MOLECULAR PHARMACOLOGY, 11, 280-286

Structurally Specific Effects of Some Steroid Anesthetics on Spin-Labeled Liposomes

DAVID K. LAWRENCE[1] AND EDWARD W. GILL[2]

University Department of Pharmacology, South Parks Road, Oxford, England

(Received August 23, 1974)

SUMMARY

LAWRENCE, DAVID K. & GILL, EDWARD W. (1975) Structurally specific effects of some steroid anesthetics on spin-labeled liposomes. *Mol. Pharmacol.*, 11, 280–286.

The effects of a range of steroids related to a potent intravenous anesthetic, 3α-hydroxy-5α-pregnane-11,20-dione, on the molecular mobility and local polarity of ultrasonically dispersed vesicles of lecithin and cholesterol (liposomes) were investigated using a nitroxide-labeled dipalmitoyllecithin as a molecular probe. The anesthetics 3α-hydroxy-5α-pregnane-11,20-dione, 3α-hydroxy-5α-pregnan-20-one, butobarbitone, and octanol were found to cause fluidization of the lipid bilayer at concentrations approximating those attained during anesthesia *in vivo*, and the magnitude of the effect was linearly related to the drug concentration. When allowance was made for differences in molecular volume, it was found that these four molecules produced a degree of fluidization approximately the same as that found previously to be produced by halothane, at a given partial volume of drug in the liposomes. On the other hand, the steroids 3β-hydroxy-5α-pregnane-11,20-dione, 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5α-pregn-16-ene-11, 20-dione, and 3α,11α-dihydroxy-20,20-ethylenedioxy-5α-pregnane, which are inactive as anesthetics, produced much less disordering of the lipid bilayer. The correlation of anesthetic potency with ability to disorder spin-labeled liposomes suggests the use of this technique for drug screening. The especially large difference between the effects of the 3α- and 3β-hydroxy isomers of 5α-pregnane-11,20-dione showed that phospholipid/cholesterol bilayers are capable of a high degree of structural discrimination, and lends support to the hypothesis that the lipid phase of nerve cell membranes is the site of action of all general anesthetics.

INTRODUCTION

General anesthetic properties are exhibited by a wide range of low molecular weight volatile compounds, and most discussions of the molecular mechanism of anesthesia, in particular those suggesting the lipid phase of the membrane as the site of action, have concentrated attention on the general physical properties of anesthetic molecules, such as their size, polarizability, and lipid solubility, rather than on the presence or absence of specific chemical groupings (1). On the other hand, the involatile or so-called "fixed" anesthetics (2), such as the barbiturates and steroids, exhibit far greater dependence of potency on quite minor stereochemical and structural changes. In such cases the rela-

This work was supported by the Medical Research Council.

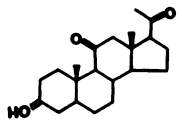
[1] Recipient of a schoarship for Training in Research Methods. Present address, I. C. I. Plant Protection, Ltd., Jealott's Hill Research Station, Bracknell, Berkshire, England.

[2] To whom requests for reprints should be addressed.

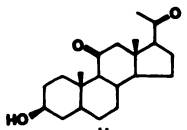
Copyright © 1975 by Academic Press, Inc.
All rights of reproduction in any form reserved.

tively simple "lipid solubility" hypothesis has been discarded in favor of a more specific interaction with some more complex structure, such as the matrix of side-chain groups of a receptor protein (3, 1), although most discussions of the structure-action relationships of the "fixed" anesthetics have concentrated on their pharmacodynamic properties and they do not appear to have received the same amount of theoretical study as have the volatile anesthetics. With the techniques at present available it is not possible to distinguish between these two sites of action by experiments on the complex lipoprotein membrane of intact cells, and indeed there is no reason a priori for supposing that all anesthetics should have the same basic molecular mode of action. The object of this work was nevertheless to determine whether simple protein-free liposomes, which appear to serve as effective model systems for the effects of the volatile anesthetics (4, 5), could also exhibit the same degree of molecular specificity in their interactions with more complex molecules, as does the living cell.

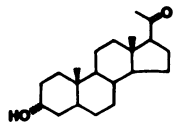
The steroids are a particularly interesting group of compounds in this context, in that they are highly lipophilic and do not contain any ionizable groups, and would be expected to penetrate readily into any lipid phase. Atkinson *et al.* (6) have described a wide range of steroids possessing structurally specific anesthetic activity; in particular 3α-hydroxy-5α-pregnane-11,20-dione (I, Alphaxolone) is a very potent anesthetic, whereas the isomeric 3β-hydroxy compound (II, Betaxolone) in inactive. A similar, though smaller, difference in activity is found between 3α- and 3β-hydroxy-5α-pregnan-20-one (III and IV), and since these isomeric pairs do not differ significantly in their lipid solubility or molecular volume, they constitute an interesting test for any model of anesthetic behavior.



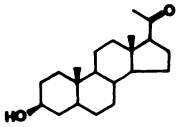
I



II

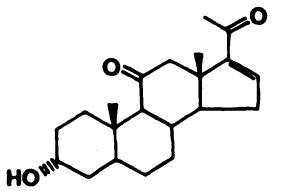


III

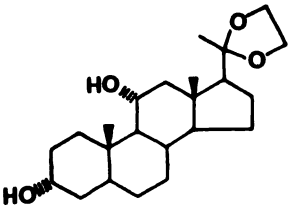


IV

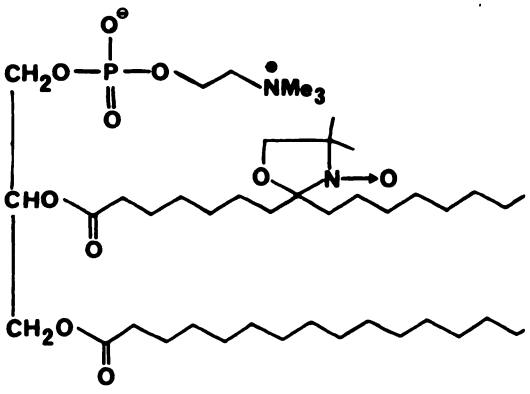
Ultrasonically dispersed suspensions of lecithin and cholesterol have been shown to consist of spherical fragments of bilayer [liposomes (7)], and these have been used as models of the lipid phase of cell membranes. The electron spin resonance signal of a stable free radical is strongly dependent on its mobility and hence, in a condensed phase, on the constraints imposed by adjacent molecules (8). Hubbell and McConnell (9) described the synthesis of a nitroxide-labeled dipalmitoyl lecithin (VII) and its use as a probe of the immediate environment of the spin label when incor-



V



VI



VII

porated into liposomes. Trudell *et al.* (4) used such spin-labeled liposomes to show that the volatile anesthetics halothane and methoxyfluorane cause fluidization of the liposome bilayer, and that this effect, like anesthesia, could be reversed by the application of external pressure (5).

The object of this work, therefore, was to determine whether barbiturates, steroids, and other involatile, high molecular weight anesthetics would produce a similar fluidization of spin-labeled liposomes, and whether the effect would show the same dependence upon chemical structure as does anesthesia.

MATERIALS AND METHODS

Drugs and reagents. 3α-Hydroxy-5α-pregnane-11,20-dione (Alphaxolone, I), 3β-hydroxy-5α-pregnane-11,20-dione (Betaxolone, II), 3α-hydroxy-5α-pregnan-20-one (III), 3β-hydroxy-5α-pregnan-20-one (IV), 3α-hydroxy-5α-pregn-16-ene-11,20-dione (V), and 3α,11α-dihydroxy-20,20-ethylenedioxy-5α-pregnane (VI) were generously donated by Glaxo, Ltd., and butobarbitone, by May and Baker, Ltd. Egg lecithin (grade I) and synthetic L-3-α-dipalmitoyl lecithin were obtained from Koch-Light Laboratories; specially pure cholesterol was obtained from British Drug Houses. The spin-labeled dipalmitoyl lecithin (VII) was synthesized as described previously (9).

Electron spin resonance techniques. All ESR spectra were recorded on a JEOL PES-1 ESR spectrometer at a temperature of 22° ± 1°, using X-band radiation at 9.45 ± 0.05 GHz. The detector modulation width was kept low to avoid artificial broadening of the signal. Calibration was done with 1,1-diphenylpicrylhydrazyl and Mn^{2+}.

Liposomes were made by sonicating lecithin (20 mg), containing approximately 1% spin-labeled lecithin, and cholesterol (9 mg) with 0.9% NaCl (0.5 ml) at full power in an MSE 100-W disintegrator for 5 min. The liposomes so prepared had a molar ratio of lecithin to cholesterol of 1:0.9, and separate batches were made from dipalmitoyl lecithin and natural egg lecithin, which contained not only palmitoyl chains but also unsaturated oleic and linoleic chains. To avoid variation between samples, the lecithin-cholesterol mixture was made up in bulk as a solution in chloroform (40 mg/ml in lecithin), aliquots (0.5 ml) of which were evaporated in a stream of oxygen-free nitrogen. Drugs were added in chloroform solution before evaporation, giving an intimate mixture which was then evaporated and sonicated. The fairly stable, translucent suspensions were used within 4 hr of sonication, and the spectrum of a sample containing no drug was measured after every two or three samples in order to check the constancy of the signals. The concentration of the drug in the liposomes was expressed as millimoles per mole of lecithin, and it was assumed that all the added drug was in the lipid phase of the suspension. In order to allow for the different molecular volumes of different drugs, these concentrations were multiplied by the calculated molal volume (10) to give the drug level as milliliters per mole of lecithin.

Calculation of results. A typical ESR spectrum is illustrated in Fig. 2. It has been shown (9) that the two outer extrema are separated by $2T_{\parallel}$, the hyperfine interaction parallel to the symmetry axis, and have, to a good approximation, the line shapes of the absorption curves for a perfectly oriented ensemble of radicals with the applied field parallel to the symmetry axis. The values of $T_{\perp}$, the hyperfine interaction perpendicular to the symmetry axis, can be established from the separation of the inner hyperfine extreme ($2T_{\perp}$, Fig. 2). The polarity of the environment around the nitroxide can be estimated from the hyperfine isotropic interaction, a', where

$$a' = \tfrac{1}{3}(T_{\parallel} + 2T_{\perp})$$

since the splittings are slightly dependent on the polarity, increasing with increasing polarity. The motion of the nitroxide group is conveniently expressed by the order parameter, S (9), where

$$S = \frac{(T_{\parallel} - T_{\perp})}{(T_{zz} - T_{xx})} \cdot \frac{a}{a'}$$

$T_\parallel$ and $T_\perp$ are the hyperfine interactions parallel and perpendicular to the major symmetry axis under the experimental conditions, T_{zz} and T_{xx} are the hyperfine interactions along the z and x axes, respectively, in pure crystals of the nitroxide-labeled compound, and

$$a = \tfrac{1}{3}(T_{zz} + T_{xx} + T_{yy}), \qquad T_{xx} \cong T_{yy}$$

The values for T_{zz} and T_{xx} were taken from those found for the *N*-oxyl-4,4-dimethyloxazolidine derivative of 5α-cholestan-3-one in a single crystal of cholesteryl chloride (11) (T_{zz} = 86 ± 2 MHz, $T_{xx} = T_{yy}$ = 16.2 ± 2 MHz). Theoretical justification of the validity of these expressions is given by Hubbell and McConnell (9), but in the present work S and a' were determined from the spectra and treated empirically, since it was shown previously (4, 5, 9) that S is a meaningful measure of the nitroxide group mobility.

RESULTS

The results are expressed in terms of the "order parameter" (S) (9), a composite function which gives a measure of the degree of flexing of the segment of the palmitic acid chain to which the nitroxide group is attached (Table 1), and the isotropic hyperfine interaction (a'), which is a measure of the polarity of the environment immediately around the spin label (Table 2). The change in order parameter produced by a given concentration of any drug was the same using either dipalmitoyl- or egg lecithin liposomes and so, although some readings were taken for each drug using egg lecithin, most were obtained using diplamitoyllecithin, since this gave reproducible liposomes more easily and had a known chemical structure. The effect of each drug is expressed as the change of the order parameter from the value without drug, and results with both types of lecithin are combined.

It was found that the potent anesthetics 3α-hydroxy-5α-pregnane-11,20-dione, the monoketone (III), butobarbitone and octanol all produced a marked decrease in the order parameter, i.e., an increased fluidity of the liposome bilayer, the extent of the disordering depending linearly on the membrane concentration of the drug. In Fig. 1 the abscissa represents the molal concentration of the anesthetic multiplied by the calculated molal volume (10), giving

TABLE 1

Effects of various drugs on disordering of egg and dipalmitoyl lecithin/cholesterol liposomes

Results are expressed as changes in the order parameter, S; the S value for spin-labeled liposomes in the absence of added drug was 0.734 ± 0.006. Each value is the mean of four to six readings ± standard deviation.

Drug	Concentration	Partial volume	$-\Delta S$
	mmoles/mole lecithin	*(ml/mole lecithin) × 10*	
Alphaxolone (I), 200 ml	60	120	0.033 ± 0.010
	120	240	0.064 ± 0.017
	240	480	0.131 ± 0.010
	480	960	0.287 ± 0.035
Betaxolone (II), 200 ml	60	120	0.002 ± 0.005
	120	240	0.009 ± 0.007
	240	480	0.012 ± 0.007
	480	960	0.039 ± 0.016
Steroid (III), 199 ml	120	238	0.042 ± 0.011
	240	476	0.083 ± 0.011
	480	952	0.156 ± 0.008
Steroid (IV)	120	238	0.034 ± 0.008
	240	476	0.059 ± 0.012
	480	952	0.092 ± 0.008
Steroid (V), 197 ml	240	475	0.003 ± 0.007
	480	950	0.005 ± 0.005
Steroid (VI), 226 ml	220	498	0.004 ± 0.008
	440	996	0.004 ± 0.007
Butobarbitone, 126 ml	120	150	0.036 ± 0.006
	240	300	0.069 ± 0.008
	480	606	0.119 ± 0.007
Octanol, 93 ml	250	232	0.045 ± 0.008
	500	464	0.092 ± 0.009

a measure of the partial volume of each drug in the liposomes rather than its concentration. At a given partial volume each of the potent anesthetics produced a considerable degree of molecular disorder of the liposomes. Anesthesia is said to occur at a membrane drug concentration of about 40 mmoles/kg of dry membrane, or 3 ml/kg of dry membrane (1), equivalent to about 10–20 ml/mole of lecithin, so it can be seen that significant disordering of the bilayer occurs at doses equal to those required for anesthesia *in vivo*. Also included in Fig. 1 (dashed line) are the results obtained by Trudell *et al.* (4) for halothane, which shows that when correction is made for the molal volume the magnitude of the effect is roughly the same as that of 3α-hydroxy-5α-pregnane-11,20-dione, for example, although an exact comparison could not be made because of the different ratios of lecithin to cholesterol used and the difference in the temperature at which the experiments were carried out.

TABLE 2

Isotropic hyperfine component (a') for dipalmitoyl lecithin/cholesterol liposomes (1:0.9 mole ratio) and effects of various concentrations of 3α- and 3β-hydroxy-5α-pregnane-11,20-dione

Each result represents the mean of three readings ± standard deviation.

Drug	Concentration	a'
	moles/mole lecithin	*MHz*
None		40.1 ± 0.2
Alphaxolone (I)	60	39.9 ± 0.3
	120	39.8 ± 0.3
	240	39.4 ± 0.2
	480	39.3 ± 0.2
Betaxolone (II)	60	40.1 ± 0.3
	120	40.1 ± 0.2
	240	40.0 ± 0.2
	480	39.9 ± 0.3

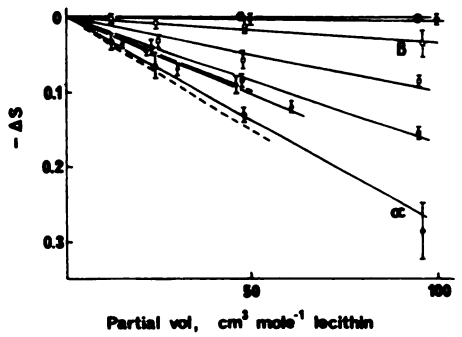


FIG. 1. *Change in order parameter (S) of lecithin/cholesterol liposomes plotted against partial drug volume in membrane*

● and ○, 3α- and 3β-hydroxy-5α-pregnane-11,20-dione (I and II, marked α and β, respectively; molal volume, 200 ml); ■ and □, 3α- and 3β-hydroxy-5α-pregnan-20-one (III and IV, respectively; molal volume, 199 ml); ×, 3α-hydroxy-5α-pregn-16-ene-11,20-dione (V; molal volume, 197 ml); ◐, 3α,11α-dihydroxy-20,20-ethylenedioxy-5α-pregnane (VI; molal volume, 226 ml); ▲, butobarbitone (molal volume, 126 ml); Δ, octanol (molal volume, 93 ml). Also shown is the line for halothane (- - -) obtained by Trudell *et al.* (4). Each value represents the mean of four to six readings ± standard deviation; readings with egg lecithin and dipalmitoyllecithin are combined.

In contrast to those results, the steroids such as the β-hydroxy-ketones (II and IV) and the pregnene (V), which possess very little anesthetic activity, and the ketal (VI), which is convulsant at high doses,[3] produced much less disordering. A particularly striking difference was observed between 3α-hydroxy-5α-pregnane-11,20-dione (I; α in Fig. 1) and its 3β isomer (II; β in Fig. 1). The ESR spectra produced by each of these compounds at a given concentration are shown superimposed in Fig. 2.

As was found to be the case with the volatile anesthetics (4), the isotropic hyperfine component, a', decreased with the order parameter, although the change was small compared with the experimental variation, indicating that the local environment of the spin label becomes less polar as the drug concentration is increased (Table 2).

DISCUSSION

One widely accepted theory of the molecular mechanism of action of general anesthetics suggests that they penetrate into, and cause a physical expansion of, the lipid phase of the nerve cell membrane [see review by Seeman (1)], although to explain why such an expansion should result in a reduction of synaptic transmission would require a greater knowledge of the molecu-

[3] B. Davis, personal communication.

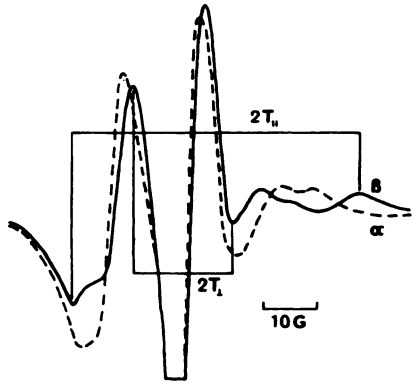


FIG. 2. *Electron spin resonance spectra of spin-labeled dipalmitoyl lecithin/cholesterol liposomes*

Liposomes contained either 3α- (marked α) or 3β- (marked β) hydroxy-5α-pregnane-11,20-dione at a concentration of 240 mmoles/mole of lecithin. The large difference in splitting between the two spectra is evident.

lar organization and function of the membrane than we possess at present. Seeman (1) has pointed out that anesthetics produce an increase in membrane volume approximately 10 times greater than the physical volume occupied by the anesthetic molecules dissolved in the lipid phase. Trudell and co-workers (4) used electron spin resonance techniques to measure the mobility of the hydrocarbon chains of phospholipid bilayers, and found that anesthetics increased the molecular freedom of the hydrocarbon core of the bilayer. The cell membrane is a lipid bilayer–protein complex, and anesthetic molecules could interact with any or all of the membrane components (3), but the hydrocarbon area of the lipid bilayer is one obvious site, accounting for the well-known correlation of anesthetic potency with lipid solubility and molecular volume. Over the volumes occupied by the low molecular weight anesthetics, the lipid phase would be virtually isotropic, and no marked dependence of activity on specific structural groupings or chirality [e.g., halothane (12)] would be expected.

The structure-action relationships exhibited by the nonvolatile anesthetics are more complex, however; for example, the enantiomers of hexobarbital produce different degrees of hypnosis in the rat at equal brain concentrations (13). In any series of compounds, anesthetic potency would be expected to depend on the ease of penetration to the active site, a property which correlates well with lipid solubility, though in a rather complex way (14), and on molecular size, compounded with any structure-dependent changes in ability to expand and disorder the lipid phase of the cell membrane. Comparisons of model systems with behavior in living organisms are most easily made with isomeric pairs of compounds, where these first two properties would be expected to be the same. Comparisons between two such pairs of steroids, differing only in the configuration of a hydroxyl group, show that the ability to disorder the hydrocarbon phase of the liposome bilayer correlates very well with anesthetic potency, with the active isomer producing a marked increase in disorder, whereas the inactive isomer does not. A similar disordering effect was produced by several other anesthetics, e.g., butobarbitone, octanol, and halothane. The magnitude of the effect is dependent on molecular size, and when, as suggested by Mullins (15), liposome concentrations are multiplied by the molal volume of the drug, roughly the same degree of disorder is produced by all the anesthetics at a given partial volume in the liposomes. The similarity between the effects of the volatile anesthetics and the high molecular weight, involatile anesthetics on liposome disordering, and the good correlation observed with potency *in vivo*, make it unnecessary to invoke the idea of a separate protein "receptor" to account for the greater structural specificity shown by the latter group of compounds.

The degree of structural specificity exhibited by a phospholipid/cholesterol bilayer would be expected to depend on the size of the molecule penetrating into it. For a small molecule, the hydrocarbon core of the bilayer would display no more structural selectivity than the simple model systems such as octanol/water, which is frequently cited in discussions of anesthetic behavior. It is known, however, that the structure of the hydrocarbon phase of the bilayer is complex: the hydrocarbon chains are not closely packed but display different degrees of disorder toward the

center of the membrane (9), and cavities are thought to be present (16). The steroid anesthetics are large molecules compared to the thickness of the lipid bilayer, and one steroid molecule at right angles to the plane of the membrane would penetrate halfway through the bilayer (17). The ring system of the two 3-hydroxy-5α-pregnane-11,20-dione isomers is in the all-*trans* configuration and is approximately planar, the isomers differing only in the configuration of the 3-hydroxy group. The active 3α-isomer has its hydroxyl group in the axial conformation, approximately at right angles to the plane of the rings, whereas in the inactive isomer that group is equatorial and approximately coplanar. One possible rationalization of the difference in activity between the two compounds would be that the formation of a hydrogen bond between the hydroxyl and polar groups at the bilayer surface causes a difference in the orientation of the two molecules in the bilayer.

The degree of disorder in natural membranes, the deviation from close packing of hydrocarbon chains, and the resultant production of cavities would depend on the nature of the fatty acids constituting the membrane (16). In this respect it is interesting that no significant difference in disordering effect was observed between egg lecithin and the fully saturated dipalmitoyl lecithin. The increase in disorder produced by the intrusion of a large anesthetic molecule would depend on its conformation in a complex way: unlike the conventional view of the interaction of antagonists with drug receptors, the disordering of the lipid phase, and the resultant anesthetic effect, would be dependent on the awkwardness of fit into the hydrocarbon matrix of the membrane.

Cholesterol, an important component of cell membranes, is chiral, and this, together with the asymmetry of lecithin, imposes a degree of chirality on the lipid phase of the membrane system. This would not be expected to be significant for small molecules, and in one of the few studies made on optically active volatile anesthetics it was found that the two enantiomers of halothane were equipotent (12). However, as with general structural effects, it is anticipated that the chirality of the membrane would be more important in interactions with large molecules such as the barbiturates, and marked differences in potency between enantiomeric pairs of barbiturates have been reported (13). Unfortunately, enantiomeric pairs of steroid anesthetics are not available, but it is expected that they would also exhibit stereospecificity in their biological effects.

From all this it is to be expected that it would be more difficult to predict the anesthetic potency of a large lipophilic molecule than it is for low molecular weight volatile compounds (18). The method of measuring the degree of liposome membrane disorder by ESR techniques would seem to be a useful screen for anesthetic activity, particularly when combined with measurements of lipid solubility.

REFERENCES

1. Seeman, P. (1972) *Pharmacol. Rev.*, **24**, 583–655.
2. Patel, A. R. (1970) in *Medicinal Chemistry* (Burger, A., ed.), p. 1314, Wiley/Interscience, New York.
3. Featherstone, R. M. & Muehlbaecher, C. A. (1963) *Pharmacol. Rev.*, **15**, 97–121.
4. Trudell, J. R., Hubbell, W. L. & Cohen, E. N. (1973) *Biochim. Biophys. Acta*, **291**, 328–334.
5. Trudell, J. R., Hubbell, W. L. & Cohen, E. N. (1973) *Biochim. Biophys. Acta*, **291**, 321–327.
6. Atkinson, R. M., Davis, B., Pratt, M. A., Sharpe, H. M. & Tomich, E. G. (1965) *J. Med. Chem.*, **8**, 426–432.
7. Wilkins, M. H. F., Blaurock, A. E. & Engelman, D. M. (1971) *Nat. New Biol.*, **230**, 72–76.
8. Keith, A. D., Sharnoff, M. & Cohn, G. E. (1973) *Biochim. Biophys. Acta*, **300**, 379–419.
9. Hubbell, W. L. & McConnell, H. M. (1971) *J. Am. Chem. Soc.*, **93**, 314–326.
10. Bondi, A. (1964) *J. Phys. Chem.*, **68**, 441–451.
11. Hubbell, W. L. & McConnell, H. M. (1969) *Proc. Natl. Acad. Sci. U. S. A.* **63**, 16–22.
12. Kendig, J. J., Trudell, J. R. & Cohen, E. N. (1973) *Anesthesiology*, **39**, 518–524.
13. Wahlstrom, G., Buch, H. & Buzello, W. (1970) *Acta Pharmacol. Toxicol.*, **28**, 493–498.
14. Hansch, C., Steward, A. R., Anderson, S. M. & Bentley, D. (1968) *J. Med. Chem.*, **11**, 1–11.
15. Mullins, L. (1954) *Chem. Rev.*, **54**, 280–323.
16. Shah, D. O. & Schulman, J. H. (1967) *J. Lipid Res.*, **8**, 215–226.
17. Vandenheuvel, F. A. (1963) *J. Am. Oil Chem. Soc.*, **40**, 455–471.
18. Ferguson, J. (1964) *Chem. Ind.*, 818–824.