THE USE OF ZWITTERIONIC SURFACTANTS IN THE AGAROSE CHROMATOGRAPHY OF BIOLOGICAL MEMBRANES

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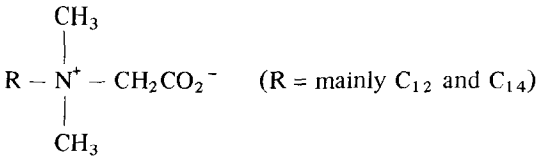
1. Introduction

The use of surfactants in the separation and analysis of the components of biological membranes is widespread [1]. Several groups have investigated the agarose chromatography of surfactant-treated membrane material, using an eluant which itself contains the surfactant; such studies have been made on the plasma membrane of rat liver cells [2], human erythrocyte membranes [3–7], nuclear membranes [8], and the milk fat-globule membrane [9,10]. The chemical classes of surfactant used in such work have been limited: sodium dodecyl sulphate [6,10], sodium deoxycholate [3], Triton X-100 (*p*-isooctylphenoxy-polyethoxyethanol) [4,5,7,8] or a mixture of these [2,9] being most commonly employed.

In the course of an investigation of the milk fat-globule membrane, a study was made of the effects on the membrane of a number of unusual surfactants; these included the zwitterionic alkylbetaine, Empigen BB*:



$$R - \overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{\overset{|}{\underset{|}{N^+}}}} - CH_2CO_2^- \qquad (R = \text{mainly } C_{12} \text{ and } C_{14})$$

This surfactant is able to dissociate the milk fat-globule membrane at low concentration and under exceedingly mild conditions. In this note we report some of the results using this material, including a comparison with the other commonly-used surfactants, and a brief evaluation of its use for the study of human erythrocyte and rat plasma membranes.

* Empigen BB is a Trade Mark of the Marchon Division of Albright and Wilson, Ltd.

2. Materials and methods

2.1. *Membrane preparation*

Milk fat-globule membrane was prepared by a similar method to that of Swope and Brunner [11]. Unwashed cream from a pooled milk was churned, and the buttermilk thus obtained brought to 0.1 M in citrate buffer pH 6.8; this was centrifuged at 12 000 *g* for 30 min at 4°C. The supernatant was centrifuged further, at 25 000 *g* for 2.75 hr at 4°C; the pellet was washed in distilled water, and centrifuged down again.

Haemoglobin-free red blood-cell membranes were obtained by the method of Moldow et al. [12], and plasma membrane from rat liver according to Stein, Widnell and Stein [13]. These membranes were homogenised by a Potter homogeniser before subjecting them to any further treatment.

2.2. *Enzyme assays*

Alkaline phosphatase was estimated by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate in 30 mM glycine buffer, pH 10.4 at 25 C. The molar extinction coefficient of *p*-nitrophenol at 410 nm is 13.3×10^3 M^{-1} cm^{-1} [14]. Xanthine oxidase was measured by the production of uric acid from xanthine in 25 mM phosphate buffer, pH 7.5 at 25°C, using an extinction coefficient at 290 nm of 12.3 ×

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10^3 M^{-1} cm^{-1} [15]. 5′-Nucleotidase was assayed by measuring the release of inorganic phosphate from 10 mM AMP at pH 8.5 and 37 C [16]. The (Mg^{++}, Na^+, K^+)-activated ATPase of red blood-cell membranes was also measured by release of phosphate ions, this time from 4.5 mM ATP in 50 mM tris buffer pH 7.3 at 37°C; the solution also contained 0.8 M KCl and 50 mM $MgCl_2$.

2.3. *Effects of surfactants on enzymic activities*

A surfactant (Empigen BB, sodium deoxycholate, sodium dodecyl sulphate or Triton X-100) was added to freshly-prepared milk fat-globule membrane (protein concentration, 18 mg ml^{-1}) to a concentration of 0.1% (w/v). The sample was diluted 1 to 10 in 0.05 M Tris, pH 8.0, containing 0.1% (w/v) of the appropriate surfactant. An untreated sample, similarly diluted in 0.05 M tris, pH 8.0, was used as a control. The samples were incubated at 4°C, and their alkaline phosphatase, xanthine oxidase and 5′-nucleotidase activities were measured at intervals over a total period of 16 days.

2.4. *Column chromatography*

The membranes were washed in 0.05 M Tris, pH 8.0, and samples were either applied to an agarose column directly, or were treated with 0.1% (w/v, approx. 3 mM) Empigen BB, agitated mechanically and left at 4 C for 18 hr before chromatography.

All samples were chromatographed at 4°C, on a 45 X 2.5 cm column of Bio-Rad A-150 m agarose; a flow rate of 6 ml hr^{-1} was adopted, and fractions of 2.4 ml were collected. The eluant was 0.05 M Tris, pH 8.0, but in separations of surfactant-treated samples, Empigen BB was added to give a concentration of 0.1% (w/v). A sample of milk fat-globule membrane was also treated and chromatographed in the presence of 0.1% (w/v) Triton X-100. The column was monitored by absorbance at 280 nm, and protein was estimated by the Lowry method [17], using bovine serum albumin as a standard.

3. Results

3.1. *Effect of surfactants on enzymic activities*

Sodium dodecyl sulphate inhibited all three of the enzymes assayed, causing almost complete loss of activity. Xanthine oxidase was the most resistant to treatment by this surfactant. The other surfactants were more selective and less inhibitory in their effect (fig.1); indeed, over the first 2–3 days, the length of time it would normally take to complete the column chromatography, activities were sometimes enhanced. This enhancement was least marked with deoxycholate Empigen BB and Triton X-100 gave moderate enhancements, and, over the long term, moderate retentions

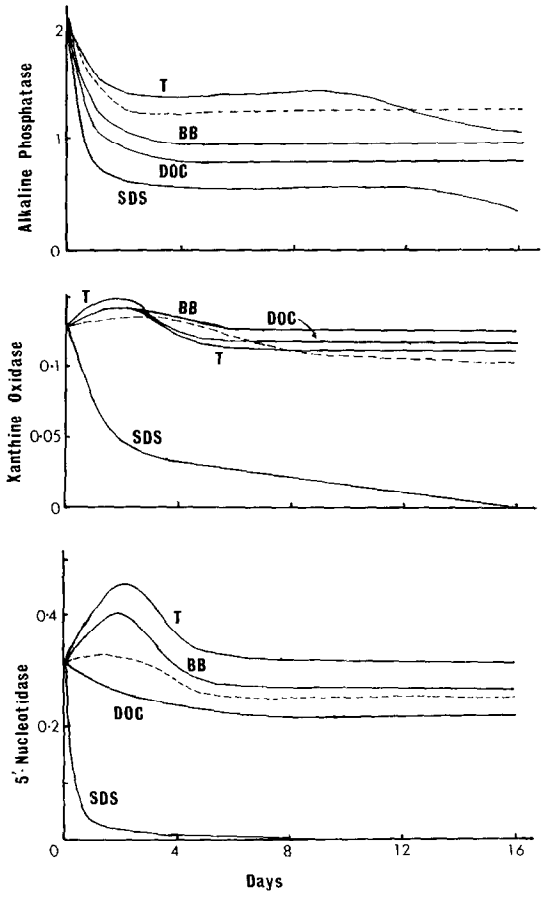

Fig.1. Effect of surfactants on activities of milk fat-globule membrane enzymes. Samples (1.8 mg protein ml^{-1}) were incubated at 4°C in 0.05 M Tris pH 8.0 containing 0.1% w/v sodium dodecyl sulphate (SDS) sodium deoxycholate (DOC), Triton X-100 (T), Empigen BB (BB) or no surfactant (dotted line). Activities are expressed in μmol product formed mg $protein^{-1}$ min^{-1} at 25°C (for alkaline phosphatase and xanthine oxidase) or 37°C (for 5′-nucleotidase).

of activities. Triton X-100, however, was the only surfactant which maintained the alkaline phosphatase activity at that of the control.

3.2. *Column chromatography*

3.2.1. Milk fat-globule membrane

Predictably, with no treatment, most of the protein eluted in a large peak coincident with the void volume of the column (fig.2). This fraction contained most of the xanthine oxidase and 5'-nucleotidase activities, but a little xanthine oxidase also appeared in a small protein shoulder which eluted after the main peak. Alkaline phosphatase activity was too low to measure. A similar preparation treated and eluted with a buffer containing 0.1% (w/v) Triton X-100 produced an identical pattern of protein concentration. Enzymic activities were also similar except that there was considerable alkaline phosphatase activity coincident with the main protein peak (maximum activity = 3.2 μmol *p*-nitrophenol produced min^{-1} ml^{-1} at 25°C).

Treatment and chromatography using Empigen BB produced a quite different protein pattern (fig.3). The second peak, sharp and well-separated, contained

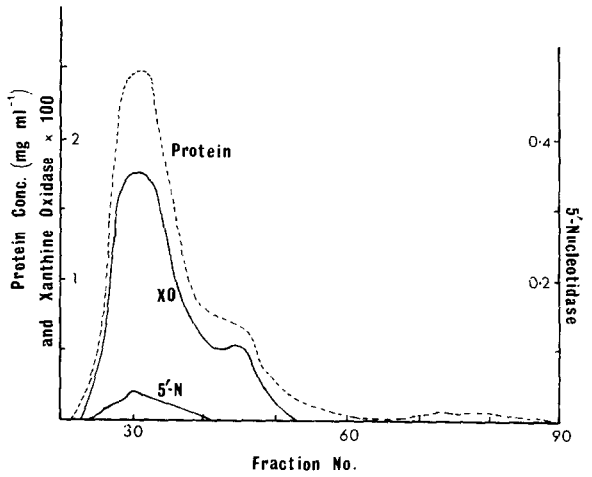

Fig.2. Chromatography of milk fat-globule membrane (12 mg protein) at 4°C on a 45 × 2.5 cm column of Bio-Gel A 150 m (1% agarose). The eluant was 0.05 M Tris pH 8.0, flow rate 6 ml hr^{-1} and the fraction size 2.4 ml. Activities are expressed in μmol product formed mg $protein^{-1}$ min^{-1} at 25°C for xanthine oxidase (XO), and at 37°C for 5'-nucleotidase (5'-N).

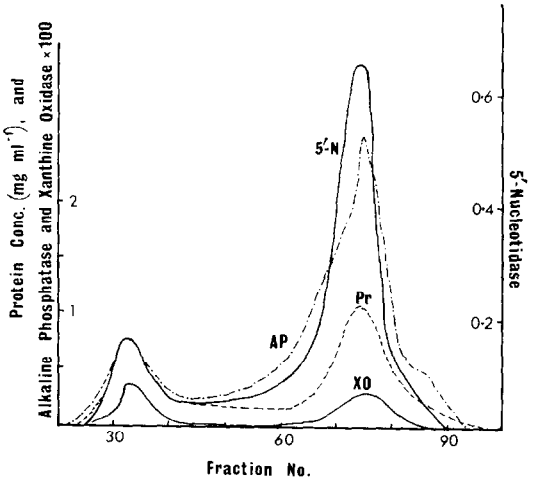

Fig.3. Chromatography of milk fat-globule membrane (12 mg protein) at 4°C on a 45 × 2.5 cm column of Bio-Gel A 150 m (1% agarose), after treatment with 0.1% (w/v) Empigen BB. The eluant was 0.05 M Tris pH 8.0, containing 0.1% (w/v) Empigen BB, flow rate 6 ml hr^{-1} and the fraction size 2.4 ml. Pr = protein concentration. Activities are expressed in μmol product formed mg $protein^{-1}$ min^{-1} at 25°C for xanthine oxidase (XO) and alkaline phosphatase (AP) and at 37°C for 5'-nucleotidase (5'-N).

most of the enzymic activities, although a little was retained in the small void-volume peak. Compared with the untreated control, the xanthine oxidase activity was somewhat lower, but alkaline phosphatase and 5'-nucleotidase activities were greatly enhanced.

3.2.2. Red blood-cell membrane

With no surfactant treatment, the homogenised erythrocyte membranes were incompletely eluted in a large protein peak coincident with the void volume; the remainder failed to elute through the agarose. ATPase activity was confined to this peak. When treated with Empigen BB, an almost clear solution was obtained, which was eluted completely when chromatographed with the surfactant in the eluant. Two protein peaks emerged (fig.4), each of which contained ATPase; the larger, lower molecular weight peak was well separated from that at the void volume.

3.2.3. Rat liver-cell plasma membrane

The homogenised, untreated membrane preparation was cloudy and completely failed to elute through the column. Treatment with Empigen BB produced a translucent sample, which chromatographed satisfac-

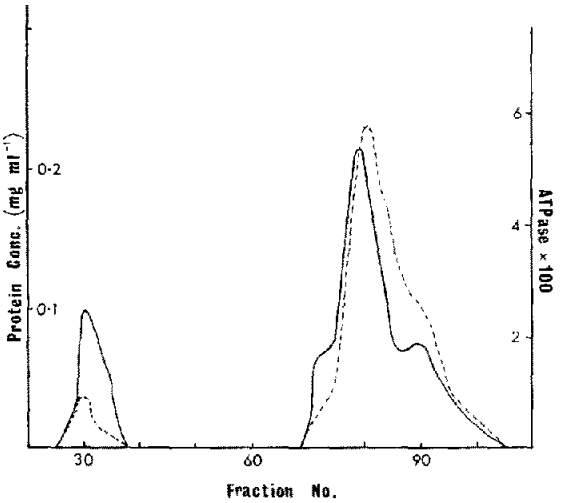

Fig.4. Chromatography of red blood-cell membranes (10 mg protein) at 4°C on a 45 × 2.5 cm column of Bio-Gel A 150 m (1% agarose), after treatment with 0.1% (w/v) Empigen BB. The eluant was 0.05 M Tris, pH 8.0, containing 0.1% (w/v) Empigen BB, flow rate 6 ml hr^{-1} and the fraction size 2.4 ml. Protein concentration (dotted line). ATPase activity, (continuous line) is expressed in μmol inorganic phosphate formed mg $protein^{-1}$ min^{-1} at 37°C.

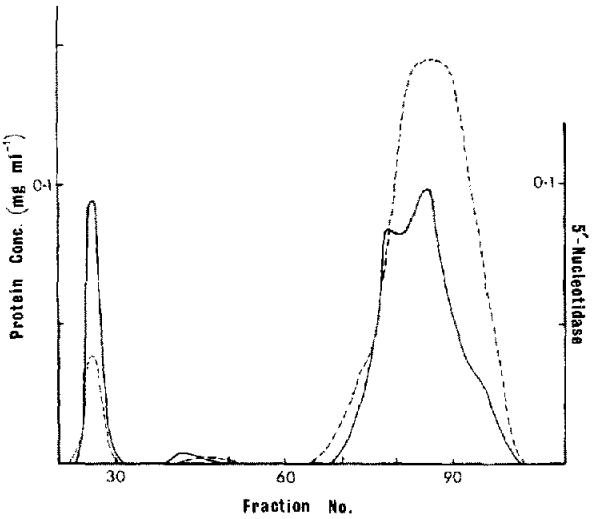

Fig.5. Chromatography of rat liver cell plasma membranes (6 mg protein) at 4°C on a 45 × 2.5 cm column of Bio-Gel A 150 m (1% agarose), after treatment with 0.1% (w/v) Empigen BB. The eluant was 0.05 M Tris pH 8.0, containing 0.1% (w/v) Empigen BB, flow rate 6 ml hr^{-1}, and the fraction size 2.4 ml. Protein concentration (dotted line); 5′-nucleotidase (continuous line) activity is expressed as μmol inorganic phosphate formed mg $protein^{-1}$ min^{-1} at 37°C.

torily to give a pattern (fig.5) similar to that given by the red blood-cell membranes. Each of the peaks contained 5′-nucleotidase activity.

4. Discussion

Ideally, the dissociation of a membrane by a surfactant should yield relatively small lipoprotein or protein fragments, and yet retain enzymic activities. The widely-used anionic surfactant, sodium dodecyl sulphate, efficiently binds to proteins [18,19], but is also a denaturant. Rapid inactivation was therefore expected and observed for all the enzymes studied in this work (fig.1). Sodium deoxycholate, also anionic, was less inactivating, but showed no tendency to maintain activities at a high value, even over the first 2–3 days.

Non-ionic surfactants, of which Triton X-100 is a typical example, have much less drastic dissociative effects [20]. Fig.1 shows that the activities of milk fat-globule membrane enzymes were preserved by Triton X-100; it was particularly effective in the case of alkaline phosphatase. However, considerably less of the protein was dissociated to a size capable of being included into the agarose employed in the chromatographic separations. In the chromatography of Triton-treated milk fat-globule membrane, the activities were maintained, but nearly all of the protein emerged at the void volume. It appears likely that the reason for its preservation of activities lies in the relatively low dissociative capability of Triton X-100.

The effects of Empigen BB on membranes differ in several respects from those of the more commonly-used surfactants. At a surfactant concentration much lower than is usually employed in such studies, considerable dissociation of the membranes occurred simply by treating the samples with 0.1% Empigen BB for 18 hr at 4°C. This was observed not only by clarification of the samples, but also by their chromatographic behaviour (figs.3–5); in each case, most of the protein was included, and emerged well after the void volume. The concentration used in this work (0.1% w/v, or approx. 3 mM) probably represents the lower limit of its effectiveness, since it is also to its critical micelle concentration of 1.8 mM [21]. In addition to this dissociative effect, enzymic activities were usually well retained after treatment with Empigen BB. Figs.3–5 show that this retention was not due to

large undissociated fragments of membrane, since considerable activities were present in the retarded, lower molecular weight peaks.

The mechanism of action of Empigen BB is by no means clear. It is a smaller molecule than Triton X-100, which may facilitate penetration of the membrane, and thus assist in its dissociation. Another factor is possibly the charge distribution of the zwitterionic surfactant, which is similar to that of membrane phospholipids such as phosphatidylcholine; the surfactant may be able to replace the phospholipids, and by mimicking them to some extent provide a suitable environment in which the membrane proteins can retain their native conformations and activities.

It thus appears that Empigen BB, at low concentration and under mild conditions, can induce a high degree of membrane dissociation together with considerable preservation of enzymic activity. It is hoped that zwitterionic surfactants may prove a useful addition to the range of dissociating agents available to those studying membrane structure and function.

Acknowledgements

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