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SPIN LABELS AS ENZYME SUBSTRATES

HETEROGENEOUS LIPID DISTRIBUTION IN LIVER MICROSOMAL MEMBRANES

A. STIER and E. SACKMANN

Department of Spectroscopy, Max-Planck-Institute of Biophysical Chemistry, Göttingen (Germany)

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SUMMARY

Phenobarbital-stimulated microsomal membranes of rabbit liver, containing the cytochrome P450–cytochrome P450 reductase hydroxylating enzyme system in high concentration, have been studied with a version of the spin label technique which uses nitroxide radicals as enzyme substrates. The reduction kinetics of a phosphate ester of tetramethylpiperidine nitroxide (TEMPO-phosphate) and of stearic acid nitroxide by the cytochrome P450 reductase has been studied as a function of the temperature. The Arrhenius plot of the reduction rate constants reveals a striking difference in the behaviour of the water-soluble TEMPO-phosphate label and the lipid-soluble fatty acid label: The activation energy of the fatty acid reduction decreases abruptly at about 32 °C from a value of 30.8 kcal/mole to a value of 8.7 kcal/mole, whereas no such break is observed in the Arrhenius plot of the TEMPO-phosphate reduction which yields a value of the activation energy of $\Delta W = 13.8$ kcal/mole in the whole temperature range investigated. Our results clearly indicate the existence of a mosaic-like structure of the membrane with the whole enzyme system being enclosed by a rather rigid phospholipid halo which is in a quasicrystalline structure below 32 °C and undergoes a crystalline–liquid crystalline phase transition at 32 °C, while the bulk lipid of the membrane is in a rather fluid state as reflected by the measured high diffusion coefficient of $D_{diff} = 11.0 \cdot 10^{-8}$ cm^2/s at 30 °C and low activation energy of diffusion of $\Delta W = 3.85$ kcal/mole of a fatty acid spin label incorporated in the membrane.

INTRODUCTION

The spin label technique applied to problems of biological membranes can in principle give information on (1) the molecular architecture and the dynamical behaviour of the membrane and (2) the chemical reactivity of the binding sites of the radicals. Nearly all spin label studies take advantage of the first possibility of information, while dealing with the question whether the lipid moiety is arranged in a bilayer

Abbreviations: TEMPO, tetramethylpiperidine nitroxide; TEMPO-phosphate, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl dihydrogenphosphate; DMSO, dimethylsulphoxide.

structure and with the evaluation of the average fluidity of the bulk of the lipid matrix (*cf.* Seelig *et al.*[1], Rottem *et al.*[2], Eletr *et al.*[3] and Jost *et al.*[4]). One of the main difficulties in the inerpretation of the results as well as one of the most interesting properties of biological membranes is a possible heterogeneous distribution of the different phospholipids throughout the membrane (lipid mosaicism; *cf.* Siekevitz[5]). This difficulty can be circumvented if biological membranes containing a definite lipid in high abundance are investigated as, for instance, membranes from mutants of *E. coli* bacteria[6]. On the other hand it is not possible as yet to obtain direct information about a possible heterogeneous lipid distribution from the observed ESR spectra.

The primary goal of this paper is to show that by using nitroxide spin label molecules as substrates it is possible to study the lipid environment of enzymes embedded within the phospholipid matrix of the membrane. As an example we studied rabbit liver microsomal membranes containing an enzyme system capable of biotransforming drugs and other chemicals. This enzyme system consists of cytochrome P450 and cytochrome P450 reductase present in a molar ratio of about 12:1 (Estabrook *et al.*[7]). It has been shown recently that this enzyme system is involved in the oxidation of the secondary amine tetramethylpiperidine to the tetramethylpiperidine nitroxide (TEMPO) radical as well as in the NADPH-dependent reduction of the TEMPO radical (Stier *et al.*[8]).

By comparing the temperature dependencies of the reduction rate of a lipophilic nitroxide radical which approaches the enzyme system by lateral diffusion within the lipid moiety of the membrane and of the reduction rate of a water soluble nitroxide, information on the physical state of the local lipid environment of the reducing enzyme can be obtained.

MATERIALS AND METHODS

Liver microsomes of rabbits pretreated with sodium phenobarbital (1 $^0/_{00}$ in drinking water) for 1 week have been prepared using standard procedures[9]. The microsomes have been washed prior to use by recentrifugation in a 0.1 M K_2HPO_4 buffer of pH 7.4 containing KCl in a final concentration of 0.05 M (KCl–phosphate buffer). 4-Hydroxy-2,2,6,6-tetramethylpiperidinooxyl dihydrogenphosphate (TEMPO-phosphate, Syva, Palo Alto, Calif., U.S.A.) was used as hydrophilic spin label and *N*-oxyl-4,4-dimethyloxazolidine derivatives of stearic acid (Syva, Palo Alto, Calif., U.S.A of the general formula I(*m*,*n*) (*cf.* Fig. 2) were used as lipophilic nitroxide radicals with I(12,3) for the measurements of the enzymic reduction and of the fluidity of the membrane and with I(1,14) for the measurement of diffusion in the membrane. The incorporation of the fatty acid labels into the microsomal membranes was achieved as follows: An appropriate amount of the label, dissolved in dimethylsulfoxide (final concentration of DMSO in the suspension 1:100, by vol.) was added to the microsomal suspension during a one second pulse of ultrasonic irradiation. The samples were then slowly rotated at 37 °C under nitrogen for 60 min. The TEMPO-phosphate label was simply added as an aqueous solution to the microsomal suspension.

The temperature dependencies of the reduction rates have been determined as follows: To the fatty acid label containing microsomal preparation NADPH (Boehringer, Mannheim, Germany) was added at low temperature (approx. 4 °C) where no appreciable reduction occurred. The samples were then heated within several seconds

to the measuring temperature where the decay of the central ESR line was recorded. In the case of the microsomal suspensions containing TEMPO-phosphate a mixing chamber attached to the cavity was used for the rapid admixture of NADPH.

The lateral diffusion rate of the fatty acid label parallel to the membrane surface has been determined from the spin exchange interaction at high label concentrations as described by Sackmann *et al.*[10] and Träuble and Sackmann[11].

ESR measurements were performed using a Varian 4502 spectrometer with a klystron as the source of microwave radiation operating at 9.5 MHz. The microwave frequency was monitored with a Hewlett–Packard 5245/L 2590 A frequency counter and the static magnetic field $\boldsymbol{H}_0$ was measured using an AEG gauss meter.

Protein was determined by the method of Lowry *et al.*[12].

RESULTS

(*1*) *Fluidity of the membrane*

Fig. 1 shows the first derivative ESR spectra of the stearic acid label I(12,3). The spectra are typically composed of an outer pair of lines separated by $2T'_{\parallel}$ and an inner pair of lines separated by $2T'_{\perp}$ and a central line in the whole temperature range between 4 and 47.5 °C. This line shape characteristic for a radical performing a rapid anisotropic motion (Hubbell *et al.*[13]) indicates that the radicals are incorporated in a lipid bilayer membrane. The intensity of the spectrum increases steadily with the temperature indicating a smooth increase of the fluidity of the bulk phospholipid matrix. This is most clearly seen from the temperature plot of the so-called order parameter S (ref. 13) of the fatty acid label which is defined by the equation

$$S = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - T_{xx}} \tag{1}$$

where T_{zz} (30.8 gauss) and T_{xx} (5.8 gauss) are the maximum and the minimum possible splittings of the outer and the inner pair of lines, respectively. The order parameter S represents a qualitative measure for the fluidity of the hydrocarbon region of the phospholipid matrix. Fig. 1 (*cf.* insert) therefore demonstrates the absence of a crystalline liquid phase transition in the bulk of the membrane lipid matrix.

(*2*) *Reduction kinetics*

The effect of the temperature on the reduction of the membrane-bound fatty acid radical I(12,3) by the cytochrome P450 reductase is most clearly seen when the ESR signal height is recorded while the temperature is continuously raised within 10 min from 5 to 45 °C. Without NADPH the signal increases smoothly with increasing temperature in accordance with the results of Fig. 1. In contrast to this behaviour the signal intensity decreases rapidly in the presence of NADPH at temperatures above 30 °C due to the rapid reduction of the nitroxide groups. At temperatures below 30 °C the shapes of the two curves differ only slightly indicating that the reduction rate is very slow.

The quantitative evaluation of the ESR signal decay at different constant temperatures shows that I(12,3) and TEMPO-phosphate are reduced in the initial reaction phase with a constant rate v according to zero-order kinetics at enzyme

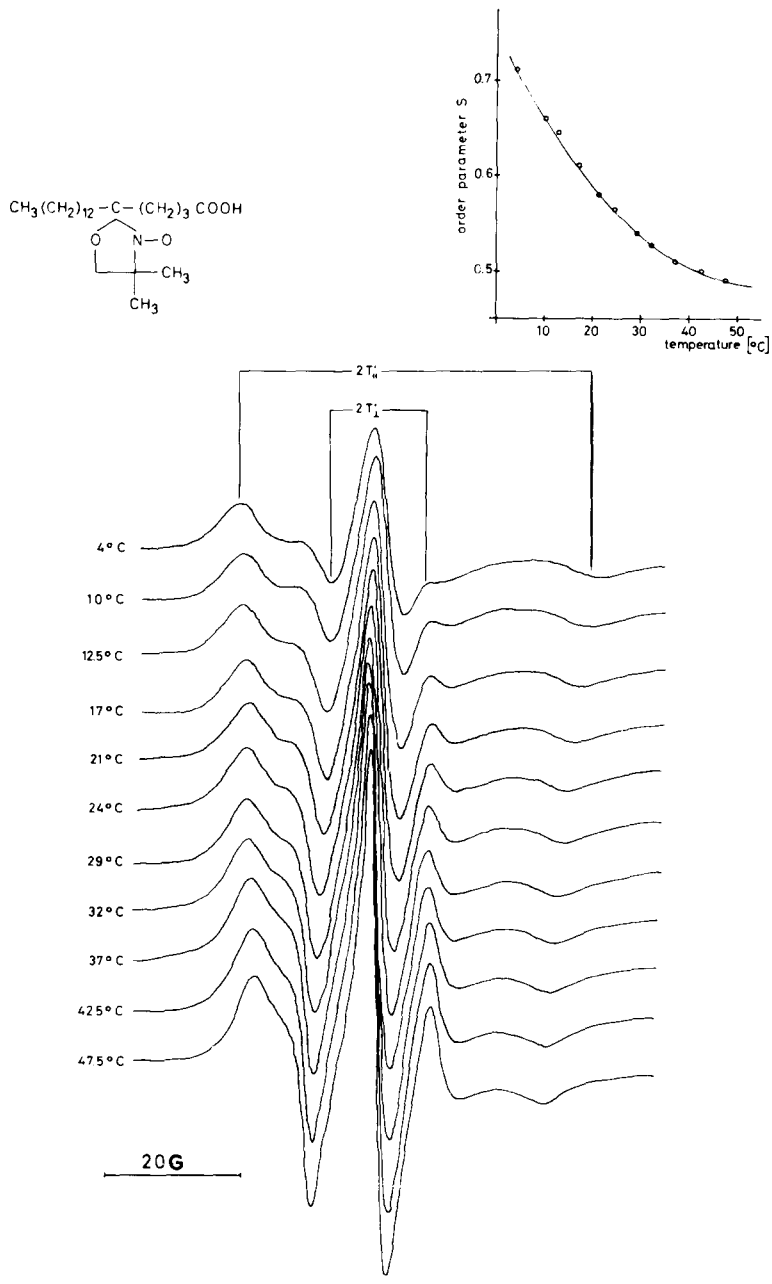

Fig. 1. Temperature dependence of the first derivative ESR spectrum of fatty acid label (I (12,3)) incorporated in microsomal vesicles (I(12,3), 3.8 mM, 42 mg/ml protein, 0.05 M sodium phosphate buffer pH 7.4). Insert: Temperature dependence of the so-called order parameter S (*cf.* Eqn 1). Note the smooth decrease of the order parameter with increasing temperature.

saturation. At 10 °C the reduction of the fatty acid label seems to be "frozen-in" as its reduction proceeds at a very low rate about 10 times more slowly than the reduction of the TEMPO-phosphate label. At 35 °C both reduction rates are similar.

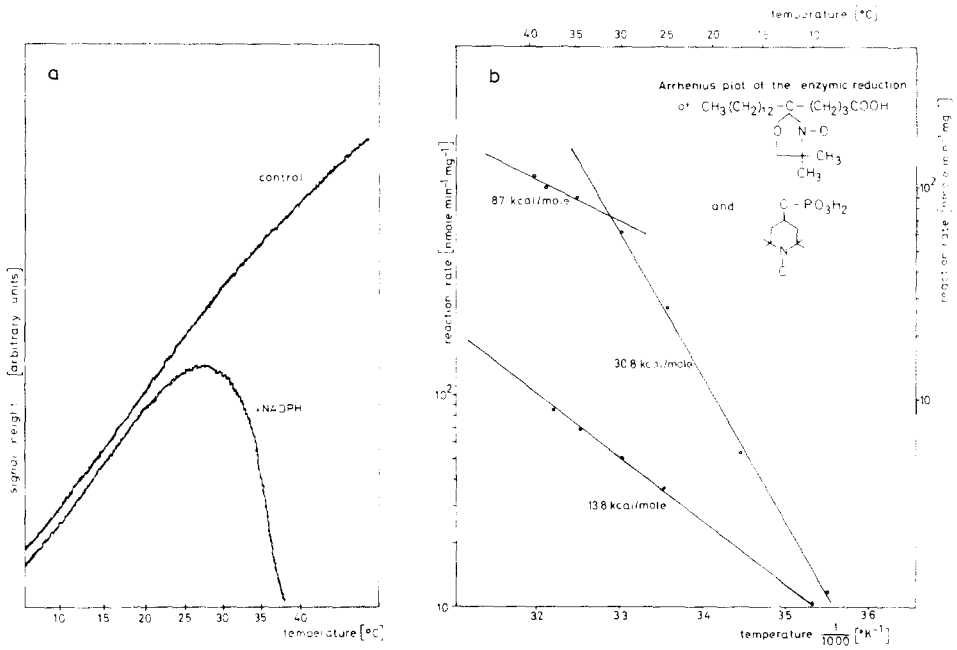

Fig. 2. Reduction of the spin labels by microsomal suspensions in the presence of NADPH. (a) The paramagnetic resonance intensity of the fatty acid label I(12,3) (3.8 mM) is recorded as a function of the temperature in the presence and in the absence of NADPH (6 mM) in the suspension (42 mg/ml protein, 0.05 M sodium phosphate buffer, pH 7.4). The rapid reduction of the nitroxide radical at temperatures above 30 °C in the presence of NADPH is clearly visible. While recording these curves the temperature has been swept with a speed of 4 °C/min. (b) Arrhenius plot of the initial reduction rates for the water-soluble TEMPO-phosphate label (●, ordinate scale on the left side, 1 mM TEMPO-phosphate, 3 mM NADPH, 8 mg/ml protein, 0.05 M sodium phosphate buffer, pH 7.4) and for the lipid-soluble fatty acid label I(12,3) (○, ordinate scale on the right side, experimental values as given under (a)), respectively. The rates for the reduction at the different temperatures are referred to the intensity of the central line of the spectrum at these different temperatures as the signal intensities rise with temperature due to the increasing mobility of the spin labels.

Fig. 2b shows the Arrhenius plot of the reduction rates of the spin labels TEMPO-phosphate and I(12,3), respectively. This figure reveals a striking difference in the temperature dependencies of the reduction rates of these two label molecules: The activation energy for the fatty acid label decreases abruptly from a rather high value of 30.8 kcal/mole at temperatures below 32 °C to a value of 8.7 kcal/mole above 32 °C. In contrast to this behaviour the activation energy for the TEMPO-phosphate reduction assumes a constant value of 13.8 kcal/mole in the whole temperature range. In other experiments of microsomal reduction of TEMPO-phosphate in which the same experimental conditions were applied as in the experiments with the fatty acid label I(12,3) (high protein content, preincubation at 37 °C in the

presence of DMSO, *cf.* Methods) an activation energy of 15.5 kcal/mole was found which was constant in the whole temperature range.

(3) Lateral diffusion

The lateral diffusion of the stearic acid label I(1,14), carrying the nitroxide group at the apolar end, within the microsomal membranes can be determined from the concentration dependence of the spin exchange frequency at high label concentrations (molar ratio label to lipid larger than $c=0.03$)[10]. The spin exchange frequency is derived from the line width of the exchange broadened central line of ESR spectra. In Fig. 3 we plotted the exchange frequency as function of the ratio $c/(1+c)$, where c is the label to lipid molar ratio. Straight lines are obtained for all temperatures studied

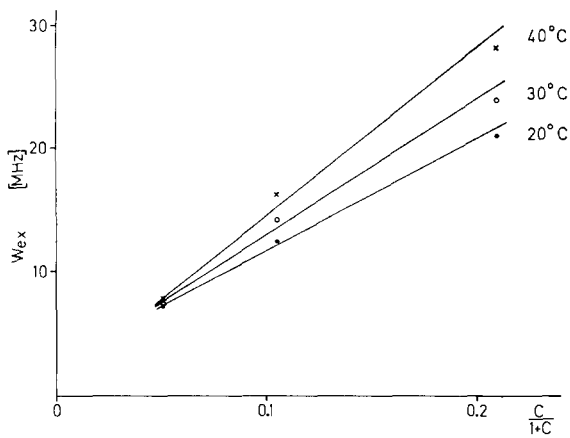

Fig. 3. Concentration dependence of the exchange frequency W_{ex} of the fatty acid nitroxide label I(1,14) in microsomal membrane suspensions (KCl–phosphate buffer, 16 mg/ml protein). c is the label to lipid molar ratio. In the case of $c>0.1$ the spectra have been corrected against the dipole–dipole broadening. The slopes of the straight lines yield the following values for the lateral diffusion coefficients with the help of Eqn 2; temp. = 20 °C, $D_{diff}=9.5\cdot10^{-8}$ cm²/s; temp. = 30 °C, $D_{diff}=11.0\cdot10^{-8}$ cm²/s; temp. = 40 °C, $D_{diff}=13.7\cdot10^{-8}$ cm²/s.

in accordance with the diffusion model[11]. Therefore, the fluidity of the bulk of the lipid matrix is high enough (1) to allow for a homogeneous distribution of the label molecules within the lipid matrix and (2) to permit a rapid lateral diffusion of the fatty acid molecules and of the phospholipids. It has been shown recently that the following relation holds between the lateral diffusion coefficient D_{diff} of a spin label and the spin exchange rate per second W_{ex} according to the diffusion model (Träuble *et al.*[11])

$$W_{ex}=\frac{4d_cD_{diff}}{3F\lambda}\frac{c}{1+c} \tag{2}$$

In this equation d_c is the critical interaction distance for the onset of the exchange interaction and F is the area occupied by one lipid molecule. λ is the length of one jump of the diffusing particles which is determined by the average distance of the lipid molecules. These characteristic parameters can not depend strongly on the nature of the lipid molecules and of the spin label and it is therefore reasonable to

assume that the characteristic values for the synthetic dipalmitoyllecithin matrix can also be used for microsomal membranes, *i.e.* we assume the values $F=58$ Å^2, $\lambda=8$ Å and $d_c=20$ Å. Using these values and identifying the experimental slopes of the straight lines in Fig. 3 with the coefficient $4\ d_c\ D_{diff}/3\ F$ in the above equation we obtained the values of the diffusion coefficient given in the text of Fig. 3. The temperature dependence of the diffusion coefficient yields a value of ΔW approx. 3.85 kcal/mole for the activation energy of the diffusion.

DISCUSSION

The ESR spectra in Fig. 1 demonstrate that the fatty acid spin label is incorporated into the microsomal membrane. If the label would be present in micellar form, one would observe a very broad (exchange broadened) one line spectrum. Furthermore, the spectra show (1) that the main part of the phospholipid matrix of microsomal membranes is in a fluid physical state even at temperatures below 32 °C and (2) that the bulk of the lipid phase does not exhibit a thermally induced phase transition above 5 °C. This result is not surprising since the microsomal membranes contain a mixture of a large number of different phospholipids possessing a high content of unsaturated hydrocarbon chains (*cf.* Dallner *et al.*[14]). Furthermore, about 8% (w/w) of the total lipid content of microsomes is cholesterol which causes considerable lowering and broadening of lipid phase transitions (Darke *et al.*[15]; Phillips *et al.*[16]). The high mobility within the phospholipid moiety of the membrane is also reflected by the rather high values of the diffusion coefficient D_{diff} which is by a factor of 4 larger than the diffusion rate in synthetic dipalmitoyllecithin membranes (Träuble *et al.*[11]). In contrast to the high membrane fluidity at low temperatures is the high activation energy for the fatty acid label reduction at temperatures below 32 °C. This result together with the appearence of a break in the Arrhenius plot indicates a thermally induced conformation change either in the reductase protein or in a smaller part of the membrane phospholipids. The first possibility has to be ruled out on account of the absence of a temperature break in the Arrhenius plot for the TEMPO-phosphate reduction.

Since an abrupt conformation change in the bulk of the lipid matrix has not been observed, our results provide clear evidence for the existence of a heterogeneous distribution of the different phospholipids in the bilayer membrane.

We conclude from our results that the reductase protein is enclosed by a halo of the phospholipid matrix which differs from the bulk lipid matrix of the membrane as it is in a quasi-crystalline state below 32 °C and undergoes a transition into a fluid state at 32 °C. Accordingly the high activation energy for the reduction of the fatty acid radical which approaches the reductase protein from the lipid phase by lateral diffusion is due to the fact that the radical must penetrate this halo of phospholipid which below 32 °C in its rigid state forms a barrier. This rigid phospholipid moiety is indicated in Fig. 4 by the inner-hatched area.

Our results provide evidence for a heterogeneous distribution of lipids in the membrane where patches of phospholipids are formed which differ in their fluidity from the bulk phase. Similar results have been obtained by Steim *et al.*[17], Engelman[18] and Metcalfe *et al.*[19] for *Mycoplasma laidlawii* membranes, McConnell *et al.*[20] for sarcosomes and H. Träuble and P. Overath, (unpublished) for *Escherichia coli*

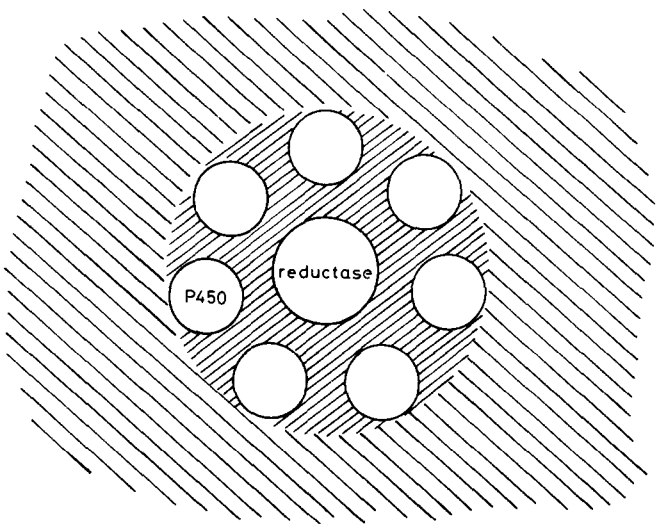

Fig. 4. Schematic representation of the incorporation of the hydroxylating enzyme system cytochrome P450–cytochrome P450 reductase embedded within the lipid matrix of the microsomal membrane. The inner-hatched area between the proteins represents the lipid moiety in a rigid quasi-crystalline state, while the outer-hatched area denotes the rather fluid bulk of the lipid matrix.

membranes. While in these studies the main part of the lipids showed a biphasic behaviour of fluidity which was used by the authors as an argument for the existence of a bilayer arrangement in those membranes, in our studies the amount of lipid showing a phase transition behaviour should be less than 20%. This part can be supposed to form a cooperative array together with the multienzyme complex of the cytochrome P450 system. In this array the metastable state of the lipid could influence not only the enzyme–enzyme interaction in the course of the reduction of cytochrome P450 which is considered to be the rate-limiting step in the biotransformation of some xenobiotics (Gillette *et al.*[21]) but also the binding of lipophilic substrates to the enzyme which reach the system by lateral diffusion within the lipid phase of the membrane as well as the off-flow of products of the enzyme reaction. Critical temperature effects on the K_m of the microsomal oxidation of aniline at 34–36 °C and of aminopyrine at 27–29 °C (Schenkman[22]) and on carbon tetrachloride-stimulated lipid peroxidation at 32 °C (Slater, T. F., unpublished) have been observed. On the other hand, Schenkman reported a single activation energy of 12 kcal/mole for the reduction of cytochrome P450 in the range of 25–40 °C. As the kinetics of the cytochrome P450 reduction is rather complex (Diehl *et al.*[23]) it seems doubtful whether the reported temperature dependence of the "initial rate" of this reduction would reveal any critical temperature effect on this enzyme–enzyme interaction in the lipid matrix of the membrane. Clearly more experimental work is necessary towards a better understanding of the molecular structure of the phospholipids enclosing those enzymes which are embedded within the supporting lipid bilayer membrane (Tanford[24]). However, the version of the spin label technique adopted in this work seems to provide a powerful tool for the investigation of those lipoprotein complexes within membranes.

REFERENCES

1 Seelig, J. and Hasselbach, W. (1971) *Eur. J. Biochem.* 21, 17–21
2 Rottem, S., Hubbell, W. L., Hayflick, L. and McConnell, H. M. (1970) *Biochim. Biophys. Acta* 219, 104–113
3 Eletr, S. and Keith, A. D. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 1353–1357
4 Jost, P., Waggoner, A. S. and Griffith, O. H. (1971) in *Structure and Function of Biological Membranes* (Rothfield, L. J., ed.), pp. 83–144, Academic Press, New York
5 Siekevitz, P. (1972) *Annu. Rev. Physiol.* 34, 117–140
6 Overath, P., Schairer, H. U. and Stoffel, W. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 606–612
7 Estabrook, R. W., Baron, J., Franklin, M., Mason, I., Waterman, M. and Peterson, J. (1972) in *The Molecular Basis of Electron Transport* (Schultz, J. and Cameron, B. F., eds), pp. 197–225, Academic Press, New York
8 Stier, A. and Reitz, I. (1971) *Xenobiotica* 1, 499–500
9 Fouts, J. R. (1971) in *Methods in Pharmacology* (Schwartz, A., ed.), Vol. 1, pp. 287–325, Appleton Century Crofts, New York
10 Sackmann, E. and Träuble, H. (1972) *J. Am. Chem. Soc.* 94, 4492–4498
11 Träuble, H. and Sackmann, E. (1972) *J. Am. Chem. Soc.* 94, 4499–4510
12 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
13 Hubbell, W. L. and McConnell, H. M. (1971) *J. Am. Chem. Soc.* 93, 314–326
14 Dallner, G. and Ernster, L. (1968) *J. Histochem. Cytochem.* 16, 611–632
15 Darke, A., Finer, E. G., Flook, A. G. and Phillips, M. C. (1971) *FEBS Lett.* 18, 326–330
16 Phillips, M. C., Williams, R. M. and Chapman, D. (1969) *Chem. Phys. Lipids* 3, 234–244
17 Steim, J. M., Trourtellotte, M. E., Reinert, J. C., Mc Elhaney, R. N. and Rader, R. L. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 104–109
18 Engelman, D. M. (1971) *J. Mol. Biol.* 58, 153–165
19 Metcalfe, J. C., Birdsall, N. J. M. and Lee, A.-G. (1972) *FEBS Lett.* 21, 335–340
20 McConnell, H. M., Wright, K. L. and McFarland, B. G. (1972) *Biochem. Biophys. Res. Commun.* 47, 273–281
21 Gillette, J. R., Davis, D. C. and Sasame, H. A. (1972) *Annu. Rev. Pharmacol.* 12, 57–84
22 Schenkman, J. B. (1972) *Mol. Pharmacol.* 8, 178–188
23 Diehl, H., Schädelin, J. and Ullrich, V. (1970) *Z. Physiol. Chem.* 351, 1359–1371
24 Tanford, C. (1972) *J. Mol. Biol.* 67, 59–74