J. Steroid Biochem. Molec. Biol. Vol. 42, No. 1, pp. 37–47, 1992
Printed in Great Britain. All rights reserved

0960-0760/92 $5.00 + 0.00
Copyright © 1992 Pergamon Press plc

THE INTERFERENCE OF POLYCHLORINATED BIPHENYLS (AROCLOR 1254) WITH MEMBRANE REGULATION OF THE ACTIVITIES OF CYTOCHROMES P-450$_{C21}$ AND P-450$_{17\alpha,lyase}$ IN GUINEA-PIG ADRENAL MICROSOMES

DAPHNE GOLDMAN* and AMINADEV YAWETZ†
Institute for Nature Conservation Research, The George S. Wise Faculty for Life Sciences, Tel Aviv University, Ramat Aviv, 69978, Israel

(Received 28 June 1991)

Summary—Regulation of cytochromes P-450 21-hydroxylase (P-450$_{C21}$) and P-450 17α-hydroxylase/C17,20-lyase (P-450$_{17\alpha,lyase}$) activities and impairment of this regulation by Aroclor 1254 was studied in guinea-pig adrenal microsomes. In a membrane depleted system, a decrease in the normally predominant, P-450$_{C21}$ activity and an increase in P-450$_{17\alpha,lyase}$ activities was observed. The same deviations were observed in intact microsomes with increase in the reaction temperature (0–40°C). Breaks in Arrhenius plots for activities of P-450$_{C21}$ and P-450$_{17\alpha,lyase}$ correlate with transition temperatures reported for the microsomal membrane. These results point to: (1) preference of a gel state membrane for catalytic expression of P-450$_{C21}$ suggesting a clustered organization of this P-450 species with reductase; (2) preference of a fluid membrane for lyase activity suggesting a random collision mechanism for reduction of P-450$_{17\alpha,lyase}$.

Aroclor 1254 introduced to reaction mixtures containing intact microsomes elicited basically the same changes as caused by depletion of the microsomal membrane or by increase in the incubation temperature. Lack of effect of Aroclor 1254 on P-450$_{C21}$ and P-450$_{17\alpha,lyase}$ activities in the membrane depleted system demonstrates that its interference with monooxygenase activities is mediated by the microsomal membrane. The similarities between altered cytochrome P-450 mediated activities in the presence of Aroclor 1254 and the deviations observed in the membrane depleted system or upon increase in the incubation temperature may suggest that this chemical exerts its impacts by influencing membrane fluidity.

INTRODUCTION

In steroidogenesis of adrenocortical microsomes, two different cytochrome P-450 species catalyze the monooxygenase reactions: P-450$_{C21}$[1] which catalyzes 21-hydroxylation and P-450$_{17\alpha,lyase}$ which catalyzes 17α-hydroxylation and, to a minor extent, C17,20 bond cleavage [1]. The major steroids secreted by the adrenal cortex are the corticosteroids. Hence, most of the 17α-hydroxy-C21-steroid produced is released from P-450$_{17\alpha,lyase}$ for further metabolism by P-450$_{C21}$ and only a limited amount is successively transformed into androgens without leaving P-450$_{17\alpha,lyase}$ [2, 3].

This laboratory has previously reported that the polychlorinated biphenyl compound (Aroclor 1254) had a selective effect on the catalytic activities of the two cytochrome P-450 species present in adrenocortical microsomes. While the activity of P-450$_{C21}$ was prominently lowered, both 17α-hydroxylation and the normally suppressed C17,20 bond cleavage activity of P-450$_{17\alpha,lyase}$ were significantly enhanced. These selective effects may lead to an abnormal state of decreased corticosteroid production and increased androgen production by the adrenal cortex [4, 5].

The Aroclor 1254-elicited alterations were observed not only in the pre-treated animals but also when the chemical was introduced to reaction mixtures prepared with adrenal microsomes from untreated animals, suggesting that

*Submitted in partial fulfilment of the requirement of Tel Aviv University for a Ph.D. degree in zoology.
†To whom correspondence should be addressed.
Abbreviations: P-450$_{C21}$, adrenal microsomal cytochrome P-450 having 21 hydroxylating activity; P-450$_{17\alpha,lyase}$ adrenal microsomal cytochrome P-450 having both 17α-hydroxylating and C17,20 bond cleaving activities (the C21 side chain cleavage cytochrome P-450); 11-DOC, 11-deoxycorticosterone; 11-D, 11-deoxycortisol; 17α-OHprogesterone, 17α-hydroxyprogesterone; A, androstenedione.

the interference of this chemical with cytochrome P-450 mediated activities in adrenal microsomes may be at a post-transcriptional, kinetic level, resulting from accumulation of this lipophilic chemical within the microsomal membrane of the steroidogenic tissue [6].

In the present study an attempt was made to further investigate the role of the microsomal membrane in mediating the effects of Aroclor 1254 on the catalytic activities of the two cytochrome P-450 species present in adrenal microsomes: $P\text{-}450_{C21}$ and $P\text{-}450_{17\alpha,lyase}$. The role of the microsomal membrane in regulating the normal catalytic expression of these cytochrome P-450 species is also considered.

EXPERIMENTAL

Materials

Aroclor 1254 was obtained from Analabs (North Haven, CT, U.S.A.). [4-C^{14}]progesterone (56 mCi/mmol) and [4-C^{14}]17α-hydroxyprogesterone (53 mCi/mmol) were purchased from Amersham (Bucks., England). NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, DTT, bovine serum albumin, Triton N-101, progesterone, 17α-hydroxyprogesterone (17α-OHprogesterone), 4-androstene-3,17-dione, 11-deoxycortisol (11-D), 11-deoxycorticosterone (11-DOC) and other steroid standards were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Reagent grade organic solvents were purchased from Merck & Co. Sodium dithionite was acquired from Baker Chemical Co. (Philipsberg, NJ, U.S.A.). HPLC grade organic solvents and sodium cholate were purchased from Fluka Chimie (Switzerland).

Preparation of the microsomal fraction

Microsomes from adrenal glands of adult (750–1000 g) male albino-guinea-pigs of a local strain were prepared by differential centrifugation. Adrenal tissues were homogenized in a Potter–Elvehjem type homogenizer with a Teflon pestle, in 10 vol of a phosphate buffer system (K_2HPO_4 0.1 M, pH 7.4) containing 20% glycerol and 1 mM each of EDTA and DTT (buffer A). The homogenates were centrifuged in a Sorvall RC 5 superspeed refrigerated centrifuge at 12,000 g for 20 min to remove nuclei, unbroken cells and mitochondria. The precipitate was discarded and the supernatant was centrifuged in a Beckman L5 50 refrigerated ultracentrifuge at 105,000 g for 60 min. The pellet was suspended in buffer A and recentrifuged at 105,000 g for 60 min to obtain the microsomal pellet.

Enzyme assays

The reaction mixtures for the assays of cytochrome P-450 mediated metabolism of progesterone or 17α-OHprogesterone contained about 1 nmol of adrenal microsomal cytochrome P-450; 0.1 M K_2HPO_4 pH 7.4; 20% glycerol; 1 mM each of EDTA and DTT; 2.6 mM NADP; 52.5 mM glucose-6-phosphate; 2.5 U of glucose-6-phosphate dehydrogenase and 2.2 mM $MgCl_2$ in a final volume of 1.15 ml. When the effect of Aroclor 1254 was studied, 13.3 μM of Aroclor 1254 dissolved in acetonitryl (20 μl) were mixed with the microsomal suspensions prior to addition of other reaction components. Control reactions received an equivalent volume of acetonitryl. After 5 min preincubation for temperature equilibration the reactions were initiated by addition of [4-C^{14}]progesterone or [4-C^{14}]17α-OHprogesterone and were carried out in a shaker at the selected temperature. After 10 min incubation, the reactions were terminated and steroids extracted into 9 ml dichloromethane containing internal standards. The dichloromethane phase was dried under a stream of nitrogen and residues were resuspended in HPLC grade methanol.

Steroid separation

Labeled steroids were separated and identified by reverse phase HPLC. Steroid substrates and products were separated on a Microbondapak C18 column with a methanol–water (60:40, v/v) solvent system. The flow rate was maintained at 1 ml/min (Waters model 6000A pump) and the eluant was monitored at 206 nm by a Jasco Uvidec 100 V u.v. spectrophotometer. Eluted steroids were identified by comparing their retention times to those of known standards and were quantitated by their radioactivity relative to that of the substrate introduced into reaction.

Effect of temperature

The temperature dependence of progesterone and 17α-OHprogesterone metabolism was investigated within the range of 0–40°C with intervals of 5°C. The assays were carried out as described under enzyme assay with 0.417 μM of either [4-C^{14}]progesterone or [4-C^{14}]17α-OHprogesterone in the presence or absence of 13.3 μM Aroclor 1254.

Effect of detergent on progesterone metabolism

Adrenal microsomes suspended in 0.8 ml of K_2HPO_4 (0.1 M, pH 7.4) were mixed at 0–4°C with increasing concentrations (0.01–0.8%, w/v) of sodium cholate. After 20 min, reaction, components other than substrate were added and after preincubation for 5 min at 15°C, the reactions were initiated by the addition of 0.522 μM [4-C^{14}]progesterone. The reactions were carried out for 10 min at 15°C. This temperature was chosen since above 20°C there occurred an infliction in the Arrhenius plots.

Effect of removal of the microsomal membrane

Components of the adrenal microsomal monooxygenase system were solubilized by suspending adrenal microsomes in buffer A containing 0.4% (w/v) of sodium cholate. Solubilization was carried out at 0–4°C for 20 min with constant mixing. The membrane fraction was removed by centrifugation at 105,000 *g* for 1 h. The supernatant was dialyzed overnight (4°C) against 2 × 0.5 l of buffer A containing 0.004% Triton N-101. The dialyzed fraction was then used for the enzyme assays. The assay for progesterone or 17α-OHprogesterone metabolism was carried out using 0.104–0.417 μM of [4-C^{14}]progesterone or [4-C^{14}]17α-OHprogesterone at 15°C for 10 min, in the presence or absence of 13.3 μM Aroclor 1254. Triton N-101 was added to reach a final concentration of 0.008%. Other components of the reaction mixtures were the same as described under enzyme assay.

Miscellaneous procedures

Cytochrome *P*-450 content was determined from the carbon monoxide binding difference spectrum (450–490 nm) of dithionite reduced microsomes, using an extinction coefficient of 91 $mM^{-1} \times cm^{-1}$ [7]. Measurements were made on a Jasco Uvidec-610 doublebeam spectrophotometer. The protein content was measured by the method of Bradford [8] using bovine serum albumin as the standard.

Statistical analysis

The data were analyzed by multiway analysis of variance and regression analysis.

RESULTS

The effect of detergent on catalytic activities of P-450_{C21} and P-$450_{17\alpha,lyase}$

In a preliminary stage, we investigated to what extent the catalytic expression of the two species of adrenal microsomal cytochrome *P*-450 is modified by gradual disruption of the intact membrane structure. This was achieved by incubating adrenal microsomes with increasing quantities of sodium cholate.

Figure 1 describes the effect of increasing concentrations (0.01–0.8%) of sodium cholate on the adrenal microsomal cytochrome *P*-450 mediated production of steroids from progesterone. The upper graph shows the overall production of metabolites as a function of detergent concentration. From the curve it is evident that up to a concentration of 0.2%, sodium cholate had no effect on the total production of metabolites. Above 0.2% there was a prominent decrease in total metabolite formation, indicating progressive disruption of the microsomal membrane structure. Retention of the catalytic activity of hepatic microsomal cytochrome *P*-450b treated with up to 0.2% of this anionic detergent has been reported by Dutton *et al.* [9].

The lower graph shows production of the various metabolites with increasing concentrations of sodium cholate relative to steroid

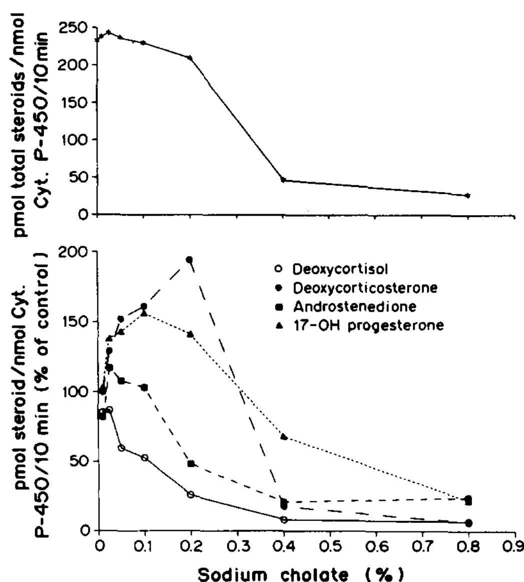

Fig. 1. Effect of sodium cholate concentration on cytochrome *P*-450 dependent metabolism of progesterone. Upper panel, total metabolite formation as a function of sodium cholate concentration. Lower panel, formation of the various metabolites at the given concentration of sodium cholate relative to steroid production in the absence of detergent. Results are the means of two separate determinations. Preparation of microsomes, the assay for monooxygenation of progesterone and the procedure for determining the effects of increasing concentrations of sodium cholate were performed as described under 'Experimental'. The concentration of progesterone was 0.522 μM and the concentration of sodium cholate varied as shown. The incubation temperature was 15°C.

production in the absence of detergent. Partial disruption of the membrane (sodium cholate <0.2%) selectively affected the catalytic activities of the two species of cytochrome P-450 as reflected by production of the various metabolites of progesterone: formation of 11-DOC and 17α-OHprogesterone increased; production of 11-D was severely lowered and androstenedione (A) production was basically unaffected up to 0.1% and above this concentration was lowered.

The effect of removal of the microsomal membrane

. The possibility that the changes elicited by Aroclor 1254 on progesterone metabolism are mediated by the microsomal membrane was investigated by comparing the effects of Aroclor 1254 on progesterone metabolism in intact microsomes to that in a membrane depleted system containing the cytochrome P-450 system solubilized from microsomes and reconstituted with 0.008% of the non-ionic detergent. Triton N-101 as a substitute for the lipid requirement of the adrenal microsomal electron transport system.

In intact microsomes total metabolite formation was significantly lowered ($P < 0.001$) as a result of introduction of Aroclor 1254 (Fig. 2, left panel). Such an effect was not detected in the membrane depleted system (Fig. 2, right panel). In a solubilized system to which no Triton N-101 was added, total metabolite formation

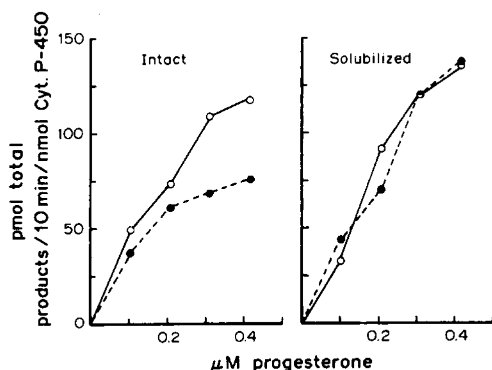

Fig. 2. Total metabolite production from progesterone by intact microsomes and by solubilized microsomal components in the presence and absence of Aroclor 1254. Open circles, in the absence of Aroclor 1254. Closed circles, in the presence of 13.3 μM Aroclor 1254. Results are the means of three separate determinations. Preparation of microsomes, the procedure for solubilization and reconstitution of the microsomal electron transfer components and the assay for progesterone metabolism were performed as described under 'Experimental'. The concentration of progesterone varied as shown. The incubation temperature was 15°C.

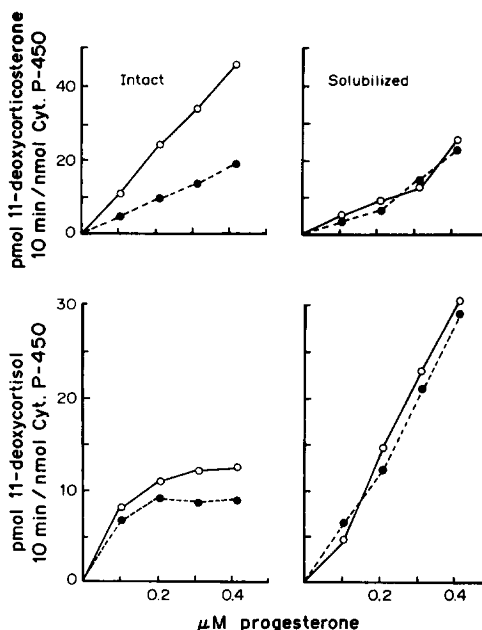

Fig. 3. Production of 11-DOC and 11-D from progesterone by intact microsomes and by solubilized microsomal components in the presence and absence of Aroclor 1254. Open circles, in the absence of Aroclor 1254. Closed circles, in the presence of 13.3 μM Aroclor 1254. Results are the means of three separate determinations. Preparation of microsomes, solubilization and reconstitution of the microsomal electron transfer components and the assay for metabolism of progesterone were performed as described under 'Experimental'. Progesterone concentration varied as shown. The incubation temperature was 15°C.

was 47 pmol/10 min/nmol P-450, which is only 35% of overall metabolite production by intact microsomes. Reconstitution using Triton N-101 (0.008%) fully restored the overall cytochrome P-450-mediated catalytic activity indicating that this non-ionic detergent compensated for the lipid requirement of the adrenal microsomal electron transport system as has been demonstrated by others [10, 11].

Figure 3 describes the effect of Aroclor 1254 on production of 11-DOC and 11-D in intact microsomes and in the membrane depleted system. Introduction of Aroclor 1254 to adrenal microsomal reaction mixtures resulted in a significant decrease in the production of both corticosteroids ($P < 0.001$ for 11-DOC and $P < 0.05$ for 11-D). In the membrane depleted system, production of these steroids was not different in the absence or presence of Aroclor 1254 indicating that removal of the microsomal membrane totally obliterated the inhibitory effect of Aroclor 1254 on activity of $P\text{-}450_{C21}$.

In the absence of Aroclor 1254, removal of the membrane resulted in a 2-fold decrease ($P < 0.001$) in production of 11-DOC and a

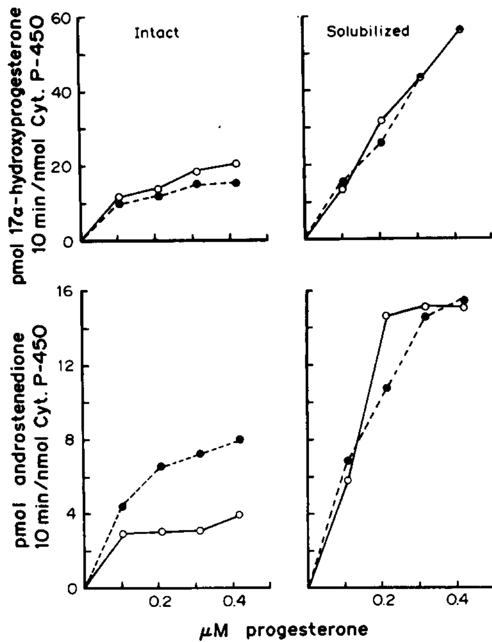

Fig. 4. Production of 17α-OHprogesterone and A from progesterone by intact microsomes and by solubilized microsomal components in the presence and absence of Aroclor 1254. Open circles, in the absence of Aroclor 1254. Closed circles, in the presence of 13.3 μM Aroclor 1254. Results are the means of three separate determinations. Preparation of microsomes, solubilization and reconstitution of the microsomal electron transfer components and the assay for metabolism of progesterone were performed as described under 'Experimental'. Progesterone concentration varied as shown. The incubation temperature was 15°C.

nearly 3-fold increase ($P < 0.01$) in the production of 11-D.

Figure 4 describes the effects of Aroclor 1254 on the 2 catalytic activities of P-$450_{17\alpha,\text{lyase}}$ represented by the production of 17α-OHprogesterone and A. Aroclor 1254 present in the incubation mixtures had no significant effect on production of the steroid intermediate 17α-OH-progesterone but resulted in a greater than 2-fold increase ($P < 0.01$) in the C17,20 bond cleaving activity represented by production of A. Removal of the microsomal membrane abolished this effect so that in the solubilized system, Aroclor 1254 caused no change in production of either of the steroids.

In the absence of Aroclor 1254, removal of the microsomal membrane resulted in a 3-fold increase ($P < 0.01$) in the 17α-hydroxylating activity and a 4-fold increase ($P < 0.001$) in the C17,20 bond cleaving activity of P-$450_{17\alpha,\text{lyase}}$, as represented by production of 17α-OHprogesterone and A, respectively.

The Aroclor 1254-elicited alterations in the activities of P-450_{C21} and in the C17,20 bond cleaving activity of P-$450_{17\alpha,\text{lyase}}$ were further studied by using the intermediate steroid 17α-OHprogesterone as a substrate in both the intact and membrane depleted systems. Results are shown in Fig. 5. In intact microsomes, Aroclor 1254 had an inhibitory effect ($P < 0.001$) on the production of 11-D. This is consistent with the results obtained when using progesterone as the substrate (Fig. 3). No effect could be detected for Aroclor 1254 on the production of A from 17α-OHprogesterone by intact microsomes, which stands in contrast to the inductive effect observed for Aroclor 1254 on production of this steroid when using progesterone as a substrate (Fig. 4).

In good agreement with the results obtained for metabolism of progesterone, no effect could be observed for Aroclor 1254 on production of either 11-D or A from 17α-OHprogesterone after removal of the microsomal membrane (Fig. 5).

In the membrane depleted system to which no Aroclor 1254 was added, production of 11-D and A from 17α-OHprogesterone was not different from that in the reactions containing intact microsomes. This stands in contrast to the increase measured in production of both products upon removal of the membrane, when using progesterone as substrate.

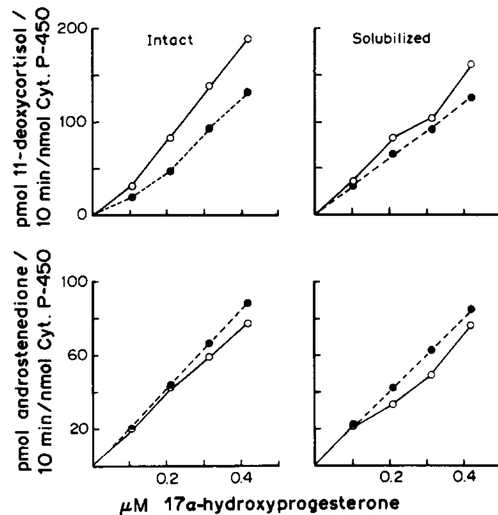

Fig. 5. Production of 11-D and A from 17α-OHprogesterone by intact microsomes and by solubilized microsomal components in the presence and absence of Aroclor 1254. Open circles, in the absence of Aroclor 1254. Closed circles, in the presence of 13.3 μM Aroclor 1254. Results are the means of two separate determinations. Preparation of microsomes, solubilization annd reconstitution of the microsomal electron transfer components and the assay for metabolism of 17α-OHprogesterone were performed as described under 'Experimental'. 17α-OHprogesterone concentration varied as shown. The incubation temperature was 15°C.

Temperature dependence of the catalytic activities of $P\text{-}450_{C21}$ and $P\text{-}450_{17\alpha,lyase}$

Arrhenius plots for production of 11-DOC and 11-D from progesterone show a definite break at 20 and 21.5°C, respectively (Fig. 6). Above the breakpoints there was a decrease in activity, which was very pronounced for 11-DOC (Fig. 6). Consistent with its marked inhibitory effect on production of 11-DOC (Fig. 3), Aroclor 1254 caused a decrease in production of this steroid at all temperatures studied, although this inhibitory effect became less pronounced with increase in the temperature of the reactions (Fig. 6). An inhibitory effect of Aroclor 1254 was likewise observed on production of 11-D, at temperatures below the breakpoint. Within this range, the extent of inhibition decreased with rise in temperature, and above the breakpoint, a change in the trend of the effect of Aroclor 1254 occurred (Fig. 6).

The temperature of the breaks in Arrhenius plots for production of both the corticosteroids was not altered by Aroclor 1254.

Arrhenius plots for the production of 17α-OHprogesterone and A show two breakpoints for each steroid: at 13.5 and 30°C for 17α-OH-progesterone and at 10 and 26.5°C for A (Fig. 7). A change in the trend of the Arrhenius plot was observed for 17α-OHprogesterone at each of the breaks. (Fig. 7). Differing significantly from the other metabolites of progesterone, production of A continuously increased with the rise in temperature (Fig. 7).

In good agreement with the previously observed inductive effect of Aroclor 1254 on production of 17α-OHprogesterone and A (Fig. 4), both catalytic activities increased when Aroclor 1254 was present in the reaction mixtures (Fig. 7). Aroclor 1254 had no effect on the temperatures of the two breakpoints in the Arrhenius plot for A formation (Fig. 7), consistent with observations made for 11-DOC and 11-D. Slight but detectable alterations in the temperature of the breakpoints could be observed only in the production of the intermediate steroid 17α-OHprogesterone (Fig. 7).

The Arrhenius plots for production of 11-D from 17α-OHprogesterone, in the absence and presence of Aroclor 1254 are shown in Fig. 8. These plots show similarities to those made when using progesterone as substrate, namely: one breakpoint, an inhibitory effect of Aroclor 1254 at temperatures below the breakpoint and a change in the trend of its effect above this, as well as no effect of Aroclor 1254 on the temperature of the break. The discrepancy between the Arrhenius plots for production of 11-D when using progesterone or 17α-OHprogesterone is attributed to the temperature of the breakpoint, which was much lower when 17α-OHprogesterone served as the substrate (15.4°C as opposed to 21.5°C, Figs 8 and 6, respectively). Only one break could be detected in the Arrhenius plot of A production from 17α-OHprogesterone, at 27.1°C (Fig. 8), which correlates well with the high breakpoint detected when A was produced from progesterone (26.5°C, Fig. 7). In

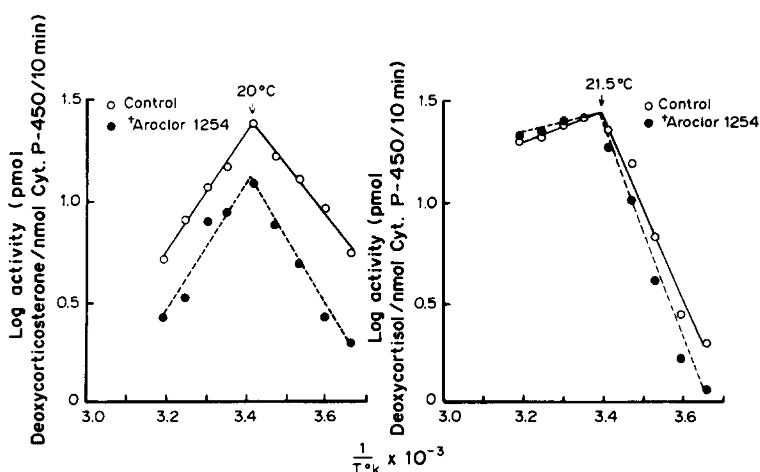

Fig. 6. Arrhenius plots for the production of 11-DOC and 11-D from progesterone in the presence and absence of Aroclor 1254. Open circles, in the absence of Aroclor 1254. Closed circles, in the presence of 13.3 μM Aroclor 1254. Results are the means of two separate determinations. Preparation of microsomes, the procedure for determination of the temperature dependence and the assay for metabolism of progesterone were performed as described under 'Experimental'. The concentration of progesterone was 0.417 μM and the temperature ranged from 0–40°C.

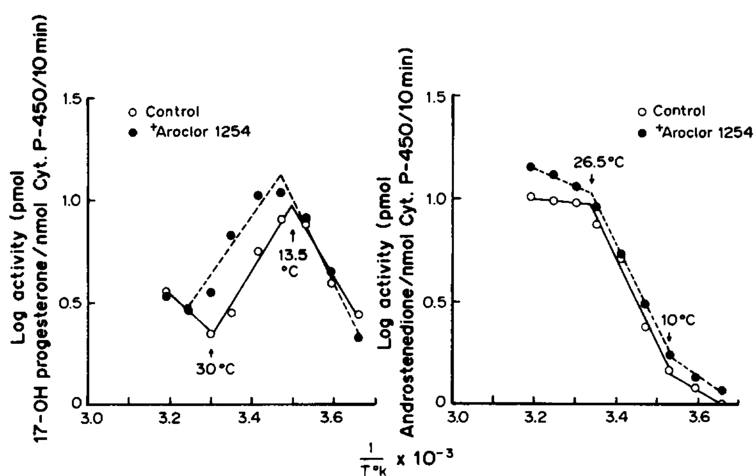

Fig. 7. Arrhenius plots for the production of 17α-OHprogesterone and A from progesterone in the presence and absence of Aroclor 1254. Open circles, in the absence of Aroclor 1254. Closed circles, in the presence of 13.3 μM Aroclor 1254. Results are the means of two separate determinations. Preparation of microsomes, the procedure for determination of the temperature dependence and the assay for metabolism of progesterone were performed as described under 'Experimental'. The concentration of progesterone was 0.417 μM and the temperature ranged from 0–40°C.

contrast to its inductive effect on A formation from progesterone, no such effect could be observed for Aroclor 1254 on the direct production of this steroid from 17α-OHprogesterone (Fig. 8).

DISCUSSION

Results previously obtained point to the possibility that the interference of Aroclor 1254 with cytochrome P-450-mediated metabolism of progesterone in adrenal microsomes is at a post-transcriptional level, although altered composition of adrenal microsomal P-450 isozymes could not be excluded [6]. The present study aimed to obtain insight concerning the impact of Aroclor 1254 on regulation of the activities of the adrenal microsomal cytochrome P-450 species: P-450_{C21} and P-$450_{17\alpha,lyase}$.

The essential role of the microsomal membrane in regulating the catalytic expression of P-450_{C21} and P-$450_{17\alpha,lyase}$ was demonstrated by the effects of the anionic detergent sodium cholate on production of the steroid metabolites (Fig. 1). The results observed in the presence of lower than 0.2% sodium cholate may be interpretated as an increase in those metabolic products that require one monooxygenation step for

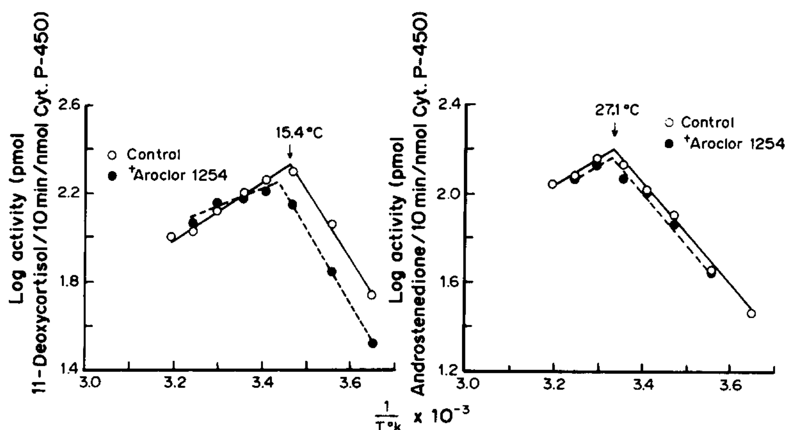

Fig. 8. Arrhenius plots for the production of 11-D and A from 17α-OHprogesterone in the presence and absence of Aroclor 1254. Open circles, in the absence of Aroclor 1254. Closed circles, in the presence of 13.3 μM Aroclor 1254. Results are the means of two separate determinations. Preparation of microsomes, the procedure for determination of the temperature dependence and the assay for metabolism of 17α-OHprogesterone were performed as described under 'Experimental'. The concentration of 17α-OH-progesterone was 0.417 μM and the temperature ranged from 0–40°C.

their formation, namely 11-DOC and 17α-OH-progesterone, while formation of A which requires 2 monooxygenase cycles carried out by the same enzyme remained unaffected up to 0.1% and then decreased and 11-D, whose formation requires 2 cycles of monooxygenation involving both species of cytochrome P-450 was severely lowered.

Upon removal of the microsomal membrane by solubilization of microsomal components and reconstitution with detergent, the changes observed in the catalytic activities were different from those described above. In this membrane depleted system, there was a decrease in the enzymatic activity of P-450_{C21} paralleled by an increase in the enzymatic activities of P-$450_{17\alpha,lyase}$ (Figs 3 and 4). Thus, the production of 17α-OHprogesterone, A were significantly elevated while production of 11-DOC was drastically reduced. The increase in production of 11-D may be the outcome of the increased 17α-hydroxylating activity of P-$450_{17\alpha,lyase}$ indicating that in intact microsomes this catalytic step is the rate-limiting stage in production of 11-D from progesterone. These selective effects on the activity of the two hemoproteins, resulting from removal of the membrane, could not be demonstrated when 17α-OHprogesterone served as the substrate (Fig. 5). Since formation of 11-D from 17α-OHprogesterone is dependent solely on activity of P-450_{C21}, removal of the microsomal membrane is not expected to result in an increase in its production, as was the case. On the other hand, an increase in the production of A from 17α-OHprogesterone in the membrane depleted system could be expected as a result of the increased lyase activity of P-$450_{17\alpha,lyase}$. The deviation from this most probably reflects the lower affinity of P-$450_{17\alpha,lyase}$ for 17α-OHprogesterone as compared to its affinity for progesterone. Consecutive C17,20 bond cleavage of the steroid intermediate 17α-OH-progesterone by P-$450_{17\alpha,lyase}$ in preference to utilization of free diffusing 17α-OHprogesterone demonstrated by Samuels *et al.* [12], is also very likely a reflection of such a difference in the affinity of P-$450_{17\alpha,lyase}$ for the two substrates.

The decrease in production of 11-DOC from progesterone in the membrane depleted system, indicates the necessity of the intact membrane structure for catalytic expression of P-450_{C21} while the drastic increase in production of both 17α-OHprogesterone, A and 11-D from progesterone suggests that catalytic expression of P-$450_{17\alpha,lyase}$ is not at its maximum capacity within the intact membrane.

The observation that in the membrane depleted system Aroclor 1254 exhibited no effects on the metabolism of either progesterone or 17α-OHprogesterone whereas in intact microsomes metabolism of these substrates was selectively altered points to the possibility that the interference of this chemical with monooxygenase activities of P-450_{C21} and P-$450_{17\alpha,lyase}$ is mediated by the microsomal membrane.

In intact microsomes the effects observed for Aroclor 1254 on the activity of the two species of cytochrome P-450 bear resemblance to those elicited by removal of the membrane, namely a significant decrease in the activity of P-450_{C21} as represented by the formation of 11-DOC from progesterone and 11-D from 17α-OHprogesterone and a significant increase in the production of A reflecting increased activity of P-$450_{17\alpha,lyase}$. The decrease in production of 11-D in the presence of Aroclor 1254 may indicate that the decrease in P-450_{C21} activity is more prominent than the increase in the 17α-hydroxylating activity of P-$450_{17\alpha,lyase}$. The apparent decrease in production of 17α-OHprogesterone may very likely be the result of the pronounced increase in lyase activity, elicited by Aroclor 1254, which pulls the metabolic pathway towards androgen production.

The possibility that Aroclor 1254 exerts its effects by altering membrane fluidity was investigated by comparing the Arrhenius plots for each of the metabolites produced from progesterone and 17α-OHprogesterone in the presence and absence of Aroclor 1254.

The temperature of the break in the Arrhenius plots for production of 11-DOC and 11-D (20–21.5°C) is similar to the temperature of the break in the Arrhenius plot for O-dealkylation of 7-ethoxycoumarin by hepatic microsomes [13], and to the break in the Arrhenius plot for the slow phase of reduction of hepatic microsomal P-450 [14]. This temperature correlates well with one of the temperatures of lipid phase transition reported for the microsomal membrane [15, 16]. The decrease in activity of P-450_{C21} above the transition temperature points to preference of a gelstate membrane for catalytic expression of P-450_{C21}. On the other hand, the continuous increase in A production with the rise in temperature supports preference of fluid membrane organization for the C17,20 bond cleaving activity of P-$450_{17\alpha,lyase}$. The temperature of the break in the Arrhenius plot for

A production (26.5–27.1°C) also correlates with a reported break at around 25°C [16]. 17α-OH-progesterone is an intermediate steroid, which may account for the complicated effect of temperature on its production, since the amount measured reflects not only its formation but also further metabolism to either 11-D or A. The break at 30°C in the Arrhenius plot of production of this steroid also correlates well with another documented phase transition temperature [15–17]. Since production of 11-D reflects 17α-hydroxylase activity as well as 21-hydroxylase activity, the limited decrease in its production above the transition temperature in contrast to the severe decrease in 11-DOC formation at respective temperatures, may be the result of increased activity of P-$450_{17\alpha,\mathrm{lyase}}$ when the membrane is in the fluid state.

The results of the present study stand in good agreement with reduction of cytochrome P-450 as the rate limiting step for enzyme activity [18, 19] even though reduction of P-450 was not directly assessed. Furthermore, the drastic reduction in overall cytochrome P-450-mediated activities resulting from removal of the microsomal membrane is also consistent with the role of lipid in facilitating formation of catalytically active complexes of cytochrome P-450 and reductase [20]. Although Triton N-101 restored overall monooxygenation to the solubilized components (Fig. 2), deviations in the catalytic activities of both species of cytochrome P-450 indicates that the specific organization of the electron transfer components within the intact membrane is what determines their expression.

The results obtained appear to support both a cluster organization [14, 16, 21] as well as a random collision model [11, 19, 22–24] for the monooxygenase activities of adrenal microsomes. The temperature dependence of 21-hydroxylating activity, namely the decrease in formation of 11-DOC from progesterone upon transition to a fluid state, points to preference of a gel-state membrane for expression of P-450_{C21} which would support a clustered organization of this species of cytochrome P-450 with reductase. Formation of stable complexes of P-450_{C21} and NADPH–cytochrome P-450 reductase purified from bovine adrenal microsomes, supporting 21-hydroxylation of progesterone, has been demonstrated by Kominami *et al.* [25]. On the other hand, the continuous increase in A formation, with rise in temperature, may indicate preference of a fluid membrane for lyase activity, suggesting that P-$450_{17\alpha,\mathrm{lyase}}$ is a limited participant of the complexes with reductase, rather its reduction is largely dependent on random collision with free diffusing reductase molecules during translational movement in the membrane. By increasing the temperature and thus the fluidity of the membrane, the following changes are brought about: initially the clusters may grow in size [14], thus including molecules of P-$450_{17\alpha,\mathrm{lyase}}$, and with transition to a fluid membrane, disintegration of the predominantly P-450_{C21} : reductase clusters would increase the ratio of available, free diffusing reductase molecules as well as enhance the lateral movement within the membrane. The outcome of these changes would be an increase in the chances for interaction between molecules of P-$450_{17\alpha,\mathrm{lyase}}$ and reductase. Hence, the effect of raising the temperature is expected to lower the activity of P-450_{C21} and increase the activities of P-$450_{17\alpha,\mathrm{lyase}}$, as was observed.

Release of the electron transfer proteins from the phospholipid environment elicited a significant decrease in 21-hydroxylating activity. This is the effect expected to occur upon solubilization, if in the intact membrane P-450_{C21} is complexed with reductase. On the other hand, removal of the membrane resulted in a drastic increase in the catalytic activities of P-$450_{17\alpha,\mathrm{lyase}}$. This is also the expected outcome if, in the intact membrane, this enzyme is a minor participant, if at all, of the complexes with reductase, and the method by which it is reduced is by random collision with those reductase molecules not associated with the clusters. Solubilization would thus release reductase from these complexes so the ratio of reductase to P-$450_{17\alpha,\mathrm{lyase}}$ would be significantly increased, supporting greater monooxygenation by this enzyme.

The assumption for above discussion on the influence of temperature and membrane on catalytic expression of P-450_{C21} and P-$450_{17\alpha,\mathrm{lyase}}$ is that reductase is a rate limiting factor in the microsomal membrane of the adrenal cortex and that the relative access of each species of P-450 to reductase is what controls the metabolic pathway. Limitation of reductase in adrenocortical microsomes has been affirmed by Yanagibashi and Hall [26] who demonstrated that limited lyase activity in adrenal microsomes compared to that in testicular microsomes is a result of 3 to 4 times lower content of adrenal microsomal reductase. The results presented herein provide a mechanism for the relative accessibility of the two species of adrenal

microsomal cytochrome P-450 to reductase. Clustering of P-450_{C21} with reductase would explain the natural predominance of corticosteroid production and suppression of lyase activity in adrenocortical microsomes.

The existence of multiple breaks in Arrhenius plots points to a heterogenous membrane in which there exist both non-fluid as well as fluid areas. Heterogenous distribution of lipids in the microsomal membrane, permitting coexistence of rigid and fluid regions has been documented [16, 27]. The selective organization of P-450_{C21} and P-$450_{17\alpha,lyase}$ within the membrane, supported by the results of this study, may result from differences in affinities, favoring complexing of P-450_{C21} over P-$450_{17\alpha,lyase}$ with reductase or, alternatively, from differential preference of the various protein components for their lipid surrounding, assuming a heterogenous lipid composition of the membrane.

Interference of Aroclor 1254 with membrane regulation of the catalytic activities of cytochrome P-450 supported by the results of this study would require accumulation of this chemical within the steroidogenic membrane, as could be expected for this highly hydrophobic chemical. Accumulation of polychlorinated biphenyls within steroidogenic tissues including the adrenals has been confirmed [28, 29]. The similarities between altered enzyme activities in the presence of Aroclor 1254 and the deviations observed in the membrane depleted system as well as when the temperature of the reactions was elevated, may suggest that this chemical interferes with the extent of fluidity of the membrane. If accumulation of Aroclor 1254 within the membrane increases the fluidity, this would be expected to elicit an increase in P-$450_{17\alpha,lyase}$ activities as opposed to a decrease in the P-450_{C21} activity assuming the preference of a rigid structure for activity of P-450_{C21} as opposed to a fluid structure for activities of P-$450_{17\alpha,lyase}$. Furthermore, a decrease in the extent of the effects of Aroclor 1254 with increased fluidity of the membrane (rise in temperature) would also be expected. The results obtained concerning the trend as well as the extent of the alterations elicited by Aroclor 1254 stand in good agreement with the above, supporting the possibility that accumulation of this chemical within the steroidogenic membrane alters the physical state of the membrane which determines the arrangement (coupling) and hence, the activity of the monooxygenase enzyme system. Increased fluidity of membrane lipids by hydrophobic molecules such as camphor has been demonstrated [14]. Drugs such as morphine derivatives and butobarbitone have been shown to lower the temperature of phase transition of lecithin membrane vesicles [30, 31]. Hence, it appears, that in the process of interaction with microsomal membrane lipids, hydrophobic xenobiotics might alter the interaction between the components of the cytochrome P-450-mediated system, thus changing their catalytic activities. Such an effect has been proposed for phenobarbitol type inducers of P-450, which, resulting from extensive contact with the endoplasmic reticulum membrane, may cause a phase shift in the lipids and thus a decoupling of the cytochrome P-450 system [27].

REFERENCES

1. Hall P. F.: Cytochromes P-450 and the regulation of steroidogenesis. *Steroids* **48** (1986) 131–196.
2. Nakajin S., Shinoda M. and Hall P. F.: Purification and properties of the 17-hydroxylase from microsomes of pig adrenal. A second C21 side-chain cleavage system. *Biochem. Biophys. Res. Commun.* **111** (1983) 512–517.
3. Nakajin S., Shinoda M., Haniu M., Shively J. E. and Hall P. F.: C21 steroid side chain cleavage enzyme from porcine adrenal microsomes. *J. Biol. Chem.* **259** (1984) 3971–3976.
4. Goldman D. and Yawetz A.: PCB induced alteration of steroid hormone biosynthesis in male guinea-pig adrenal microsomes. In *Cytochrome P-450: Biochemistry and Biophysics* (Edited by I. Schulster). Taylor & Francis (1989) pp. 833–836.
5. Goldman D. and Yawetz A.: Effect of exposure to Aroclor 1254 on cytochrome P-450 mediated metabolism of progesterone in male and female guinea-pigs. In *Environmental Quality and Ecosystem Stability. Vol. IV/B—Ecosystem Stability* (Edited by E. Spanier). ISEEQS Publication (1989) pp. 497–506.
6. Goldman D. and Yawetz A.: The interference of Aroclor 1254 with progesteronc metabolism in guinea-pig adrenal and testes microsomes. *J. Biochem. Toxic.* **5** (2) (1990) 99–107.
7. Omura T. and Sato R.: The carbon monoxide binding pigment of liver microsomes. *J. Biol. Chem.* **239** (1964) 2370–2378.
8. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analyt. Biochem.* **72** (1976) 248–251.
9. Dutton D. R., McMillen S. K. and Parkinson A.: Purification of rat liver microsomal cytochrome P-450b without the use of nonionic detergent. *J. Biochem. Toxic.* (1989) 131–145.
10. Lu A. Y. H., Levin W. and Kuntzman R.: Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds and endogenous substrates. *Biochem. Biophys. Res. Commun.* **60** (1974) 266–272.
11. Dean W. L. and Gray R. D.: Relationship between state of aggregation and catalytic activity for cytochrome P-450LM2 and NADPH–cytochrome P-450 reductase. *J. Biol. Chem.* **257** (1982) 14679–14685.

12. Samuels L. T., Bussmann L., Matsumoto K. and Huseby R. A.: Organization of androgen synthesis in the testis. *J. Steroid Biochem.* **6** (1975) 291–296.
13. Duppel W. and Ullrich V.: Membrane effects on drug monooxygenation activity in hepatic microsomes. *Biochim. Biophys. Acta* **426** (1976) 399–407.
14. Peterson J. A., Ebel R. E., O'Keeffe D. H., Matsubara T. and Estabrook R. W.: Temperature dependence of cytochrome *P*-450 reduction. A model for NADPH–cytochrome *P*-450 reductase: cytochrome *P*-450 interaction. *J. Biol. Chem.* **251** (1976) 4010–4016.
15. Narisimhulu S.: Thermotropic transitions in fluidity of bovine adrenocortical microsomal membrane and substrate–cytochrome *P*-450 binding reaction. *Biochim. Biophys. Acta* **487** (1977) 378–387.
16. Yang C. S.: The organization and interaction of monooxygenase enzymes in the microsomal membrane. *Life Sci.* **21** (1977) 1047–1058.
17. Stier S. and Sackman E.: Spin labels as enzyme substrates. Heterogenous lipid distribution in liver microsomal membranes. *Biochim. Biophys. Acta* **311** (1973) 400–408.
18. Gigon P. L., Gram T. E. and Gillette J. R.: Studies on the rate of reduction of hepatic microsomal cytochrome *P*-450 by reduced nicotinamide adenine dinucleotide phosphate: effect of drug substrates. *Molec. Pharmac.* **5** (1969) 109–122.
19. Taniguchi H., Imai Y. and Sato R.: Protein–protein and lipid–protein interactions in a reconstituted cytochrome *P*-450 dependent microsomal monooxygenase. *Biochemistry* **26** (1987) 7084–7090.
20. Muller-Enoch D., Churchill P., Fleischer S. and Guengerich F. P.: Interaction of liver microsomal cytochrome *P*-450 and NADPH–cytochrome *P*-450 reductase in the presence and absence of lipid. *J. Biol. Chem.* **259** (1984) 8174–8182.
21. Franklin M. R. and Estabrook R. W.: On the inhibitory action of mersalyl on microsomal drug oxidation: A rapid organization of the electron transport chain. *Archs Biochem. Biophys.* **143** (1971) 318–329.
22. Miwa G. T., West S. B., Huang M. and Lu A. Y. H.: Studies on the association of cytochrome *P*-450 and NADPH–cytochrome *c* reductase during catalysis in a reconstituted hydroxylating system. *J. Biol. Chem.* **254** (1979) 5695–5700.
23. Becker J. T., Meehan T. and Bartholomew J. C.: Fatty acid requirements and temperature dependence of monooxygenase activity in rat liver microsomes. *Biochim. Biophys. Acta* **512** (1978) 136–146.
24. Miwa G. T. and Lu A. Y. H.: The association of cytochrome *P*-450 and NADPH–cytochrome *P*-450 reductase in phospholipid membranes. *Archs Biochem. Biophys.* **234** (1984) 161–166.
25. Kominami S., Hara H., Ogishima T. and Takemori S.: Interaction between cytochrome *P*-450 (*P*-450C21) and NADPH–cytochrome *P*-450 reductase from adrenocortical microsomes in a reconstituted system. *J. Biol. Chem.* **259** (1984) 2991–2999.
26. Yanagibashi K. and Hall P. F.: Role of electron transport in the regulation of the lyase activity of C21 side-chain cleavage *P*-450 from porcine adrenal and testicular microsomes. *J. Biol. Chem.* **261** (1986) 8429–8433.
27. Stier A.: Lipid structure and drug metabolizing enzymes. *Biochem. Pharmac.* **25** (1976) 109–113.
28. Brandt I.: Tissue localization of polychlorinated biphenyls. Chemical structures related to pattern of distribution. *Acta Pharmac. Toxic.* **40** (Suppl. II) (1977) 1–108.
29. Biessmann A.: Effects of PCBs on gonads, sex hormone balance and reproduction processes of Japanese Quail *Coturnix coturnix japonica* after ingestion during sexual maturation. *Archs Environ. Contam. Toxic.* **10** (1982) 653–662.
30. Lawrence D. K. and Gill E. W.: Structurally specific effects of some steroid anesthetics on spin-labeled liposomes. *Molec. Pharmac.* **11** (1975) 280–286.
31. Cater B. R., Chapman D., Hawes S. M. and Saville J.: Lipid phase transitions and drug interactions. *Biochim. Biophys. Acta* **363** (1974) 54–69.