BIOORGANIC CHEMISTRY 7, 437–445 (1978)

The Effect of Some Alkyl Derivatives of Cholesterol on the Permeability Properties and Microviscosities of Model Membranes[1,2]

A. K. LALA, H. K. LIN,[3] AND KONRAD BLOCH

The James Bryant Conant Laboratories, Harvard University, Cambridge, Massachusetts 02138

Received February 16, 1978

The properties of lecithin liposomes or vesicles containing a variety of sterols have been studied by measuring either the release of entrapped glucose or determining microviscosity by fluorescence depolarization of the probe diphenylhexatriene. Sterols containing alkyl substituents at C_3, C_4, or C_{14} were less effective in reducing glucose permeability or increasing microviscosity than cholesterol. 19-Norcholesterol was also less effective than cholesterol in raising membrane viscosity. These results support the hypothesis that the selective biological demethylation of lanosterol to a planar (α-face) structure optimizes the ability of the sterol molecule to condense the lipid phase of the membrane bilayer. Removal of an angular methyl group (C_{19}), a rare event in biological systems, has the opposite effect.

It is known from a variety of studies that sterols profoundly affect the physicochemical properties of both natural and artificial membranes (*1*). Cholesterol, the principal animal sterol, ordinarily predominates in sterol-containing membranes from animal cells (erythrocytes, plasma membranes) (*2*). Few attempts appear to have been made to replace cholesterol in natural membranes by structurally related sterols. On the other hand, since artificial membranes readily incorporate a wide variety of cholestane derivatives, useful model systems are available for defining some of the structural determinants for sterol effects on membrane fluidity or microviscosity. In general, only sterols having the planar A/B *trans* fused ring system of cholestane, an unsubstituted equatorial 3-hydroxyl group, and a branched aliphatic side chain alter membrane properties in the same manner as cholesterol (*3*).

Evidence based on a variety of physical techniques has been interpreted as showing that cholesterol entering the membrane aligns alongside the extended acyl chains of phospholipid, the isooctyl side chain pointing toward the monolayer interior and the sterol hydroxyl group toward the water–bilayer interface (*1*, *4*, *5*). Hydrogen bonding between the hydrophilic OH group and some polar element of the phospholipid head group at the membrane interface, such as the carbonyl ester oxygen, is another feature

[1] Dedicated to Professor William S. Johnson on the occasion of his 65th birthday.

[2] Supported by grants from the National Institutes of Health, the National Science Foundation, and the Eugene Higgins Fund of Harvard University.

[3] Present address: New York University Medical Center, New York, New York 10016.

0045–2068/78/0074–0437$02.00/0

of currently popular models (*6*, *7*). This view is supported by the invariable occurrence of free sterols in membranes, but it lacks direct experimental proof.[4]

The α-face of the rigid cholestane ring system is planar, and from space-filling models it is evident that this planarity is critical if not essential for the hydrophobic, fluidity-determining interactions between sterol and the proximal segment of the phospholipid acyl chains. Nonplanar sterols such as coprostane derivatives (A/B *cis*, kinked) do not duplicate the condensing effects of cholesterol on artificial membranes (*3*).

Certain aspects of sterol evolution, structure and function were considered in a recent speculative paper from this laboratory (*11*). The point was emphasized that during the late stages of cholesterol biosynthesis the three methyl substituents linked to the sterol α-face are invariably removed, whereas the two angular methyl groups at C_{18} and C_{19} are retained. Lanosterol or partially demethylated intermediates do not occur intracellularly in significant amounts (*12*). Removal of methyl groups at C_{14} and C_4, resulting in a planar α-face, was therefore attributed to evolutionary pressures rendering the sterol molecule more effective for membrane function in higher organisms. To test this hypothesis we have compared the glucose permeability of artificial membranes containing either cholesterol, lanosterol, or various methyl-substituted sterols. Both liposomes and vesicles prepared by sonication were used for this purpose. Moreover, microviscosities of membranes containing various sterols were determined by measuring fluorescence depolarization with diphenylhexatriene as a probe.

MATERIALS AND METHODS

Cholesterol (Sigma) was purified by way of the 5,6-dibromide and dried *in vacuo* before use. Commercial lanosterol (Sigma) was either purified by way of the 24,25-dibromide (*13*) or isolated after acetylation and chromatography on silica gel tlc plates impregnated with 12.5% silver nitrate. Lanosterol obtained by either method was more than 97% pure judged by gas–liquid chromatography. Lanosten-7-ol, a gift from Dr. R. B. Woodward, was 97% pure (glc) after two crystallizations from methanol–chloroform. 4,4-Dimethylcholesterol was synthesized according to Woodward *et al.* (*14*). Lophenol (4α-methylcholest-7-en-3β-ol) and 14-α-methylcholestanol were gifts from Dr. C. Djerassi. The following compounds were supplied by Drs. J. Nelson and T. W. Spencer: 4β-methylcholestan-3-β-ol, 4α-ethyl-4β-methyl-5α-cholestan-3β-ol, 4β-ethyl-4α-methyl-5α-cholestan-3β-ol and 4,4-diethyl-5α-cholestan-3β-ol. Their purities were 97, 87, 96 and 98%, respectively. 19-Norcholesterol was kindly provided by Dr. J. Mathieu. 3-α-Methylcholestanol was synthesized by the method of Barton *et al.* (*15*). Egg yolk lecithin was purified by a slight modification of the procedure of Singelton *et al.* (*16*). Sterols were tested for purity by gas–liquid chromatography with a Perkin–Elmer Model-900 instrument. A disc integrator was used to measure peak areas. The

[4] We have recently investigated this problem by ^{13}C nmr spectroscopy of vesicles prepared from dipalmitoyl-lecithin labeled with ^{13}C at the ester carbonyl groups. When cholesterol was incorporated into such vesicles only negligible changes in chemical shift, a slight increase in T_1 and a decrease in T_2 were observed (A. K. Lala, unpublished). Similar results have recently been obtained by others (*8–10*). These data do not support the postulated H-bonding of cholesterol to carbonyl ester oxygen.

column (2 ft) was packed with 3% OV-17 and operated at 235°C with a flow rate of 40 ml/min.

Preparation of Membranes

Liposomes containing sterols were prepared from egg lecithin and 5 mol% dicetylphosphate and assayed for release of trapped glucose by the method of Demel *et al.* (*3*). After dialysis to remove bulk external glucose, aliquots of the dialyzed liposomes were assayed enzymatically for entrapped glucose before and after incubation for 1 hr at 40°C. Lecithin vesicles were prepared by dissolving 20 μmol of phospholipid and the desired quantity of test sterol in chloroform. After evaporation of the solution to dryness *in vacuo*, 2 ml of a 1 m*M* solution of [1-^{14}C]glucose (2×10^6 cpm) in 10 m*M* NaCl were added to the residue, and the suspension was sonicated for 30 min at 4°C under nitrogen in a Branson sonicator equipped with a microtip. The sonicated solution was centrifuged at 105,000 *g* for 30 min at 4°C to sediment the larger multilamellar vesicles. Tests for oxidized unsaturated fatty acids in the remaining supernatant (*17*) were negative. The vesicle-containing solutions were applied to 15-ml columns of Sephadex G-50 (medium) that had been equilibrated with 1 m*M* glucose in 10 m*M* NaCl. Vesicles containing trapped [^{14}C]glucose eluted in the void volume (3 ml). A 1-ml aliquot of this solution was dialyzed against 10 ml of 1 m*M* glucose in 10 m*M* NaCl for 1 hr at 37°C. The amount of [^{14}C]glucose remaining inside the dialysis bag before and after dialysis was counted in a scintillation counter. Incorporation of all test sterols into the vesicles (void volume fraction of Sephadex G-50 chromatography) was complete. No sterol could be detected by glc in the sediment after centrifugation at 105,000 *g*. Data (Fig. 1) are expressed as percentages of glucose released per hour = 100 $(1 - G_A/G_B)$ where G_B and G_A are the amounts of glucose retained in vesicles before and after 1 hr of incubation, respectively.

Microviscosities were determined in an Elscint microviscosimeter, Model MV-1A. Egg lecithin and various quantities of sterol were dissolved in chloroform, and the solution was evaporated to dryness. To the residue 2 ml of 10 m*M* NaCl were added, and the suspension was sonicated for 30 min at 4°C under nitrogen in a Branson sonicator. To 1 ml of this sonicate (1 m*M* in lecithin) was added 1 ml of a 1 μM solution of diphenylhexatriene (DPH) in 10 m*M* NaCl, and the mixture was kept at 25°C for 1 hr. Fluorescence depolarization of DPH was then measured, and microviscosities were calculated according to Shinitzky and Inbar (*18*).

RESULTS

Experiments with Liposomes

In confirmation of the observations of Demel *et al.* (*3*), our results show a progressive retardation of the exit of entrapped glucose with increasing cholesterol content of the lecithin liposomes. At a molar 1 : 1 ratio of cholesterol to phospholipid liposomes only 5% of glucose was released in the course of 1 hr, compared to a 50% glucose release from sterol-free liposomes. Under the same conditions incorporation of lanosterol retards glucose exit only slightly (from 50 to 43%). The reduced lanosterol derivatives,

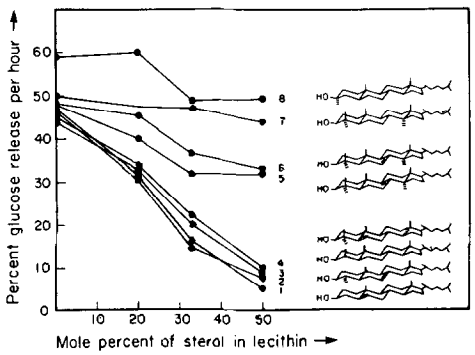

FIG. 1. Effect of alkyl derivatives of cholesterol on the release of glucose from lecithin liposomes after incubation for 1 hr at 40°C. Liposomes were prepared from egg lecithin and varying amounts of the following sterols: (1) cholesterol, (2) lophenol (4α-methylcholest-7-en-3β-ol), (3) 4-β-methylcholestanol, (4) 4,4-dimethyl cholesterol, (5) 24,25-dihydrolanosterol, (6) lanost-7-enol, (7) lanosterol, and (8) 3-α-methylcholestanol. The data for (8) were obtained with lecithin vesicles. The results represent averages of two or more experiments.

lanosten-7-ol and 24,25 dihydro lanosterol, restrain glucose release somewhat more than lanosterol, but it is clear that all the trimethylcholestane derivatives lower glucose permeability much less than cholesterol.

In the biological conversion of lanosterol to cholesterol, the methyl substituent at C_{14} is the first to be removed (*19*). Therefore sterols lacking the 14-α methyl group were chosen next for testing membrane behavior. 4,4-Dimethylcholesterol and the epimeric 4-monomethyl sterols, 4α-methyl-cholesten-7-ol (lophenol) and 4β-methylcholestanol, reduced glucose exit only somewhat less effectively than cholesterol (Fig. 1). While the precision of the method is probably too low for assessing the observed differences between the 4-methyl sterols and cholesterol, microviscosity data (see below) suggest that the membrane responses to 4,4-dimethylcholesterol and cholesterol are not identical. Increasing the size of the alkyl substituent at C_4 has a more marked effect. Thus, glucose exit from liposomes containing 4,4-diethylcholestanol occurs at approximately half the rate as from sterol-free membranes, i.e., about twice as rapidly as from membranes containing cholesterol (Table 1). When C_4 carries ones

TABLE 1

EFFECT OF 4,4'-DIALKYL STEROLS ON THE RELEASE OF GLUCOSE FROM LECITHIN LIPOSOMES[a]

Sterol	Glucose release (%) [1 hr at 40°C]
None	46.5
4,4-Diethylcholestanol	28.9
4α-Ethyl, 4β-methylcholestanol	16.5
4α-Methyl, 4β-ethylcholestanol	14.1

[a] As prepared, the liposomes contained sterol and lecithin in a molar ratio of 1:1. For details see experimental procedures.

methyl and one ethyl substituent, the reduction of glucose permeability approaches that shown by 4,4-dimethylcholesterol (Table 1).

Experiments with Lecithin Vesicles

Lecithin vesicles containing cholesterol, lanosterol, or 4,4-dimethylcholesterol gave qualitatively the same results as the corresponding liposome preparations. However, in the vesicle system the effects of cholesterol on glucose release were less marked than in liposomes (*20*). Nevertheless the difference in glucose permeability between vesicles containing cholesterol and vesicles containing lanosterol were still significant. The percentages of glucose released in 1 hr were 60% for sterol-free vesicles, 27% for membranes containing cholesterol (1 : 1), and 42% for membranes containing lanosterol (1 : 1). One alkyl-substituted sterol, 3α-methyl-cholestanol, was tested only in the vesicle system. Incorporation of this compound into the membrane produced no detectable reduction of glucose permeability (Fig. 1).

Microviscosity

Incorporation of increasing amounts of cholesterol into phosphatidyl choline vesicles progressively raises membrane microviscosity measured by fluorescence depolarization (*18*). With certain qualification this technique permits an independent appraisal of membrane "fluidity" as a function of membrane composition.[5] In Figs. 2 and 3 are shown the microviscosity changes of lecithin vesicles caused by incremental quantities of cholesterol and various cholestane derivatives. Cholesterol, above 20 mol%, raises the microviscosity sharply, whereas lanosterol and 3α-methylcholestanol, at all concentrations tested, cause only minor changes in microviscosity. The effect of 4,4-dimethylcholesterol on microviscosity significantly exceeds that of lanosterol, but is clearly less than that of cholesterol. This result distinguishes the behavior of the 4,4-dimethyl sterol from cholesterol much more clearly than the marginally different effects of these two sterols on glucose permeability (Fig. 1). The membrane response to the monoalkyl derivative 14-α-methyl cholestanol is of special interest. Up to 33% mol% this sterol raises microviscosity no more than lanosterol, the 4,4,14-trimethylcholestane derivative. At the highest concentration tested (42 mol%) there is some viscosity increase, but it remains far below that afforded by cholesterol. Also included in Fig. 2 are data for vesicles containing 19-norcholestanol. The results show that a sterol lacking one of the angular methyl groups (β-face demethylation) is substantially less effective in raising membrane viscosity than cholesterol itself. The principal conclusion that emerges from the data presented is that the presence or absence of alkyl substituents at the two faces of the cholestane ring system profoundly effects the physicochemical behavior of phospholipid vesicles. If cholesterol is the prototype of a functionally

[5] Recent reports (*21–23*) have questioned the validity of microviscosity data obtained from fluorescence depolarization measurements. Microviscosity is derived from Perrin's law of isotropic depolarization, which is strictly valid only in the case of exponential decay of both emission anisotropy and total emission. Nanosecond time-dependent fluorescence depolarization measurements of diphenylhexatriene in artificial membranes and oils indicate that decays are nonexponential. Thus the relationship between the steady-state fluorescene depolarization observed, as in the present study, and microviscosity may be more complex. Nevertheless microviscosity measurements of membranes containing different sterol structures may be valid for a qualitative comparison of membrane fluidity, but no significance should be attached to the absolute values of η given in Figs. 2 and 3.

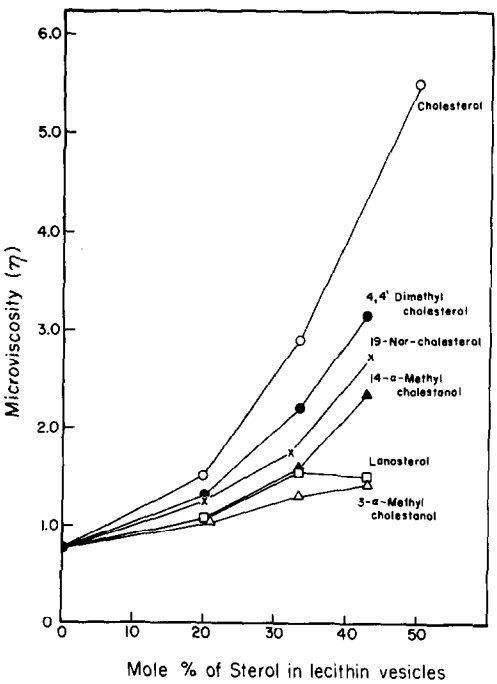

FIG. 2. Changes in microviscosity of egg lecithin vesicles containing increasing concentrations of various sterols. For details see experimental procedure.

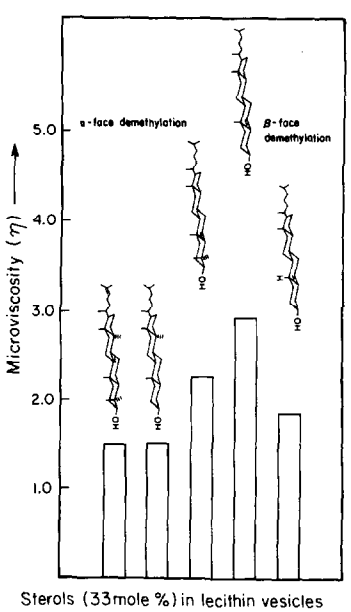

FIG. 3. Effect of sterol structure on microviscosity. The vesicles contained 33 mol% sterol. Data taken from Fig. 2.

competent membrane sterol, then this competence is diminished either by the presence or addition of extra methyl groups at the sterol α-face or elimination of an angular (β-face) methyl substituent.

DISCUSSION

Previous studies examining the relationship between sterol structure and function in artificial membranes have focused on the stereochemistry of the A/B ring junction, the requirement for a free and equatorial 3-hydroxy group, and the need for a branched aliphatic side chain (*3*). Moreover it has been widely assumed, although not stated explicitly, that the perfectly planar α-face of the fused cholestane system (provided by the axial hydrogens at C_3, C_5, C_1, C_7, C_{12}, and C_{14}) is essential for intimate hydrophobic interactions with phospholipid acyl chains (*1*). It necessarily follows from currently favored membrane models that substitution of any of the axial in-plane hydrogens by a bulkier axial group will weaken sterol–phospholipid interactions. The present experiments with artificial membranes were designed to test this prediction.

We have chosen two widely used approaches for determining sterol effects on artificial membranes: (i) the exit rate of glucose trapped in lecithin vesicles or liposomes [Much of the already existing knowledge of membrane behavior as modulated by sterols stems from this technique (*3*).]; and (ii) changes in microviscosity as manifested by rates of fluorescence depolarization of the probe dihexphenylhexatriene.

In interpreting our results we assume that glucose permeability and fluorescence depolarization both measure membrane fluidity, i.e., the mobility of bulk membrane lipids. Sterols introduced into liquid–crystalline membranes will render the system less fluid if the hydrophobic interactions between the sterol ring system and phospholipid acyl chains are sufficiently strong to immobilize the lipid components. Cholesterol is the standard of reference for sterol effects on membranes, not only because it (or side-chain-modified cholestane derivatives) is the predominant sterol component in eucaryotic membranes, but also because no other sterol, natural or synthetic, exceeds the ability of cholesterol to control membrane fluidity.

Lanosterol, the biological cholesterol precursor, and the synthetic 3α-methylcholestanol have the least effects of any sterols tested on either glucose permeability or microviscosity of lecithin vesicles. Neither of these two sterols solidifies the membrane. The structural feature that is shared by these two sterols is axial α-methyl substitution at positions which, according to the model, will disturb sterol α-face interactions. The behavior of two other compounds strengthens the conclusion that the failure of lanosterol to change membrane fluidity is primarily due to the obstructing methyl substituent at C_{14}. 14-α-Methylcholestanol increases vesicle microviscosity only slightly and to the same extent as lanosterol, at least at the lower sterol concentrations. Second, the behavior of 4,4-dimethylcholesterol is clearly distinct from that of lanosterol; i.e., when the 14-α methyl group is removed from the trimethylcholestane structure, the molecule becomes more "cholesterol-like", though not as effective as cholesterol itself in changing membrane fluidity (Figs. 1 and 2). Space-filling models suggest an explanation. The gem-dimethyl groups at C_4 of lanosterol and 4,4-dimethylcholesterol (4α-axial and 4β-equatorial) are located above the sterol α-plane and will therefore not interfere with fatty acyl-chain packing. Nevertheless, substituents

at C_4 appear to prevent optimal lipid–lipid interactions in regions of the membrane yet undefined. In accord with this view, 4,4-diethylcholestanol decreases glucose permeability much less than the 4,4-dimethyl compound, presumably because the bulkier ethyl substituents interfere more severely sterically with hydrophobic interactions that would otherwise occur, e.g., packing of fatty acyl chains to the sterol β-face. Since the conformation of the ring system and the orientation of the hydroxyl group are the same for lanosterol and cholesterol, we conclude that the inert behavior of lanosterol in artificial membranes is attributable primarily to the 14-methyl group which protrudes from the otherwise planar α-face, and to a lesser extent to the methyl substituents at C_4. The ^{13}C-nmr spectra observed for vesicles containing either cholesterol, lanosterol, or 4,4-dimethylcholesterol can be similarily interpreted (*20*). Mixed cholesterol–lecithin vesicles display no resonances attributable to sterol carbon atoms. The same is true for vesicles containing 4,4-dimethyl cholesterol. However, the ^{13}C-nmr spectra of lanosterol-containing vesicles show several resonances assignable to quaternary or methyl carbons. The spectroscopic data therefore indicate that lanosterol is much more mobile in the phospholipid bilayer than cholesterol or 4,4-dimethyl cholesterol, in agreement with the membrane permeability and microviscosity data presented here.[6]

The effect of 19-norcholesterol on vesicle microviscosity further defines the structural features that optimize sterol–phospholipid interactions in membranes. A molecule lacking an angular methyl group between rings A and B raises the viscosity far less than cholesterol. Thus, the bulk provided by at least one of the two angular alkyl substituents at the β-face appears to contribute significantly to the immobilization of cholesterol in the membrane and, by inference, enhances the effectiveness of fatty acyl chain binding to the sterol β-face.

Lanosterol, 4,4-dimethyl sterols, and 4-mono-methyl sterols are the normal cholesterol precursors in the animal organisms, but ordinarily they are not major membrane components. When lanosterol instead of cholesterol accumulates, e.g., in mutant lines that are defective in oxidative demethylation, such cells are not viable; they rapidly lyse and die (*25*). This observation supports our hypothesis that the metabolic removal of the "extra" methyl groups of lanosterol from the α-face occurred in response to evolutionary pressures yielding a sterol structure uniquely effective for membrane function. The rationalization of the cholesterol architecture can now be extended to some features of the sterol β-face. The fact that the angular methyl groups enhance viscosity and that they are retained, unlike methyl groups at the α-face, illustrates the remarkable efficiency and selectivity of processes that tailor a molecule for optimal biological function.[7]

[6] It is outside the scope of this paper to consider interactions between sterols and proteins in natural membranes. However, one observation by London *et al.* (*24*) is relevant to the present discussion. Employing the monolayer technique, these investigators have studied the binding of Folch–Lees protein, a major myelin protein, to cholesterol and various other sterols. They find that lanosterol and nonplanar sterols such as coprostanol are much less effective than cholesterol in increasing the surface pressure of monolayers injected with Folch–Lees protein. Thus the planarity of the α-face of cholesterol seems to be important also for sterol–protein binding.

[7] We are aware of the natural occurrence of 19-norsterols in primitive marine organisms such as sponges (*26*) and gorgonions (*27*). They appear to be formed by demethylation of cholesterol (*28*). It is not clear, however, whether these compounds are the major membrane sterols in these primitive animal cells. If they are, it could be argued that this modification was abandoned during the later evolution of animal cells.

Finally we wish to point out that our results are in accord not only with the usual membrane models that specify intercalation of sterol monomers between two fatty acyl chains, but also with the possible existence of sterol dimers.

Also, we recognize that membranes are dynamic, not static systems. Hence, any structural model that specifies atomic cholesterol–phospholipid interactions is a representation of events averaged statistically over time.

REFERENCES

1. R. A. DEMEL AND B. DEKRUYFF, *Biochim. Biophys. Acta* **457**, 109 (1976).
2. R. A. DEMEL, L. L. M. VANDEENEN, AND B. A. PETHICA, *Biochim. Biophys. Acta* **135**, 11 (1967).
3. R. A. DEMEL, K. R. BRUCKDORFER, AND L. L. M. VANDEENEN, *Biochim. Biophys. Acta* **255**, 321 (1972).
4. Y. K. LEVINE AND M. H. WILKINS, *Nature New Biol.* **230**, 69 (1971).
5. D. L. WORCESTER AND N. P. FRANKS, *J. Mol. Biol.* **100**, 359 (1976).
6. H. BROCKERHOFF, *Lipids* **9**, 645 (1974).
7. C. HUANG, *Nature (London)* **259**, 242 (1976).
8. P. L. YEAGLE AND R. B. MARTIN, *Biochem. Biophys. Res. Commun.* **69**, 775 (1976).
9. A. W. M. LANCEE-HERMKENS AND B. DEKRUYFF, *Biochim. Biophys. Acta* **470**, 141 (1977).
10. C. F. SCHMIDT, Y. BARENHOLZ, C. HUANG, AND T. E. THOMPSON, *Biochemistry* **16**, 3948 (1977).
11. K. BLOCH, "Reflections in Biochemistry" (A. Kornberg, B. L. Horecker, L. Cornudella and J. Oro, Eds.), pp. 143–150, Pergamon Press, Oxford, 1976.
12. P. B. SCHNEIDER, R. B. CLAYTON, AND K. BLOCH, *J. Biol. Chem.* **229**, 175 (1957).
13. K. BLOCH AND J. URECH, *Biochem. Prep.* **6**, 32 (1958).
14. R. B. WOODWARD, A. PATCHETT, D. H. R. BARTON, D. A. J. IVES, AND R. B. KELLY, *J. Chem. Soc.*, 1131 (1957).
15. D. H. R. BARTON, A. DA, S. CAMPOS-NEVES, AND R. C. COOKSON, *J. Chem. Soc.*, 3500 (1956).
16. W. S. SINGELTON, M. S. GRAY, M. L. BROWN, AND J. L. WHITE, *J. Amer. Oil Chem. Soc.* **42**, 53 (1965).
17. R. A. KLEIN, *Biochim. Biophys. Acta* **210**, 486 (1970).
18. M. SHINITZKY AND M. INBAR, *Biochim. Biophys. Acta* **433**, 133 (1976).
19. F. GAUTSCHI AND K. BLOCH, *J. Amer. Chem. Soc.* **79**, 684 (1957).
20. P. L. YEAGLE, R. B. MARTIN, A. K. LALA, H. K. LIN, AND K. BLOCH, *Proc. Nat. Acad. Sci. USA* **79**, 4924 (1977).
21. L. A. CHEN, R. E. DALE, S. ROTH, AND L. BRAND, *J. Biol. Chem.* **252**, 2163 (1977).
22. S. KAWATO, K. KINOSITA, JR., AND A. IKEGAMI, *Biochemistry* **16**, 2319 (1977).
23. R. E. DALE, L. Y. CHEN, AND L. BRAND, *J. Biol. Chem.* **252**, 7500 (1977).
24. Y. LONDON, R. A. DEMEL, Y. S. M. GEURTS VAN KESSEL, P. ZAHLER, AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta* **332**, 69 (1974).
25. T. V. CHANG, C. TELAKOWSKI, W. A. VANDENHEUVEL, A. ALBERTS, AND P. R. VAGELOS, *Proc. Nat. Acad. Sci. USA* **74**, 832 (1977).
26. L. MINALE AND G. SODANO, *J. Chem. Soc. Perkin Trans. 1*, 1888 (1974).
27. S. POPOV, R. M. K. CARLSON, A. WEYMANN, AND C. DJERASSI, *Tetrahedron Lett.*, 3491 (1976).
28. M. DE ROSA, L. MINALE, AND G. SODANO, *Experentia* **31**, 758 (1975).