Glucose Catabolism in Brain

INTRACELLULAR LOCALIZATION OF HEXOKINASE*

David M. Parry‡ and Peter L. Pedersen§
From the Department of Biological Chemistry, Laboratory for Molecular and Cellular Bioenergetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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A major energy source in brain is glucose, which is committed to metabolism by hexokinase (Type I isozyme), an enzyme usually considered to be bound to the outer mitochondrial membrane. In this study, the subcellular location of hexokinase in brain has been rigorously investigated.

Mitochondrial fractions containing hexokinase (>500 milliunits/mg protein) were prepared by two different procedures, and then subjected to density gradient centrifugation before and after loading with barium phosphate, a technique designed to increase the density of the mitochondria. The gradient distribution patterns of both unloaded and loaded preparations show that brain hexokinase does not distribute exclusively with mitochondrial marker enzymes. This is particularly evident in the loaded preparations where there is a clear distinction between the peak activities of hexokinase and mitochondrial markers. The same observation was made when the mitochondrial fraction of either untreated or barium phosphate-loaded mitochondria was subjected to titration with digitonin. In fact, at concentrations of digitonin, which almost completely solubilize marker enzymes for both the inner and outer mitochondrial membranes, a significant fraction of the total hexokinase remains particulate bound. Electron microscopy confirmed that particulate material is still present under these conditions.

Significantly, hexokinase is released from particulate material only at high concentrations of digitonin which solubilize the associated microsomal marker NADPH-cytochrome c reductase. Glucose 6-phosphate, which is known to release hexokinase from the brain "mitochondrial fraction" also releases hexokinase from this unidentified particulate component.

These results on brain, a normal glucose utilizing tissue, differ from those obtained previously on highly glycolytic tumor cells where identical subfractionation procedures revealed a strictly outer mitochondrial membrane location for particulate hexokinase (Parry, D. M., and Pedersen, P. L. (1953) J. Biol. Chem. 258, 10904-10912). It is concluded that in brain, hexokinase has a greater propensity to localize at nonmitochondrial receptor sites than to those known to be associated with the outer mitochondrial membrane.

Past work in this and other laboratories has shown that the hexokinase activity of highly glycolytic cancer cells is (a) markedly elevated (1, 2), (b) less resistant to glucose 6-phosphate inhibition (3), and (c) bound in large amounts (50-90%) to the mitochondrial fraction (3-6) where it has preferred access to ATP generated by oxidative phosphorylation (7). Rigorous subfractionation studies, including digitonin fractionation and density gradient centrifugation, before and after loading the mitochondria with barium phosphate (4-6) have shown that hexokinase activity associated with the tumor mitochondrial fraction is indeed bound to the mitochondria and is not bound to contaminating membranes. Additional studies have shown that tumor hexokinase is bound to the outer mitochondrial membrane (3-5) where the protein "porin" appears to constitute at least one part of the receptor complex (8).

In contrast to highly glycolytic tumor cells, the hexokinase activity of normal tissues is quite low (1, 2). Subfractionation studies conducted in this laboratory on kidney (9) and liver (6) show that the particulate form of hexokinase distributes with microsomal markers rather than mitochondrial markers. These results on normal tissues differ from many reports on brain (for a review see Ref. 10) where the particulate location of the Type I enzyme is usually regarded as exclusively mitochondrial. However, evidence attributing this particulate activity to a mitochondrial location has not been rigorous. In fact, in submitochondrial fractionation studies with digitonin, only a few concentrations of digitonin were used (11) or the release of microsomal markers was not documented (12, 13). Although somewhat convincing, the immunochemical approach (13) has not been able to distinguish between hexokinase bound to mitochondria and that bound to closely associated structures (i.e. microsomes or cytoskeletal proteins). Finally, localization of hexokinase based solely on density gradient centrifugation has not eliminated all contaminating particles (14). Certainly, purified brain hexokinase has the capacity to bind to the outer mitochondrial membrane of rat liver mitochondria (15), but these findings alone cannot be taken as evidence for the enzyme's subcellular location in its tissue of origin.

As past work in this laboratory has revealed striking differences between the particulate distribution of hexokinase in normal and transformed tissues (5, 9), it seemed important to examine rigorously the distribution of this important enzyme in brain which, similar to tumor cells, utilizes glucose as a major fuel source.

EXPERIMENTAL PROCEDURES

Materials

Male CD albino rats (obtained from Charles River Breeding Laboratories, Wilmington, MA) were used in all experiments. The

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‡ Present address: Dept. of Clinical Chemistry, St. Boniface Hospital, 499 Tache Ave., Winnipeg, Manitoba R2H 2A6, Canada.

§ To whom correspondence should be addressed.
animals were fed Charles River RMH 1000 rat chow (Agway, Inc., Swarbucks, NY) and given water, ad libitum. Sucrose was purchased from J. T. Baker Chemical Co, and digitonin from Behring Diagnostics. Bovine serum albumin was fatty acid-free fraction V from Sigma. Substrates, cofactors ancillary enzymes, and inhibitors were obtained from Sigma or Boehringer Mannheim. All other chemicals were of analytical grade.

**Methods**

**Preparation of Mitochondria—**Animals were killed by cervical dislocation. Skin was cut away from the skull and reflected forward. The skull was separated from the supraspinatus was inserted into the foramen and the skull cut along its lateral sutures to completely expose the brain. The entire brain was scooped from the calvarium with a chilled spatula and placed into (unless otherwise indicated) ice-cold isolation medium (H-medium) containing 0.2 M d-mannitol, 0.07 M sucrose, 5 mM Hepes, pH 7.4, and 0.1% BSA. The composition of the isolation medium varied according to experimental design. The entire procedure of killing the animal and removal of brain tissue was accomplished within 10 s.

The combined collection of brain tissue (from several rats) was washed with isolation medium, weighed, cut into small pieces with scissors, and then homogenized in a Potter-Elvehjem glass homogenizer. The homogenate was then filtered through a Teflon filter (clearance 0.25 mm) at about 1500 rpm in 10 volumes of isolation medium.

Two procedures were employed for the preparation of mitochondria. Mitochondrial preparation I was obtained by the following procedure based on the procedure described by Ozawa et al. (16). The brain tissue homogenate (as obtained above) was centrifuged at 600 $\times$ g for 5 min and the resulting supernatant carefully decanted and saved. The pellet was resuspended in isolation medium and homogenized again in a Potter-Elvehjem glass homogenizer with a “tight” fitting Teflon pestle (clearance, 0.1 mm) at about 1500 rpm in about half the initial volume of isolation medium. After centrifugation of the resuspended pellet at 600 $\times$ g for 8 min, the resulting pellet was discarded and the supernatant mixed with the previous supernatant and centrifuged at 10,000 $\times$ g for 10 min. The pellet was saved on ice and the decanted supernatant centrifuged again at 10,000 $\times$ g for 10 min. The supernatant was discarded and the pellet resuspended along with the previous pellet in 40 ml of isolation medium and centrifuged at 10,000 $\times$ g for 10 min. The supernatant was discarded and the pellet washed several times with isolation medium to remove the loosely packed white layer on top of the tightly packed brown layer.

The washes were discarded and the pellet resuspended in about 40 ml of isolation medium and centrifuged at 600 $\times$ g for 8 min. The pellet was discarded and the decanted supernatant centrifuged at 10,000 $\times$ g for 10 min. The resulting supernatant was discarded, and the medium resuspended again in 40 ml of isolation medium and the remaining pellet resuspended in the final appropriate volume of isolation medium.

Mitochondrial preparation II was obtained by the procedure described by Clark and Nicklas (17) except that the isolation medium employed contained 0.21 M d-mannitol, 0.07 M sucrose, 5 mM Hepes, pH 7.4, 0.5 mM EDTA and 0.1% BSA (unless otherwise indicated). This method utilizes a discontinuous Ficoll density gradient procedure based on the procedure described by Clark and Nicklas (17) except that the isolation medium varied according to experimental design.

**Density Gradient Centrifugation—**Step and linear gradients were made with sucrose solutions. Centrifugation was performed at 2-5 °C using a Beckman SW 65.1 rotor in a Sorval OTD-50 ultracentrifuge. Details of centrifugation and gradients are given in the legends to tables and figures. Fractions were collected through a hole in the bottom of the centrifuge tube.

**Loading Mitochondria with Barium Phosphate—**Mitochondrial preparation II was incubated at a protein concentration of 1mg/ml for 10 min at 50 °C under the following conditions. For submaximal loading of mitochondria, the incubation medium contained 200 mM mannitol, 5 mM succinic acid, 2 mM KHPO, 1 mM ATP (Tris salt), and 0.5 mM BaCl2 adjusted to pH 7.4 with Tris. The incubation medium for maximal loading of mitochondria contained 250 mM sucrose, 10 mM Hepes, 10 mM succinate, 4 mM KHPO, 3 mM BaCl2, 1.5 mM ATP (dipotassium salt) adjusted to pH 7.4 with KOH. Immediately after incubation, the suspension was chilled on ice for 4 min and then centrifuged at 20,000 $\times$ g for 4 min. The pellet was gently resuspended in 0.25 M sucrose (plus 0.1 mM EGTA after submaximal loading) and fractionated by density gradient centrifugation.

**Treatment with Digitonin—**The mitochondrial preparations were incubated with increasing concentrations of digitonin in the isotonic media and at the protein concentrations described in the figure legends. Incubation was on ice for 15 min with constant stirring, immediately after which the digitonized suspensions were diluted 10-fold with the same isotonic medium (but without digitonin). The diluted suspensions were homogenized gently with three strokes of a Teflon pestle (manually) in a small glass homogenizer. Aliquots were removed and centrifuged at 20,000 $\times$ g for 15 min. The resulting pellets were resuspended in 2 ml of isolation medium and assayed for the enzymes remaining. Mitochondrial preparations subjected to the same treatment but in the absence of digitonin served as controls.

**Treatment with Glucose 6-Phosphate—**Prior digitonin treatment of the mitochondrial preparation was at digitonin concentrations of 0.2 and 1.2 mg/ml for 15 min on ice. The digitonized mitochondrial preparation was centrifuged at 20,000 $\times$ g for 15 min and the pellets resuspended in medium containing 0.21 M d-mannitol, 0.07 M sucrose, 5 mM Hepes, pH 7.4, 0.5 mM EDTA, and 0.1% w/v bovine serum albumin. Subsequent treatment was carried out by incubation with 2 mM glucose 6-phosphate at 30 °C for 10 min, followed by centrifugation at 20,000 $\times$ g for 15 min. The pellets were resuspended and either untreated (first treatment) or incubated again with glucose 6-phosphate for up to two additional consecutive times, centrifuging and resuspending after each incubation (second and third treatments). A mitochondrial preparation subjected to the same treatment but in the absence of glucose 6-phosphate served as controls.

**Assays—**Lactate dehydrogenase was assayed as described previously (5). All other enzyme assays were according to the methods reported elsewhere (5, 9). One milliunit is defined as the number of nanomoles of substrate converted to product in 1 min. The locations of the marker enzymes employed are generally well established (18, 19).

**Brain Hexokinase—**Mitochondrial suspensions, obtained after incubation of mitochondrial preparation II without and with different concentrations of digitonin, were processed for electron microscopy as described by Pedersen et al. (21). Briefly, the particulate components were fixed with 2% glutaraldehyde followed by 2% osmium tetroxide. After dehydration, the specimens were embedded in an epoxy-based resin mixture, sectioned, and post-stained with uranyl acetate and lead citrate. Sections were examined in a Zeiss EM 10 electron microscope.

**RESULTS**

**Comparison of Mitochondrial Preparations I and II—**Two procedures were employed to isolate brain mitochondria. The major difference between the two procedures as emphasized in detail under “Experimental Procedures” is the use of density gradient centrifugation in mitochondrial preparation II. Consistent with a number of previous studies on brain hexokinase (For a review see Ref. 10), it can be seen in Table I that both methods result in a “mitochondrial fraction” which contains significant amounts of hexokinase. The values obtained of 520 milliunits/mg for preparation I and 679 milliunits/mg for preparation II are considerably higher than that characteristic of the mitochondrial fractions of other normal tissues like liver and kidney which exhibit values of ~1 and 43 milliunits/mg, respectively (6, 9). Although preparation II, relative to preparation I, is somewhat enriched in both hexokinase and the mitochondrial marker enzymes, monoamine oxidase and succinate-cytochrome c reductase, it is important to note that this preparation still contains significant contamination of the mitochondrial fraction with synaptosomes. It seems clear from these studies that commonly used...
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Specific activities of hexokinase and marker enzymes in mitochondrial preparations I and II

Mitochondria were prepared by two methods. Preparation I was obtained by differential centrifugation only, while preparation II involved the use of density gradient centrifugation on a discontinuous Ficoll gradient, both as described under "Experimental Procedures." Specific activities are expressed as means with the number of values in parentheses.

| Enzyme assayed          | Mitochondrial preparation |
|-------------------------|--------------------------|
|                         | I                        | II                       |
| Hexokinase              | 520.6 (4)                | 679.1 (5)                |
| Mitochondrial enzymes   |                          |                          |
| Monoamine oxidase       | 5.3 (3)                  | 8.5 (4)                  |
| Succinate-cytochrome c reductase | 166.7 (2) | 281.3 (2) |
| Microsomal enzyme       |                          |                          |
| NADPH-cytochrome c reductase | 4.3 (2)     | 2.8 (3)                  |
| Cytosolic enzyme        |                          |                          |
| Lactate dehydrogenase   | 645.8 (3)                | 435.5 (3)                |

TABLE II

Latency of hexokinase in mitochondrial preparation I

Hypotonic treatment was performed by centrifuging mitochondrial preparation I (3.8 mg/ml) at 5000 × g for 10 min and resuspending the pellet in water at the initial volume. After incubating on ice for 10 min, the mitochondrial suspension was diluted 8-fold with H-medium, centrifuged at 5000 × g for 10 min and then resuspended in H-medium at the initial volume. A control was treated identically except that H-medium was used in place of water. Detergent treatments were performed by incubating mitochondrial preparation I on ice for 15 min with different detergents. Controls were incubated in the absence of detergent. Hexokinase was assayed under isotonic conditions.

| Mitochondrial preparation I | % increase in hexokinase activity |
|-----------------------------|----------------------------------|
| Water                       | 58.4                             |
| Tween 90 (5%)               | 107.1                            |
| Triton X-100 (0.5%)         | 115.2                            |
| Lubrol (1 mg/ml)            | 110.0                            |

Preparation schemes for brain mitochondria, whether based solely on differential centrifugation (preparation I) or on both differential centrifugation and density gradient centrifugation (preparation II) are not of sufficient purity to definitively ascribe the localization of a given enzyme to the mitochondria per se. Specifically, as it concerns the localization of hexokinase, these studies indicate that microsomes, mitochondria, and synaptosomes are all potential localization sites.

Latency of Hexokinase Associated with the "Mitochondrial Fraction"—To test for latency, the hexokinase activity of mitochondrial preparation I was assayed in the presence of 0.23 M sucrose. About 50% of the total activity present is assasayable under this condition. The latency of hexokinase in brain mitochondrial preparations has been attributed to its entrapment in synaptosomes or pinched-off nerve endings formed during tissue disruption. Assay of total hexokinase activity requires pretreatment of the mitochondrial preparation with membrane-disrupting techniques. Hypotonic treatment (i.e., osmotic shock) performed as described in the legend to Table II, exposed about 60% of the latent hexokinase activity present. Pretreatment of the mitochondrial preparation separately with three different detergents increased assayable activity slightly over 100%. These findings indicate that synaptosomes or pinched-off nerve endings containing entrapped hexokinase are present in the brain mitochondrial fraction.

Particulate Location of Hexokinase Associated with the Mitochondrial Fraction—The particulate association of brain hexokinase within the mitochondrial fraction was investigated initially by subjecting preparation II to two sucrose gradient centrifugation procedures, based on those designed by Whittaker and Barker (22) for the subcellular fractionation of brain tissue. In the first procedure, a two-step sucrose gradient was used to resolve the mitochondrial preparation into a mitochondrially enriched fraction and into two other fractions enriched in the contaminants, synaptosomes and myelin. The percent distribution of hexokinase and lactate dehydrogenase (a cytoplasmic marker) in each of these fractions is reported in Table III. As expected, the proportion of lactate dehydrogenase is highest in the contaminating synaptosomal fraction. Hexokinase, on the other hand, is distributed in approximately equal proportions in the mitochondrial and synaptosomal fractions. An increase in the hexokinase to lactate dehydrogenase ratio in the mitochondrial fraction emphasizes that synaptosomal contamination alone cannot account for the particulate association of brain hexokinase in the mitochondrial preparation.

In the second procedure, a linear sucrose gradient was employed in an attempt to better resolve mitochondrial preparation I. As seen in Fig. 1, the distribution of hexokinase in the resultant fractions overlaps with that of mitochondria (monoamine oxidase and succinate-cytochrome c reductase), synaptosomes (lactate dehydrogenase), and microsomes (NADPH-cytochrome c reductase). It can be seen that almost 50% of the total hexokinase activity fails to sediment with the mitochondrial markers (compare Fig. 1, A and B) and the remaining activity overlaps significantly with the microsomal marker (compare Fig. 1, A and C). Also in Fig. 1, heterogeneity is observed in the distribution of the mitochondrial markers, an observation which is consistent with that reported by others (23–25).

Although the above results on mitochondrial preparations I and II show that a significant portion of the total hexokinase activity in brain does not distribute with mitochondrial markers, it cannot be concluded whether the remaining activity is of mitochondrial or nonmitochondrial origin.

Effect of Loading Mitochondria with Barium Phosphate on the Particulate Location of Hexokinase—To further assess to what extent mitochondria serve as a particulate locus for

| Enzyme assayed          | Myelin Synaptosomes Mitochondria |
|-------------------------|---------------------------------|---------------------------------|
| Hexokinase              | 4.4 50.2 45.4                   |
| Lactate dehydrogenase   | 19.4 64.8 29.8                  |
| Ratio                   | 0.4 0.8 2.0                     |

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Brain hexokinase, mitochondrial preparations were treated with barium phosphate immediately prior to density gradient centrifugation. Barium and phosphate are taken up by mitochondria when incubated, in vitro, in the presence of a metabolic energy source. Accumulation of these ions within the mitochondria in the presence of a saturating amount of barium (maximal loading).

Two experimental approaches were taken. One was to restrict mitochondrial enzymes, monoamine oxidase and succinate-cytochrome c reductase, to the more dense region of the gradient, it does not peak (i.e. in fraction 6) with the mitochondrial marker enzyme.

Reported in Table IV are the results obtained after maximal loading of mitochondria with barium phosphate. Mitochondria were loaded submaximally with barium phosphate as described under “Experimental Procedures.” Fractionation was carried out by layering 0.5 ml of the treated mitochondrial suspension on top of a gradient of 0.45 m steps of 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, and 2.5 m sucrose and centrifuged at 115,000 × g for 3 h. Following centrifugation, the entire gradient was removed and the remaining pellet resuspended in 1 ml of 0.25 m sucrose. Values in parentheses indicate percentage of the corresponding specific activities of the starting mitochondrial preparation. Results are typical of two experiments.

Table IV

| Enzyme assayed | Specific activity | % distribution |
|----------------|------------------|----------------|
|                | Gradient Pellet  | Gradient Pellet |
| Hexokinase     | 662.8 (101.6)    | 66.7 33.3      |
| Monooamine oxidase | 2.0 (27.0)     | 7.0 93.0       |
| Succinate-cytochrome c reductase | 212.1 (83.0) | 44.4 55.6 |
| NADPH-cytochrome c reductase | 1.4 (58.3) | 57.8 42.2 |
| Lactate dehydrogenase | 863.7 (218.1) | 97.6 2.4 |

Finding difficult to reconcile with a mitochondrial location for this enzyme. It is important to note also that 42.2% of the activity of the microsomal enzyme, NADPH-cytochrome c reductase was recovered in the pellet, and similar to hexokinase, its specific activity was not markedly altered.

Correlation of Hexokinase Release with Microsomal Release on Digitonin Fractionation of the Mitochondrial Preparations—At this point it seemed important to employ an alternative approach. Therefore, digitonin was used to fractionate the mitochondrial preparations. The results obtained with preparation I are illustrated in Fig. 3. As seen in panel A, synaptosomal membranes are relatively sensitive to disruption by digitonin as evidenced by the release of lactate dehydrogenase at the low digitonin concentration. The biphase response in the release of adenylate kinase probably represents the disruption of synaptosomes at low digitonin concentrations (releasing cytosolic adenylate kinase) and the disruption of the outer mitochondrial membrane at higher concentrations (releasing the adenylate kinase from the inter-
were obtained in the presence of EDTA and in the absence of
and D, in the presence of H-medium plus 1
plus 0.1% BSA and at a protein concentration of 2 mg/ml. Pan& C
preparation I was treated with increasing concentrations of digitonin,
following conditions. Panels A and B, in the presence of H-medium
as described under “Experimental Procedures,” in the presence of the
concentration of 0.15
protein concentration of 1.3 mg/ml. Panels E and F, in the presence
concentration of 1.4 mg/ml. The remaining particulate material was
assayed for adenylate kinase (A), lactate dehydrogenase (O, hexoki-
that no hexokinase is released on disruption of the synapto-
somal membrane (i.e. along with lactate dehydrogenase re-
release
of glucose B-phosphate. In panel B, the mitochondria were treated
with sufficient digitonin to remove also the outer membrane enzyme
loading with barium phosphate. The remaining particulate material was
assayed for adenylate kinase (A), lactate dehydrogenase (C), hexoki-
(\texttimes), monoamine oxidase (O), succinate cytochrome c reductase
(\Delta), NADH-cytochrome c reductase (∅), and NADPH-cytochrome c reductase (©). Results are expressed as percentage of activity re-
covered in the pellet from control.

membrane space). That hexokinase is highly resistant to
digonin solubilization, even after the outer mitochondrial
membrane enzyme monoamine oxidase has been almost com-
pletely released, is evident in panel R. (It will be noted also
that no hexokinase is released on disruption of the synapto-
somal membrane (i.e. along with lactate dehydrogenase re-
lease panels A and B) indicating that hexokinase within the
synaptosomes is particulate.)

To ensure that the resistance of hexokinase to solubilization
by digitonin is not simply a consequence of rebinding (after
initial solubilization) to newly exposed particulate binding
sites, digitonin fractionation was carried out also under
conditions which are unfavorable for hexokinase binding. The
digonin fractionation profiles in panels C and D of Fig. 3
were obtained in the presence of EDTA and in the absence of
BSA. For those in panels E and F, fractionation was carried
out in isotonic KCl containing EDTA. Only about 30% of the
hexokinase activity remains particulate bound in the presence
of isotonic KCl compared with the same material in the
presence of isosmotic sucrose/mannitol. Under both experi-
mental conditions, hexokinase was still highly resistant to
digonin solubilization. This response of hexokinase to
digonin fractionation appears to correlate best in both cases
with the fractionation pattern obtained for the microsomal
enzyme, NADPH-cytochrome c reductase (panels D and E).
Still apparent are the distinct dissociations between the
digonin fractionation profiles of hexokinase and all the mito-
chondrial marker enzymes. In fact, 70% of the hexokinase
activity was recovered in the pellet even though almost all of
the inner mitochondrial membrane enzyme succinate-cyto-
chrome c reductase had been released (Fig. 3, panels C and
E).

Digitonin fractionation of mitochondrial preparation II

![Fig. 3. Digitonin fractionation of mitochondrial prepara-
tion I under different experimental conditions. Mitochondrial
preparation I was treated with increasing concentrations of digitonin,
as described under “Experimental Procedures,” in the presence of the
following conditions. Panels A and B, in the presence of H-medium
plus 0.1% BSA and at a protein concentration of 2 mg/ml. Panels C
and D, in the presence of H-medium plus 1 mM EDTA and at a
protein concentration of 1.5 mg/ml. Panels E and F, in the presence
of 0.15 M KCl, 5 mM Hepes, and 0.5 mM EDTA and at a protein
concentration of 1.4 mg/ml. The remaining particulate material was
assayed for adenylate kinase (\textA), lactate dehydrogenase (\textC), hexoki-
(\texttimes), monoamine oxidase (\textO), succinate cytochrome c reductase
(\textDelta), NADH-cytochrome c reductase (∅), and NADPH-cytochrome c reductase (©). Results are expressed as percentage of activity re-
covered in the pellet from control.](http://www.jbc.org/)

![Fig. 4. Digitonin fractionation of mitochondrial prepara-
tion II before and after purification of the mitochondria by
loading with barium phosphate. Digitonin fractionation was car-
rried out, as described under “Experimental Procedures,” in the
presence of H-medium plus 0.1% BSA and 0.5 mM EDTA. Panels A and
B show fractionations of the mitochondrial preparation II at a protein
concentration of 1.5 mg/ml. Panels C and D show fractionation of
barium phosphate loaded mitochondria at a protein concentration of
3.8 mg/ml. Mitochondria were loaded maximally with barium phos-
te as described under “Experimental Procedures.” The remaining
particulate material was assayed for adenylate kinase (\textA), monoamine
oxidase (\textO), hexokinase (\texttimes) succinate-cytochrome c reductase (\textDelta),
NADPH-cytochrome c reductase (∅), and succinate dehydrogenase
(©). Results are expressed as percentage of activity recovered in the
pellet from control.](http://www.jbc.org/)

![Fig. 5. Release of hexokinase from mitochondrial prepara-
tion I by glucose 6-phosphate after treatment with digi-
tonin. In panel A, the mitochondrial preparation was pretreated with
a low concentration of digitonin (0.2 mg/mg protein) to first expose
that fraction of the total hexokinase enclosed within synaptosomes.
After centrifugation, the mitochondria were treated with glucose 6-
phosphate as described under “Experimental Procedures.” After glu-
cose 6-phosphate treatment, the particulate material was assayed for
hexokinase (\texttimes), monoamine oxidase (\textO), and succinate cytochrome c
reductase (\textDelta). Results are expressed as percentage of activity re-
covered in control mitochondria treated identically but in the absence
of glucose 6-phosphate. In panel B, the mitochondria were treated
with sufficient digitonin to remove also the outer membrane enzyme
monoamine oxidase, prior to glucose 6-phosphate treatment.](http://www.jbc.org/)
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**FIG. 6.** Electron micrographs of mitochondrial preparation II before and after treatment with digitonin. Panel A, mitochondrial preparation II before digitonin treatment. Panel B, mitochondrial preparation II after treatment with a low concentration of digitonin (0.2 mg/mg protein) followed by centrifugation to isolate the mitochondrial pellet. Panels C and D, treatment of mitochondrial preparation II with 1.4 and 2.0 mg digitonin/mg protein, respectively, followed by high speed centrifugation to isolate particulate material. In all cases, treatment with digitonin and subsequent centrifugation was carried out as described under “Experimental Procedures.” The pellets obtained after centrifuging the digitonin-treated mitochondrial preparation and a control were assayed for hexokinase and monoamine oxidase activity and processed for electron microscopy as described previously. The ratio of the percentage activity of hexokinase to that of monoamine oxidase increased from 1 in control preparation (panel A) to 10 in the digitonin treated preparation (panel B). Magnification is ×13,200.

Yielded similar results (Fig. 4, panels A and B) with hexokinase release correlating best with that of the microsomal enzyme. Even after further purification of the mitochondria (i.e. barium phosphate-loaded mitochondria) the release of hexokinase on digitonin fractionation still correlated better with the release of the microsomal enzyme than the mitochondrial enzymes (Fig. 4, panels C and D).

**Site Specificity of Particulate Hexokinase after Treatment with Digitonin**—Glucose 6-phosphate is known to solubilize particulate-bound hexokinase in a variety of tissues (4–6, 9). Failure of digitonin to solubilize hexokinase in the brain mitochondrial fraction described here might suggest that hexokinase had been released during cell fractionation procedures and then rebound very tightly to nonspecific sites. To test this possibility, the brain mitochondrial fraction was subjected to successive treatments with glucose 6-phosphate. Such treatments completely released bound hexokinase while leaving unaffected the binding of monoamine oxidase or succinate-cytochrome c reductase. When sufficient digitonin was used to first remove the outer membrane marker monoamine oxidase, glucose 6-phosphate addition still induced hexokinase release in soluble form (Fig. 5, panel B).

These results with glucose 6-phosphate render it unlikely that the resistance of hexokinase to digitonin solubilization (Figs. 3 and 4) is due to its release and nonspecific rebinding during cell fractionation.

**Electron Microscopic Analysis of the Brain Mitochondrial Fraction before and after Treatment with Digitonin**—Electron microscopy was used to examine the membrane disrupting effect of digitonin. Shown in panel A of Fig. 6 is an electron micrograph of mitochondrial preparation II. Evident are synaptosomes, some myelin material, and numerous mitochondria, the outer membranes of which are intact. The effects of digitonin can be observed in panels B–D. After incubation of the mitochondrial preparation with a low concentration of digitonin (0.20 mg/mg protein), most synaptosomes were ruptured and the myelin material somewhat disrupted, but mitochondria remained intact (panel B). At higher digitonin concentrations (1.4 and 2.0 mg/mg protein), essentially all structures are severely disrupted leaving membrane fragments which can still be pelleted at 12,000 × g (panels C and D). It is these fragments which retain substantial hexokinase activity but essentially no monoamine oxidase activity.
RESULTS

Results presented in this paper show that the mitochondrial fraction of brain, whether prepared by a differential centrifugation or a density gradient procedure, is highly enriched in hexokinase activity. However, in contrast to the very high hexokinase activity found in the mitochondrial fraction of tumor cells (6, 7), the hexokinase activity associated with the mitochondrial fraction of brain could not be shown to distribute strictly as a mitochondrially localized enzyme. This was demonstrated in five different types of experiments: 1) by density gradient centrifugation in sucrose (Fig. 1); 2) by submaximally loading the mitochondrial preparation with barium phosphate followed by density gradient centrifugation (Fig. 2); 3) by maximally loading the mitochondrial preparation with barium phosphate and sedimenting the mitochondria (Table IV); 4) by subjecting the untreated mitochondrial preparation to digitonin subfractionation (Fig. 3); and 5) by subjecting the barium phosphate-loaded mitochondrial preparation to digitonin subfractionation (Fig. 4). In fact, in the latter three types of experiments the hexokinase activity correlated most closely with that of the microsomal marker NADPH-cytochrome c reductase.

These observations extend earlier findings from this laboratory which show that particulate hexokinase found in the mitochondrial fractions of liver and kidney subfractionate with microsomal marker enzymes rather than with mitochondrial markers (6, 9). It now seems highly unlikely that the correlation of hexokinase with microsomal markers is fortuitous or that hexokinase in all three normal tissues is localized on a select population of mitochondria that is more resistant to digitonin solubilization than the mitochondria containing the marker enzymes assayed. It is interesting to compare results obtained here with those reported by Dobrani et al. (12) in which digitonin fractionation was used also to monitor hexokinase release from brain mitochondria. These workers concluded that hexokinase resides in brain mitochondria as a porin-hexokinase complex located in a cholesterol-free membrane domain together with inner membrane components. This conclusion was made despite the fact that hexokinase and porin followed different release patterns upon titration with digitonin and microsomal marker enzyme release was not documented (12). Also, experiments were not carried out to establish whether hexokinase becomes enriched in the heavy mitochondrial fraction after preloading the mitochondria with barium phosphate. We show in the present study that it does not (Table IV).

Microsomal marker enzyme release was also not monitored in the recent studies of Kottke et al. (13), where the conclusion that hexokinase resides at contact sites between inner and outer mitochondrial membranes was based largely upon an immunochromatographic approach. Unfortunately, the latter approach would not be able to distinguish between hexokinase bound to contaminating membrane fragments and contact sites.

It is well established that the outer mitochondrial membranes of both liver and hepatoma mitochondria contain a hexokinase receptor complex (8, 15) which may consist, in part, of the outer membrane voltage-dependent anion channel protein or "VDAC" (26, 27), more commonly referred to as porin. Porin is present also in brain mitochondria (12). Significantly, brain hexokinase, of which there is only one isozyme (Type I) (25), has been shown to readily bind to the hexokinase receptor of liver mitochondria (15). In fact, the latter receptor site was discovered in liver mitochondria by using brain hexokinase (15). With these thoughts in mind, we suggest that in brain and other normal tissues more than one receptor (or receptor complex) for hexokinase is present, one associated with the outer mitochondrial membrane, and one with a nonmitochondrial component (membrane or membrane-cytoskeletal element). It seems clear from experiments described in this study that the Type I hexokinase in brain has a greater affinity for the nonmitochondrial receptor than the receptor associated with the outer mitochondrial membrane. In highly glycolytic tumor cells where hexokinase subfractionates with mitochondria rather than nonmitochondrial contaminants (3, 6), we suggest that either the nonmitochondrial receptor is absent or structurally altered. Consequently, hexokinase binds in tumor cells only to the mitochondrial receptor. An alternative explanation is that in tumor cells the Type II isozyme that predominates (28) may have a greater affinity for the mitochondrial than the nonmitochondrial receptor.

As yet, we have not identified the nonmitochondrial component which appears to be the preferential site of much of the hexokinase activity which is found bound in normal tissues. The simplest explanation is that hexokinase is bound to a cellular membrane or a membrane-cytoskeletal network which in turn sediments with the mitochondria. Significantly, the N-terminal region of the Type I brain enzyme has been shown by Polakis and Wilson (29) to consist of mostly hydrophobic amino acids. Also, there have been several reports that some glycolytic enzymes of brain do interact with actin, (30, 31) and other reports suggesting that actin interacts with mitochondria (32).

Further experiments are required to identify the nonmitochondrial component of normal tissues involved in hexokinase binding.

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