

*PROTEIN CONFORMATION IN CELL MEMBRANE PREPARATIONS
AS STUDIED BY OPTICAL ROTATORY DISPERSION
AND CIRCULAR DICHROISM**

BY JOHN LENARD† AND S. J. SINGER

DEPARTMENT OF BIOLOGY, UNIVERSITY OF CALIFORNIA, SAN DIEGO (LA JOLLA)

Communicated by Martin D. Kamen, October 20, 1966

To elucidate the basic structure of biological membranes, experimental evidence concerning the detailed molecular organization of the proteins of membranes is required. This evidence has been almost totally lacking or ambiguous up to the present time. The Cotton effects due to the peptide bond ultraviolet absorption bands, as studied by ORD and CD, have recently proved extremely valuable in characterizing the molecular conformations of polypeptides and soluble proteins. In this paper, we report the results of ORD and CD studies in the ultraviolet on membrane preparations from human red blood cells and from *B. subtilis* in several solvent media.

Materials and Methods.—Membrane preparations: Red blood cell membranes were prepared by the method of Dodge *et al.*¹ from fresh human blood. Heme analyses¹ of several preparations showed uniformly that no more than 0.5% of the total protein present was hemoglobin. Membrane preparations from *B. subtilis*, strain 168, were prepared essentially by the procedure of Salton and Ehtisham-Ud-Din.² A low-speed centrifugation step was inserted to remove bacterial debris before the 39,000 × *g* centrifugation that brought down the membranes.³ Microscopic examination of both of these membrane preparations showed them to consist almost entirely of whole membranes. Solutions of each membrane preparation in 2-chloroethanol were prepared at 4° from lyophilized portions of the preparations.

Lipids were removed from red cell membranes by an isopropanol-chloroform extraction procedure.⁴ The precipitate which remained (mainly the protein portion of the membrane) was about 50% soluble in 2-chloroethanol. After removal of the solvent from the extracted membrane lipids, these were dissolved in 2-chloroethanol for ORD study. Lipid concentrations were determined by the organic phosphorus assay of Bartlett.⁵

Reagents: Reagent grade 2-chloroethanol from several commercial sources was purified by distillation under reduced pressure. Sufficient concentrated hydrochloric acid was added to the purified 2-chloroethanol to give a pH of 2.69–2.71 when 1 vol of 2-chloroethanol was diluted with 9 vol of water.⁶

ORD and CD measurements and calculations: ORD and CD measurements were carried out at room temperature with a Durrum-Jasco UV-5 instrument.⁷ To calculate $[m']$, the reduced mean residue rotation, and θ , the mean molar ellipticity, the protein concentration is required. This was determined from the OD₂₈₀ of 2-chloroethanol solutions prepared from measured amounts of the original membrane suspension. The OD₂₈₀ of the 2-chloroethanol solution was related to the protein content by total acid hydrolysis and amino acid analysis on a modified Beckman 120 analyzer.⁸ The protein concentration was then calculated by summing all the amino acid residues and assuming a mean residue weight of 114.

The refractive indices of the aqueous media were assumed to be identical to pure water.⁹ For 2-chloroethanol, the refractive index measurements of Foss and Schellman¹⁰ were extrapolated into the wavelength region of interest.

Results: The ORD spectrum of intact red cell membranes in dilute phosphate buffer is shown in Figure 1. For comparison, the spectra are shown of poly-L-lysine in the α -helix, β , and random coil conformations.¹¹ The membrane spectrum shows a peak and trough at wavelengths similar to the α -helix spectrum with the interesting exception that these wavelengths are shifted about 2 m μ toward the red. Parameters of the ORD spectra are listed in Table 1. The CD spectrum of intact

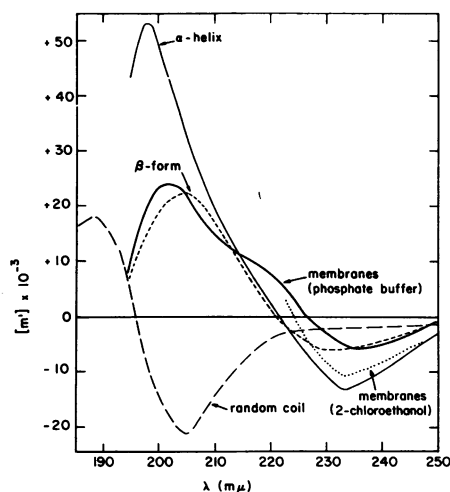


FIG. 1.—ORD spectra of red blood cell membranes suspended in 0.008 *M* phosphate buffer, pH 7.7 (thick solid line) and dissolved in 2-chloroethanol (dotted line). The ORD spectra of poly-L-lysine¹¹ in the α -helical (thin solid line), β - (small dashes), and random coil (large dashes) forms are plotted for comparison.

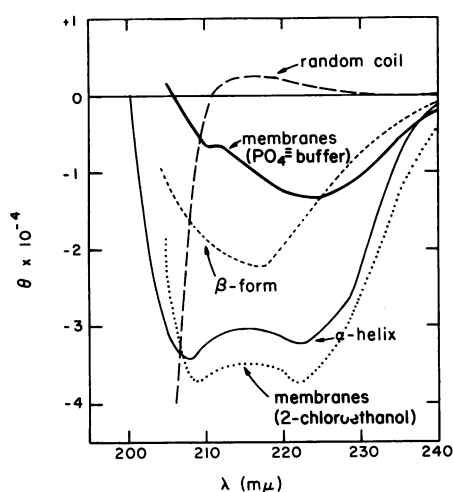


FIG. 2.—CD spectra of red blood cell membranes suspended in 0.008 *M* phosphate buffer, pH 7.7 (thick solid line) and dissolved in 2-chloroethanol (dotted line). The CD spectra of poly-L-lysine¹¹ in the α -helical (thin solid line) and β - (small dashes) forms, and of random-coil poly-L-glutamic acid¹⁴ (large dashes) are plotted for comparison.

red cell membranes in aqueous buffer (Fig. 2; Table 1) also most nearly resembles that of a partially α -helical protein, with the exceptions that: (a) again, about a 2- $m\mu$ shift to longer wavelengths is observed; and (b) the relative intensities of the two CD bands are altered. No Cotton effects were observed in either the ORD or CD spectra in the region from 240 to 300 $m\mu$.

Also listed in Table 1 are the distinguishing features of the ORD and CD spectra of *B. subtilis* membranes. The two kinds of membrane preparations exhibit spectra which are remarkably similar.

Before discussing the significance of these findings, it is necessary to document: (a) that these spectra do not reflect any artifacts arising from the turbidity of the membrane suspensions, and (b) that the spectra are in fact due to the protein conformation in the membrane and do not contain significant contributions from other optically active membrane components.

Two separate controls have eliminated the possibility that turbidity has produced artifacts in the observed spectra. First, BSA was added to each membrane suspension in sufficient amount that it made the dominant contribution to the observed rotation. Neither the position nor the magnitude of the ORD trough at 233 $m\mu$ and the peak at 198–199 $m\mu$ characteristic of BSA¹² were affected by the presence of the membranes. As a second control, the red cell membranes were suspended in a medium containing 90 per cent glycerol. The refractive index of 90 per cent glycerol is much higher than that of aqueous buffer, with the result that the membranes give an optically clear suspension in this solvent. This clear suspension showed ORD and CD spectra quite similar in their over-all characteristics to those obtained in the turbid aqueous suspension (Table 1). Related experiments were

TABLE 1
ORD AND CD CHARACTERISTICS OF MEMBRANE PREPARATIONS; COMPARISON WITH SYNTHETIC POLYPEPTIDES^{11, 14}

Material	Solvent	ORD Parameters				CD Parameters	
		λ_{in}	$[\eta]_{\text{in}} \times 10^{-4}$	$\lambda_{\text{crossover}}$	$[\eta]_{\text{max}} \times 10^{-4}$	λ_{min}	$\theta_{\text{min}} \times 10^{-4}$
Poly-L-lysine ¹¹	H ₂ O, pH 11 (α -helix)	232	-14.5	~222	+55.5	222.5	-33
	H ₂ O, pH 11 (β -form)	230	-6.3	~222	+23	208	-35
	H ₂ O, pH 7 (random coil)	205	-21.5	~196	+18.5	217.5	-23
Poly-L-glutamic acid ¹⁴	H ₂ O, pH 7 (random coil)	235-235.5	-5.8	226-7	+23.9	~200	~-50
	Human red cell membranes	234.5-235	-6.1	224-5	+28.3	224	-13.2
	90% Glycerol-10% 0.008 M phosphate, pH 7.7†	233	-6.2	220-1	+26.0	210	-6.1
<i>B. subtilis</i> membranes	0.05 M SLS	233-4	-10.4	224-5		223-4	-16.9
	100% 2-Chloroethanol	235-237.5	-5.2	227	+21.1	222	-12.9
	0.15 M NaCl 0.008 M † phosphate, pH 7.7	235-237	-7.2	226	+16.2	224	-7.8
<i>B. subtilis</i> membranes	75% glycerol, † 25% 0.15 M NaCl, 0.008M phosphate, pH 7.7	233	-6.4	221-2	+51.5	210-1	-10.1
	0.05 M SLS	233	-6.4	221-2		222	-20.7
	100% 2-Chloroethanol	233	-10.7	224		208	-23.8
						221-2	-32.4
						211	-28.5

* ORD measurements from 200 to 250 m μ and all CD measurements made with a 10-mm cell, 0.00245 gm protein/dl; ORD measurement from 190 to 220 m μ made with a 1-mm cell, 0.0098 gm protein/dl.

† Refractive index of glycerol taken as 1.49 at 235 m μ , and 1.76 at 200 m μ .

‡ All measurements made in a 1-mm cell, 0.0193 gm protein/dl.

performed with *B. subtilis* preparations, and similar results were obtained (Table 1). These controls demonstrate that turbidity has no profound influence on the spectra.

Red cell membranes contain three general classes of optically active materials: proteins, lipids, and polysaccharides. Polysaccharides account for only a small fraction of the total material in the membrane. The ORD characteristics of some blood group polysaccharides have been studied by other workers,¹³ and are quite different from those of the whole membrane. It thus seems unlikely that oligosaccharide contributes significantly to the membrane spectrum. To eliminate lipids as significant contributors, the lipids of red blood cells were extracted⁴ and dissolved in 2-chloroethanol. No significant optical rotation could be demonstrated in this solution, at the same concentration at which measurements on the intact membranes were made, down to 215 $m\mu$. Further, the ORD spectrum of the 2-chloroethanol-soluble portion of the nonlipid residue, chiefly protein, was identical to that of a 2-chloroethanol solution prepared from whole membranes. Thus, the ORD and CD spectra of these membrane preparations arise entirely from the protein in the membrane.

The ORD and CD characteristics of membranes dissolved in 2-chloroethanol (Figs. 1 and 2) and in 0.05 *M* SLS are listed in Table 1. In both media the membrane protein spectra are more typical of α -helical contributions; the shifts toward longer wavelengths which characterize the intact membrane preparations in aqueous buffer are absent.

Discussion.—Protein conformation: The ORD and CD spectra of the intact membrane preparations in aqueous buffer show features which strongly suggest the presence of some helical conformation in the membrane protein. In particular, the appearance of the two minima in the CD spectra is highly significant. The CD minima at 208 and 222 $m\mu$ observed with typically α -helical polypeptides and proteins have been attributed¹⁴ to the $\pi^0 - \pi^-$ and $n_1 - \pi^-$ electronic transitions of the peptide group, respectively. The presence of the two minima is completely dependent upon the helical arrangement of the peptide groups, although their relative magnitudes vary with the polymer and solvent. On the other hand, both CD minima for the aqueous membrane suspensions occur at slightly longer wavelengths than are typical for the α -helix, as does the minimum in the ORD spectrum. These spectral shifts are not artifacts of the turbidity of the suspensions, and they are uncommon enough to be noteworthy. They may be due to one of three possibilities: (1) the superposition upon the helical Cotton effect of Cotton effects at higher wavelengths, as has been observed with poly-L-tyrosine;¹⁵ (2) the presence of a polypeptide helix other than the α -helix;¹⁶ (3) the presence of an α -helix with spectral properties perturbed by neighboring lipid or protein in the membrane. The first possibility is eliminated by the fact that no appreciable Cotton effect is observed in the wavelength region from 240 to 300 $m\mu$. The second possibility cannot be eliminated, but there is no evidence that with ordinary polypeptides and proteins helical conformations other than the α -helix have thermodynamic stability and real existence.^{16, 17} With regard to the third possibility, it seems unlikely that the spectral perturbations are simply a solvent effect of surrounding lipid in view of the finding of Scanu¹⁸ that the ORD spectrum of a serum lipoprotein shows the 233- $m\mu$ minimum typical of the α -helix. On the other hand, that protein-protein interactions may be important was suggested to us by Cassim and Yang¹⁹ who observed

that the ORD and CD spectra of α -helical poly-L-glutamic acid undergo similar small red shifts on aggregation of the polypeptide chains in acid solutions. Strong dipole interactions between adjacent oriented helices might measurably perturb the spectral properties of these helices.²⁰ This suggestion is consistent with the observations that in 2-chloroethanol or in aqueous detergent the red shift is absent, since the membranes are molecularly dispersed in these media.

A reasonable conclusion, therefore, is that the Cotton effects observed with the aqueous membrane preparations reflect the presence of polypeptide α -helices which are in a special local environment which produces the red shift. The special environment could include adjacent interacting helices.

It is difficult to quantitate the fraction of the protein which is in the helical form in the intact membrane. From the value of $[m']$ of about -5000 to -6000 at the minimum of the ORD spectrum, and the still uncertain value of $-14,000$ to $-18,000$ for $[m']_{233\text{ m}\mu}$ for a completely α -helical polypeptide,^{11, 21-23} one may estimate that one quarter to one third of the membrane protein is helical. The remainder of the membrane protein is probably primarily in the random-coil form. The observed ORD spectrum of the aqueous membrane suspensions (Fig. 1) can be reasonably well accounted for as a summation of α -helical and random coil contributions; particularly significant in this connection is the inflection in the spectrum at about $220\text{ m}\mu$.¹¹ A mixture of only α -helix and β -form would not give such an inflection, as is clear from Figure 1. This is consistent with the fact that Maddy and Malcolm²⁴ found no evidence for a β -form in infrared spectral studies of D_2O suspensions of red cell membranes.

Structure of membranes: It is a remarkable fact that closely similar ORD and CD spectra are obtained with membrane preparations from such diverse sources as red blood cells and *B. subtilis*. In preliminary studies, the ORD spectra of membranes of *Mycoplasma laidlawii* (PPLO)²⁵ have also exhibited a pronounced minimum at about $235\text{ m}\mu$. Similar ORD spectra showing a red shift in the minimum have been obtained by other workers with plasma membranes from tumor cells²⁶ and chloroplast-lamellae fragments.²⁷ These spectra occur so frequently with membrane preparations, and yet are so unusual with simple protein systems, that they appear to reflect some common characteristic feature of the proteins of biological membranes. Therefore, we suggest that these spectra may be largely due to structural proteins, perhaps related to those which Green and his colleagues^{28, 29} consider to be ubiquitous and predominant protein components of all membranes. Such structural proteins might well be expected to exhibit similar conformational characteristics and interactions in different membranes. On the other hand, if the helical structures were associated with peripheral rather than the structural proteins of the membranes, one might expect these to vary widely in physical properties among different membranes.

The Danielli-Davson model,³⁰ as modified and refined by Robertson,³¹ is currently widely accepted as representing the basic ultrastructure of biological membranes, although the experimental basis of this acceptance has recently been seriously questioned.^{32, 33} In this model, the phospholipid is arranged as a bimolecular leaflet in a continuous two-dimensional sheet, and the protein is distributed as extended polypeptide chains on the top and bottom surfaces of the sheet. The protein is assumed to be held to the lipid primarily by electrostatic interactions.

If we incorporate in this model the proposition that about one quarter to one third of the structural protein of the membrane is α -helical, the model may be schematically represented as in Figure 3a.

On the other hand, this model is not consistent with recent information about the structure of proteins and other macromolecules. It is evident that hydrophobic interactions³⁴ play a key role in determining, for example, the specific conformations of globular proteins in water solution. This has been most clearly demonstrated with the two proteins, myoglobin and hemoglobin,³⁵ whose molecular structures have been determined in nearly complete detail. It has therefore been suggested^{28, 36, 37} that the native structures of membranes are likewise primarily determined by hydrophobic, rather than electrostatic, interactions involving their protein and lipid components. The Danielli-Davson-Robertson model does not maximize hydrophobic interactions. With the structural protein entirely in the unfolded state on the exterior surfaces of the membrane, a large fraction of nonpolar amino acid side chains would be exposed to the water phase, a condition which is thermodynamically unfavorable.³⁴ Furthermore, the helices shown in Figure 3a should have amino acid sequences in which approximately every second amino acid residue is ionic in order to interact both with water on the outer exposed surface of the helix and with the ionic heads of lipid molecules on the inner embedded surface of the helix. It seems unlikely that α -helices would be stable under such circumstances.³⁵

At the present time there is insufficient information available to validate any particular model of membrane structure. However, it may be useful conceptually to consider the alternative scheme shown in Figure 3b. This model is radically different from the Danielli-Davson-Robertson model in several respects, and in our opinion is more consistent with the principles of macromolecular structure as they are currently understood. It is proposed that: (1) The ionic and polar heads of the lipid molecules, together with all of the ionic side chains of the structural protein, are on the exterior surfaces of the membrane in van der Waals contact with the bulk aqueous phase. (2) Sequences of the structural protein consisting predominantly of nonpolar side chains are in the interior of the membrane, together with the hydrocarbon tails of the phospholipids and the relatively nonpolar lipids such as cholesterol. In particular, the helical portions of the protein are interior, where they are stabilized by hydrophobic interactions. There is no definite evidence, however, to indicate whether or not single polypeptide chains traverse the entire thickness of the membrane. (3) Structural proteins are characterized by unique amino acid sequences which specifically adapt them to interact with the lipid components of the

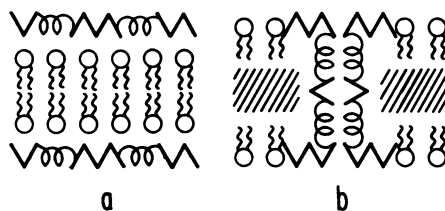


FIG. 3.—(a) The Danielli-Davson-Robertson unit membrane, as modified to include the data presented in this communication. The proteins on the outer surfaces of the membrane consist of helical (α) and random-coil (β) portions. The polar lipids (ϕ) are oriented in a bimolecular leaflet with their polar heads (\circ) facing out. (b) A generalized membrane as described in the text. Protein and lipids are symbolized as in (a). The cross-hatched areas are assumed to be occupied by relatively nonpolar constituents (hydrophobic amino acid residues or lipids). Single polypeptide chains are drawn to transverse the entire membrane, but there is no evidence bearing on this point.

membranes and the aqueous environment; the over-all conformations of the structural proteins are determined by these interactions.³⁶ (4) A structure such as shown in Figure 3b might be organized into subunits which could aggregate in two dimensions to form an intact membrane.³²

A detailed discussion of this model will be given elsewhere. Here, however, it is of interest to point out the relevance of the ORD and CD results with membranes dissolved in 2-chloroethanol. In this solvent, the membrane proteins are molecularly dispersed,³⁸ and are found to be substantially more helical (Figs. 1 and 2) than in their native conformations in intact membranes in aqueous solution. This is strikingly parallel to results obtained with simple globular proteins, such as ribonuclease,³⁹ in 2-chloroethanol and water. The same conclusions can apply in both cases:³⁶ (1) there are no intrinsic structural restraints preventing the membrane proteins, or ribonuclease, from adopting a much more helical conformation than they exhibit in their native physiological state; and therefore (2) the particular conformation shown by the native protein is probably the most thermodynamically favorable one, as determined by the sum of its interactions with the molecular environment.

Summary.—ORD and CD spectra of aqueous suspensions of membranes of human red blood cells and of *B. subtilis* have been obtained and validated. The spectra show features which indicate that about one quarter to one third of the membrane protein is in a helical conformation, with the remainder most likely in the random-coil form. Certain unusual aspects of these protein spectra are observed with several different membrane systems, and suggest that a common protein structural feature is involved. The observations are interpreted in terms of a model of membrane structure which is consistent with recent information about the conformation of proteins and other macromolecules.

Abbreviations used: ORD, optical rotatory dispersion; CD, circular dichroism; SLS, sodium lauryl sulfate; BSA, bovine serum albumin.

* This work was supported by grants AI-04255 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, and B6-1466E from the National Science Foundation.

† Advanced research fellow of the American Heart Association.

¹ Dodge, J. T., C. Mitchell, and D. J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).

² Salton, M. R. J., and A. F. M. Ehtisham-Ud-Din, *Australian J. Exptl. Biol. Med. Sci.*, **43**, 255 (1965).

³ We are indebted to Dr. Frank Young, Scripps Clinic and Research Foundation, La Jolla, California, for instruction and assistance in preparing these membranes.

⁴ Rose, H. G., and M. Oklander, *J. Lipid Res.*, **6**, 428 (1965).

⁵ Bartlett, G., *J. Biol. Chem.*, **234**, 466 (1959).

⁶ The acidity of the 2-chloroethanol was essential to its solvent properties. 2-Chloroethanol which gave a pH of 3.3 in 9 vol of water did not dissolve the membranes. Ethanol containing HCl was also ineffective as a solvent for membranes.

⁷ Preliminary ORD observations were made on a Bendix Polaromatic instrument. We are indebted to Prof. H. C. Urey and Dr. B. Nagy for the use of the instrument. We are also grateful to Dr. J. T. Yang for helping us with some preliminary CD measurements with the Durrum instrument in his laboratory.

⁸ Dus, K., S. Lindroth, R. Pabst, and R. M. Smith, *Anal. Biochem.*, **14**, 41 (1966).

⁹ Dorsey, N. E., *Properties of Ordinary Water Substance in all Its Phases*, ACS Monograph Series no. 81 (1940), p. 287, as quoted by Peterson, H. A., and J. F. Foster, *J. Biol. Chem.*, **240**, 3858 (1965)

¹⁰ Foss, J. G., and J. A. Schellman, *J. Chem. Eng. Data*, **9**, 551 (1964).

- ¹¹ Sarkar, P. K., and P. Doty, these PROCEEDINGS, **55**, 981 (1966).
- ¹² Blout, E. R., I. Schmier, and N. S. Simmons, *J. Am. Chem. Soc.*, **84**, 3193 (1962).
- ¹³ Beychok, S., and E. A. Kabat, *Biochemistry*, **4**, 2565 (1965).
- ¹⁴ Holzwarth, G., and P. Doty, *J. Am. Chem. Soc.*, **87**, 218 (1965).
- ¹⁵ Fasman, G. D., E. Bodenheimer, and C. Lindblow, *Biochemistry*, **3**, 1665 (1964).
- ¹⁶ Donahue, J., these PROCEEDINGS, **39**, 470 (1953).
- ¹⁷ Saludjian, P., and V. Luzzati, *J. Mol. Biol.*, **15**, 681 (1966).
- ¹⁸ Scanu, A., these PROCEEDINGS, **54**, 1699 (1965).
- ¹⁹ Cassim, J., and J. T. Yang, personal communication.
- ²⁰ Tinoco, I., and C. A. Bush, *Biopolymers Symp.*, **1**, 235 (1964).
- ²¹ Samejima, T., and J. T. Yang, *J. Mol. Biol.*, **8**, 863 (1964).
- ²² Harrison, S. C., and E. R. Blout, *J. Biol. Chem.*, **240**, 299 (1965).
- ²³ Breslow, E., S. Beychok, K. D. Hardman, and F. R. N. Gurd, *J. Biol. Chem.*, **240**, 304 (1965).
- ²⁴ Maddy, A. H., and B. R. Malcolm, *Science*, **153**, 213 (1966).
- ²⁵ A gift of Dr. Mary Pollock, Scripps Clinic and Research Foundation, La Jolla.
- ²⁶ Wallach, D. F. H., and E. R. Blout, personal communication.
- ²⁷ Ke, B., *Arch. Biochem. Biophys.*, **112**, 554 (1965).
- ²⁸ Green, D. E., H. D. Tisdale, R. S. Criddle, P. Y. Chen, and R. M. Bock, *Biochem. Biophys. Res. Commun.*, **5**, 109 (1961).
- ²⁹ Richardson, S. H., H. O. Hultin, and D. E. Green, these PROCEEDINGS, **50**, 821 (1963).
- ³⁰ Danielli, J. F., and H. A. Davson, *J. Cellular Comp. Physiol.*, **5**, 495 (1935).
- ³¹ Robertson, J. D., in *Cellular Membranes in Development*, ed. M. Locke (New York: Academic Press, Inc., 1964), p. 1.
- ³² Green, D. E., and J. F. Perdue, these PROCEEDINGS, **55**, 1295 (1966).
- ³³ Korn, E. D., *Science*, **153**, 1491 (1966).
- ³⁴ Kauzmann, W., *Advan. Protein Chem.*, **14**, 1 (1959).
- ³⁵ Perutz, M. F., J. C. Kendrew, and H. C. Watson, *J. Mol. Biol.*, **13**, 669 (1965).
- ³⁶ Singer, S. J., *Advan. Protein Chem.*, **17**, 1 (1962).
- ³⁷ Benson, A. A., and S. J. Singer, in *Abstracts*, 150th National Meeting, *Amer. Chem. Soc.*, Atlantic City, N. J. (1965), p. 8c.
- ³⁸ Lenard, J., and S. J. Singer, unpublished observations.
- ³⁹ Doty, P. in *Biophysical Science*, ed. J. L. Oncley (New York: Wiley, 1959), p. 112.