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Brain Hexokinase: Immunohistochemical Localization at the Light Microscopic Level*

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ABSTRACT: The indirect fluorescent antibody technique was used to localize hexokinase at the light microscopic level. Antibodies were produced in rabbits to soluble bovine brain hexokinase. Evidence of purity was obtained from agar gel diffusion and the precipitin test. The antiserum completely inhibited soluble hexokinase but only partially (60–70%) inhibited the activity associated with intact mitochondria or with the outer membrane fraction of mitochondria. When these antibodies were used to demonstrate the presence of hexokinase in thin sections of brain cortex, bright fluorescent granules were observed in the cytoplasm of nerve cells and in the intervening tissue. These corresponded in size and distribution to mitochondria demonstrated either by the succinate-dependent reduction of tetranitro blue tetrazolium or a classical supravital staining method. An unsuccessful attempt was made to confirm these observations in the electron microscope using the peroxidase-conjugated antibody technique.

Although it is generally believed that mitochondria contain the enzymes necessary to oxidize pyruvate completely to carbon dioxide and water, the location of the glycolytic enzymes has been the subject of many conflicting reports. In 1956, Gallagher *et al.* reported that brain mitochondria, unlike those isolated from other sources, possessed the ability to oxidize glucose completely to carbon dioxide and water. On the other hand, the preparations of Brody and Bain (1952) and Aldridge (1957), did not possess this ability. The subsequent preparation of brain mitochondria which are relatively free of contaminating microsomes, nerve ending particles, and glycolytic capacity has led to the conclusion that glycolysis is probably not a function of brain mitochondria (Løvtrop and Svennerholm, 1963; Beattie *et al.*, 1964; Ruscak *et al.*, 1967). Nevertheless, the work of Crane and Sols (1953) and Johnson (1960) has revealed that 75–90% of the hexokinase activity of brain homogenates is sedimented with the mitochondrial fraction, whereas the other glycolytic enzymes are present in the soluble fraction. Basford *et al.* (1964) have shown that the addition of mitochondria to the supernatant fraction of brain homogenates results in a severalfold stimulation of glycolytic activity which can be duplicated by the addition of yeast hexokinase alone. Furthermore, direct assay revealed that both hexokinase and phosphofructokinase were concentrated with the mitochondria. The association of both of the ATP-requiring enzymes of glycolysis with the mitochondria might be a way of allowing more efficient integration of ATP production and glucose utilization. This type of mechanism would be particularly important in brain where the major source of energy is supplied by blood glucose.

The uncertainty of the results obtained from studies involving the differential centrifugation of brain homogenates has prompted this investigation into the *in situ* location of hexokinase at the light microscopic level in thin sections of brain cortex. In the following paper (Craven *et al.*, 1969), evidence for the association of hexokinase with the outer mitochondrial membrane will be presented.

Materials

The following materials were obtained commercially: glucose 6-phosphate dehydrogenase (type 5), ATP, TPN^+, and EDTA from Sigma Chemical Co., St. Louis, Mo.; Sephadex gels from Pharmacia, Uppsala, Sweden; DEAE-cellulose (Cellex D) from Bio-Rad Laboratories, Richmond, Calif.; tetranitro-BT[1] from Nutritional Biochemicals Corp., Cleveland, Ohio; bovine serum albumin from Armour Pharmaceutical Co., Kankakee, Ill.; Freund's complete adjuvant and fluorescein isothiocyanate from Baltimore Biological Laboratories, Baltimore, Md.; Ouchterlony double-diffusion plates, pattern B from Hyland Laboratories, Los Angeles, Calif.; and Tris from General Biochemicals, Chagrin Falls, Ohio. The sheep antirabbit 7S γ-globulin was the generous gift of Dr.

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† The data presented are taken from the dissertation of P. A. C. offered in partial fullfillment of the requirements for the Ph.D. degree.

[1] Abbreviation used is: tetranitro-BT, tetranitro blue tetrazolium.

Stuart Sell. All other reagents of analytical grade were obtained from Fisher Scientific Co., Pittsburgh, Pa.

Methods

Hexokinase activity was measured at room temperature in a system in which glucose 6-phosphate production was coupled to TPNH formation in the presence of glucose 6-phosphate dehydrogenase. The procedure used is described by Schwartz and Basford (1967). Enzyme samples were diluted in 1% (w/v) bovine serum albumin unless otherwise stated. One unit of enzyme activity is defined as the formation of 1 μmole of glucose 6-phosphate/min under the above conditions.

Pure soluble beef brain hexokinase was prepared by the method of Schwartz and Basford (1967). It had a specific activity of 80 units/mg.

Protein was determined by the biuret method of Gornall *et al.* (1949). Particulate samples were solubilized by the addition of sodium deoxycholate.

Preparation of Antiserum. New Zealand white rabbits were immunized with a total of 5 mg each of soluble bovine brain hexokinase in Freund's complete adjuvant. The first injections were given in each of two rear footpads; 1 week later two injections were given in each of the two rear thighs. The animals were bled 3 weeks later from the marginal ear vein and the antiserum was stored in small aliquots at $-20°$.

Purification of Antiserum. DEAE-cellulose columns were prepared according to the method of Peterson and Sober (1962). A volume of 20 ml of serum could be fractionated on a 2×10 cm column which had previously been equilibrated with 0.0175 M phosphate buffer (pH 6.3) (Levy and Sober, 1960). The 7S γ-globulin is not adsorbed to the column and is eluted by further washing with the phosphate buffer. Alternatively 0.01 M potassium phosphate buffer (pH 7.6) (Tomasi and Kunkel, 1964) could be used and was found to be more reliable. The phosphate buffers were prepared by the method of mole fractions according to the table given by Green and Hughes (1955).

Preparation of Conjugated Antibody. The procedure used was the same as that described by Nairn (1964). The ratio of fluorochrome to protein was 0.05. After dialysis against phosphate-buffered saline (pH 7.2), the conjugate was washed through a 2×40 cm Sephadex G-25 column which had previously been equilibrated with the buffered saline. This procedure successfully removed unconjugated fluorochrome.

Purification of Conjugated Antibody. A homogenate of brain cortex containing 25 g of tissue in 70 ml of 0.1 M phosphate buffer (pH 6.8) was lyophilized in a Thermovac freeze-drying apparatus. For adsorption of antiserum, 100 mg of the dry material was mixed with 1 ml of antiserum for 2–3 hr. The adsorbent was removed by centrifugation at 2130*g* for 15 min (Servall SS34 rotor)[2] and washed once with phosphate-buffered saline, pH 7.2.

Preparation of Tissue for Fluorescent Microscopy. Cryostat sections were cut on an International-Harris Model CT cryostat. Small cubes of cortex, 2–5 mm on each side, were frozen to the cryostat chuck in Dry Ice at $-79°$. Sections were cut at $-15°$ and between 6 and 12 μ thick. They were picked up on a cold microscope slide and allowed to air dry for 30 min to assure firm adherence of the section. The slides were immersed in cold 95% ethanol for 10 min, air dried, and rehydrated with a drop of phosphate-buffered saline (pH 7.2). They were incubated for 30 min in a moist atmosphere at room temperature in antihexokinase 7S γ-globulin and then in fluorescein-labeled antirabbit γ-globulin. After each incubation they were washed ten to twelve times in buffered saline. The sections were mounted in 25% phosphate-buffered glycerin (pH 8.0) and examined in a Leitz Ortholux fluorescence microscope. Photographs were taken with Kodak, Tri X Pan film.

Tetranitro-BT-Staining Technique Small pieces of cortex, 2–3 mm on each side, were prefixed in graded formaldehyde in Hank's basic salt solution for 2–4 min each. The concentrations of formaldehyde used were 0.7, 1.3, and 2.0%. The tissue was cut in the cryostat (4 μ thick) and transferred without thawing to the incubation mixture at 37° containing 10 mg of tetranitro-BT and 1 mmole of succinate in 20 ml of 0.5 M phosphate buffer (pH 7.6). This procedure has been described previously by Walker and Seligman (1963). Photographs were taken with Kodak, Constant Process Ortho film.

The Supravital Staining of Mitochondria. Freshly obtained cubes of beef brain cortex were fixed for several days with Regaud's fixative and stained with Altmann's aniline acid fuchsin. The procedure followed is described by Lillie (1954).

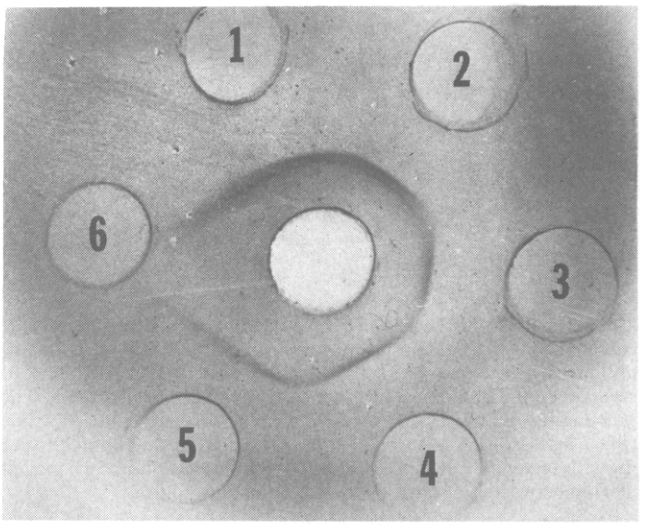

FIGURE 1: Ouchterlony double-diffusion plate. The inner well contains pure soluble beef brain hexokinase. Five of the outer wells contain decreasing concentrations of antihexokinase serum. The sixth well is empty. The plate was stained with naphthalene black.

Results

Properties of the Antiserum to Hexokinase. The serum of rabbits injected with pure soluble beef brain hexokinase was found to contain antibodies to hexokinase. The appearance of a single precipitin band in an Ouchterlony double-diffusion experiment indicates the existence of only one antigen–antibody system (Figure 1). When a crude soluble hexokinase preparation was allowed to diffuse against the antiserum, there was similarly just one precipitin band formed (not shown). Figure 2 shows the results of a precipitin test on the antiserum. The presence of only one sharp absorption peak in a precipitin

[2] All centrifugal forces are given as the average gravitational force. For calculation using the SS34 rotor, a radius of 3 in. was used.

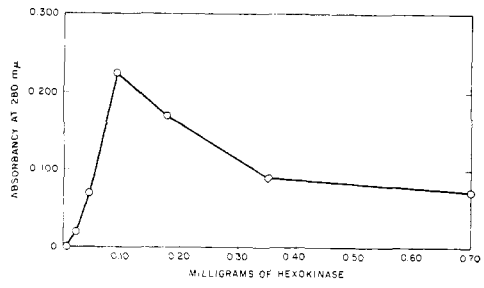

FIGURE 2: Precipitin test. Increasing concentrations of pure soluble beef brain hexokinase in 0.5 ml were added to 0.5-ml portions of antihexokinase or normal rabbit serum. The tubes were allowed to stand at room temperature for 2 hr and then at 0–4° overnight. The precipitates were washed three times in phosphate-buffered saline (pH 7.2), dissolved in 0.5 ml of sodium hydroxide, and diluted to 1.5 ml with water. The curve represents the difference in absorbancy at 280 mμ between the control and experimental solutions.

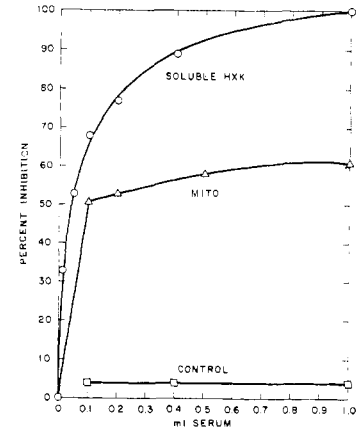

FIGURE 3: Comparison of the inhibition of pure soluble beef brain hexokinase and intact mitochondria by antiserum to hexokinase. The control indicates the per cent inhibition observed when normal rabbit serum was added to soluble hexokinase. The serum was preincubated with the enzyme sample, diluted in 1% bovine serum albumin, for 15 min before addition to the standard assay cuvet.

test again suggests the purity of the antigen–antibody system. At equivalence, there were 0.18 mg of hexokinase and 0.33 mg of antibody in the precipitate. The amount of antibody in the precipitate was calculated by subtracting that fraction of the optical density reading due to the adsorption of the antigen. The extinction coefficient[3] of hexokinase at 280 mμ was assumed to be 5.53 (Schwartz and Basford, 1967), and that for γ-globulin, 13.8 (Misaka, 1966). If a molecular weight of 96,000 is assumed for hexokinase (Grossbard and Schimke, 1966), this corresponds to a 1:1 mole ratio of antigen to antibody.

The antiserum completely inhibited soluble hexokinase activity but only partially (60–70%) inhibited the activity associated with whole mitochondria (Figure 3). When normal rabbit serum was substituted for antiserum, there was no appreciable change in the reaction rate. When mitochondria were being examined for enzyme inhibition, it was essential first to dilute the sample for assay in 1% bovine serum albumin. This is due to the fact that serum albumin has an activating effect on mitochondrial hexokinase which is probably related to its ability to bind inhibitory fatty acids. This point will be discussed in the following paper.

It was determined by electrophoresis against a polyvalent antirabbit serum that the antibodies were in the 7S γ-globulin fraction of rabbit serum. Figure 4 shows the results obtained during a routine preparation of 7S γ-globulin on DEAE cellulose. The effluent was shown to contain only 7S γ-globulin by immunoelectrophoresis and retained its ability to inhibit hexokinase activity. The highly purified specific antibody was then used to localize hexokinase in thin sections of bovine brain cortex using the indirect fluorescent antibody technique. Fluorescein was conjugated to sheep antirabbit 7S γ-globulin. The fluorescent conjugate yielded only one band in an Ouchterlony double-diffusion plate when allowed to diffuse against whole rabbit serum. It was purified by adsorption with lyophilized brain cortex. One adsorption was sufficient to remove all the nonspecific fluorescence observed when the unpurified conjugate was incubated with thin sections of cortex.

Localization of Hexokinase at the Light Microscopic Level. Tissue was prepared as described under Methods. Thorough washing with ten to twelve changes of phosphate-buffered saline (pH 7.2) was necessary to remove most of the nonspecific fluorescence observed in control preparations. The controls consisted of incubation with the fluorescent conjugate alone, substitution of normal rabbit 7S γ-globulin for antihexokinase 7S γ-globulin, or a blocking experiment in which tissue is incubated with antihexokinase, unconjugated antirabbit γ-globulin, and then antirabbit γ-globulin conjugated to fluorescein. All the control slides had a diffuse fluorescence of lesser intensity than the experimental ones. The fluorescence observed in the blocking control was not as weak as the other controls because of some exchange of labeled and nonlabeled antirabbit γ-globulin. In a typical fluorescent micrograph showing the distribution of hexokinase in cerebral cortex, there are bright fluorescent granules in the cytoplasm of neuronal cell bodies and in the intervening tissue (Figure 5). Mitochondria were demonstrated in similar sections using the succinate-dependent tetranitro-BT-staining technique (Figure 6). The distribution of mitochondria is similar to the distribution of fluorescent granules in the previous photograph. When succinate was left out of the incubation mixture no staining was observed. In addition, cysteine-reduced tetranitro-BT did not adsorb to the tissue slice. Figure 7 demonstrates a similar distribution of mitochondria in similar sections of beef brain cortex fixed for several days in Regaud's fixative and stained with Altmann's aniline–acid fuchsin. The significance of the presence of granular staining outside of the cytoplasm of neurona lcell bodies is discussed below; however conclusions will be drawn only on the basis of the particulate staining found in the cytoplasm of well-defined cell bodies.

The Peroxide-Conjugated Antibody Technique. Since mitochondria are only just visible in the light microscope it seemed desirable to study the intracellular distribution of hexokinase at the electron microscopic level. The peroxidase-labeled antibody technique developed by Nakane and Pierce (1967) had several apparent advantages as compared with the ferritin-labeled antibody technique; however, in our hands it was unsuccessful. Several different procedures for freezing, fixing,

[3] The extinction coefficient is defined as the absorption of a 1% solution having a 1-cm light path.

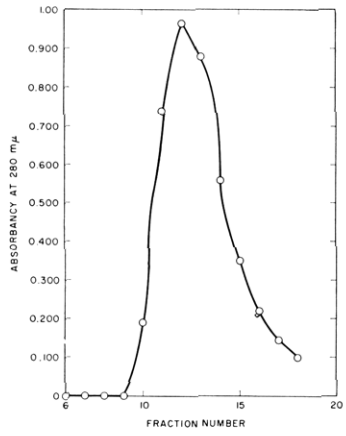

FIGURE 4: The elution pattern of rabbit 7S γ-globulin from DEAE-cellulose. The buffer used was 0.0175 M potassium phosphate (pH 6.3).

and washing the tissue were tried, none of which resulted in the penetration of labeled antibody and the removal of non-specifically adsorbed conjugate. In particular, glutaraldehyde-fixed tissue was unsuitable as it resulted in a very strong non-specific adsorption of peroxidase at concentrations as low as 2 mg of peroxidase/ml. The use of electrophoretically pure peroxidase, containing only the isoelectric component at pH 7, in the preparation of the conjugate, and the purification of the conjugate by DEAE-cellulose chromatography and tissue adsorption resulted in an over-all decrease in the intensity of the staining but did not result in a significant difference between the control and experimental preparations.

Discussion

The fact that antiserum only partially inhibited mitochondrial hexokinase activity could be interpreted in several ways. First, a portion of the enzyme could be inside the mitochondria and inaccessible to a large antibody molecule. However, as demonstrated in the following paper (Craven *et al.*, 1969),

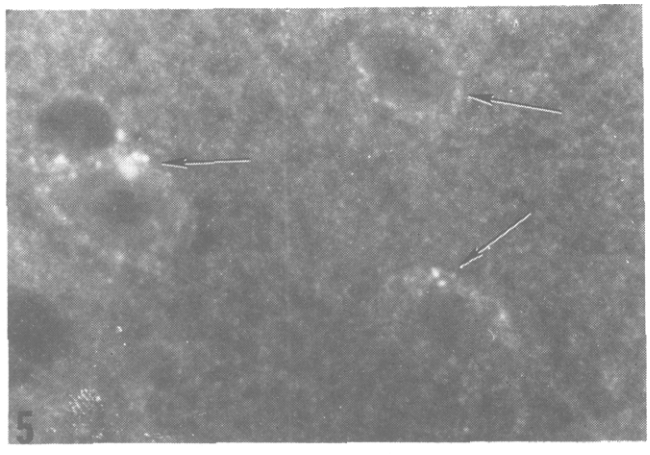

FIGURE 5: Fluorescent micrograph demonstrating the distribution of hexokinase in bovine brain cortex, 1500×. Bright fluorescent granules are present in the cytoplasm of nerve cell bodies (arrows) and in the intervening tissue.

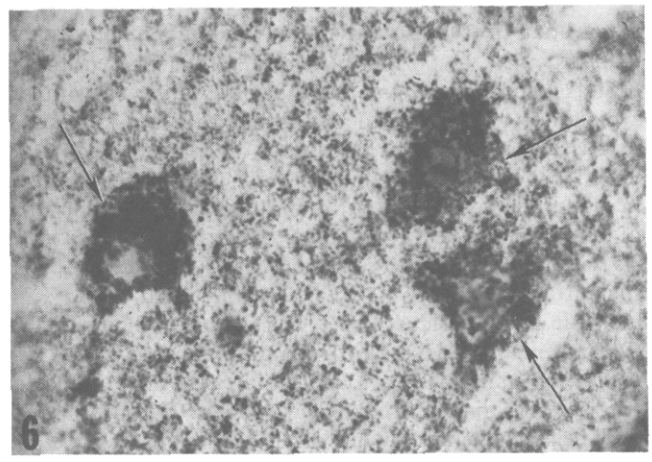

FIGURE 6: Photomicrograph of a section from a different cube of bovine brain cortex stained with succinate-dependent tetranitro-BT to demonstrate mitochondria, 1150 ×. The distribution of granules in the nerve cell bodies (arrows) and intervening tissue is the same as in Figure 5.

outer membranes prepared according to the procedure of Sottocasa *et al.* (1967) are still only 66% inhibitable. If the hypothesis were true, one would expect that the outer membrane would be completely inhibited by antiserum. The second possibility is that brain mitochondria might contain more than one antigenically distinct form of the enzyme and during the solubilization and purification of the enzyme only one of these isozymes is isolated, and the antiserum only reacts with this isozyme. Katzen and Schimke (1965) have recently investigated the pattern of hexokinase isozymes in various rat tissues. They find that the major portion of rat brain hexokinase is isozyme I, named for its mobility on starch gel electrophoresis, but that there is a small amount of isozyme II present as well. Isozyme II comprises only 1–2% of the total

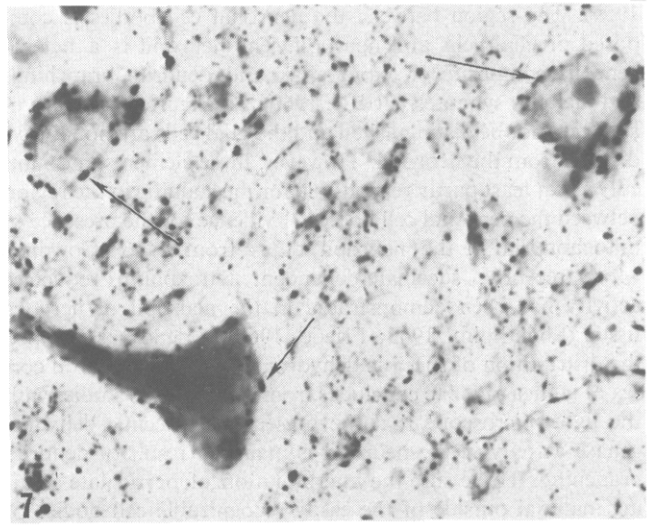

FIGURE 7: Photomicrograph of a section from a third cube of bovine brain cortex, fixed in Regaud's fixative and stained with Altmann's aniline–acid fuchsin to demonstrate mitochondria. The distribution of granules in the cytoplasm of nerve cell bodies (arrow) and in the intervening tissue is the same as in Figures 5 and 6, 1300 ×.

of rat brain hexokinase activity; however it is more unstable than isozyme I. A portion of our antiserum was sent to Dr. Schimke, and it was found that the antiserum reacts with isozyme I from rat brain but does not react with any of the other four isozymes isolated from various other rat tissues (R. T. Schimke, personal communication). This fact might be used in support of the hypothesis. On the other hand, solubilization of mitochondrial hexokinase by a variety of methods such as 0.5 M $MgCl_2$, 0.2 mM glucose 6-phosphate, 1% Triton X-100, or sonic irradiation yields a soluble enzyme which is 100% inhibitable and a residual pellet which is 60–70% inhibitable (P. Craven and R. E. Basford, 1967, unpublished data). This pattern of inhibition is not compatible with the existence of two isozymes, one inhibitable and one not. If both isozymes were being solubilized by one or all of these procedures one would expect that the solubilized enzyme would not be inhibited by 100%. If only isozyme I were being solubilized one would expect a decrease in the extent of inhibition of the residual pellet. Therefore, a third hypothesis is favored, that is, that a portion of the enzyme might be bound to the membrane in such a way that the antigenic groups are not accessible to antibody.

Mitochondria were demonstrated in sections of brain cortex using the succinate-dependent tetrazolium-staining technique. At first glance the presence of reduced tetrazolium deposits in the region between the neuronal cell bodies is surprising. The possibility that cell breakage has extruded fragmented microsomes or mitochondria into an extracellular space must be considered. The tissue used to demonstrate succinic dehydrogenase activity was fixed in graded formalin and frozen to the cryostat chuck before leaving the slaughter house. This method of fixation results in the preservation of cell structure as well as a high level of staining (Walker and Seligman, 1963). In addition, another more classical method of demonstrating mitochondria without the use of frozen sections, namely Regaud's fixative and stain, resulted in the same distribution of mitochondria (see Figure 7). In explanation, it must be pointed out that there is no major extracellular space in brain (Schultz *et al.*, 1957; Horstmann and Meves, 1959). The region between the neuronal cell bodies is composed of glial cells and neuropil. The neuropil is a heterogeneous composite of axon, glial, and dendrite branchings and includes synapses (Friede, 1960). In Figures 5 and 6 synapses cannot be distinguished and the glial cells are not always distinct from the neuropil. However, these elements undoubtedly are at least partly responsible for the staining in the region between the neuronal cell bodies. Evidence for the presence of mitochondria in the neuropil dates from 1916 (Cowdry). More recently, succinate-dependent tetrazolium reductase activity has been demonstrated in the neuropil of nervous tissue (Söderholm, 1965; Friede, 1960). Friede has mapped the distribution of succinic dehydrogenase in the cerebral cortex of guinea pig. He concludes from comparative studies with the light microscope that the presence of succinic dehydrogenase activity in the neuropil is mainly a result of dendrite branching. To be sure the interpretation of particulate staining material outside of the easily recognizable cell bodies of neurons is not easily made at the resolution of the present technique. Neither is it necessary at this time. For this reason we must emphasize the particulate staining observed in the cytoplasm of neuronal cell bodies.

A granular fluorescent pattern has previously been used to localize a glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase, in rat kidney (Emmart *et al.*, 1963a) and developing skeletal muscle cells of chick, mouse, and human embryonic myoblasts grown in culture (Emmart *et al.*, 1963b). These workers observed bright fluorescent granules similar in size and distribution to mitochondria. In the case of developing muscle cells, the pattern of fluorescence progressed from diffuse to granular and this result was interpreted as an increased involvement of mitochondria in glycolytic metabolism as the cell matures. In the present study brain hexokinase exhibited a granular fluorescent pattern and the size and distribution of granules was the same as the size and distribution of mitochondria as demonstrated by the succinate-dependent tetranitro-BT staining technique and supravital staining. The results are consistent with those obtained by the differential centrifugation of brain homogenates and with the hypothesis that brain hexokinase is located on the mitochondrial membrane. Further work is needed to confirm these results at the level of the electron microscope.

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Brain Hexokinase. The Preparation of Inner and Outer Mitochondrial Membranes*

Patricia A. Craven,† Peter J. Goldblatt,‡ and R. E. Basford

ABSTRACT: Three different methods were used to prepare inner and outer membranes of brain mitochondria. The digitonin and phospholipase A methods were relatively unsuccessful. In the former case, the outer membrane marker enzyme, monoamine oxidase, was not released into the outer membrane fraction over a range of concentrations from 0.11 to 0.66 mg of digitonin per mg of mitochondrial protein. In the latter case, 60% of the outer membrane marker rotenone-insensitive reduced diphosphopyridine nucleotide–cytochrome *c* reductase was lost during the incubation with phospholipase A and the portion of the enzyme which was recovered remained in the inner membrane fraction even after prolonged digestion. On the other hand, the combined swelling, shrinking, and sonication method gave more satisfactory results. The sonication procedure resulted in a 1.5–2-fold activation of hexokinase activity which could be duplicated by diluting the mitochondrial suspension in 1% bovine serum albumin. Biochemical assay revealed that between 70 and 75% of the outer membranes were removed. Approximately 63% of the total hexokinase activity was associated with the outer membrane fraction while 12% of the activity was solubilized during the process. The increase in the specific activity of hexokinase was the same as that for the outer membrane marker, rotenone insensitive reduced diphosphopyridine nucleotide–cytochrome *c* reductase. Biochemical and electron microscopic data are presented which indicate that rotenone insensitive reduced diphosphopyridine nucleotide–cytochrome *c* reductase is not a microsomal contaminant. The results are discussed with reference to the problems and limitations of enzyme localization.

The presence of hexokinase on the outer membrane of brain mitochondria is indicated by the success of the fluorescent antibody technique in revealing the sites of hexokinase activity in thin sections of cortex (Craven and Basford, 1969) and by the ease with which it can be solubilized and rebound (Rose and Warms, 1967; Wilson, 1968). Wilson (1968) has suggested the presence of a latent hexokinase activity on the inner membrane of brain mitochondria based on the harsh treatment needed to activate this activity. However, as demonstrated in the present report, the same activity can be revealed by dilution of the mitochondria in 1% bovine serum albumin as by sonication or detergent treatment. Fractionation of serum albumin with methanol at −10° to remove fatty acids and decanol, a detergent used in the preparation of serum albumin, had no effect on its ability to activate latent hexokinase activity. It seemed desirable to separate the mitochondria into inner and outer membranes and assay these fractions under conditions of maximal hexokinase activity.

Methods have been described for the separation of the inner and outer membranes of rat liver (Sottocasa *et al.*, 1967;

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† The data presented are taken from the dissertation of Patricia A. Craven offered in partial fulfillment of the requirements for the Ph.D. degree.

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