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CONSTRAINT ON THE SUBSTRATE CYTOCHROME *P*-450 BINDING REACTION IN BOVINE ADRENOCORTICAL MICROSOMES AT PHYSIOLOGICAL TEMPERATURE

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Summary

The addition of cholate to the microsomes at 37.5°C resulted in a striking decrease in the apparent substrate dissociation constant (K_s') and its temperature dependency. The microsomal membranes depleted of 80% of the lipids preserved the temperature dependency of the K_s and exhibited breaks in the Van't Hoff plot at the characteristic temperature of the lipids phase transition. The results indicate that the cytochrome *P*-450 is considerably restrained from expressing its maximum substrate binding potential at physiological temperature. In addition, the results indicate that the majority of the lipids apparently do not play a significant role in imposing constraint on the substrate-cytochrome *P*-450 binding reaction and in the temperature dependency of the K_s.

Introduction

Evidence suggesting that constraint on membrane-bound enzyme activity is an important factor for the control of membrane function, is slowly accumulating [1—3]. In vitro activation of membrane-bound enzymes and changes in the affinity of membrane components for their ligands by the presence of detergents has been reported in several instances [3,4]. As one of the possible interpretations of the phenomenon, Changeux [3] has suggested that the detergent releases a membrane constraint created either by membrane lipids or proteins or both which may stabilize the molecule in an alternative conformation. The concept of membrane lipids specifically restraining the maximum

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene, ANS, 1-anilinonaphthalene-6-sulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

catalytic activity of enzymes is an intriguing one with regard to the control of membrane function. The demonstration by Dodd [1] that cardiolipin reversibly inhibits isolated glutamate dehydrogenase suggests that lipid-dependent constraint on a membrane enzyme is feasible. The liver enzyme glucose-6-phosphatase and UDPglucuronyl transferase have been reported [5] to be specifically inhibited by phospholipids. In the case of guinea-pig liver microsomal glucuronyl transferase, the latency could be removed by mechanical treatment, freezing and thawing, phospholipase C treatment, all of which alter lipid-protein and lipid-lipid interactions [4]. On the other hand, phospholipase C treatment of hepatic microsomes resulted in the loss of binding of type I substrates to the cytochrome *P*-450 [6,7]. Furthermore, the addition of phospholipids to a detergent-solubilized and purified *P*-450 resulted in an increase in the affinity of the substrate for the cytochrome [8]. These results can be interpreted as indicating that the lipids are required for the binding of the lipid-soluble substrates to the cytochrome, not restraining the binding reaction. But treatment of membranes with lipid-solubilizing agents could make them develop an artificial need for phospholipids for their activity. Furthermore, Cater et al. [10] have reported that treatment of hepatic microsomes with cholate inhibited the hydroxylation reaction catalysed by hepatic microsomal *P*-450, while lecithin restored the activity. Therefore, solubilization of membranes with lipid-solubilizing agents can have opposing effects, i.e. destruction of latency of the enzyme, on the one hand, and inhibition of the enzyme owing to removal of lipids which may be required for the activity, on the other, complicating matters for quantitative evaluation of phospholipid dependency or constraint.

In bovine adrenocortical microsomes, a study of the effect of temperature on the substrate-cytochrome *P*-450 binding reaction and the membrane fluidity has shown that changes in the fluidity affect the affinity of the substrate to the cytochrome [11 *,12]. Although these results do not indicate whether or not there is an obligatory requirement for lipids in the binding reaction, they definitively indicate that the physical state of the membrane lipids is an important factor in determining the affinity of the substrate to the cytochrome.

In the present paper, results are presented which indicate that at physiological temperature, the membrane-bound cytochrome *P*-450 is considerably restrained from expressing its maximum potential substrate affinity. In addition, the results indicate that bulk lipids may not play a role in imposing the constraint.

Materials and Methods

Chemicals. Fatty acid-free bovine serum albumin; 17-α-hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione) and sodium cholate were purchased from Sigma Chemical Company, RNAase-free sucrose from Schwartz/Mann Company. The fluorescence probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-anilinonaphthalene-6-sulfonate (ANS) were purchased from Aldrich Company and K and K Labs, respectively.

* In Ref. 11, a mistake is to be pointed out. In Fig. 2A the y-axis should read $1/K_S \times 10^6$ M^{-1} instead of $1/K_S \times 10^7$ M^{-1}.

Preparation of the microsomal fraction. The procedure for preparation of the cortex tissue, homogenization of the tissue, isolation of the microsome fraction and further fractionation by a discontinuous sucrose density gradient (1.2, 0.9, and 0.4 M) centrifugation were exactly as previously described [12]. The band which was collected between 0.9 M and 1.2 M sucrose was used in the experiments reported, unless otherwise indicated.

Preparation of the lipid-depleted P-450-containing preparation. Aqueous suspension of microsomes were lyophylized and depleted of their lipids by extracting them with anhydrous *n*-butanol followed by anhydrous acetone, as previously described [13]. The lipid-depleted powder was suspended in 0.12 M buffered (0.0025 M Hepes, pH 7.4) sucrose by homogenizing. The suspension was centrifuged over 1.2 M sucrose. The supernatant was 1 : 1 diluted and again centrifuged at 75 000 × *g* for 1 h. The supernatant after the second centrifugation which contained 0.4 nmol of *P*-450 (-91 $cm^{-1} \cdot mM^{-1}$ for *P*-450 · CO) and 220 nmol of phosphorous/mg of protein was used in the experiment reported. The microsomes contain about 1000 nmol of phosphorous/mg of protein.

Extraction of lipids from the microsomal preparation. The procedure is essentially according to Folch et al. [14], exactly as described previously [11].

Determination of the apparent substrate dissociation constant K_s. The substrate-produced type I difference spectrum of cytochrome *P*-450 characterized by a trough at 421 nm, a peak at 388—390 nm and an isosbestic point at 407 nm was used as the criterion for the binding of the substrate to the cytochrome. The assay system and the procedure for equilibrating the assay system at different temperatures, titrating with the substrate by a semimicro technique, recording of the absorbancy difference ($\Delta A_{407-412nm}$) and calculation of the dissociation constant have been described previously in detail [11].

Measurement of fluidity of the microsomal preparations. Changes in fluidity of the microsomal preparations were measured by the technique of fluorescence polarization. The hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) [15,16] and the amphiphilic probe 1-anilinonaphthalene-6-sulfonate (ANS) [17] were used to detect fluidity changes. The polarization measurements were made at different temperatures using an Elscint fluorometer. This instrument is equipped to measure polarization directly with a high degree of precision. Temperature was controlled by means of a thermoelectric heating-cooling system.

In all experiments reported the temperature was measured with a copper-contant thermocouple attached to a digital readout with a readability of 0.1°C.

Fluorescence life time measurements. Fluorescence life times were measured on an Ortec Photon-counting Fluorescence life time instrument equipped with an air spark gap-type flash lamp as described by Vanderkooi et al. [18]. Corning glass filter with 350 nm band-pass was used for excitation and a 420 nm cut-off filter for emission. The decay of fluorescence was defined by 265 data points. Fluorescence life-times were calculated by a computor by fitting either a single or double exponential, as the case may be. The data were plotted on a Tetronix Plotter.

Analysis of the temperature profiles of fluorescence polarization and the substrate dissociation constant. When the data seemed to be describable by

more than one model based on visual examination, the best model was selected by a computer-assisted polynomial regression analysis of the data and comparison of the different models by the application of the '*F*' test (variance ratio test). In the segmented models described, when the break point between two segments could not be ascertained by the '*F*' test, the decision was made to include the last point at each end of a given segment only if the deviation of the point from the theoretical line through the remaining points in the segment was less than twice the standard error of the estimate.

Results

The effect of cholate on the substrate-cytochrome p-450 binding reaction in bovine adrenocortical microsomes

Section A of Fig. 1 (curves 1—3) shows reciprocal plots of the data obtained by titrating the cytochrome *P*-450 with the substrate 17-α-hydroxyprogesterone at the three different temperatures 18, 29 and 37.5°C. The linearity of the plots indicates that the substrate-cytochrome binding reaction apparently follows the classical Michaelis-Menten kinetics at all temperatures and the range of

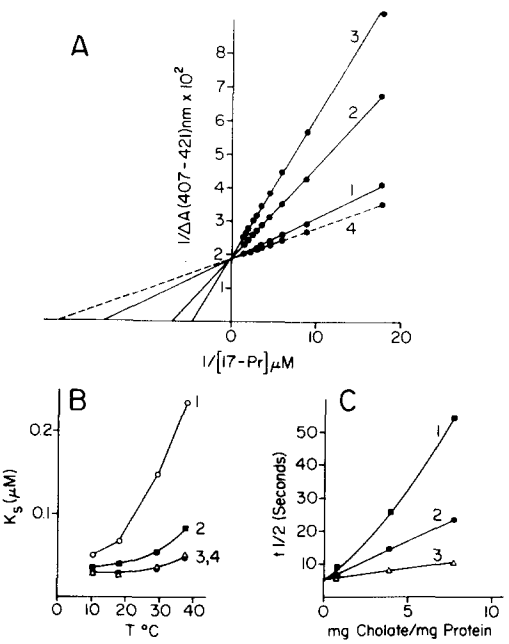

Fig. 1. The effect of cholate on the substrate-cytochrome *P*-450 binding reaction. The assay system (3.0 ml) consisted of 1.5% fatty acid-free bovine serum albumin containing glycylglycine buffer (pH 7.4; 0.25 M RNAase-free sucrose and 0.62 mg of the microsomal protein). The K_S was determined by the semimicro titration technique as described under Materials and Methods. (A) Typical Lineweaver-Burk plots of the titration data. Curves 1—3 at 18, 29 and 37.5°C, respectively. Curve 4 at 37.5°C in the presence of 3.8 mg of cholate/mg of the microsomal protein. The lines were calculated by the least-square method. (B) The effect of cholate on the temperature dependency of the K_S. Curve 1 in the absence, and curves 2—4 in the presence of 0.73, 3.8 and 7.7 mg of cholate/mg of protein. (C) The effect of cholate on the rate of development of the substrate-produced spectral change. The time required for the development of half-maximum spectral change ($T_{1/2}$) is plotted as a function of cholate concentration. The concentration of the substrate was 0.0311 μM.

substrate concentration tested (0.02—0.6 μM). As previously reported [11,12], the K_s increased, with increase in temperature as indicated by the decrease in the intercept on the x-axis. The y-intercept for all the three curves remain the same, indicating that the temperature has no detectable effect on the maximum absorbancy change ($\Delta A_{407-421nm}$) which represents maximum binding. The addition of the ionic detergent cholate at 37.5°C (curve 4) strikingly decreased the K_s without altering the maximum binding. The addition of the lipids extracted from the microsomes at a detergent to lipid ratio of 0.8 (mg/mg) resulted in 60% reversal of the activating effect of the detergent (not shown in the figure). These results indicate the absence of irreversible denaturation of the enzyme by the detergent.

Section B of Fig. 1 shows the effect of different concentrations of the detergent cholate on the temperature dependency of the dissociation constant. The 'K_s' S' obtained from such plots as shown in section A are plotted as a function of temperature. Curve 1 was obtained in the absence of cholate. The addition of the detergent (0.73 mg/mg protein, curve 2) at 37.5°C resulted in a nearly three-fold decrease in the K_s from 0.235 to 0.083 μM. At the higher concentration (3.8 mg/mg protein, curve 3) of the detergent, the decrease was nearly five-fold from 0.235 to 0.05 μM. Further increase in the detergent concentration (7.7 mg/mg protein, curve 4) was without further effect. The concentration of the detergent required for half-maximum decrease in the K_s was approximately 0.2 mg/mg of the microsomal protein (Fig. 3, section B; curve 1). The lowest value obtained for the K_s at 37.5°C (0.05 μM) is very close to that obtained at 10°C in the absence of the detergent. In addition to decreasing the K_s the detergent largely eliminated the temperature dependency, as indicated by a much smaller increase in the dissociation constant from 0.03 μM at 10°C to 0.05 μM at 37.5°C. This decrease in temperature dependency represents a decrease in the overall enthalpy between 10 and 37.5°C from 9.9 kcal · mol^{-1} in the absence of the detergent to 3.2 kcal · mol^{-1} in its presence. At 37.5°C the free energy increased from 9 kcal · mol^{-1} to 11 kcal · mol^{-1}.

The detergent while decreasing the substrate dissociation constant increased the time required for spectral change to occur upon addition of the substrate (Fig. 1, section C). The figure shows the time required for half-maximum spectral change ($t_{1/2}$) upon addition of a given concentration of the substrate, as a function of cholate concentration at the three temperatures 18, 29 and 37.5°C. This effect of the detergent decreased with increase in temperature, with very little effect remaining at 37.5°C.

The effect of depleting the microsomes of the lipids on the temperature dependency of the substrate dissociation constant

Since the lipid-perturbing agent cholate largely eliminated the temperature dependency of the substrate dissociation constant, it was of interest to find out the effect of removing the microsomal lipids in the absence of detergents on the temperature dependency. Fig. 2 shows the Van't Hoff plot of the substrate dissociation constant ($1/K_s$) in the P-450-containing preparation which is 78% depleted of the microsomal lipids/mg of protein. The K_s was determined at temperature intervals of 2—3°C. Similarly to the case of the microsomes, the

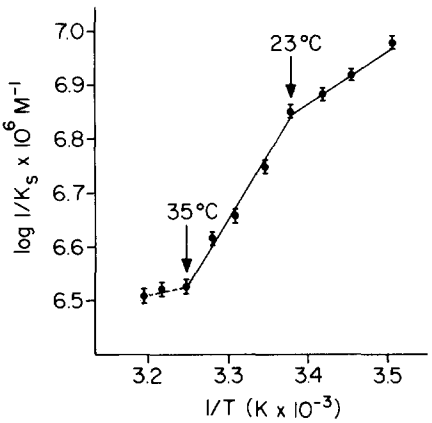

Fig. 2. Van' Hoff plot of the substrate association constant in the lipid-depleted *P*-450 preparation. The assay system was composed of 2.0 ml of the albumin-containing glycylglycine buffer (pH 7.4) described previously [11] and 1.0 ml (1.5 mg protein) of the lipid-depleted preparation. K_s was determined as previously described [11]. The lines were calculated by least-square method. Statistical analysis of the data which lead to the segmented model shown is described in the text. The bars represent standard error of determination of the K_s calculated from five identical titrations at a single temperature (18°C) and expressed as percent of the mean.

K_s increased with increase in temperature. Analysis of the data as described in Materials and Methods indicated that in the segmented model shown in Fig. 2, N (number of points) = 3 and 5 for segments I and II is a reliable description of the first eight points (from $1/T(K) \times 10^{-3}$ = 3.506—3.249) with a definite break close to 23°C. Attempts to find either polynomial or segmented models to include the remaining two points (T(K) = 3.219 and 3.197) were not successfull. However, the present analysis suggests the possibility of a sudden change in the slope of the line close to 35°C. Although more data points are necessary to be certain of the exact position and the number of breaks, it is interesting that the two breaks (23°C and 35°C) suggested by the statistical analysis of the present data are reasonably close to the two characteristic temperatures (35°C and 23—25°C) of the lipid phase transition in the lipid-depleted preparation (Fig. 4, section B). The possible existence of a third break corresponding to the characteristic temperature of the lipid phase transition around 18°C cannot be eliminated.

The K_s values in the depleted preparation increased from 0.1 μM to 0.31 μM by increasing the temperature from 12 to 37.5°C where as in the microsomes, it increased from 0.058 μM to 0.24 μM. Whether or not the higher values in the depleted preparation is due to lipid depletion or due to some effects of organic solvent treatment of the microsomes is not clear. However, it is interesting that smaller increase in the depleted preparation (3.1-fold as opposed to 4.2-fold in the microsomes) is consistent with the smaller decrease in the fluorescence polarization of DPH. In the depleted preparation the polarization decreased from 0.36 at 12°C to 0.29 at 37.5°C as opposed to 0.267—0.165 in the microsomes (Fig. 4, section A).

It should be pointed out that the maximum amount of lipids that could be extracted by the present extraction procedure is about 80% of the total lipids.

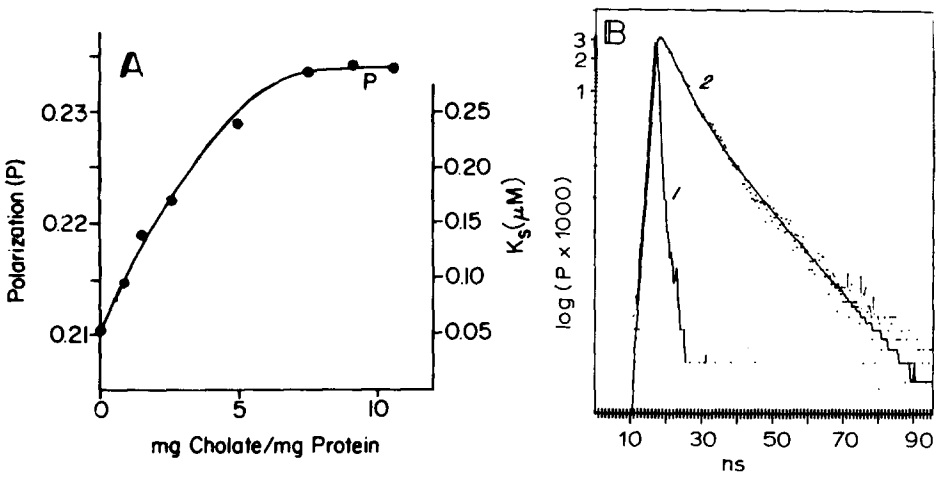

Fig. 3. The effect of cholate on ANS fluorescence in the microsomal membranes. (A) On the steady-state fluorescence polarization. The reaction medium consisted of 3.0 ml of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.5 mg of the microsomal protein and 10^{-5} M ANS. The temperature was 37.5°C. (B) Decay of ANS fluorescence at the temperature of the cuvette chamber. Reaction medium as in (A). Curve 1, ·····, lamp flash; curve 2, ·····, experimental points. ———, computer fit.

This figure is within the range of values (75—90%) reported as bulk lipids in several biological membranes [19]. In addition, it is reported that those lipids which interact with the membrane proteins are mainly acidic phospholipids [19]. Accordingly, acidic phospholipids phosphatidylserine plus phosphatidylinositol account for 52% of the total lipids in the depleted preparation as opposed to 14.9% in the original microsomes [20]. Therefore, it is considered that the residual lipids in the depleted microsomes are strikingly enriched by the boundary lipids.

The effect of cholate on the fluidity of the microsomal membranes

The increase in the affinity of the substrate to the cytochrome *P*-450 with decrease in temperature is related to the decrease in the fluidity of the microsomal membranes [11,12]. Since addition of cholate to the microsomes at the higher temperatures results in an increase in the affinity, the effect of the detergent on the fluidity of the membranes was examined. Since the affinity of the substrate increases with decrease in temperature and the uptake of steroids by lipids is drastically reduced below the phase transition temperatures [33], it is assumed that the substrate-cytochrome binding may be influenced by more peripheral regions of the bilayer. Therefore, a fluorescent probe which is supposed to report on the more peripheral region was selected. The probe 1-anilino-8-naphthalene sulfonate (ANS) is considered to occupy the head group region of the bilayer, the non-polar fluorescent group penetrating to only a short distance between the fatty acid chains [21,22]. In addition fluorescence polarization of ANS has been shown to be sensitive to dipalmitoyl phosphatidylcholine phase transition [23,24] indicating that this probe can detect fluidity changes. In the adrenal microsomes, the steady-state fluorescence

polarization increased from 0.19 at 40°C to 0.24 at 10°C. The addition of cholate to the microsomes resulted in a significant increase (12—17%) in the polarization (Fig. 3A). The polarization remained unaltered upon dilution of the labelled microsome/buffer mixture regardless of whether the detergent is present or not. This indicates the absence of detectable interference from possible changes in the scatter. The fluorescence enhancement of ANS in cholate/buffer mixture was less than 5% of that in the microsomal membranes. In addition the small enhancement remained unaltered upon increasing the concentration of the detergent. Therefore, any contribution from ANS-cholate interaction to the observed increase in the polarization is considered insignificant. The concentration of the detergent required for half-maximal increase in the polarization was approximately 2.5 mg/mg protein which is ten times that required for half-maximum decrease in the substrate dissociation constant.

Since steady-state measurements result in bulk average parameter and ANS is known to bind to lipids as well as proteins, it was necessary to resolve the two types of binding. Therefore, fluorescence life time measurements were made. Fig. 3B shows fluorescence decay of ANS in the microsomal membranes following a nanosecond light pulse, as function of time. The data could be described by a double exponential (solid line, Fig. 3B) indicating the existence of at least two different species of ANS. The two species are characterized by emission life times of 4.67 and 11.43 ns. In the lipids extracted from the microsomes, fluorescence of ANS exhibits a single exponential decay with a life time of 4.77 ns. This value is nearly equal to the life time of the short life time species in the microsomal membranes. The existence of two life times as well as the correspondence of the short life time to that in the lipids extracted from the microsomes is very similar to that reported for ANS in erythrocyte and mitochondrial membranes [23]. While the short life time species may represent the lipid phase in these membranes, the long life time species has been argued to be due to ANS binding to proteins at the 'region where proteins and lipids interact'. The addition of cholate to the microsomes, while decreasing the lifetime (from 4.67 to 3.23 ns) of the short life time species, was without effect on the long life time species. However, the experimental points deviated from the double exponential fit toward the end of the decy curve indicating that treatment of the microsomes with the detergent could have resulted in development of a new binding site for ANS.

Fluidity of the lipid-depleted preparation

Fig. 4A shows fluorescence polarization of the hydrophobic probe DPH as a function of temperature in the microsomes (dots) and lipid-depleted *P*-450 preparation (circles). Increase in temperature from 12°C to 37.5°C resulted in a decrease in polarization of DPH from 0.265 to 0.162 in the microsomes and from 0.365 to 0.295 in the lipid-depleted preparation. The higher values in the case of the depleted preparation indicates that the residual lipids are more immobilized than in the microsomes. This would be expected if the residual lipids are mainly those which are in close association with the protein. However, they are not completely immobilized as indicated by the detectable lipid phase transition (Fig. 4B). The figure shows the increase in fluorescence polarization of the hydrophobic probe DPH in the lipid-depleted preparation as

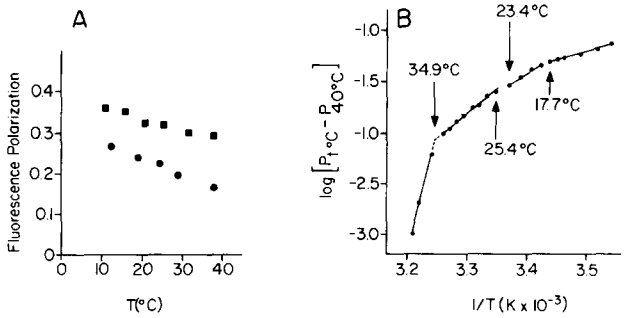

Fig. 4. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in the microsomal preparations. 3 ml of the assay system consisted of 0.03 M Tris buffer (pH 7.4) plus either the lipid-depleted *P*-450 preparation (2.5 mg protein) or the microsomes (1.5 mg protein). The procedure is as described under Materials and Methods. (A) Comparison of the lipid-depleted *P*-450 preparation (■) and the microsomes (●). (B) Lipid phase transitions in the lipid-depleted *P*-450 preparation. In order to accentuate the characteristic temperatures, the difference between polarization at a given temperature and that at 40°C ($P_t - P_{40°C}$) has been used. Statistical analysis of the data which lead to the above segmented model is described in the text.

the temperature was decreased from 40°C. The results of statistical analysis of the data which lead to the segmented model shown in the figure are as follows: the two segmented models $N = 3$, 8, 4, and 6 (Fig. 4B) and $N = 3$, 7, 5 and 6 for segments I, II, III and IV (figure), respectively, were found to be far better than the best polynomial (6th degree) to describe the data. This was indicated by the high 'F' values of 43 and 40 for the two segmented models versus the polynomial, respectively. These values are considerably higher than the theoreticaly value of 9.07 for 99% level of certainty. Similar application of the 'F' test to compare the two segmented models indicated that the difference between the two models was insignificant. However, it was found that the deviation of the 8th point in segment II (Fig. 4B) from the theoretical line through the remaining points in each segment, was 6.2 times the standard error of estimate when included in segment III and was 1.8 times when included in segment II. Therefore, $N = 8$ and 4 were assigned for segments II and III. By similar statistical reasoning, segments I, III and IV were drawn. This model indicates a break in the curve at 35°C, a second break between 23°C and 25°C and a third around 18°C.

Discussion

As suggested by Changeux [3] in the case of nicotinic receptors, the activating effect of cholate on the steroid-cytochrome *P*-450 binding reaction can be interpreted as the detergent releasing a constraint on the cytochrome caused either by the membrane lipids or proteins or both.

A precise description of the mechanism of constraint or release of the constraint by the detergent is difficult at the present time. However, the results can be discussed in the light of the present knowledge of structure-function relationships in biological membranes. Evidence gained in the last few years has shown that physical state of the lipids has marked effects on the activity of

membrane-bound proteins [21—23]. Studies with bacterial membranes have provided reasonable direct evidence of coupling between certain membrane systems (transport systems) and the degree of order or fluidity in the membrane lipids [22,23]. It is suggested that conformational transitions in the membrane proteins are mediated through lipid phase transitions. In the present study, the striking decrease in the apparent substrate dissociation constant (K_s) upon addition of cholate to the microsomes at 37.5°C indicates that a physiological temperature the membrane-bound cytochrome *P*-450 is considerably restrained from expressing its maximum substrate binding potential. In the presence of the detergent, the increase in K_s caused by the addition of the lipids extracted from the microsomes, indicates that the effect of the detergent is reversible and that changes in lipids are probably involved in th mechanism of action of the detergent.

According to the fluid mosaic model of Singer and Nicholson [18] for membrane structure, a small portion of the phospholipids is thought to specifically interact with the proteins. This specific interaction results in appropriate conformation for the membrane-bound enzymes enabling them to interact with their substrates and other ligands. In the microsomal membranes, the substrate dissociation constant (K_s) for cytochrome *P*-450 undergoes abrupt changes at the characteristic temperatures of the lipid phase transition [11,12]. It is reported [28] that at these temperatures lipid-protein interactions are maximally affected. Therefore, although no direct evidence is as yet available, it is reasonable to assume that protein conformation change mediated by the lipid phase transition is responsible for the observed changes in the K_s. Then the effects of cholate on the K_s may be interpreted as follows: since the lowest value for K_s (0.05 μM) at 37.5°C attained in the presence of the detergent is very close to that obtained by decreasing the temperature to 10°C in the absence of the detergent, it is possible that the protein conformation at 37.5°C in the presence of the detergent is similar to that at 10°C in its absence. However, the presence of the detergent largely eliminated the temperature dependency of the K_s indicating that the detergent has caused profound changes in the components of the proteolipid systems which are involved in the substrate-cytochrome *P*-450 binding reaction.

While the addition of cholate to the microsomes strikingly decreased the K_s and its temperature dependency, removal of 80% of the microsomal lipids had little effect on the K_s and its temperature dependency. In the depleted preparation, increasing the temperature from 12 to 37.5°C resulted in a 3.1-fold increase in the K_s (0.1—0.31 μM) whereas in the microsomes the increase was 4.2-fold (from 0.058 to 0.24 μM). The smaller increase in the K_s in the depleted preparation is consistent with the smaller decrease in fluidity as indicated by the smaller decrease in the fluorescence polarization of DPH (from 0.36 to 0.29), than that in the microsomes (from 0.267 to 0.165). Although the depleted preparation appears to be less fluid as indicated by the higher polarization, the residual lipids are capable of undergoing functionally related phase transitions. This is indicated by the temperatures at which breaks occurred in the Van't Hoff's plot of the K_s being very close to the characteristic temperatures of the lipid phase transition as detected by the fluidity probe DPH. Assuming that the lipid-depleted preparation is considerably enriched in

the boundary lipids, these results can be interpreted as the boundary lipids playing a significant role in determining the affinity of the substrate to the cytochrome *P*-450. Similarly close association of the protein with the lipids may also be necesary for rapid conformational changes in the cytochrome upon binding to its substrate as indicated by the striking decrease in the rate of type I spectral change by the presence of the detergent. That the substrate-produced type I spectral change is associated with conformation change in the cytochrome has been demonstrated in the case of hepatic microsomal *P*-450 [29] and the purified cytochrome from *Pseudomonas putida* [29]. In hepatic microsomes, it is suggested that phospholipids are requirred for the cytochrome to assume active conformation.

Examination of the effect of cholate on the membrane structure by fluorescence probe analysis using the probe ANS indicated that the detergent increases the fluidity of the lipid phase. This is evidenced by the decrease in the life time (from 4.67 to 3.23 ns) of the short life time species of ANS in the microsomal membranes. That this species in the microsomes represents the lipid phase is indicated by the life time (4.77 ns) being very close to that in the lipids extracted from the microsomes. Assuming (for reasons discussed in Results) that the long life time species (11.43 ns) represents the probe binding to protein or to the 'region where proteins and lipids interact' the lack of effect of the detergent on this species may indicate that the motional characteristics around the probe is the same before and after treatment with the detergent. The increase in the steady-state fluorescence polarization of ANS while increasing the fluidity of the lipids phase would be consistent if the detergent has caused conformational change in the probe-bound protein. The detergent could cause conformational change either directly interacting with the protein or fluidizing the lipids around it which could decrease lipid-protein interaction stripping the lipids from the proteins. That cholate is capable of stripping the lipids from proteins has been reported in the case of Ca^{2+} transport ATPase.

The results of present experiments indicate that at physiologic temperature, the membrane-bound cytochrome *P*-450 is considerably restrained from expressing its maximum substrate binding potential. In addition, although the results do not shed light on the precise mechanism whereby cholate releases the constraint, they indicate that the detergent has fluidizing effect on the lipid phase and plausibly resulting in conformation change in the microsomal proteins. The present result that the ionic detergent decreases the K_s and the previous result (Ref. 11 *) that the non-ionic detergent increases the K_s indicates that the substrate affinity can be either increased or decreased by way of lipid perturbation. One possible mechanism for the perturbations in the lipid phase to be transmitted to the protein would be by way of lipid-protein interactions. The possibility that the substrate affinity can be altered at constant temperature offers a mechanism whereby cortexolone (the hydroxylated product) can be restored to optimum levels by increasing the substrate affinity under conditions in which the availability of 17-hydroxyprogesterone is low and by decreasing the affinity when the substrate concentration is high.

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* See footnote on p. 458.

Acknowledgements

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