In view of the close relationship claimed by most authors between the K^+-activated phosphatase and the cation transport system, it is intriguing that the (Na^+-K^+)-independent ATPase activity, whose meaning to the transport ATPase is not yet clear, is enhanced, and the ouabain-sensitive activity is inhibited by Ca^{2+} (ref. 6) in the same range of concentrations that affects the phosphatase.

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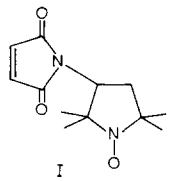
A spin-label study of erythrocyte membranes

In 1965, STONE *et al.*[1] introduced the technique of spin-labelling of polypeptides and proteins with paramagnetic nitroxide radicals and showed that structural and kinetic information can be obtained from the ESR spectrum of the spin label. Since then, quite a few applications of this technique have appeared in the literature[2–4]. In our laboratory, we have been interested in studying the physical properties of phospholipids[5] and the nature of molecular interactions in synthetic lipid–protein systems[6] and in biological membranes[7,8]. We have recently examined a series of spin-labelled proteins complexed with phospholipids in aqueous systems and in isooctane, by ESR spectroscopy[5]. The results obtained have given us clues about binding between proteins and phospholipids and have prompted us to extend the spin-label technique to natural membranes. In this communication we report preliminary results obtained from spin-labelled erythrocyte membranes examined by ESR spectroscopy.

Human erythrocyte ghosts were prepared from 1–2-week-old blood-bank blood (group A, Rh + ve) by the method of DODGE, MITCHELL AND HANAHAN[9], and were freed from traces of haemoglobin by exposure to pH 8 at low ionic strength followed by desalting. The water-washed stroma suspension was adjusted to pH 7.4 by washing with 20 mosM sodium phosphate buffer. Protein was determined by the ninhydrin procedure of MOORE AND STEIN[10] using crystalline bovine serum albumin as a reference standard.

The spin label, *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide (I) was

synthesised by Dr. M. T. A. EVANS of this laboratory. This substance is known to label SH and NH_2 groups of proteins[11].



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For labelling erythrocyte membrane, 1 mg of the spin label was added to membrane (protein, 10 mg) in 5–10 ml of 20 mosM sodium phosphate buffer (pH 7.4). The mixture was stirred in an ice bath for at least 2 h and was centrifuged at 39000 rev./min for 15 min. The pellet was washed twice with buffer and resuspended to a concentration of approx. 5 mg/ml membrane protein. After dialysis against 200 vol. of 20 mosM buffer (pH 7.4) for 4 h, the preparation was used in all experiments as a spin-labelled membrane.

Blocking of SH groups was carried out by *N*-ethylmaleimide prior to spin-labelling. About 1 mg of *N*-ethylmaleimide was added per 10 mg membrane protein, and the mixture was stirred gently for 6 h. After dialysis overnight against 20 mosM sodium phosphate buffer (pH 7.4) (200 vol.) and centrifugation at 39000 rev./min the pellet was resuspended in buffer and spin-labelled as above.

Lipid extraction of the spin-labelled membrane was carried out by the method of REED *et al.*[12]. The lipids were dried under vacuum and swollen in buffer to the volume of the original sample.

In order to examine the effects of pH, spin-labelled membrane was divided into several aliquots and washed with distilled and deionised water by ultracentrifugation at 39000 rev./min to yield a number of pellets. Each pellet was resuspended separately in appropriate acetate, phosphate or Tris buffer to yield suspensions having final pH's between 3.5 and 8.6. Lower and higher pH suspensions were made with 0.01 M HCl and Tris base, respectively. The ionic strength was maintained at 0.01. The final protein concentration was 5 mg/ml. Between pH 3.5 and 4.5 the material was maximally aggregated, as judged by visual aggregation titres. Below and above these pH values, the turbidity decreased progressively. The aggregated samples were mixed vigorously on a vortex stirrer prior to sampling for ESR spectroscopy to obtain homogeneous specimens.

ESR spectra were obtained at 9.5 kMHz using a Varian V-4502 X-band spectrometer. The theory relating to molecular tumbling of the spin label to the appearance of its ESR spectrum is now well established[2,13].

The ESR spectrum of spin-labelled erythrocyte membrane is shown in Fig. 1a. The spectrum consists of two components; (i) one of three narrow lines arising from spin label which can tumble freely, and (ii) a component of three broad lines from spin label which is immobilised for times greater than or equal to about 10^{-8} sec, which is the reciprocal of the anisotropy of the hyperfine interaction of the spin label. The spin label, therefore, can distinguish between two sites of binding in the erythrocyte membrane.

The lipid extract of the membrane exhibited only a trace of paramagnetic material, the major part of the signal being in the lipid-free residue. Amino-sugars

present in the carbohydrates of the membrane are reported to be N-acetylated[14] and are therefore not expected to be available as sites for binding of the spin label. The major part of the spin label therefore appears to be attached to the membrane protein.

The spectrum of erythrocyte membrane which was treated with *N*-ethylmaleimide before spin-labelling is shown in Fig. 1b. The almost complete absence of the broad component from this spectrum indicates that the broad component results from the spin-labelling of SH groups. This situation compares directly with the work of GRIFFITH AND MCCONNELL[11] who found that the broad component of the ESR spectrum of spin-labelled bovine serum albumin could be removed by prior treatment with *N*-ethylmaleimide which has a high degree of specificity for blocking SH groups. Erythrocyte membrane is known to contain a number of titratable SH groups[15]. At least some of these SH groups appear to be at "interior" sites in the membrane where, although the spin label can penetrate, it cannot tumble freely. The narrow component of the spectrum is probably produced by the reaction of spin label with NH_2 groups (*e.g.*, ε-NH_2 groups of lysine).

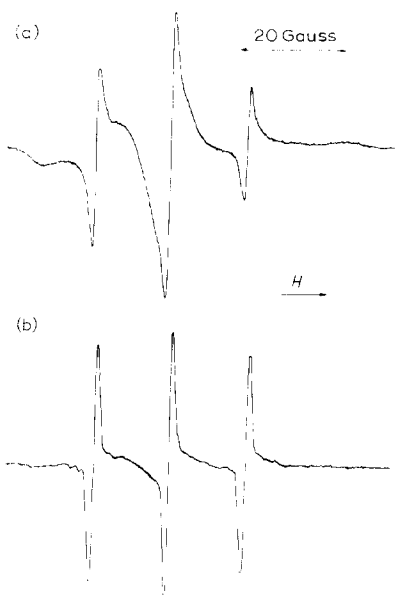

Fig. 1. a. ESR spectrum of spin-labelled erythrocyte membrane. b. ESR spectrum of erythrocyte membrane spin-labelled after treatment with *N*-ethylmaleimide.

The effect of pH on the ESR spectrum of spin-labelled erythrocyte membrane is shown in Fig. 2. The intensity of the narrow low-field hyperfine line was taken as an arbitrary measure of the amount of freely-tumbling spin label. The amount of freely-tumbling spin label is seen to be minimum between pH 3.5 and 4.5 and is maximum below pH 2 and above pH 10. At extreme pH values, the broad component of the membrane spectrum completely disappears and the intensity of the narrow component increases correspondingly.

The minimum in the intensity of the narrow component corresponds to the isoelectric region of the membrane protein[14]. If we consider the narrow component

of the ESR spectrum to arise from spin label at the charged sites of the membrane protein, any interaction at these sites, due to charge equalisation at the isoelectric point, would be expected to inhibit the tumbling of the spin label (see ref. 6). This would cause a broadening of the spectrum and a resulting loss of narrow line intensity.

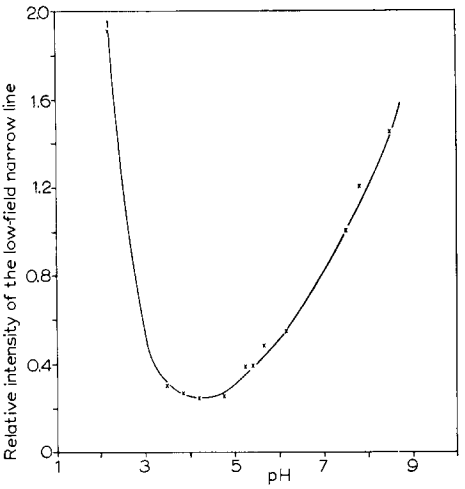

Fig. 2. Graph of intensity of low-field hyperfine line of spin-labelled erythrocyte membrane against pH (pH 7 intensity = 1).

The decrease in the broad component of the spectrum at extreme pH values (>9.5, <1) reflecting a reduction in the inhibition to the motion of the spin label at the interior sites, indicates depolymerisation of the membrane.

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