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LECTINS AS BIOCHEMICAL AGENTS FOR THE ISOLATION OF SEALED MEMBRANE VESICLES OF DEFINED POLARITY

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Summary

The asymmetric distribution of carbohydrate on biological membranes has provided the basis for the development of lectin-affinity methodology which permits the isolation of sealed, inside-out membrane fractions from heterogeneous populations of vesicles.

Optimal conditions for these separations have been assessed employing purified right-side-out and inside-out vesicles derived from the plasma membrane of human erythrocytes as a model system. In this special case, homogeneous populations of defined polarity can be produced by varying the ionic conditions during formation of the vesicles. Surface-specific enzymic markers exist also for monitoring the integrity and orientation of a given population.

Multivalent lectins such as wheat germ agglutinin and soya bean agglutinin, which induce direct agglutination of erythrocyte membrane fragments containing accessible carbohydrate residues, selectively remove more than 90% of right-side-out and non-sealed membrane from a mixed population, a reaction which is inhibited by GluNAc or GalNAc, respectively.

Non-agglutinating lectins, e.g. concanavalin A, immobilized on an inert matrix such as Sepharose 4B, may be employed to adsorb out specifically vesicles with exposed glycopeptides on their surface. In this technique, it is necessary normally to remove the non-sealed membranes on Dextran density gradients prior to the final preparation of inside-out vesicles on Con A-Sepharose.

Finally, selective immunoprecipitation of fragments with accessible sugars may also be achieved after treatment with a non-agglutinating lectin (concanavalin A) followed by incubation with anti-concanavalin A IgG which promotes

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Abbreviation: SDS, sodium dodecyl sulphate.

rapid aggregation of membrane containing exposed receptors for the lectin.

These procedures should prove generally suitable for the isolation of tightly-sealed, inside-out membrane populations in a variety of biological systems. Pure populations of vesicles, exhibiting reversed polarity, are valuable in surface-labelling studies for investigating the structure, function and transmembrane distribution of integral membrane proteins/glycoproteins.

Introduction

In recent years, there has been increasing recognition of the important role of carbohydrate in biological membranes. For most mammalian cells, this component comprises 1—10% of the total membrane mass, the bulk being covalently associated with protein [1]. Integral glycoprotein(s) and glycolipid(s) are now implicated in a wide variety of major cell surface phenomena including hormone-receptor responses [2], antigenic determination [3], immunological mechanisms [4] and transport functions [5,6].

Early studies on red blood cell plasma membranes established that their total sialic acid content was located on the outer surface and was susceptible to treatment by neuraminidase [7]. Subsequently, it was ascertained by electron microscopy using specific staining techniques, including ferritin-conjugated lectins [8,9], that all the carbohydrate is situated exclusively on the exterior surface of the cell.

The asymmetric distribution of carbohydrate is a common, if not yet a clearly universal, feature of biological membranes, and can be extended to include intracellular elements e.g. rough and smooth endoplasmic reticulum [10] and nuclear membrane [11]. In these instances, the glycosylated regions of the integral membrane glycoproteins never appear to be in direct contact with the cytoplasm of the cell. In rough endoplasmic reticulum, the orientation of glycoconjugates determines that the carbohydrate is confined to the surface opposite to bound ribosomes, i.e. facing into the intra-cisternal space. Virtanen and Wartiovaara [11] consider that in nuclei the carbohydrate-containing material is located only between the double membrane of the nuclear envelope. These observations are consistent with current views for the mode of synthesis, processing and transport of membane and/or secretory glycoproteins [12,13].

A special property of erythrocyte plasma membranes is their ability to form sealed homogeneous populations of inside-out or right-side-out vesicles by manipulation of the ionic environment [14]. In other cell types, isolation of plasma membrane yields mixed populations of vesicles, together with non-sealed or 'scrambled' membrane formed by fusion of fragments of opposite polarity.

In red blood cells, good criteria exist also for assessing the quality of preparations based mainly on the crypticity of enzymes known to be located on either the inner or outer surface of the bilayer [15]. Acetylcholinesterase (EC. 3.1.1.7) and sialic acid are useful markers for the outer surface while glyceraldehyde-3-phosphate dehydrogenase (EC. 1.2.1.12) and NADH-cytochrome *c*

reductase are associated with the cytoplasmic side of the membrane in intact cells.

The capacity to isolate pure inside-out populations is particularly advantageous for topographical studies aimed at detecting transmembraneous proteins/glycoproteins [16], as the exterior surface can always be 'tagged' specifically in the intact cell [17] or organelle [18]. Using red blood cell membrane as a model system, we have developed three related procedures, based on the specific interaction of lectins with membrane carbohydrate, for the isolation of inside-out vesicles from heterogeneous populations of membrane fragments. The quality of these preparations is similar to those generated by conventional methods in this system. These techniques should prove generally applicable to other biological membranes where it is not possible to induce controlled sealing of membrane fragments in a defined orientation as is the case for red cell membranes.

Materials and Methods

Preparation of right-side-out and inside-out fragments of plasma membrane from human erythrocytes

Human red blood cells (outdated) were obtained from the Blood Bank, Western Infirmary, Glasgow. Purified plasma membrane 'ghosts' were employed to produce Mg^{2+}-sealed right-side-out or inside-out vesicles essentially as described by Steck and Kant [15]. However, isolation of the tightly sealed membrane fragments in 8% (w/v) Dextran T-70/5 mM phosphate buffer, pH 8.0, was achieved by upward flotation at 100 000 $\times g$ for 2 h at 4°C. Non-sealed membrane was pelleted under these conditions. This procedure prevented physical trapping of non-sealed material at the Dextran/sample interface which may have occurred in the original method where the sample was overlaid on the Dextran density barrier.

On occasions, sealing was induced in the presence of 20 μCi thymidine 5′-[^{32}P]triphosphate (2000—3000 Ci/mmol) or [*methyl*-^{3}H]thymidine 5′-triphosphate (40—60 Ci/mmol), the Radiochemical Centre, Amersham, U.K., and the isolated fractions were washed 5 times in 0.1 M NaCl/1 mM PP_i/20 mM Tris-HCl, pH 7.5, to remove extraneously bound TTP.

Criteria for integrity and polarity of membrane vesicles from red cell plasma membrane

Right-side-out and inside-out populations of membrane fragments were assessed routinely by specific enzymic markers [15]. Glyceraldehyde-3-phosphate dehydrogenase is located exclusively on the cytoplasmic face of the membrane, whereas acetylcholinesterase activity is confined to the exterior surface. Consequently, since their substrates are non-penetrating, their activities are latent in vesicles of the appropriate polarity unless the membrane is subjected to treatment with detergents, e.g. Triton X-100.

Lectin sources

Wheat germ agglutinin and soya bean agglutinin were purified by affinity chromatography on chitin and Sephadex G-50 columns [19] and Sepharose-

N-caproylgalactosamine columns [20], respectively. Wheat germ agglutinin appeared as a single band on 5.6% (w/v) and 10% (w/v) polyacrylamide gels with a subunit molecular weight of 18 000. Three bands were observed in soya bean agglutinin preparations with the major species (approx. 80% of the Coomassie blue stain) of M_r 30 000, corresponding to the subunit polypeptide of the lectin. Purified concanavalin A was purchased from Sigma (G.B.) Ltd. and Con A-Sepharose 4B from Pharmacia (G.B.) Ltd.

Antiserum to concanavalin A

Antibody production was stimulated in Dutch rabbits by subcutaneous, multiple-site injection of 1 mg concanavalin A in Freund's complete adjuvant. After 4—6 weeks, a similar regime was followed using Freund's incomplete adjuvant. This treatment was repeated after only 10—12 days and antiserum collected 10—12 days later by bleeding from an ear vein. Further stimulation of antibody production, by injection of 0.5—1.0 mg concanavalin A in incomplete adjuvant, was always performed 10—13 days prior to subsequent bleedings.

All antisera yielded a single, continuous precipitin line in the standard Ouchterlony double-diffusion test [21]. In all cases, 100—150 μl of crude antisera gave optimal precipitation of 10 μg lectin. Purification of the IgG fraction was achieved by ion-exchange chromatography on DEAE-cellulose [22].

Results

Fig. 1 demonstrates the effect of non-ionic detergent on the activities of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase, specific markers for the outer and inner surfaces of the red cell plasma membrane, in standard preparations of right side-out vesicles. As expected, acetylcholinesterase activity (Fig. 1B) is almost fully expressed in these fragments. In contrast, glyceraldehyde-3-phosphate dehydrogenase is stimulated routinely 6—9-fold (Fig. 1A) on addition of Triton X-100. In analogous experiments on inside-out vesicles (Fig. 2), acetylcholinesterase activity (Fig. 2A) increases 5—10-fold in the presence of Triton X-100, whereas only minor detergent effects [15] can be measured on glyceraldehyde-3-phosphate dehydrogenase activity (Fig. 2B). The figures for the degree of accessibility of these enzymes in the two preparations are in close agreement with those reported originally [15]. The efficiency of sealing and quality of purification is observed also in cases where [^{3}H]TTP or [^{32}P]TTP is trapped inside membrane fragments during the process of vesiculation. If the band containing sealed membrane vesicles is harvested from the Dextran density barrier along with the pellet which comprises largely non-sealed material, the radioactivity associated with each fraction can be determined. For right-side-out (96 836 cpm/mg) and inside-out (99 686 cpm/mg) vesicles, the specific activity of the samples in this experiment is 21-fold and 17-fold higher, respectively, than that of the non-sealed pellets (4647; 5743 cpm/mg). These data indicate that TTP is present in the interior of the vesicles and is not bound to their external surfaces.

Confirmation of this result is obtained in Fig. 3. In intact vesicles (Fig.

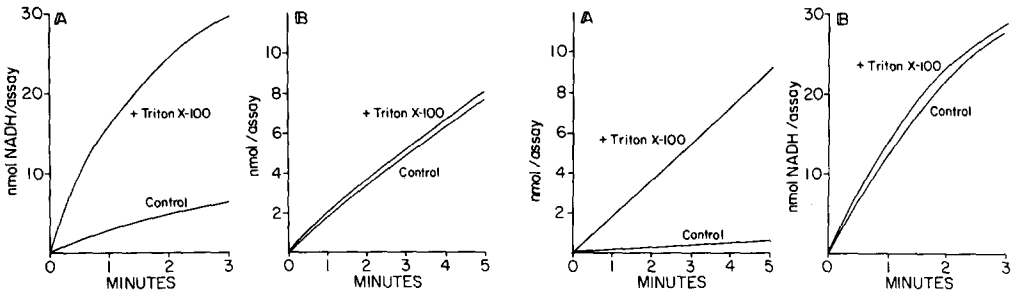

Fig. 1. Crypticity of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase in Mg^{2+}-sealed right-side-out vesicles from erythrocyte plasma membrane. Sealed right-side-out membrane fragments were prepared by the method of Steck and Kant [15]. Untreated samples (2—10 μg) were assayed for glyceraldehyde-3-phosphate dehydrogenase (A) or acetylcholinesterase activity (B), and compared to samples pre-treated for 30 s with 0.1% (v/v) Triton X-100. Enzymatic activity was measured spectrophotometrically at 20°C at 340 nm or 412 nm, respectively.

Fig. 2. Crypticity of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase in inside-out vesicles of erythrocyte plasma membrane. Inside-out vesicles (10—20 μg), prepared as described in Materials and Methods, were assayed for the accessibility of acetylcholinesterase (A) or glyceraldehyde-3-phosphate dehydrogenase (B) as described in Fig. 1.

3A), [^{3}H]TTP appears at the void volume of a Sephadex G-50 column along with the sealed membrane material. On disruption with Triton X-100 however, the membrane proteins are still excluded from the column as large protein-Triton X-100 micelles while [^{3}H]TTP now elutes in its expected position as a low molecular weight component corresponding to the total column volume (Fig. 3B).

Direct agglutination of membrane populations by lectins

Sealed populations of right-side-out vesicles derived from human red blood cell plasma membranes are tested for their capacity to be agglutinated by three lectins, namely concanavalin A, wheat germ agglutinin and soya bean agglutinin. The patterns of agglutination are found to be very similar to those noted previously for intact human erythrocytes. Thus, concanavalin A induces no significant aggregation at concentrations of up to 1 mg/ml, whereas wheat germ and soya bean lectins cause rapid and complete aggregation of right-side-out vesicles. An example is shown in Fig. 4 in which treatment with soya bean agglutinin elicits quantitative (90—95%) precipitation of right-side-out vesicles as assessed by the disappearance of acetylcholinesterase activity from the supernatant fraction. The presence of GalNAc, the specific monosaccharide inhibitor of soya bean lectin, completely prevents this reaction.

The value of this approach for the selective isolation of inside-out vesicles from heterogeneous populations of membrane is indicated in Fig. 5. Equal amounts of right-side-out and inside-out vesicles, prepared in the presence of [^{32}P]TTP or [^{3}H]TTP, respectively, are mixed prior to treatment with increasing concentrations of soya bean lectin. The agglutinated material is pelleted firmly by centrifugation at 400 × *g* for 10 min before estimation of the ^{3}H and ^{32}P isotope remaining in the supernatant fraction on a Beckman Biogamma T.M. Counter. It is apparent that the right-side-out population

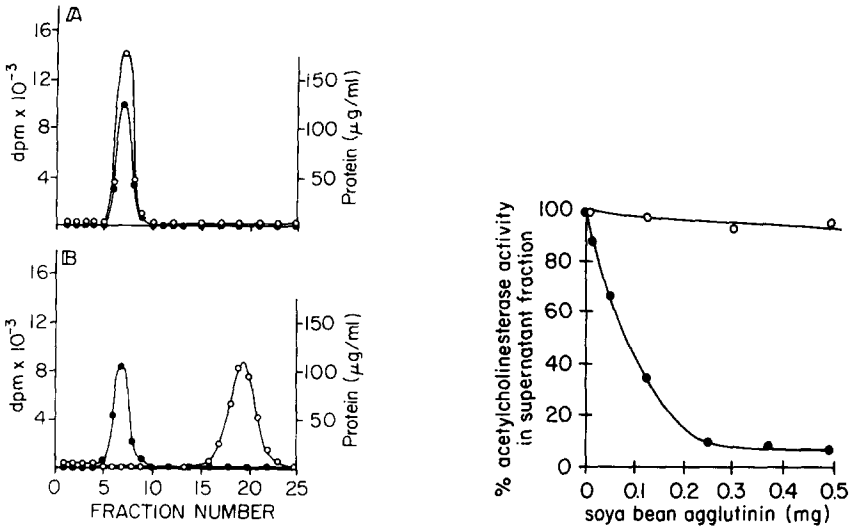

Fig. 3. Incorporation of [γ-^{32}P]TTP in sealed vesicles. A standard sample of Mg^{2+}-sealed right-side-out vesicles was formed in the presence of 20 μCi [γ-^{32}P]TTP. The washed fragments (Materials and Methods) were applied to a 1.5 cm X 10.0 cm Sephadex G-50 (Fine) column in 0.1 M KCl/20 mM Tris-HCl buffer, pH 8.0 (A). An identical sample (B) was run in the same buffer with the addition of 1% (v/v) Triton X-100. 1 ml fractions were collected for assay of protein (•) and radioactivity (○).

Fig. 4. Specific agglutination of sealed, right-side-out vesicles by soya bean agglutinin. Right-side-out vesicles (480 μg) were incubated for 30 min at 20°C with increasing amounts of soya bean agglutinin in the presence (○) or absence (•) of 20 mM GalNAc. The incubation mixture (1 ml) contained 0.1 M KCl/0.2 mg/ml bovine serum albumin/20 mM Tris-HCl, pH 7.5. Samples were centrifuged at 400 X g for 10 min and acetylcholinesterase activity remaining in the supernatant fraction (pre-treated with 0.1% (v/v) Triton X-100) determined as described before.

(^{32}P-labelled) is selectively removed under these conditions and there is no equivalent decrease in the amount of inside-out fragments (^{3}H-labelled) remaining in suspension. Optimal conditions for selective agglutination require a low concentration of membrane (1 mg/ml) and the presence of bovine serum albumin (0.2 mg/ml) and 0.1 M KCl [23] in the incubation medium; otherwise appreciable non-specific agglutination can occur, particularly with wheat germ agglutinin which is a basic protein.

Instead of producing radioactively labelled vesicles, the quality of separation may be monitored also on a more routine basis, by enzymic criteria, by observing the extent of removal of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase activity from the supernate on lectin treatment. As shown in Fig. 6, there is a marked decrease in the level of acetylcholinesterase in suspension after incubation with wheat germ agglutinin. No corresponding loss of glyceraldehyde-3-phosphate dehydrogenase is found in the same sample. Both assays are conducted in the absence of detergents: under these conditions acetylcholinesterase activity is principally associated with right-side-out vesicles while the bulk of the glyceraldehyde-3-phosphate dehydrogenase is accessible on inside-out vesicles.

Selective adsorption of membranes containing accessible carbohydrate

In many instances, lectins will interact specifically with glycoprotein recep-

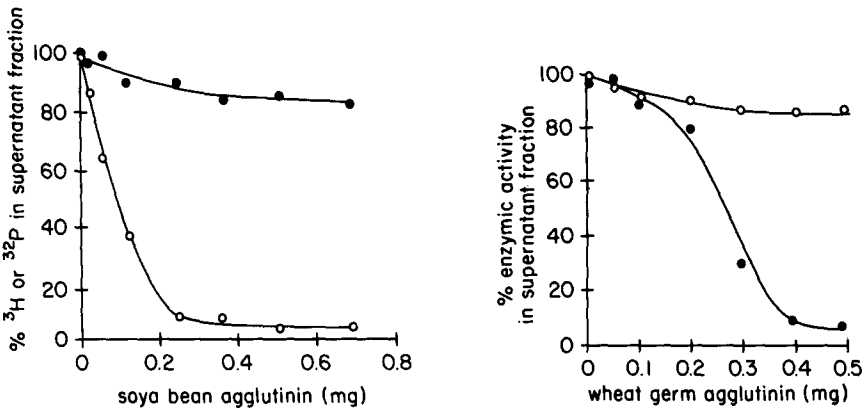

Fig. 5. Selective precipitation of right-side-out vesicles by soya bean agglutinin from a heterogeneous population of sealed membrane fragments. Standard samples of right-side-out and inside-out vesicles were prepared in the presence of 20 μCi [γ-^{32}P]TTP or [*methyl*-^{3}H]TTP, respectively. The washed pellets were mixed in a ratio of 2 : 1 on a protein basis, and 280 μg amounts incubated with increasing amounts of lectin, employing the incubation conditions described in Fig. 4. After centrifugation at 400 × *g* for 10 min, residual ^{32}P radioactivity (right-side-out fragments, ○) and ^{3}H radioactivity (inside-out fragments, ●) in the supernatant fraction was determined. Control samples, incubated as above, were spun at 28 000 × *g* for 20 min to pellet the vesicles completely. Only 4—6% of the radioactivity was released into the supernate in these samples, indicating that there is no significant leakage of TTP from the vesicles during the experiment.

Fig. 6. Analysis of selective removal of right-side-out vesicles by enzymatic criteria. Standard preparations of right-side-out and inside-out vesicles (2 : 1 ratio) were treated as in Fig. 4 with varying amounts of wheat germ agglutinin. After centrifugation, the supernatant fraction was monitored for the disappearance of acetylcholinesterase (●) or glyceraldehyde-3-phosphate dehydrogenase activity (○).

tor(s) on the surface of the plasma membrane without inducing agglutination, e.g. concanavalin A, lentil lectin in human red blood cells. For these cases, vesicles with accessible carbohydrates may be adsorbed out conveniently on immobilized lectin-affinity columns. Table I reveals the separation of vesicles of opposite polarity on Con A-Sepharose 4B. The quality of the original right-side-out and inside-out preparations, as indicated by the percentage of maximum acetylcholinesterase activity (±Triton X-100) is expressed as percent accessibility [15].

The mixed population for application to the column has 61.9% accessibility of its acetylcholinesterase activity in the absence of detergent. The unretarded fraction which appears in the void volume resembles closely the original inside-out vesicles in the crypticity of its acetylcholinesterase activity (11.8% accessibility). This sample also represents 31.4% of the applied protein, equivalent to the proportion of inside-out material in the original mixed population. No bound membrane could be eluted by inclusion of 0.2 M methyl-α-D-glucoside in the column buffer. Further addition of 1% (v/v) Triton X-100 to disrupt the vesicles results in almost quantitative recovery of protein and enzymic activity.

A useful variation on this approach is to perform selective immunoprecipitation of membrane fragments, pre-treated with lectin to interact with available glycoprotein receptors. Specific agglutination may then be induced with anti-lectin antibody (IgG). This procedure is particularly suitable in systems where

TABLE I

SEPARATION OF RIGHT-SIDE-OUT AND INSIDE-OUT MEMBRANE VESICLES ON CON A-SEPHAROSE 4B

Purified right-side-out and inside-out fragments were mixed as indicated and the percentage accessibility (acetylcholinesterase activity minus detergent X 100/activity plus detergent) determined as described previously [14]. The sample of mixed vesicles (430 μg) was applied to a 2 ml column of Con A-Sepharose 4B in 0.1 M NaCl/20 mM Tris-HCl, pH 8.0. The column was preequilibrated in this buffer containing bovine serum albumin (1 mg/ml) to minimize non-specific binding. The flow rate was maintained at 2 ml/h while fractions were collected. Adsorbed membrane was eluted by inclusion of 1% (v/v) Triton X-100 and 0.2 M methyl-α-D-glucoside in the original buffer. Total protein and enzymatic activity in the unretarded and eluted peaks was determined after pooling the appropriate fractions.

Sample	Protein (μg)	Acetylcholinesterase		
		—Triton X-100 (nmol/min per assay)	+Triton X-100	Accessibility (%)
Original fractions				
Right-side-out vesicles	298	233.8	279.4	83.7
Inside-out vesicles	119	20.3	110.3	18.4
Mixture	430	238.9	386.0	61.9
Con A-Sepharose fractions				
Unretarded fraction	135	16.2	136.9	11.8
Eluted fraction	318	—	198.5	—
%Recovery	105.3	—	86.9	—

the lectin itself does not cause direct agglutination and the preparation contains significant amounts of non-sealed membrane.

A heterogeneous population of red blood cell plasma membrane fragments is incubated in the presence of concanavalin A. Free and non-specifically bound lectin is removed by washing in 0.1 M KCl/0.2 mg/ml bovine serum albumin/

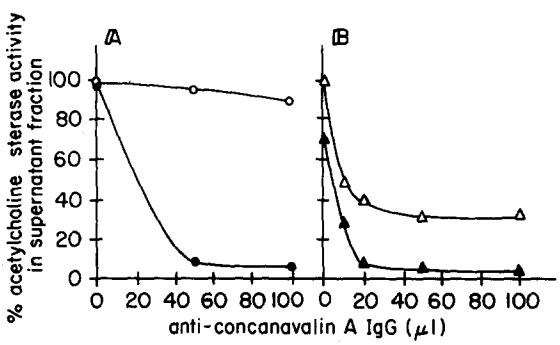

Fig. 7. Specific immunoprecipitation of membrane fragments with accessible carbohydrate by concanavalin A and anti-concanavalin A IgG. Approximately 2 mg of right-side-out and inside-out vesicles were treated separately with 1 mg concanavalin A at 20°C for 30 min in 0.1 M KCl, 0.2 mg/ml bovine serum albumin and 20 mM Tris-HCl, pH 7.5, before washing thoroughly (3X) in the same medium to remove excess and non-specifically bound concanavalin A. A 2 : 1 mixture of the fragments (425 μg) were incubated with increasing amounts of anti-concanavalin IgG (B) at 25°C for 30 min. After removal of the agglutinated material (400 X *g* for 2 min), acetylcholinesterase activity minus detergent (▲) or plus 0.1% (v/v) Triton X-100 (△) was measured as before. Control assays (+detergent) on pure inside-out (○) or pure right-side-out (●) populations in the supernate following standard immunoprecipitation are shown in Fig. 7A. All enzymic activities are expressed as a percentage of acetylcholinesterase activity of the original sample(s) in the presence of 0.1% (v/v) Triton X-100.

20 mM Tris-HCl, pH 7.5, before treatment with anti-concanavalin A IgG. The extent of precipitation with increasing anti-concanavalin A IgG levels is followed by assaying the disappearance of acetylcholinesterase in the supernatant fraction with or without Triton X-100 (Fig. 7B). No significant enzymic activity is detectable in the final supernatant fraction in the absence of detergent, although approximately 40% of the original activity is detected on addition of Triton X-100. The cryptic nature of the acetylcholinesterase activity in the supernate, at this stage a 9—10-fold stimulation is induced by detergent treatment, suggests strongly that it is the right-side-out population which is removed by the immunoprecipitation.

This is confirmed in control experiments, monitoring the effects of anti-concanavalin A IgG treatment on individual right-side-out and inside-out populations (Fig. 7A). Little or no precipitation of the latter preparations can be induced by incubation with concanavalin A/anti-concanavalin A IgG. However, in the case of right-side-out vesicles, only a small amount (5—10%) of residual acetylcholinesterase activity can be detected in the supernate.

Discussion

Many of our current concepts on membrane structure and organization as encompassed in the Fluid-Mosaic model are based on our knowledge of the erythrocyte plasma membrane [24]. This reflects mainly the inherent technical advantages of this system in that (a) large amounts of pure membrane are easily obtainable, (b) the membrane has a relatively simple polypeptide composition as analyzed on SDS-polyacrylamide gels, and (c) sealed, homogeneous populations of defined polarity can be isolated for topographical studies.

The present communication deals with the development of methods for the separation of inside-out vesicles from mixed populations of membrane fragments. These procedures should prove generally applicable to all biological membranes where there is a unilateral distribution of carbohydrate on the exterior surface. Assessment of these techniques, using erythrocyte membranes as a model, suggests that inside-out vesicles obtained in this manner are comparable in quality to those produced by conventional means. Thus populations which are 90% or more inside-out, based on the inaccessibility of enzymic markers, can be obtained in both cases.

Fractionation of sealed and non-sealed membrane vesicles

In the original procedure [15], separation of residual non-sealed plasma membrane from right-side-out or inside-out vesicles is achieved by centrifugation through a Dextran T-70 density gradient. Sealed fragments are observed to band at lower densities since trapping of H_2O in their internal phase appears to alter their buoyancy, their exact equilibrium density point being determined by their size. This step is obviated in the direct agglutination and indirect immuno-precipitation methods described in this paper. Lectins will interact equally well with either right-side-out or non-sealed membrane containing accessible glycoprotein receptors.

For isolation of inside-out populations on affinity columns, it is still necessary to remove unsealed material by other means. This important point has

been shown by Walsh et al. [25] in the fractionation of lymphocyte plasma membrane vesicles on Con A-Sepharose 4B columns. These workers observed that a proportion of the vesicles appear to maintain a general 'inside-out' orientation but are not tightly-sealed. Thus, although they do not interact with this lectin bound to agarose beads or ferritin-concanavalin A, the glycoprotein receptors are accessible to free concanavalin A. Similarly, glycopeptides can be released from their surfaces only with free trypsin and not the immobilized derivative. In lymphocyte membranes, the unretarded inside-out fraction on Con A-Sepharose columns is not agglutinated by specific antisera directed against the outer surface of the membrane, after prior removal of non-sealed material on Dextran T-10 gradients.

Technical aspects of the separations

It should be emphasized that isolation of high quality vesicles by these procedures is not a trivial operation and it is desirable to have proper criteria, e.g. enzymic markers or surface-specific antisera, to monitor the purity of preparations routinely. Care should be taken not to overload the Con A-Sepharose 4B columns which only have a limited capacity to bind vesicles (approximately 250 μg protein/ml in this case), presumably because vesicles do not penetrate the gel matrix, only interacting with available concanavalin A molecules on the surface. Low flow rates (2—10 ml/h) or stopping the column for 10—20 min are essential for complete retention of appropriate vesicles.

Non-specific absorption or aggregation can be minimized by the presence of bovine serum albumin (0.2 mg/ml) and intermediate ionic strength (0.1 M KCl) [23]. For immuno-precipitation studies, it has been observed that incomplete precipitation of vesicles will occur in conditions of antibody excess. It is therefore important to regulate vesicle concentrations precisely during incubation with lectins and anti-lectin sera.

These types of preparations should prove suitable for researchers interested in detecting proteins located exclusively on the interior surface of biological membranes or possible transmembrane proteins which are ideal candidates for roles in a variety of transport or receptor functions. We are currently using these procedures in selective labelling studies of mitochondrial membranes to define major transport systems of the inner membrane at a molecular level.

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