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SOME PROPERTIES OF ALKALI-EXTRACTED RED CELL GHOST MEMBRANES

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The properties of integral membrane proteins obtained by dilute alkali extraction of red cell ghosts were examined. A variety of conditions promoted the disulfide-mediated aggregation of integral membrane proteins, principally band 3. Procedural modifications which minimized aggregation were the use of EDTA and *S*-alkylation. Integral membrane proteins were solubilized under non-denaturing conditions using Brij 36T, a lauryl polyoxyethylene ether with an NMR-determined average chain length of 8.2 (oxyethylene) units. Detergent gel filtration revealed a chromatographic shoulder due to aggregated band 3 when membrane proteins were not alkylated. Analyses of the column profile also revealed a discrete peak for sialoglycoproteins and two phosphate peaks, an early one due to phospholipid and a later one not identified, but probably due to phosphoinositide.

Introduction

Among membrane constituents, the integral or intrinsic proteins present the most formidable characterization problems because of their insolubility and tendency to aggregate. Most studies have utilized strong depolymerizing agents to isolate them. The relatively mild method of Steck and Yu [1], which by brief exposure at 0°C to pH 12 water yields a preparation stripped of peripheral components from ghost membranes, has been very useful.

We have recently been employing the integral proteins obtained according to Ref. 1 as starting material in the isolation of antigenic membrane constituents. The use of antibody ligands necessitated the rigorous exclusion of reducing agents. It soon became apparent that under a variety of experimental manipulations significant degrees of aggregation occurred which under reducing conditions were masked. This communication deals with the identification of those factors which promote, and of some which inhibit, this behavior.

Materials and Methods

Red cell membranes. Ghosts were obtained by hypotonic lysis of washed fresh blood, essentially according to the procedure of Fairbanks et al. [2].

Alkaline extraction procedures. Two modifications of the Steck and Yu [1] procedure were employed. Method A: This was carried out as described in Ref. 1. Method B: Ghosts (3 mg protein per ml) were preincubated in 5 mM sodium phosphate, pH 8.0, containing 1 mM EDTA for 10 min at 0°C. This was followed by addition of N_2-bubbled 0°C water containing 1 mM EDTA, to yield a final protein concentration of 0.3 mg/ml. After 20 min stirring at 0°C the suspension was spun at 27 000 × *g* for 30 min. The membrane pellet was washed with 5 mM sodium phosphate/1 mM EDTA (pH 8.0). Method C: As in B, but after the centrifugation the pellet was washed in a solution comprising 5 mM sodium phosphate/1 mM EDTA/5 mM iodoacetamide. Following re-centrifugation the pellet was resuspended in the same solution at a final protein concentration of 3 mg/ml and incubated

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at 0°C with stirring for 20 min in the dark. The alkylated, extracted membranes were washed with cold 5 mM sodium phosphate, pH 8.0, containing 1 mM EDTA.

Detergent gel filtration. Alkaline extracted membranes were mixed by vortexing to a final protein concentration of about 1 mg/ml with column buffer comprising 0.5% (w/v) Brij 36T/20 mM Tris-HCl/ 1 mM EDTA, pH 7.4. After incubating for approx. 15 min at 37°C the mixture was spun at 100 000 × *g* for 60 min. The supernatant was collected and introduced by upward flow into a 450 ml bed volume Sepharose CL-4B water-jacketed column at 15°C. Column monitoring was at 280 nm, where the detergent did not interfere.

Analytical procedures. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done in 1% sodium dodecyl sulfate/8 M urea gels with 20 : 1 monomer-to-crosslinker ratio according to Fairbanks et al. [2]. Phosphate analyses with and without ashing at 180°C in 70% $HClO_4$ were done by the method of Chen et al. [3]. Sialic acid analyses following hydrolysis in 0.05 M H_2SO_4 at 80°C for 1 h was carried out on all column fractions by the method of Warren [4]. Lipid thin-layer chromatography was by methods previously published [5]. Extraction of lipid was as in Folch et al. [6]. Lactoperoxidase-catalyzed iodination and the assays of glucose oxidase and lactoperoxidase activities were done according to Hubbard and Cohn [7]. Protein estimations were according to Lowry et al. [8] with bovine serum albumin as standard. NMR spectra were recorded in a Varian HR-220 spectrometer.

Chemicals. All enzymes employed were from Worthington. Electrophoresis reagents and supplies were obtained from BioRad. Brij 36T was from Emulsion Engineering (Elk Grove Village, IL). Chromatography media were from Pharmacia. All other chemicals were reagent grade or the best available commercial grade.

Results

As shown in Fig. 1, where only the original method of Steck and Yu [1] was employed, there is faint aggregation evident even in fresh preparations (but not at pH 12) when analyzed in the absence of reductant (Fig. 1., gel 3). Very marked aggregation occurs with time at any temperature (Fig. 1, gels 4–6). There was no tendency to aggregate further if the proteins were frozen in gel electrophoresis detergent sample solution.

Aggregation was also evident after typical iodination conditions [7], as shown in Fig. 1, gel 7. To minimize the exposure to H_2O_2 generated by glucose oxidase, enzymatic iodinations were performed where the ratio of enzymatic activity units of lactoperoxidase over glucose oxidase was five. As gels 8 and 9 in Fig. 1 show, however, aggregation still occurred. This was the usual finding after iodination but, occasionally, analyses performed in the presence of reductant indicated that degradation had also occurred (Fig. 1, gel 10). It is important to emphasize that in the presence of reductant the material shown in Fig. 1, gels 3–9, appeared identical to Fig. 1, gel 2 (gels with reductant not shown).

Various modifications of the extracting procedure were introduced to control aggregability. Since sulfhydryl oxidations tend to be trace-metal-catalyzed, EDTA was included during the extraction. The presence of EDTA during the extraction alone did not significantly alter the degree of aggregation found with method A. Preincubation with N_2-bubbled EDTA solution prior to alkaline EDTA extraction (Method B), however, was more effective (data not shown). The least aggregation was detected when the proteins were alkylated (data not shown). The efficiency of alkaline extraction with Methods A, B and C was comparable. It was 55.7 ± 6.09 S.D. (n = 13), 50.3 ± 11.56 (n = 3), and 53.4 ± 5.33 (n = 9) for Methods A, B and C, respectively, expressed as percentages of residual protein present after extraction.

Integral membrane proteins, as obtained following alkaline extraction by Methods A, B and C as described above, were characterized by gel filtration after non-ionic detergent solubilization using Brij 36T, a lauryl polyoxyethylene ether, According to the manufacturer (personal communication, ICI Americas, Inc.), Brij 36T has a melting point of 30°C, a critical micelle concentration of 0.1 mM and an HLB number * equal to 14.1. Based on the starting stoichiometry of reactants, its synthesis

* HLB number, hydrophile-lipophile balance number.

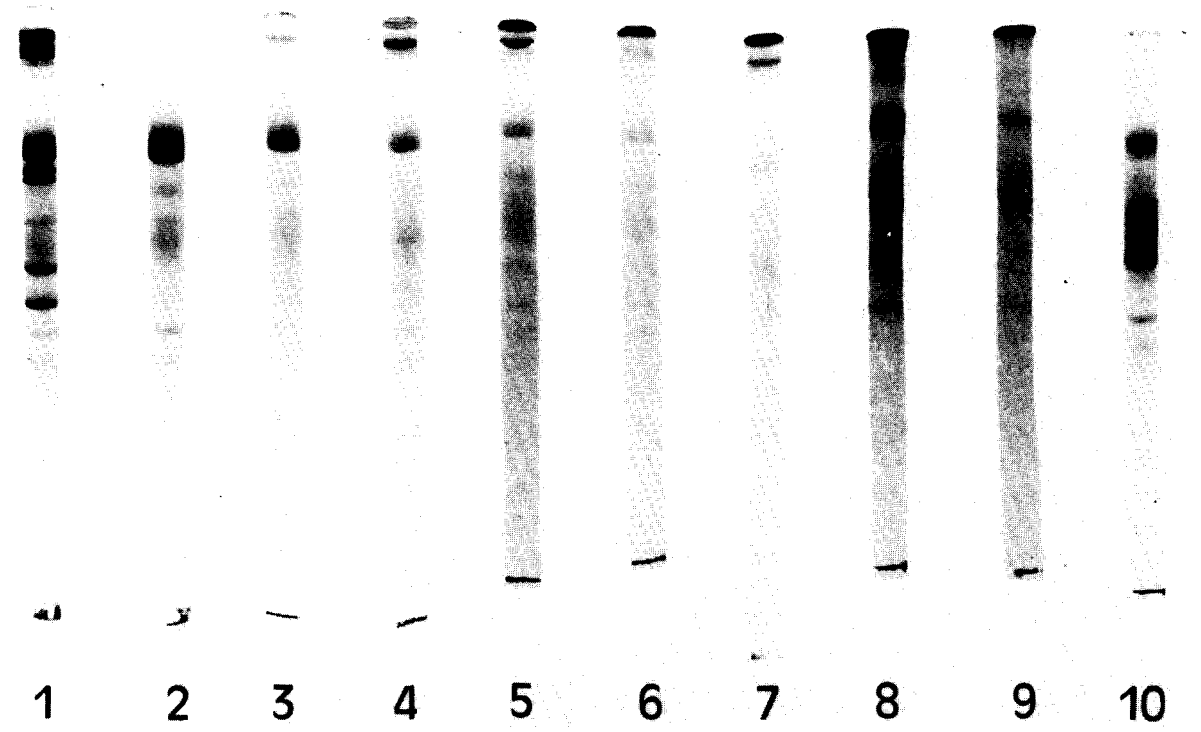


Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of alkali extracted ghosts prepared according to Ref. 1. About 20 μg protein per gel. Gel 1, control ghosts; gel 2, fresh preparation after alkali extraction, reduction with 40 mM dithiothreitol; gel 3, same as gel 2, no dithiothreitol; gels 4–6, effect of temperature, no dithiothreitol; gel 4, after incubation for 1 h at 37°C; gel 5, after storage at 4°C for 2 days; gel 6, after storage at −20°C for 2 days; gels 7–9, effect of iodination, no dithiothreitol; gel 7, standard conditions, same units of lactoperoxidase and glucose oxidase [7]; gel 8, 50 mU lactoperoxidase: 10 mU glucose oxidase; gel 9, 500 mU lactoperoxidase: 100 mU glucose oxidase; gel 10, effect of iodination, reducing conditions with 40 mM dithiothreitol.

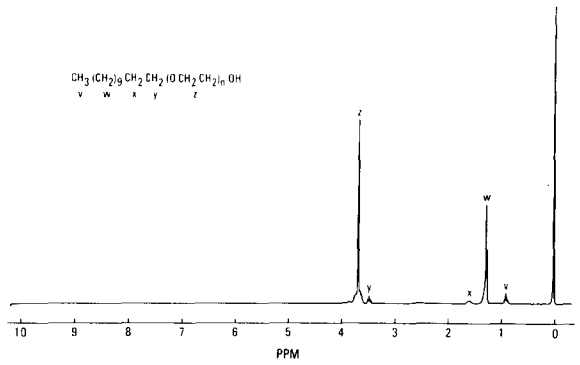
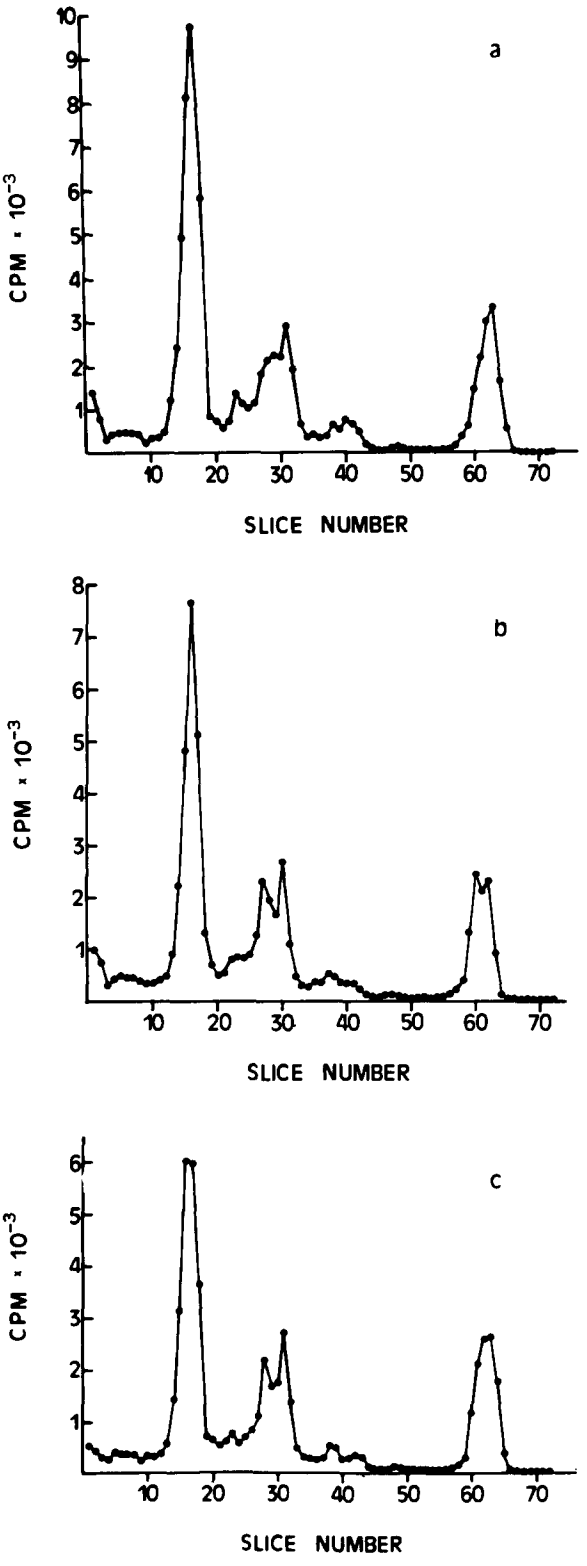


Fig. 2. ^1H NMR 220 MHz spectrum of Brij 36T at approximate 5% (w/v) in $^2HCCl_3$. Chemical shifts are referenced to internal TMS. The spectrum was recorded at 2500 Hz sweep width. Spectral assignments are based on those of Ribeiro and Dennis [9].

should theoretically yield an average chain length of $n = 10$ oxyethylene units. Our analyses of a 1 l production lot did not produce that average chain length. Quantitative analyses by proton integration of expanded NMR spectra (not shown), based on the constant proton contribution by the lauryl moiety, indicated an average chain length of 8.2, i.e., $C_{12}E_{\bar{n}} = 8.2$. As shown in Fig. 2, the NMR spectrum of Brij 36T is virtually identical to that of authentic $C_{12}E_8$ (analyzed but not shown; published by Ribeiro and Dennis [9]). To our knowledge, this makes Brij 36T the only polydisperse nonionic detergent for which the pure monomer (i.e., $C_{12}E_8$) corresponding closely to its average constituent (i.e., $C_{12}E_{\bar{n}} = 8.2$) has recently become available commercially both in non-radioactive (Nikko Chemicals, Tokyo)



and radiolabeled (Research Products International, Elkgrove Village, IL) forms.

The efficiency of alkaline extracted ghost solubilization by Brij 36T was variable, ranging from 60 to 90%. However, as shown in Fig. 3, there was no evidence of preferential solubilization before or after 100 000 × *g* centrifugation (Fig. 3b and c).

The results of gel filtration of Brij 36T-solubilized alkaline-extracted proteins obtained by Method A are shown in Fig. 4. The column profile obtained was essentially identical to that obtained after Method B (i.e., EDTA-modified alkaline extraction did not prevent the formation of aggregated protein at fraction 46). Method C (alkylated membrane proteins) did not show the shoulder peak occurring at about fraction 46, but was otherwise identical.

The sialoglycoproteins banded sharply around fraction 58, as shown by sialic acid analysis and as demonstrated by periodic acid-Schiff gels (not presented). Two phosphate peaks were detected in the profile (fraction 28 was the void volume), one at about fraction 63 which was associated with major phospholipids by thin-layer chromatography and a second one at about fraction 80 which has not been definitively identified. The P_i of the peak centered at fraction 80 was twice as acid labile as that of the peak centered at fraction 63, based on P_i analyses with and without prior ashing of samples. In the region of fraction number 80 absorbance at 280 nm was also observed. However, the absorption spectrum of that region (not shown) indicated the absorption maximum occurring at about 255 nm and a 280/260 absorbance ratio equal to 0.70. A Lowry analysis of the column profile (not shown) indicated that, while for the most part it could be superimposed upon the 280 nm profile, there was very little Lowry reactivity associated with the peak at fraction 80. Consequently, it may not be a major protein area (see Discussion).

Fig. 3. Analysis of ^{125}I-labeled alkaline extracted ghosts in the presence of 40 mM dithiothreitol. (a) Control integral proteins according to Ref. 1; (b) after solubilization at about 1 mg/ml in 0.5% Brij 36T/20 mM Tris-HCl pH 7.4/1 mM EDTA; (c) same as (b) after 100 000 × *g* centrifugation; analysis of supernatant shown. Tracking dye migration was to slice number 65. The large peak preceding it was labeled lipid.

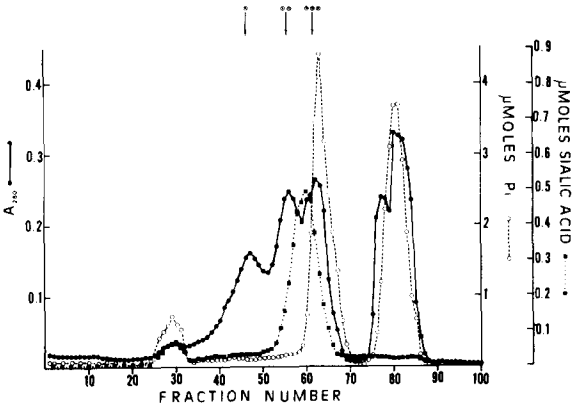


Fig. 4. Gel filtration of solubilized integral membrane proteins obtained according to Ref. 1. Approx. 16 mg protein were loaded on to the column, and 5 ml fractions were collected. Void volume was at fraction number 28, flow rate was about 0.3 ml/min. Symbols above first, second and third included peaks (from left to right) refer to column locations where gel electrophoresis shown in Fig. 5 was carried out.

The membrane protein fraction obtained by Method B (data not shown) displayed greater aggregation (in the absence of reductant) before gel filtration than material obtained by Method C (not shown). Material obtained by Method A did not enter the 5.6% acrylamide gels used in the absence of reductant under these same conditions (not shown).

Fig. 5 shows Coomassie Blue stain absorbance profiles of electrophoretic gels derived from material in gel filtration column locations indicated in Fig. 4 by arrows below dotted circles. In Fig. 5 the three vertical rows from left to right show gel results after alkaline extraction by Methods A, B and C, respectively. Where indicated on the figure, the gels in Fig. 5 were run in the presence of dithiothreitol. Except for the alkylated membrane protein (Method C, right vertical row), the polypeptides did not significantly enter 5.6% acrylamide gels in the absence of dithiothreitol.

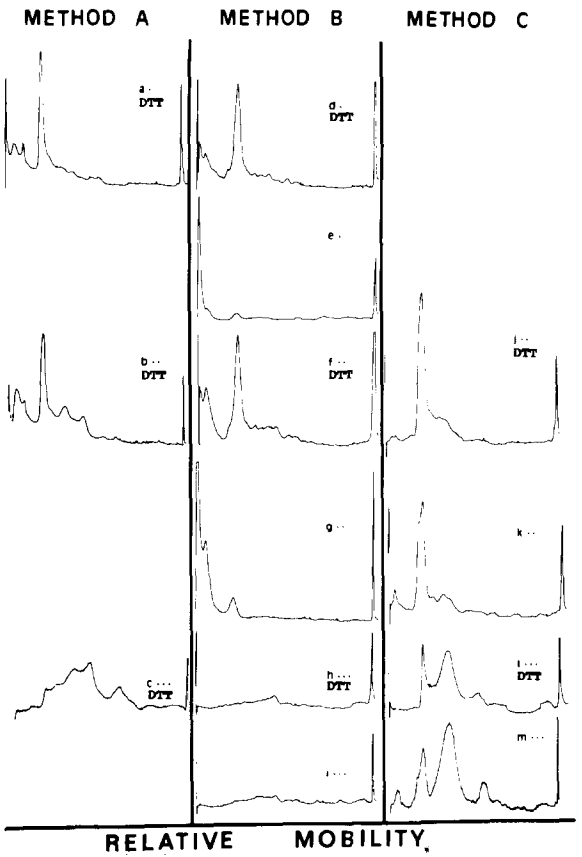


Fig. 5. A_{546} absorbance profiles of Coomassie Blue-stained gels obtained from fractions in column profile indicated by symbols (see also text). Left vertical row shows scans from integral proteins obtained as in Ref. 1 (Method A). Middle vertical row indicates profiles of integral proteins obtained in the presence of EDTA (Method B) and the right vertical row shows alkylated integral membrane proteins (Method C). Gels a, b, c, d, f, h, j, l: with 40 mM dithiothreitol; gels e, g, i, k, m: without dithiothreitol.

Discussion

The isolation of integral membrane proteins by alkali stripping of peripheral constituents according to the method of Steck and Yu [1] is efficient, convenient and, in contrast to some methodologies, relatively mild. The procedure, with slight or no modifications, has been widely employed [10–16]. In one paper [17], gels showing alkaline extracted ghosts in the presence and absence of reductant were shown but aggregation was not the subject of that investigation. It is unclear how widely recognized the phenomenon is. In at least one report what is probably -SH-mediated aggregation has been interpreted as residual spectrin. Moreover, in other investigations the preparation (for valid reasons) has been electrophoretically analyzed in the presence of reductant, where aggregation would go undetected. Although this report is concerned with the human red

cell membrane, we note that alkaline extraction is being applied to other membrane preparations [18, 19] and that the aggregation of enriched intrinsic membrane protein fractions by sulfhydryl oxidation has recently been reported in *Micrococcus* [20].

Alkali treatment of proteins has been widely studied [21,22], primarily because of its importance to food technology. Aggregation has frequently been observed [23]. In the red cell membrane the cross-linking effects of -SH oxidizing agents has been described by Haest et al. [24]. Moreover, in several ultrastructural studies the aggregation of intramembranous particles has been reported after the removal of peripheral proteins [14,16,25]. Studies by Gerritsen et al. [14,16] on the aggregation of intramembranous particles following alkaline extraction of ghost membranes implicitly recognized the aggregability of base-extracted membranes.

Some factors influencing disulfide-mediated aggregation of proteins have been the subject of investigations not involving the red cell membrane. Aggregation as a function of temperature has been reported [26]. Freezing injury in plants has often been explained on the basis of freezing-induced disulfide aggregation [27]. Similarly, lactoperoxidase-catalyzed oxidation of protein sulfhydryls has been reported [28]. In addition, degradation following iodination has also been shown [29,30].

Since alkaline-extracted ghost membranes consist predominantly of band 3 and sialoglycoproteins and since the latter lack sulfhydryls [31], it is clear that the subject of this investigation is primarily a study of band 3 sulfhydryl behavior. This area has been studied extensively [32,33]. It is unclear if the disulfide-mediated aggregation of band 3 in this report reflects a release of submembrane constraints following the removal of spectrin, as has been suggested to explain the aggregation of intramembranous particles that follows non-alkaline removal of peripheral proteins [25]. It has been recently demonstrated that mobility of integral membrane proteins can occur in human erythrocytes [34]. This finding possibly extends the relevance of results obtained in ghosts and membrane fractions to the intact cell.

Sulfhydryl oxidation by non-ionic detergents has been reported [35,36]; however, we did not detect significantly enhanced aggregation by the addition of Brij 36T. This detergent was selected after Simon et al. [37] reported that, out of many mild detergents tried, it alone was successful in solubilizing a functional opiate receptor. We learned subsequently that Gitler [38,39] applied it to red cell membranes in 1972 in experiments in which he showed the retention of enzyme activities and the preservation of the oligomeric state of membrane proteins. The mildness of Brij 36T in affecting oligomeric proteins has recently been confirmed [40].

The region emerging at about fraction 80 of the gel filtration analysis has not been identified. Several considerations, however, have led us to conclude that it probably represent polyphosphoinositides. The human red cell membrane, unlike that of other species, is highly enriched in phosphoinositides [41]. The relative acid lability of phosphate present would be consistent with a sugar phosphate. As indicated under Results, the absorption maximum was at 255 nm. This could be explained by alkali-induced isomerization and conjugation of polyene fatty acyl moieties. The low Lowry reactivity found could be explained by the well known ability of peroxidized lipids to give a strong reaction in the assay [42]. The 280/260 ratio and the fact that alkali-stripped membranes were involved probably rule out a nucleotide phosphate. A low molecular weight phosphoprotein cannot be rigorously excluded [43].

Alkali-extracted red cell ghost membranes represent a useful starting material in the study of integral membrane protein structure and function. As this investigation has shown, however, it will be important to consider the role, if any, of sulfhydryl oxidation-reduction before final conclusions can be arrived at in studies involving their use.

Acknowledgements

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References

1 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
2 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
3 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
4 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
5 Victoria, E.J., van Golde, L.M.G., Hostetler, K.Y., Scherphof, G.L. and van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 239, 443–457
6 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
7 Hubbard, A.L. and Cohn, Z.A. (1972) J. Cell Biol. 55, 390–405
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
9 Ribeiro, A.A. and Dennis, E.A. (1977) J. Phys. Chem. 81, 957–963
10 Abbott, R.E. and Schachter, D. (1976) J. Biol. Chem. 251, 7176–7183
11 Zoccoli, M.A. and Lienhard, G.E. (1977) J. Biol. Chem. 252, 3131–3135
12 Goheen, S.C., Gilman, T.H., Kauffman, J.W. and Garvin, J.E. (1977) Biochem. Biophys. Res. Commun. 79, 805–814
13 Grinstein, S. and Rothstein, A. (1978) Biochim. Biophys. Acta 508, 236–245
14 Gerritsen, W.J., Verkley, A.J., Zwaal, R.F.A. and van Deenen, L.L.M. (1978) Eur. J. Biochem. 85, 255–261.
15 Galletti, P., Paik, W.K. and Kim, S. (1978) Biochemistry 17, 4272–4276
16 Gerritsen, W.J., Verkleij, A.J. and van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 555, 26–41
17 Steck, T.L., Ramos, B. and Strapazon, E. (1976) Biochemistry 15, 1154–1161
18 Neubig, R.R., Krodel, E.L., Boyd, N.D. and Cohen, J.B. (1979). Proc. Natl. Acad. Sci. USA 76, 235–239
19 Citri, Y. and Schramm, M. (1980). Nature 287, 297–300
20 Muñoz, E. and Estrugo, S.F. (1979). FEMS Microbiol. Lett. 6, 235–239
21 Nashef, A.S., Osuga, D.T., Lee, H.S., Ahmed, A.I., Whitaker, J.R. and Feeney, R.E. (1977) J. Agr. Food Chem. 25, 245–251
22 Florence, T.M. (1980) Biochem. J. 189, 507–520
23 Ishino, K. and Kudo, S. (1980). Agr. Biol. Chem. 44, 1259–1266
24 Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977). Biochim. Biophys. Acta 469, 226–230
25 Elgsaeter, A. and Branton, D. (1974) J. Cell. Biol. 63, 1018–1030
26 Pace, M. and Dixon, J.E. (1979). Int. J. Peptide Protein Res. 14, 409–413
27 Levitt, J. (1962). J. Theor. Biol. 3, 355–391
28 Thomas, E.L. and Aune, T.M. (1977). Biochemistry 16, 3581–3586
29 Zweig, M., Heilman, Jr., C.J. and Hampar, B. (1979) Virology 94, 442–450
30 Jackson, D.C. (1980) J. Immunol. Meth. 34, 253–260
31 Furthmayr, H. (1978) J. Supramol. Struct. 9, 979–995
32 Reithmeier, R.A.F. and Rao, A. (1979) J. Biol. Chem. 254, 6151–6155
33 Steck, T.L. (1979) J. Supramol. Struct. 8, 311–324
34 Fowler, V. and Branton, D. (1977) Nature 268, 23–26
35 Giotta, G.J. (1976) Biochem. Biophys. Res. Commun. 71, 776–782
36 Chang, H.W. and Bock, E. (1980) Anal. Biochem. 104, 112–117
37 Simon, E.J., Hiller, J.M. and Edelman, I. (1975) Science 190, 389–390
38 Gitler, C. (1972) in Biomembranes (Manson, L.A., ed.), Vol. 2, pp. 41–73, Plenum, New York
39 Gitler, C. (1972) in Hibernation and Hypothermia. Perspectives and Challenges (South, F.E., Hannon, J.P., Willis, J.R., Pengelley, E.T. and Alpert, N.R., eds.), pp. 239–280, Elsevier, Amsterdam
40 Lakshmi, T.S. and Nandi, P.K. (1978) Int. J. Peptide Protein Res. 12, 197–203
41 Verpoorte, J.A. and Palmer, F.B. St.C. (1974). Int. J. Biochem. 5, 69–77
42 Eichberg, J. and Mokrasch, L.C. (1969). Anal. Biochem. 30, 386–390
43 Gaetjens, E. (1976) Biochemistry 15, 50–54