Biochimica et Biophysica Acta, 426 (1976) 697–702

BBA 77242

ON THE USE OF THE SPIN LABELING TECHNIQUE IN THE STUDY OF ERYTHROCYTE MEMBRANES

D. A. BUTTERFIELD*, **, C. C. WHISNANT and D. B. CHESNUT*

P. M. Gross Chemical Laboratory, Duke University, Durham, N.C. 27706 (U.S.A.)

(Received August 1st, 1975)

SUMMARY

ESR spectra and scanning electron micrographs of human erythrocytes spin labeled with the conventional stearic acid nitroxide substituted at the 5-position have been obtained over a range of label-to-lipid ratios. While morphological changes as previously reported (Bieri, V. G., Wallach, D. F. H. and Lin, P. S. (1974) Proc. Natl. Acad. Sci. U. S. 71, 4797–4801) are reproduced, it is shown that at label-to-lipid ratios of 1 : 10 or less the basic ESR spectrum is not significantly affected. At low label concentrations the spin labeling technique is a viable one and can be used to investigate membrane properties.

INTRODUCTION

The spin labeling technique has been well established as a valuable tool in the elucidation of biological systems since its introduction by McConnell and his co-workers in the mid-1960's. The multitude of publications and review articles (for example refs. 1–4) shows that it has been applied with success to a variety of systems. Its limitations have been studied, its short-comings exposed and, generally, its cautious use has been heeded. Newer techniques such as improved NMR studies [5] have been carried out, which involve a lower magnitude of perturbation; still, the utility of the spin label technique remains. Any foreign probe molecule will perturb a system, but if the labeling technique is properly handled much valuable information is derivable.

Recently, Bieri et al. [6] have carried out a study in which the perturbing effects of nitroxide spin labels on the morphology of human erythrocytes are investigated. Some of the more common membrane spin labels were studied over a range of concentration and clearly observable changes in the external morphology of the cell were observed. There is no question of the general validity of this investigation, but it did seem to us that it did not address the apprproiate question insofar as the electron

Abbreviation: 5-doxylstearic acid, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxozolidinyloxyl.

* Requests for reprints may be addressed to either of these authors.

** NIH Postdoctoral Fellow, 1974–75. Present address: Department of Chemistry, University of Kentucky, Lexington, Ky. 40506, U.S.A.

spin resonance technique is concerned. Since these authors did not include the ESR spectra of the altered cells, it is possible that the changes they observed may be interpreted by others as invalidating this particular use of ESR. The question we wish to address in this short paper is whether the information contained in the ESR spectra at the low label concentrations usually employed is or is not reflective of at least some of the true physical attributes of the erythrocyte membrane. According, we have reinvestigated human erythrocytes in a range of label concentrations overlapping that of Bieri et al. [6]. Our findings suggest that the morphological changes observed are not reflected in the ESR spectra at low label concentrations and, more importantly, that whatever model of molecular motion one wishes to employ to analyse the ESR results valid data are obtainable at sufficiently low molar ratios of spin label-to-lipid.

EXPERIMENTAL

Freshly drawn heparinized blood from a single healthy male donor was used in all experiments. The blood was centrifuged at $3500 \times g$ for 10 min and washed with phosphate-buffered saline (150 mM NaCl, 5 mM phosphate, pH 8.0) four times, care being taken to remove the buffy coat.

A thin film of label (Syva Associates) was prepared by placing 0.25 ml of chloroform containing various concentrations of 5-doxylstearic acid in a small vial and evaporating the chloroform with a stream of N_2. 0.5 ml of a 50 % hematocrit solution of cells in phosphate-buffered saline (containing $2.7 \cdot 19^9$ cells [7]) was added and the vial shaken at room temperature for 15 min. ESR spectra were recorded on a standard Varian V-4502 12-inch system with care being taken to avoid modulation and saturation broadening.

1 h after labeling, each cell suspension was centrifuged to remove unlysed cells, and the amount of hemoglobin in the supernatant was determined by measuring the absorbance at a wave length of 541 nm in a Beckman DB-G spectrophotometer. The fraction of cells lysed was calculated from the hemoglobin released by 100 % lysis of the appropriate volume of packed cells with hypotonic phosphate buffer.

In order to observe the labeled erythrocytes by scanning electron microscopy two drops of the spin-labeled cells were fixed in 10 ml of isotonic 5 mM phosphate buffer (pH 7.2) containing 2 % gluteraldehyde. The fixed cells were washed twice with de-ionized water and once with propylene oxide, air dried at room temperature and shadowed with gold/palladium (60 : 40). The micrographs were obtained at magnifications of 1000–7000 × with a JELCO model JSM-S1 scanning electron microscope.

RESULTS AND DISCUSSION

We have chosen to discuss our experimental arrangement in terms of the spin label-to-lipid ratio, a physically important quantity; in our investigation we cover a range of 1 : 83–12 : 1, while Bieri et al. [6], in their study, employ ratios of 1 : 25 500–39 : 1. Since the label employed is not soluble in buffer to any significant extent and since none of it remains in the vial after treatment with the cells, we presume all the label to be incorporated into the cell membrane. We use the averaged data given by van Deenen and de Gier [8] for lipid composition of human erythrocytes and, in order to calculate a molecular ratio, use cholesterol, phosphatidylcholine and *N*-acetyl-

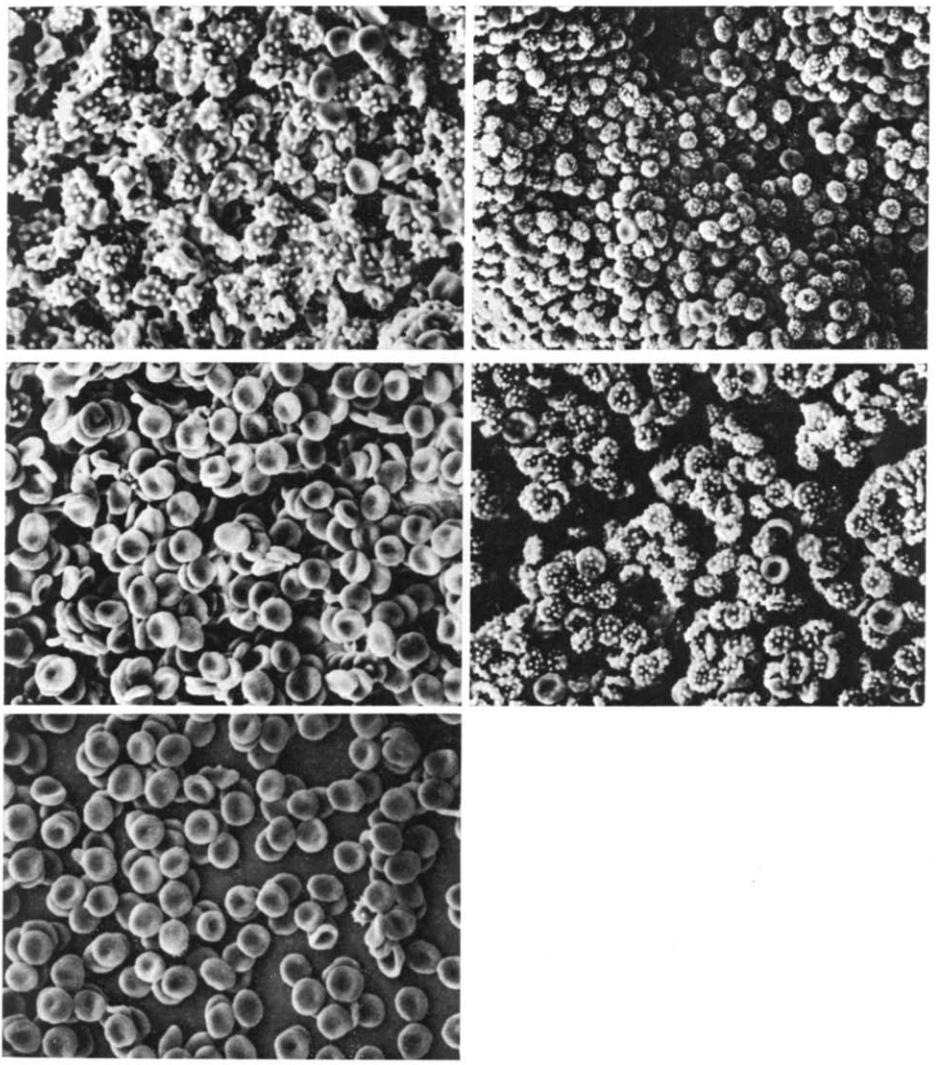

Fig. 1. Scanning electron micrographs (700× magnification) of human erythrocytes labeled with 5-doxylstearic acid. The label-to-lipid molar ratios illustrated are (top row, left to right) 1 : 17, 1 : 1.7; (middle row, left to right) 1 : 83, 1 : 8.3; (bottom row) 0 (control).

neuramic acid to obtain mean molecular weights of the three major membrane lipid constituents, namely cholesterol, phospholipids and glycolipids.

The scanning electron micrographs shown in Fig. 1 confirm the results obtained by Bieri and co-workers [6] as a function of the increase in concentration of spin label accessible to the cell membrane. Morphological changes are somewhat apparent in the 1 : 83 ratio region and are definitely present in the 1 : 17 case. At higher label concentrations, the cells not only contain increasingly more protuberances per cell but also begin to lose their discoid shape in favor of a more nearly spherical one. As indicated in Table I, which describes the qualitative presence of both morphologi-

TABLE I

QUALITATIVE PRESENCE OF MORPHOLOGICAL CHANGES (SCANNING ELECTRON MICROSCOPY EFFECTS), HEISENBERG EXCHANGE EFFECTS AND CELL LYSIS IN HUMAN ERYTHROCYTES AS A FUNCTION OF THE SPIN LABEL-TO-LIPID RATIO

Label-to-lipid ratio	Scanning electron microscopy effects	Exchange effects	Degree of lysis
1 : 83	Slight	No	~ 2 %
1 : 17	Definite, most cells modified	No	~ 2 %
1 : 8.3	All cells modified	No	~ 2 %
1 : 1.7	All cell modified	Yes	~ 10 %
1.2 : 1	No intact cells	Yes	~ 100 %
12 : 1	No intact cells	Strong exchange	~ 100 %

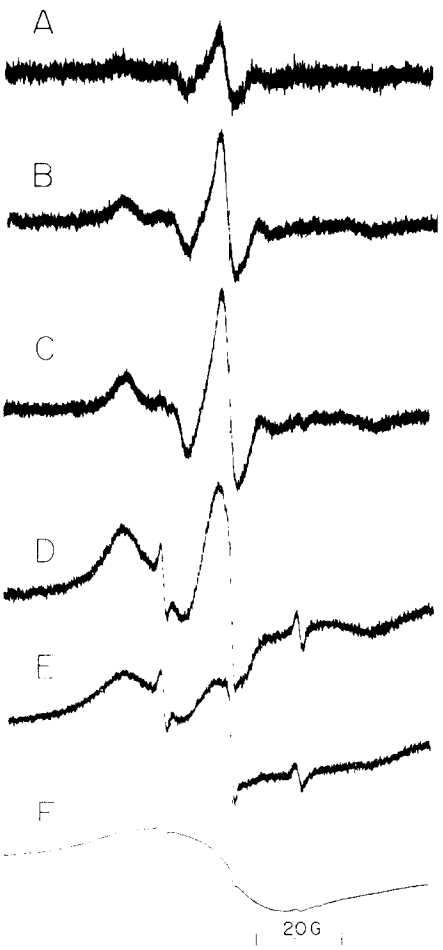

Fig. 2. ESR spectra of 5-doxylstearic acid incorporated in intact human erythrocytes with label-to-lipid molar ratios of 1 : 83 (A), 1 : 17 (B), 1 : 8.3 (C), 1 : 1.7 (D), 1.2 : (E) and 12 : 1 (F).

TABLE II

ESR SPECTRAL PARAMETERS AS A FUNCTION OF THE SPIN LABEL-TO-LIPID RATIO IN HUMAN ERYTHROCYTES

All parameters are in gauss (G) except the dimensionless order parameter S. The standard deviations are 0.1–0.2 G and approximately 0.02 for S. S is calculated as $(T'_{\parallel} - T'_{\perp})/(T_{\parallel} - T_{\perp})_{x1} \cdot a_{x1}/a$ using the crystal parameters of Jost et al. [11]. a is the isotropic coupling constant $(1/3\ TrT)$ and $0.5\ \Delta H_{+1}$ is the half-width at half-maximum amplitude of the low field peak.

Label-to-lipid ratio	$T'_{\parallel}$	$T'_{\perp}$	S	a	$0.5\ \Delta H_{+1}$
1 : 83	29.2	8.8	0.70	15.6	–
1 : 17	28.8	9.1	0.68	15.7	3.2
1 : 8.3	29.0	9.3	0.67	15.8	3.4
1 : 1.7	28.9	–	–	–	5.2
1.2 : 1	–	–	–	–	7.9
12 : 1	–	–	–	–	–

cal and ESR effects, complete cell lysis is observed following the 1 h incubation period for spin label-to-lipid ratios greater than 1 : 1. Indeed, at these higher concentrations lysis is apparent after a few minutes.

Fig. 2 shows the ESR spectra as a function of the label-to-lipid ratio over the range studied and exhibits the most important point to be made in this work. Exchange effects are clearly present when the label-to-lipid ratio is 1 : 1.7 or higher. Extensive exchange is present in the most concentrated solution having a ratio of 12 labels per lipid molecule. The inception of the exchange appears to coincide with extensive lysis of the cell and is also accompanied by the enhanced appearance of the so-called liquid lines superimposed on the spectrum of the immobilized label. An examination of the spectra where the label-to-lipid ratio is 1 : 8 and lower clearly indicates that the qualitative appearance of the spectrum is unchanged in that range of spin label concentration.

Table II gives the usually measured spectral parameters [9]. The inception of exchange is also noted by an inability to measure $T_{\perp}$ and an increase in the half width of the low field peak. In the low label concentration range the ESR parameters are essentially constant. There is some possible indication of a trend in the order parameter which, if real, appears to result from a systematic variation in $T_{\perp}$. It is conceivable that the apparent decrease in S with increasing label concentration is due to an increasing fluidity of the membrane. Our basic point, however, is not to interpret the present results in terms of any particular model but rather to point our that, so long as one has a label-to-lipid ratio less than 1 : 10, the ESR parameters are correctly reflecting basic properties of the membrane. Regardless of the possible significance of slight trends in the low-concentration region, it is obvious that an extrapolation to infinite dilution of the spin label is not going to result in significant spectral changes or in modification of the measured parameters.

The morphological changes in the cell appearance and, in certain cases, the resulting lysis are probably a result of the high concentration of the amphiphilic spin label. For example, the data of Helenius and Simmons [10] show that a ratio of about four sodium dodecyl sulfate molecules per lipid molecule is sufficient to solubilize membranes. They also indicate, however, on the basis of permeability studies in the

presence of detergents and from binding studies of fluorescent probes and anesthetics (both soluble amphiphiles), that if sufficiently small amounts of detergent are added they will be incorporated into the membrane bilayer without disrupting it. Our results, which indicate the lack of any significant change in the ESR spectral parameters of the 5-doxylstearic acid label at label : lipid ratios of 1 : 10 or less, are consistent with this observation.

The morphological changes induced in human erythrocytes by labeling with 5-doxylstearic acid illustrate the need to accompany spin labeling results whenever possible with data comparing the relevant properties of the labeled and unlabeled systems. Nevertheless, our results indicate that the validity of the spin labeling method as applied to cell membranes cannot be invalidated by morphological changes alone and, indeed, if one works at sufficiently low label concentrations where exchange effects are absent the technique remains a viable one.

ACKNOWLEDGEMENTS

We are indebted to Mr. Roy Weiss for performing the scanning electron microscopy. This work was supported in part by a National Science Foundation grant (GP-22546, D. B. C.), the Duke University National Institutes of Health Biomedical Support grant (C. C. W. and D. B. C.) and a National Institutes of Health NINDS grant (1 F22 NSO 1364-01, D. A. B.).

REFERENCES

1 McConnell, H. M. and McFarland, B. C. (1970) Q. Rev. Biophys. 3, 91–136
2 Jost, P., Waggoner, A. S. and Griffith, O. H. (1971) in Structure and Function of Biological Membranes (Rothfield, L. I., ed.), pp. 83–144, Academic Press, New York
3 Schreier-Muccillo, S. and Smith, I. C. P. (1973) Prog. Surface Membrane Sci., in the press
4 Keith, A. D., Sharnoff, M. and Cohn, G. E. (1973) Biochim. Biophys. Acta 300, 379–419
5 Seelig, J. C. and Niederberger, W. (1974) Biochemistry 13, 1585–1588
6 Bieri, V. G., Wallach, D. F. H. and Lin, P. S. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4797–4801
7 Villee, C. A. (1963) Biology, 4th edn., p. 250, W. B. Saunders Co., Philadelphia
8 Van Deenen, L. L. M. and de Gier, J. (1974) in The Red Blood Cell (Surgenor, D. M., ed.) 2nd edn., pp. 147–212, Academic Press, New York
9 Hubbell, W. L. and McConnell, H. M. (1971) J. Am. Chem. Soc. 93, 314–326
10 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
11 Jost, P., Libertini, V. C., Hebert, C. and Griffith, O. H. (1971) J. Mol. Biol. 59, 77–98