BBA 75886

A STUDY OF INTACT HUMAN ERYTHROCYTES AND THEIR GHOSTS USING STEARIC ACID SPIN LABELS

FRANK R. LANDSBERGER[a*], JOHN PAXTON[b] AND JOHN LENARD[a**]

[a] *Division of Endocrinology, Sloan-Kettering Institute for Cancer Research, New York, N.Y. 10021 and* [b] *Varian Associates, Springfield, N.J. 07081 (U.S.A.)*

(Received August 23rd, 1971)
(Revised manuscript received December 14th, 1971)

SUMMARY

The electron spin resonance spectra of intact human red cells and isolated red cell ghosts labelled with stearic acid spin labels have been compared. No differences can be detected in the lipid phase of these two systems within the accuracy of the measurements. However, the bovine serum albumin–spin label complex used to label the membranes is strongly adsorbed to isolated ghosts, but not to intact red cells. It is suggested that at least part of the adsorption arises from increased accessibility of the inner surface of the isolated ghosts to spin-labelled bovine serum albumin.

INTRODUCTION

The use of fatty acid spin labels of the Structure I (m,n) (Fig. 1) to investigate membrane structure has already yielded much important new information. Some facts which have been demonstrated using these spin labels (and phospholipids containing them) are the following: (1) the label is incorporated into several biological membranes and phospholipid bilayers in such a way that the hydrocarbon chain is preferentially oriented perpendicular to the plane of the structure surface[1–3]; (2) a marked flexibility gradient exists in bilayers and biological membranes, going from considerable rigidity close the surface to considerable fluidity in the interior[2–4]; (3) the rigidity of different biological membranes and synthetic bilayers can vary considerably and is influenced by the specific lipid composition of the system, especially the content of cholesterol and unsaturated fatty acids[5,6]; (4) various small molecules cause measurable alterations in the structure of red cell membranes[7]. The striking parallel between the fatty acid spin label spectra obtained from synthetic bilayers and biological membranes has provided confirmatory evidence for the existence of a bilayer structure in a number of biological membranes[2,5] and has also provided the basis for concluding that both oncogenic and non-oncogenic lipid-containing viruses possess bilayer structures[8,9].

It has recently been observed that the membranes of intact red cells differ markedly from isolated ghosts in their reactivity toward certain small covalently

* Present address: Department of Chemistry, Indiana University, Bloomington, Ind., U.S.A.
** Established Investigator of the American Heart Association.

bound molecules and in their susceptibility to digestion by hydrolases[10–13]. These observations raise questions regarding the extent to which membrane isolation alters the structure of the red cell membrane. In this paper we report observations on the spin-labelled lipid phase of red cell membranes in the intact cell and in the isolated ghosts. We conclude that the parameters measured by three stearic acid spin labels, I (12,3), I (1,14) and I (5,10) are not altered by isolation or by the different ionic strength buffers which are generally used to suspend intact cells and isolated ghosts. Some differences in the spectra of spin-labelled cells and ghosts are observed. It is shown that these differences arise from adsorption of the bovine serum albumin–spin label complex (used to introduce the spin label) to the ghosts but not to the intact cells.

MATERIALS AND METHODS

Human red cells were isolated from freshly drawn heparinized blood, washed in isotonic phosphate buffer several times, and the buffy coat removed by aspiration. Membranes were prepared by the method of Dodge *et al.*[14] and by the method of Steck *et al.*[15], both procedures giving identical results. Spin labels were purchased from Synvar, Palo Alto, Calif. and bovine serum albumin (less than 0.01 % fatty acid) from Sigma Chemical Co., St. Louis, Mo. Red cells and ghosts were spin-labelled by exchange from bovine serum albumin[2] as described by Landsberger *et al.*[8]. After labelling the samples were washed 3–5 times by suspension in appropriate buffer and recentrifugation.

The membrane protein spectrin[16] and most of the adsorbed bovine serum albumin were separated from the rest of the membrane protein and lipid by incubating the membranes with 9 vol. of 0.1 mM EDTA at pH 8 and 37°C for 15 min[17]. This procedure solubilizes spectrin and causes the membrane to break down into small closed vesicles which can be seen in the phase contrast microscope. Spectra were obtained at room temperature using a Varian E-12 electron spin resonance spectrometer. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out as previously described[18,19].

RESULTS

In Fig. 1, Spectra a, b and c, respectively, are of intact red cells, ghosts in isotonic buffer and ghosts in hypotonic buffer after incorporation of the indicated spin labels. With I (12,3) the three spectra appear identical, although very careful comparison reveals that the width of the outermost peaks is greater for ghosts than for cells. With I (1,14), on the other hand, the spectra of the ghosts show major new peaks on the high and low field sides. These peaks are indistinguishable from those in the spectrum of the bovine serum albumin–spin label complex which was used to label the membranes (Fig. 1f). That these peaks do arise from the complex is suggested by separating the membrane into two fractions by treatment with dilute EDTA. This procedure is known to solubilize the high molecular weight protein spectrin from the red cell membrane, while leaving the remaining proteins and all the lipids as small, closed sedimentable vesicles[17].

As shown by the gels in Fig. 2, a substantial amount of bovine serum albumin is adsorbed to the spin-labelled membranes (Fig. 2b), and most of this is released

into the supernatant upon treatment with EDTA (Fig. 2d). The spectra of the pellet and the supernatant (after concentration by lyophilization) of spin-labelled membranes after EDTA treatment is shown in Fig. 1, Spectra d and e. The supernatant in each case has a spectrum (Fig. 1e) which is essentially identical with that of the spin-label–bovine serum albumin complex (Fig. 1f). Superimposed on the 'broad line' spectrum in Fig. 1e are three sharp lines which arise from freely tumbling spin label. These 'liquid lines' are also seen in varying amounts in all the other spectra in Fig. 1.

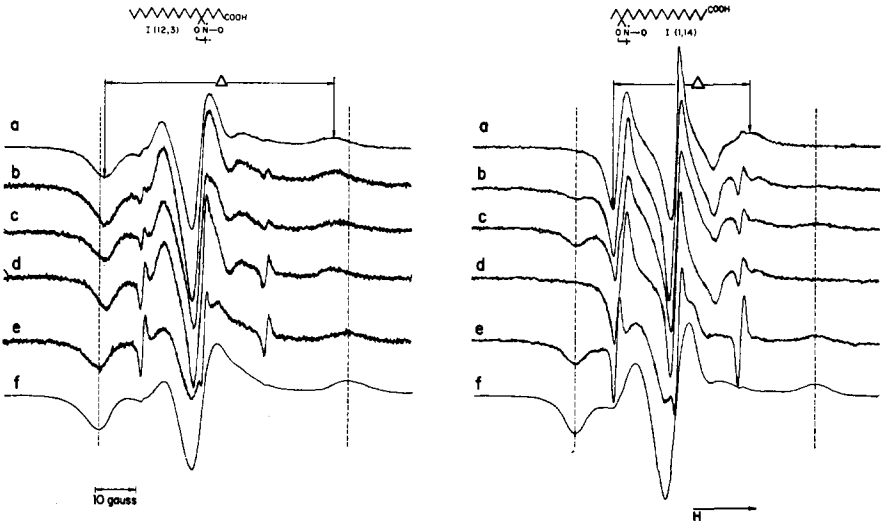

Fig. 1. Spectra of various red cell and ghost preparations labelled with I (12,3) and I (1,14) as indicated. (a) Intact red cells in isotonic phosphate buffer; (b) isolated ghosts in isotonic phosphate buffer; (c) isolated ghosts in hypotonic (0.005 M) phosphate buffer; (d) pellet obtained from isolated ghosts after treating with dilute EDTA (see text) and centrifuging; (e) supernatant obtained from isolated ghosts after treating with dilute EDTA (see text), centrifuging, and concentrating by lyophilization; (f) spin-labelled bovine serum albumin. Δ is the splitting between the low and high magnetic field peaks of the "broad line" spectrum. Vertical lines are drawn for comparison through the outermost peaks of f.

The spectra of the EDTA pellets (Fig. 1d) show no contribution from the bovine serum albumin–spin label complex, and bovine serum albumin is absent, or present in very small amounts, in the corresponding gels (Fig. 2c). These pellets contain all the membrane lipid and have spectra which are strikingly similar to those obtained from whole red cells and from intact ghosts once the contribution from the spin label–bovine serum albumin has been subtracted.

It may be noted that an alternative interpretation of the spectra in Fig. 1 might be that the spin label is bound directly to spectrin, giving rise to spectra which are indistinguishable from that of the bovine serum albumin–spin label complex, and that the presence of the bovine serum albumin in the EDTA superantant is fortuitous. This possibility has been ruled out by the following experiment. Whole red cells were labelled with I (1,14) as described, and ghosts were prepared from them. These ghosts were re-incubated with I (1,14) which had been dispersed as a film in the bottom of a test tube, in the absence of bovine serum albumin. This incubation increased the amount of spin label incorporated into the membrane approximately 15-fold, but in none of the samples was the spectrum detected which we have assigned

to the bovine serum albumin–spin label complex. Thus, spectrin-bound spin label is not responsible for the observed spectra.

Comparison of the spectra of I (1,14) labelled ghosts in isotonic and hypotonic buffers (Fig. 1, Spectra b and c) show that more spin label–bovine serum albumin appears to be bound in hypotonic buffer than in isotonic buffer. Furthermore, in

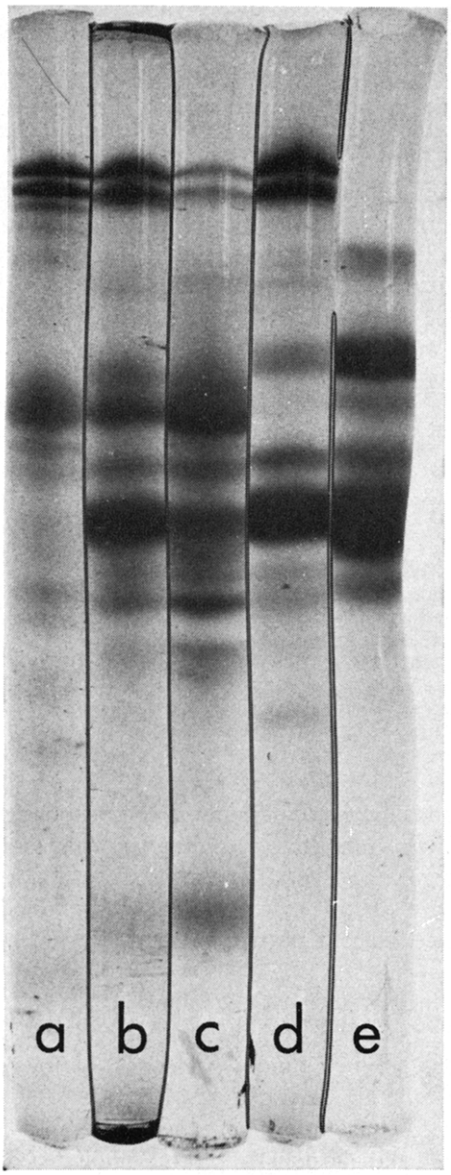

Fig. 2. Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate of various isolated ghost preparations. (a) Ghosts before spin labelling; (b) ghosts after spin labelling with I (12,3); (c) pellet obtained from spin-labelled ghosts after treating with dilute EDTA (see text) and centrifuging; (d) supernatant obtained from spin-labelled ghosts after treating with dilute EDTA (see text) and centrifuging; (e) spin-labelled bovine serum albumin.

other experiments, it has been found that binding of the spin label–bovine serum albumin complex in hypotonic buffer can be increased (as measured by increase in the intensity of the outer spectral peaks) by reacting the ghosts with $5 \cdot 10^{-4}$ M *N*-ethylmaleimide before spin labelling[19].

In Table I are shown the spectral splittings calculated for whole red cells and for the lipid phase of isolated ghosts. The splittings for each spin label are identical within experimental error. Several observations on red cells and isolated ghosts using spin label I (5,10) likewise revealed no detectable differences.

TABLE I

SPECTRAL SPLITTINGS, Δ (SEE Fig. 1) FROM WHOLE RED CELLS AND ISOLATED GHOSTS LABELLED WITH I (12,3) AND I (1,14)

	Spectral splitting (gauss)	
	I (12,3)	*I (1,14)*
Intact cells	55.4 ± 0.3	33.0 ± 0.5
Ghosts	56.2 ± 0.8	31.9 ± 0.8

DISCUSSION

In this paper we have presented evidence that the interior of the lipid phase of red blood cell membranes is not measurably altered by the procedures used to prepare and isolate ghosts. On the other hand, isolated ghosts bind bovine serum albumin tightly while intact red cells do not. This binding may be due to the accessibility of the inner surface of the membrane to large molecules, or to an alteration of the membrane surface, or both. Increased accessibility of the bovine serum albumin–spin label complex to the inner surface of the isolated ghosts is suggested by the fact that *N*-ethylmaleimide-treated ghosts show enhanced binding. Since about one-half of the reactive sulphydryl groups of the membrane are in spectrin[19], and since spectrin is known to be located on the inner surface of the membrane[20,21], at least part of the bovine serum albumin–spin label complex is assumed to be adsorbed on the inner surface. This thus provides an additional indication that the inner surface of the isolated ghost is freely available to large molecules in the medium, in this case protein molecules which have no hydrolytic activity.

From the data in Table I, two observations can be made. First, the superposition of the spectrum from the bovine serum albumin–spin label complex on the membrane spectrum does not alter the observed spectral splittings arising from membrane-bound spin label. Second, the adsorption of the bovine serum albumin–spin label complex to the membrane does not measurably alter the structure of the membrane lipid phase. As noted above, the outermost peaks of the I (12,3) labelled ghosts (Fig. 1, Spectra b and c) are slightly broader than those from the intact cells (Fig. 1a) due to the contribution from the bovine serum albumin–spin label complex. The possibility of this kind of spectral alteration by adsorption of the spin-labelled bovine serum albumin to membranes should be borne in mind in future studies.

ACKNOWLEDGEMENTS

This research was supported in part by Public Health Service grant CA-08748, National Science Foundation grant 6B-19797 and a grant from the Cystic Fibrosis Research Foundation.

REFERENCES

1 L. J. Libertini, A. S. Waggoner, P. C. Jost and O. H. Griffith, *Proc. Natl. Acad. Sci., U.S.*, 64 (1969) 13.
2 W. L. Hubbell and H. M. McConnell, *Proc. Natl. Acad. Sci., U.S.*, 64 (1969) 20.
3 P. Jost, L. J. Libertini, V. C. Hebert and O. H. Griffith, *J. Mol. Biol.*, 59 (1971) 77.
4 W. L. Hubbell and H. M. McConnell, *J. Am. Chem. Soc.*, 93 (1971) 314.
5 S. Rottem, W. L. Hubbell, L. Hayflick and H. M. McConnell, *Biochim. Biophys. Acta*, 219 (1970) 104.
6 H. M. McConnell and B. G. McFarland, *Ann. N.Y. Acad. Sci.*, in the press.
7 W. L. Hubbell, J. C. Metcalfe, S. M. Metcalfe and H. M. McConnell, *Biochim. Biophys. Acta*, 219 (1970) 415.
8 F. R. Landsberger, J. Lenard, J. Paxton and R. W. Compans, *Proc. Natl. Acad. Sci., U.S.* 68 (1971) 2579.
9 F. R. Landsberger, R. W. Compans, J. Paxton and J. Lenard, *J. Supramol. Structure*, in the press.
10 M. S. Bretscher, *J. Mol. Biol.*, 58 (1971) 775.
11 W. W. Bender, H. Garan and H. C. Berg, *J. Mol. Biol.*, 58 (1971) 783.
12 M. S. Bretscher, *Nature New Biol.*, 231 (1971) 229.
13 B. Roelofson, R. F. A. Zwaal, P. Comfurius, C. B. Woodward and L. L. M. Van Deenen, *Biochim. Biophys. Acta*, 241 (1971) 925.
14 J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
15 T. L. Steck, R. S. Weinstein, J. H. Straus and D. F. H. Wallach, *Science*, 168 (1970) 255.
16 S. L. Marchesi, E. Steers, V. T. Marchesi and T. W. Tillack, *Biochemistry*, 9 (1970) 50.
17 G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2606.
18 J. Lenard, *Biochemistry*, 9 (1970) 1129.
19 J. Lenard, *Biochemistry*, 9 (1970) 5037.
20 V. T. Marchesi, E. Steers, T. W. Tillack and S. L. Marchesi, in G. A. Jamieson and T. J. Greenwalt, *Red Cell Membrane, Structure and Function*, Philadelphia, Pa., 1969, p. 117.
21 G. L. Nicholson, V. T. Marchesi and S. J. Singer, *J. Cell Biol.*, 51 (1971) 265.