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EFFECT OF CHOLESTEROL AND PROTEIN CONTENT ON MEMBRANE FLUIDITY AND 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN MITOCHONDRIAL INNER MEMBRANES OF BOVINE ADRENAL CORTEX

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The steroid biosynthetic enzymes in the adrenal cortex are localised in endoplasmic reticulum and mitochondrial membranes. For some of the enzymes in endoplasmic reticulum the activity appears to be modulated by lipid fluidity, (21-hydroxysteroid hydroxylase and 3β-hydroxysteroid dehydrogenase). A mechanism for the regulation of corticosteroid biosynthesis mediated by the membrane fluidity has been suggested. Therefore a study of the mitochondrial inner membrane of the bovine adrenal cortex has been undertaken in comparison with a previous study of the endoplasmic reticulum. The kinetic parameters of the 3β-hydroxysteroid dehydrogenase were studied as a function of pH and temperature. No thermal transition can be observed in the Arrhenius plot for this enzyme in contrast with the results obtained for the microsomal enzyme. Membrane fluidity using, as fluorescent probes, diphenylhexatriene and a set of *n*-(9-anthroyloxy)fatty acids has been also studied as a function of temperature with or without addition of cholesterol. No thermal transition in the lipid phase can be observed. The addition of cholesterol to total mitochondrial membrane as to a lipid extract of the membrane decreases fluidity to the same extent as it does with microsomes. The presence of a large amount of protein in mitochondria has an effect which is additive to that of the cholesterol.

Introduction

In the steroidogenic tissues and especially in the adrenal cortex, steroid biosynthesis is performed via a metabolic pathway involving membrane-bound enzymes either in the endoplasmic reticulum or in the mitochondrial membranes [1].

In the endoplasmic reticulum membranes of the bovine adrenal cortex, the activities of the 21-hydroxysteroid hydroxylase [2] and also of the 3β-hydroxysteroid dehydrogenase [3,4] are modulated by the lipid fluidity, whereas the activity of the 3-oxosteroid Δ^4–Δ^5 isomerase is not [5]. This last enzyme is, however, dependent on phospholipids for its activity [6]. These observations suggest either the existence of lipid microdomains or different lipid-protein interactions due to the protein structures. Therefore the hypothesis of a possible mechanism for the regulation of steroid biosynthesis mediated at some limiting steps by the 'membrane fluidity' has been suggested [3–5]. Membrane

Abbreviations: $NADH,H^+$, nicotinamide adenine dinucleotide (reduced form); PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; Tris, tris(hydroxymethyl)aminomethane; *n*-AS, *n*-(9-anthroyloxy)stearic acid; *n*-AP, *n*-(9-anthroyloxy)palmitic acid; cytochrome $P\text{-}450_{scc}$, specific for the side-chain cleavage of cholesterol.

fluidity is a function of the chemical composition of the membrane: protein/phospholipid ratio, cholesterol/phospholipid ratio, unsaturation of the fatty acyl chains [7–9] and has been involved in a number of membrane functions [10].

Nothing is known about the membrane fluidity of the inner mitochondrial membrane of the adrenal cortex despite the fact that this membrane is very important in the overall steroid biosynthesis. Indeed, one of the limiting steps of the biosynthesis seems to be, according to recent publications [11,12], the redistribution of cholesterol in this membrane to reach the cytochrome $P\text{-}450_{scc}$ binding site. A knowledge of the dynamic structure of the inner mitochondrial membrane of the adrenal cortex and the influence of cholesterol is thus relevant to the understanding of the regulation processes in steroid hormone biosynthesis.

The present study of the mitochondrial internal membrane of the bovine adrenal cortex was undertaken using a similar approach as that for the previous study of the endoplasmic reticulum of the same gland [3–5]. The effect of pH and temperature on the enzymatic parameters of the 3β-hydroxysteroid dehydrogenase was studied, this enzyme being present in both membranes.

The membrane fluidity was studied using the fluorescence anisotropy technique with different probes: the all *trans*-1,6-diphenyl-1,3,5-hexatriene and a set of *n*-(-anthroyloxy)fatty acids (2-, 7-, 9- and 12-(9-anthroyloxy)stearic acid and 16-(9-anthroyloxy)palmitic acid). These latter probes have been studied in model systems in our laboratory [13,14] and allow a probing of the membrane at different levels [15,16].

It will be shown that in contrast to the data obtained with the microsomes for the 3β-hydroxysteroid dehydrogenase, no thermal transition can be observed in the lipid bilayer of mitochondrial internal membrane or in the activity of the 3β-hydroxysteroid dehydrogenase. However, the low content of cholesterol in the mitochondrial internal membrane and its high protein content play an important role for the difference in fluidity from endoplasmic reticulum and perhaps in the enzymatic activity of the 3β-hydroxysteroid dehydrogenase.

Materials and Methods

Chemicals. Steroids were a kind gift from Roussel-Uclaf (Romainville, France). Organic solvents were purchased from Merck (Darmstadt, F.R.G.). Anthroyloxy fatty acid derivatives were from Molecular Probes (Pleno, U.S.A). NAD was from Sigma (Grade III). Digitonin was from Eastman Kodak. All others reagents were of the highest grade commercially available.

Mitochondrial membrane preparations. Mitochondria were prepared from fresh adrenal bovine cortex according to Hogeboom [17]. Internal membranes of mitochondria were prepared according to the method of Levy, modified by Schnaitman et al. [18,19] with digitonin incubation. The preparation controlled by electron microscopy showed a high degree of purity in agreement with the enzymatic activities measured by Satre [20]. The preparations were stored in liquid nitrogen.

Enzyme assay for the 3β-hydroxysteroid dehydrogenase was carried out as described in Ref. 4 by measuring the fluorescence due to the formation of NADH at 35°C in 0.1 M Tris-HCl (pH 8.5). For succinate dehydrogenase activity the method used was that of Green et al. [21] modified by Earl and Korner [22].

Protein content was estimated by the Lowry procedure as modified by Markwell et al. [23].

Lipid extraction from mitochondrial internal membrane was carried out according to the method of Folch et al. [24] as modified by Rouser and Fleischer [25] with a minor modification: the protein is filtered on a sintered glass filter No. 2 instead of centrifuged.

Liposomes were obtained after evaporation of the lipid chloroform-methanol extract under vacuum overnight. The samples were hydrated in 50 mM Tris-HCl/100 mM NaCl (pH 8.5) and vortexed five times for 30 s at room temperature. Addition of cholesterol was performed by adding a solution in chloroform (5 mg/ml) to the chloroform-methanol extract of the lipids. The mixture was evaporated and hydrated as above.

Cholesterol content. Extraction of the cholesterol from the membrane was performed according to Rose and Oklander [26] with chloroform/isopropyl alcohol (7:11). The cholesterol content was

estimated by gas chromatography using cholesterol and cholesterol acetate as standards. Alternatively the enzymatic method described in Ref. 27 was used. For the modification of the cholesterol content of the mitochondrial internal membrane, an ethanolic solution (5 mg/ml) of cholesterol was added directly to the mitochondrial preparation. To determine the amount of added cholesterol incorporated into the mitochondrial internal membrane, the cholesterol supplemented preparation was washed with 20 volumes of water and centrifuged at $10000 \times g$ for 10 min. The cholesterol was measured both in the supernatant and after extraction in the mitochondria.

Labeling of membranes and liposomes by diphenylhexatriene. A solution of diphenylhexatriene (10^{-3}M) in tetrahydrofuran was used. The molar ratio diphenylhexatriene/lipid never exceeded 1/500.

Labeling of membranes with anthroyloxy fatty acid derivatives. Membranes were incubated 1 h at room temperature with 1 to 2 μl of 2 mg/ml tetrahydrofuran solution of 2-, 7-, 9- and 12-(9-anthroyloxy)-stearic acids and 16-(9-anthroyloxy)-palmitic acid. Probe/phospholipid molar ratio was approx. 1/160.

Fluorescence measurements. Fluorescence polarization measurements were performed with a T format SLM 8000 apparatus equipped for photon-counting and thermostatically controlled with a water circulatory device [5].

The nanosecond time-resolved emission measurement was obtained with a single photocounting fluorometer (Applied Photophysics system SP7 and Ortec system system 9200 electronic device). For diphenylhexatriene the excitation wavelength was set at 360 nm. Emission light was collected through a Schott KV408 cut-off filter.

In this series of experiments the excited-state lifetime τ and the steady-state anisotropy r_s of diphenylhexatriene incorporated in the membrane as a function of temperature were measured. From these experimental data a value of ρ, the rotational correlation time, was calculated using the Perrin equation for isotropic medium as a first approximation.

$$\frac{r_0}{r_s} - 1 = \frac{3\tau}{\rho}$$

where r_0 is the intrinsic anisotropy in propylene glycol at $-60°C$, $r_0 = 0.384$ at 360 nm excitation wavelength, τ is the mean lifetime of the excited state of the fluorophore.

Since the values obtained for r_s/r_0 are between 0.33 and 0.70, a value of S (the average orientational order parameter) can be calculated according to Pottel et al. [28] from

$$\frac{r_\infty}{r_0} = S^2 = \frac{4}{3}\frac{r_s}{r_0} - 0.28$$

for diphenylhexatriene, a rod-like molecule with both transition moments parallel to the long axis.

The analysis of the results was performed as described previously [4].

For the anthroyloxy fatty acids the excitation wavelength was set at 367 nm. The same cut-off filter was used for the emission light and estimation of ρ was performed according to Perrin equation since in fluid membranes no residual anisotropy (r_∞) can be evidenced [13].

Results

Chemical analysis of mitochondrial internal membrane from bovine adrenal cortex

According to Wang et al. [29], the protein content is higher in the mitochondrial internal membrane of the adrenal cortex compared to the endoplasmic reticulum. The present results agree with these data: phospholipid/protein (w/w) was 0.33 in the mitochondria, 0.6–0.7 [4,5] for endoplasmic reticulum. The cholesterol/lipid molar ratio was 0.03–0.04 compared to 0.17 in the endoplasmic reticulum.

Kinetic parameters of the 3β-hydroxysteroid dehydrogenase

There is a significant difference between kinetic parameters of the mitochondrial and of the endoplasmic reticulum enzyme as shown in Table I. The K_m is lower and the V_m higher for the latter.

In the mitochondrial internal membrane preparations a progressive decrease of the steroid dehydrogenase activity has been observed as a function of time: total inactivation occurred after about 5 weeks. For the succinate dehydrogenase activity of the same preparations, measured as a

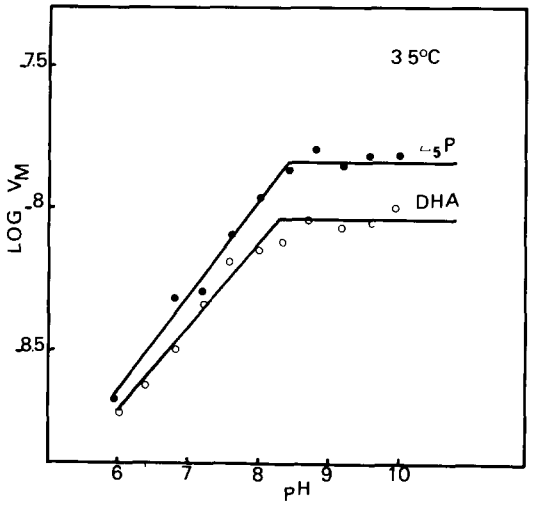

Fig. 1. Dixon plot of V_m vs. pH for dehydroepiandrosterone (DHA) and pregnenolone (Δ_5P) as substrates (at 35°C).

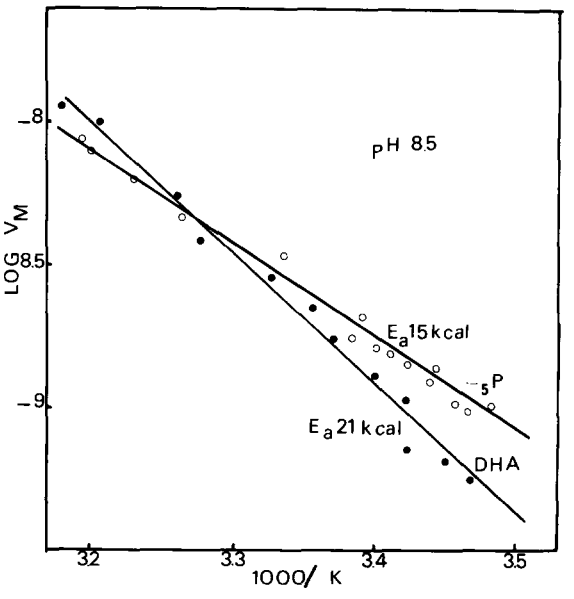

Fig. 2. Arrhenius plot of V_m vs. $1/T$ for dehydroepiandrosterone (DHA) and pregnenolone (Δ_5P) as substrates (at pH 8.5).

marker enzyme of the mitochondrial internal membrane, there is no change in the initial value over the same period of time (0.05 ± 0.01 μmol/min per mg).

Enzymatic activities as a function of pH and temperature

Fig. 1 gives the Dixon plot of log V_m vs. pH for the 3β-hydroxysteroid dehydrogenase activity measured with dehydroepiandrosterone and pregnenolone as substrates. pK_{ES} is between 8.0 and 8.2 for both substrates (at 35°C) which is in the same range as the values found for the endoplasmic reticulum [5]. At pH 8.5, the dehydrogenase activity measured as a function of temperature with the same substrates gives a straight line for the Arrhenius plot between 16 and 37°C (Fig. 2). This is in contrast with the endoplasmic reticulum enzyme [4]. The activation energy is 21 kcal/mol for the mitochondrial internal membrane enzyme with dehydroepiandrosterone as substrate and 15 kcal/mol with pregnenolone.

Fluorescence studies of membrane fluidity

(1) Diphenylhexatriene as fluorescent probe. The

TABLE I

KINETIC PARAMETERS OF THE 3β-HYDROXYSTEROID DEHYDROGENASE IN MITOCHONDRIA AS COMPARED WITH ENDOPLASMIC RETICULUM

The results are the extreme values obtained with a series of ten experiments.

	K_m (μM)		V_m (nmol/min per mg)	
	for dehydroepiandrosterone	for NAD	for dehydroepiandrosterone	for pregnenolone
Mitochondria	3 -4	5.5-6	4-10	
Mitochondrial inner membrane [a]	5 -8	3 -4	2-11	4.5-6.5
Endoplasmic reticulum	0.1-0.5	2 -6	19-25	32

[a] The protein content of the external membrane is 3.7% of the total protein content of the mitochondria [20].

TABLE II

AVERAGE ORIENTATIONAL ORDER PARAMETER OF DIPHENYLHEXATRIENE IN MITOCHONDRIAL INNER MEMBRANES AND IN ENDOPLASMIC RETICULUM

Results given are the mean of five measurements.

Temperature (°C)	*S*				
	Mitochondrial inner membrane			Endoplasmic reticulum	
	No cholesterol added	With cholesterol added		a	b
		9%	16%		
5	0.712	0.754	0.811	0.677	0.749
20	0.569	0.652	0.761	0.608	0.621
30	0.480	0.563	0.702	0.528	–
37	0.423	0.485	0.649	0.497	0.437

[a] Values obtained by anisotropy decay measurements [21].

[b] Values calculated according to Pottel et al. [28].

TABLE III

AVERAGE ORIENTATIONAL ORDER PARAMETER OF DIPHENYLHEXATRIENE IN LIPID EXTRACTS FROM MITOCHONDRIAL INNER MEMBRANES AND ENDOPLASMIC RETICULUM

Results are the means of six measurements.

Temperature (°C)	*S* for extracted lipids of				
	Mitochondrial inner membrane			Endoplasmic reticulum	
	No cholesterol added	With cholesterol added		a	b
		12%	16%		
5	0.599	0.632	0.689	0.654	0.675
20	0.415	0.434	0.518	0.528	0.603
30	0.262	0.293	0.394	0.424	0.352
37	0.185	0.203	0.305	0.395	0.281

[a] Values obtained by anisotropy decay measurements [21]

[b] Values calculated according to Pottel et al. [28].

results of the steady-state anisotropy measurements are summarized on Table II. It appears that the values of ρ and S, calculated as described under Materials and Methods, with mitochondrial internal membrane are significantly lower than those calculated with the endoplasmic reticulum. This agrees with the lower cholesterol content. Addition of cholesterol (cholesterol/phospholipid 9–12%) to the membranes indeed leads to an increase in both values, which reach those obtained with the endoplasmic reticulum.

As was observed in the case of the endoplasmic

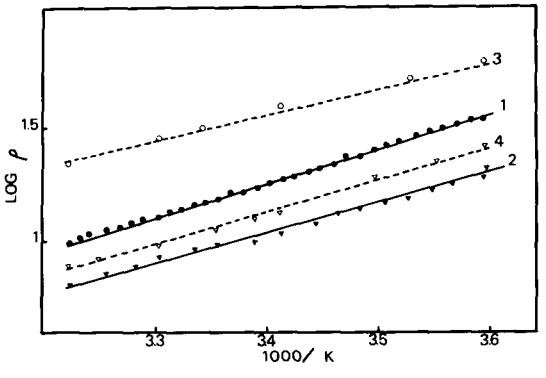

Fig. 3. Arrhenius plot of the rotational relaxation time of diphenylhexatriene in: 1 mitochondrial internal membrane (MIM); 2, extracted lipids of MIM; 3, MIM + 12% cholesterol; 4, extracted lipids of MIM + 12% cholesterol.

reticulum [4] the lipid extracts of the inner mitochondrial membranes show lower values of ρ and S over the whole temperature range as compared with the initial membranes themselves. The temperature dependence of S is more pronounced in the lipid extract than in the total membranes. On the other hand, the lipid extract from mitochondrial internal membrane presents lower values of ρ and S than those of the endoplasmic reticulum lipid extract (Table III and Fig. 3). The effect of cholesterol addition is weaker with the lipid extract than with the total membranes over the whole temperature range. The Arrhenius plot of log ρ vs. $1/T$ is linear over the whole range of temperature for the total membranes and also for the lipid extract.

(2) n-(9-Anthroyloxy)fatty acid derivatives. The profile of the rotational relaxation time (ρ) of these fluorophores as a function of the substitution position (Fig. 5A) exhibits a maximum at C_7, as was found with the endoplasmic reticulum [5]. This pattern cannot be ascribed to intrinsic properties of the probes themselves as demonstrated by studies in isotropic solvents [13], except for 16-AP which undergoes a faster rotation as compared with the other probes. The values of ρ vs. carbon number are always slightly lower in the mitochondria than in the endoplasmic reticulum. The maximum difference occurs at C_9–C_{12}. Removal of the proteins depresses the ρ values at any level of the fatty acyl chains (Fig. 5B). The maxi-

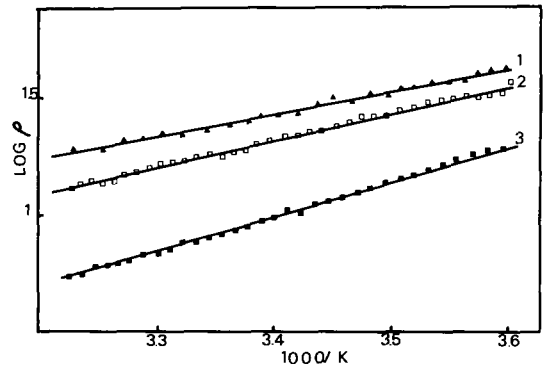

Fig. 4. Arrhenius plot of the rotational relaxation time of 1, 7-(anthroyloxy)stearic acid; 2, 2-(anthroyloxy)stearic acid; 3, 16-(anthroyloxy)palmitic acid in mitochondrial internal membrane.

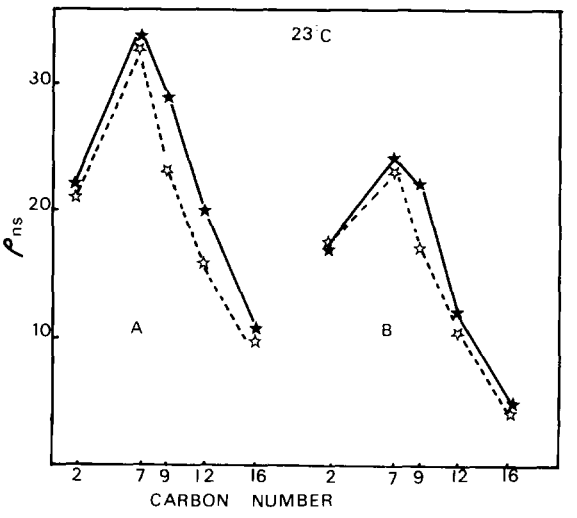

Fig. 5. Rotational relaxation time of *n*-(9-anthroyloxy)fatty acid derivatives at 23°C; (A) in the mitochondrial (— — —) and endoplasmic reticulum (———) membranes; (B) in the extracted lipids of mitochondrial (— — —) and endoplasmic reticulum (———) membranes. Data for endoplasmic reticulum were taken from Ref. 5.

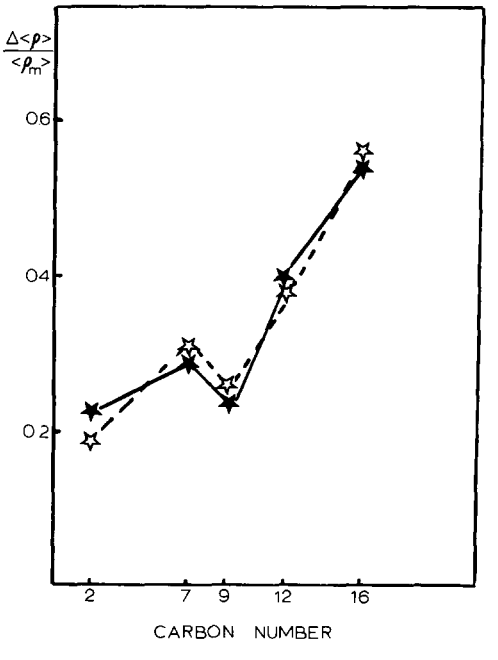

Fig. 6. Relative difference $\Delta\langle\rho\rangle/\langle\rho_m\rangle$ between the values of the rotational relaxation time obtained in complete membranes ($\langle\rho_m\rangle$) of endoplasmic reticulum (———) and of mitochondrial internal membrane (— — —) and derived liposomes ($\langle\rho_l\rangle$). $\Delta\langle\rho\rangle = \langle\rho_m\rangle - \langle\rho_l\rangle$. Data for endoplasmic reticulum were taken from Ref. 5.

mum difference between the mitochondrial lipid extract and the endoplasmic reticulum extract now occurs at the C_9 level. The effect of the proteins in rigidifying the membrane lipids appears to be maximum in the hydrophobic part of the membrane (Fig. 6). The global effect of proteins is the same with the two types of membrane for every substitution position (Fig. 6). But the effect of the proteins on the ρ value with mitochondria even with a higher protein content, is not stronger than that with endoplasmic reticulum, since the value of ρ in both lipid extracts are about the same except in the C_9 region, where the cholesterol is known to affect the bilayer.

Discussion

In the present study differences between the lipid dynamics of mitochondrial inner membrane and endoplasmic reticulum membranes of bovine adrenal cortex are demonstrated by using a variety of fluorescent probes. Such differences could be explained mainly on the basis of the respective cholesterol and/or protein contents of the two membranes. Indeed their phospholipid composition is very similar, the only difference being for the alkenyl acyl-glycerophosphoethanolamines which are present to a higher extent in the endoplasmic reticulum and for the cardiolipins which are more concentrated in the mitochondria, as it has been found with other tissues. The degree of unsaturation of the acyl chains is comparable, both kinds of membranes being rich in arachidonic acid especially in the case of the alkenyl acyl-glycerophosphoethanolamine and diacyl-glycerophosphoinositol [30]. The mitochondrial inner membrane contains twice as much protein as the endoplasmic reticulum whereas its cholesterol content is only one-fifth. The influence of the proteins is demonstrated by the large decrease (56% at 37°C) of the average orientational order parameter S of diphenylhexatriene in the lipid extract of the mitochondrial internal membrane as compared with the total membranes. In the endoplasmic reticulum this decrease was only 20% [4]. The influence of the proteins on the lipid dynamics is more pronounced in the deepest hydrophobic lipid region probed by the 16-AP. This was also observed in the endoplasmic reticulum [5]. It is therefore likely that in both types of membranes the proteins are highly hydrophobic and interact preferentially with the more fluid part of the acyl chain of phospholipids. However, in the total membranes the lower protein content of the endoplasmic reticulum appears to be compensated by a higher cholesterol content.

The influence of cholesterol is first emphasized comparing the data obtained with the lipid extracts of the two types of membrane. The higher cholesterol content of the endoplasmic reticulum may account for the lower fluidity of its lipid extract mainly in the C_7–C_9 region as detected by the *n*-(9-anthroyloxy)fatty acid probes. In model systems the same region of the bilayer appears to be perturbed by the addition of cholesterol [14]. The same effect is also shown in the experiments where cholesterol was added both to total mitochondrial inner membrane and lipid extract. In these experiments, the lipid fluidity is decreased by cholesterol addition to the total mitochondrial internal membrane but the value attained is lower than that found with the endoplasmic reticulum despite the fact that a smaller amount of cholesterol was added as compared with the amount present in the microsomes. On the other hand, the fluidity decrease evoked by cholesterol is more important in the total internal membranes than in their lipid extracts. Cholesterol and proteins therefore have an additive effect on the average lipid fluidity in the inner mitochondrial membrane of bovine adrenal cortex.

The thermal dependence of the rotational relaxation time of the different probes employed in this study does not show any lipid phase transition either in the total mitochondrial internal membrane or in the lipid extract with or without added cholesterol. The activation energy of the 3β-hydroxysteroid dehydrogenase is also constant over the whole temperature range studied. This is in contrast with the results obtained for the 11β-hydroxylase in the total mitochondria by Kimura et al. [31]. The Arrhenius plot for this enzymatic activity exhibited two slopes with a break around 24°C. A global lipid phase transition cannot account for the different behavior of two enzymes embedded in the same biological membrane but a protein conformational modification [32] of the multi enzymatic system of the 11β-hydroxylase is

one possibility of explanation. It is also worthwhile remarking that the 3β-hydroxysteroid dehydrogenase in the endoplasmic reticulum exhibited two values for the reaction activation energy with a break at 30°C in the Arrhenius plot at pH 8.5. Although it is not known if the endoplasmic reticulum and the mitochondrial enzymes are identical molecules and although the actual presence of this enzyme in the inner membranes has been discussed [33,34] it appears that: (i) the similarity of pH dependency of their respective maximum velocity and (ii) the similar order of magnitude of the activation energy of the reaction are in favor of analogous enzymes actually being present in the two membranes. Therefore the interaction between the membrane lipids and the enzymatic proteins are likely to be different in the mitochondrial internal membrane and in the endoplasmic reticulum as far as the 3β-hydroxysteroid dehydrogenase is concerned.

The low value of V_m and of $1/K_m$ for the mitochondrial enzyme seems to indicate that in the mitochondria the 3β-hydroxysteroid dehydrogenase is not the effective enzyme in the biosynthetic pathway. The rapid inactivation of the enzyme in the inner membrane preparation could be due to the location of the NAD site in the matrix of the inner membrane [35] and could be an indication of a secondary role for this enzyme in the biosynthesis of corticosteroids. The function of the dehydrogenase-isomerase complex is crucial for transformation of pregnenolone to corticosteroids via progesterone. The inhibition or a low activity of the enzymatic complex could completely block the biosynthesis and leads to pathological situations [36]. Furthermore the regulation of this biosynthesis, which is known to be subject to control by adrenocorticotropic hormone and also by the cortisol level in blood, should be very precise at all levels. The possibility of a regulation through membrane lipid dynamics is suggested in the endoplasmic reticulum by the correlation between lipid dynamics and the activation energy of the enzymatic reaction. This hypothesis is in line with recent results demonstrating that adrenocorticotropic hormone modifies the lipid composition in a time-dependent way compatible with it having a key role in the regulation of cortisol synthesis [37].

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