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Mitochondrial boundary membrane contact sites in brain: points of hexokinase and creatine kinase location, and control of Ca^{2+} transport

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The location of hexokinase at the surface of brain mitochondria was investigated by electron microscopy using immuno-gold labelling techniques. The enzyme was located where the two mitochondrial limiting membranes were opposed and contact sites were possible. Disruption of the outer membrane by digitonin did not remove bound hexokinase and creatine kinase from brain mitochondria, although the activity of outer membrane markers and adenylate kinase decreased, suggesting a preferential location of both enzymes in the contact sites. In agreement with that, a membrane fraction was isolated from osmotically lysed rat brain mitochondria in which hexokinase and creatine kinase were concentrated. The density of this kinase-rich fraction was specifically increased by immuno-gold labelling of hexokinase, allowing a further purification by density gradient centrifugation. The fraction was composed of inner and outer limiting membrane components as shown by the specific marker enzymes, succinate dehydrogenase and NADH-cytochrome-*c*-oxidase (rotenone insensitive). As reported earlier for the enriched contact site fraction of liver mitochondria the fraction from brain mitochondria contained a high activity of glutathione transferase and a low cholesterol concentration. Moreover, the contacts showed a higher Ca^{2+} binding capacity in comparison to outer and inner membrane fractions. This finding may have regulatory implications because glucose phosphorylation via hexokinase activated the active Ca^{2+} uptake system and inhibited the passive efflux, resulting in an increase of intramitochondrial Ca^{2+}.

Introduction

Several investigators have shown that by far the major portion of hexokinase activity in brain was associated with the mitochondrial fraction (for a review, see Ref. 1). Furthermore, the existence in brain mitochondria of creatine kinase activity comparable to that of heart has been described [2]. The possibility that a mitochondrial location of these kinases might facilitate access to ATP generated by the oxidative phosphorylation was examined for hexokinase in brain [3] and liver [4,5] and for creatine kinase in heart mitochondria (for a review, see Ref. 6). These authors postulated a functional coupling of the mitochondrial-bound hexokinase and the creatine kinase to the oxidative phosphorylation.

Recent work has provided structural evidence for this functional compartmentation: (a) it has been observed that the outer mitochondrial mem-

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brane is permeable to ADP exclusively along a pore protein [7]; (b) the pore protein, described as a voltage-dependent anion-selective pore by Colombini [8], was proved to be identical [9,10] with the hexokinase binding protein characterized by Felgner [11]; (c) the outer and inner membranes form intimate contacts which vary with the metabolic states of the mitochondria [12]. In view of these findings we postulated the existence of a microcompartment in the contact sites allowing a direct coupling of the peripheral-bound hexokinase to the adenylate translocator [13]. Isolation and characterization of the contact sites from liver mitochondria resulted in a preferential location of the hexokinase porin complex within these sites at the mitochondrial surface [14]. This finding may explain the ineffectivity of digitonin treatment to remove surface-bound hexokinase activity from the mitochondrial fraction, which has been described for mitochondria from liver [14], brain [15], heart [16] and kidney [17]. Since it has been shown that digitonin does not remove the outer mitochondrial membrane completely [18,19] hexokinase may also bind to remnants of the outer membrane which remain attached in the contacts with the inner membrane.

The location of hexokinase in the contact sites of brain mitochondria would also explain why some authors located hexokinase at the outer mitochondrial membrane [20], while others assumed a location at the outer side of the inner membrane [21] because of tight binding of the enzyme to their isolated inner membrane fraction.

The preferential binding of hexokinase in the contact sites led us to suggest that these structures may be of principal importance for functional coupling of kinases to the inner compartment and, therefore, also to expect location of creatine kinase in these mitochondrial areas. Furthermore, we have postulated that the contact sites between the two boundary membranes are also important in channeling metabolites and ions to the inner mitochondrial compartment [13]. In fact, Panfili and Sandri [22] assumed that the active Ca^{2+} uptake system of brain mitochondria may be located in the contact/hexokinase binding sites because the uptake and Na^{+}-dependent efflux of Ca^{2+} was impaired upon removal of porin-bound hexokinase in brain mitochondria.

Using hexokinase as a specific marker enzyme, we therefore attempted to characterize in brain mitochondria the location and structure of the contact sites by immuno-electron microscopy. Moreover, we tried to isolate the contact sites from brain mitochondria to study their function in Ca^{2+} uptake and to analyze the location of creatine kinase.

Materials and Methods

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

Experimental animals. Male rats of the Chbb-THOM strain (200–250 g body weight) were used. They were fed with a standard diet of Altromin in Lage, F.R.G.

Assays. Hexokinase (EC 2.7.1.30), adenylate kinase (EC 2.7.4.3) and creatine kinase (EC 2.7.3.2) were determined photometrically according to Bücher et al. [23]. The assays of succinate dehydrogenase (EC 1.3.99.19) and NADH cytochrome-*c* reductase (EC 1.6.2.2) rotenone insensitive were carried out as described by Sottocasa et al. [24]. Glutathione transferase (EC 2.5.1.18) was determined as described by Habig et al. [25] and acetylcholine esterase (EC 3.1.1.7) according to Ellman et al. [26].

Isolation of mitochondria. Mitochondria were isolated from rat brain according to Rehncrona et al. [27] by differential centrifugation in a medium (designated below as mannitol/sucrose medium) containing 0.25 M mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.4) and 0.1% BSA fatty acid free.

Preparation of submitochondrial fractions. Osmotic lysis and fractionation of the lysed brain mitochondria was performed as described recently [14] in a linear sucrose density gradient varying from a density of 1.22 to 1.06. In some experiments the peak fractions of rotenone insensitive NADH cytochrome *c* reductase (outer membrane), hexokinase (contact sites), and succinate dehydrogenase (inner membrane) were pooled and the membranes were concentrated by centrifugation of the diluted fractions for 1 h at 200 000 × *g*. The pellets were resuspended in 0.25 mM sucrose, 10 mM Hepes (pH 7.4) medium.

Isolation of hexokinase type I. Hexokinase I was

purified from rat brain according to Chou and Wilson [28].

Preparation of antibodies. Antibodies active against hexokinase I were raised in rabbits by injection of isolated hexokinase I as described recently [29]. The immunoglobulin fraction was prepared from the serum by ammonsulfate precipitation. The antibody fraction inhibited the activity of free hexokinase I completely and approx. 50% of that of the bound enzyme.

Cholesterol determination. Cholesterol was determined according to Barham et al. [30].

Preparation of protein-A with gold particles. Homogeneous colloidal gold particles with a diameter of 6.3 nm were prepared with a mixture of tannic acid and citrate as reducing agent according to Slot and Geuze [31]. The colloidal gold was labelled with protein-A (Pharmacia). The amount of protein-A necessary to coate the gold sol was determined by the salt-induced flocculation test described by Karisberger and Rosset [32].

Indirect immuno gold assay for hexokinase I antibody binding to the mitochondria. Brain mitochondria were suspended in mannitol/sucrose medium without EGTA. 40 μl of the suspension containing 4 mg/ml of mitochondrial protein were incubated for 10 min at room temperature with 50 μl antibody. The antibody solution contained 9.7–17.5 mg/ml of protein and 1% bovine serum albumine (BSA). After centrifugation, 30 s in a tabletop centrifuge, the samples were suspended in mannitol/sucrose medium and were washed two further times in the same medium by centrifugation. The final sediment was resuspended in 40 μl mannitol/sucrose medium and incubated as above with 40 μl protein-A-gold (approx. 0.2 μg/ml). The samples were subsequently centrifuged, washed and resuspended as above. The mitochondria were subjected to fixation. Incubation with unspecific antibodies was performed in the same way.

In separate experiments 300 μl aliquots of the contact site fraction from the density gradient were either incubated with 300 μl hexokinase specific antibodies (27 mg/ml) or with mannitol/sucrose medium. After centrifugation for 20 min at 400000 $\times g$ in a Beckman TL-100 ultracentrifuge both samples were washed, resuspended in the same volume and incubated with 200 μl protein-A gold (4.3 nm, absorbance$_{520} = 0.51$) as above. Subsequently, the fractions were recentrifugated on a discontinuous (40 : 45 : 50, w/v) sucrose density gradient.

Fixation and embedding. All mitochondrial pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (ph 7.4) and postfixed with 2% osmium. The fixed samples were embedded in Spurr's epoxy resin [33], thin sectioned and stained with hot alcoholic uranyl acetate [34]. The samples were examined in a Siemens IA electron microscope. The quantitative evaluation was performed using a Kontron MOP Am2 picture analyzing system connected to a Hewlett-Packard 9825 calculator.

Treatment of isolated mitochondria with digitonin. Brain mitochondria suspended in mannitol/sucrose medium contained a protein concentration of 30 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated for 30 s at room temperature with concentrations of digitonin ranging from 100 to 700 μg/mg of protein. The suspension was subsequently centrifuged for 1 min in a tabletop centrifuge. The supernate was then removed and the sediment was resuspended in sucrose medium.

Determination of Ca^{2+} uptake and efflux. Calcium movements were followed spectrophotometrically at 665–685 nm (in a dual-wavelenth recording spectrophotometer) using purified arsenazo III (2,2′-(1,8-dihydroxy-3,6-disulfo-2,7-naphthalene-bis azo) bisbenzenarsonic acid), as suggested by Vallieres et al. [35]. For further details and experimental conditions see legends to the figures.

Ca^{2+}-binding measurements. Ca^{2+}-binding was determined according to Gratzer and Beaven [36] using purified arsenazo III. Measurements of total and free Ca^{2+} concentrations were derived spectrophotometrically at 665–685 nm by back-titration with EDTA. Details are given in Fig. 5. Data were plotted according to Scatchard.

Results

Activity of hexokinase in isolated mitochondria from brain and liver cells

Mitochondria were isolated from rat liver and brain. Both types of mitochondria were well cou-

pled and exhibited a P/O ratio of 1.6–1.8 and an acceptor control index of 6–8 with succinate as substrate. The activity of hexokinase in the mitochondrial fraction from liver was increased to approx. 30 mU/mg by rebinding of isolated hexokinase I. The glucose phosphorylation by bound hexokinase was investigated in liver and brain mitochondria in mitochondrial isolation medium using an optical test system. The ATP was provided either by direct addition or indirectly via oxidative phosphorylation from succinate, phosphate, and ADP (Table I). The apparent K_m for ATP generated via oxidative phosphorylation was 10 times lower compared to that with external ATP. At concentrations of ADP higher than 0.5 mM, glucose phosphorylation was inhibited. The initial hexokinase I activity with 2 mM external ATP in brain mitochondria and liver mitochondria (saturated with isolated isozyme I) was comparable. When ATP was provided by the oxidative phosphorylation, glucose phosphorylation in both types of mitochondria decreased by 50% of the activity with 0.2 mM external ATP and 20% compared to that with 2 mM ATP. These results suggested that the mitochondrial bound hexokinase was able to generate significant amounts of glucose 6-phosphate from intramitochondrial ATP, no matter whether the enzyme was originally bound as in brain mitochondria or artificially attached as in liver mitochondria.

Effect of hexokinase activity on Ca^{2+} uptake and efflux

We had observed that glucose 6-phosphate which desorbes and inhibits hexokinase also affected Ca^{2+} movements in brain mitochondria [22]. Preincubation of brain mitochondria for 60 s with 2 mM glucose 6-phosphate released about 30% of the bound hexokinase, but did not change the mitochondrial integrity. This was carefully examined according to a previous investigation by measuring the mitochondrial membrane potential by means of safranin [22]. In spite of an unchanged membrane potential incubation with glucose 6-phosphate reduced the active Ca^{2+} uptake and Na-dependent passive efflux to 40% and 50%, respectively (Table II). The efflux of Ca^{2+} remained reduced when the glucose 6-phosphate was added after Ca^{2+} accumulation. This led us to suggest that the Ca^{2+} efflux might be controlled by glucose 6-phosphate. In order to distinguish between glucose 6-phosphate effects on hexokinase activity and Ca^{2+} transport we used the activity of bound hexokinase to produce low amounts of this compound close to the mitochondrial surface. This was possible as shown in Table I when glucose was present and the oxidative phosphorylation was active. In this case we observed a two-fold activation of the active Ca^{2+} accumulation and a very pronounced (5-fold) reduction of Ca^{2+} efflux (Fig. 1). According to the activity of the

TABLE I

KINETIC PROPERTIES OF BOUND HEXOKINASE I IN LIVER AND BRAIN MITOCHONDRIA

Hexokinase I was bound to isolated liver mitochondria by 10 min incubation at room temperature in the presence of 5 mM Mg^{2+} and 5 mM glucose. The mitochondria were centrifuged. The pellet was rinsed and resuspended in sucrose medium. The activity of mitochondrial hexokinase was determined by a direct optical test system in mitochondrial isolation medium. The assay system contained 4 mM $MgCl_2$, 4 mM phosphate, 5 mM NADP, 2 mM glucose, 0.5 U glucose-6-phosphate dehydrogenase and 5 μM rotenone. Activity of glucose phosphorylation was determined either by addition of 2 mM and 0.2 mM ATP or via the oxidative phosphorylation with 10 mM succinate and 0.2 mM ADP. Km for ATP and ADP were determined by Lineweaver-Burk plots.

Substrate	Activity (mU/mg)		K_m (μM)	
	brain mitochondria	liver mitochondria	brain mitochondria	liver mitochondria
ATP			135.0±14	93.0±1.0
2.0 mM	30.0 ±8.7	26.5±2.7		
0.2 mM	12.5 ±5.4	14.0±2.4		
ADP (0.2 mM)	6.34±4.2	7.5±3.6	11.9±9.3	15.5±9.4

TABLE II

INFLUENCE OF GLUCOSE-6-PHOSPHATE ON HEXOKINASE DESORPTION AND Ca^{2+} MOVEMENTS IN BRAIN MITOCHONDRIA

Isolated rat brain mitochondria were incubated for 1 min at 22°C in the absence and in the presence of 2 mM glucose 6-phosphate, both before and after Ca^{2+} accumulation. The uptake and efflux of Ca^{2+} were followed spectrophotometrically as described in Materials and Methods. The cuvette contained (3 ml final volume) 1.5 mg of mitochondrial protein in 0.1 M mannitol, 25 mM Tris-HCl (pH 7.4), 50 mM KCl, 3 mM potassium phosphate (pH 7.4), 5 mM glutamate and 5 mM malate. 0.2 μM ruthenium red and 15 mM Na^+ were added to elicit the Ca^{2+} efflux. 30 ng-ions of Ca^{2+} were added to each sample. Hexokinase (HK) activity was determined in the pellet and supernate after rapid centrifugation of the cuvette content at the end of spectrophotometric recording. HK data are shown as % of activity in the mitochondrial preparation.

Conditions	HK released (%)	(ng-ions/min per mg protein)	
		Ca^{2+} uptake	Ca^{2+} efflux
Control	10	225	15.0
+ G-6-P before Ca^{2+} accumulation	41	85	7.2
Control	10	221	16.2
+ G-6-P after Ca^{2+} accumulation	38	221	9.5

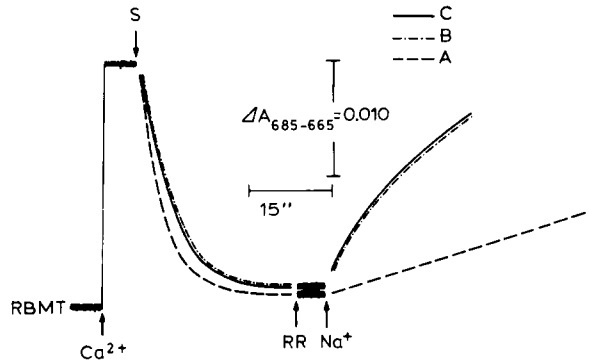


Fig. 1. Effect of hexokinase activity on Ca^{2+} uptake and Na^+-dependent efflux in isolated rat brain mitochondria. Isolated rat brain mitochondria (RBMT) containing approx. 100 mU/mg of hexokinase were incubated for 1 min at 22°C in the presence of 3 mM potassium phosphate (pH 7.4) before addition of Ca^{2+} and substrates (C). In experiment (A) the assay contained 0.2 mM ADP and 0.5 mM glucose, in experiment (B) the assay contained the same as in (A) +2.0 U glucose-6-phosphate dehydrogenase and 3 mM NADP. The uptake and efflux of Ca^{2+} was followed spectrophotometrically by the use of Arsenazo III as described in Materials and Methods. The cuvette (3 ml final volume) contained 1.5 mg of mitochondrial protein in 0.1 M mannitol, 25 mM Tris-HCl (pH 7.4), 50 mM KCl, 3 mM potassium phosphate, 5 mM glutamate and 5 mM malate (S). 0.2 μM ruthenium red (RR) and 15 mM Na^+ were added to elicit the Ca^+ efflux. 30 ng-ions of Ca^{2+} were added in each sample.

structure-bound hexokinase under these conditions (Table I) this inhibition of Ca^{2+} efflux was caused by approx. 40–50 nmol of glucose 6-phosphate which is by far not enough to desorbe significant amounts of hexokinase from the mitochondrial membrane. When the glucose 6-phosphate produced by hexokinase was immediately removed (in the presence of glucose 6-phosphate dehydrogenase and NADP), the effect of hexokinase activity on Ca^{2+} movements was abolished (Fig. 1).

Immuno-electron microscopic localization of hexokinase at the mitochondrial surface and contamination by synaptosomes

By applying the gold-labelling technique and electron microscopy we attempted to localize the bound hexokinase at the surface of brain mitochondria. As compared to the binding of unspecific antibodies from control serum, the antibodies against hexokinase I bound with significantly higher affinity to the mitochondrial outer membrane, while in both cases, a significantly lower binding to contaminating synaptosomes was observed (Table III, Fig. 2B). Determined by electron microscopy we found, relative to the number of mitochondria, a neglectable contamination by endoplasmic reticulum but 15% contamination by synaptosomes (Fig. 2B arrow heads), although, by measuring activity of acetylcholine esterase, we calculated 5% and by determination of lactate dehydrogenase only 1.3% of synaptosomal contamination (legend Table III). These differing data show that it may not be very reliable to calculate the contamination from the activity of representative enzyme activities, as used in many published experiments. To determine the distribution of hexokinase at the mitochondrial surface we analyzed 46 mitochondria like in Fig. 2A, which showed a separation between both limiting membranes. The number of gold grains bound to the outer membrane close to, or separated from, the

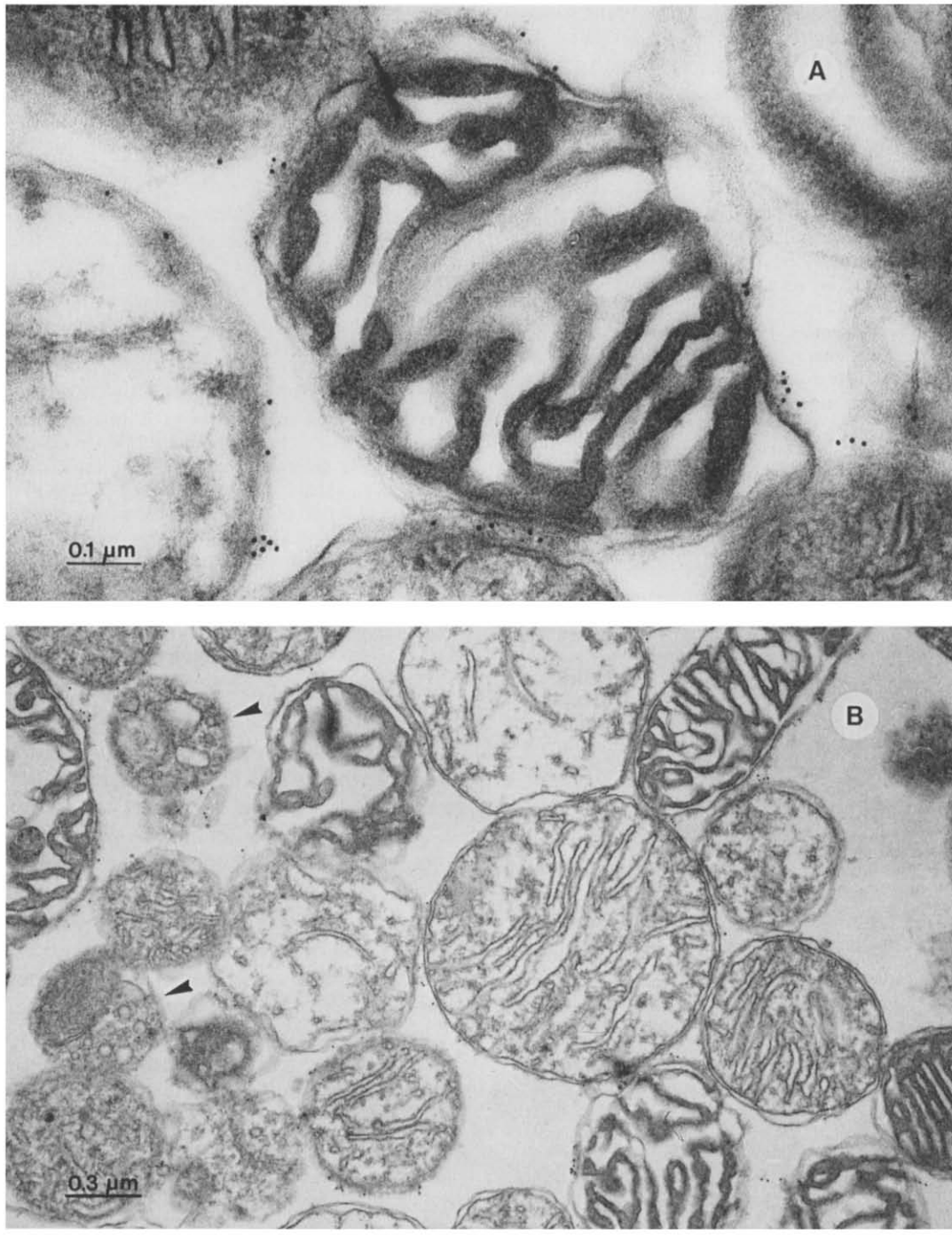


Fig. 2. Thin section of rat-brain mitochondria and the inner membrane subfraction. (A) Thin section of an isolated brain mitochondrium which was decorated with antibodies against hexokinase I and subsequently treated with a protein-A-gold conjugate as described in Materials and Methods. Gold grains are concentrated at the surface of the mitochondrium where both limiting membranes are apposed. Bar = 0.1 μm. (B) Thin section of the isolated mitochondrial fraction from brain showing a contamination by synaptosomes (arrows). Bar = 0.3 μm.

TABLE III

LOCALIZATION OF HEXOKINASE IN THE MITOCHONDRIAL FRACTION OF RAT BRAIN

Mitochondria were incubated as described in Materials and Methods with antibodies against hexokinase I which were subsequently decorated with protein-A-gold: expt. 1, absorbance$_{520\,nm}$, approx. 6.0; expt. 2, absorbance$_{520\,nm}$, approx. 0.8. As control, mitochondria were incubated in the same way with unspecific antibodies (present in the control serum) and protein-A-gold. The glutaraldehyde, osmium fixed samples were thin sectioned and examined in the electron microscope. The data show mean and standard deviation of the number of gold grains per μm bound to mitochondria and to contaminating synaptosomes. In addition the number of gold grains bound per free and attached outer membrane is given. Data in brackets are the numbers of organelles used for evaluation. The probability that the experimental groups (labelled with letters) are not statistically different is expressed as $P\%$ and was calculated by the Mann-Whitney-U-test [53]. A contamination by synaptosomes relative to the number of mitochondria of 15% was determined by electron microscopy; according to activity of lactate dehydrogenase and of acetylcholine esterase the contamination was 1.5% and 5%, respectively.

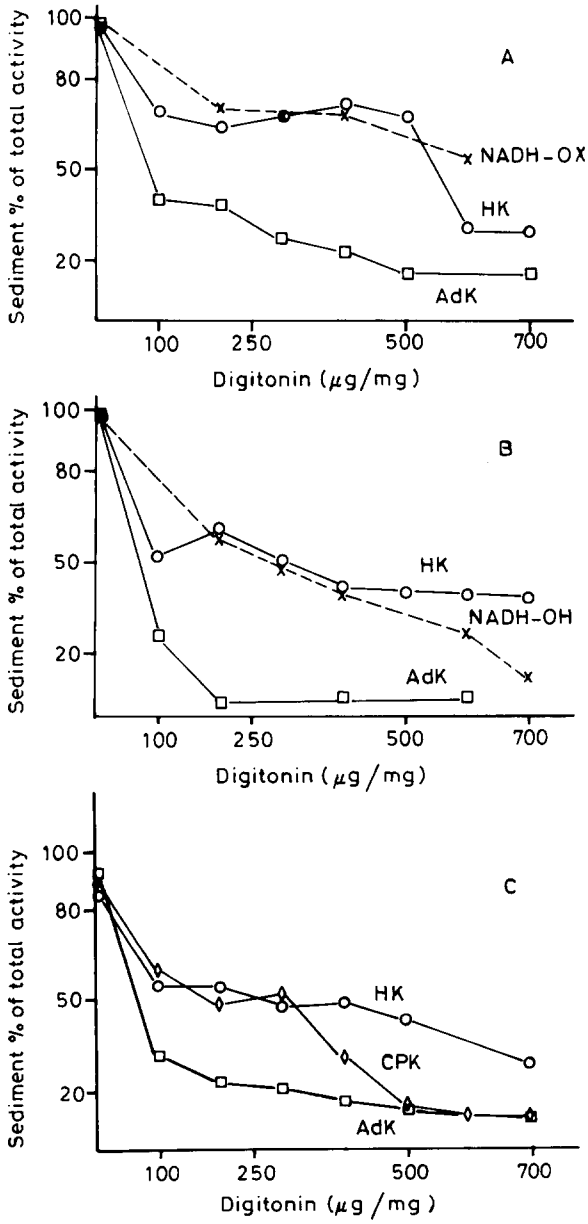
	Grains per μm	Length of total membrane evaluated (μm)
Experiment 1		
Attached boundary membranes	5.4 ± 3.1 (46,a)	
Separated boundary membranes	3.5 ± 4.7 (46,b)	72.5
	$P\%$ (a,b) $= 3 \cdot 10^{-3}$	31.1
Experiment 2		
Antibody specific, mitochondria	3.9 ± 2.4 (63,c)	
Antibody unspecific, mitochondria	1.1 ± 0.9 (63,e)	
Antibody specific, synaptosomes	0.4 ± 0.5 (36,d)	
	$P\%$ (c,d) $= 5.6 \cdot 10^{-12}$	
Antibody unspecific, synaptosomes	0.7 ± 0.8 (14,f)	
	$P\%$ (e,f) $= 3.3$	

inner membrane was counted. There was a significantly higher concentration of gold grains at the attached surface of the outer membrane than at the separated surface (Table III). Compared to the activity of hexokinase associated with the mitochondrial fraction (Table I) the density of gold grains appeared relatively low. Based on 30 U/mg of specific activity of hexokinase (molecular mass, 100 kDa) and a mitochondrial surface of 170 cm^2 per mg protein [37], we calculated 63 molecules of hexokinase at the surface of a 70 nm section. At this surface we counted 9–10 gold grains which means a 15% efficiency of labelling. This value agrees with the efficiency observed with post-embedding decoration on tissue sections which was between 10 and 40% [38]. The reason for the low efficiency may be severalfold: firstly, not all hexokinase may be labelled by the antibodies, and secondly, it can be assumed that label is lost during the dehydration and embedding procedure.

Fractionation of the outer mitochondrial membrane by digitonin

In view of the above findings the outer membrane of brain mitochondria appeared to have a heterogenous composition concerning the binding of hexokinase: the area of the outer membrane located close to the inner membrane may be different compared to the area beyond of the contacts. By the use of digitonin it was possible to separate between these two parts of the outer membrane. This reagent fragments the outer membrane so that the free part becomes liberated and the other part remains attached via the contacts to the inner membrane [18,19]. When phosphorylating brain mitochondria (which have a high frequency of contacts) were exposed to increasing digitonin concentrations, activity of adenylate kinase, located between the two limiting membranes, was decreasing while more than 50% of rotenone-insensitive NADH cytochrome *c* reductase and hexokinase activity was still bound to the mitochondria (fig. 3A and B). This suggested that, although the outer membrane became disrupted, large parts of it remained bound to the inner membrane together with hexokinase. To investigate the involvement of contact sites in this attachment of the outer membrane and hexokinase, we repeated the same experiment in the presence of glycerol which reduces the contacts [12]. Under these conditions significantly more hexokinase and rotenone-insensitive NADH cytochrome *c* reductase were removed from the sediment (Fig. 3B). Hexokinase is known to bind to the outer membrane; adenylate kinase and creatine kinase, however, are thought to be dissolved within the

outer mitochondrial compartment. When we compared the effect of digitonin on the liberation of creatine kinase with adenylate kinase (Fig. 3C) we observed a retarded desorption of creatine kinase, suggesting a different organization of the two intermembrane enzymes. It should be pointed out that this experiment was done under non-phosphorylating conditions, state 1, where the mitochondria have a lower frequency of contacts [12]. This explains the lower amount of hexokinase activity in the sediment of Fig. 3C compared to Fig. 3A. Irrespective of these differences the experiment (Fig. 3C) shows that the release of creatine kinase by digitonin resembles that of hexokinase and not that of adenylate kinase.



Isolation and characterization of the contact fraction from osmotically lysed brain mitochondria

Membranes of osmotically lysed liver mitochondria were recently separated into three fractions by centrifugation on a continuous sucrose density gradient: an outer membrane fraction containing high activity of monoamine oxidase, an inner membrane fraction characterized by high activity of succinate dehydrogenase activity, and an intermediate fraction characterized by high activity of glutathione transferase and hexokinase [14]. When the same method was applied to osmotically lysed brain mitochondria we searched for a fraction with high hexokinase activity relative to the outer membrane and crista membrane marker enzymes. The activity profiles of specific marker enzymes in the gradient (Fig. 4) revealed a membrane fraction (at a density of 1.15 g/ml) in which most of the hexokinase and creatine kinase activity was concentrated. This fraction, probably containing inner boundary membrane and the contact sites, also had high glutathione transferase

Fig. 3. Desorption of bound hexokinase and creatine kinase by digitonin in isolated brain mitochondria differing in frequency of contact sites. Mitochondria isolated from rat-brain phosphorylating (A). (5 mM succinate, 1 mM ADP, 4 mM $MgCl_2$, 4 mM potassium phosphate) were incubated for 30 s in mannitol/sucrose medium with increasing concentrations of digitonin and subsequently centrifugated. Adenylate kinase (AdK), hexokinase (HK) and rotenone-insensitive NADH cytochrome-*c* reductase (rotenone insensitive NADH-OX) were determined in the sediment. The frequency of contact sites between the two boundary membranes was reduced in (B) by addition of 30% glycerol to phosphorylating mitochondria. (C) Liberation of creatine kinase (CPK) and adenylate kinase from mitochondria, in state 1, at different digitonin concentrations. The data are the mean of five experiments in A, two experiments in B and three experiments in C. Mean and standard deviations of HK at 500 μg digitonin are 65.2±5.7% (A) 40.1±0.1% (B). Mean of HK at 300 μg digitonin are 48.8±4.1% (C), and 52.2±8.0% (CPK).

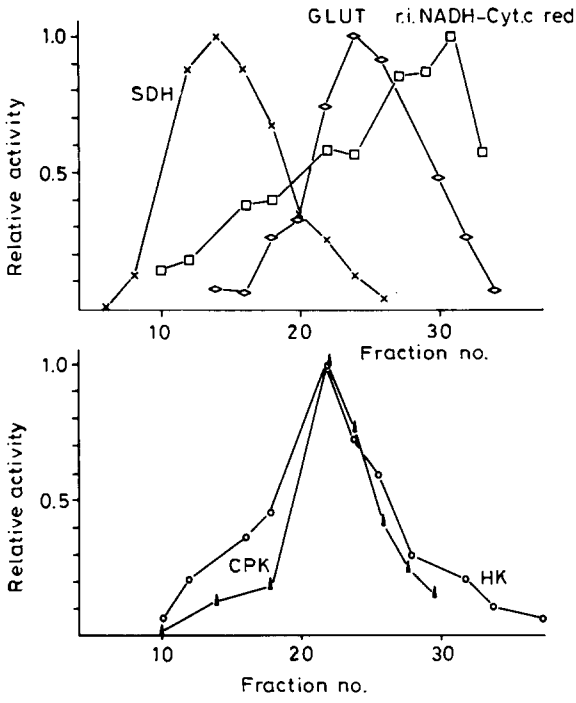


Fig. 4. Distribution of enzymes in a density gradient separating subfractions of osmotically lysed rat brain mitochondria. Rat brain mitochondria were disrupted by osmotic shock and sonication. The subfractions were separated on a 23 ml continuous density gradient varying between 1.22 and 1.06 g/ml. The gradient, and 6.5 ml overlay and 4 ml 70% sucrose cushion was divided into 40 fractions, starting from the bottom. In these fractions the distribution of hexokinase (HK) and creatine kinase (CPK) was analyzed, lower pannel, in correlation to the activity profiles of marker enzymes of outer membrane (rotenone-insensitive NADH cytochrome *c* reductase = r.i. NADH-Cyt *c*-RED), contact sites (glutathione transferase = GLUT) and inner membrane (succinate dehydrogenase = SDH). The enzyme activities (U/ml) are expressed relative to the maximum activity in the peak fraction of the respective enzyme and are mean values of four different experiments.

activity. According to the distribution of rotenone-insensitive NADH cytochrome *c* reductase, outer membrane was concentrated on top of the gradient (at a density of 1.13 g/ml). The fractions (at a density higher than 1.17 g/ml), where succinate dehydrogenase representative for crista membranes was present, contained little activity of hexokinase and creatine kinase.

Composition of the contact site fraction as analyzed by specific antibodies against hexokinase

It could not be excluded that the presumptive contact fraction from brain mitochondria was contaminated by synaptosomes and endoplasmic reticulum and therefore contained hexokinase and creatine kinase activity. Furthermore, it was possible that the fraction was accidentally composed of free inner and outer membrane vesicles of the same density. In order to separate specifically contact sites from contaminating other membrane components, we used hexokinase as a tool. This enzyme according to the results presented in Fig. 2A and Table III is exclusively located at the surface of mitochondria with preference for the contact sites [14] and was not observed at the surface of synaptosomes. By using the protein-A gold assay for antibodies bound to hexokinase I (as in Fig. 2A) we increased the density of the contact fraction which was obtained from the density gradient. The antibody-labelled fraction was subsequently recentrifugated on a second discontinuous sucrose gradient which was composed of the highest density (50%) in the first gradient (Fig. 4) the density of the inner membrane fraction (45%) and 40% sucrose, which is a slightly higher density than that of the contact sites fraction (36%). The activity profiles of outer (rotenone insensitive NADH cytochrome *c* reductase) and inner (succinate dehydrogenase) membrane marker enzymes, hexokinase and creatine kinase in this gradient coincided and were shifted to higher density compared to a gradient loaded with the unlabelled contact fraction (Fig. 5). This suggested that the two boundary membranes were linked together and the two kinases were specific components of this fraction.

Ca^{2+} binding capacity of the isolated gradient fractions

It was observed that the activity of hexokinase impaired both the active Ca^{2+} uptake and Na^{+}-dependent passive release in brain mitochondria (Table II and Fig. 1) probably via production of glucose 6-phosphate close to the Ca^{2+} transport system. Therefore, the three membrane fractions from the density gradient which differed in hexokinase activity were analyzed for Ca^{2+} binding. It was found that the contact fraction had a higher Ca^{2+}-binding activity as compared to the inner and outer membrane fractions (Fig. 6). However, the binding constant for Ca^{2+} appeared to be the same in all fractions of the gradient. This

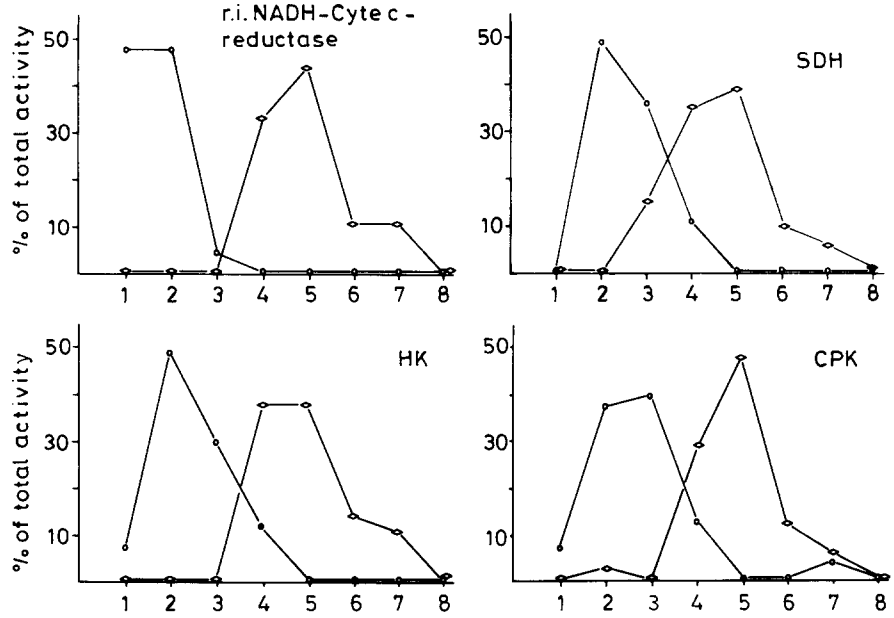


Fig. 5. Distribution of contact site-specific enzymes in a density gradient after immuno-gold labelling of hexokinase. The isolated contact site fraction (Fig. 4) was decorated with antibodies against hexokinase I. This fraction as well as an untreated one were subsequently incubated with a protein-A-gold conjugate. Both fractions were centrifuged on a discontinuous density gradient containing 40% (fraction 2–3), 45% (fraction 4–5) and 50% (fraction 6–7) sucrose w/v. Fraction 1 represents the volume of the sample and fraction 8 is the pellet fraction. The activities of enzymes (names abbreviated as in Fig. 4) are expressed as % of total activity in the gradient. Open circles = enzyme activity profiles in the gradient loaded with unlabelled contact fraction.

is not surprising if one considers the fact that the Ca^{2+} binding protein calvectin which is responsible for these results is in part readily soluble from osmotically treated mitochondria and may therefore be distributed in small amounts within all the gradient fractions.

Cholesterol content of the isolated gradient fractions

The different effect of digitonin on the outer membrane within and beyond the contact sites suggested a lower amount of cholesterol in the contact fraction where hexokinase was bound. Analysis of the mitochondrial membrane subfractions from the density gradient revealed indeed a 3-times lower cholesterol content in the hexokinase-rich fraction compared to the outer membrane-containing fraction, while the inner membrane fraction contained the lowest amount of cholesterol (Table IV). However, it appeared inconsistent to deduce from these data a reduction of cholesterol in the outer membrane of the contact sites while withholding the presence of inner membrane phospholipids, which dilute the outer membrane lipids. A calculation on the basis of marker enzymes did not solve the problem because they may not be randomly distributed within

TABLE IV

CHOLESTEROL CONTENT OF MITOCHONDRIAL SUBFRACTIONS OF RAT BRAIN

The membrane fractions from the density gradient (Fig. 4) were analyzed for cholesterol as described in Methods. Data are mean values of six different experiments. The molar ratios were calculated by using the phospholipid concentration per mg of protein determined in the liver membrane subfractions: 327 nmole for the inner membrane, 771 nmole for the outer membrane, and 638 nmole in the contact fraction.

Fraction	Cholesterol	
	nmole per mg protein	c/PLP (mol%)
Swollen and sonicated mitochondria	85.3 ± 24.5	13.3
Outer Membrane	102.3 ± 51.6	13.2
Contact sites	33.0 ± 6.2	5.2
Inner Membrane	10.8 ± 3.9	2.8

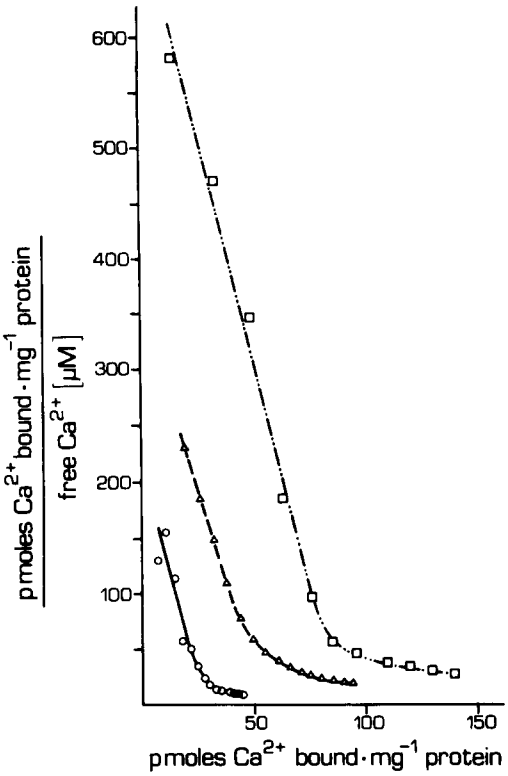


Fig. 6. Scatchard plot of Ca^{2+} binding to subfractions of rat brain mitochondria. The subfractions, obtained by density gradient centrifugation as shown in Fig. 4 were analyzed, after exhaustive dialysis, for Ca^{2+} binding as described in Materials and Methods. The assays contained in a 3 ml cuvette 0.5–1 mg protein per sample, 0.16 mM arsenazo III, 50 mM Tris-HCl (pH 8.3) and sufficient free Ca^{2+} to obtain an absorbance of 0.07. Aliquots of 5 nmol EDTA were repeatedly added to the assays until titration was complete. □, contact sites, △, outer membrane, ○, inner membrane.

the two membranes. On the whole we arrived at the conclusion that the data did not proof but may suggest a lower cholesterol content in the outer membrane of the contact sites. The relatively high colesterol content of the osmotically lysed mitochondria may result from the synaptosomal contamination which was subsequently reduced by density gradient centrifugation.

Ultrastructural characterization of the gradient fractions

The pooled outer-membrane, contact, and inner-membrane fractions were fixed with glutaraldehyde and osmium tetroxide, embedded in epoxy resine and thin sectioned. The outer membrane fraction (Fig. 7A) was composed almost homogeneously of small, single-walled vesicles, whereas in the contact site fraction (Fig. 7B) more often double-walled vesicle (marked with asterisk) were observed. As was shown for the contact site fraction from liver mitochondria, these structures may represent right-side-out outer membrane vesicles enwrapping inner membrane. The inner-membrane fraction was enriched of crista-like structures (Fig. 7C). All fractions including the contact site fraction were slightly contaminated by small synaptosomes filled with synaptic vesicles. These synaptosomes did, however, not contain mitochondrial structures. These results, together with those presented in Fig. 5, suggest that the slight synaptosomic contamination of the contact site fraction could neither account for the activity of hexokinase and creatine kinase nor for the presence of mitochondrial inner and outer membrane marker enzymes.

Discussion

Nonrandom distribution of kinases at the surface of brain mitochondria

Contacts between mitochondrial boundary membranes, first described by Hackenbrock [40] have been analyzed to be dynamic structures which are regulated by the mitochondrial metabolism [12] and by hormones [41]. We were able to enrich and characterize contacts from osmotically lysed liver mitochondria [14]. Compared to the pure outer membranes, the outer membrane component in the contacts contained a pore/hexokinase binding protein with a higher capacity to bind hexokinase. Likewise the outer membrane contact areas of brain mitochondria appeared to be distinct from areas beyond the contact zones. This could be deduced from the nonrandom distribution of gold-labelled hexokinase antibodies (Table II and Fig. 2A), and the reduced desorption of the enzyme by treatment with digitonin (Fig. 3) [15]. In liver mitochondria this reagent removes specific domains of the outer membrane and leaves parts of it in the contacts unaffected [18,19]. The involvement of contact sites in the binding of hexokinase was further supported from the fact that reduction of contacts resulted in a more effec-

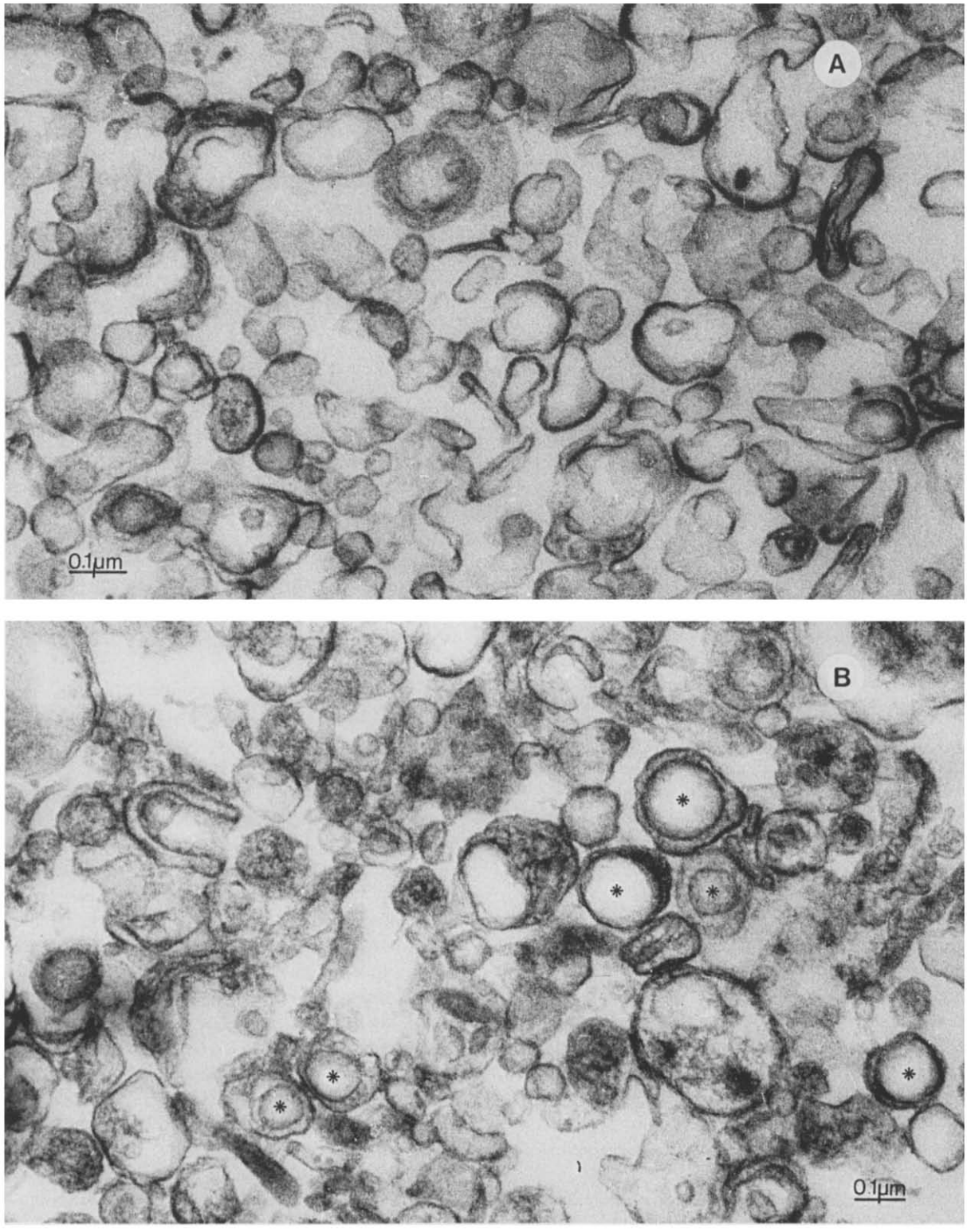


Fig. 7. Thin sections of membrane subfractions of osmotically lysed brain mitochondria. The peak fractions of rotenone-insensitive NADH-cytochrome *c* dehydrogenase (outer membrane = A), hexokinase (contact sites = B) and succinate dehydrogenase (inner membrane = C) were pooled and concentrated by centrifugation. Samples of the centrifuged material, suspended in sucrose medium, were fixed with 2.5% glutaraldehyde and 2% osmium followed by conventional embedding in Spurr's epoxy resin. Double-walled vesicles are marked by asterisks. Bar = 0.1 µm.

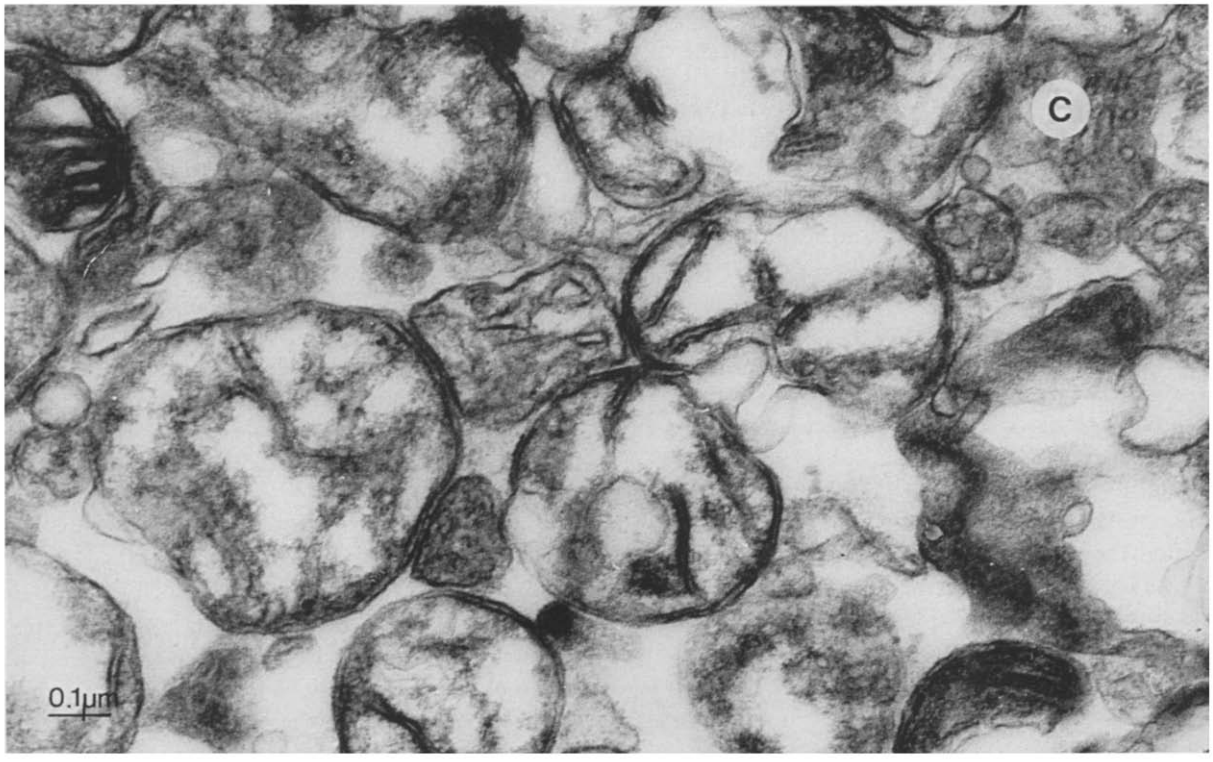


Fig. 7. (continued).

tive desorption of the enzyme by digitonin together with parts of the outer membrane (Fig. 3). Concerning location at the mitochondrial periphery, creatine kinase is comparable to adenylate kinase, however, the enzyme was incompletely removed by digitonin treatment. This suggested that creatine kinase, like hexokinase, could be bound in the contact sites.

The reduced effect of digitonin on the outer membrane in the contact sites may be explained by a lower cholesterol content of the isolated contact fractions of brain (Table IV) and liver [14].

In conclusion these findings indicated that the formation of contacts result in a heterogeneous lipid and protein composition of the outer membrane and a nonrandom distribution of intermembrane enzymes.

Isolation and characterization of the contact sites

The specific composition of the outer membrane in the contacts was recently used to identify the contact fraction is a discontinuous density gradient which separated subfractions of brain mitochondria [42]. In agreement with these findings it was possible in the present investigation (Fig. 4) to separate a fraction which contained most of the hexokinase and creatine kinase activity together with a high glutathione transferase activity. This fraction was located between the activity peaks of outer-membrane (NADH cytochrome *c* reductase, rotenone insensitive) and inner-membrane (succinate dehydrogenase) marker enzymes. Comparable results were recently obtained in the liver [14], suggesting that the hexokinase containing fraction from brain mitochondria resembles the contact fraction obtained from liver mitochondria. The specific composition of the contact fraction was, furthermore, verified by immuno gold labelling of bound hexokinase. This increased the density of hexokinase but upon recentrifugation of the contacts on a second density gradient the activity of the enzymes representative for inner and outer

membrane fragments as well as creatine kinase became not separated from the activity of hexokinase. These results strongly support the assumption of a complex between hexokinase and the two boundary membranes and furthermore exclude hexokinase binding to synaptosomal and microsomal contaminants (Fig. 5). In the latter case, inner and outer membrane would have remained on top of the second gradient. A significant amount of hexokinase was always present in the outer membrane fraction, which is not astonishing because hexokinase binds also to the pore protein in the isolated outer membrane but with a lower capacity [9,14]. This localization of hexokinase in the contact zones and to some extent also in the pure outer-membrane fraction explains the results of Vallejo et al. [21], who located the enzyme at the outer side of the inner membrane; on the other hand, it agrees with the results of Craven et al. [20] who located the enzyme at the outer membrane.

The electron-microscopic characterization (Fig. 7B) of the contact fraction revealed a slight contamination by synaptosomes. However, these synaptosomes did not contain mitochondrial structures, suggesting that this contaminant could not account for the high hexokinase and creatine kinase activity present in the contacts. The double-walled vesicle structure, relatively frequent in the contact fraction, resembled that of the liver, where proteolytic analysis and freeze fracturing served to assume an inner-membrane vesicle inside a right-side-out outer-membrane vesicle [14].

Metabolic functions of kinases in the contact sites

The preferential binding of hexokinase in the contact sites of liver and brain mitochondria suggests that these sites play a principal role in coupling of peripheral kinases to the adenine nucleotide translocator [13]. Yet creatine kinase was also located in the contacts although the enzyme is known to bind to cardiolipin at the surface of the inner membrane [43]. This fact points to the importance of the outer membrane for the regulation of the mitochondrial creatine kinase as was previously postulated [44,45]. Based on the fact that the ionic conductivity of the pore is voltage dependent [8,9] a regulation of the pore conductivity by the inner-membrane potential may be taken into consideration. However, this would be possible exclusively inside the contact sites.

A functional coupling of hexokinase [3,4] and creatine kinase [6,45–48] to the translocator has been already suggested by several authors. It would increase the effective concentrations of adenine nucleotides near the target enzymes which is of special importance because the translocator has the same binding constants for ADP and ATP [49]. This coupling of kinases, therefore, provides a mechanism for the transfer of high energy phosphate from the mitochondrial compartment to the cytosol in the presence of a high phosphorylation potential outside the mitochondria, while maintaining highly activated oxidative phosphorylation.

Localization of the Ca^{2+} transport system in the contacts

When the Ca^{2+} binding of the different subfractions from the density gradient was studied all fractions appeared to have the same binding constant of $1.2 \cdot 10^{-7}$ M, which resembles that of the Ca^{2+} binding protein described earlier [50]. However, the highest capacity for Ca^{2+} binding was by all means present in the contact containing fraction (Fig. 6), suggesting that the Ca^{2+} binding protein was concentrated in this fraction. The Ca^{2+} binding protein appears to be involved in active and passive Ca^{2+} movements [50]. Thus the contact sites may be also responsible for Ca^{2+} import and export processes. Such close neighbourhood of hexokinase and Ca^{2+} binding protein posed the question whether both systems can interact with each other. Indeed, Ca^{2+} movements across the inner membrane were affected by hexokinase activity. The active Ca^{2+} uptake was reduced when hexokinase was desorbed but was activated when the enzyme was phosphorylating in the presence of glucose (Table II and Fig. 1). The passive efflux system became inhibited by glucose 6-phosphate when either produced in situ by hexokinase activity or when added to the test system (Table II and Fig. 1). Both effects of the hexokinase activity, the activation of uptake and inhibition of efflux would serve to increase the intramitochondrial Ca^{2+} level. This may be of regulatory importance because Ca^{2+} activates the pyruvate dehydrogenase, NAD-specific isocitrate

dehydrogenase, and ketoglutarate dehydrogenase in mammalian mitochondria [51]. A feed-forward activation of citric acid cycle activity might, thus, occur by hexokinase activity via elevation of intramitochondrial Ca^{2+}. On the other hand a decrease of glucose 6-phosphate would liberate Ca^{2+} from the mitochondria which could increase glycogenolysis (although of limited importance in brain) via activation of phosphorylase kinase [52]. So far this possible regulation of Ca^{2+} uptake by hexokinase activity has not been demonstrated in mitochondria other than brain.

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