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ISOLATION OF A SEALED HOMOGENEOUS POPULATION OF INNER MEMBRANE FRAGMENTS WITH INVERTED ORIENTATION FROM RAT LIVER MITOCHONDRIA USING SPECIFIC LECTIN IMMUNOPRECIPITATION

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Summary

A novel method for the isolation of well-defined populations of inside-out vesicles from rat liver mitochondria is described. The technique utilizes specific immunoprecipitation of vesicles with accessible carbohydrate residues from a mixed population of inner membrane fragments using wheat germ agglutinin and anti-wheat germ agglutinin IgG. The unprecipitated fraction comprises 30—50% of the original population and exhibits little or no cytochrome *c* oxidase activity as estimated with exogenous cytochrome *c* as substrate. Addition of deoxycholate to promote membrane disruption results in an 8—10-fold increase in enzymic activity compared to only 1.5—2.0-fold stimulation in standard preparations of submitochondrial particles. It is concluded that the lectin affinity-purified membranes represent a sealed homogeneous (90—95% pure) population of inside-out inner membrane vesicles.

Introduction

It is commonly assumed that vesicles prepared from the mitochondrial inner membrane by sonication or pressure disruption are 'inside-out' with respect to the intact organelle. Evidence cited in favour of this hypothesis is both of a structural (location of F_1 ATPase, extraction and antibody studies

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Abbreviations: NacGlc, *N*-acetyl glucosamine; MOPS, 4-morpholinepropanesulphonic acid.

of cytochrome *c*) and functional nature (proton uptake) [1]. However, despite these criteria it is still unclear whether submitochondrial particles represent a sealed homogeneous preparation of inside-out vesicles or whether this represents the only active fraction in a mixed population also containing non-sealed membranes.

Disruption of cells or organelles leads in general to the formation of a mixed population containing inside-out, right-side-out and non-sealed fragments with perhaps some 'scrambled' membrane induced by the fusion of vesicles of opposite sidedness. In order to examine the detailed topography of the inner mitochondrial membrane, a great deal of work has been performed by surface-specific labelling of mitochondria depleted of their outer membrane (C-side) and submitochondrial particles prepared by sonic disruption (M-side) [2—4]. However, the ambiguity in the homogeneity of submitochondrial particles is of particular concern as certain groups of experimenters have failed to demonstrate any sizeable differences in the labelling of the M- and C-sides of the inner membrane with the aid of membrane-impermeant probes [5]. Further, other workers have interpreted a reduction in labelling of polypeptide components, e.g. in complex III, as indicating their absence from a particular membrane surface [6]. In order to estimate the homogeneity of populations of submitochondrial particles from rat liver mitochondria, we have assayed for crypticity of a particular marker enzyme known to be located on a specific side of the bilayer. Cytochrome *c* oxidase satisfies this criterion. Since cytochrome *c*, the substrate for this enzyme, has its binding site on the cytoplasmic side of the membrane and does not readily penetrate the lipid bilayer, assay of enzymic activity with externally added cytochrome *c* in the presence or absence of detergents [7] can provide clear indication of the orientation and degree of sealing of submitochondrial particles in any given preparation.

The asymmetric distribution of carbohydrate is a common feature of plasma membranes, e.g. red blood cell [8] and lymphocyte membrane [9]. These observations have now been extended to include intracellular membranes such as the rough and smooth endoplasmic reticulum [10]. It has been reported that isolated mitochondria contain 1—2% carbohydrate by weight [11] and its presence on the surface of the mitochondrial membrane has been demonstrated by direct agglutination with lectins [12,13]. We have used wheat germ agglutinin, a lectin which has a binding specificity for *N*-acetyl glucosamine (NAcGlc), to establish the presence of the latter saccharide moieties on the cytoplasmic surfaces of both the inner and outer membranes.

In this paper we will present evidence that pure (90—95%) preparations of inside-out submitochondrial particles can be isolated by taking advantage of the fact that wheat germ agglutinin interacts only with those membranes in which the binding sites are accessible (right-side-out inner membrane vesicles/non-sealed fragments). The unagglutinated membrane fraction (inside-out vesicles) has a very low cytochrome *c* oxidase activity, which can be activated on rupture of the membrane by detergents, indicating that the cytochrome *c* binding site is inside the permeability barrier.

Materials and Methods

Isolation and purification of rat liver mitochondria

Rat liver mitochondria were isolated from starved 180—200 g rats of the Wistar strain according to the method of Chance and Hagihara [14] in 0.225 M mannitol/0.15 M sucrose/2 mM MOPS/400 μM EGTA, pH 7.4.

The mitochondria were purified on a discontinuous sucrose gradient consisting of 2.0 ml of 54%, 6.5 ml of 45%, 6.5 ml of 39% and 5.5 ml of 20% (w/w) sucrose [15]. The purified mitochondria (band of density 1.19 g/cm^3) was harvested after centrifugation at 49 000 × *g* for 2 h and pelleted at 6500 × *g* for 10 min after slow dilution in isolation medium.

Preparation of mitoplasts

Purified mitochondria isolated from the liver of a single rat were swollen in 10 mM Tris-phosphate buffer, pH 7.5, at 4°C for 5 min. The mitochondria were then shrunk in 0.3 M sucrose/2 mM ATP/2 mM $MgSO_4$/1 mM phenylmethylsulphonyl fluoride. The mitochondria were then pelleted by centrifugation at 6500 × *g* for 10 min. The mitochondria were resuspended in 0.12 M KCl/20 mM MOPS, pH 7.4, buffer and washed once in the same buffer prior to treatment with digitonin (0.20 mg/mg protein) for 15 min on ice [16]. The reaction was terminated by diluting the sample into the ionic strength buffer and pelleting the mitochondria by centrifugation at 8000 × *g* for 10 min. The mitoplast fraction was finally washed once in 0.12 M KCl/20 mM MOPS, pH 7.4.

Preparation of submitochondrial particles

A similar procedure to the preparation of mitoplasts was followed up to the swelling and shrinking stage. The mitochondria were then sonicated for 2 × 30-s intervals at 3.5 A using a Branson sonicator with a microtip probe. The sonicated preparation was separated on a discontinuous sucrose gradient. The inner membrane pellet was harvested from the gradient and washed twice by pelleting in 50 mM phosphate buffer, pH 7.5, at 100 000 × *g* for 40 min.

Assessment of polarity of inner membrane vesicles

Cytochrome *c* oxidase activity was estimated as described in Ref. 7 in 3.0 ml of assay medium containing 60 mM KCl/0.25 M sucrose/10 mM Tris-HCl, pH 7.4/25 mM ascorbate/100 μM cytochrome *c*/3 μM rotenone/1 μM 2,4-dinitrophenol. Latent enzymic activity in the vesicle populations was stimulated by addition of 0.3% (w/v) deoxycholate.

Purification of inside-out inner membrane vesicles with no accessible carbohydrate

Inner membrane vesicles resuspended in 100 mM phosphate buffer, pH 7.5, were prepared from purified rat liver mitochondrial inner membrane (20 mg protein) by sonication for 2 × 30-s, followed by centrifugation at 100 000 × *g* for 40 min. The final preparation (10 mg protein) was incubated with 2 mg wheat germ agglutinin for 30 min at 25°C. Excess lectin was removed by washing twice in 100 mM phosphate, pH 7.5. Lectin-treated

vesicles were subsequently incubated for 30 min at 25°C and overnight at 4°C with increasing amounts of purified anti-wheat germ agglutinin IgG (rabbit) or IgG from normal rabbit serum. Phenylmethylsulphonyl fluoride (1 mM) was added to inhibit protease activity. Samples were then centrifuged at 1000 × *g* for 10 min to remove agglutinated vesicles. Purified inside-out inner membrane vesicles were finally pelleted at 100 000 × *g* from the supernatant fraction.

Lectin sources

Wheat germ agglutinin was purified by affinity chromatography on chitin and Sephadex G-50 columns [17]. Wheat germ agglutinin appeared as a single band on 10.0% (w/v) polyacrylamide gel with a subunit molecular weight of 18 000.

Iodination of wheat germ agglutinin

Wheat germ agglutinin was iodinated essentially as described in Ref. 18 in the presence of 0.3 M NAcGlc. The iodinated lectin was dialyzed against 0.1 M sodium phosphate buffer, pH 7.2, containing 1 mM KI at 4°C for three changes.

Preparation of antiserum

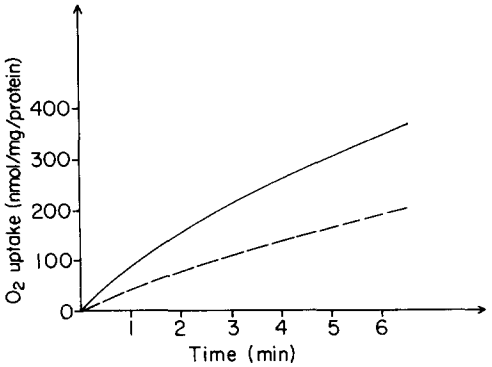
Antibody production was stimulated in Dutch rabbits by subcutaneous multiple-site injections of 1 mg wheat germ agglutinin in Freund's complete adjuvant. This treatment was repeated 4—6 weeks later with incomplete adjuvant and again 10—12 days later. Antiserum was collected 12 days later by bleeding from an ear vein. Before subsequent bleedings rabbits were always injected 10—12 days previously with 0.5—1.0 mg lectin in incomplete adjuvant. All antisera yielded a single, continuous precipitin line in the standard Ouchterlony double-diffusion test [19]. (Optimal precipitation was obtained with 10 μg lectin for 100 μl anti-lectin IgG). Purification of the IgG fraction was achieved by ion-exchange chromatography on DEAE-cellulose [20].

Results

Stimulation of cytochrome c oxidase activity in a sonicated inner membrane preparation on addition of deoxycholate

A valuable technique for assessing the purity of sealed mitochondrial inner membrane populations, is by measurement of cytochrome *c* oxidase activity which should be latent in sealed inside-out submitochondrial particles using externally added cytochrome *c* as substrate.

Cytochrome *c* oxidase activity was estimated in a sample of the submitochondrial particle suspension as described in Fig. 1. On addition of 0.3% (w/v) deoxycholate enzymic activity was stimulated routinely only 1.5—2.0-fold, indicating that only 30—50% of the total cytochrome *c* binding sites are inaccessible in a normal preparation. A similar result of approx. 2.0—2.2-fold stimulation of cytochrome *c* oxidase activity with exogenous cytochrome *c* in the presence of detergent was also obtained with a freshly prepared submitochondrial preparation from pigeon breast mitochondria. Such data are



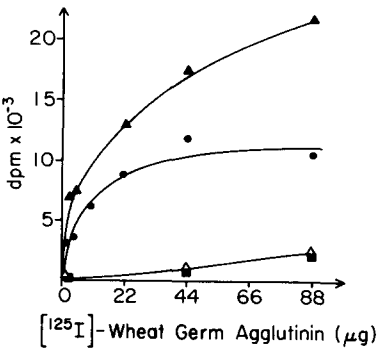


Fig. 1. Crypticity of cytochrome *c* oxidase activity in rat liver mitochondrial inner membrane vesicle populations. Inner membrane vesicles (10 mg) were prepared from rat liver mitochondria as described in Materials and Methods and finally resuspended in isolation medium. An aliquot of the membrane suspension (80—100 μg) was assessed for cytochrome *c* oxidase activity in the absence (- - - - - -) and in the presence of 0.3% (w/v) deoxycholate (———).

Fig. 2. Binding studies of ^{125}I-labelled wheat germ agglutinin to exposed lectin binding receptors in rat liver mitochondria and mitoplasts. Rat liver mitochondria and mitoplasts were prepared as described in Materials and Methods and finally washed and resuspended in 0.12 M KCl/20 mM Tris-HCl, pH 7.2. ^{125}I-labelled wheat germ agglutinin binding studies were carried out at 25°C for 20 min simultaneously in siliconized Eppendorf tubes on intact rat liver mitochondrial fractions (0.3 mg protein) (●) or mitoplasts (0.3 mg protein) treated with 0.2 mg digitonin/mg protein (▲). Samples were maintained in the ionic strength buffer throughout the procedure, before washing (four times) in the same buffer containing 1 mM KI. In a control experiment, 0.1 M NAcGlc was present during the lectin incubation and washing stages (△). An additional control experiment adopted an identical procedure, except that addition of the mitochondrial sample was omitted. The empty washed tubes were counted for residual radioactivity bound to the tube (■).

clearly not consistent with submitochondrial particles representing a uniformly sealed inside-out preparation of vesicles.

Studies of lectin binding to rat liver mitochondria and mitoplasts

Although the presence of carbohydrate on the mitochondrial membranes has been firmly established, very little is known abouts its precise location within the organelle. Using radio-iodinated wheat germ agglutinin as an external ligand for carbohydrate residues we have detected the presence of *N*-acetyl glucosamine residues on the external surfaces of both inner and outer membranes. ^{125}I-labelled wheat germ agglutinin binding studies were performed on mitochondria and mitoplasts that were resuspended in ionic strength buffer as described in Fig. 2. The data show that the number of binding sites for wheat germ agglutinin in mitochondria treated with digitonin (0.20 mg digitonin/mg protein) is almost double that present in intact mitochondria. Inhibition of binding to the lectin receptors was carried out by including 0.1 M *N*-acetyl glucosamine in the assay.

Preparation of a purified population of inside-out vesicles by selective immunoprecipitation of right-side-out vesicles and non-sealed membrane fragments with wheat germ agglutinin and anti-wheat germ agglutinin IgG

The previous result clearly demonstrates that submitochondrial particles

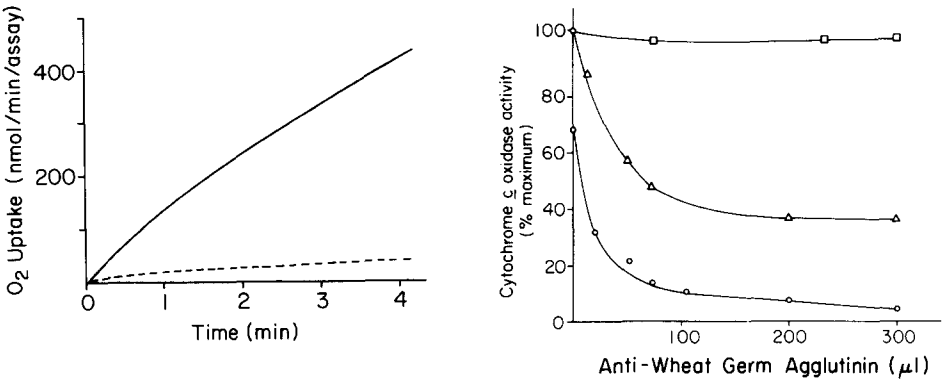


Fig. 3. Stimulation of latent cytochrome *c* oxidase enzymic activity in a purified inside-out vesicle population on addition of deoxycholate. Submitochondrial particles prepared from the mitochondrial inner membrane (5 mg) were treated with wheat germ agglutinin (2 mg) as described in Materials and Methods. An aliquot (1 mg protein) was immunoprecipitated with 300 μl anti-wheat germ agglutinin as described. The immunoprecipitate was centrifuged at 1000 × *g* for 10 min and residual cytochrome *c* oxidase activity in the supernatant fraction was estimated in the absence (- - - - -) and in the presence (———) of 0.3% (w/v) deoxycholate as described in Ref. 7.

Fig. 4. Selective immunoprecipitation by wheat germ agglutinin and anti-wheat germ agglutinin IgG of mitochondrial inner membrane vesicles containing exposed carbohydrate residues. Mitochondrial inner membrane vesicles were prepared and treated with wheat germ agglutinin as described in Materials and Methods. Aliquots of the lectin-bound protein fraction (1 mg) were immunoprecipitated with increasing quantities of anti-lectin IgG. The immunoprecipitates were centrifuged and residual cytochrome *c* oxidase activity estimated as described in Fig. 3. ○, vesicles incubated with anti-wheat germ agglutinin IgG; △, vesicles incubated with anti-wheat germ agglutinin IgG treated with 0.3% (w/v) deoxycholate before assay; □, vesicles incubated with normal IgG + 0.3% (w/v) deoxycholate before assay. All results were expressed as percentage of cytochrome *c* oxidase activity of the untreated submitochondrial particles and in the presence of 0.3% (w/v) deoxycholate.

prepared from rat liver are not of uniform conformation, in conflict with previous conclusions [1]. By employing different methodology, based on the premise that the carbohydrate in mitochondria is asymmetrically distributed, purification of inside-out vesicles from a mixed submitochondrial population by selective immunoprecipitation of all membrane fragments and vesicles with accessible carbohydrate was achieved.

A mixed population of purified mitochondrial inner membrane vesicles was prepared by sonication. The vesicles were treated with lectin and immunoprecipitated with anti-lectin IgG as described in Fig. 3. The quality of inside-out vesicles remaining in the supernatant fraction was assessed by measuring the latency of cytochrome *c* oxidase activity in the supernatant fraction. When 0.3% (w/v) deoxycholate was added to the supernatant fraction it resulted in a 9—10-fold stimulation in cytochrome *c* oxidase activity, indicating that the inside-out population is between 90—95% pure. In comparison, enzymic activity in the original submitochondrial population (see Fig. 1) is stimulated 1.5—2.0-fold with deoxycholate, showing that only 30—50% of the total cytochrome *c* binding sites are inaccessible in a normal preparation.

It can be tentatively concluded from this experiment that a large fraction of the submitochondrial population has accessible carbohydrate and can be

TABLE I

RECOVERY OF CYTOCHROME *c* OXIDASE ACTIVITY IN THE MITOCHONDRIAL FRACTIONS AFTER LECTIN AND ANTI-LECTIN IgG TREATMENT

Inner membrane vesicles (5 mg) were prepared from purified rat liver mitochondrial inner membrane and treated with wheat germ agglutinin (2 mg) as described in Materials and Methods. Excess lectin was removed by washing the vesicles in 100 mM phosphate buffer, pH 7.5. Lectin-treated vesicles (1 mg protein aliquots) were subsequently incubated for 30 min at 25°C and overnight at 4°C with either anti-wheat germ agglutinin IgG, normal IgG or phosphate buffer. Phenylmethylsulphonyl fluoride (1 mM) was added to inhibit protease activity. Samples were centrifuged at 1000 × *g* for 10 min to remove agglutinated vesicles. Cytochrome *c* oxidase activity was estimated in the absence and in the presence of deoxycholate (0.3% w/v) in the total sample or in the supernatant and pellet fractions in the case of specific immunoprecipitation.

Sample (Original fractions)	Cytochrome *c* oxidase (nmol O_2 / min per assay)		% Accessibility
	—Deoxycholate	+Deoxycholate	
Submitochondrial particles	772.2	1425.5	54.2
Submitochondrial particles + wheat germ agglutinin	760.0	1382.4	55.0
Submitochondrial particles + wheat germ agglutinin + normal IgG	797.2	1416.3	56.2
Submitochondrial particles + wheat germ agglutinin + anti-wheat germ agglutinin IgG			
supernatant fraction	81.4	667.7	12.2
precipitated fraction	668.6	691.7	96.6
%Recovery	94.1	96.0	—

selectively precipitated with wheat germ agglutinin and anti-wheat germ agglutinin IgG. The submitochondrial particles remaining in the supernatant fraction contain no exposed carbohydrate, while the cytochrome *c* binding site must also be inaccessible indicating that these vesicles are sealed and 'inside-out'.

To confirm this hypothesis and to test the possibility that the apparent latency of cytochrome *c* oxidase in purified submitochondrial populations is not caused by selective inhibition of enzymic activity by wheat germ agglutinin or IgG, we have quantified the recovery of cytochrome *c* oxidase in the supernatant and immunoprecipitation fractions (see Table I). The data clearly show that there is complete recovery of enzymic activity, demonstrating that the presence of wheat germ agglutinin and IgG has no significant effect on the assay for cytochrome *c* oxidase. The data also indicate that the two populations (supernatant and pellet fractions) respond in characteristic fashion to deoxycholate treatment. The cytochrome *c* oxidase is cryptic in nonprecipitated vesicles, being stimulated 8—10-fold on addition of detergent. Deoxycholate treatment of the corresponding pelleted vesicles, in contrast, elicits little or no response in associated cytochrome *c* activity. It is also clear that the inside-out submitochondrial population comprises approx. 40% of the total population, consistent with the 1.5—2.0-fold increases in cytochrome *c* oxidase (±deoxycholate) observed in crude preparations.

The data in Fig. 4 demonstrates the effects of increasing amounts of anti-wheat germ agglutinin IgG on the course of the immunoprecipitation. A rapid

increase in the latency of cytochrome *c* oxidase activity in submitochondrial particles is observed, indicating that with increased precipitation, vesicles with exposed cytochrome *c* binding sites are removed selectively. Maximal precipitation occurs at 100—300 μl of anti-wheat germ agglutinin IgG at which stage approx. 40% of the total cytochrome *c* oxidase (+deoxycholate) remains in the supernatant fraction. In the absence of excess quantities of anti-lectin IgG (greater than 300 μl) an increase of cytochrome *c* oxidase activity in the supernatant fraction was observed (data not shown) suggesting that the quantity of anti-lectin IgG for optimum precipitation of carbohydrate bound vesicles is 100—300 μl.

Discussion

In order to determine the orientation of protein within the lipid bilayer it is convenient to have access to defined populations of inside-out vesicles. Comparative labelling studies on vesicles of defined polarity can be used to ascertain the topography within the membrane and, in particular, identify transmembrane proteins. From a historical standpoint, it has been documented that sonication causes a fragmentation and 'pinching off' of the cristae, giving rise at least in part to vesicles which the outer surface corresponds to the original inner surface of the cristae. This is visualized by the position of the repeating ATPase units (F_1) on electron micrographs and other functional criteria, such as proton uptake instead of the ejection observed for intact mitochondria, which suggest that the vesicles have an opposite polarity to intact mitochondria [22]. However, although these criteria confirm the presence of 'inside-out' vesicles, they do not necessarily reflect homogeneity of the entire population, perhaps representing the active fraction in a mixed vesicle preparation.

In the case of beef heart mitochondria which have a very convoluted inner membrane comprising 50—60% (w/w) of the total mitochondria protein (as compared to 15—25% w/w in rat liver), it has been reported [22] that sonication gives rise to a homogeneous population of inside-out vesicles. However, in pigeon breast mitochondria, where the inner membrane is also heavily invaginated, sonication does not appear to give rise to a homogeneous population of submitochondrial particles as estimated by cytochrome *c* oxidase activity. It seems doubtful therefore that in the inner membrane vesicles prepared from rat liver mitochondria, which do not have such a convoluted cristal structure, that submitochondrial particles represent a sealed homogeneous population of inside-out vesicles. Evidence citing that submitochondrial particles in fact represent a scrambled population comes from Chance et al. [23], who reported that approx. 40% of cytochrome *c* and c_1 was rapidly oxidised by ferricyanide as compared to over 90% in intact mitochondria.

Smith and Ragan [5] have used at least two criteria for the homogeneity of their submitochondrial particles from beef heart. However, neither of the assay systems used (NADH oxidation; ascorbate reduction of cytochrome *c*) give an indication of the degree of 'sealing'. Other workers [24] have used an immobilised cytochrome *c* column to isolate inside-out vesicles. A possible draw-back of this technique is that a vesicle may not be sealed and yet be incapa-

ble of binding external immobilized cytochrome *c*. Consequently, the vesicle would not be retained on the column. The latter point has been discussed by Walsh et al. [25] with respect to their attempts to isolate inside-out vesicles from lymphocyte plasma membranes on an immobilized concanavalin A column. The data of Walsh et al. [25] demonstrate that although the isolated inside-out vesicles satisfied all their experimental criteria, the vesicles were permeable to concanavalin A and trypsin. The same vesicles, however, were impermeable to concanavalin A and trypsin when the sizes of the ligands were increased by attachment of ferritin and Sepharose, indicating that the vesicles were 'leaky'.

In the inner mitochondrial membrane where good criteria exist for assessing 'sidedness' a procedure has been developed for the isolation of inside-out vesicles from a mixed membrane population. This technique is based on the ability of lectins to interact with specific monosaccharides in the carbohydrate chains of membrane glycoproteins. The polarity of the vesicles isolated after lectin immunoprecipitation is estimated by measuring cytochrome *c* oxidase activity with exogenous cytochrome *c* as substrate in the presence and in the absence of detergent. This assay system gives an indication of membrane orientation as well as degree of sealing of vesicles towards cytochrome *c*.

An important corollary from this experiment is the asymmetric distribution of carbohydrate on the external surface of the inner membrane, as inside-out vesicles are neither absorbed nor precipitated by lectins. Although Andreu et al. [26] have reported the presence of carbohydrate in the subunits of the F_1 ATPase as detected by periodic acid-Schiff staining, there still appears to be much controversy about the result. Recently Satav et al. [27] have shown an inhibition of yeast mitochondrial ATPase by concanavalin A. The same lectin has no effect on beef heart ATPase. Similarly, Nalin et al. [28] have reported a complete lack of carbohydrate in the beef heart F_1 ATPase as detected by the method of Andreu et al. [26] suggested that the result of the latter authors is an artifact of the method employed.

The idea employed in this paper for isolating inside-out vesicles would favour the results of Nalin et al. [28] as it supports the hypothesis of an asymmetric distribution of carbohydrate on the external surface of the membrane. Alternatively, if the ATPase is glycosylated it can be unequivocally concluded that it possesses no wheat germ agglutinin binding sites.

We are currently using this technique to prepare vesicle populations of defined orientation for investigation into the topography of the various respiratory chain complexes by performing surface-labelling studies. However, this technique may not be suitable for preparing large quantities of purified material such as would be required in functional studies.

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