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Enrichment and biochemical characterization of boundary membrane contact sites from rat-liver mitochondria

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A subfraction of mitochondrial membranes was prepared from osmotically lysed rat liver mitochondria by density gradient centrifugation which contained the inner boundary membrane and the contact sites between this membrane and the outer membrane. The fraction was composed of inner and outer limiting membrane components as shown by the presence of specific marker enzymes, monoamine oxidase and glycerolphosphate oxidase. Surface proteolysis analysis, studies of cytochrome *c* permeability, and electron microscopy revealed the localization of the inner membrane component within a right-side-out outer membrane vesicle. Moreover, the outer membrane component in this fraction exhibited a higher capacity to bind hexokinase and had a higher specific activity of glutathione transferase than the pure outer membrane. In freeze-fracture analyses the fraction showed fracture plane deflections which may be specific for hydrophobic interactions between the two membranes.

Introduction

Cells fixed by rapid freezing contain mitochondria which, unlike other organelles, show an irregular freeze-fracture face characterized by frequent changes of the fracture plane [1]. This phenomenon is preserved during isolation of the mitochondria and is interpreted as a deflection of the fracture plane between the interiors of the two boundary membranes. It has been suggested that these deflections occur in zones of semifusion between the two membranes [2] and are probably the contacts described in thin sections by Hackenbrock [3].

To quantify the frequency of membrane contacts, we analyzed the frequency of fracture plane deflections in mitochondria of different functional states. An increase in contacts has been previously observed in phosphorylating (state 3) mitochondria compared to both energized (state 4) and freshly isolated (state 1) mitochondria [4]. This increase, upon transition into state 3, depends on the degree of coupling (5) and therefore, appears to correlate with the regulation of oxidative phosphorylation.

In view of the finding that the contacts are dynamic structures, we suggest that they play a principle role in the regulation of mitochondrial metabolism. As shown by immunocytochemical techniques, a fraction of outer membrane porin, which has a high affinity for hexokinase, is localized almost exclusively in regions where the boundary membranes are closely apposed [6].

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Hexokinase, when bound to the pore in the contacts, may serve to create a microcompartment facilitating a direct exchange of ATP and ADP between the enzyme and the compartment of oxidative phosphorylation [7]. Such a functional coupling would increase the effective concentrations of metabolites near the target enzymes and therefore, maintain high activity rates. In addition, it may provide a mechanism for the transfer of high-energy phosphate from the mitochondrial compartment to the cytosol. Using this mechanism, the cell could then keep a constant, high phosphorylation potential outside the mitochondria while maintaining highly activated oxidative phosphorylation.

After treatment with digitonin, the boundary membrane contacts are preserved [8,9] and peripheral proteins in the contact region of the outer membrane remain attached to the mitoplasts [10]. Using this treatment, we observed specific properties of the outer membrane region in the contact area. Compared to the pure outer membranes the outer membrane component in the contacts has a higher specific activity of glutathione transferase and contains a pore protein which appears to have a higher capacity to bind hexokinase. In addition the inner membrane forming the contacts has been shown to differ from the crista membrane. This inner boundary membrane has a high activity of glycerolphosphate oxidase, while that of cytochrome oxidase and ATPase is low [11,12].

On the basis of the above findings we were able to enrich and characterize contacts from osmotically lysed mitochondria in the inner boundary membrane fraction. The fraction is composed of inner and outer boundary membranes.

Materials and Methods

All chemicals were purchased from Boehringer-Mannheim and Merck-Darmstadt, F.R.G.

Enzyme assays. The determination of monoamine oxidase (EC 1.4.3.4), succinate dehydrogenase (EC 1.3.99.1) and glycerol-3-phosphate oxidase (EC 1.1.99.5) was carried out as described recently [12]. The glutathione transferase (EC 2.5.1.18) was determined according to Habig et al. [13], the ATPase (EC 3.6.1.3) according to Pullmann et al. [14], the adenylate kinase (EC 2.7.4.3) and the hexokinase (EC 2.7.1.1) in agreement with Bücher et al. [15]. Rotenone-insensitive NADH-(EC 1.6.2.2) and NADPH-cytochrome *c* reductase (EC 1.6.2.a) was determined according to Sottocasa et al. [16], Glucose-6-phosphate phosphatase (EC 3.1.3.9) according to Swanson [17], and acid phosphatase (EC 3.1.3.2) according to Bergmeyer [18].

Preparation of mitochondria and microsomes from rat liver. Liver mitochondria from 10 rats (250 g body weight) were isolated by differential centrifugation in 0.25 M sucrose. 10 mM Hepes (pH 7.4). The mitochondrial sediment was washed two times using $6000 \times g$ and $3000 \times g$ (Sorvall, rotor SS-34) for sedimentation.

The first postmitochondrial supernatant was centrifuged 15 min at $15\,000 \times g$ and the resulting supernatant 45 min at $200\,000 \times g$. The pellet containing the microsomal fraction was resuspended in sucrose medium.

Preparation of submitochondrial particles. The purified mitochondria were exposed to a swelling and shrinking procedure by incubation in 72 ml of 10 mM phosphate buffer (pH 7.4), with subsequent addition (after 20 min incubation) of 24 ml 60% sucrose. After 20 min incubation portions of 25 ml were sonicated with a Branson Sonifier B-15 (3×30 s at level 5.5), followed by centrifugation at 9000 rpm for 15 min in a Sorvall SS-34 rotor. The supernatant was layered above a 20 ml linear sucrose density gradient, varying from a density of 1.22 to 1.06 g/ml at 4°C and was centrifuged for 20 h in a Sorvall Rotor TV-850 at 47000 rpm. Subsequently the gradient was divided into either 19 or 40 fractions. The fractions were characterized by different marker enzymes. Pooled fractions 8 and 9 (the glutathione transferase peak) from the original gradient were recentrifuged on a less steep sucrose density gradient (1.20 to 1.06 g/ml), to determine homogeneity of the contact fraction.

Binding of hexokinase I to submitochondrial particles. Hexokinase from rat brain was prepared as described by Chou and Wilson [19]. To compare the capacity of the outer membrane and contact fraction for the binding of hexokinase I, the fractions containing 0.25 mg protein were added to sucrose medium: 0.25 M sucrose, 10 mM Hepes (pH 7.4), with addition of 10 mM $MgCl_2$, 5 mM

glucose and 1.25 U hexokinase, in a total volume of 2 ml. After 15 min incubation at room temperature, the samples were diluted 1 : 1 with sucrose medium and centrifuged at 48 000 rpm for 90 min in a Sorvall 50 Ti rotor. Subsequently, the pellets were resuspended in sucrose medium. Hexokinase activity was determined photometrically and the amount of the outer membrane in the fractions was calculated from the monoamine oxidase activity and the porin concentration. The latter was determined densitometrically in the SDS-polyacrylamide gel electrophoresis.

Purified mitochondria were osmotically lysed in the presence and absence of 1.5 mM dinitrophenol. The mitochondrial membranes were subsequently incubated for 20 min at room temperature in 10 ml sucrose medium with 0.5 U hexokinase, 4 mM $MgCl_2$ and were centrifuged as described above on a sucrose gradient. The different gradient fractions were analyzed for specific marker enzymes and hexokinase activity.

Surface proteolysis analysis. To study the sideness of the outer membrane and the orientation of the two membrane components in the contact fraction, the fraction was treated with different trypsin concentrations (0, 0.01, 0.05, 0.10, 0.20 mg trypsin/mg protein). The incubation for 15 min at 4°C occurred in: 20 mM Hepes, 100 mM NaCl and 2 mM $MgCl_2$ (pH 7.4), followed by the addition of the trypsin inhibitor. Subsequently the different samples underwent SDS-polyacrylamide gel electrophoresis, which was performed as described by Laemmli [20].

Localization of the inner membrane in the contact fraction. Based on the fact that the mitochondrial outer membrane is impermeable to cytochrome *c*, externally added cytochrome *c* should not react with components inside an outer membrane vesicle. The contact fraction was incubated for 20 min at 4°C with 0–400 μg digitonin/mg protein in a medium of 0.25 M sucrose, 10 mM Hepes (pH 7.4) and subsequently centrifuged. The reduction of external cytochrome *c* by succinate dehydrogenase was determined.

Activation of glutathione transferase. The activation of membrane bound glutathione transferase by *N*-ethylmaleimide was performed according to Morgenstern and DePierre [21]. The contact fraction and mitochondrial outer membrane incubated in 0.1 M phosphate buffer (pH 7.4) and 1 mM *N*-ethylmaleimide for 5 min at room temperature. The glutathione transferase activity was determined, and compared with a control sample which had not been treated with *n*-ethylmaleimide.

Specific antibodies. Antibodies reactive against the isolated glutathione transferase from rat liver endoplasmatic reticulum were obtained from R. Morgenstern, Arrhenius Laboratory, Stockholm. These antibodies reacted as well with the enzyme in the outer mitochondrial membrane.

Electrotransfer and immunodecoration. These were performed as described by Rott and Nelson [22].

Assay of protein concentration. Protein was determined by the method of Lowry et al. [23].

Freeze-fracture analysis. Different fractions were centrifuged and the pellets were subjected to rapid cryofixation as recently described [24], without chemical fixation or cryoprotectives. The samples were broken in a Balzers 360M freeze-etch device at -120°C and $2 \cdot 10^{-7}$ Torr, followed by Pt/C and C shadowing.

For electron microscopy, a Siemens 101 instrument was used at 80 kV. Morphological evaluations were performed using a Kontron MOP Am2 picture analysing system. The nomenclature of the exposed membranes follows that of Branton et al. [25].

Results

Purification of the mitochondrial fraction

To reduce contamination of the mitochondrial fraction by microsomes, the mitochondria were washed two further times and sedimented with reduced *g*-forces. This procedure reduced the activity of the reticulum marker enzyme, glucose-6-phosphate phosphatase, to 0.03% of the total activity in the homogenate, while 12% of succinate dehydrogenase activity remained in the mitochondrial fraction (Table I).

Isolation and characterization of the contact fraction from osmotically lysed mitochondria

Membranes of osmotically-shocked liver mitochondria can be separated into three fractions of different functions: (1) an outer boundary membrane characterized by high monoamine oxidase

TABLE I

CONTAMINATION OF THE MITOCHONDRIAL FRACTION BY ENDOPLASMIC RETICULUM AND LYSOSOMES

Rat liver was homogenized in 0.25 M sucrose, 10 mM Hepes (pH 7.4). The homogenate was centrifuged in a rotor SS-43 Sorvall for 10 min at $500 \times g$. The supernatant was centrifuged for 10 min at $10000 \times g$ resulting in the first mitochondrial sediment (Pellet I). The sediment was resuspended in sucrose medium and subjected to a low speed centrifugation as above and a high speed centrifugation at $6000 \times g$. The resulting sediment was resuspended in sucrose medium and centrifuged for 10 min at $3000 \times g$ (mitochondria). Protein, monoamine oxidase (MAO), succinate dehydrogenase (SDH), glucose-6-phosphate phosphatase (G-6-Pase), and acid phosphatase (S-Pase) were determined as described in Methods.

Fraction	Protein (%)	MAO (%)	SDH (%)	G-6-Pase (%)	S-Pase (%)
Homogenate	100	100	100	100	100
Pellet I	5	17	11	1	3
Mitochondria	1	2	12	0.03	0.4

activity [26], (2) an inner boundary membrane characterized by high glycerolphosphate oxidase activity [11,12], and (3) the crista membranes characterized by high succinate dehydrogenase and ATPase activity [11,12]. Therefore, to isolate the contacts between the two boundary membranes, we searched for a fraction containing high glycerolphosphate oxidase and monoamine oxidase activity relative to the crista membrane marker enzymes. The activity profiles of specific marker enzymes in a gradient (Fig. 1A) revealed a membrane fraction (in gradient fractions 8 and 9, density 1.15 g/ml) with a high activity of glycerolphosphate oxidase relative to succinate dehydrogenase and a high activity of monoamine oxidase relative to ATPase. These fractions (8 and 9), probably containing inner boundary membrane and the contact sites, also had high glutathione transferase activity. According to their content of specific marker enzymes, fractions 3–5 of the gradient (density zone of 1.2 g/ml) contained enriched crista and inner boundary membranes, whereas the outer membrane was concentrated in fraction 12 and 13 (density zone 1.10 g/ml).

If one assumes that the two mitochondrial boundary membranes are tightly connected in the contact fraction, the inner and outer membrane parts in this fraction should not be separable. Therefore, the pooled fractions 8 and 9 from the gradient were recentrifuged on a less steep sucrose density gradient. The activity profiles of specific marker enzymes (Fig. 1B) indicated no separation of inner and outer membrane components. This suggests that the different membrane vesicles are connected. Alternatively, the fraction may contain separated outer and inner membrane vesicles with the same density. The protein distribution in the two density gradients (Fig. 2A) revealed two peaks in the first gradient, representing the inner and outer membrane, whereas the second gradient contained only one protein peak. Additional protein material found on top of the second gradient varied between different preparations and represents soluble protein (Fig. 2B).

Distribution of microsomes and mitochondrial membranes in the density gradient

Glucose-6-phosphate phosphatase activity in the mitochondrial fraction was too low to be determined in the different gradient fractions (Table I). Therefore, we measured rotenone insensitive NADH-cytochrome *c* reductase distribution in the gradient. However, NADH- and NADPH-cytochrome *c* reductase are also present in the outer membrane of mitochondria [16,27]. Thus, we analyzed the distribution of free microsomes in the gradient (Fig. 3). The microsomes migrated to gradient fractions of high density similar to the mitochondrial inner membrane components and were clearly separated from the fractions containing the contact sites. When the gradient was loaded with mitochondrial membrane fragments, the activity profile of NADH-cytochrome *c* reductase coincided with the distribution of monoamine oxidase. This suggests that the contact fraction was not contaminated by microsomal membranes. Microsomes and mitochondrial membranes were preincubated with 4 mM $MgCl_2$ as in the hexokinase binding experiments.

Freeze-fracturing analysis of the contact and outer membrane fraction

The fraction containing inner boundary membrane and the presumptive contacts was fixed by the rapid freezing technique, and freeze-fractured. In cross fractures often vesicles were observed

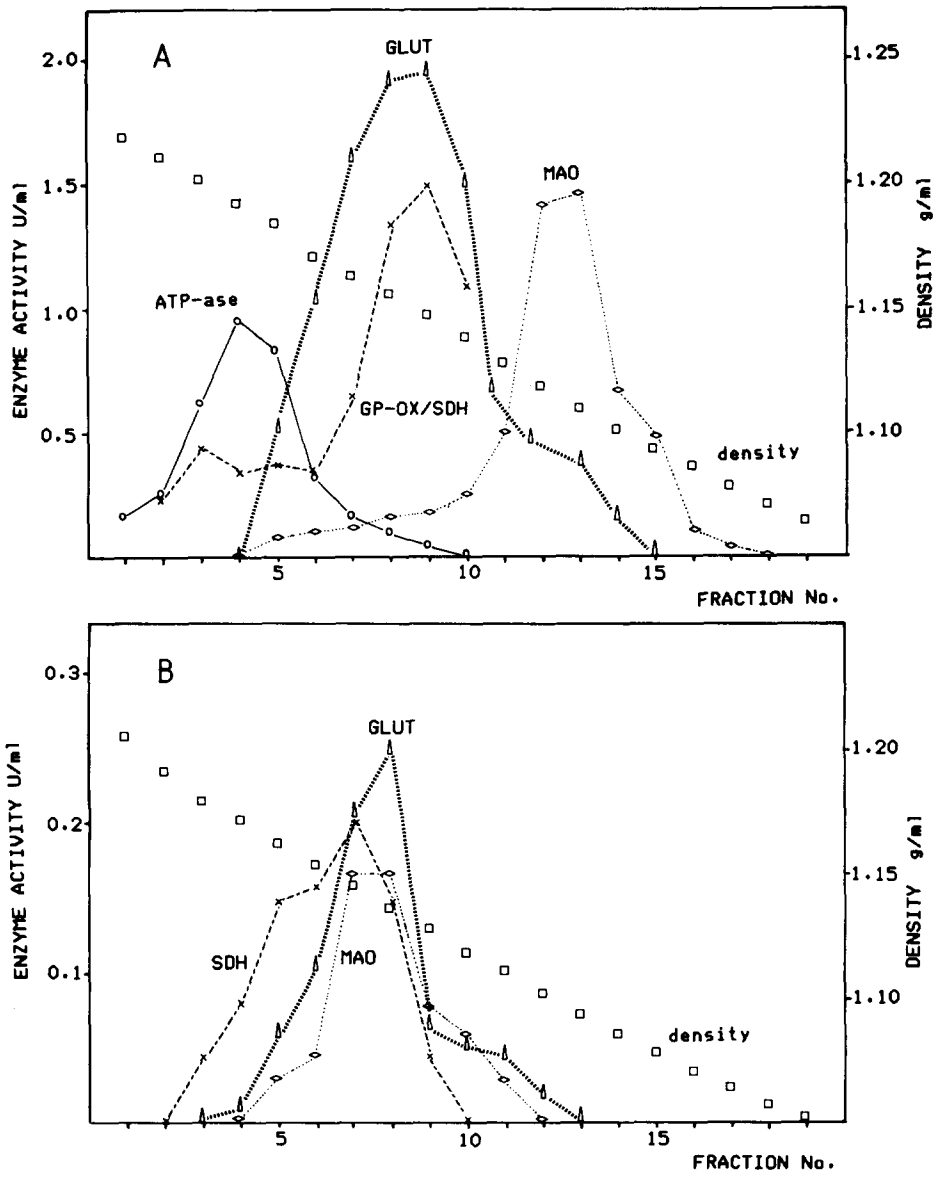

Fig. 1. Separation of mitochondrial membrane fragments on a density gradient after osmotic shock. (A) Liver mitochondria were exposed to a swelling, shrinking and sonification procedure and were subsequently centrifuged on a linear sucrose density gradient varying from a density of 1.22 to 1.06 g/ml. (B) Fractions 8 and 9 from the gradient in (A) were recentrifuged on a linear density gradient varying from a density of 1.20 to 1.06 g/ml. The activity of inner and outer membrane enzymes was determined in the different fractions of the gradient: SDH, succinate dehydrogenase; ATPase, oligomycin-sensitive ATP synthetase; GP-OX, mitochondrial glycerolphosphate oxidase; MAO, monoamine oxidase; GLUT, glutathione transferase. Enzyme activity is given as U/ml: ATPase times 1, GLUT times 2.5, MAO times 10, GP-OX activity times 10 is shown relative to SDH activity in the respective fraction (mU/ml fraction 4 SDH: 420, GP-OX: 10.5; fraction 8 SDH: 10, GP-OX: 1.3).

containing smaller vesicles (arrows Fig. 4A) and occasionally the membranes of these inner and outer vesicles appeared attached (Fig. 4A).

Because most cellular membranes contain intermembraneous particles that are asymmetrically distributed between the two membrane leaflets, it

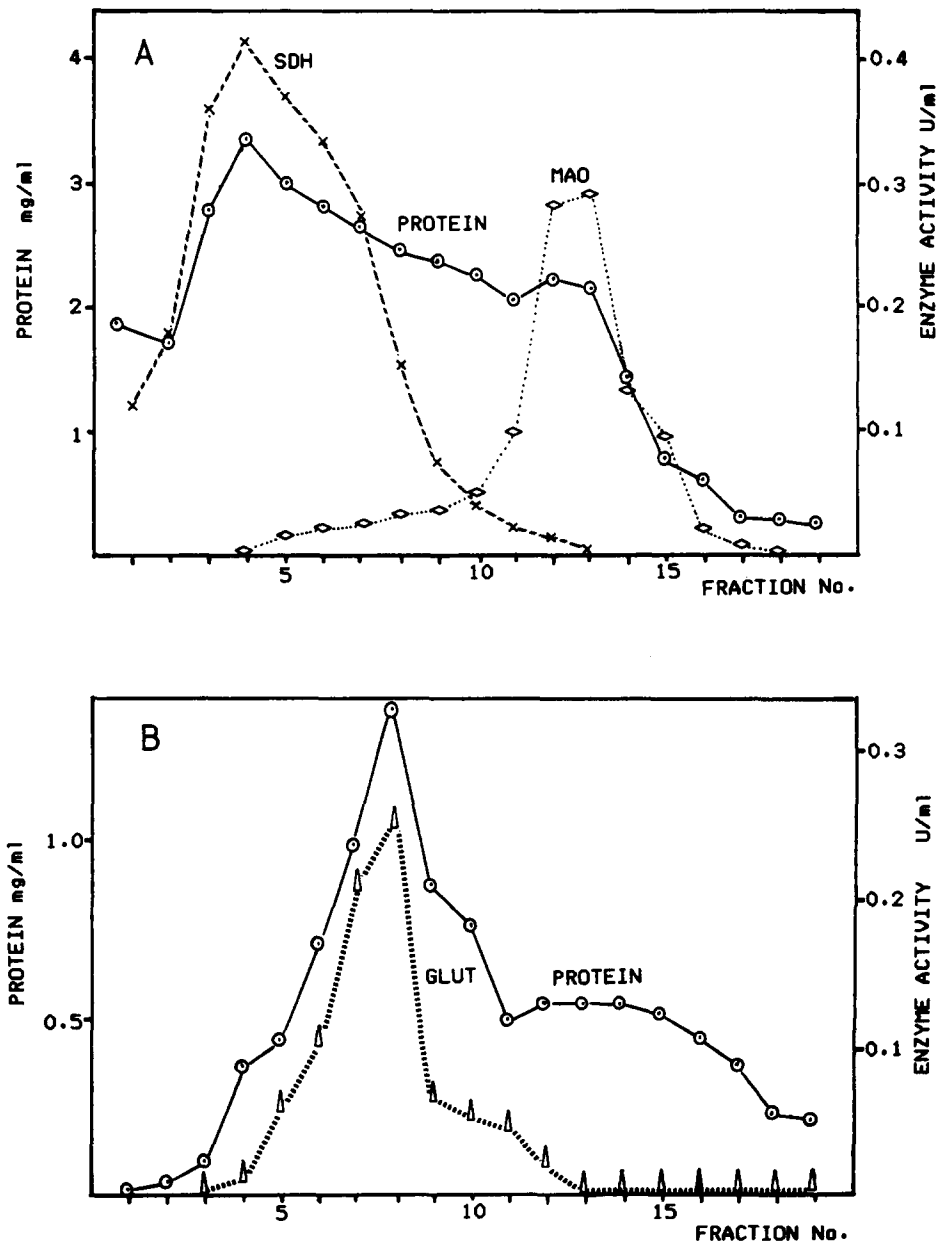

Fig. 2. Protein distribution in the density gradients (Fig. 1) after separation of mitochondrial membrane fragments. The protein distribution in the density gradients A and B shown in Fig. 1 is compared to the activity profiles of succinate dehydrogenase (SDH), monoamine oxidase (MAO), and glutathione transferase (GLUT). Activity is expressed as U/ml, MAO times 2.

is possible to determine the sidedness of a membrane vesicle. In the present study the convex fracture face of the outer membrane fraction in most cases exposed a smooth leaflet representing the exoplasmic face, while the concave fracture face was covered with particles (representing the protoplasmic face). This correspondence to the physiological orientation of the outer membrane and quantitative analysis revealed 78% of right-side-out vesicles (Table II). The convex fracture

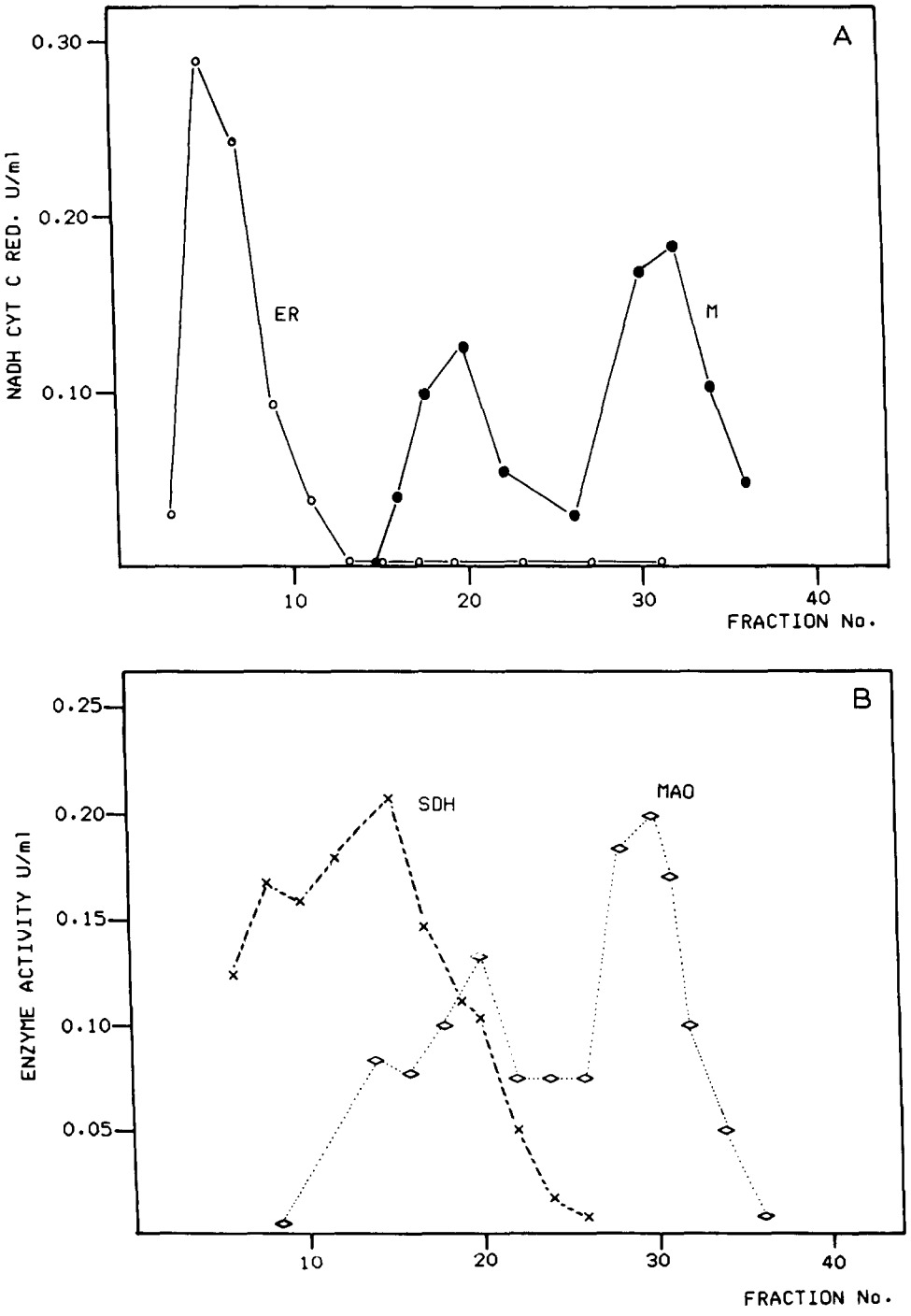

Fig. 3. Distribution of microsomal and mitochondrial membranes in the density gradient. Mitochondria were osmotically lysed as in Fig. 1. Microsomes were isolated from the postmitochondrial supernatant. The microsomal (ER) and mitochondrial membranes (M) were incubated for 20 min at room temperature with 4 mM $MgCl_2$ and centrifuged on a linear sucrose density gradient as in Fig. 1. The distribution of rotenone-insensitive NADH-cytochrome *c* reductase in the two density gradients is shown in (A) and monoamine oxidase (MAO) in a gradient loaded with mitochondrial membranes (B). The activity of monoamine oxidase is expressed in U/ml times 10.

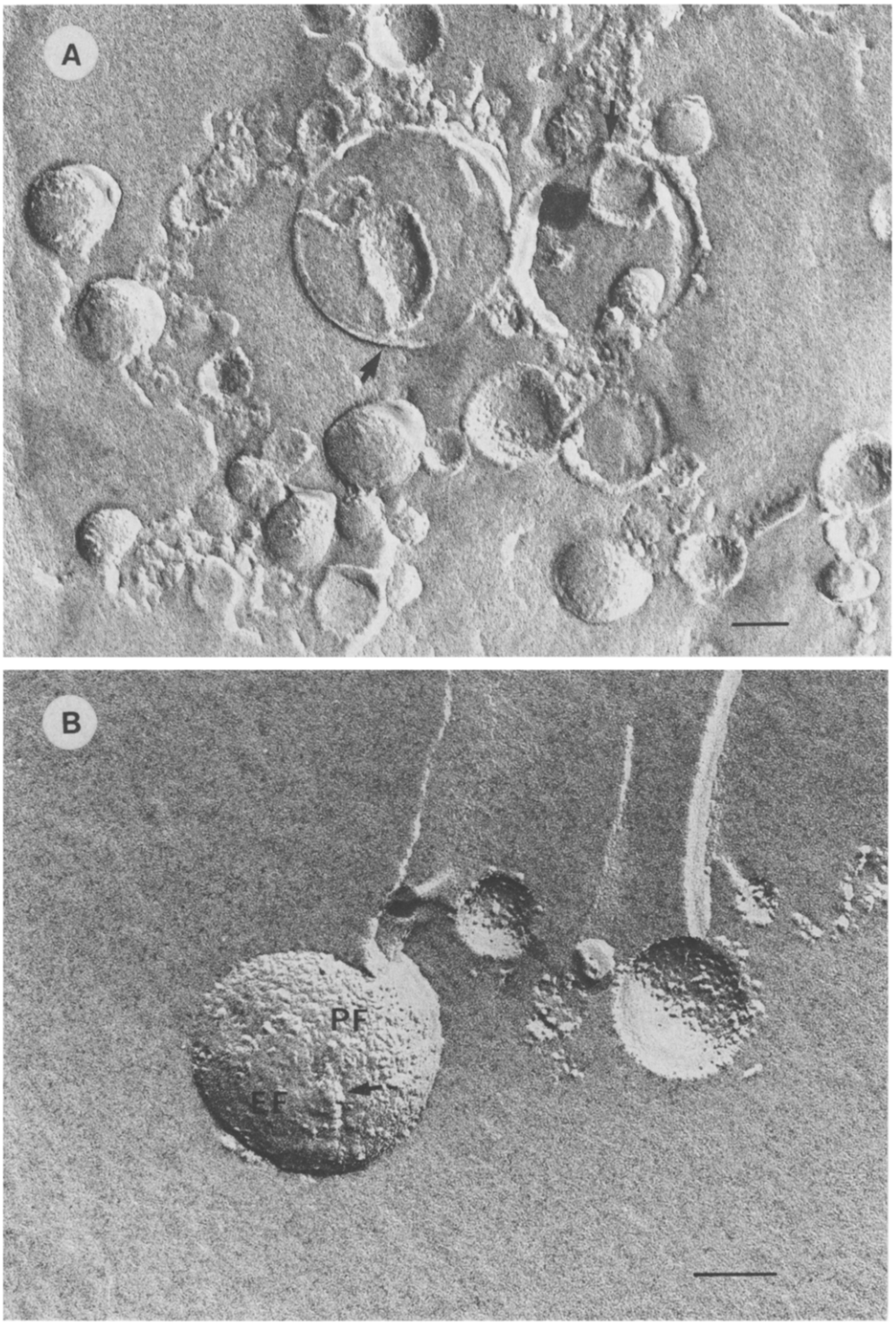

Fig. 4. Freeze-fracture of the contact fraction. (A) Many of the convex fractures expose a smooth fracture face corresponding to the exoplasmic face of the outer membrane. In cross fractures (arrows) vesicles inside vesicles are visible which appear sometimes attached. (B) Vesicle exhibiting different fracture planes. The fracture deflects from the exoplasmic face (EF) of the outer membrane to the protoplasmic face (PF) of the inner membrane. Bar = 0.1 μm.

TABLE II

SIDEDNESS ANALYSIS OF THE CONTACT AND OUTER MEMBRANE FRACTION

According to the relative particle densities in the outer membrane the number of right-side-out (RO) and inside-out (IO) vesicles in the outer membrane and contact fraction was determined in freeze-fractured samples. The data give additional information about the orientation of the inner and outer membrane components in the contact fraction.

Membrane fraction	Total area (μm^2)	Relative area (%)	Number of vesicles	Average vesicle area (μm^2)
Outer membrane				
IO	0.639	21.68	24	0.027
RO	2.308	78.32	111	0.021
Contact fraction				
IO	0.443	15.28	21	0.021
RO	2.456	84.72	107	0.023

faces of the corresponding contact fraction also exposed a smooth leaflet (Fig. 4A) which is specific for the exoplasmic face of the outer membrane. Because 85% of the vesicles were right-side-out outer membrane (Table II), it appears that the inner membrane fragments in this fraction were always enclosed in outer membrane vesicles.

Occasionally the fracture plane deflected from the outer membrane vesicle to a second inner vesicle and exposed a particle-rich leaflet which most likely was the protoplasmic face of the inner membrane (Fig. 4B). The observed deflection of the fracture plane is specific for the hydrophobic interactions of the two boundary membranes in intact mitochondria.

Electrophoretic characterization of the gradient fractions

SDS-polyacrylamide gel electrophoresis of the different gradient fractions revealed specific polypeptide patterns (Fig. 5, top). The distribution of several polypeptides was determined densitometrically in the different gradient fractions (Fig. 5, bottom). A 45 kDa polypeptide was enriched in the contact fraction, and the 32 kDa polypeptide,

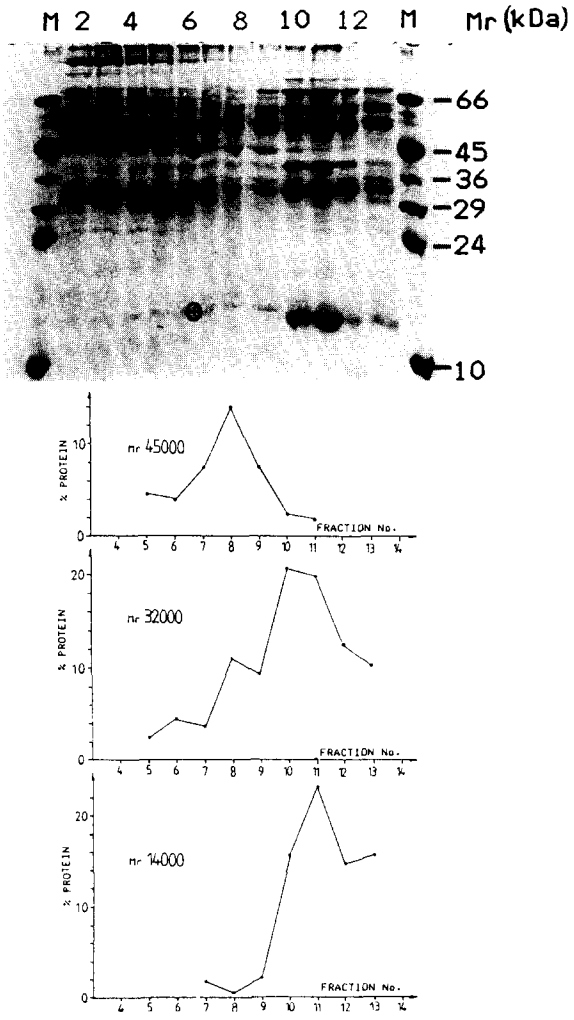

Fig. 5. Densitometer distribution profile of polypeptides in the different density gradient fractions separated on SDS-polyacrylamide gel electroporesis. Samples were withdrawn from the density gradient shown in Fig. 1 and were employed on a 12.5% Laemmli gel. The gel was stained with Coomassie brilliant blue. The lanes were scanned on a soft laser densitometer (LKB ultroscan). The intensity of the polypeptide bands was determined by the integrator of the densitometer. Porin, M_r 32000; glutathione transferase, M_r 14000.

representing the pore protein [28], showed a small peak in the contact fraction and a larger one in the outer membrane fraction. The 14 kDa polypeptide, representing glutathione transferase [29], appeared to be localized in the outer membrane fractions. Because glutathione transferase activity in the gradient was maximal in the contact frac-

tion, the highest activity and the maximal protein concentration did not coincide.

Activation and immunological identification of glutathione transferase

The glutathione transferase was, therefore additionally identified in the gradient fractions using specific antibodies. Polypeptides of the various gradient fractions were phosphorylated by cAMP dependend protein kinase. After SDS electrophoresis the polypeptides were transblotted from gels to cellulose nitrate sheets. Autoradiography and decoration with antibodies against glutathione transferase was performed with the same sheet. The 14 kDa polypeptide became phosphorylated and was decorated by specific antibodies against glutathione transferase (Fig. 6). Determined by densitometry, the contact fraction contained a low concentration of this polypeptide but a high enzyme activity. Based on these values we calculated a 40-fold higher specific activity of glutathione transferase in the contacts compared to the outer membrane. Morgenstern and DePierre [21] found that glutathione transferase in the membranes of endoplasmatic reticulum in contrast to the soluble glutathione transferases could be activated by *N*-ethylmaleimide. In the contact fraction glutathione transferase was not further activated by *N*-ethylmaleimide, whereas there was a 3- to 4-fold activation of the enzyme in the outer membrane by *N*-ethylmaleimide, Table III).

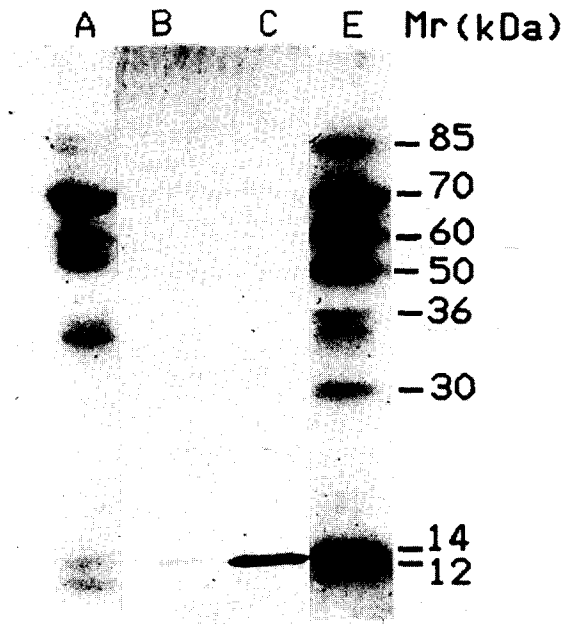

Fig. 6. Identification of glutathione transferase by immuno decoration. Samples of the contact (A,B) and outer membrane (C,E) fractions were phosphorylated by cAMP dependent protein kinase and run on a SDS-polyacrylamide gel electrophoresis. The gel was transferred to a nitrocellulose transferase (lane B and C) and analyzed by autoradiography (lane A and E). The molecular masses of the polypeptides are given as kDa. The 14 kDa polypeptide is phosphorylated and reacts with the specific antibodies in both fractions.

Surface proteolysis analysis

Proteolytic analysis was applied to study the sidedness of the outer membrane and the orientation of the two membrane components in the contact containing fraction. In agreement with previous observations in yeast [30], the 14 kDa polypeptide was exposed to the outer surface of the outer membrane in intact liver mitochondria and was sensitive to proteolysis. Treatment of intact liver mitochondria with different trypsin concentrations resulted in a decrease of the 14 kDa polypeptide and an increase of a proteolytic product of 12 kDa (Fig. 7). Similar results were obtained by trypsination of the isolated outer membrane (Fig. 8), suggesting the outer membrane vesicles in this fraction were right-side-out. In addition, the tryptic degradation of a second outer membrane component (M_r 68000) was observed. Exposure of this polypeptide to the surface of the outer membrane of liver mitochondria has

TABLE III

ACTIVATION OF GLUTATHIONE TRANSFERASE BY *N*-ETHYLMALEIMIDE IN THE OUTER MEMBRANE AND CONTACT FRACTION

Glutathione transferase activity was determined in the outer membrane and contact fraction before and after activation by *N*-ethylmaleimide (NEM) according to Morgenstern et al. [21].

	Outer membrane (mU/mg protein)	Contact fraction (mU/mg protein)
−NEM	1.6±0.7	8.3±0.4
+NEM	5.4±0.3	8.2±0.4

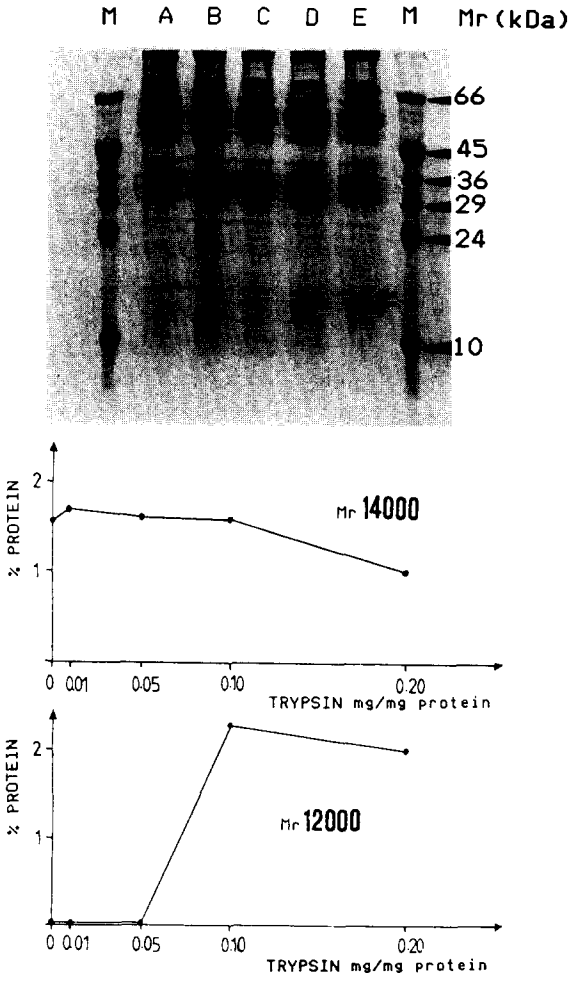

Fig. 7. Proteolytic analysis of the surface of intact mitochondria. Intact liver mitochondria were treated with increasing concentrations of trypsin as described in methods. After incubation the mitochondria were removed by centrifugation and subjected to SDS gel electrophoretic separation of the polypeptides (top, lane M, molecular weight markers; M_r and kDa; lane A, control; lanes B–E, different trypsin concentrations shown underneath). The concentration of the 14 kDa polypeptide and the degradation product of 12 kDa were determined in the different lanes by densitometry (bottom).

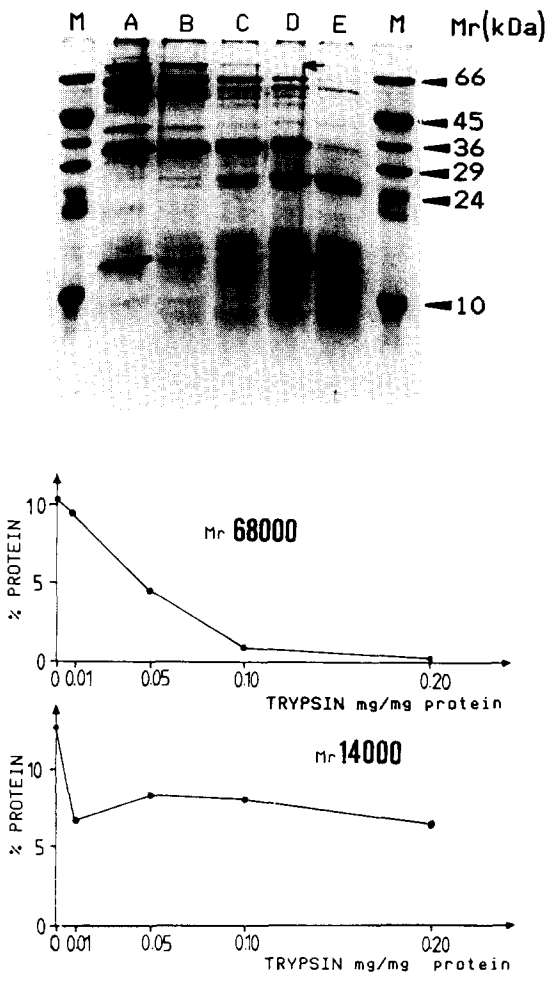

Fig. 8. Proteolytic surface analysis of the outer membrane fraction. The outer membrane fraction from the density gradient was treated with increasing trypsin concentrations as described in Methods. After incubation the polypeptides of the outer membrane were separated by SDS-polyacrylamide electrophoresis (top, lane M, molecular weight markers; M_r in kDa; lane A, control; Lane B–E, different trypsin concentrations shown underneath). Two polypeptides of 68 kDa and 14 kDa at the outer surface were sensitive to proteolysis. The degradation was followed by densitometric scanning of the different lanes (bottom).

been recently described [31]. Surface proteolysis of the contact fraction resulted in the degradation of both the 68 kDa and the 14 kDa polypeptide. This indicates the outer membrane in this fraction is susceptible to proteolysis and has a regular right-side-out orientation. It is interesting that a 45 kDa polypeptide, which was specifically enriched in the contact fraction (Fig. 5), was also sensitive to proteolysis and appears to be exposed to the outer surface in the contacts (Fig. 9).

Orientation of the inner membrane vesicles in relation to the outer membrane

It is known that the right-side-out, outer mem-

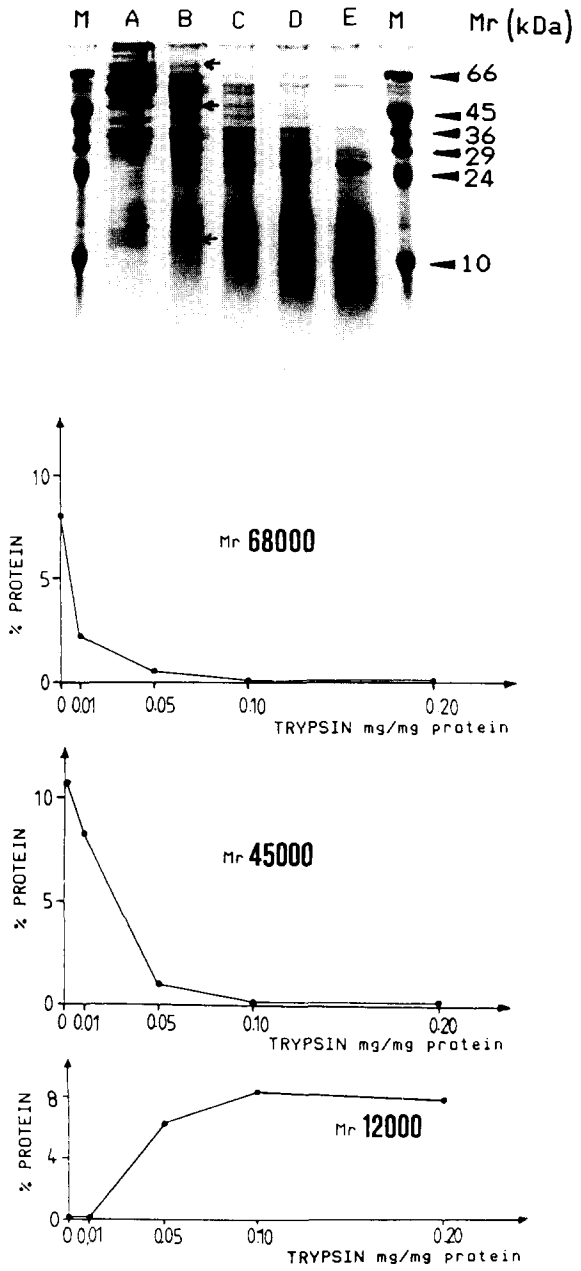

Fig. 9. Proteolytic surface analysis of the contact fraction. The contact fraction from the density gradient was subjected to tryptic digestion as described in Methods and in Figs. 7 and 8. Top, SDS-polyacrylamide gel electrophoresis of the fraction incubated with different trypsin concentrations (lane M, molecular weight markers; M_r in kDa; lane A, control; lane B–E, different trypsin concentrations shown underneath). Bottom, densitometric determination of the concentrations of a 68 kDA and 45 kDa polypeptide which became degraded and a 12 kDa product of the 14 kDa polypeptide.

brane vesicles are sealed in yeast [30]. Thus, electron transfer from succinate dehydrogenase in the inner membrane to externally added cytochrome *c* may answer the question as to whether the outer membrane enwraps the inner membrane or if the two membranes are attached side by side. A greater reduction of external cytochrome *c* by succinate dehydrogenase was observed in intact mitochondria when the outer membrane became permeable to adenylate kinase and cytochrome *c* following treatment with digitonin (Fig. 10A). Similar results were obtained when this assay was applied to succinate dehydrogenase in the contact containing fraction (Fig. 10B). These data suggest that approximately one third of the inner membrane was accessible to cytochrome *c* before the addition of digitonin, whereas two thirds of the inner membrane component may be located inside the outer membrane vesicles. A 3-times higher digitonin concentration per mg of protein was necessary to permeabilize the outer membrane in the contact fraction. This may be explained by a lower content of cholesterol per mg of protein in the contact fraction compared to the outer membrane (legend Fig. 10).

Functional characterization of the contacts in intact mitochondria

The treatment of mitochondria with digitonin fragments the outer membrane and leaves parts of the membrane attached in the contacts with inner boundary membrane [8–10]. Therefore, analysis of the attached part of the outer membrane in the contact regions was possible. Using increasing concentrations of digitonin, caused disruption of the outer membrane and liberated adenylate kinase and monoamine oxidase activity (not shown) into the supernatant (Fig. 11A), whereas bound hexokinase remained in the sediment (Fig. 11B). Significant amounts of a M_r 32000 polypeptide (which migrated to the same position as isolated pore/hexokinase-binding protein in the co-electrophoresis) were detached from the mitochondria following this treatment (Fig. 11A). This may suggest that hexokinase preferentially binds to the pore protein in the contact region. To verify the importance of the contacts for hexokinase binding, we repeated the same experiment in the presence of an uncoupler (dinitrophenol, which re-

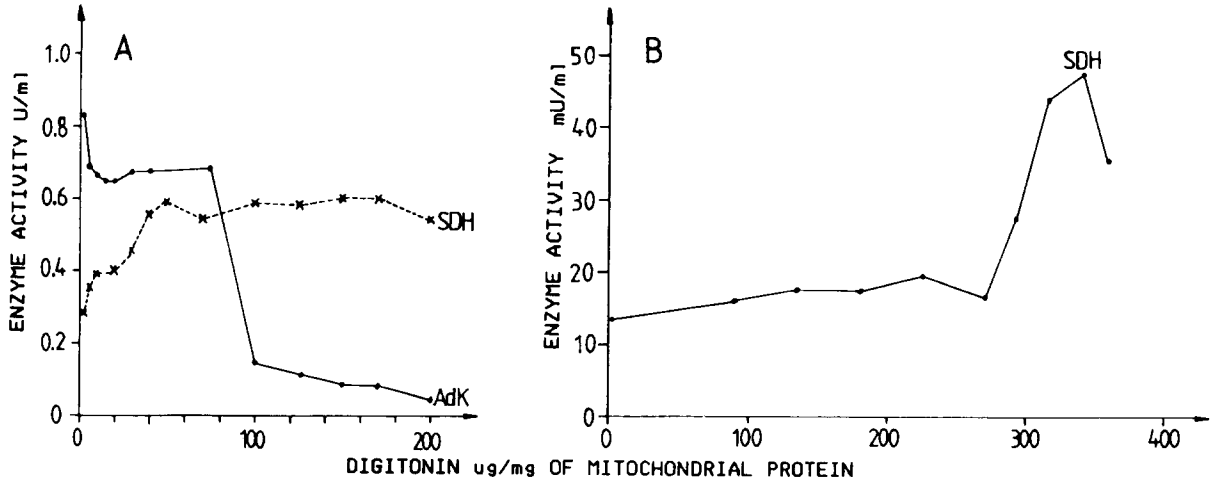

Fig. 10. Localization of the inner membrane fragments in the contact fraction. The accessibility of succinate dehydrogenase in the inner membrane to external cytochrome *c* was determined by following the reduction of externally added cytochrome *c* by succinate dehydrogenase (A) in intact mitochondria, (B) in the isolated contact fraction. The outer membrane was permeabilized by increasing concentrations of digitonin, which liberated adenylate kinase (AdK). Activity of succinate dehydrogenase (SDH) in (A) was multiplied by 3. Cholesterol nmol/mg of protein: outer membrane 82.7, contact fraction 62.9.

duces the contacts. Under these conditions, hexokinase is also removed from the sediment by digitonin (Fig. 11B).

In a similar series of experiments, we observed that the activity of glutathione transferase in the mitoplast fraction remained constant although significant amounts of the 14 kDa polypeptide, representing the enzyme [29], were liberated into the supernatant (Fig. 11A). This suggests that the outer membrane component in the contacts contains a high specific activity of glutathione transferase, which was also observed in the isolated contact fraction.

Binding of hexokinase to the contact fraction

In view of the above findings it appears that the contacts have a higher binding capacity for hexokinase which agrees with electron microscopic binding studies using gold-labelled hexokinase [6]. We, therefore, compared the capacity of the outer membrane and the contact fractions for binding of hexokinase type I under saturating conditions (Table IV). The amount of outer membrane present in the contact fraction was calculated from the monoamine oxidase activity and the porin concentration, determined densitometrically. The outer membrane in the contact fraction had a 3- to 5-times higher hexokinase binding capacity compared to the pure outer membrane suggesting that the pore in the contacts may have a different structure which has a higher capacity to bind hexokinase.

TABLE IV

BINDING OF HEXOKINASE TO THE OUTER MEMBRANE AND CONTACT FRACTION

The contact and outer membrane fractions were incubated with hexokinase isoenzyme I as described in methods. The activity of bound hexokinase and monoamine oxidase was determined photometrically, the concentration of pore protein was estimated densitometrically in the gel electrophoreses of the fractions. The values obtained in the contact fraction are expressed relative to the values determined in the corresponding outer membrane.

Expt. No.	A Hexokinase	B Monoamine oxidase	C Porin	Ratio A/B	Ratio A/C
1	0.17	0.04	–	4.3	–
2	0.43	0.08	–	5.4	–
3	0.61	0.11	0.18	5.5	3.4
4	0.24	0.05	–	4.8	–
5	0.90	0.17	–	5.3	–
			Mean:	5.1	(±0.5)

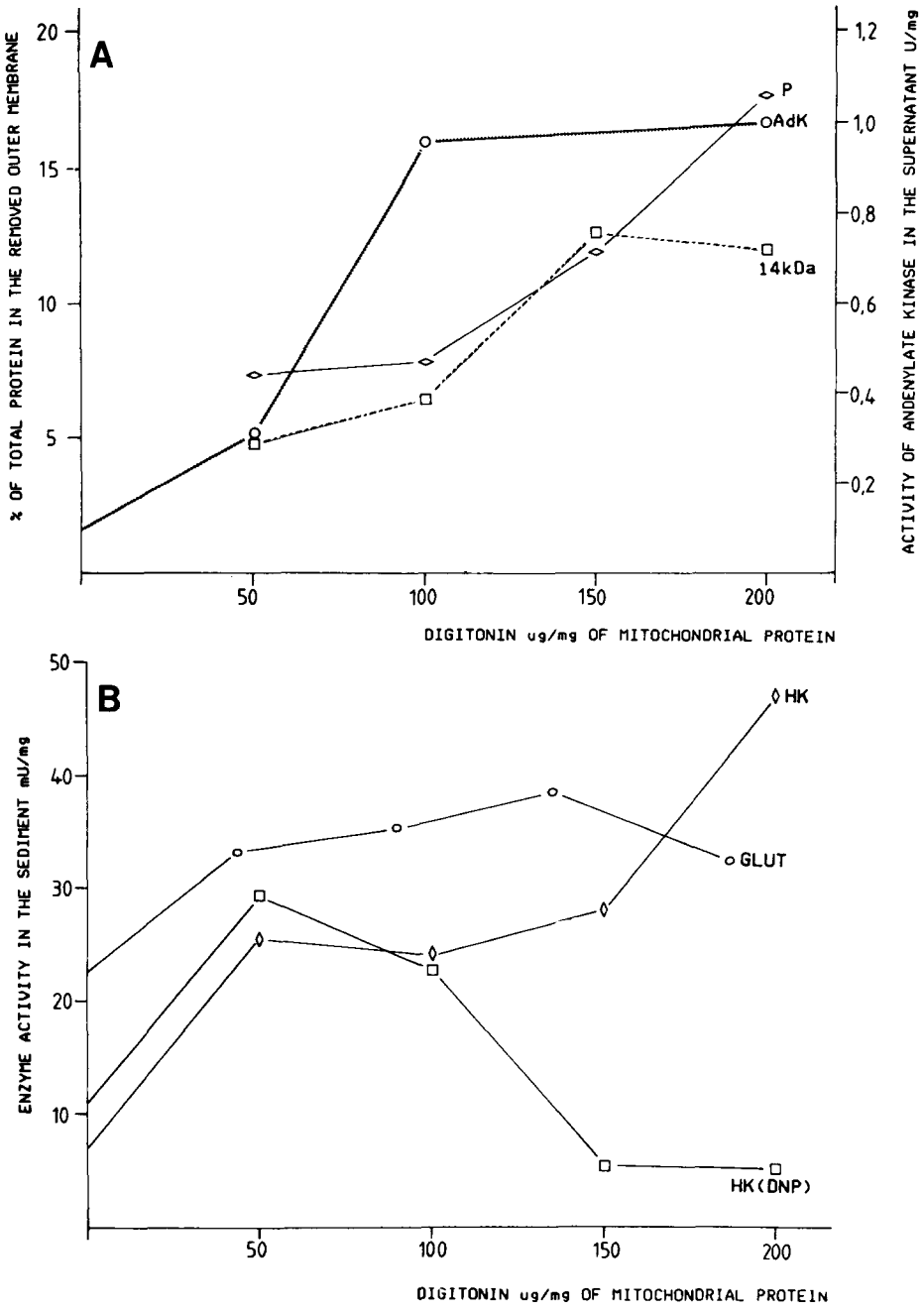

Fig. 11. Characterization of boundary membrane contacts in mitoplasts. (A) Isolated liver mitochondria were incubated with increasing concentrations of digitonin and subsequently centrifuged as described in Methods. The outer membrane liberated into the supernatant by digitonin was removed by high speed centrifugation and was subjected to SDS-polyacrylamide gel electrophoresis. The concentration of the pore (P) and 14 kDa protein in the gels was determined by densitometry. Activity of adenylate kinase (AdK) in the supernatant was determined by optical test. (B) Isolated liver mitochondria were treated with digitonin in the presence and absence of 3 mM dinitrophenol (DNP) and subsequently centrifuged as in (A). Hexokinase (HK, HK-DNP) and glutathione transferase (GLUT) activity was determined in the sediment.

Distribution of hexokinase in the density gradient

Osmotically lysed mitochondria were incubated with isolated hexokinase I to mark the contact fraction. Because the outer membrane has a lower capacity to bind hexokinase, non-saturating amounts of hexokinase were used to reduce the binding of the enzyme to the free outer membrane. The membrane subfractions were subsequently separated on the density gradient. The activity profile of hexokinase in the latter experiment differed from that with free hexokinase (Fig. 12A). The membrane-bound hexokinase was found in the fractions containing the inner boundary membrane and the contact sites which is characterized by the presence of glycerolphosphate oxidase and monoamine oxidase activities (Fig. 12B).

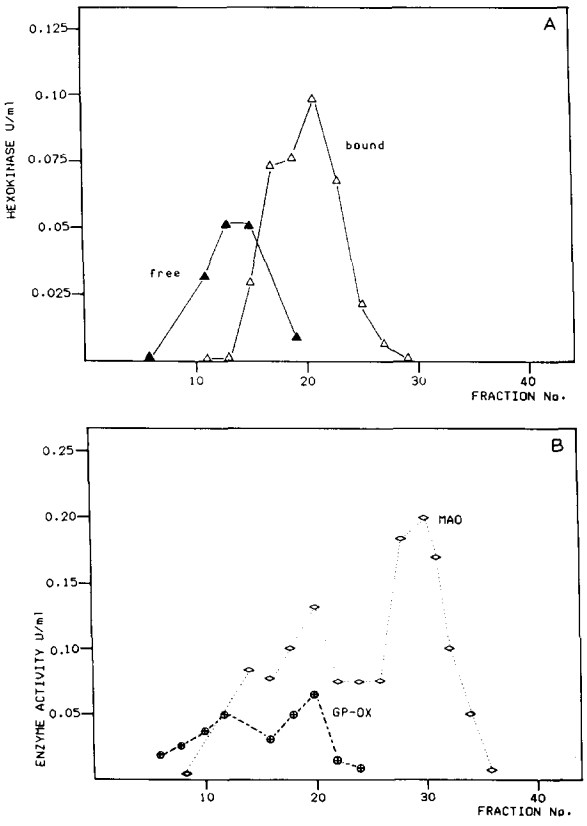

Fig. 12. Distribution of free and membrane bound hexokinase in the density gradient. Mitochondria were disrupted by osmotic shock and incubated for 20 min at room temperature with 0.5 U of isolated hexokinase I and 4 mM $MgCl_2$. Subsequently the mitochondrial membranes and free hexokinase were centrifuged on a sucrose density gradient. The activity profiles of free and bound hexokinase are shown in (A) and compared to the distribution of monoamine oxidase (MAO, times 10) and glycerolphosphate oxidase (GP-OX, times 10) (B).

Effect of uncoupling on the contact fraction

The number of contact sites was reduced by

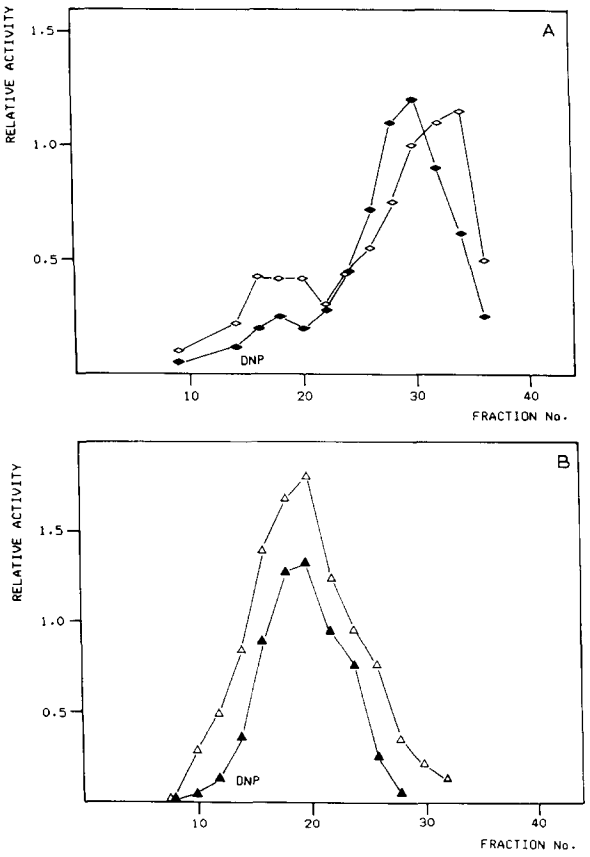

Fig. 13. Effect of uncoupling on the distribution of monoamine oxidase and hexokinase in the density gradient. Isolated mitochondria were uncoupled by addition of 1.5 mM dinitrophenol (DNP). These mitochondria as well as untreated ones were osmotically lysed and afterwards incubated with hexokinase as in Fig. 12. The membrane subfractions were separated on a linear density gradient as in Fig. 1. The activity of monoamine oxidase (A), and hexokinase (B) in the different gradient fractions are expressed relative to the activity in the suspension which was layered on top of the gradient. Recoveries: monoamine oxidase: .99%, +DNP 93.5% ($n = 2$), hexokinase: $196 \pm 52\%$, +DNP $156 \pm 61\%$ ($n = 4$). The high recovery of hexokinase is due to activation of the enzyme by binding to the membrane.

uncoupling of the isolated mitochondria with 1.5 mM dinitrophenol. Subsequently, uncoupled and untreated mitochondria were osmotically lysed and incubated with isolated hexokinase I. As above, non-saturating amounts of hexokinase were used to reduce the binding of the enzyme to free outer membrane. The membrane subfractions were separated on a density gradient. The activity of monoamine oxidase and hexokinase in the different gradient fractions was based on the activity in the membrane suspension which was layered on top of the gradient. Uncoupling led to a significant decrease of the relative activity of monoamine oxidase and hexokinase in the fractions containing the inner boundary membrane and the contact sites (Figs. 13A and B). The recovery of enzyme activities in the presence and absence of dinitrophenol was the same, excluding inhibition of monoamine oxidase and hexokinase by dinitrophenol. This was not true for the activity of glutathione transferase. The assay system of this enzyme appeared negatively influenced by dinitrophenol. Therefore, the observed reduction of glutathione transferase activity in the contacts upon uncoupling is difficult to interpret.

Discussion

Specific properties of the inner and outer membrane components in the fraction containing the contact sites

Contacts between the two mitochondrial boundary membranes have been characterized in freeze-fractured mitochondria to be dynamic structures which are positively correlated to the degree of coupling. However, when the mitochondria are not completely uncoupled, some contacts are still present [4,5].

Separation of osmotically lysed mitochondria on a linear density gradient reveals a membrane fraction containing inner and outer boundary membrane fragments. These cannot be separated by recentrifugation on a shallower second gradient (Fig. 1). The fraction has a 40-fold higher specific activity of glutathione transferase, and bind 3- to 5-times more hexokinase compared to the outer membrane (Table IV). In addition, this fraction contains a specific polypeptide (M_r 45000) of unknown function which appears to be partially integrated into the membrane and partially exposed to the outer surface of the outer membrane (Fig. 9). The inner membrane component of the contact fraction contained a relatively high activity of glycerolphosphate oxidase and low activity of ATPase and cytochrome oxidase. Thus, the contact fraction resembles a fraction which has been previously described as the inner boundary membrane and is observed at comparable density in the sucrose density gradient [12]. A formation of contact sites can be expected with the inner boundary membrane. Therefore, the amount of outer membrane components present in the fractions containing this membrane might indicate the concentration of contacts. Indeed, if the contacts are reduced by uncoupling, a significant reduction of the outer membrane marker enzyme monoamine oxidase in the inner boundary membrane fraction is observed (Figs. 12 and 13).

The outer membrane contact areas are distinct from areas beyond the contact zones. This can be deduced from the effect of digitonin on intact mitochondria, which removes the outer membrane but leaves parts of it in the contacts unaffected. Hexokinase can still bind to the latter part of the outer membrane in mitoplasts (Fig. 11). When membranes of osmotically lysed mitochondria are incubated with free hexokinase under non-saturating conditions, the enzyme preferentially moves to the density gradient fractions containing the contact sites (Fig. 12). Since the outer membrane contains even more pore/binding protein for hexokinase, this finding suggests that the pore protein may assume different functional or conformational states in different regions of the outer membrane. Indeed, single-channel conductance measurements of purified porin, reconstituted into planar bilayers, indicate that this molecule can form at least two kinds of pores differing in conductance [28]. Furthermore, cross-linking experiments with isolated outer membrane of yeast mitochondria show that the pore can adopt four different oligomeric structures [32]. From our electron microscopic observations with gold-labelled antiporin antibodies, it appears that this antibody specifically recognized the porin structure present in the outer membrane at the sites of contact where hexokinase was preferentially bound [6]. To explain why hexokinase can also bind to isolated

outer membrane, we assume that the porin structure exhibiting a higher binding capacity for hexokinase is also present in the isolated membrane but to a lower extent compared to the contact sites [32].

Relative orientation of inner and outer membrane components in the contact fraction

In agreement with previous observations in yeast [30], the 14 kDa polypeptide was found to be exposed to the outer surface of liver mitochondria because it was sensitive to trypsination. Exposure of a second outer membrane component (M_r 68000) to the surface of rat liver mitochondria has been recently described [31]. In this investigation the tryptic degradation of these polypeptides (M_r 14000 and 68000) was observed in the contact fraction and the pure outer membrane fraction from the gradient. This indicates that the outer membrane in both fractions has a regular right-side-out orientation.

To answer the question as to whether the outer membrane enwraps the inner membrane or if the two membranes are attached side by side, we studied electrotransfer from succinate dehydrogenase to externally added cytochrome *c*. It is known that the outer membrane is impermeable to cytochrome *c* [33] in intact mitochondria. Therefore, a greater reduction of external cytochrome *c* by succinate will occur following treatment with digitonin. Similar results were obtained in the present study when this assay was applied to the contact fraction, and suggested that the outer membrane vesicles in the contact fraction were sealed and two thirds of the inner membrane fragments were inside the outer membrane vesicles (Fig. 10). This view of membrane orientation is additionally supported by a binding of hexokinase to the contact fraction because this enzyme binds to the outer surface of the outer membrane in intact mitochondria. Upon incubation with non-saturating concentrations of hexokinase, we observed no binding to the fraction containing the free outer membrane but, because of higher binding capacity, exclusively to the contact fraction (Figs. 12 and 13). This suggests that the contact fraction does not contain free outer membrane but presumably only that part of the membrane which is connected to the inner boundary membrane.

Likewise, freeze-fracture analysis of the contact fraction reveals similar results concerning the sidedness and orientation of the inner and outer membrane. The normal orientation of the different layers in Fig. 4 can be identified according to the relative particle densities of the four faces of the two mitochondrial membranes [34,35]. In addition, fracture plane deflections are visible between the two boundary membranes which specify the presence of hydrophobic membrane contacts. The function of the glutathione transferase in the contacts is at present not known. However, the increased capacity for hexokinase binding in the contact sites may be important for a direct adenine nucleotide exchange between peripheral kinases and the ATP/ADP translocating system [7].

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