47°C. But examination of the plates several days after their return to 25°C revealed that the fungus had resumed growth at the points where sterol had been applied, but not elsewhere.

To confirm this observation, Difco potato-dextrose agar plates were inoculated, near the edge, with fungus and incubated at 30°C for 16 hours until the colonies were 45 to 50 mm in diameter. On the advancing edge of the colony, toward the center of the plate, a few crystals of cholesterol or β-sitosterol were placed. The plates were incubated at 30°C for 2 hours and then placed in test chambers in pairs (one of the pair with, the other without, sterol) and bottom-to-bottom, with thermocouples taped between the plates at the sites of sterol application. Temperatures were recorded on an eight-point Brown potentiometer during the test periods. Following each test period the plates were returned to 25° or 30°C, and the extent of subsequent growth was recorded periodically.

The organism, without sterol, could resume growth after up to 2 hours at 43° to 44°C. With sterol, growth was stimulated; after exposure for 3 hours at 43° to 44°C or for shorter periods at higher temperatures, growth was resumed only at the points of sterol application. In these experiments the cultures took 1.5 hours to reach the 43°C level. In later work, where cultures were heated to 43°C within 2 minutes, the fungus was barely able to withstand 10 minutes at the test temperature without sterol.

At the low-temperature limits, a series of cultures on the same agar did not survive 48 hours' storage at 1° to 2°C unless provided with a suitable sterol.

The temperatures which the mycelium of this fungus, with and without sterols and other selected nutrients, can survive after heating and cooling at various rates have not been determined.

R. H. HASKINS

Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan

References and Notes

1. R. H. Haskins, A. P. Tulloch, R. G. Micetich, *Can. J. Microbiol.* **10**, 187 (1964).
2. J. W. Hendrix, *Science* **144**, 1028 (1964); C. G. Elliott, M. R. Hendrie, B. A. Knights, W. Parker, *Nature* **203**, 427 (1964); H. W. Klemmer and J. F. Lenney, *Phytopathology* **55**, 320 (1965); J. W. Hendrix, *ibid.* **55**, 790 (1965).
3. R. H. Haskins, *Can. J. Microbiol.* **9**, 451 (1963).
4. NRC contribution 8787.

7 September 1965

Protein Conformations in the Plasma Membrane

Abstract. *Infrared spectroscopy and optical rotatory dispersion have been used to test theories of structure of membrane protein. No evidence has been found to support the view that adjacent to the lipid there is a monolayer of protein in the β-conformation. The extracted protein appears to be a fairly typical globular protein with a low α-helical content.*

The classical Harvey-Danielli model (*1*) for the plasma membrane has formed the basis of discussion of its structure for the last 20 to 30 years. While the model was never intended to explain fully the varied behavior of the membrane, it remains the most acceptable basis for a theory of membrane structure. The state of the protein and the nature of its interaction with the lipid remain, however, almost totally unknown. The proposals of Danielli and his co-workers (*2*) were dependent on the current views on the conformation of proteins at interfaces. They cautiously concluded that proteins "on coming into contact with an oil-water interface, unroll into thin sheets, in a reversible manner" and that the membrane consisted of "a continuous film of lipoidal molecules, of which the two outermost layers are so orientated that the hydrated polar groups are in the oil-water interfaces, with a layer of protein molecules adsorbed on both of these interfaces" (*1*). Globular proteins were then believed to be adsorbed on these layers of "unrolled" or spreading proteins. Later workers have ascribed conformational states—usually the β-conformation—to the layers of protein associated with the lipid; for example, Kavanau (*3*) postulated that they "consist of unfolded and uncoiled fabric proteins in an extended β-conformation, possibly resembling a pleated sheet, with average spacings between the backbones of about 4.7 to 4.9 Å," together with some incompletely unfolded and uncoiled segments which provide regions of potential extensibility (*3a*).

The speculations on the arrangement of protein in the membrane have been greatly influenced by studies on the surface chemistry of proteins which implied that interfacial forces destroy the secondary and tertiary structure of proteins, producing interfacial films of unfolded open-chain molecules lacking any helical regions (*4*). However, infrared spectroscopy, and the measurement of rates of deuterium exchange in monolayers of synthetic polypeptides show that α-helices can exist at interfaces (*5*). Consequently, the whole question of the conformation of the protein in the membrane must be reopened bearing in mind that, since the conformation any protein adopts at any given time is contingent upon its environment, the conformation of membrane protein in vivo requires the presence of its associated lipid. As the techniques available for the study of conformation are mostly confined to substances in true solution or to crystals, the possibilities of a direct approach to membrane-protein conformation are limited.

Infrared spectroscopy is one of the few techniques yielding information on protein conformation that is applicable to the intact membrane, albeit in the form of a dry film. This technique reveals the presence of β-protein in the film by the position of the amide-I and -II bands which in the β state are observed at about 1630 and 1520 cm^{-1} as contrasted with about 1660 and 1540 cm^{-1} given by an α-helix or a random coil (*6*). The infrared spectrum of a film of hemoglobin-free ghosts of ox erythrocytes (*7*), dried in air at 20°C on a barium fluoride plate is shown in Fig. 1*a*. The amide-I band is at about 1660 cm^{-1} with no trace of a component at 1630 cm^{-1}. The amide-II band, while not so reliable for diagnostic purposes, also shows no indication of a β-conformation.

The ghost spectrum may be compared with that of a dry film prepared from a solution of the ghost protein obtained by *n*-butanol treatment of the ghost (*8*) (Fig. 1*b*, solid line). The differences between these two are attributable to the lipid component of the ghosts which can be estimated from a spectrum of the extracted lipid (Fig. 1*b*, broken line). The lipid makes only a small contribution to the spectrum of the ghost except in the region of the CH-stretching frequencies about 2850 cm^{-1}. About 50 percent of the extracted protein can be converted to a state possessing a β-form spectrum by heating with 50 percent ethanol at 70°C for 3 minutes (Fig. 1*c*).

It might be argued that the amount of β-protein required to form a layer on the lipid interface is, relative to other proteins in the membrane, too small to be detected by this method.

That this is not the case is shown by calculation of the amount of β-protein required to cover the lipid of the membrane when the lipid is arranged as a bimolecular layer. The phospholipid, which makes up 70 percent of the total lipid, (28 percent of the ghost by weight) will—if it is assumed that each molecule in the bilayer occupies an area of 70 Å^2 (*9*) and has a mean molecular weight of 900—give a total surface area of $2.1 \times N$ Å^2 (N, Avogadro's number) per 100 g ghost material. The remaining 30 percent of the lipid is mostly cholesterol, which (if cross-sectional area is 36 Å^2 per molecule) can add a further maximum value of $1.1 \times N$ Å^2 to the total area of the lipid. The actual contribution of cholesterol is probably less in that (i) the area of a mixed monolayer of phospholipid and cholesterol is less than the sum of the areas of the two pure components (*10*) and (ii) the cholesterol molecules might undergo hydrogen-bonding together, giving a complex solvated in the hydrocarbon chains of the phospholipid bilayer. The second possibility seems to have been overlooked by many workers. We are therefore left with a total surface area for the lipid of about $3 \times N$ Å^2. As the area per amino acid residue of a protein monolayer is about 17 Å^2, in either α- or β-conformation, the protein, which accounts for about 60 percent by weight of the ghost, would (if the mean residue weight of the protein is 120) form a monolayer of $8.5 \times N$ Å^2 per 100 g ghost. Therefore, 35 percent of the protein of the ghost would have to be in the β-conformation to form a complete layer over the lipid; if present, this percentage is high enough to be detected by infrared spectroscopy. The protein layer contiguous with the lipid has been considered insoluble, yet over 90 percent of the total ghost protein may be obtained in aqueous solution by butanol treatment of the ghosts (*8*).

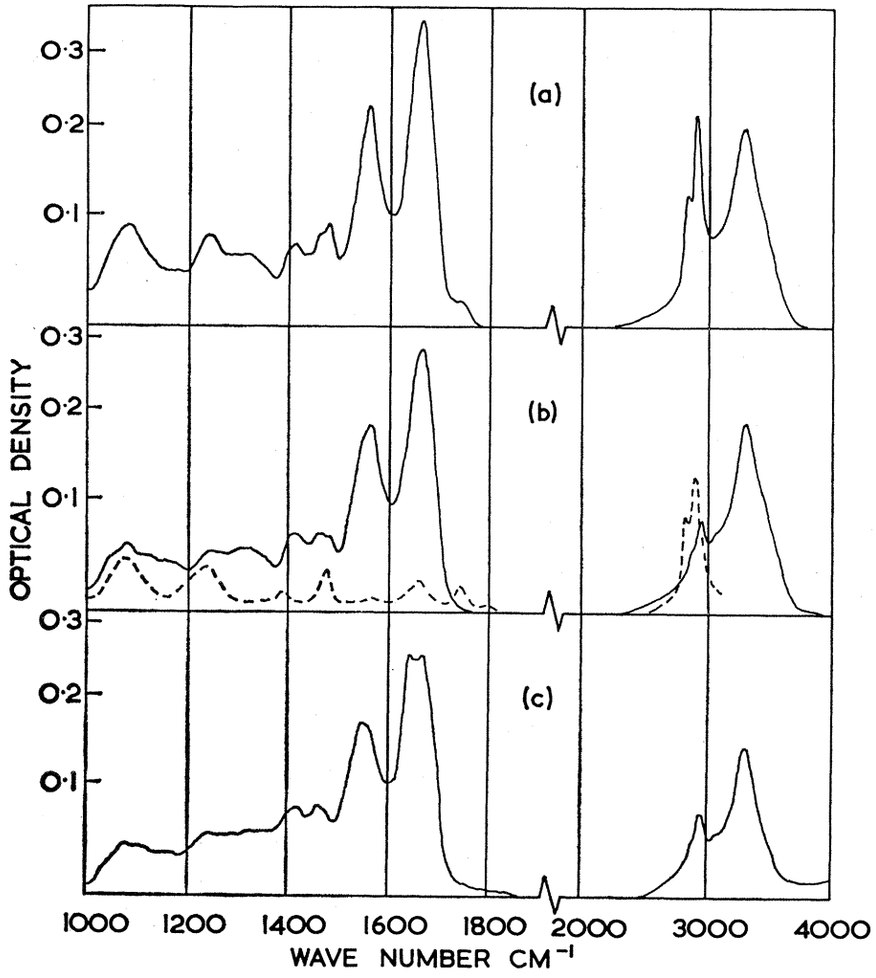

Fig. 1. Infrared spectra of air-dried specimens. (*a*) Red-cell ghosts. (*b*) Solid line, extracted ghost protein; broken line, estimated relative contribution of lipid to spectrum of ghost. (*c*) Ghost protein denatured with ethanol, showing about 50 percent of β-conformation.

The infrared analysis, while it excludes a significant amount of the β-form, does not distinguish between the α-helix and the random chain; but the amount of α-helix in the membrane, or, to be more precise, the membrane protein when it is dissolved in water, can be estimated from the optical rotatory dispersion of the aqueous solution. Removal of lipid from the protein may well change the latter's conformation from the state in vivo, although neither the butanol treatment nor the air drying used for preparing the infrared films would be expected to result in a decrease of any β-form which may have been present. Solutions of the protein in phosphate buffer at pH 7.0 (ionic strength 0.1) have therefore been examined with a polarimeter (Bellingham and Stanley Polarmatic 62) over the range 588 mμ to 246 mμ. The results can be fitted to the equation for proteins developed by Moffitt and Yang (*11*) with $\lambda_0 = 216$ mμ. For a mean residue weight of 120 the constant b_0, which can be interpreted as proportional to α-helical content, is found to be -90 compared to about -535 if the α-helix content was 100 percent, with $\lambda_0 = 216$ mμ. The most straightforward, but not unique, interpretation of these results is therefore that the protein contains about 17 percent of α-helix. However, the presence of about 8 percent by weight of sugars (sialic acid, hexosamine, and hexose), a trace of lipid, and possibly a small amount of the β-form not detectable by infrared spectroscopy, may cause this figure to be only a rough estimate.

These observations suggest that the membrane protein is a fairly typical globular protein perhaps modified by its sialic acid residues. How and to what extent the α-helical and random-coil components interact with the lipid still remain largely an open question, but the supposition of an extensive array of the protein in the β-conformation adjacent to the lipid is no longer justifiable. This removes a rather stable and perhaps intractable element from certain models, a trend in tune with recent concepts of the dynamic interrelation between different micellar states in the lipoidal phase.

A. H. Maddy
B. R. Malcolm
Departments of Molecular Biology and Zoology, University of Edinburgh, Edinburgh, Scotland

References and Notes

1. E. M. Harvey and J. F. Danielli, *Biol. Rev. Cambridge Phil. Soc.* **13**, 319 (1938).
2. J. F. Danielli and E. M. Harvey, *J. Cell. Comp. Physiol.* **5**, 483 (1934); J. F. Danielli, *Cold Spring Harbor Symp. Quant. Biol.* **6**, 190 (1938). H. Davson and J. F. Danielli, *The Permeability of Natural Membranes* (Cambridge Univ. Press, Cambridge, England, 1952).
3. J. L. Kavanau, in *Structure and Function of Biological Membranes* (Holden-Day, San Francisco, 1965), vol. 1, p. 146.

3a. Note added in proof: The protein conformation postulated by F. A. Vandenheuvel, *J. Amer. Oil Chem. Soc.* **42**, 481 (1965), in the myelin sheath is not a true β-conformation since it does not involve intermolecular peptide hydrogen bonds which are an essential feature of the β-conformation.

4. D. F. Cheeseman and J. T. Davies, *Advance. Protein Chem.* **9**, 439 (1954).
5. B. R. Malcolm, *Nature* **195**, 901 (1962); ———, in *Surface Activity and the Microbial Cell* (Society of Chemical Industry Symposium No. 19, London, 1965).
6. A. Elliott, E. M. Bradbury, A. R. Downie, W. E. Hanby, in *Polyamino Acids, Polypeptides and Proteins*, M. A. Stahmann, Ed. (Univ. of Wisconsin Press, Madison, 1962), p. 255.
7. J. T. Dodge, C. Mitchell, D. J. Hanahan, *Arch. Biochem. Biophys.* **100**, 119 (1963).
8. A. H. Maddy, *Biochim. Biophys. Acta* **88**, 448 (1964); ———, *ibid.*, in press.
9. V. Luzatti and F. Husson, *J. Cell Biol.* **12**, 207 (1962).
10. L. de Bernhard, *Bull. Soc. Chim. Biol.* **40**, 161 (1958).
11. W. Moffitt and J. T. Yang, *Proc. Nat. Acad. Sci. U.S.* **43**, 596 (1956).
12. We thank Dr. J. C. P. Schwarz, University of Edinburgh, for the use of the polarimeter. One of us (A.H.M.) is indebted to the Nuffield Foundation for financial support.

11 October 1965

Chronic Infection of Rodents by Machupo Virus

Abstract. *Machupo virus, the etiologic agent of human hemorrhagic fever in Bolivia, induced chronic asymptomatic infection in laboratory hamsters and colonized individuals of the peridomestic, wild, South American rodent,* Calomys callosus. *Viruria was detected for more than 500 and 150 days, respectively, in the two species. Chronic viremia was shown only for* Calomys. *Virus-neutralizing substances were present in parenterally infected adult animals, but not in animals born to, and in contact with, an infected female. Chronic infection in wild rodents may be an important mechanism in the natural history of Machupo and related virus infections.*

Machupo virus was first isolated in 1963 from the spleen of a patient from northeastern Bolivia who died of hemorrhagic fever (*1*). This agent was subsequently found to be serologically related to Junin virus, which has been associated with a similar hemorrhagic syndrome in Argentina (*2*), and to Tacaribe virus, originally recovered from bats on the island of Trinidad (*3*). Tacaribe virus, it had been previously demonstrated, shares antigens with Junin virus (*4*).

Table 1. Recovery of Machupo virus from parenterally infected adult hamsters at various times (in days) after inoculation. Symbols: +, virus isolated; −, virus not isolated; n, no test or unsatisfactory test.

3	6	8	9	10	12	14	22	509
				Urine				
−	−	+	+	+	+	+	+	+
				Feces				
−	n	−	+	−	+	−	n	n

Junin virus was isolated several times from wild rodents in Argentina (*5*), and it has been reported that arthropods might be important in transmission of infection (*6*). Machupo virus was recovered repeatedly from the small pastoral mouse *Calomys callosus* Rengger, 1830, captured in and near homes in the 1963 epidemic center of San Joaquin, Bolivia (*7*), but repeated attempts to detect the virus in many different hematophagous arthropods were unsuccessful.

In the course of laboratory studies with the Machupo virus in infant mice and hamsters, for which the agent had been shown to be pathogenic after parenteral inoculation, it was noted that uninoculated hamster dams frequently ate one or more of their offspring at about the time that virus-induced illness appeared in the litters. This interval was usually 6 to 10 days after administration of virus. Female white mice rarely cannibalized their young under similar circumstances. In two instances female hamsters became grossly ill with tremors and intermittent convulsions 19 and 21 days after they had eaten one or more infants of their demonstrably infected litters. Machupo virus was isolated from brain and spleen of both mothers by inoculation of infant hamsters. Complement-fixing (CF) antibodies to the virus also were detected in their serums. Fourteen asymptomatic hamster dams were bled 30 days after inoculation of their offspring with virus, and these serums were tested for CF antibodies. Each of these animals had eaten one or more of the infected infants. All serums contained CF antibodies for Machupo virus. These findings suggested that virus had been transmitted from infant to adult and that virus might be detectable for a considerable interval in infected adult animals.

To test this hypothesis, hamsters 5 to 6 weeks old were inoculated intraperitoneally (IP) with 10^4 $IHLD_{50}$ (infant-hamster lethal dose, 50 percent effective) of Machupo virus (strain Carvallo), that had been passed twice in hamsters. Urine and fecal specimens from asymptomatic individuals were obtained and assayed for virus in infant hamsters. Observations are summarized in Table 1. Fecal specimens were often toxic for infant hamsters; thus the results do not exclude the possibility of significant excretion of virus in feces. Urine samples obtained weekly from two animals uniformly continued to yield virus (509 days at this writing, 1 August 1965). Virus-neutralizing and CF antibodies were present 12 weeks after inoculation and were detected in similar titer in serums taken 46 weeks after infection. Systematic attempts to demonstrate viremia early in the course of infection were not made, but tests with whole blood were negative 32 and 47 weeks after inoculation.

In order to extend these observations a laboratory colony of *Calomys callosus* was established from 13 adult animals captured in San Joaquin, Bolivia. The original animals were subsequently killed, and their tissues were shown to be free of Machupo virus. Ten animals of the third laboratory generation, 5 to 7 weeks old and equally divided by sex, were inoculated intraperitoneally with $10^{5.0}$ $IHLD_{50}$ of the same virus pool used to infect the hamsters. All animals remained asymptomatic. Viruria was regularly demonstrable after the 15th day after inoculation. Titration of samples at intervals revealed continuous presence of 10^3 to 10^5 $IHLD_{50}$ of virus per milliliter of urine. Blood from seven of eight animals tested 6 weeks after virus inoculation contained virus, but neutralizing antibodies were detected in only one rodent at this time. At 20 weeks after inoculation six of seven animals had virus-neutralizing antibody titers from 1 : 4 to 1 : 256, and three of the positives had detectable viremia. Viruria was also present and has persisted through 153 days without end point.

Another pertinent observation was made as follows: in the course of an attempted virus titration experiment, a female *Calomys* delivered five infants 10 days after intraperitoneal injection of $10^{5.0}$ $IHLD_{50}$ of Machupo virus. These animals remained with the mother for 9 weeks; during this time