BRAIN MITOCHONDRIA

II. The Relationship of Brain Mitochondria to Glycolysis

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ABSTRACT

A mitochondrial fraction prepared from calf brain cortex possessed negligible glycolytic activity in the absence of the enzymes of the high speed supernatant fraction. When mitochondria were added to a supernatant system supplemented with optimal amounts of crystalline hexokinase, a 20 per cent stimulation of glycolysis was observed. The supernatant fraction produced minimal amounts of lactate in the absence of exogenous hexokinase; the addition of mitochondria doubled the lactate production. The substitution of glycolytic intermediates for glucose as substrates as well as the addition of exogenous glycolytic enzymes to the supernatant fraction or supernatant fraction plus mitochondria indicated that the mitochondria contributed mainly hexokinase and phosphofructokinase. By direct assay of all of the enzymes of the glycolytic pathway, only hexokinase and phosphofructokinase were shown to be concentrated in the mitochondrial fraction. All other glycolytic enzymes were found to exhibit higher total and specific activities in the supernatant fraction.

Although it is generally agreed that brain mitochondria, like mitochondria from other tissues, carry out citric acid cycle oxidations and oxidative phosphorylation, there are varying reports in the literature concerning the relationship of the enzymes of glycolysis to the mitochondrial fraction of brain. Hesselbach and Du Buy (1, 2) showed that brain mitochondria convert glucose and glycolytic intermediates to lactate both aerobically and anaerobically. Similarly, Gallagher et al. (3) demonstrated the complete oxidation of glucose and glucose-6-phosphate to carbon dioxide and water by brain mitochondria. On the other hand, Balázs and Lagnado (4) reported that only 10 per cent of the total glycolytic activity of rat brain was associated with their mitochondrial preparation.

Johnson (5) and Brunngraber and Abood (6) have indicated that most of the glycolytic enzymes are found in the soluble portion of homogenates of brain but that hexokinase is concentrated in the mitochondria. Brunngraber and Abood (6) also reported that the addition of mitochondria to a supernatant fraction in the presence of optimal amounts of exogenous hexokinase caused a twofold stimulation of lactate formation. The mitochondria alone glycolyzed at 25 per cent of the rate of the supernatant fraction.

Contrary to the above reports, Brody and Bain (7) and Aldridge (8) concluded that the brain mitochondrial preparations used in their studies possessed no glycolytic activity. Whittaker (9, 10) discovered that the crude mitochondrial fraction from brain contained particles derived from the pinching-off of nerve endings (NEPs) which were easily separable from the mitochondria by density gradient centrifugation. The presence of NEPs which must contain entrapped soluble cytoplasm may explain the 7 to 28 per cent of soluble glycolytic enzymes which Johnson (5) observed in the

mitochondrial fraction. The association of lactic dehydrogenase with the NEPs has been confirmed (10).

The preparation of beef brain mitochondria described in the previous paper (11) was shown to be relatively free of contamination by non-mitochondrial particulate material and contained mitochondria which exhibited enzymic and morphological properties comparable to those of mitochondrial preparations from other tissues. Studies on the glycolytic capacity of this mitochondrial fraction are reported here. Some aspects of the work have been reported (12, 13).

MATERIALS AND METHODS

SOURCE OF MITOCHONDRIA AND SOLUBLE ENZYMES: Brain mitochondria were prepared by Method I of the previous paper (11). The supernatant fraction from a 30-minute centrifugation of the homogenate at 105,000 g was used as the source of glycolytic enzymes.

Type III hexokinase (150,000 KM units/gm), crystalline hexokinase (1.3 $\times$ 10^6 KM units/gm), crystalline aldolase, 3-phosphoglycerate kinase, and triosephosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Missouri, Glucose-6-phosphate dehydrogenase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, enolase, pyruvate kinase, and lactic dehydrogenase were obtained from California Corporation for Biochemical Research, Inc., Los Angeles, Phosphofructokinase was partially purified (specific activity: 2.37 μmole/minute/mg protein) from rabbit muscle by the method of Ling et al. (14).

ENZYMIC ASSAYS: Glycolysis was determined manometrically with 95 per cent N_2-5 per cent CO_2 as the gas phase in the following medium: ATP[1], 1 μmole; $MgCl_2$, 20 μmole; KH_2PO_4, 20 μmole; nicotinamide, 60 μmole; DPN, 1 μmole; $KHCO_3$, 50 μmole; glucose, 25 μmole; mitochondrial and supernatant fractions and hexokinase (see Tables I, II, IV, and V); final volume, 2.5 ml, pH 7.4. The flasks were gassed for 10 minutes at 30°C and the reaction initiated by the addition of glucose and hexokinase from the side arm. The CO_2 evolved as glycolysis progressed was used as an estimate of lactate formation. After 60 minutes, 0.2 ml of 75 per cent TCA was added to each flask and the protein-free filtrate assayed for lactate by the method of Barker and Summerson (15).

[1] The abbreviations used are: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN and TPNH, oxidized and reduced triphospho-pyridine nucleotide, respectively; EDTA, ethylene-diaminetetraacetate; TCA, trichloroacetic acid; and NEPs, nerve ending particles.

Hexokinase activity was measured spectrophotometrically (Zeiss model M4Q II spectrophotometer) in a system in which glucose-6-phosphate production was coupled to TPNH formation in the presence of glucose-6-phosphate dehydrogenase. The glucose-6-phosphate dehydrogenase preparation was diluted to 1 mg per ml in 0.15 M potassium glycylglycine buffer, pH 8.0, and stored at −20°C. The reaction cuvette at 30°C contained in a final volume of 1 ml: 15 μmole ATP, 25 μmole glucose, 80 μmole Tris, pH 8.0, 3 μmole EDTA, 19 μmole $MgCl_2$, 1 μmole TPN, and 5 μg of glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 0.1 ml of a tissue sample diluted with 10^{-3} M EDTA, pH 7.0. A similar assay procedure has been developed by Bennet et al. (16).

The remaining glycolytic enzymes were assayed spectrophotometrically (Cary model 14 spectrophotometer) by measuring the absorbancy change at 340 mμ due to oxidation or reduction of pyridine nucleotide. When it was necessary to couple the enzyme being assayed to a reaction involving pyridine nucleotide, purified coupling enzymes were added in excess. Preliminary control experiments without tissue were performed to insure that the coupling enzymes were not contaminated with the particular enzyme being assayed. As an additional control, the change in absorbancy at 340 mμ was determined for each enzyme in the absence of substrate and subtracted from that obtained in its presence. Tissue suspensions were stored at 0°C and diluted prior to addition to the assay medium. The activities were expressed as μmole of substrate consumed per minute per mg of protein at 27°C.

Phosphofructokinase, aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase were assayed by the method of Wu and Racker (17). Triosephosphate dehydrogenase was assayed in the forward reaction by the method of Wu and Racker (17), and in the reverse reaction by the method of Johnson (5). Glucose-6-phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase were assayed by the method of Johnson (5), and lactic dehydrogenase by the method of Kornberg (18).

All substrates obtained as the barium salts were converted to potassium salts by addition of an equivalent amount of sulfate ion and adjusted to pH 7.5 with KOH.

Protein was determined by the method previously described (11).

RESULTS

MITOCHONDRIAL STIMULATION OF GLYCOLYSIS BY THE SUPERNATANT FRACTION: The mitochondrial fractions alone exhibited very little glycolytic activity (Table I).

Less than 5 per cent of the glycolytic activity of the supernatant fraction, S, was present in the mitochondrial fraction, R_4, as determined by a comparison of the specific activities. Maximal linear glycolytic rates (μmole lactate/mg protein/hour) in the presence of exogenous hexokinase were obtained with 3 to 4 mg of S protein or 3 to 7 mg of R_4 protein per flask.

The low glycolytic activity observed in R_4 could be due to suboptimal amounts of one or more en-

TABLE I

Glycolytic Activity of Subcellular Fractions

Fraction	Protein added to each flask	Specific activity* μmole lactate produced/mg protein/hr
	mg	
Supernatant, S	2.7	3.15
	3.5	3.46
Mitochondria, R_4	3.7	0.20
	7.0	0.16
	9.0	0.14

* Each figure represents the mean specific activity calculated from the amount of lactate produced in three reaction vessels containing the standard glycolytic assay medium after a 60-min. incubation.

TABLE II

The Effect of Exogenous Hexokinase

KM Units	μmole lactate/hr	
	S	S plus R_4
Type III hexokinase		
0	2.8	7.8
15	5.6	8.7
30	9.4	10.4
50	10.5	13.2
75	12.1	12.6
95	10.1	9.5
Crystalline hexokinase		
0	4.3	10.1
10	6.3	13.6
30	13.0	17.4
60	16.5	19.4
100	17.4	18.2

Each flask contained 3 mg of S protein and, when added, 4 mg of R_4 protein.

zymes of the glycolytic scheme. In agreement with the work of Brunngraber and Abood (6), fraction S required exogenous hexokinase for optimal glycolytic activity, as shown in Table II. The addition of 4 mg of R_4 to fraction S stimulated lactate formation to about 250 per cent of the rate of S alone, but increased lactate formation only slightly (4 to 20 per cent) when the optimal amount of Sigma, type III hexokinase was present. Excess hexokinase exerted a slight inhibitory effect whether the source of glycolytic enzymes was S alone or S plus R_4. Crystalline hexokinase yielded similar results (Table II), although no inhibition of glycolysis was observed at the highest concen-
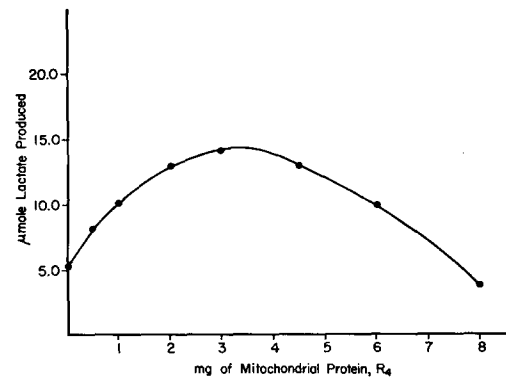

FIGURE 1 The effect of the mitochondrial fraction on the glycolytic activity of the supernatant fraction. Standard assay medium as indicated in the text, supplemented with 25 KM units of Sigma Type III hexokinase.

tration of hexokinase used. Addition of R_4 to S caused a twofold stimulation of lactate formation in the presence of low concentrations of hexokinase, but less than a 20 per cent stimulation in the presence of optimal amounts of hexokinase. Thus, most of the enhancement of the glycolytic activity of S was due to hexokinase in R_4.

Fig. 1 shows the effect of increasing amounts of R_4 on the glycolytic activity of S in the presence of 25 KM units of type III hexokinase. A threefold stimulation was observed with 2 to 4 mg of R_4, whereas larger amounts produced an inhibition of lactate production.

DISTRIBUTION OF HEXOKINASE IN FRACTIONS OF THE HOMOGENATE: Since it has been reported that hexokinase is primarily located in the mitochondrial or particulate fraction of brain homogenates (5, 6, 19), this enzyme was assayed in all of the fractions of the homogenate

(11). As indicated in Table III, a large percentage of the hexokinase activity is found in the initial residue which contains a large proportion of the mitochondria along with nuclei and debris (11). The enzymic activity is distributed throughout the remaining fractions, although an eightfold increase in specific activity was observed in R_4.

USE OF GLYCOLYTIC INTERMEDIATES AS SUBSTRATES: The presence of glycolytic enzymes other than hexokinase in R_4 was indicated by the use of various intermediates of the glycolytic pathway as substrates (Table IV). A 50 to 70 per cent increase in lactate formation was observed

a particular enzyme, then addition of that enzyme to the assay medium containing S should reproduce the stimulation of glycolysis by R_4. Table V shows that an approximately 30 per cent increase in lactate formation was observed upon the addition of exogenous aldolase, triosephosphate dehydrogenase, or phosphoglycerate kinase, using suboptimal amounts (25 KM units) of Type III hexokinase. Since suboptimal levels of hexokinase were used the per cent increase in lactate production afforded by R_4 was greater than would have been observed with optimal amounts of hexokinase (cf. addition of aldolase to a system supplemented

TABLE III

Distribution of Hexokinase

Fraction*	Total activity‡	% of Total	Specific activity
Homogenate	530	100	0.098
R_1	276	52	0.135
S_1	253	48	0.172
R_2	188	35.5	0.315
S_2	53	10	0.043
R_3	74.9	14	0.458
S_3	51.6	9.7	0.114
R_4	59.5	11	0.688
S_4	2.7	2	0.061

* For explanation of symbols, see paper I (11).
‡ μmole glucose-6-phosphate produced/min. at 30°C.
§ μmole glucose-6-phosphate produced/min./mg protein at 30°C.

TABLE IV

Glycolytic Intermediates as Substrates

	μmole lactate produced/hr.			
		S plus R_4		
Substrate	S	2.5 mg	3.8 mg	5.1 mg
Glucose-6-phosphate	9.2	13.8	—	15.6
Fructose-1,6-diphosphate	5.9	7.1	10.1	—
Glyceraldehyde-3-phosphate	3.9	4.0	5.1	6.3
α-Glycero-phosphate	2.3	2.8	2.9	3.3

Each flask contained 3 mg of S protein plus the indicated amount of R_4 protein. All substrates were substituted for glucose in equimolar amounts in the standard glycolytic medium.

when R_4 was added to fraction S with glucose-6-phosphate as substrate. Fraction R_4 stimulated glycolytic activity 20 to 70 per cent with fructose diphosphate as substrate, although S alone produced less lactate with this substrate than with glucose. The addition of R_4 to S caused a 30 to 60 per cent increase in lactate formation with glyceraldehyde-3-phosphate as substrate and a 40 per cent increase with phosphoglyceric acid as substrate at the highest level of R_4 used.

ADDITION OF INDIVIDUAL GLYCOLYTIC ENZYMES TO S AND S PLUS R_4: In order to investigate more fully the stimulation afforded by R_4, the individual enzymes of the glycolytic scheme were added to either S alone or S plus R_4 in the presence of optimal levels of hexokinase. If R_4 stimulated lactate formation because it contained

with crystalline hexokinase). The stimulation afforded by aldolase, triosephosphate dehydrogenase, and phosphoglycerate kinase, however, was approximately the same whether these enzymes were added to S plus R_4 or to S alone.

Similarly, the addition of 20 μg of aldolase to either a system supplemented with crystalline hexokinase or one using glucose-6-phosphate as substrate caused a 20 per cent stimulation of lactate formation with both S and S plus R_4 as the source of glycolytic enzymes.

The addition of 80 μg of phosphofructokinase to fraction S containing optimal amounts of crystalline hexokinase, however, produced a stimulation in lactate formation equal to that produced by 4 mg of R_4. Addition of the same amount of phosphofructokinase to S plus R_4 produced no further stimulation.

TABLE V

Addition of Glycolytic Enzymes

Type of hexokinase	Enzyme added	Fraction of brain homogenate	µg of enzyme added				
			0	20	60	80	120
			µmole lactate produced/hr.				
Type III	Triosephosphate dehydrogenase	S	8.0	10.8	11.0		10.9
		S + R₄	12.9	18.2	18.0		14.2
	Aldolase	S	5.0	5.4	6.8		10.1
		S + R₄	11.3	13.1	14.1		19.4
	Phosphoglycerate kinase	S	7.3	9.9	10.0		—
		S + R₄	10.7	11.5	12.2		—
Crystalline	Aldolase	S	13.4	15.0			
		S + R₄	15.0	17.7			
	Phosphofructokinase	S	15.3			23.3	
		S + R₄	23.7			24.0	

Each flask contained 3 mg of S protein and 4 mg of R₄ protein where indicated, with 25 KM units of Type III or 60 units of crystalline hexokinase.

TABLE VI

Distribution of Glycolytic Enzymes

Enzyme	Homogenate		Supernatant, S		Mitochondria, R₄	
	%	Sp. Act.*	%	Sp. Act.*	%	Sp. Act.*
Hexokinase	100	0.508	17	0.343	14	4.08
Glucose-6-phosphate isomerase	100	0.162	54	0.269	1.1	0.053
Phosphofructokinase	100	0.460	68	1.18	8.6	2.92
Aldolase	100	0.054	40	0.100	0.48	0.019
Triosephosphate isomerase	100	0.035	59	0.063	2.6	0.026
α-Glycerophosphate dehydrogenase	100	0.014	55	0.018	4.3	0.013
Triosephosphate dehydrogenase	100	0.042	51	0.103	1.5	0.041
Phosphoglycerate kinase	100	0.477	20	0.843	0.28	0.078
Phosphoglycerate mutase	100	0.228	57	0.494	0.39	0.066
Enolase	100	0.218	35	0.349	0.71	0.115
Pyruvate kinase	100	0.122	53	0.217	0.53	0.051
Lactic dehydrogenase	100	0.278	57	0.956	0.24	0.064

* µmole of substrate consumed/min./mg of protein at 27°C.

DISTRIBUTION OF GLYCOLYTIC ENZYMES: The distribution of hexokinase discussed earlier (Table III) indicated that sufficient information on distribution could be obtained from a comparison of the total and specific activities of the homogenate and fractions S and R_4. The remaining glycolytic enzymes were assayed in these three fractions. As indicated in Table VI, for each enzyme studied, a very low percentage of the enzymic activity of the homogenate was associated with R_4. Except for hexokinase and phosphofructokinase, the specific activity of the glycolytic enzymes in the mitochondrial fraction was lower than or equal to that of the homogenate. For each enzyme other than the two kinases, the highest specific activity and per cent of the total activity were located in fraction S.

DISCUSSION

The mitochondrial fraction, R_4, prepared from calf brain by Method I of Stahl *et al.* (11) exhibited negligible glycolytic activity in the absence of the enzymes of the high speed supernatant fraction, S. Lactate formation by S in the presence of exogenous hexokinase was increased by the addition of R_4. The extent of stimulation was dependent on the type of hexokinase preparation used. Brunngraber and Abood (6), using Sigma Type V hexokinase, have shown a twofold increase in lactate formation by the addition of mitochondria. We found that the addition of 4 mg of R_4 caused a two- to threefold stimulation in the presence of Sigma Type III hexokinase; 6 mg of R_4 inhibited lactate formation. When optimal amounts of crystalline hexokinase were used, only a 20 to 25 per cent stimulation of glycolytic activity was observed upon addition of R_4. In the absence of exogenous hexokinase, the addition of R_4 to S caused a 250 per cent increase in glycolytic activity, suggesting the presence of hexokinase in fraction R_4.

The use of several intermediates of the glycolytic pathway as substrates indicated the steps at which the mitochondrial fraction stimulated lactate formation. When glucose-6-phosphate, the product of the initial step involving hexokinase, was used as substrate, the same increase in lactate formation occurred as was observed in the presence of optimal amounts of hexokinase. In the presence of optimal amounts of R_4 protein, as indicated in Fig. 1, a 50 to 70 per cent increase in lactate formation was observed with glucose-6-phosphate and fructose-1,6-diphosphate as substrates. A 25

to 30 per cent increase in glycolytic activity occurred at these protein concentrations with glyceraldehyde-3-phosphate and α-glycerophosphate as substrates. These results suggested that only small amounts of glycolytic enzymes beyond aldolase in the glycolytic scheme were present in fraction R_4.

Brunngraber and Abood (6) have concluded that aldolase present in their mitochondrial preparation was responsible for the stimulation of lactate formation by the supernatant fraction supplemented with exogenous hexokinase. Johnson (20), however, has criticized the experimental procedure on which these findings were based. In our system, the addition of R_4 to S in the presence of optimal amounts of exogenous hexokinase and aldolase produced a 25 per cent stimulation of glycolytic activity. Likewise, the addition of triosephosphate dehydrogenase or phosphoglycerate kinase to a system containing S and R_4 supplemented with hexokinase also stimulated lactate formation. The addition of phosphofructokinase to fraction S supplemented with hexokinase reproduced the stimulation of lactate formation provided by fraction R_4. Addition of R_4 to the above system provided no further stimulation of lactate production. We conclude, therefore, that the stimulatory effect of R_4 on the glycolytic activity of S was due to phosphofructokinase and hexokinase in fraction R_4.

Johnson (5) has reported that 75 per cent of the total hexokinase of rat brain homogenates was present in the mitochondrial fraction. Each of the other glycolytic enzymes occurred mainly (70 to 90 per cent) in the supernatant fraction. Abood *et al* (21) confirmed the presence of the glycolytic enzymes in rat brain mitochondria by solubilization of the enzymes with a detergent. They concluded that some, but not all of the glycolytic enzymes were loosely bound to the mitochondria. Balázs and Lagnado (4), however, have concluded that their mitochondrial preparation contains 35 per cent of the total hexokinase, 12 per cent of the aldolase, 9 per cent of the triosephosphate dehydrogenase, and 11 per cent of the lactic dehydrogenase. Our mitochondrial fraction showed the highest specific activity in the hexokinase assay, but in contrast to the results of Johnson (5) and Balázs and Lagnado (4), only 14 per cent of the hexokinase activity of the homogenate was present in the mitochondrial fraction. The low percentage, however, is in agreement with the low yield of mitochondria obtained by the method used (11).

The only other enzyme which showed an increased specific activity in the mitochondrial fraction was phosphofructokinase. This unusual subcellular location of the two ATP-requiring enzymes of the glycolytic scheme could be an adaptation allowing efficient integration of ATP production and utilization for the metabolism of glucose.

All other glycolytic enzymes were present in trace amounts in the mitochondrial fraction. This activity may be explained by the presence in R_4 of NEPs which Whittaker (10) has suggested must contain small amounts of entrapped soluble cytoplasm. The possibility does remain that these enzymes might be loosely bound to the mitochondria *in vivo* and lost during the isolation procedure without major alteration of the oxidative and phosphorylative properties of the particles. Cohen (22) has reported that two types of mitochondrial fractions can be prepared from guinea pig or rabbit brain. One fraction, P-1, required the addition of crude yeast hexokinase for net phosphorylation with glucose as substrate. The other fraction, P-2, carried out active aerobic glycolysis without crude yeast hexokinase. In the presence of exogenous hexokinase, the aerobic glycolysis in P-2 was inhibited and the phosphorylative efficiency increased. No data were given on morphological integrity or contamination by non-mitochondrial particles for the two fractions.

The data presented here indicate that calf brain mitochondria, which are comparable to mitochondria from other tissues with respect to enzymic and morphological integrity and degree of contamination, are deficient in most of the enzymes of glycolysis. It remains to be shown whether hexokinase and phosphofructokinase are an integral part of the mitochondrion or merely follow the same fractionation scheme as the mitochondria.

Note added in proof: In a recent paper, Drs. R. Tanaka and L. G. Abood (*J. Neurochem.*, 1963, 10, 571) have shown that rat brain mitochondria, subjected to subfractionation by the technique of gradient centrifugation in a medium of Ficoll-sucrose-EDTA, exhibit no glycolytic activity.

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