Infrared Spectra and the Chain Organization of Erythrocyte Membranes

Abstract. *Changes in the intensity of the 720 cm^{-1} band were followed in the infrared spectra of the human erythrocyte membrane and its total lipid extract, recorded at various temperatures. These changes are interpreted in terms of the configuration of the lipid hydrocarbon chains within the membrane. Effects of temperature, sonication, removal of lipids, and organic solvents on the amide I and II bands, associated with protein conformations, were also studied.*

In recent years (*1–3*) infrared spectroscopy has been employed to provide information about the protein conformation in cellular membranes. Use has been made of the C=O stretching frequency due to the amide I band at 1652 cm^{-1} and 1628 cm^{-1}. The band at 1652 cm^{-1} is associated with α-helical or random coil conformation, or both, while that at 1628 cm^{-1} is correlated with the β-configuration of polypeptides and proteins (*4, 5*).

Maddy and Malcolm (*1, 2*) showed, from the infrared spectra of air-dried films prepared from aqueous suspensions of ox erythrocyte ghosts or ghost protein, that no β-conformation was detectable. Wallach and Zahler (*3*) reported similar results for plasma-membrane fragments of Ehrlich ascites cells. We confirm these earlier observations, but also in this report emphasize additional features of membrane infrared spectra, including those pertaining to the lipid chain organization.

Human erythrocyte ghosts were prepared according to the method of Dodge *et al.* (*6*), fragmented by exposure to *p*H 8 at low ionic strength, desalted by dialysis, and freeze-dried. This material was sedimentable at 20,000*g* in 30 minutes from aqueous suspension. Sonic dispersion of freeze-dried membrane in H_2O was carried out with a soniprobe (*7*) at 20 kc/sec for 3 minutes in a lusteroid tube surrounded by ice-cold H_2O. This reduced the membrane to particles of microsomal dimensions which could be sedimented at 105,000*g* in 1 hour. Total lipid extract was obtained by the method of Reed *et al.* (*8*). Phospholipid was prepared by passing total lipid extract through a silicic acid column according to Borgstrom's method (*9*).

Spectroscopic samples were prepared by evaporating, in a vacuum desiccator, 2 to 3 mg of material, either as aqueous suspension or in organic solutions, layered on silver chloride windows made by impressing a metal mold (diameter, 1.5 cm) on disposable strips, to provide a uniform cross-sectional area of the dried films. Lipids were removed from the aqueous films by dipping them in a mixture of chloroform and methanol (1 : 1) for 30 minutes, rinsing them with the same solvent, and evaporating traces of the solvent under vacuum. Spectra were recorded on a Grubb-Parsons GS4 double-beam grating spectrometer, first at room temperature and subsequently between −110° to +110°C, without changing the position of the film in the optical path. A variable temperature cell (Research and Industrial Instrument Co.) was used for temperature studies.

To understand the molecular construction of cellular membranes, it is necessary to have more detailed information about the organization of the lipid hydrocarbon chains. This can be provided by the absorption band which occurs at 720 cm^{-1} (13.9 μ), which has been assigned to the main methylene (CH_2) rocking mode (*10*). It occurs in the spectra of many long-chain molecules and is present in both liquid and crystalline phases. It appears when there are four or more connected $\cdot CH_2$ groups organized in an all-*trans* planar configuration (*11, 12*). (We are referring to the planarity of the carbon skeleton of the hydrocarbon chains of the lipid and not to unsaturation.) The lipid components of erythrocyte membranes contain both saturated and unsaturated long-chain fatty acid residues so that there is considerable opportunity for this condition to be met. Presence or absence of the 720 cm^{-1} band in the spectra of biological membranes thus provides an indication of the organization of the lipid chains. We have therefore examined the changes in the intensity of the 720 cm^{-1} band with temperature in the infrared spectra of erythrocyte membranes and compared these changes with those obtained with the total lipid extracts.

A quantitative comparison of the infrared spectra of the membrane and its total lipid extract at room temperature is shown in Fig. 1. The spectrum of the lipid extract is typical of that obtained when phospholipids are in a liquid-crystalline organization (*13*). There are no differences in the peak positions associated with the polar groups of the lipid in the spectra of the lipid extract and the erythrocyte membrane. In particular the frequencies associated with the phosphate group at about 1225 cm^{-1} occur at the same frequency within the limits of experimental measurement.

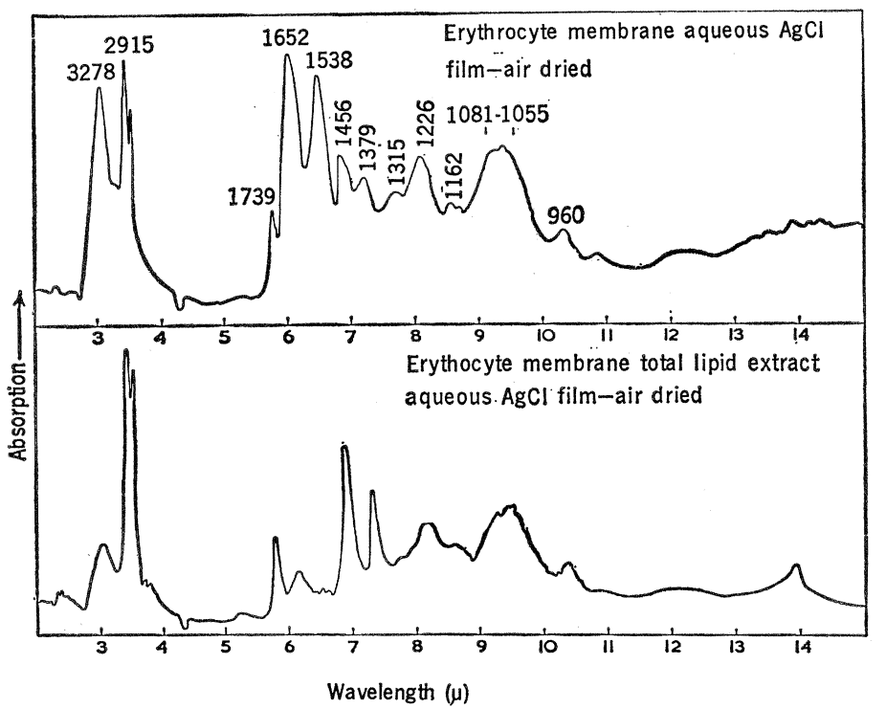

Fig. 1. Quantitative comparison of infrared spectra of the membrane and its lipid extract. Peak positional values are expressed per centimeter.

The 720 cm^{-1} band is prominent in the spectrum of the lipid extract but is extremely weak in the spectrum of the original membrane. Spectral changes associated with change of temperature of the membrane and of the total lipid extract are shown in Figs. 2 and 3. Cooling of the films below 0°C causes an increase in the intensity of the 720 cm^{-1} band in both spectra. Increase in intensity is more pronounced in the spectrum of the lipid extract, but at −110°C the band is quite prominent in the membrane spectrum. (The spectrum of a membrane film from which the lipid has been removed does not show a band at this position even at −110°C, confirming that it is specific for membrane lipid.) In the spectra of the lipid extracts, the 720 cm^{-1} band is distinguishable up to 55°C but gradually disappears at higher temperatures. The same variation in intensity occurs with membrane phospholipid alone. (All these changes are reversible.) Similar results are obtained whether membrane films are cast from H_2O or D_2O, and whether lipid films are prepared from organic solvents or from swollen aqueous suspensions.

Quantitative comparisons of the infrared spectra are not easy because of the difficulties in preparing samples of equivalent hydration and thickness. However, our results indicate: (i) At room temperature within the membrane there is little all-*trans* planar character of CH_2 groups of the lipid hydrocarbon chains. (This fact needs to be considered in future models of the erythrocyte membranes. The 720 cm^{-1} band is much more prominent in the spectrum of myelin.) (ii) At any given temperature at or below room temperature, there is less all-*trans* planar character within the membrane than in the corresponding lipid extract. (iii) The all-*trans* planar character of the CH_2 groups is temperature-dependent. (This assumes that the conclusions obtained with air-dried membranes can be compared with membranes in vivo.)

If our quantitative comparison is valid, can we explain the greater disorder of the chains in the membrane? The lipids in the membrane may be less tightly packed than in the isolated total lipid. A looser lipid packing would allow more movement of the methylene groups to flex and rotate away from the *trans* configuration. Another reason may be that the lipid hydrocarbon chains in the membrane tend to adopt conformations other than the all-*trans* conformation because of hydrophobic association with apolar amino acid residues of the membrane protein. The latter explanation could be consistent with some of our recent nuclear magnetic resonance spectroscopic results (*14*) that show a broadening of the chain signal which we believe to be the result of lipid-protein interaction (*15*). These possibilities are not necessarily exclusive and could be complementary.

When we examine the bands associated with protein conformation, we find in the infrared spectra of films cast from aqueous membrane supensions or from sonic dispersions (Figs. 1 and 2) at temperatures ranging from −110° and +110°C, a symmetrical amide I band, near 1652 cm^{-1}. This temperature variation does not produce any detectable extended β-configuration.

When lipid is removed from the membrane the spectrum shows, as expected, that the ester carbonyl stretching frequency at 1739 cm^{-1} is removed. (Other bands that diminish in intensity

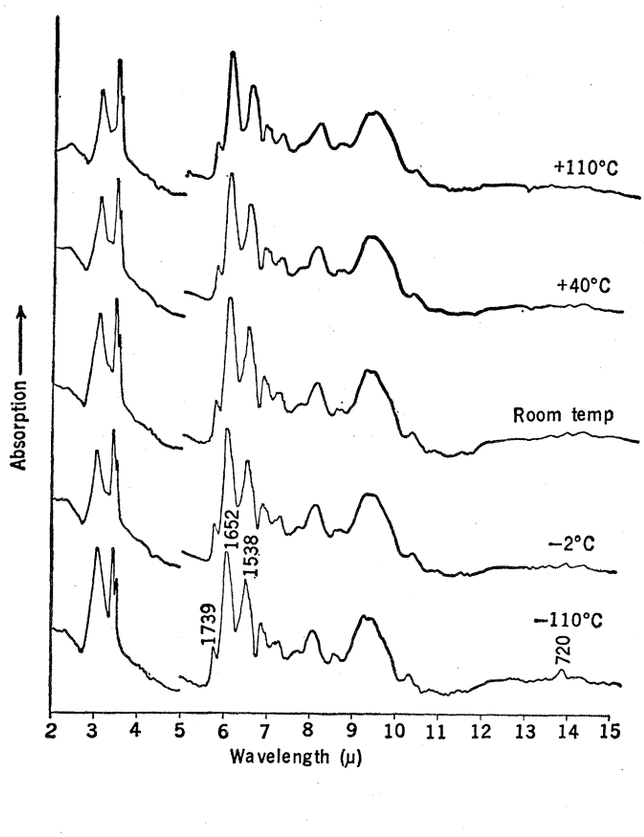

Fig. 2 (left). Infrared spectra of dried films of erythrocyte membranes at different temperatures.

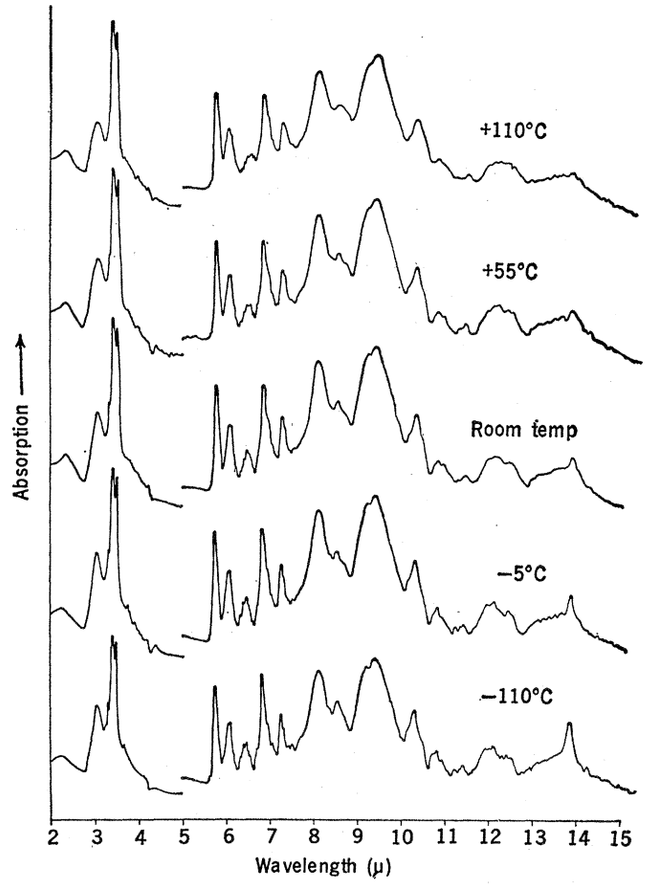

Fig. 3 (right). Infrared spectra of total lipid extract of erythrocyte membranes at different temperatures.

are those due to CH stretching at 2915 cm^{-1}, CH_2 and CH_3 bending between 1456 to 1459 cm^{-1}, P=O stretching between 1350 to 1250 cm^{-1}, P−O−C vibrations about 1050 to 980 cm^{-1}, and the amide II band near 1540 to 1550 cm^{-1}.) A reduction of the amide I band is observed only in the spectrum of films prepared from sonic aqueous membrane dispersion from which the lipid has been removed. It is possible that some sphingomyelin, which also contributes to absorption in the amide I and amide II regions, is less easily removed from the larger membrane fragments.

Heating the films, after removal of lipids, to temperatures as great as +150°C does not produce a detectable transition to a β-conformation. This may indicate that some residual lipid tightly bound to protein can protect the membrane protein from transition by thermal denaturation to a β-conformation. Bulk extraction of membrane fragments with a mixture of chloroform and methanol (1 : 1), according to the method of Reed *et al.* (*8*), leaves a residue which, when examined by infrared spectroscopy, shows a broad asymmetrical amide I band with peaks at 1652 cm^{-1} and a shoulder at 1628 cm^{-1}. Membrane films cast from organic solvents, for example, 2-chloroethanol, pyridine, or dimethyl formamide (Fig. 4), show a marked splitting of amide I band with peaks at 1652 and 1628 cm^{-1}, indicating a transition of a great portion of membrane protein to a β-configuration. A similar effect is observed with dimethyl sulfoxide or formic acid.

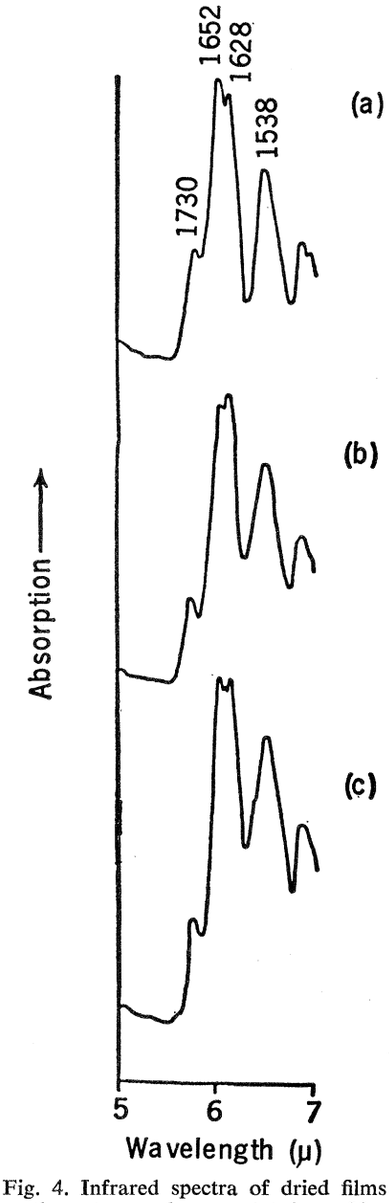

Fig. 4. Infrared spectra of dried films of erythrocyte membranes cast from 80 percent 2-chloroethanol (a), dimethylformamide (b), and pyridine (c). All were recorded at room temperatures.

Another feature of the infrared spectrum of the membrane is a shoulder at 1711 cm^{-1} associated with a lipid carbonyl group. The infrared spectrum of the lipid extract does not show this band. The presence of this band may be associated in some way with the interaction of lipid and protein. Further studies of the infrared spectra of other membranes will be useful in providing information about their structure.

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16. We thank Mrs. S. Glaister for assistance with the infrared spectra.

11 October 1967

Actinomycin D: Inhibition of Phospholipid Synthesis in Chick Embryo Cells

Abstract. *When chick embryo fibroblasts are exposed to actinomycin D for 30 minutes to 3 hours, there is progressive inhibition of phospholipid synthesis, so that by 3 hours the inhibition is 40 percent. All phospholipids are affected. Since this inhibition is twice as great as the inhibition of protein synthesis, some effects of actinomycin D previously ascribed to decreased protein synthesis must be reevaluated.*

Although widely used as an inhibitor of DNA-dependent RNA synthesis (*1*), actinomycin D has many effects which are not clearly related to this inhibition. These include inhibition of the replication of the RNA viruses poliovirus (*2*), influenza virus (*3*), and reovirus (*4*); inhibition of the replication of the RNA bacteriophage MS2 (*5*); interference with the assembly of the DNA bacteriophage T4 (*6*); inhibition of intestinal amino acid transport (*7*); and stimulation of the breakdown of messenger RNA (*8*). The rate of protein synthesis falls in cells treated with actinomycin D (*9*); this decrease is thought to be secondary to inhibition of RNA synthesis. Thus, the effects of actinomycin D on viral replication and amino acid transport have been ascribed to decreased protein synthesis. We now report that actinomycin D also inhibits phospholipid synthesis, and that the inhibition of phospholipid synthesis greatly exceeds the inhibition of protein synthesis in our system.

Chick embryo fibroblasts were prepared as previously described (*10*). They were exposed to actinomycin D (0.5 μg/ml) for 30 minutes to 3 hours, washed with Eagle's medium, and incubated with various radioactive substrates for 30 minutes; the cells were then scraped from the glass. The phospholipids were extracted by the method of Folch *et al.* (*11*). The incorporation of uracil-H^3 into RNA, and of leucine-H^3 into protein, was measured by the Schmidt-Tannhauser procedure (*12*).

Actinomycin D inhibited the incorporation of $^{32}P_i$ into phospholipid (Fig. 1). The magnitude of the inhibition increased with time of exposure to actinomycin D. Phospholipid synthesis was decreased by 10 percent in cells treated with actinomycin D for 15 minutes and by 25 percent in cells treated for 30 minutes, whereas protein synthesis was