Biochemical Pharmacology, Vol. 25, pp. 109–113. Pergamon Press, 1976. Printed in Great Britain.

COMMENTARY

LIPID STRUCTURE AND DRUG METABOLIZING ENZYMES

ANTON STIER

Max-Planck-Institut für biophysikalische Chemie, 34 Göttingen, Germany

A multienzyme system consisting of at least 4 different enzymes (the cytochromes P-450 and b_5 and their corresponding reductases) is involved in mixed function hydroxylation, the phase I reactions of the biotransformation of drugs and other xenobiotics in the endoplasmic reticulum of the liver. At least in microsomes of phenobarbital stimulated animals the mixed function hydroxylation system accounts for 40% of the total integral membrane proteins. In addition the membrane contains several enzymes carrying out the phase II reactions, for example the UDP-glucuronyl transferase. Thus this membrane represents a 2-dimensional compartment in the cell with a dense packing of many enzymes involved in catalyzing consecutive steps of biotransformation reactions. This imposes on the kinetics of those reactions some features deviating from the kinetics of enzymes homogeneously distributed in the 3 dimensional plasma space of the liver cell as shown by model calculations: For example the mere reduction in dimensionality could favour under certain conditions the diffusion of substrates to their biotransformation site [1], the immobilization on the membrane of 2 enzymes catalyzing a sequence of reactions could enhance the rate of end product formation [2] or the dense packing of the enzymes could make the Michaelis–Menten equation inapplicable [3]. Furthermore a certain topography of the 4-enzyme system could specifically channel the flow of electrons during a mixed function hydroxylation. Thus the question (crucial for the regulation of the system) whether the transfer of the first or the second electron is rate-limiting for the overall reaction might be a matter of structural organization of the whole multienzyme complex in the membrane. The membrane lipids form the matrix of the 2-dimensional compartment. What is their special role with regard to the structural arrangement of the biotransformation enzymes and their peculiar kinetics?

Phase separation in lipid membranes

Recently concepts have been elaborated on the dynamics of the phospholipids in membranes based on the cooperativity of the supramolecular structure of lipid bilamellae. One of the most important properties learned from experiments with artificial bilamellar phospholipid membranes and verified for some biological membranes is the occurrence of phase separation equilibria [4]. The different lipid components of membranes might not be randomly distributed within the membrane, rather areas could occur that have been separated from the bulk mixture and exist in a different physical state. This physical state determines the arrangement of multienzymes embedded in the lipid matrix as well as the conformational states of the individual enzymes, whereas a conformational change of a receptor protein initiated by the binding of a transmitter, a hormone or drug might induce a phase separation. Thus a local disturbance on the membrane could affect the whole membrane structure. Two different functions in the membrane could be linked by this long range cooperativity of lipids, a stimulus on the one functional site being transduced to a qualitatively different functional site and even being amplified by the linking of multiple secondary functional sites to the receptor (e.g. [5]). Calcium [6] and cholesterol [7] have been shown to induce phase separation. It is tempting to extrapolate from these concepts of lipid dynamics to an understanding of the biological function of the endoplasmic reticulum. The following discussion of recent results on the structure of lipids in isolated liver microsomal membranes, mainly obtained by techniques of ESR- and NMR-spectroscopy [8], may be confined to the questions (a) whether the present stage of our knowledge of lipid structure of microsomes allows for the application of these concepts, and (b) what principles we can deduce for the understanding of the mechanisms of biotransformation reactions.

Rigid and fluid lipid areas in liver microsomes

Some of the lipids of microsomal membranes form lamellae existing in a state of high fluidity: The ESR-spectra of a stearic fatty acid spinlabel incorporated into microsomal membranes of rabbits are characteristic for the fatty acid molecule undergoing rapid anisotropic rotation [9]. An order parameter ($S = 0{\cdot}71$ at 23°C) as a measure of lipid fluidity can be evaluated (c.f. *cit.* [10]), which lies in the lower range of order parameters measured so far by this method in biological membranes (e.g. [11]; sarcosomes 0·8, human erythrocytes 0·83, human lymphocytes 0·8). The order parameter continuously decreases with temperature showing that the fluidity of the lipid continously increases. But the fluidity of the membrane not only allows for a high rotational mobility of the fatty acid spinlabel but also for a high rate of lateral diffusion within the membrane. From the concentrapion dependence of the spin exchange frequency at high label concentrations (molar ratio label to lipid > 0·03), diffusion constants D_{diff} 40°C of 14×10^{-8} cm^2/sec and D_{diff} 30°C 10×10^{-8} cm^2/sec have been determined which means a diffusion rate of about 3–4 μm/sec [9]. Thus the distribution of lipophilic compounds within the confines of the endoplasmic reticulum within a liver cell might effectively compete with a random distribution through the plasma space. This satisfies one of the conditions for

B.P. 25/2—A

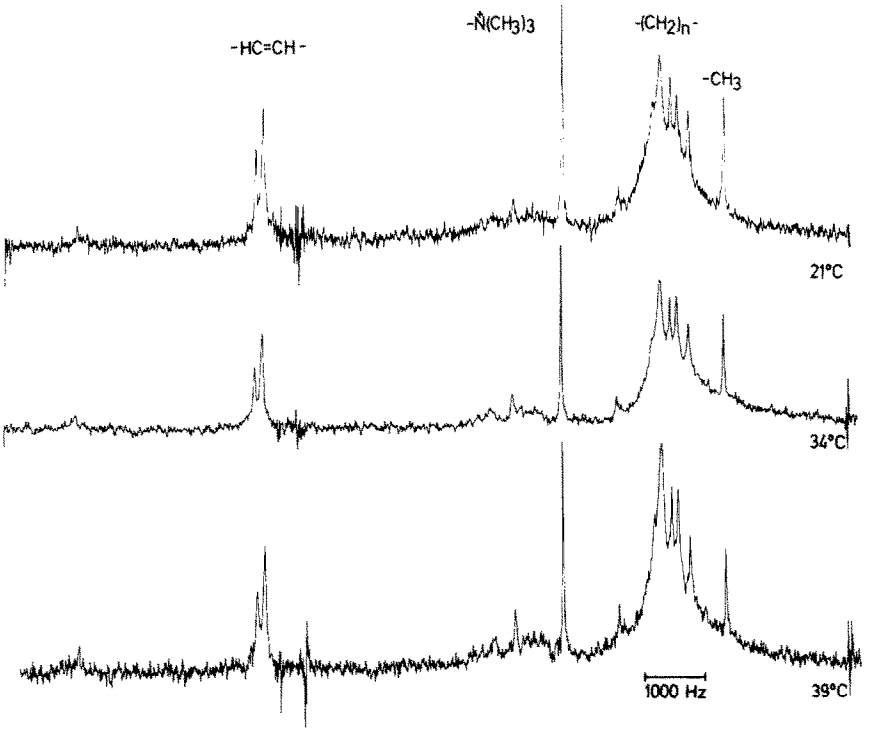

Fig. 1. Natural abundance C^{13} NMR spectra of phenobarbital stimulated rabbit liver microsomes (30 mg/ml protein) at different temperatures. Spectra were recorded by Fourier transform technique on a Bruker WH 270 spectrometer with accumulation of 90,000 pulses. Note the increase and sharpening of the methylene-resonances at the expense of the underlying broad band in the spectrum at 39°C.

the applicability of a kinetic model (reduction of the diffusional space to 2 dimensions [1]) previously mentioned.

The lipids we can examine by the spinlabel experiments mentioned do not represent the total lipid of the membrane. Other membrane areas exist composed of lipids which are not as easily accessible to the spinlabel due to their low fluidity: Proton magnetic resonance spectra show, underlying the sharp resonances of the cholinomethyl-, methylen- and methylgroups of lipids in a highly fluid state, broad bands that are nearly absent in microsomal lipid extracts, redispersed by ultrasonication, or in microsomal preparations to which cholate has been added in a molar ratio to the lipids of 1 to 2·5. This immobilized part of the membrane lipids shows a critical increase of its fluidity with rising temperature around 36°C, as can be seen from the temperature dependence of the intensities of the proton- and the C^{13}-resonances (see Fig. 1) of the fatty acid methylene groups by NMR-spectroscopy. Above 36°C a markedly higher amount of small lipophilic molecules dissolve in the membrane as shown by the measurement of the partitioning of the spinlabel 2,2,6,6-tetramethylpiperidine-1-oxyl between the aqueous phase and microsomal membranes.

Lipids exhibiting this kind of a phase transition are associated with the cytochrom P-450: The order parameter (S) of a stearic acid spinlabel incorporated into a cytochrome P-450 preparation, in which the bulk of the lipids and proteins of the membrane had been detached with cholate and separated by affinity chromatography on AH-sepharose 4B (ca. 50% of total protein in this preparation is represented by cytochrome P-450), is much higher ($S = 0{\cdot}86$ at 23°C) than the order parameter observed for the spinlabel incorporated into the extracted and resuspended lipids of the same cytochrome P-450 preparation ($S = 0{\cdot}63$ at 23°C), and exhibits an abrupt change at about 36°C. Apparently the remaining protein of this membrane fraction, presumably cytochrome P-450 imposes a high degree of order on the attached lipid, and the lipoprotein complex changes its conformation at about 36°C.

Other experiments with intact microsomes give further evidence for the existence of an area of immobilized lipids surrounding the cytochrome P-450 system: Spinlabels can be reduced by NADPH in microsomal preparations probably by the cytochrome P-450-reductase [12]. The activation energy for reduction of a fatty acid spinlabel decreases abruptly from a value of 30·8 kcal/mole at temperature below 32°C to a value of 8·7 kcal/mole above 32°C, whereas the activation energy for the reduction of a hydrophilic spinlabel is constant (13·8 kcal/mole) in the whole temperature range [9]. The high activation energy at low temperature for the reduction of the lipophilic spinlabel which appraoches the reductase protein from the lipid phase by lateral diffusion within the membrane is due to the fact that this substance must

penetrate a barrier of phospholipid in rigid state, whereas the polar spinlabel by-passes the barrier approaching the enzyme from the water phase.

Apparently the proposal for the applicability of the concepts of the lipid dynamics as outlined above is satisfied: The lipids in the endoplasmic reticulum membrane are heterogeneously distributed. Several lines of experimental evidence suggest that an area of lipids in a rigid state is associated with the cytochrome P-450 system whereas other membrane lipids are arranged in a highly fluid bilayer.

Coupling and decoupling of the cytochrome P-450 system

From the functional relationship of the components of the cytochrome P-450 system (cytochrome P-450 and cytochrome P-450 reductase) and their molar ratio of occurrence in the membrane, in accordance with the experiments on the NADPH dependent reduction of the fatty acid spinlabel, a rosette-like assemblage of these components, as shown schematically in Figure 2, might be assumed as the most simple model of structural organization of the multienzyme complex. The model might be extended by the inclusion of the transfer chain of the second electron (cytochrome b_5 and the corresponding reductase) [13] which might amplify but not definitely modify the principles that can be derived from the application of the concepts of lipid dynamics. The lipid halo is visualized as rigidly coupling the components of the multienzyme system.

It can be calculated, on the assumption of bimolecular circles of rigid lipids surrounding the cytochrome P-450 system in addition to the lipid between the enzyme subunits, for the system representing 20% of the total microsomal protein that the lipid of the halo in the rosette model accounts for 35% of the total microsomal lipid which is in fair agreement with the NMR-spectroscopic measurements. The conformational transition temperature is related to the physiological temperature. That means the halo or the lipoprotein complex as a whole is in a metastable state. The border of the halo with the surrounding membrane lipids constitutes a phase boundary. Therefore the halo is part of a phase which is in a dynamic equilibrium with other parts of the membrane. Structural changes in the bulk of the membrane or in the lipoprotein complex of the cytochrome P-450 system itself, induced by substrate binding or reduction of the cytochrome [14], might shift the phase equilibrium in either direction. As a consequence the subunits of the multienzyme complex may be coupled or decoupled. Such a phase equilibrium might be the basis for a functional linkage between the cytochrome P-450 complex and other biological functions of the endoplasmic reticulum. Ca^{2+}, Mg^{2+} and endogenous steroids may shift the equilibrium and metabolic transformation of the membrane lipids and lipid peroxidation could influence the coupling–decoupling dynamics. Sex [15], age, nutrition [16] and diurnal dependent variations in drug biotransformation reactions might be related to variations in lipid metabolism. These variations might be controlled within certain limits, as the cytochrome P-450 is itself involved in the metabolism of lipids and cholesterol (see [17]) and thus could regulate its own phase-dependent activity. The consequences for the biotransformation might be quantitative and qualitative: In the coupled state the flavoprotein can be assumed to act predominantly as a cytochrome P-450-reductase and the cytochrome as a hydroxylase whereas in the decoupled state the flavoprotein could act as a hydroxylase [18] and the cytochrome as a peroxidase [19]. Substrate, ligand and inhibitor binding could be different in the coupled and decoupled states. Thus multiple forms of cytochrome P-450, as differentiated by the application of binding criteria [20], might in reality represent a single form in varying lipid environments.

Drug interactions with lipid membranes

Drugs could influence the coupling–decoupling dynamics in two ways. They could trigger a phase separation by binding to the cytochrome P-450 (see above) or by interaction with secondary binding sites in the bulk of the membrane lipids. Recent spinlabel experiments with artifical lipid membranes have demonstrated that drugs can interact quite specifically with membranes. A correlation of the anaesthetic potency of steroids with the capability to decrease the order parameter of spinlabelled liposomes was observed [21]. A shift to lower temperatures of the phase transition of lecithin membrane vesicles caused by morphine derivatives appeared to be related to their structure [22]. Also butobarbitone [21], several tricyclic antidepressants [22] and high concentrations of inhalational anaesthetics [23] destabilize the crystalline lattice of lecithin membranes thus weakening the cooperativity of the lipids. On the contrary

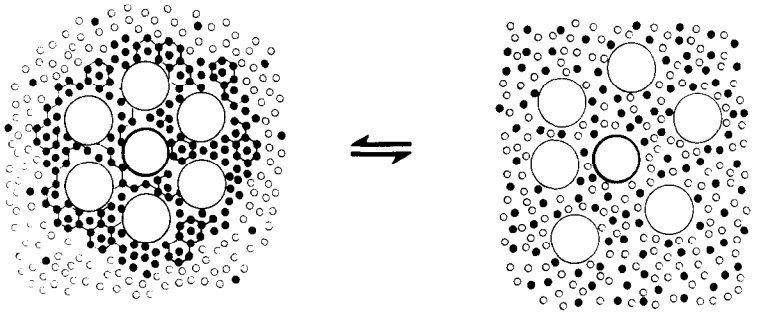

Fig. 2. Hypothetic model of the coupling and decoupling of the cytochrome P-450 system enzyme components (large heavy circles: cytochrome P-450 reductase; large light circles: cytochrome P-450) dependent on the phase separation of lipids (for simplicity and 2 different types of lipids are drawn: small open and closed circles) as viewed from above the membrane.

halothane in a lower concentration range where clinical anaesthesia is produced stabilized a lecithin membrane [24]. Digitoxigenin binds specifically to the hexagonal lattice of the polar headgroups of lecithin membranes in their crystalline state [25]. The binding site having a shape complementary to that of this molecule meets some of the structural features required by cardioactive steroids. The binding stabilizes the hexagonal lattice and shifts the phase transition to higher temperature. Thus digitoxigenin exerts a procooperative effect on these membranes. As a conclusion from these results we might differentiate membrane active agents into procooperatively and anticooperatively acting groups. These two groups might be expected to shift phase equilibria in different directions.

Thus drugs might by their interaction with microsomal membrane lipids influence the coupling–decoupling equilibrium of the cytochrome P-450 system. These effects exerted from secondary binding sites in the membrane could complement or cancel the primary effects elicited by binding to cytochrome P-450. Depending on concentration of substrate and on the ratio of affinity to the binding sites a biphasic effect might be observed. Biphasic and dual effects on drug biotransformation are well known to be exhibited by substances acting as activators and/or inhibitors on drug biotransformation. The simultaneous presence of two or more drugs in the liver could produce a combination of effects on lipid structure thus causing drug interaction on the level of biotransformation to be a highly complex phenomenon.

Many of the products of phase I biotransformation reactions are stronger amphiphiles than their parent compounds and therefore stronger phase shifting agents. Depending on the type of interaction with lipids (procooperative or anticooperative) and site (cytochrome P-450 system inclusive the lipid halo or bulk lipid) product inhibition or product activation of the biotransformation might be expected. *In vivo* however the products might be trapped by the subsequent phase II reactions and transported vectorially through the membrane due to the sidedness of distribution of phase I and phase II enzymes. The functional linkage between the cytochrome P-450 system and UDP-glucuronyl transferase could be lipid mediated. Some of the effects known to be exerted by various factors on the activity of this enzyme [26] can be considered as lipid phase shifts.

The common denominator in the activity of the many phenobarbital type stimulators of the mixed function hydroxylation system is long time contact with the endoplasmic reticulum membrane which might induce a persisting phase shift in the membrane lipids resulting in decoupling of the cytochrome P-450 system. This long term phase shift could disturb many membrane functions thereby eliciting a host of compensatory responses.

Microsomes—restraints of a model

Microsomes are only artificial models for the *in vivo* biotransformation reactions. This statement seems to be trivial. A thorough delineation of the limitations of the model however appears to be intricate. From the concepts of lipid dynamics as outlined above we might expect the versatility of the biotransformation system being severely restrained by many *in vitro* conditions. The absence of the cell plasma, containing factors acting directly (like steroids, ions, proteins, nucleic acids) or indirectly (like endogenous inhibitors of lipid peroxidation [27], regulators of membrane lipid metabolism) on the lipid structure, might be one of the limiting conditions whose consequences are difficult to be evaluated unequivocally. The accumulation of products of phase I reactions in some cases, temperature, ionic composition or unduly high substrate amounts added together with solvents belong to conditions which might shift the system to a stable 'standard'-state in which it responds reproducibly but in a manner nonrelevant to the *in vivo* situation. On the other hand the evaluation of kinetic parameters of a single selected biotransformation reaction in a way which neglects the implications of the peculiar kinetics of the multienzyme system localized in the 2-dimensional compartment of a membrane might not reveal the complexity of reactions involved. Furthermore as the lipid dynamics influences the biotransformation reactions qualitatively as well as quantitatively the investigation of the whole pattern of products formed appears to be necessary to get a better insight into the mechanisms of the microsomal biotransformation reactions.

The results outlined above indicate that the microsomal membrane exists in a metastable state at the physiological temperature; that means that it is more sensitive to phase shifting influences at this temperature than for example at 30°C at which temperature it might have been shifted thermotropically to an artificial stable state. This metastability constitutes the most critical property inherent in the system restraining the value of microsomes as an *in vitro* model.

REFERENCES

1. G. Adam and M. Delbrück, in *Structural Chemistry and Molecular Biology* (Eds. A. Rich and N. Davidson), p. 198. W. H. Freeman, San Francisco (1968).
2. R. Goldman and E. Katchalski, *J. theor. Biol.* **32**, 243 (1971).
3. S. Cha, *J. biol. Chem.* **245**, 4814 (1970).
4. E. J. Shimshick and H. M. McConnell, *Biochemistry* **12**, 2351 (1973).
5. O. F. Schmitt, D. M. Schneider and D. M. Crothers (Eds.), *Functional Linkage in Biomolecular Systems*, Raven Press, New York (1975).
6. S. J. Ohnishi and T. Ito, *Biochem. biophys. Res. Commun.* **51**, 132 (1973).
7. E. Oldfield and D. Chapman, *Fedn. Eur. Biochem. Soc. Lett.* **23**, 285 (1972).
8. A. Stier and R. Rösen, in preparation.
9. A. Stier and E. Sackmann, *Biochim. biophys. Acta* **311**, 400 (1973).
10. P. Jost, L. J. Libertini, V. C. Hebert and O. H. Griffith, *J. mol. Biol.* **59**, 77 (1971).
11. C. F. Chignell and D. A. Chignell, *Biochem. biophys. Res. Commun.* **62**, 136 (1975).
12. A. Stier and I. Reitz, *Xenobiotica* **1**, 499 (1971).
13. M. R. Franklin and R. W. Estabrook, *Archs. Biochem. Biophys.* **143**, 318 (1971).
14. F. C. Yong, T. E. King, S. Oldham, M. R. Waterman and H. S. Mason, *Archs Biochem. Biophys.* **138**, 96 (1970).
15. H. Belina, S. D. Cooper, R. Farkas and G. Feuer, *Biochem. Pharmac.* **24**, 301 (1975).
16. W. P. Norred and A. E. Wade, *Biochem. Pharmac.* **21**, 2887 (1972).

17. A. H. Conney and R. Kuntzman, in *Hdb. exp. Pharmacol., Vol. XXVIII/2* p. 401 Springer, Berlin Heidelberg New York (1971).
18. S. D. Aust, D. L. Roerig and T. C. Pederson, *Biochem. biophys. Res. Commun.* **47,** 1133 (1972).
19. F. F. Kadlubar, K. C. Morton and D. M. Ziegler, *Biochem. biophys. Res. Commun.* **54,** 1255 (1973).
20. J. Werringloer and R. W. Estabrook, *Archs Biochem. Biophys.* **167,** 270 (1975).
21. D. K. Lawrence and E. W. Gill, *Moll. Pharmac.* **11,** 280 (1974).
22. B. R. Cater, D. Chapman, S. M. Hawes and J. Saville, *Biochim. biophys. Acta* **363,** 54 (1974).
23. J. R. Trudell, W. L. Hubell and E. N. Cohen, *Anesthesiology* **291,** 321 (1973).
24. P. Rosenberg, H. J. Eibl and A. Stier, *Mol. Pharmac,* in press (1975).
25. R. Rösen, U. Fricke and A. Stier, *Nauyn-Schmiedeberg's Arch. Pharmacol.,* submitted for publication, (1975).
26. G. W. Lucier, O. S. McDaniel and H. B. Matthews, *Archs Biochem. Biophys.* **145,** 520 (1971).
27. T. Kamataki, N. Ozawa, M. Kitada, H. Kitagawa and R. Sato, *Biochem. Pharmac.* **23,** 2485 (1974).