Biochemical Pharmacology, Vol. 34, No. 5, pp. 663–668, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
© 1985 Pergamon Press Ltd.

INTERACTION OF LOCAL ANAESTHETICS WITH CYTOCHROME OXIDASE STUDIED WITH FLUORESCENCE QUENCHING

A. M. CASANOVAS,* C. LABAT, PH. COURRIERE and J. OUSTRIN

Laboratoire de Biophysique et Biomathématiques, Faculté des Sciences Pharmaceutiques, 35 chemin des Maraîchers, 31062 Toulouse Cédex, France

(*Received* 8 *June* 1984; *accepted* 27 *August* 1984)

Abstract—The interaction of a series of eight local anaesthetics with cytochrome oxidase chosen as a membrane model protein has been studied with fluorescence technique using quinacrine as a fluorescent probe.

The existence of hydrophobic interactions with a non polar region of cytochrome oxidase complex has been shown.

The ability of the drug molecules to displace quinacrine bound to cytochrome oxidase correlate as closely with their anaesthetic potency as with their octanol–water partition coefficient.

Our results are in good agreement with a recent model of local anaesthetic action on nerve membranes presenting a site of anaesthesia including both lipid binding and protein binding environments.

Several theories have been proposed to define a molecular mechanism of local anaesthetic action on nerve membranes [1, 2]. A recent model suggests a heterogenous anaesthesia site which could include both lipid binding and protein binding environments [1]. For a better understanding of drug–membrane interaction it is necessary to account for both the drug's affinity for membrane lipids and for the eventual functional effects of the drug on membrane proteins. Therefore we have been interested by the interaction of a series of local anaesthetics with different molecular structures and pharmacological activity with a model system. For this study, we chose cytochrome oxidase, which forms a good model membrane system with definite components and functional properties. Cytochrome oxidase preparations contain a protein and phospholipid component. Boundary lipids exist around the protein and are directly associated to its effectiveness [3–5]. Cytochrome oxidase complex is included in the electron transport chain located in the inner mitochondrial membrane; its biological function is to transfer electrons from cytochrome *c* to oxygen.

In an earlier study [6] we correlated the physicochemical parameters (pK_a, octanol–water partition coefficient) of twelve local anaesthetics with their activity. Similar results already obtained in this field have led to hypotheses of anaesthetic action based on their interactions with the lipid moiety of nerve membranes [7, 8].

In a recent report [9] the inhibition of cytochrome oxidase activity by eight local anaesthetics has been described. A linear relationship has been recognized between the anaesthetic activity of infiltration and the affinity for the enzyme. The inhibition appears to depend on both electrostatic interactions with the protein and hydrophobic interactions between anaesthetics and oxidase associated phospholipids. The drugs investigated were: procaine, parethoxycaine, lidocaine, prilocaine, carticaine, bupivacaine, pramocaine and quinisocaine.

In the present paper we present a more in-depth study of the interactions of these drugs with cytochrome oxidase complex. We used two techniques: ultrafiltration and fluorescence quenching. Ultrafiltration let us to determine the binding-parameters of quinisocaine to the protein. This method failed for the other molecules which presented too weak a concentration of bound-ligand to be accurately measured. The majority of drug–protein interactions have therefore been studied by the latter technique. These techniques are described in the next section.

Materials. Cytochrome oxidase, quinacrine and procaine were purchased from the Sigma Chemical Co. (St. Louis, MO). The other anaesthetics were kindly furnished by Pharmaceutical Laboratories.

Fluorescence measuring technique. This technique uses quinacrine as a fluorescent probe. The fluorescence intensity of quinacrine is enhanced by the presence of cytochrome oxidase. A similar fluorescence change has been observed for the binding of quinacrine to submitochondrial membrane particles by Massari *et al.* [10]; they concluded that the enhancement effect reflects a lower polarity or higher viscosity of the fluorophore environment suggesting a binding of quinacrine to a non polar region of the membrane. Moreover the same authors have observed [11] that the binding of quinacrine to phospholipid vesicles induced an enhancement of quinacrine fluorescence at low dye/site ratio. Greenberg *et al.* [12] used the enhancement of quinacrine fluorescence in presence of axonal membranes to study the binding of quinacrine to these membranes and suggested that two models can be conceived for

* To whom all correspondence should be addressed.

the binding site: either it is a protein, or it is at the level of interaction of some protein and its phospholipid environment (perhaps annular lipid).

On account of the observations of Massari [11] and Greenberg [12] and of the structure of cytochrome oxidase (single boundary layer of immobilized lipid surrounding the protein complex intercalated into lipid bilayer [3, 4]), the modification of the quinacrine fluorescence can be imputed to the interaction of quinacrine with phospholipids associated to the protein. We report here that local anaesthetics modify this interaction by decreasing the fluorescence enhancement effect and we try to quantify the hydrophobic interactions of the drugs with cytochrome oxidase.

A similar technique has already been used by other authors [12, 13] to study the effect of local anaesthetics on membrane-bound quinacrine. The fluorescence decrease has been explained by a competition between quinacrine and local anaesthetics for the same binding sites. Moreover the effectiveness of the displacement of bound quinacrine from membrane fragments correlates with the nerve blocking potency of the drugs.

All measurements were performed at room temperature (20°) using an Aminco Bowman spectrofluorimeter. Quartz fluorescence cells with a cross-section (10 × 10 mm) were filled with 2.5 ml of solution. Excitation of quinacrine was at 350 nm and emission was read at 500 nm.

All the solutions were prepared in 67 mM phosphate buffer at pH 7. Absorbances were maintained below 0.1 to avoid an inner filter effect; therefore we chose, for cytochrome oxidase, a concentration of 100 μg of protein/ml. The quinacrine concentration was 11 μM; this concentration is located in the linear part of the fluorescence intensity/quinacrine concentration plot, to avoid quenching due to aggregation of quinacrine molecules in solution.

The protein solution was added to quinacrine, in the absence or in the presence of local anaesthetic, and left to stabilize for 10 min before fluorescence measuring. The presence of protein causes an enhancement of quinacrine fluorescence intensity of about 25% at the concentrations above mentioned.

Otherwise, it was verified that neither protein nor drugs, at concentrations used, exhibit any detectable fluorescence. Samples were purged with high purity nitrogen to avoid oxygen quenching.

Ultrafiltration measuring technique. The molecular weight of cytochrome oxidase is about one thousand times higher than that of quinisocaine. Such a difference in molecular size allows the use of an ultrafiltration method to study the binding equilibrium between the protein and the local anaesthetic. Experiments were carried out through a direct ultrafiltration method [14, 15]. We used a 13 mm dia. stirred cell, Millipore XX01310, filled with 2 ml of protein–ligand mixture. A pressure of 1.8 bar was applied to solution. We operated with a pellicon membrane of a nominal molecular weight limit of 25,000. It has been verified that the anaesthetic molecules cross the membrane without any retention. The concentration of ligand in the filtrate, noted C_f, has been determined by u.v. spectrometry. It remained constant during filtration and represents the concentration of free ligand in the cell. The concentration of bound molecules, noted C_b, was deduced by subtraction of C_f from the total concentration of quinisocaine in the initial mixture.

We chose the Scatchard representation (16) to describe the binding equilibrium:

$$\nu / C_f = K_A n - K_A \nu \quad , \text{with } \nu = C_b/C_p,$$

where C_p is the concentration of protein, n is the number of sites on the protein molecule, K_A is the association constant.

The value of K_A is deduced from the slope of the plot of ν/C_f v ν, the value of n from the intercept of the line with abscissa. All the experiments were made at room temperature (20°) for a protein concentration of 0.348 μM and a concentration of ligand varying in the 0.02–0.1 mM range.

RESULTS AND DISCUSSION

We have observed that the fluorescence enhancement of quinacrine bound to protein decreases in presence of local anaesthetics. In this study we shall consider the quenching of quinacrine situated in the lipid phase of cytochrome oxidase complex, by local anaesthetics molecules. I and I_0 are the fluorescence intensities in the presence and absence of a quencher. The decrease of fluorescence of quinacrine cannot be described as a true quenching phenomenon, so we used the Stern–Volmer equation: $(I_0/I)-1 = K(Q)$ where (Q) is the quencher concentration and K the quenching constant, as a simple way to quantify the results. Figures 1 and 2 illustrate the variation of $(I_0/I)-1$ as a function of (Q) for the series of molecules investigated. The values of K obtained from these plots are reported in Table 1.

At high concentrations the Stern–Volmer plots are non linear for quinisocaine, pramocaine and par-

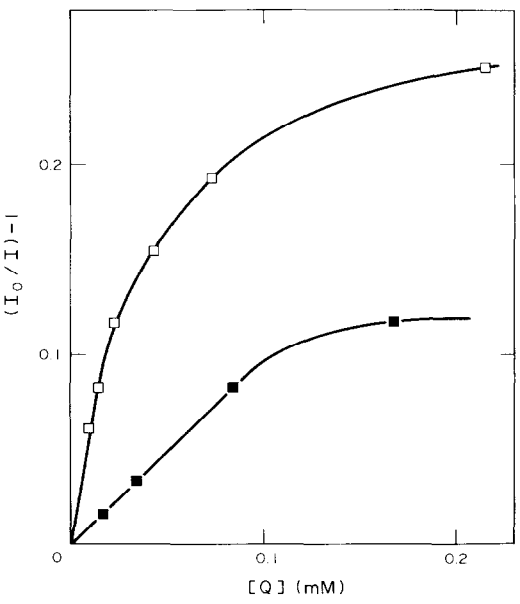

Fig. 1. Stern–Volmer plot of the quenching of cytochrome oxidase bound quinacrine by pramocaine (■) and quinisocaine (□).

Table 1. Variation of the quenching rate of local anaesthetics as a function of anaesthetic activity, octanol–water partition coefficient and affinity constant for cytochrome oxidase

Anaesthetic	Stern–Volmer constant K (M^{-1})	Standard error S_K (M^{-1})	Affinity constant K_i^{-1} (M^{-1})	Octanol–water partition coefficient Log P	Anaesthetic activity of infiltration (A.A.I.)
Procaine	1.9	0.3	78	1.4	1
Lidocaine	6.8	0.2	92.6	1.3	4.2
Prilocaine	6.5	0.6	53.8	1.1	4.5
Carticaine	8.1	0.3	166.7	1.7	6.3
Parethoxycaine	53.6	2.6	142.9	2.2	7
Bupivacaine	15.2	0.2	188.7	1.6	10.1
Pramocaine	984	2.5	555.6	2.1	17.7
Quinisocaine	5309	498	1408.5	2.5	55.8

* The affinity constant is defined as the reciprocal of the inhibition constant K_i.

ethoxycaine, in a manner which suggests saturational binding in the lipid phase of the complex. The linear plot observed for the other molecules can indicate only a simple partitioning of them into the cytochrome oxidase complex. The theories elaborated on the binding of a quencher molecule to lipid membranes [17, 18] states that such a non linear Stern–Volmer plot is better described as a binding process than a partition process. In this case the double reciprocal plot of the Stern–Volmer equation is linear and the association constant can be calculated as follows: K_A = ordinate intercept/slope. The values of K_A have been determined from the plots shown in Figs. 3, 4 for the three molecules investigated. They are reported in Table 2, as the binding parameters of quinisocaine obtained by ultrafiltration.

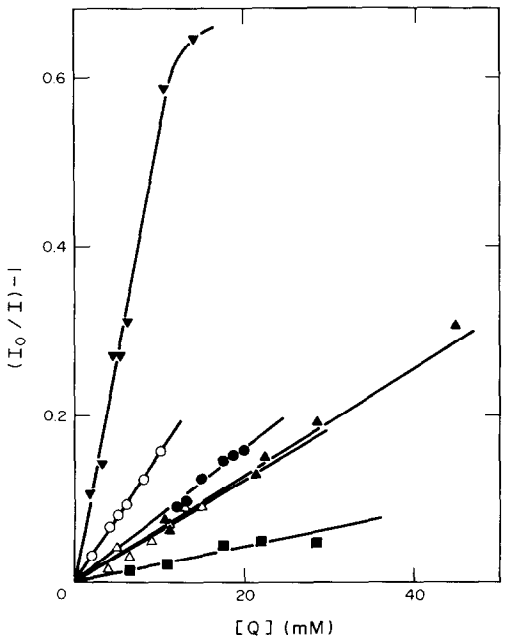

Fig. 2. Stern–Volmer plot of the quenching of cytochrome oxidase bound quinacrine by local anaesthetics procaine (■), lidocaine (▲), prilocaine (△), carticaine (●), bupivacaine (○) and parethoxycaine (▼).

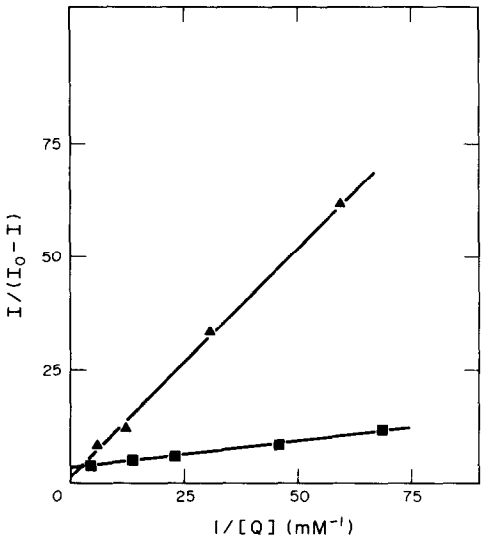

Fig. 3. Double reciprocal plot of Stern–Volmer equation for quinisocaine (■) and pramocaine (▲). The regressions are significant at 0.5%.

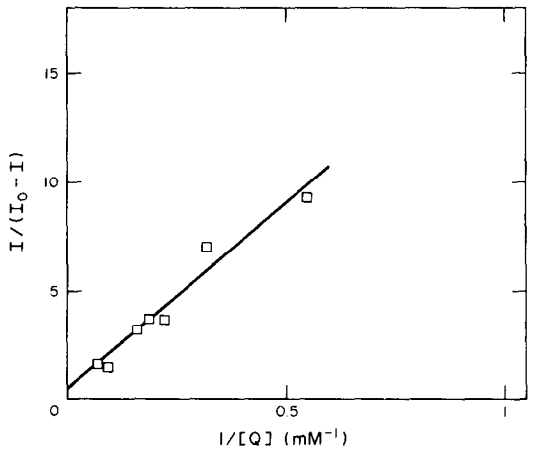

Fig. 4. Double reciprocal plot of Stern–Volmer equation for parethoxycaine. The regression is significant at 0.1%.

Table 2. Binding parameters of local anesthetics to cytochrome oxidase

Ligand	Association constant $K_A \times 10^{-3}$ (M^{-1})	Maximal binding capacity (sites/molecule of protein)	Method	Source
Quinisocaine	28.3 (19.5–37.2)*		Fluorescence	Fig. 3
	18.3 (13.5–23.1)*	256	Ultrafiltration	Fig. 5
Pramocaine	1.09		Fluorescence	Fig. 3
Parethoxycaine	0.03		Fluorescence	Fig. 4

* 95% confidence limits.

The binding study of quinisocaine to cytochrome oxidase by this method is described by the Scatchard plot shown in Fig. 5. The linearity of the Scatchard plot is significant at 0.1% and indicates a single set of binding sites in the range of concentration investigated.

As in a previous study [6] we measured the anaesthetic activity of infiltration of the same drug molecules (these values noted (A.A.I.) are reported in Table 1), we have tried to correlate the ability of the drug molecules to penetrate the lipid phase of the cytochrome oxidase complex characterized by the quenching constant K to their biological activity. This correlation is illustrated by Fig. 6 and the following relationship:

$\text{LogK} = 2.15\ \text{Log(A.A.I.)} - 0.306$, $r = 0.91$, $n = 8$, $t = 5.48$, $t_{(6)0.005} = 3.707$.

The correlation is significant at 0.5%.

The good correlation of the quenching constant K with the anaesthetic activity of infiltration (A.A.I.) confirms the results obtained from recent studies on the correlation between anaesthetic potency of drugs and their ability to interact with lipid membranes [19–21].

Then we study the role played by the liposolubility of the investigated local anaesthetics molecules in their interaction with cytochrome oxidase by testing the correlation between the constant of quenching K and the octanol–water partition coefficient LogP (cf. Table 1) measured in previous studies [6]; the results are plotted on Fig. 7 and the following relationship has been obtained:

$\text{LogK} = 2.17\ \text{LogP} - 2.11$, $r = 0.87$, $n = 8$, $t = 4.31$, $t_{(6)0.005} = 3.707$.

The correlation is significant at 0.5%.

To take into account the differences of ionization state of drug molecules we have calculated a multilinear correlation including their pK_a's values:

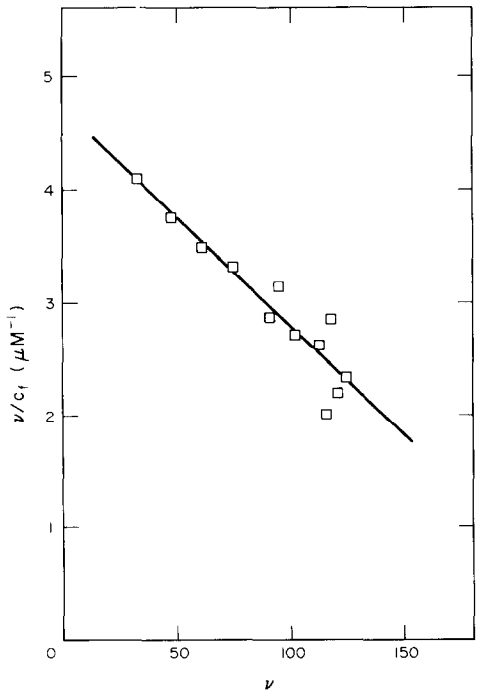

Fig. 5. Scatchard plot for quinisocaine binding to cytochrome oxidase.

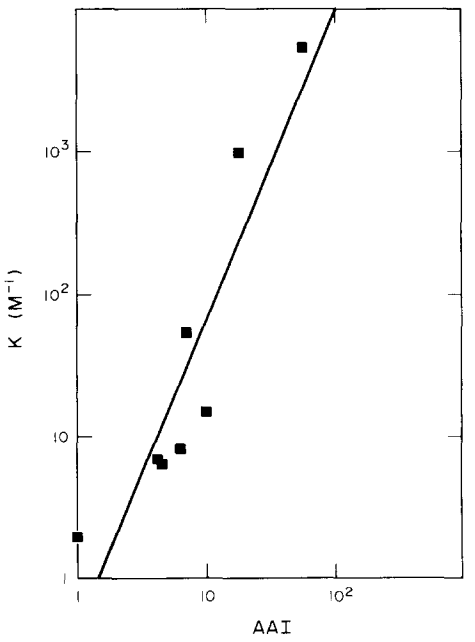

Fig. 6. Correlation between K, the quenching rate of local anaesthetics and the anaesthetic activity of infiltration (A.A.I.).

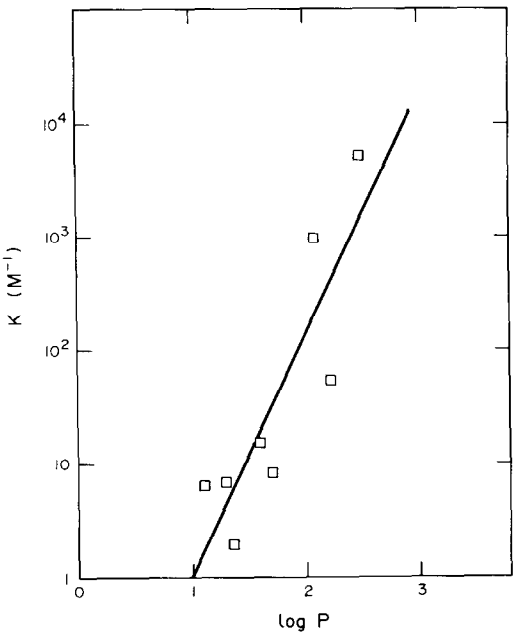

Fig. 7. Correlation between K, the quenching rate of local anaesthetics and their octanol–water partition coefficient (LogP).

$\text{LogK} = 1.57\ \text{LogP} - 0.77\ \text{pK}_a + 4.47$, $R^2 = 0.979$, $n = 8$, $F = 114.86$, $F_{(2,5)0.01} = 13.27$. The correlation is significant at 1%.

The results show that the interactions of local anaesthetics molecules with cytochrome oxidase complex are particularly significant for molecules presenting at once a high liposolubility and a low dissociation constant value (that is to say in a low ionization state).

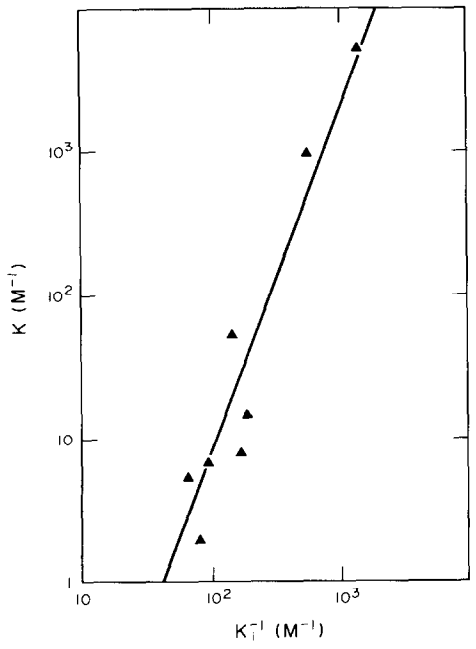

Fig. 8. Correlation between K, the quenching rate of local anaesthetics and their affinity for enzyme (K_i^{-1}).

Owing to the diversity of the ionization state of the drug molecules investigated the following comments can be drawn:

It has been found by Surewicz *et al.* [22] that charged and uncharged forms of amine local anaesthetics may be incorporated into lipid membranes. In our study, procaine, parethoxycaine and carticaine were 90% ionized and were able to quench the fluorescence of quinacrine bound to the lipid phase of cytochrome oxidase. These two results can be compared to each other; moreover it has been shown [11] that the binding of quinacrine to phospholipid vesicles is enhanced by the presence of negative charges in the phospholipids; so the quenching effect of charged anaesthetic molecules can be explained by the interaction of the drugs with charged groups of phospholipids associated to cytochrome oxidase.

Pramocaine and quinisocaine are in a lower ionization state, about 17% at pH 7; they show, however, the greatest effectiveness in their interaction with cytochrome oxidase, the greatest anaesthetic activity and octanol–water partition coefficient (cf. Table 1). We can agree with Sikaris *et al.* [23], who studied the interaction of local anaesthetics with synthetic phospholipid bilayers, that the neutral form of the molecule is the more active specie and the ability to partition into the lipid phase plays an important role. Finally we compare the results on the interaction of local anaesthetics with cytochrome oxidase obtained on one hand from fluorescence quenching of quinacrine bound to the cytochrome complex, and on the other hand from the inhibitory effect of the drugs on the cytochrome activity [9].

The agreement between these two studies is illustrated by the following correlation between the quenching constant K and the affinity constant defined as the reciprocal of the inhibition constant K_i:

$\text{LogK} = 2.4\ \text{Log}\ (K_i^{-1}) - 3.88$, $r = 0.94$, $n = 8$, $t = 6.76$, $t_{(6)0.005} = 3.707$.

The correlation is significant at 0.5% and is plotted on Fig. 8.

The results obtained from the study on the inhibitory effect of the drugs on the cytochrome oxidase activity [9] agree with those of Singer [24, 25] and led to the same conclusion: local anaesthetic reduces the enzyme turnover number by interacting, probably, with its boundary lipids. Moreover in the present study we show that the quinacrine bound to phospholipid environment of the enzyme is displaced by the drug molecules. The agreement between these two experiments supports the hypothesis of the hydrophobic interaction of the local anaesthetic with cytochrome oxidase.

In our opinion the drugs act by altering the functionally required lipid–protein interaction in cytochrome oxidase activity. Such a proposal has been made by Wang *et al.* [26] to explain how local anaesthetics affect membrane activities. Our results show that local anaesthetics interact with the model membrane protein cytochrome oxidase in a manner similar to interactions with biological membranes involving a site of action situated in the lipophilic region of the protein lipid interface.

Acknowledgements—The authors wish to express their gratitude to Professor Guy Pitet, Faculty of Pharmaceutical Sciences of Toulouse, for unrestricted access to the spectrofluorimeter of his Laboratory and his expert assistance in fluorescence experiments.

Thanks should also be sent to Bellon and Clin Midy Laboratories for the generous gift of anaesthetic drugs.

REFERENCES

1. J. R. Trudell, *Prog. Anesthesiol.* **2**, 261 (1980).
2. C. D. Richards, C. A. Keightley, I. R. Hesketh and J. C. Metcalfe, *Prog. Anesthesiol.* **2**, 337 (1980).
3. P. C. Jost, O. H. Griffith, R. A. Capaldi and G. Vanderkooi, *Proc. natn. Acad. Sci. U.S.A.* **70**, 480 (1973).
4. O. H. Griffith and P. Jost, *Ann. N. Y. Acad. Sci.* **5**, 561 (1973).
5. M. Fry and D. Green, *Biochem. biophys. Res. Commun.* **93**, 1238 (1980).
6. A. M. Casanovas, C. Labat, Ph. Courrière and J. Oustrin, *Eur. J. med. chem. Chim. Ther.* **17**, 333 (1982).
7. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
8. A. S. Janoff, M. J. Pringle and K. W. Miller, *Biochim. biophys. Acta* **649**, 125 (1981).
9. A. M. Casanovas, M. F. Malmary-Nébot, Ph. Courrière and J. Oustrin, *Biochem. Pharmac.* **32**, 2715 (1983).
10. S. Massari, P. Dell'Antone, R. Colonna and G. F. Azzone, *Biochemistry* **13**, 1038 (1974).
11. S. Massari, *Biochim. biophys. Acta* **375**, 22 (1975).
12. M. Greenberg and Tian Yow Tsong, *J. biol Chem.* **257**, 8964 (1982).
13. H. Grünhagen and J. P. Changeux, *J. mol. Biol.* **106**, 497 (1976).
14. W. F. Blatt, S. M. Robinson and H. J. Bixler, *Anal. Biochem.* **26**, 151 (1968).
15. B. Lejeune, M. Lecureuil and G. Crouzat-Reynes, *J. chim. Phys.* **70**, 789 (1973).
16. G. Scatchard, *Ann. N. Y. Acad. Sci.* **51**, 660 (1949).
17. K. R. Thulborn and W. H. Sawyer, *Biochim. biophys. Acta* **511**, 125 (1978).
18. E. A. Haigh, K. R. Thulborn, L. M. Nichol and W. H. Sawyer, *Aust. J. biol. Sci.* **31**, 447 (1978).
19. D. Papahadjopoulos, *Biochim. biophys. Acta* **265**, 169 (1972).
20. M. A. Singer, *Biochem. Pharmac.* **26**, 51 (1973).
21. F. A. Vilallonga and E. W. Phillips, *J. pharm. Sci.* **68**, 314 (1979).
22. W. K. Surewicz and W. Leyko, *J. Pharm. Pharmacol.* **34**, 359 (1982).
23. K. A. Sikaris and W. H. Sawyer, *Biochem. Pharmac.* **31**, 2625 (1982).
24. M. A. Singer, *Biochem. Pharmac.* **29**, 2651 (1980).
25. M. A. Singer, *Biochem. Pharmac.* **31**, 527 (1982).
26. H. H. Wang, J. Earnest and D. Chan, *Prog. Anesthesiol.* **2**, 483 (1980).