Pergamon

0960-0760(95)00122-0

J. Steroid Biochem. Molec. Biol. Vol. 54, No. 3/4, pp. 121–129, 1995

0960-0760/95 $9.50+0.00

Modulation of Hydroxylase and Lyase Activities of Bovine Cytochrome *P*-450$_{17\alpha}$ in Adrenal and Testicular Microsomes by a Tissue-specific Local Membrane Environment

A. Perrin, E. M. Chambaz and G. Defaye*

CEA, INSERM Unité 244, Laboratoire de Biochimie des Régulations Cellulaires Endocrines, Département de Biologie Moléculaire et Structurale, Centre d'Etudes Nucléaires, 17, rue des Martyrs, 38054 Grenoble Cedex 9, France

In steroidogenic tissues, cytochrome *P*-450$_{17\alpha}$ catalyzes both steroid 17α-hydroxylation and 17,20-lyase reactions. The ratio of the two activities, hydroxylase over lyase (H/L) depends upon the tissue of origin; this ratio is low in the testis whereas it is high in the adrenal cortex. To examine the factors responsible for this specific regulation, two approaches were followed: (i) the purified enzyme was incorporated into liposomes made of microsomal lipids of testis or adrenal cortex; and (ii) the effects of disorganization of the microsomal membrane on the activities were observed. The results show that the cytochrome 17,20-lyase activity is stimulated by the presence of lipids from testicular origin. In the adrenal microsomes, this activity appears to be dependent upon the local membrane organization. Specific component(s) associated with the neutral fraction of the microsome lipid extract may be responsible for the repression of lyase activity in the adrenal.

J. Steroid Biochem. Molec. Biol., Vol. 54, No. 3/4, pp. 121–129, 1995

INTRODUCTION

Steroidogenic tissues such as adrenal cortex and testis, convert cholesterol to active hormones by a series of reactions catalyzed by membrane bound microsomal and mitochondrial enzymes. Hydroxylations by cytochrome *P*-450 are key reactions in this biosynthesis [1]. The 17α-hydroxylase-C17,20-lyase (17-OH-L) microsomal enzymatic system comprises NADPH-cytochrome *P*-450-reductase, a flavoprotein which is ubiquitous for all microsomal hydroxylases and cytochrome *P*-450$_{17\alpha}$ which confers specificity to the reaction. This enzymatic system catalyzes the 17α-hydroxylation of both pregnenolone and progesterone leading to 17α-hydroxypregnenolone (17-OH pregnenolone) and 17α-hydroxyprogesterone (17-OH progesterone), respectively. Subsequently, the same enzyme expresses its lyase activity which cleaves the C-17,20 bond and thus converts the 21-carbon (C21) steroids to 19-carbon (C19) steroids. Since it shares a dual function, 17-OH-L plays a key role toward orienting steroidogenesis to the production of either gluco- and mineralocorticoids (C21 steroids) or androgens (C19 steroids). Accordingly, lyase activity is low in the adrenal cortex and high in testis where biosynthesis of androgens occurs. This results in different characteristic ratios of hydroxylase to lyase activities (H/L) for each organ.

Testicular [2] and adrenal [3] cytochrome *P*-450$_{17\alpha}$ have been isolated from pig, where they were found to be highly homologous proteins. In bovines, in the two organs, the hydroxylase and the lyase activities were found to be catalyzed by the same protein [4] and this agrees with the existence of a unique bovine gene encoding cytochrome *P*-450$_{17\alpha}$ [5]. This result was confirmed by the expression of bovine cytochrome *P*-450$_{17\alpha}$ cDNA in COS 1 cells, which showed that a single enzymatic polypeptide chain catalyzes both the 17α-hydroxylase and lyase activities [6]. The same gene is also expressed in human adrenal and testicular tissues [7]. Since after purification, porcine as well as bovine enzymes from both tissue origins exhibit the same H/L ratio, this suggests that the differences in 17-OH-L activities expressed *in situ* in testicular as

*Correspondence to G. Defaye
Received 29 Nov. 1994; accepted 2 Mar. 1995.

compared to adrenal intact microsomes are due to different membranous environments and arise from specific local component(s) or structural organization.

Several aspects of the regulation of the 17-OH-L have been investigated. The role of the electron flux generated by the reductase has been examined for the human system expressed in transfected COS 1 cells [8], and for isolated porcine [9] or guinea-pig [10] systems. Other studies have emphasized the possible role of the local substrate concentration near the active site of the cytochrome for the rat testicular enzyme [11, 12].

Since other hydroxylase systems like 11β-hydroxylase [13], or cholesterol side chain cleavage [14, 15] as well as 3β-hydroxysteroid-dehydrogenase/isomerase [16, 17] are affected in this way, the influence of the lipid membrane composition has been investigated. Yanagibashi and Hall [9] have shown that the origin of the lipids (from adrenal or testis microsomes) did not specifically modify the lyase activity of porcine cytochrome *P*-450$_{17\alpha}$. Nevertheless, the bovine system seems to be different from the porcine. Firstly it presents no lyase activity with 17-OH progesterone as a substrate. Secondly, the H/L ratios for the microsomal or purified cytochrome *P*-450$_{17\alpha}$ are not of the same order of magnitude in the two species.

We have previously purified cytochrome *P*-450$_{17\alpha}$ from testicular and adrenal bovine microsomes [4]. The main purpose of the present work was to determine which factors are responsible for the differences in lyase activities of the same enzyme located in two different organs, namely adrenal or testis. The lyase over hydroxylase activity ratio of the cytochrome *P*-450$_{17\alpha}$ was examined: (i) following modification of the relative reductase concentration; (ii) by incorporating the purified cytochrome into liposomes prepared with testicular or adrenal microsomal lipids; and (iii) by disturbing the structural organization of the microsomal membrane using either a detergent or the action of a lipolytic enzyme.

The present observations clearly showed that the same enzymatic protein expresses different H/L activity ratio depending upon its integration in a testicular or adrenal microsomal environment. Thus the molecular lipid environment of cytochrome *P*-450$_{17\alpha}$ appears to contribute to the overall expression of steroidogenic differentiated functions.

MATERIALS AND METHODS

Materials

[^{3}H]pregnenolone (25 Ci/mmol) was from NEN-Dupont, [^{3}H]17-OH pregnenolone was prepared from [^{3}H]pregnenolone using purified cytochrome *P*-450$_{17\alpha}$ [4]. Silica gel plates were from Merck, Darmstadt FRG. Lipase (EC 3.1.1.3), insoluble enzyme from wheat germ, was from Sigma. NADPH-cytochrome *P*-450-reductase was prepared according to [4].

Preparation of the microsomes

All operations were carried out at 4C. Calf testes and bovine adrenal glands (fasciculata-reticularis zone of the cortex) were homogenized with a Teflon homogenizer in Tris–HCl buffer (5 mM, pH 7.4) containing sucrose (275 mM). The homogenate was centrifuged at 800 ***g*** for 15 min, then at 10,000 ***g*** for 15 min to eliminate the nuclear and mitochondrial pellet, respectively, and the resulting supernatant was spun at 100,000 ***g*** for 45 min. The pellet was suspended in phosphate buffer (100 mM, pH 7.4) containing glycerol (20% v/v), EDTA (1 mM) and DTT (1 mM).

Enzymatic activities

Steroid 17α-hydroxylase and C17,20-lyase activities were determined aerobically at 30°C. The assay system was composed of 2.5 nmol of labeled substrate ([^{3}H]pregnenolone or [^{3}H]17-OH pregnenolone), glucose-6-phosphate (2.5 μmol), glucose-6-phosphate dehydrogenase (0.6 U), $MgCl_2$ (1.5 μmol), NADPH (120 nmol) and the fraction to be tested in a final volume of 500 μl of 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA. For the measure of hydroxylase activity, purified enzyme (15 pmol) or microsomes from adrenal or testes (10 μg) were added; for the lyase activity, purified enzyme (40 pmol) or adrenal (120 μg) or testes (40 μg) microsomes were added. NADPH-cytochrome *P*-450-reductase (100 pmol) was always present with the purified enzyme, and only when mentioned with the microsomes. Incubations were, respectively, 10 min for hydroxylase, and 20 min for lyase activity assay. The reactions were stopped by addition of methanol (1 ml) and chloroform (1 ml). The lower phase was evaporated to dryness and the steroids separated by TLC on silica gel plates in chloroform–ethyl acetate (1:1, v/v), using authentic pregnenolone, 17-OH pregnenolone and dehydroepiandrosterone as internal standards.

NADPH-cytochrome *P*-450-reductase activity was calculated following the reduction of cytochrome c (20 μM) in the presence of NADPH (50 μM) [18].

Action of lipase

Lipase (insoluble enzyme, 2U) was added to 100 μl (0.4 mg) of microsomal suspension and stirred at 25°C for 15 min. After centrifugation (15,000 ***g***, 2 min), the supernatant was used to measure hydroxylase and lyase activities.

Extraction of microsomal lipids

Total lipids from microsomes were extracted by chloroform–methanol (2:1, v/v) according to Dawson

[19]. The phospholipids were quantified colorimetrically by the ammonium ferrothiocyanate method [20].

Separation of neutral lipids

Separation of neutral lipids was carried out according to Kates [21]. The total lipid extract (100 mg) was evaporated to dryness under nitrogen, then 0.5 ml of chloroform was added, followed by cold acetone (5 ml), and $MgCl_2$, 6 H_2O (10% v/v in methanol, 0.1 ml). After stirring, the mixture was left in ice for 1 h, and then centrifuged (5 min, 800 ***g***). The supernatant containing the neutral lipids was separated and the pellet of phospholipids was washed with cold acetone and dried. These phospholipids now devoided of neutral lipids were redisolved in a known volume of chloroform.

Analysis of the lipids

Phospholipids and neutral lipids were analyzed by thin layer chromatography (TLC) on silica gel plates using different solvents hexane–diethylether–acetic acid (90:10:1, v/v) or chloroform–methanol–acetic acid (60:40:1). The following standards were added: phosphatidyl ethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, cholesterol, cardiolipin, 1,3- and 2,3-diacylglycerol.

Incorporation of cytochrome $P\text{-}450_{17\alpha}$ into liposomes

Two different methods were used.

Elimination of detergent by gel filtration [22]. A chloroform solution of phospholipids (10 mg), to which 0.1 mCi of [^{3}H]cholesterol was added, was evaporated to dryness under a nitrogen stream, rinsed with diethyl ether and dried under nitrogen. To the dry material 0.2 ml of "liposome buffer" (50 mM Tris–HCl, pH 7.0, containing 0.2 mM EDTA, 0.1 mM dithiothreitol and 0.1 M NaCl) with 6.5 mg of sodium cholate (1.3 %, w/v, of final volume) was added. After sonication (3 min, Sonimass apparatus, Annemasse, France), the mixture was stirred at room temperature until the lipid layer was dispersed, then the solution of purified enzyme (300 μl, 150 mg) was added while temperature was maintained at 4°C. The suspension was transferred onto a Sephadex G-50 column (1 × 15 cm), previously equilibrated with the above sonicated phospholipid mixture in order to limit adsorption of lipid material. The column was eluted with the "liposome buffer" and the collected fractions were examined for protein and radioactivity contents.

Incorporation of the enzyme in preformed vesicles. A chloroform solution of lipids (5 mg) was evaporated to dryness under nitrogen; 175 μl of "liposome buffer" were added, the tube was closed under nitrogen and sonicated for 10 min. Then the solution of enzyme (75 μl, 50–60 μg) was added and the suspension was gently stirred for 20 min at 4°C. In the two vesicle preparation procedures, the integrity of the liposomes and the incorporation of the enzyme was controlled by gel filtration through a Sephacryl S1000 gel column yielding a calculated Stokes radius of about 30 nm [23].

Dissociation of the membranes by cholate

Sodium cholate was added to a microsomal solution (20 mg protein/ml) at final concentrations of 0, 0.2, 0.6, 1, 1.4, 1.8 and 2.2% (w/v). After rapid shaking, the 17α-hydroxylase and lyase activities were measured without addition of NADPH-cytochrome *P*-450-reductase.

RESULTS

Influence of NADPH-cytochrome P-450-reductase on the hydroxylase over lyase activity ratio of cytochrome $P\text{-}450_{17\alpha}$

Purified cytochrome $P\text{-}450_{17\alpha}$. The effect of increasing the concentration of NADPH-cytochrome *P*-450-

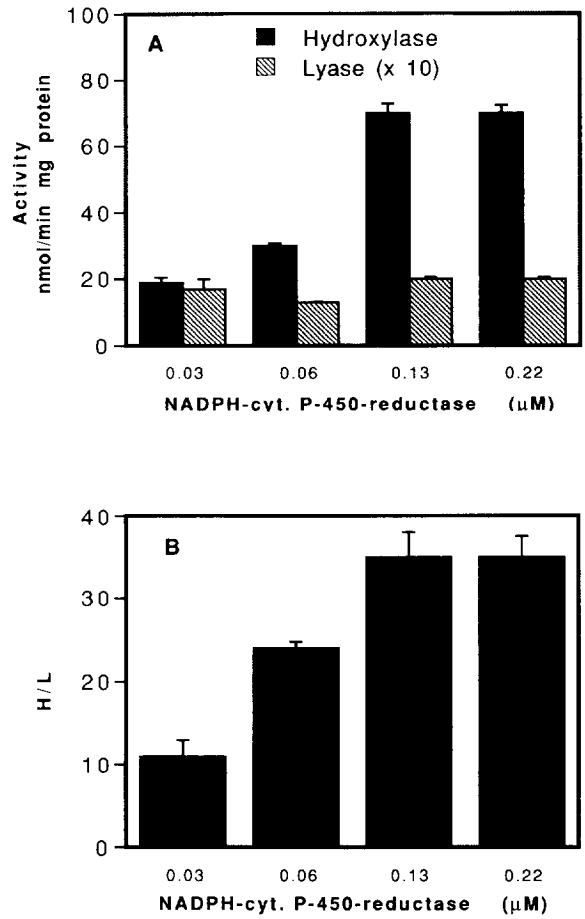

Fig. 1. (A) Purified cytochrome $P\text{-}450_{17\alpha}$ was incubated with increasing concentrations of NADPH-cytochrome *P*-450-reductase and [^{3}H]pregnenolone or [^{3}H]17-OHpregnenolone as substrates, as described in Materials and Methods, to measure hydroxylase and lyase activities. Results represent the average of duplicate measurements in the same experiment and are representative of five independent experiments (± the standard error). (B) The H/L ratio calculated from the hydroxylase and lyase activities is reported in (A).

Table 1. Influence of reductase on the 17α-hydroxylase and lyase activities of adrenal and testicular microsomes

	NADPH-cytochrome *P*-450-reductase					
	–			+		
	Hydroxylase	Lyase	H/L	Hydroxylase	Lyase	H/L
Adrenal	1.14 ± 0.08	0.014 ± 0.001	81	2.04 ± 0.1	0.015 ± 0.001	136
Testis	1.65 ± 0.06	0.24 ± 0.01	6.8	2.45 ± 0.2	0.25 ± 0.01	9.7

17α-hydroxylase and lyase activities were measured for microsomes from adrenal or testes origin without or with NADPH-cytochrome *P*-450-reductase (0.2μM). The activities are expressed in nmol/min/mg of protein.

reductase from 0.02 to 0.24 μM on the hydroxylase and lyase activities of purified cytochrome $P\text{-}450_{17\alpha}$ was measured with a reconstituted system. As shown in Fig. 1(A), increasing the concentration of the reductase resulted in stimulation of the hydroxylase activity whereas the lyase activity remained unchanged. This resulted in a progressive increase in the ratio H/L [Fig. 1(B)] which reached a plateau for reductase concentrations over 0.25 μM (data not shown).

The kinetic parameters of the hydroxylase and lyase activities were calculated with purified cytochrome $P\text{-}450_{17\alpha}$. The affinity for the reductase was higher for the lyase (K_m 0.01 μM) than for the hydroxylase (K_m 0.06 μM) activity.

Adrenal or testis microsomes. The hydroxylase and lyase activities were measured in intact microsomes of testicular or adrenal origin. The H/L ratio was always higher in adrenal (average value 63) than in testicular (average value 6) microsomes although the value from adrenal microsomes showed rather large variations. Adding excess of exogenous reductase at a concentration of about 10 K_m, to both bovine and testis microsomes gave the hydroxylase and lyase activities shown in Table 1. Hydroxylase activities in both testicular and adrenal microsomes increased about 1.5-fold whereas the lyase activities remained unchanged in both cases. This resulted in an increase in the H/L ratio which was the same for the two kinds of microsomes.

Influence of microsomal lipids on purified cytochrome $P\text{-}450_{17\alpha}$ activities

The purified cytochrome was incorporated into liposomes prepared by two different methods.

Table 2. Influence of microsomal lipids

Cytochrome $P\text{-}450_{17\alpha}$ (origin)	H/L		
	Control	Adrenal P/L	Testicular PL
Adrenal	35 ± 3	23 ± 9	2 ± 1
Testis	31 ± 2	23 ± 10	3 ± 2

Cytochrome $P\text{-}450_{17\alpha}$ was purified from adrenal and testis microsomes. The purified enzyme was incorporated into liposomes prepared with lipids from adrenal or testes origin. Hydroxylase and lyase activities were measured and the H/L ratio was calculated. Control represents the ratio for the soluble cytochrome.

Elimination of detergent. Lipid extracts were obtained from testicular or adrenal microsomes. The extracts and purified cytochrome $P\text{-}450_{17\alpha}$ were dispersed in the presence of cholate, and the detergent was removed by gel filtration. Hydroxylase and lyase activities were measured in the presence of excess reductase. Table 2 shows the H/L ratio for the purified cytochrome $P\text{-}450_{17\alpha}$, either soluble or incorporated into liposomes. These results showed that the biological origin of the lipids strongly affects these activities. Low H/L ratios were obtained from testicular lipids. Similar results were obtained with cytochrome purified either from adrenals or testes.

Although the liposomes obtained in this way were monodisperse, the method suffers some disadvantages. Firstly, large quantities of cytochrome were required and, secondly, the cytochrome which is very sensitive to cholate, in the purified form, was partly denatured during the dispersion of the phospholipids with the detergent. For these reasons, a different method of preparation was preferred which required smaller quantities of protein and in the absence of detergent.

Incorporation of the cytochrome into preformed vesicles. Reconstitution was obtained by incubation of cytochrome $P\text{-}450_{17\alpha}$ with preformed vesicles, prepared by sonication of lipid extracts from adrenal or testicular origin. The complete association of the cytochrome to the phospholipid bilayer was assessed by gel filtration on Sephadex S-1000. The hydroxylase and lyase activities are shown in Fig. 2(A). The hydroxylase specific activity was always lower in the liposomes than for the soluble enzyme. The lyase activity was increased with testicular lipids but remained unchanged with adrenal lipids. This leads to a decrease in the H/L ratio in the testicular lipid environment in line with the aforementioned data obtained with the first type of liposome preparation [Fig. 2(B)].

The phospholipids used were contained in a crude extract. In order to determine the origin and biological nature of the regulatory factor of the lyase activity in adrenals, the neutral lipids were separated from total lipids extracts from both origins. They contain essentially cholesterol, triglycerides, fatty acids and diglycerides (data not shown).

The phospholipids from adrenal cortex or testis microsomes, both with and without neutral lipids

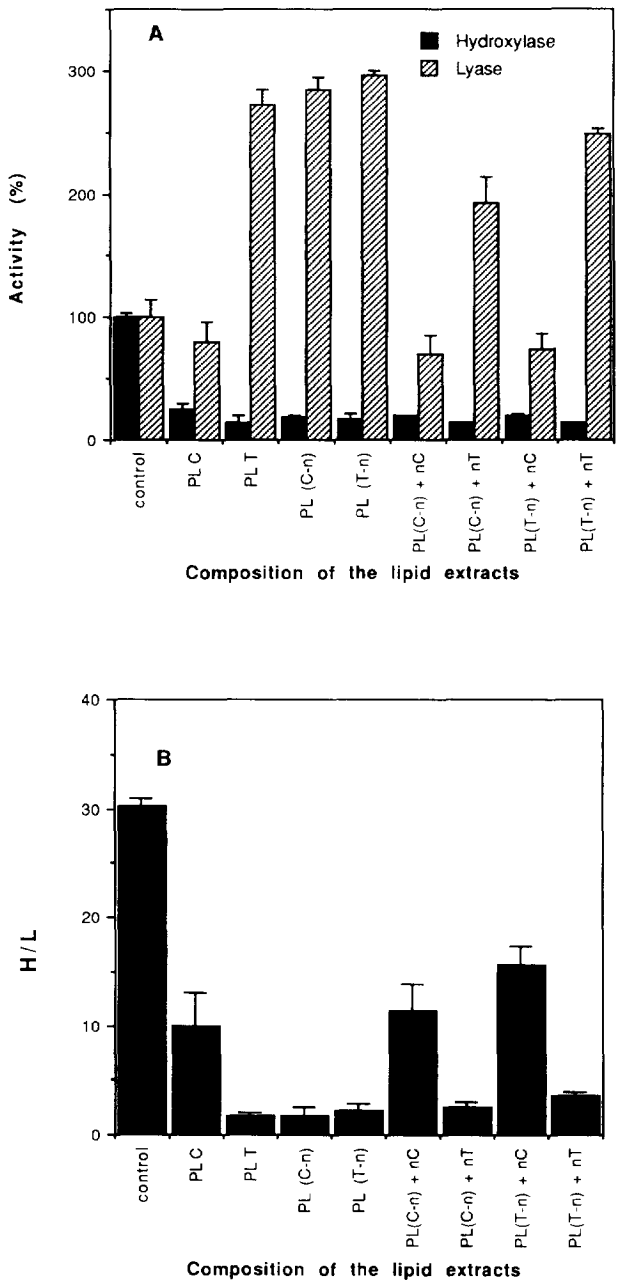

Fig. 2. Liposomes were prepared by sonication of lipid extracts then purified cytochrome $P\text{-}450_{17\alpha}$ was incorporated. Hydroxylase and lyase activities were measured as described previously. The following abbreviations for the phospholipids (PL) used in the preparation of liposomes are: PL C, PL from adrenal cortex; PLT, PL from testes; PL (C-n), PL from adrenal cortex devoided of neutral lipids; PL (T-n), PL from testes devoided of neutral lipids; PL (C-n) + nC, PL from adrenal devoided of neutral lipids plus adrenal neutral lipids; PL (C-n) + nT, PL from adrenal devoided of neutral lipids plus testes neutral lipids; PL (T-n) + nT, PL from testes devoided of neutral lipids plus testes neutral lipids; PL (T-n) + nC, PL from testes devoided of neutral lipids plus adrenal neutral lipids. (A) represents the hydroxylase and lyase activities. Each activity is the mean of triplicate measurements ±SD in the same experiment This is representative of three independent experiments. (B) corresponds to H/L ratio.

Table 3. Kinetic constants for the lyase activity

Cytochrome $P\text{-}450_{17\alpha}$	K_m (μM)
Control	0.25
Liposomes PL adrenal	2.5
Liposomes PL testis	1.8
Liposomes (PL − n) adrenal	0.71
Liposomes (PL − n) testis	0.68

Kinetic constants were measured for the lyase activity with 17-OH pregnenolone as substrate. PL adrenal, PL testis, (PL − n) adrenal and (PL − n) testis represent phospholipids from adrenal, testicular, adrenal without the neutral lipids or testicular without the neutral lipids origin, respectively.

were used to incorporate cytochrome into preformed liposomes and the two activities measured. As shown in Fig. 2(A), the removal of the neutral lipids from the adrenal phospholipids extracts has only a slight effect on the hydroxylase activity of the liposome-incorporated cytochrome $P\text{-}450_{17\alpha}$. On the contrary, the lyase activity increases dramatically. The corresponding activities assayed in liposomes made with testicular lipids showed no effect of removal of neutral lipids [Fig. 2(A)]. When the neutral lipids were absent, the corresponding H/L ratio was low and similar whatever the biological origin of the phospholipids used [Fig. 2(B)].

The enzyme kinetics measured in liposomes constituted from both crude phospholipid extracts and neutral lipids free extracts were measured and their values are given in Table 3. The K_m for 17-OH pregnenolone obtained with adrenal phospholipids was higher than that obtained with testicular phospholipids. They remained unchanged when the neutral fraction was removed.

Reconstitutions of the total lipid extracts was carried out by mixing together the neutral lipids extract with the residual phospholipid extract from which the neutral lipids have been previously removed. The neutral lipids and phospholipids were either from adrenals or testis (Fig. 2). In all cases, 17α-hydroxylase activity remained unchanged. We observed that the addition of adrenal neutral lipids to testicular or adrenal phospholipids inhibited the lyase activity and lead to a high H/L ratio as obtained with the crude phospholipid extract from adrenal cortex. The testicular neutral lipids did not have this effect on lyase activity, they only decrease slightly the two activities of the cytochrome, and the H/L ratio remained low.

Role of the microsomal membrane organization

A first approach to understanding 17-OH-L regulation was to study purified cytochrome $P\text{-}450_{17\alpha}$ in a reconstituted system containing a heterogeneous phospholipid composition. In a second approach the cytochrome was kept in its natural environment, which was subsequently perturbed either by detergents or by enzymatic reactions.

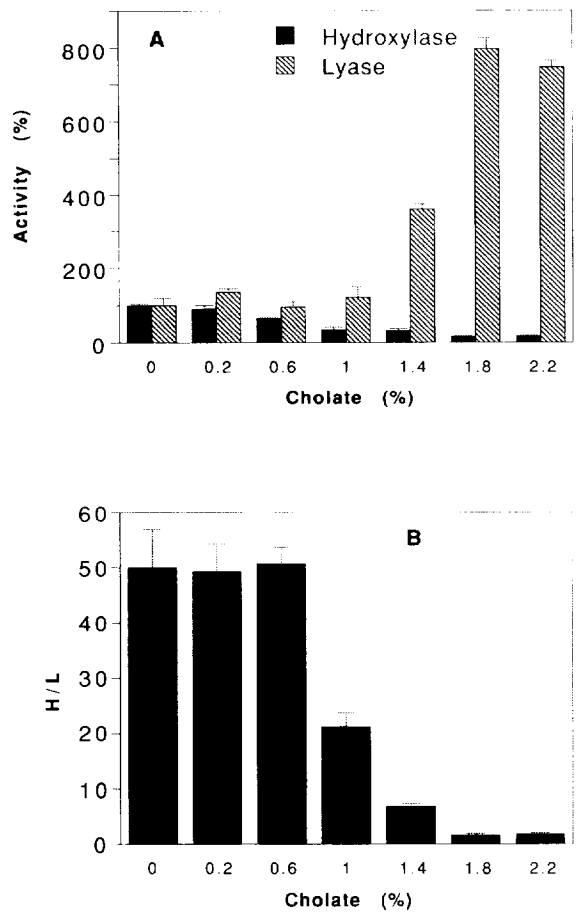

Fig. 3. To adrenal microsomes were added increasing concentrations of cholate. (A) Hydroxylase and lyase activities were measured without addition of exogenous reductase. 100% represents the two activities without cholate. They are the mean of triplicate measurements ±SD. The H/L ratio is in (B).

Dissociation of microsomes by sodium cholate. After addition of sodium cholate at concentrations from 0.2 to 2.2% (w/v) to the adrenal microsomes, enzymatic activities were measured in the absence of exogenous NADPH-cytochrome *P*-450-reductase (Fig. 3). For cholate concentrations from 0 to 0.6% cholate, 17α-hydroxylase and lyase activities remained unchanged. At higher concentrations the lyase activity increased with increasing concentrations of detergent whereas 17α-hydroxylase activity slightly decreased. This results in a significant decrease in the H/L ratio. For cholate concentrations higher than 1.8%, the two activities remained constant.

In order to be sure that these effects could be attributed to the disorganization of the microsomal membrane rather than to a loss in reductase activity in the presence of detergent, the activity of NADPH-cytochrome *P*-450-reductase was measured by its capacity to reduce cytochrome c. The total activity was fixed as the value obtained before centrifugation of adrenal microsomes to which 0.6 or 1.8% cholate had been added. After centrifugation, the quantity of reductase extracted from the membranes was measured in the supernatant. For cholate concentration between 0.6 and 1.8%, there was no detectable loss in the membrane associated reductase activity.

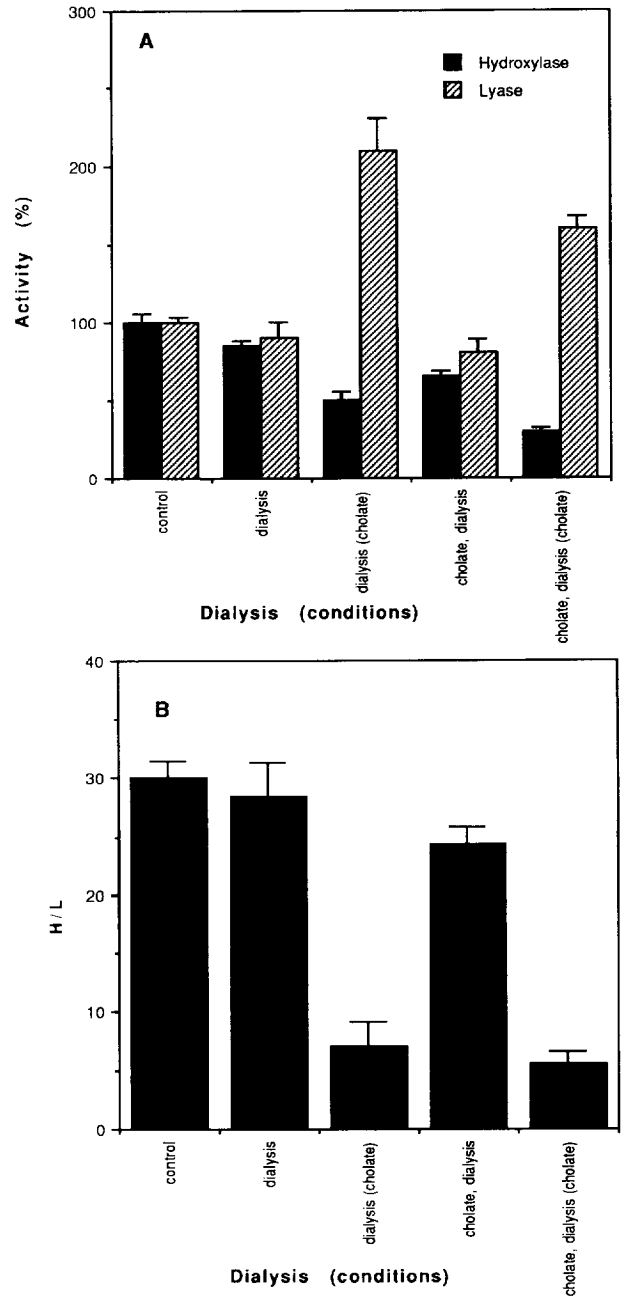

Fig. 4. Adrenal microsomes were dialyzed against buffer (dialysis) or buffer containing cholate (dialysis (cholate)); the liposomes were also dialyzed after dissociation by cholate either against buffer (cholate dialysis) or buffer containing cholate (cholate dialysis (cholate)). The concentration of cholate either for the dissociation of the microsomes or for the buffer was 1.8% (w/v). The hydroxylase and lyase activities were measured. (A) The hydroxylase and lyase activities of the adrenal microsomes are taken as 100%. This is the mean of triplicate measurements ±SD. It is representative of four independent experiments. The H/L ratio is reported in (B).

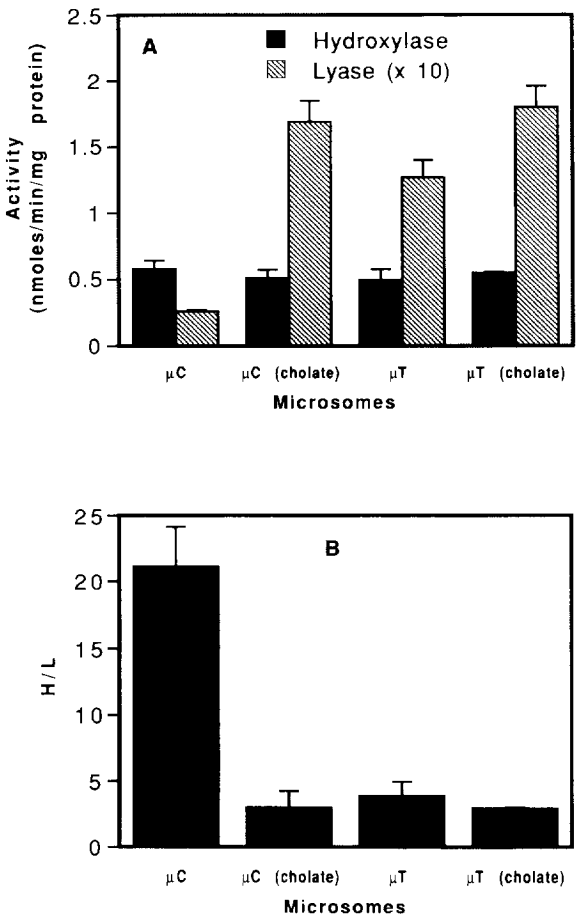

Fig. 5. (A) The hydroxylase and lyase activities were measured with adrenal cortex (μC) or testicular (μT) microsomes intact or previously dissociated with 1.8% of cholate (μC cholate, μT cholate). Each activity is the mean of triplicate measurements ±SD in the same experiment. This is representative of three independent experiments. The corresponding H/L ratio is reported in (B).

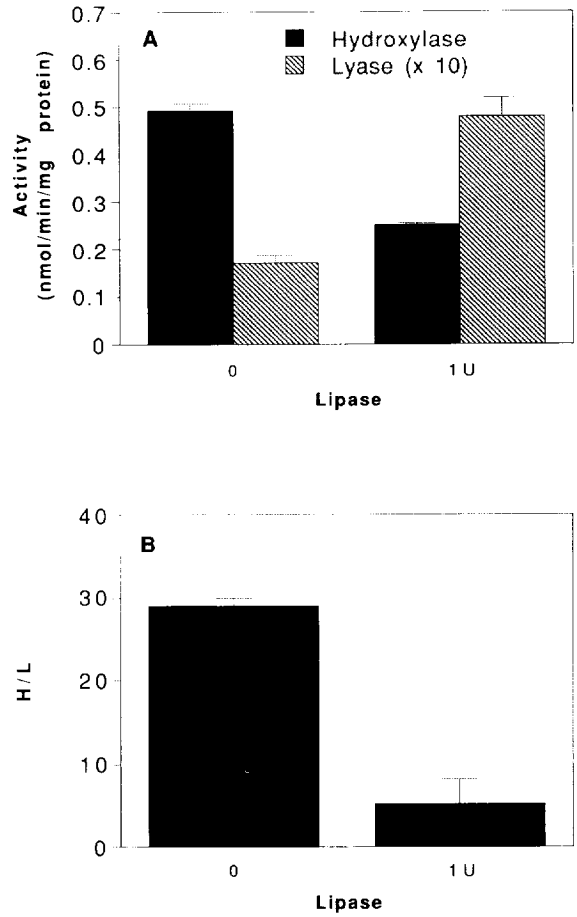

Fig. 6. Immobilized lipase (1 U) was added to adrenal liposomes during 10 min. (A) After centrifugation, the hydroxylase and lyase activities were measured in the supernatant. Each activity is the mean of triplicate measurements ±SD in the same experiment. This is representative of three independent experiments. The H/L ratio is in (B).

After dissociation of the adrenal microsomes by the cholate (1.8%), the mixture was dialyzed against buffers containing (or not) the detergent (1.8%). The results are shown in Fig. 4. The dissociation of the adrenal microsomes by cholate resulted in an increase in the lyase activity; this effect was suppressed after dialysis against a buffer without detergent. Similarly, when the microsomes were dialysed against a buffer containing the detergent, the lyase activity increased.

The same experiments were carried out with testicular microsomes at a 1.8% concentration of cholate. As illustrated in Fig. 5, the cholate treatment had no effect on the hydroxylase and lyase activities of the testicular microsomes.

Lipolytic treatment of microsomes. Since neutral lipids seem to play a significant role in the regulation of the lyase activity of cytochrome $P\text{-}450_{17\alpha}$, the microsomes were treated with a lipase which was active on the triglycerides (Fig. 6). The hydroxylase activity was decreased by about 50% when measured after treatment by one unit of lipase for 10 min. By contrast, the lipolytic treatment led to an increased lyase activity. Consequently, the action of lipase resulted in a very low H/L ratio.

When the testicular microsomes were treated with lipase under the same conditions the values of the hydroxylase and lyase activities decreased slightly in parallel, giving an unchanged H/L ratio (data not shown).

DISCUSSION

The aim of this work was to study the influence of various membrane components on the hydroxylase and lyase activities of the cytochrome $P\text{-}450_{17\alpha}$, so as to explain, and hence account for, the functional differences in these steroidogenic enzymatic activities between cortex and testicular bovine microsomes. We address the question as to why testicular microsomes show a high lyase activity as compared to adrenal

microsomes and if lyase activity is stimulated in testis or inhibited in adrenals. The effect of some membrane components, NADPH-cytochrome *P*-450-reductase and lipid composition, in addition to the general organization of the membrane, have been investigated.

Firstly, we examined the effect of NADPH-cytochrome *P*-450-reductase on the two cytochrome activities using either the purified enzyme or intact microsomes. The addition of different amounts of reductase in the reconstituted 17-OH-L system showed that increasing the concentration of the reductase resulted in an increase of 17α-hydroxylase activity by about 1.5–2-fold, whereas lyase activity remained unchanged. This agrees with the K_m values of the two activities of the cytochrome for the reductase. A similar effect was observed upon reductase addition to the microsomes: the hydroxylase activity increased slightly and there was no change in lyase activity, either with adrenal or testicular microsomal preparations. With the pig purified cytochrome *P*-450$_{17\alpha}$, Yanagibashi and Hall [9] have shown that an increased concentration of reductase favoured lyase versus hydroxylase activity. The difference between the two species may be explained by the different K_m values for the reductase. For the testicular purified pig enzyme [9], the hydroxylase activity has a higher affinity for the reductase than the lyase. Others authors [8] have also investigated the influence of the stoechiometry of the reductase and the cytochrome, after expression of the human cytochrome *P*-450$_{17\alpha}$ and NADPH-cytochrome *P*-450-reductase cDNAs in COS1 cells. In this case, lyase activity increased approx. 3-fold when the reductase was co-expressed. Recently [24] a recombinant fusion protein, containing domains of bovine cytochrome *P*-450$_{17\alpha}$ and rat NADPH-cytochrome *P*-450-reductase, has been expressed in *E. coli*. In agreement with our results, increasing concentration of reductase has no effect on lyase activity. This observation also strengthens the idea that bovine and porcine cytochrome *P*-450$_{17\alpha}$ exhibit significant differences in their enzymatic properties.

Although no effect of phospholipids was found on the hydroxylase and lyase activities of the porcine cytochrome *P*-450$_{17\alpha}$ [9], a different behaviour might be expected for the bovine enzyme and a modulation of the activities of the cytochrome by the lipid environment was then studied.

In a first approach, purified cytochrome *P*-450$_{17\alpha}$ was incorporated into liposomes prepared with lipids of adrenal or testicular origin. Small unilammelar vesicles were prepared, either by elimination of detergent after gel filtration of a mixture of lipids, protein and detergent, or by incorporation of the protein in preformed vesicles. In each case, the lyase activity was found to be high when reconstituted in a testicular lipid environment. Fractionation of the lipid extracts showed that the difference in lyase activity between the two tissues may be attributed to the neutral lipids fraction. On the contrary hydroxylase activity is not affected by the neutral lipids. The striking observation was that the neutral lipid fraction from adrenal cortex microsomes appears to contain inhibitory factor(s) of the lyase activity. After removing this lipid fraction from the total extract, adrenal phospholipids, like testicular phospholipids, stimulate cytochrome lyase activity. Furthermore, addition of this crude adrenal neutral lipids fraction to depleted phospholipids of the two organs suffices to obtain a low lyase activity.

The dependence of the lyase activity on the membrane environment was confirmed by modifying the microsomes by either a detergent or a lipolytic enzyme. The hydroxylase activity was not affected by the detergent whereas the adrenal lyase activity increased with increasing detergent concentrations (cholate). A maximum lyase activity was obtained with a concentration of cholate greater than 40 mM. At this concentration the phospholipid bilayer might be destroyed since a cholate concentration of 30 mM (1.3%) has been shown necessary to dissociate multilammelar vesicles of lecithin [25]. What happens during this dissociation? Is the loss of a soluble regulator factor responsible for these effects or does destruction of the structural organization of the membrane suffice? The increased lyase activity is not due to the concentration of cholate in the assay nor to an interaction with the reductase (data not shown). Thus the detergent does not have its effect during the enzymatic assay, but before by modification of the membrane environment. Moreover, this effect is reversible since after elimination of the detergent by dialysis, the low lyase activity of adrenal microsomes was recovered. This suggests that only dissociation of the membrane is responsible for the activation of the lyase activity in adrenal microsomes since a soluble factor might have been lost after dialysis.

In bovine, the 11β-hydroxylase and aldosterone synthase activities are also affected by the membrane components. Detergent solubilized mitochondria from the fasciculata-reticularis zone catalyse the transformation of corticosterone to aldosterone as do the mitochondria from the glomerulosa zone whereas intact mitochondria were unable to produce aldosterone [26]. It has been suggested that some specific factor(s) selectively inhibit the expression of the aldosterone synthase activity of cytochrome *P*-450$_{11\beta}$ in the membrane.

A perturbation of the membrane was also obtained by action of a lipase on the adrenal microsomes. The same results were obtained; namely an increased lyase activity after action of the lipase on adrenal microsomes. No major changes in the microsomal phospholipids were observed (after TLC analysis), only a decrease in the triglycerides content. Under the same conditions, the lipase had no effect on the testicular microsomes. The effect of phospholipases A2 and C

on the activities of cytochrome $P\text{-}450_{17\alpha}$ in pig [16] or rat [27] testicular microsomes has been studied, but no comparison with adrenal microsomes has been described.

The relative intensities of the two activities of cytochrome $P\text{-}450_{17\alpha}$ appear to be modulated by several integral membrane components. In this study, we have ignored the role of cytochrome b_5 [28] which was suggested to be involved in the electron transfer to the cytochrome. Our main purpose was to compare the adrenal and the testicular environment of the bovine cytochrome $P\text{-}450_{17\alpha}$. The results obtained provide some insights into the properties of the membrane, and emphasize the role of the lipid composition, and thus the structural organization of the cytochrome inside the membrane. The hypothesis of an inhibition of lyase activity in bovine adrenal microsomes is now demonstrated.

Although a unique and clear explanation of the regulation of the two activities of cytochrome $P\text{-}450_{17\alpha}$ is not given by these results, they do show the complexity and the multiplicity of the factors involved.

Acknowledgements—This work was supported by the Institut National de la Santé et de la Recherche Médicale (Unité 244), the Centre de l'Energie Atomique (DSV/DBMS/BRCE), the Association pour la Recherche contre le Cancer, the Fédération Nationale des Centres de Lutte contre le Cancer, the Fondation pour la Recherche Médicale and the Ligue Nationale Française contre le Cancer.

REFERENCES

1. Hanukoglu I.: Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. *J. Steroid Biochem. Molec. Biol.* **43** (1992) 779–804.
2. Nakajin S. and Hall P.F.: Microsomal cytochrome *P*-450 from neonatal pig testis. *J. Biol. Chem.* 256 (1981) 3871–3876.
3. Nakajin S.J., Shinoda M. and Hall P.F.: Purification and properties of 17α-hydroxylase from microsomes of pig adrenal: a second C21-side-chain cleavage system. *Biochem. Biophys. Res. Commun.* **111** (1983) 512–517.
4. Perrin A., Defaye G., Guidicelli C. and Chambaz E.M.: Purification and properties of steroid 17α-hydroxylase from calf testis. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 431–435.
5. Bhasker C.R., Adler B.S., Dee A., John M.E., Kagimoto M., Zuber M. X., Ahlgren R., Wang X., Simpson E.R. and Waterman M.R.: Structural characterization of the bovine CYP17 (17α-hydroxylase) gene. *Arch. Biochem. Biophys.* 271 (1989) 479–487.
6. Zuber M.X., Simpson E.R. and Waterman M.R.: Expression of bovine 17α-hydroxylase cytochrome *P*-450 cDNA in non steroidogenic (COS 1) cells. *Science* **261** (1986) 1258–1261.
7. Chung B.C., Picado-Leonard J., Haniu M., Bienkowski M., Hall P.F., Shively J.E. and Miller W.L.: Cytochrome $P\text{-}450_{17\alpha}$ (steroid 17α-hydroxylase/17-20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 407–411.
8. Lin D., Black S.M., Nagahama Y. and Miller W.L.: Steroid 17α-hydroxylase and 17,20-lyase activities of *P*-450c17: contributions of serine^{106} and P-450 reductase. *Endocrinology* **132** (1993) 2498–2506.
9. Yanagibashi K. and Hall P.F.: Role of electron transport in the regulation of the lyase activity of C_{21} side-chain cleavage *P*-450 from porcine adrenal and testicular microsomes. *J. Biol. Chem.* **261** (1986) 8429–8433.
10. Kominami S., Higuchi A. and Takemori S.: Interactions of steroids with adrenal cytochrome *P*-450 ($P\text{-}450_{17\alpha}$, lyase) in liposomes membranes. *Biochim. Biophys. Acta* **937** (1988) 177–183.
11. Kühn-Velten W.N., Bunse T. and Förster M.E.C.: Enzyme kinetic and inhibition analyses of cytochrome *P*-450XVII, a protein with a bifunctional catalytic site. *J. Biol. Chem.* **266** (1991) 6291–6301.
12. Kühn-Velten W.N., Lessmann M., Förster M.E.C. and Staib W.: Specific accumulation of 17α-hydroxyprogesterone in microsomal membranes during the process of cytochrome *P*-450(C-17)-catalysed androgen biosynthesis. *Biochem. J.* **256** (1988) 53–59.
13. Wada A., Ohnishi T., Nonaka Y., Okamoto M. and Yamano T.: Synthesis of aldosterone by a reconstituted system of cytochrome $P\text{-}450_{11\beta}$ from bovine adrenocortical mitochondria. *J. Biochem.* **98** (1985) 245–256.
14. Tuckey C.T. and Kamin H.: Kinetics of the incorporation of adrenal cytochrome $P\text{-}450_{scc}$ into phosphatidylcholine vesicles. *J. Biol. Chem.* **257** (1982) 2887–2893.
15. Lambeth D. and Kamin H.: Phosphatidylcholine vesicle reconstituted cytochrome $P\text{-}450_{scc}$. *J. Biol. Chem.* **255** (1980) 8282–8288.
16. Cooke G.M. : Phospholipases modulate immature pig testicular androgen and 16-androstene biosynthetic pathways *in vitro*. *J. Steroid Biochem. Molec. Biol.* **41** (1992) 99–107.
17. Cooke G.M.: Identification of phospholipids capable of modulating the activities of some enzymes involved in androgen and 16-androstene biosynthesis in the immature pig testis. *J. Steroid. Biochem. Molec. Biol.* **42** (1992) 151–163.
18. Hiwatashi A. and Ichikawa Y.: Physiochemical properties of reduced nicotinamide adenine dinucleotide phosphate-cytochrome *P*-450 reductase from bovine adrenocortical microsomes. *Biochim. Biophys. Acta* **580** (1979) 44–63.
19. Dawson R.M.C., Hemington N. and Lindsay D.B.: The phospholipids of the erythrocyte "ghosts" of various species. *Biochem. J.* 77 (1960) 226–230.
20. Stewart J.C.M.: Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Analyt. Biochem.* **104** (1980) 10–14.
21. Kates M.: *Laboratory Techniques in Biochemistry and Molecular Biology : Techniques of Lipidology* (Edited by T.S. Work and E. Work). North Holland Publishing Co., Amsterdam (1975) pp. 393–395.
22. Lombardo A., Defaye G., Guidicelli C., Monnier N. and Chambaz E.M.: Integration of purified adrenocortical cytochrome $P\text{-}450_{11\beta}$ into phospholipid vesicles. *Biochem. Biophys. Res. Commun.* **104** (1982) 1638–1345.
23. Nozaki Y., Lasic D.D., Tanford C. and Reynolds J.A.: Size analysis of phospholipid vesicle preparations. *Science* **217** (1982) 366–367.
24. Shet M.S., Fisher C.W., Arlotto M.P., Shackleton C.H.L., Holmans P.L., Martin-Wixtrom C.A., Saeki Y. and Estabrook R.W.: Purification and enzymatic properties of a recombinant fusion protein expressed in *Escherichia coli* containing the domains of bovine *P*-450 17α and rat NADPH-*P*-450 reductase. *Arch. Biochem. Biophys.* **311** (1994) 402–417.
25. Brunner J., Skrabal P. and Hauser H. : Single bilayer vesicles prepared without sonication physico-chemical properties. *Biochem. Biophys. Acta* 455 (1976) 322–331.
26. Ohnishi T., Wada A., Lauber M., Yamano T. and Okamoto M. : Aldosterone biosynthesis in mitochondria of isolated zones of adrenal cortex. *J. Steroid Biochem. Molec. Biol.* **31** (1988) 73–81.
27. Cooke G.M. and Robaire B.: Phospholipases modulate the rat testicular androgen biosynthetic pathway *in vitro*. *Biol. Reprod.* **39** (1988) 329–339.
28. Onoda M. and Hall P.F.: Cytochrome b_5 stimulates purified testicular microsomal cytochrome *P*-450 (C_{21} side-chain cleavage). *Biochem. Biophys. Res. Commun.* **108** (1982) 454–460.