *n* for acetylcholinesterase in membrane-like material obtained in the presence or absence of cholesterol from rats grown on the corn oil-supplemented diet are shown in Table V. As can be observed, the Hill coefficient is low only in reconstituted membrane obtained in the presence of cholesterol. The increase of cholesterol content in solubilized enzymatic preparation has no effect on the allosteric behaviour. These facts indicate that cholesterol perturbed the acetylcholinesterase behaviour only when incorporated into the membrane matrix. The fatty acid composition remained unchanged during the reconstitution experiment[6].

TABLE V

EFFECTS OF THE FORMATION OF MEMBRANE-LIKE STRUCTURE IN THE PRESENCE OF ADDITIONAL CHOLESTEROL ON THE VALUES OF n FOR ACETYLCHOLINESTERASE FROM RATS FED A CORN OIL-SUPPLEMENTED DIET

1.5 ml of 20 mM sodium phosphate (pH 7.4) containing 5.5 mg ghost protein from rats fed a corn oil-supplemented diet were treated with 1.5 ml 1% Triton X-100. Then, 2 mg of cholesterol were added to the mixture (the cholesterol was not fully solubilized). This suspension was centrifuged and dialysed for 24 h against three changes of 500 ml of 5 mM sodium phosphate (pH 6.0) containing 10 mM $MgCl_2$. The suspension obtained was centrifuged at $30000 \times g$ for 30 min and the pellet was resuspended in 100 mM sodium phosphate (pH 8.0) (Expt 2). The insoluble material was resolubilized with 0.2% Triton X-100 and centrifuged at $100000 \times g$ for 1 h (Expt 3). The control experiment was carried out in the same way without the addition of cholesterol.

Expt	*Enzyme preparation*	*Cholesterol (mg/mg protein)*		*Values of n*	
		+Cholesterol	*Control*	*+Cholesterol*	*Control*
1	$100000 \times g$ supernatant	0.32	0.23	−1.7	−1.8
2	Material reaggregated by dialysis	0.38	0.26	−1.0	−1.7
3	Supernatant after treatment of reaggregated material with Triton X-100	0.61	0.72	−1.6	−1.6

The value of $n = -1.7$ found in the supernatant after treating the cholesterol-loaded material with detergent indicates that the low value (−1.0) obtained in it was not a consequence of allosteric desensitization of the enzyme during dialysis. The increase of cholesterol content has no influence on the specific activity and K_i of the enzyme. The specific activity increased 4-fold throughout the procedure in both preparations[6].

DISCUSSION

The allosteric nature of the inhibition by F^- of the ATPases and acetylcholinesterase from rat red cells has been reported[2,6] showing that they belong to the V system according to Monod *et al.*[26]. It was demonstrated in these allosteric systems that the same enzymes were present in rats fed fat-sufficient and fat-free diets and that the decrease of the allosteric behaviour observed in fat-deficient animals could be interpreted as the uncoupling of the interaction without modifying the site for the F^- effector, due to a conformational alteration in the protein provoked by changes in the membrane lipid composition. Recent comparative studies have shown that erythrocyte membrane-bound *p*-nitrophenyl phosphatase from rats fed fat-sufficient and fat-deficient diets are both allosteric, but the distribution between the two interconvertible forms R and T, predicted by the model of Monod *et al.*[26], is different in both groups[27]. This fact has been fully elucidated with acetylcholinesterase from rats fed a fat-free diet: the release of this enzyme from the membrane provokes a reorientation in it in such a way that the stereospecific interactions with the F^- effector are reestablished[6]. The data presented here demonstrate that the changes in the distribution between the two forms R and T of the membrane-bound enzymes occur independently of the presence of essential fatty acids in the diet.

Based on the n values and fatty acid compositions obtained, several correlations were calculated. No connection was found between $(n-3)$ $(n-6)$ and $(n-9)$ fatty acid families and allosteric changes of the ATPases and acetylcholinesterase.

The correlation between the allosteric transitions of acetylcholinesterase and the linoleic acid content is in agreement with the effect reported by Walker and Kummerow[24] when studying the rate of haemolysis and $(n-6)$ fatty acid content. However, the picture that emerges from this fact is limited and insufficient for the explanation of the allosteric regulation of the membrane-bound enzymes by changes in fatty acid composition, since only acetylcholinesterase presented this behaviour. The fact that the double bond index/saturation ratio was correlated with the allosteric transitions of $(Na^+ + K^+)$-ATPase and acetylcholinesterase, might represent a more sensitive indication for the membrane as a regulator of its bound enzymes. In addition, obviously the increase of linoleic acid content affects the double bond index in the same direction and this is in accordance with the positive correlation between the values of n for acetylcholinesterase and both relations (Figs 2A and 3B).

Studies performed on the diffusion of different solutes out of liposomes prepared from lecithins indicated consistent positive correlation between the degree of unsaturation of the fatty acid residues and permeability[28-33]. Experiments with phospholipid monolayers have revealed that increasing the degree of unsaturation of the fatty acid residues results in an increase of the fluidity of the lipid film because of the rise of the area per molecule[34]. The physical studies on membranes indicate that the membrane lipids are directly related to the membrane fluidity[35-40]. Saturated fatty acids tend to render the membrane less fluid, while unsaturated fatty acids have the opposite effect. From the above considerations the double bond index/saturation ratio could be taken as an indicative of membrane fluidity. In agreement with this hypothesis, recent physical studies carried out with rats fed fat-free and fat-sufficient diet suggest that the freedom of molecular motion of a spin label in alkyl domains could be proportionate to the unsaturation index[41].

The relationship between a decrease of the allosteric transitions for the inhibition by F^- of the $(Na^+ + K^+)$-ATPase and the double bond index/saturated ratio could indicate that the condensing effect of the saturated fatty acid of the membrane increases the strength of interactions between the allosteric effector sites. In contrast, when less tightly packed membranes are obtained, an increase of the cooperativity is acquired by membrane-bound acetylcholinesterase. If an extrapolation is then permitted, one would expect that when this enzyme is separated from the membrane, the maximum absolute value of n should be obtained. In agreement with this hypothesis, solubilized acetylcholinesterase renders a value of $n=-1.6$ (Table V). In addition, values of $n=-1.0$ were obtained in experiments of the reconstitution of solubilized membranes from red cell ghosts of rats fed a fat-free diet[6].

The Mg^{2+}-ATPase appeared to be insensitive to the fluidity parameter. This fact may be interpreted in terms of a heterogeneous response to lipid–protein interaction within the membrane[39]. In addition, the allosteric behaviour of membrane-bound $(Na^+ + K^+)$-ATPase and acetylcholinesterase have comparable sensitivities to the modulation by fluidity, although their responses present strikingly opposite signs. A plausible explanation to this fact could be that acetylcholinesterase is localized on the outside of the erythrocyte membrane, whereas $(Na^+ + K^+)$-ATPase was shown to be present on the inner face[42-45].

The effect of increased cholesterol content on lipid–protein interactions in membranes were investigated by means of electron spin resonance and infrared spectroscopy[46]. These results showed that the cholesterol loading of erythrocytes led to an increase in the local viscosity of the lipid phases of the membrane without apparent effect on the protein conformation.

The changes that occur in the values of n for acetylcholinesterase in response to an increase in the compression of the membrane lipid phases mediated by cholesterol are in agreement with the correlations found between allosteric behaviour and membrane fluidity (Fig. 3). This is an additional proof on the biochemical phenomenon involved in the regulation of allosteric behaviour of some membrane-bound enzymes. Furthermore, we found an inverse shift in the values of n for the $(Na^+ + K^+)$-ATPase and acetylcholinesterase when rats grown on the corn oil-supplemented diet were fed the same diet with 1% cholesterol for 1 week. (Bloj, B., Morero, R. D., Farías, R. N. and Trucco, R. E., unpublished results).

It is interesting to note that, contrary to the findings using physical probes[46], in our case protein conformational changes are recorded when the physical properties of membrane lipids are altered. These facts indicate that the study of the allosteric changes in membranous enzymes is a sensitive tool for recording modifications in membrane lipid-protein interactions *in situ* (Fig. 3, and refs 1, 2, 5, 6, 27).

In the membrane, protein–fatty acid interactions obviously depend on the nature of the fatty acid pool, which in turn depends on lipid nutrition. The physiological significance of the relation between the allosteric behaviour of the membrane-bound enzymes to the effector F^- is at present matter of speculation[27]. It is interesting to note that the allosteric modification was found in heart and kidney microsomal ATPases from rats fed a fat-deficient diet[47], indicating that this regulation phenomenon is not confined to one tissue. In addition, the values of n for both Na^+ and K^+ activation of the $(Na^+ + K^+)$-ATPase were changed under conditions in which the fatty acid composition of the membrane was modified[1]. The latter observation, coupled with the fact that the $(Na^+ + K^+)$-ATPase is intimately associated with Na^+ and K^+ pumping in many different tissues[48], raises questions about the possible effects of membrane fluidity modifications, caused by dietary fats, on the membranous energy-dependent pumps.

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