BRAIN MITOCHONDRIA

I. Isolation of Bovine Brain Mitochondria

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ABSTRACT

Two methods of preparing a mitochondrial fraction from beef brain cortex are described. Data are presented on the rate of oxidation of substrates, P/O and respiratory control ratios, cholinesterase activity, and DNA content. Electron micrographs of isolated mitochondria and mitochondria *in situ* are shown. Comparisons are drawn between these preparations and mitochondria prepared in 0.25 M sucrose. The data on enzymic properties and contamination by non-mitochondrial material indicate that mitochondrial fractions which compare favorably with those from other tissues can be prepared from brain tissue.

There is a considerable amount of information on mitochondria from brain which suggests that in many ways they exhibit the same enzymic properties as mitochondria of other tissues. For example, mitochondrial fractions isolated from brain have been shown to carry out citric acid cycle oxidations (1–4) with concomitant phosphorylation (1, 5–7). On the other hand, there are varying reports in the literature regarding the relationship of the enzymes of glycolysis and the mitochondria of brain (3, 8–13).[1]

Brody and Bain (1) and Abood *et al.* (4) appear to have been the first to prepare mitochondrial fractions from brain, using procedures based on the method developed by Schneider and Hogeboom (14) for the isolation of liver mitochondria. Most of the subsequent work on the enzymology of brain mitochondria has been carried out on mitochondrial fractions prepared by the methods described by these workers (5, 6, 11, 13, 15) or by direct application of the Schneider-Hogeboom method (2, 3, 8, 9).

[1] For a discussion of the glycolytic capacity of brain mitochondria, see the following paper (26).

Our early attempts to prepare mitochondria from beef brain by the methods of Abood *et al.* (4) or Brody and Bain (1) indicated that the mitochondrial preparations were contaminated by non-mitochondrial particles, especially myelin fragments. This observation has also been made by Balázs and Richter (16) using the method of Brody and Bain (1).

Dahl *et al.* (17) separated a rat brain mitochondrial fraction into two subfractions, "P" which contained all of the oxidative capacity and "W" which was not characterized, by differential centrifugation in 0.25 M sucrose.

Until recently, estimates of the purity of mitochondrial preparations from brain seem to have been based mainly on visual examination under the light or phase contrast microscope. However, Petrushka and Giuditta (18) have shown by electron microscopy that rat brain mitochondrial fractions prepared in 0.88 M sucrose by the method of Hogeboom, Schneider, and Palade (19) are grossly contaminated by non-mitochondrial material.

Gray and Whittaker (20, 21) and De Robertis

et al. (22, 23) showed that crude mitochondrial fractions of guinea pig or rat brain, prepared in 0.32 M sucrose, contained myelin fragments and pinched-off nerve endings containing synaptic vesicles as well as mitochondria. The mitochondrial fraction could be separated by density gradient centrifugation into subfractions which were relatively pure with respect to subcellular particle type. The mitochondrial fractions obtained by sucrose density gradient centrifugation were characterized by electron microscopy and succinic dehydrogenase content. Other enzymic properties were not reported.

It seemed worthwhile, therefore, to develop a method of fractionation of brain homogenates designed to permit the separation of a mitochondrial fraction which (*a*) was relatively pure with respect to subcellular particle type and (*b*) contained mitochondria which satisfied the criteria for enzymic and morphological integrity which have been established for mitochondria from other tissues.

This communication describes the preparation and properties of such a fraction from beef cerebral cortex. Some aspects of the work have been reported earlier (24, 25). The following paper (26) deals with the glycolytic capacity of this mitochondrial preparatinn.

During the preparation of this manuscript, a short publication by Løvtrup and Zelander appeared (27) in which they reported some of the results of an investigation of the properties of brain mitochondria isolated by various procedures. The quality of the preparations was judged by electron microscopy as well as by enzymic and chemical means. They concluded that rat or rabbit brain mitochondria prepared in 0.44 M sucrose exhibited the most acceptable enzymic and morphological properties. A comparison of the mitochondrial fraction of Løvtrup and Zelander with mitochondrial fractions prepared by the methods described here is presented in the Discussion.

MATERIALS AND METHODS

PREPARATION OF THE MITOCHONDRIAL FRACTION, METHOD I: Undamaged calf or beef brains were obtained from kosher-killed animals at the slaughter house. The brains were removed within 5 to 10 minutes after death of the animals and placed in a polyethylene bag containing ice cold medium. The bag and contents were stored in ice for transport to the laboratory. The average lapse of time between death of the animals and the arrival of specimens at the laboratory was 30 minutes.

In the 5°C cold room, the cerebral hemispheres were removed from the brains and the meninges were removed with forceps. The gray matter was scraped from the cortices with a dull spatula, leaving behind as much of the white matter as possible without the spending of undue time for careful dissection. Two brains yielded about 100 gm of wet tissue. The tissue was homogenized (ten passes of the pestle) in a 200 ml capacity teflon and glass homogenizer, 0.004 to 0.006 inch clearance (Kontes Glass Co., Vineland, New Jersey), using 2 ml of medium A/gm of wet tissue. The composition of medium A included 0.4 M sucrose, 0.001 M ethylenediaminetetraacetate (EDTA), and 0.02 per cent polyethylene sulfonate (PES,[2, 3] all adjusted to pH 6.8 to 7.0.

After the tissue had been homogenized in the teflon and glass homogenizer, it was more thoroughly homogenized (10 to 15 passes of the pestle) in a ground-glass homogenizer, 0.004 to 0.006 inch clearance, and the suspension passed through cheesecloth. Medium A was added to give a suspension of 10 ml of medium/gm of original tissue. The homogenate in medium A was centrifuged at 2000 *g* for 20 minutes[4] in the International PR-2 centrifuge.

The low speed centrifugation was repeated once to yield a supernatant fluid, S_1, and a residue, which, when combined with the initial residue, was designated R_1. S_1 was centrifuged at 12,000 *g* for 15 minutes in a Sorvall, Model SS 1, or Spinco Model L, centrifuge to yield a crude mitochondrial fraction, R_2, comprised of a tan lower layer and a white fluffy upper layer, and a supernatant fluid, S_2. Fraction R_2 was then homogenized in 6 ml of medium F/gm of original tissue, and centrifuged at 12,000 *g* for 30 minutes. The composition of medium F was the same as that of medium A, except that Ficoll (a high molecular weight carbohydrate) was added to a final concentration of 8

[2] The synthetic anticoagulant PES was a substitute for heparin, introduced by Birbeck and Reid (28) to prevent agglutination in the preparation of liver mitochondria.

[3] The abbreviations used are: EDTA, ethylenediaminetetraacetate; PES, polyethylene sulfonate; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GDP, guanosine diphosphate; DPN, diphosphopyridine nucleotide; INT, 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium; ChE, cholinesterase; ACh, acetylcholine; DNP, 2,4-dinitrophenol.

[4] All centrifugal forces are given as the maximal gravitational force, g_{max}.

per cent (w/v). The resulting supernatant fluid, S_3, was decanted from the brown pellet, R_3, which was usually visibly free of contaminating, white fluffy material.[5]

The mitochondrial fraction, R_3, was washed by homogenization in 4 ml of medium A/gm of original tissue and centrifugation at 12,000 *g* for 15 minutes, to yield the final mitochondrial fraction, R_4, and supernatant fluid, S_4.

In the preparation of the tissue for homogenization, if the entire cerebrum was used without removal of as much of the white matter as possible, a much higher percentage of "fluff" was obtained in fraction R_2 and more time was required for resuspension and centrifugation in medium F than was spent in gross dissection, to obtain a comparable degree of purity.

PREPARATION OF THE MITOCHONDRIAL FRACTION, METHOD II: Method II was essentially the same as Method I, except that digestion by the bacterial proteinase, Nagarse (introduced by Hagihara, 29, for isolation of heart mitochondria), was substituted for the second homogenization of the cortical tissue. The homogenized tissue, obtained as described in Method I prior to the homogenization in the all glass homogenizer, was adjusted to pH 6.9 to 7.0 with a few drops of 2 M Tris, pH 10.8, 1 mg of Nagarse/gm of tissue added, and the mixture stirred at 0–4°C for 15 minutes. The pH of the suspension was maintained throughout the digestion by the addition of more Tris, if necessary. The suspension was passed through cheesecloth, diluted with medium A (10 ml/gm of original tissue), and the preparation continued as described for Method I.

The average yield of mitochondria (Method I or II) from 100 gm of tissue (wet weight) was 100 mg of protein (biuret). The over-all recovery of mitochondria from the homogenate by either method, based on succinic–INT reductase activity (30), was only 7 to 10 per cent. About 60 per cent of the total activity appeared in R_1. Attempts to improve the yield of mitochondria by fractionation of R_1 or by more drastic homogenization of the tissue have been unsuccessful.

The supernatant fraction, S_2 (Method I or II), contained the major portion of the fluffy material, microsomes, and soluble components of the homogenate. The presence of the fluffy material prevented a clear cut separation of microsomes from the homogenate. S_2 was separated either into S_5 (soluble components) and R_5 (microsomes and, fluff) by centrifugation at 105,000 *g* for 60 minutes or into a fluff fraction, R_6, with some microsomes entrapped, and a supernatant fraction, S_6, containing mainly microsomes and soluble material, by centrifugation at 46,900 *g* for 60 minutes. S_6 was further fractionated into R_7 and S_7 (microsomal and supernatant fraction, respectively) by centrifugation at 105,000 *g* for 60 minutes. The white fluffy fractions R_5 or R_6 plus R_7 may correspond to fraction W of Dahl *et al.* (17).

For comparison, mitochondria from beef brain were prepared in 0.25 M sucrose by the method of Schneider and Hogeboom (14).

ANALYTICAL METHODS: Protein was determined by the biuret method, essentially as described by Gornall *et al.* (31), using bovine serum albumin as a standard. Samples of particulate preparations were precipitated by the addition of an equal volume of cold 20 per cent TCA, allowed to stand for 20 to 30 minutes in ice, centrifuged, and the pellet washed once with 5 to 10 volumes of cold 10 per cent TCA. The resulting pellet was dissolved in a minimum volume of 3 per cent NaOH, adjusted to a convenient volume with water, and aliquots taken for protein determination. In addition to biuret reagent, deoxycholate was added to a final concentration of 0.67 per cent to insure optical clarity. The values for milligrams of mitochondrial protein may be converted to milligram N (Kjeldahl determination) by multiplying by 0.082. The low value of the conversion factor is due in part to the fact that mitochondrial protein, dissolved in NaOH and deoxycholate under the same conditions as described for the biuret determination, absorbs appreciably at 540 mμ in the absence of biuret reagent.

Nucleic acids were extracted and determined in the manner recommended by Hutchison and Munro (32). Ribonucleic acid was determined by its absorbancy at 260 mμ or as ribose by the procedure of Schmidt, Thannhauser, and Schneider (see reference 33), using yeast RNA as a standard. Deoxyribonucleic acid was estimated by the method of Burton (34), using salmon sperm DNA as a standard.

Cholinesterase activity was measured by the null-point potentiometric titration method of Smith *et al.* (35), with ACh as substrate.

Oxidative capacity was determined manometrically or polarographically. The composition of the manometric assay medium was: KH_2PO_4, 25 μmole; cytochrome *c*, 5×10^{-3} μmole; DPN, 0.25 μmole; ATP, 1.0 μmole; glucose, 12 μmole; hexokinase (Sigma Chemical Co., St Louis, Missouri, type III, 0.5 mg or crystalline, 23 to 30 KM units); $MgCl_2$, 20 μmole; dialyzed serum albumin, 10 mg; sucrose, 360 μmole; substrate 50 to 60 μmole; mitochondrial protein, 3 to 5 mg; final volume, 2.5 ml, pH 7.4.

Phosphorylative efficiency in the manometric determination was calculated from the disappearance of inorganic phosphate as determined by the Dryer

[5] If R_3 was not free of fluffy material, it was resuspended and homogenized in medium F and centrifuged at 12,000 *g* for 30 minutes, to yield fraction R'_3, and supernatant fluid, S'_3.

modification (36) of the method of Fiske and Subbarow (37), or by the appearance of glucose-6-phosphate as determined with glucose-6-phosphate dehydrogenase (38).

In experiments where crystalline hexokinase was used, the ammonium sulfate was removed from the suspension by dialyses against 0.001 M phosphate buffer, pH 7.4, or by passage through Sephadex G 50. Optimal amounts of either type III or ammonium sulfate-free crystalline hexokinase yielded comparable P/O ratios calculated by either of the above methods.

Polarographic determinations of oxidative phosphorylation were carried out using a vibrating oxygen electrode (Oxygraph, Gilson Medical Electronics, Middleton, Wisconsin), according to the method of Chance and Williams (39). The polarizing voltage was maintained at −0.65 v with respect to the saturated calomel electrode. The amplitude of oscillation was set at the highest value for maximum response and sensitivity. The assay medium (40) contained 600 μmole mannitol, 20 μmole Tris, 20 μmole KCl, 8 mg dialyzed bovine serum albumin, 20 μmole phosphate, 25 μmole substrate and 2.5 to 4.5 mg mitochondrial protein; final volume, 2.0 ml, pH 7.4. The P/O ratios were calculated as ADP/O from the oxygen uptake in microatoms during the active state of respiration and micromoles of ADP added. The ADP/O ratios were confirmed by determination of the amount of ATP formed from ADP using the luciferase assay (41).

Respiratory control ratios were calculated as the rate of oxidation in the presence of phosphate acceptor (glucose and hexokinase or ADP) divided by the rate in the absence of acceptor.

Mitochondrial pellets and samples of cortex were fixed for electron microscopy in buffered 1 per cent osmium tetroxide, pH 7.4, at 4°C for 1 hour, dehydrated with increasing concentrations of ethanol in water (60, 80, 95, and 100 per cent) and subsequently immersed in dry acetone for 1 hour. Small pieces from various areas of the pellet were imbedded in Vestopol W (42) for 3 hours and transferred to capsules containing Vestopol W plus 1 per cent initiator and activator, and polymerized at 48°C for 24 to 48 hours. Thin sections, cut on a Porter-Blum microtome, were stained with lead hydroxide (43) and examined in either a Philips Model 100B or 200 electron microscope.

CHEMICALS AND SPECIAL REAGENTS: Desiccated firefly tails, crystalline and type III hexokinase, α-glycerophosphate, cytochrome *c*, and 2-(*p*-iodophenyl) - 3 - *p* - nitrophenyl - 5 - phenyltetrazolium were obtained from Sigma Chemical Co.; DL-β-hydroxybutyric, malic, and α-ketoglutaric acids, and glucose-6-phosphate dehydrogenase from California Corporation for Biochemical Research, Los Angeles, Ficoll and Sephadex G 50 from Pharmacia, Rochester, Minnesota and Nagarse, crystalline bacterial proteinase 1800 P.U.N./mg, Enzyme Development Corp., New York. Polyethylene sulfonate (average molecular weight, 5900) was obtained from Dr. M. Speeter, The Upjohn Co., Kalamazoo, Michigan, and from Dr. R. K. Thoms, Lloyd Bros., Cincinnati, Ohio. The authors are indebted to Dr. Speeter for suggesting the use of PES and to both him and Dr. Thoms for their generosity in supplying the compound. Amytal (Eli Lilly and Co., Indianapolis, Indiana) was recrystallized twice from ethanol-water. Seconal was a gift from Eli Lilly and Co. All other chemicals were commercial products, reagent grade or better.

RESULTS

ELECTRON MICROSCOPY: During the evolution of the two methods described for the preparation of brain mitochondria, electron microscopy was used as a guide for the evaluation of the quality of the preparations. The morphology of the isolated mitochondria was compared with that of mitochondria *in situ*, and contamination by nonmitochondrial material was compared with that seen in mitochondrial fractions prepared in varying concentrations of sucrose (18, 25, 27).

An electron micrograph of a thin section of beef cerebral cortex (Fig. 1) fixed by immersion in buffered osmium tetroxide indicates that the mitochondria in brain tissue obtained from the slaughter house have not undergone more extensive morphological changes than are observed in sections of brain from laboratory animals (18). The maintenance of the fine structure of the brain as indicated by the preservation of mitochondria and myelin, in material fixed approximately 30 minutes after the death of the animal, confirms the observations of Ito (44) on the effects of postmortem change on the fine structure of the cell.

Electron micrographs of the mitochondrial fractions prepared by Methods I and II are shown in Figs. 2 and 3, respectively. They indicate that either Method I or II yields a mitochondrial fraction which is much less contaminated by nonmitochondrial particles, especially myelin fragments, than has been reported in previous studies utilizing varying concentrations of sucrose as the isolation medium (18, 25, 27). Examination of Figs. 2 and 3 reveals that the mitochondrial fractions prepared by either Method I or II are not completely free of nerve ending particles; *cf.* electron micrographs of Gray and Whittaker (21) and De Robertis *et al.* (22, 23). The mitochondria

obtained by either method possess a "zebra" appearance, making it difficult to resolve their membranous components.

OXIDATIVE PHOSPHORYLATION: The rate of oxygen uptake, P/O and respiratory control ratios for several substrates, using beef brain mitochondria prepared by Methods I and II, are shown in Table I. All data listed in the table were calculated from manometric determinations. Included for comparison are the rate of oxidation and P/O ratio observed with pyruvate and malate, using beef brain mitochondria prepared in 0.25 M sucrose by the method of Schneider and Hogeboom (14).

The P/O ratios obtained with pyruvate + malate, glutamate, and succinate + amytal are reasonably close to the theoretical values, using mitochondria prepared by either method. With α-ketoglutarate as substrate, ratios approaching four were obtained occasionally but not routinely; the addition of exogenous GDP did not improve the phosphorylation. The P/O ratio observed with α-glycerophosphate as substrate was only 50 to 60 per cent of theoretical (but see polarographic measurements, Table II) and β-hydroxybutyrate was not oxidized by the mitochondrial preparation (Method II).

The specific rate of oxygen uptake (microatom O/minute/milligram protein) may be taken as an indication of the relative purity of preparations, *i.e.* the higher the rate, the less non-mitochondrial protein has been included in the mitochondrial fraction. By use of the rate of oxygen uptake with pyruvate as a criterion, it can be seen that the degree of purity of the mitochondrial fraction prepared by Method II is 3.5 times greater than that of the 0.25 M sucrose mitochondrial fraction and 1.5 times greater than that of the Method I fraction. The rates of oxidation of all substrates tested, except α-ketoglutarate, were higher with the Method II preparation than with the Method I fraction with little change in P/O ratios. The respiratory control ratios indicate that both Methods I and II yield mitochondria which are fairly "tightly coupled." Voss *et al.* (40) have recently demonstrated respiratory control with succinate, α-ketoglutarate, and glutamate in rat brain mitochondria.

The phosphorylative ability of R_4 was not stable to freezing ($-20°C$) and thawing but was stable

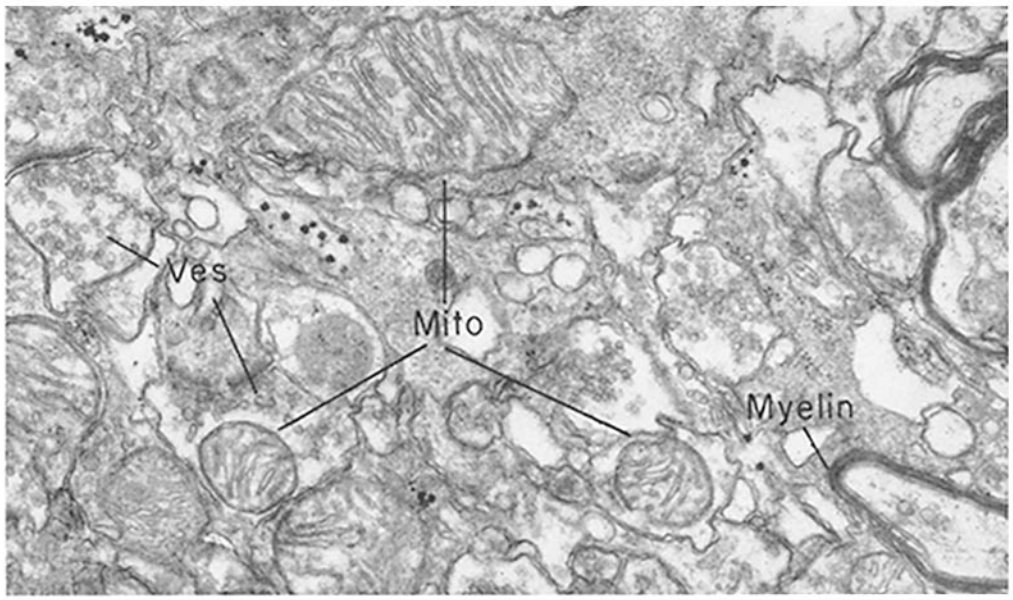

FIGURE 1 An electron micrograph of bovine brain cortex, fixed in buffered osmium tetroxide and stained with lead hydroxide ½ hour after death of the animal. The mitochondria, myelin, and synaptic vesicles (arrows) are well preserved. Abbreviations: mitochondria, *Mito*; vesicles, *Ves*. × 23,000.

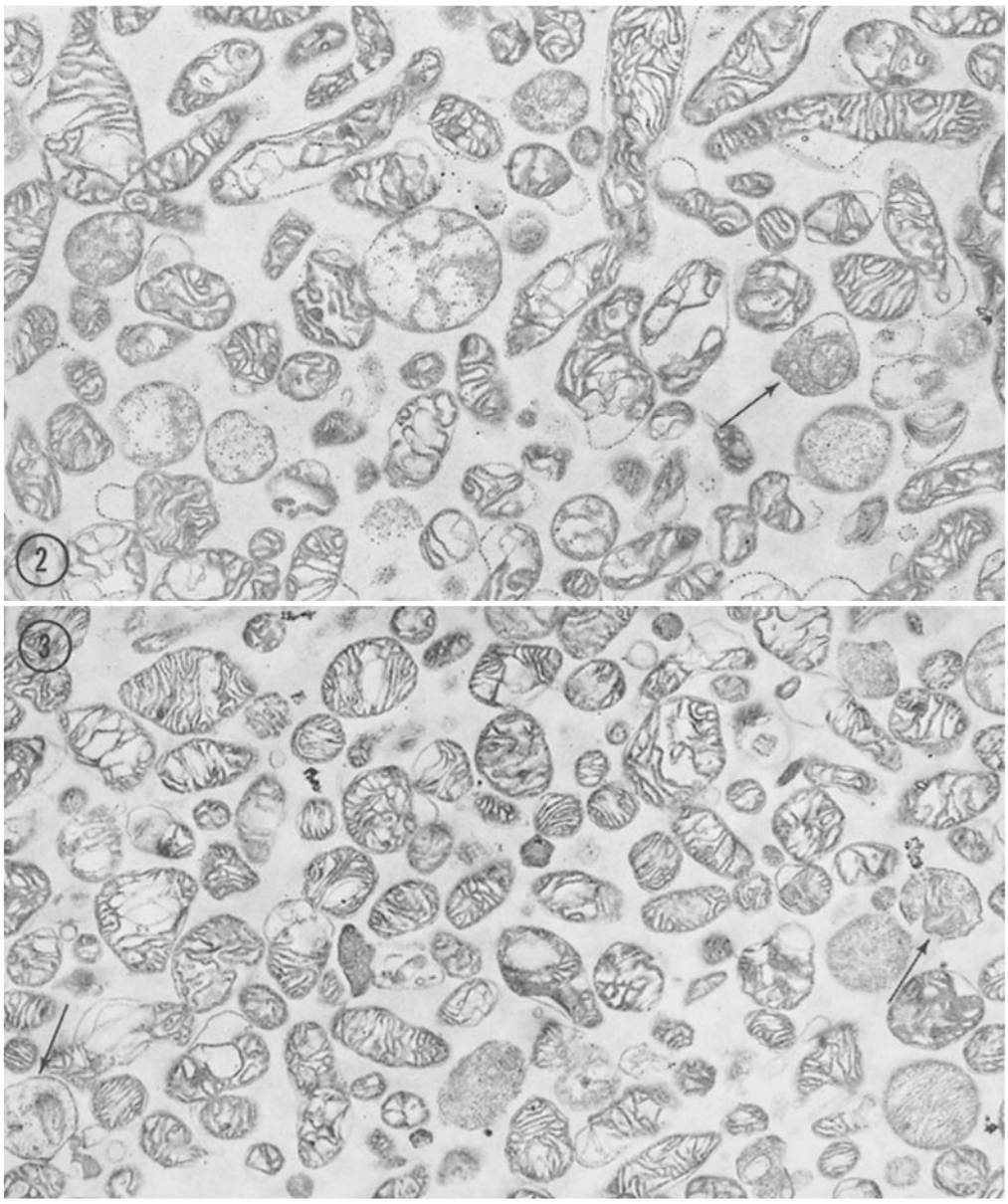

FIGURE 2 Mitochondrial fraction prepared by Method I. A variety of mitochondrial profiles can be observed. The punctate appearance of the membranes is an artifact resulting from the lead hydroxide stain. A particle probably derived from a nerve ending, with an entrapped mitochondrion and synaptic vesicles, is indicated by the arrow. × 11,000.

FIGURE 3 Mitochondrial fraction prepared by Method II. The preparations are free of myelin contamination. Nerve ending particles are indicated by arrows. Stained with lead hydroxide. × 11,000.

(decrease in P/O, 0 to 5 per cent) after storage in ice for 24 hours. After storage in ice for 48 to 72 hours, the P/O ratios diminished 25 per cent, and after 5 days, no phosphorylation could be demonstrated.

Hatefi *et al.* (45) have reported that beef heart mitochondria exhibit no respiratory control with succinate as substrate if amytal is added to prevent oxidation of DPNH produced by further oxidation control (see also Table II). Upon the addition of 20 μmole of succinate, oxygen was consumed at the rate of 0.020 μatom/minute/mg protein. After all of the ADP was phosphorylated, the rate dropped to 0.011 μatom O/minute/mg protein. A second addition of ADP produced a two-fold increase in the rate and a respiratory control ratio of two. The ADP/O ratio was calculated to be 1.95.

TABLE I

Oxidative and Phosphorylative Ability of Mitochondrial Preparations from Bovine Brain

Method of preparation	No. of experiments*	Substrate	Rate‡	P/O§	R.C.‖
0.25 M sucrose	2	Pyruvate + 3 μmole malate	0.045 ± .009	3.06 ± 0.44	—
Method I	8	Pyruvate + 3 μmole malate	0.103 ± 0.034	2.94 ± 0.42	3–10
	3	Glutamate	0.078 ± 0.022	3.08 ± 0.47	3–5
	3	α-Ketoglutarate	0.057 ± 0.022	2.47 ± 0.66	4–5
	5	Succinate + 2.5 μmole amytal	0.165 ± 0.022	1.93 ± 0.17	3–4
Method II	6	Pyruvate + 3 μmole malate	0.157 ± 0.039	3.02 ± 0.03	3–6
	4	Glutamate	0.154 ± 0.066	2.86 ± 0.20	4–5
	4	α-Ketoglutarate	0.053 ± 0.031	2.71 ± 0.32	2–6
	5	Succinate	0.137 ± 0.031	2.18 ± 0.34	2–3
	2	Succinate + 7.5 to 10 μmole amytal	0.214 ± 0.027	1.39 ± 0.17	2–4
	3	α-Glycerophosphate	0.078 ± 0.024	1.78 ± 0.29	1–2
	1	DL-β-Hydroxybutyrate	0.005	0	—

* Each manometric experiment consisted of two "zero time" flasks, two flasks without glucose and hexokinase, and two to four experimental flasks using the standard medium as indicated in the text.
‡ μAtom of oxygen uptake per min. per mg protein at 30°C ± standard deviation.
§ Ratio of phosphate disappearance to oxygen uptake ± standard deviation.
‖ Respiratory control ratio.

of malate. These investigators have suggested, therefore, that respiratory control is associated only with the DPNH oxidase system of beef heart mitochondria. That this is not the case in beef brain mitochondria is indicated in Table I (manometric experiments) and shown by the polarographic trace in Fig. 4. Seconal was used instead of amytal, since it is more water soluble and equally effective as an inhibitor of DPNH oxidation (46). In the presence of 2 μmole of seconal, the oxidation of pyruvate + malate was inhibited 90 per cent and there was no respiratory

The requirements for oxidation and phosphorylation and the effects of some inhibitors with succinate, pyruvate, and α-glycerophosphate as substrates, calculated from polarographic measurements similar to those shown in Fig. 4, are listed in Table II. The addition of dialyzed bovine serum albumin was required for phosphorylation as measured polarographically but not manometrically. Further stimulation of the rate with succinate as substrate was observed upon the addition of $MgCl_2$ (47) or cytochrome *c*. Only a very slow rate of oxygen uptake and no phosphorylation

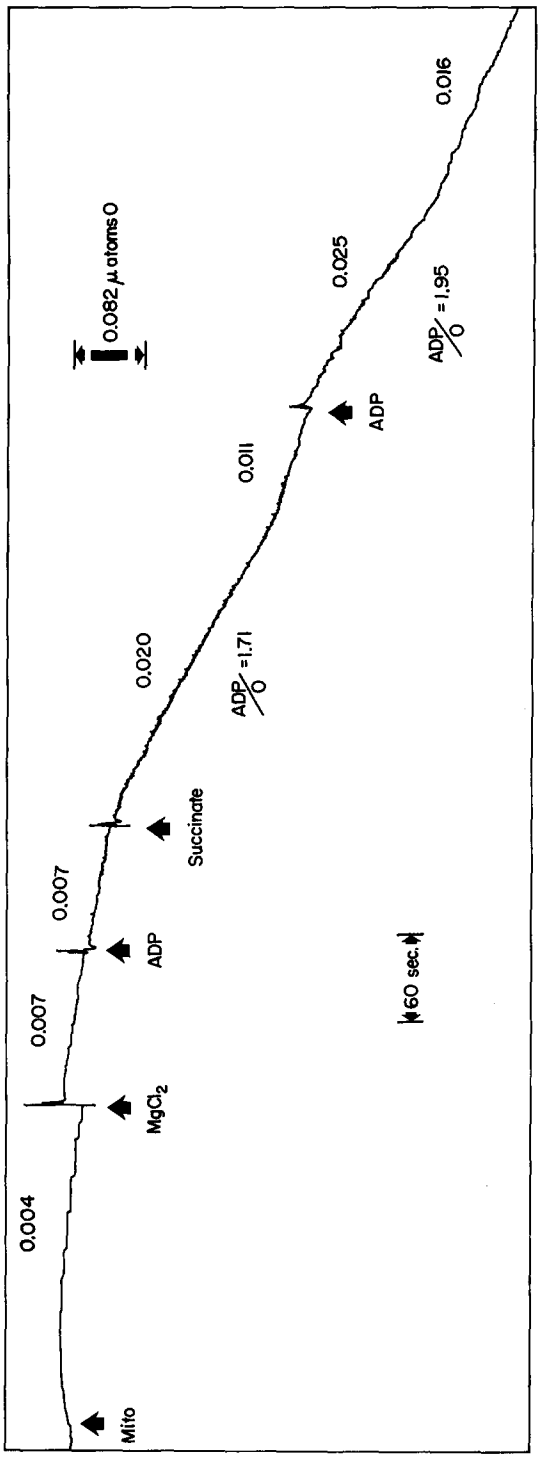

FIGURE 4 Polarographic trace showing respiratory control with *succinate in the presence of seconal*. Mitochondria, Method II, (2.6 mg protein) were added, as indicated by the arrow, to the standard medium (see text) with pyruvate + *malate and 2 μmole seconal*. Also added, as indicated by the arrows, were: $MgCl_2$, 20 μmole; ADP, 0.318 μmole; and succinate, 2.5 μmole. Temperature, 26°C. Rates are indicated on top of the trace in μatom O/minute/mg protein; ADP/O ratios, below the trace.

TABLE II

Requirements for Optimal Phosphorylation and Respiratory Control and Effect of Inhibitors

Substrate	Additions or deletions*	ADP/O	Rate μAtom O/min/mg protein +ADP	Rate μAtom O/min/mg protein −ADP	R.C.‡	No. of Experiments
Pyruvate	None	0	0.009	0.009	—	1
	+ Malate	2.53	0.070	0.016	4.5	5
	+ Malate + DPN	2.11	0.098	0.021	4.7	1
	+ Malate + $MgCl_2$	2.42	0.058	0.014	4.3	1
	+ 2nd addition of ADP	2.64	0.039	0.011	3.5	1
	+ DNP	0		0.051	—	2
	− BSA	0	0.013	0.008	—	1
	+ Seconal ± $MgCl_2$	0	0.007	0.007	—	3
Succinate	None	1.69	0.110	0.047	2.4	6
	+ Cytochrome *c*	1.79	0.134	0.059	2.3	2
	+ $MgCl_2$	1.60	0.149	0.035	4.3	3
	− BSA	0	0.046	0.041	—	2
	+ Seconal	1.49	0.111	0.053	2.1	6
	+ Seconal + $MgCl_2$	1.43	0.117	0.041	2.8	3
	+ 2nd addition of ADP	1.93	0.089	0.024	3.7	2
	+ Pyruvate, malate, and seconal first, then succinate	1.08	0.031	0.021	1.5	2
	+ 2nd addition of ADP	1.79	0.037	0.016	2.3	2
	+ Pyruvate, malate, seconal, and $MgCl_2$ first; then succinate	1.47	0.019	0.012	1.5	2
	+ 2nd addition of ADP	2.03	0.024	0.015	1.6	2
	+ DNP	0		0.063	—	1
α-Glycerophosphate	None	1.39	0.062	0.041	1.5	2
	− BSA	0	0.027	0.030	—	1
	+ EDTA	0	0.057	0.049	1.2	1
	+ $MgCl_2$	1.67	0.068	0.027	2.7	3
	+ EDTA + $MgCl_2$	1.48	0.051	0.017	3.0	3
	+ 2nd addition of ADP	2.39	0.043	0.012	3.6	2
DPNH	None	0	0.016	0.018	—	2

Mitochondria isolated by Method II and assayed polarographically, standard medium as indicated in the text, temperature, 25°C.

* Additions where indicated: Seconal, 2 μmole; $MgCl_2$, 20 μmole; Cytochrome *c*, 4.2×10^{-3} μmole; Malate, 1.5 μmole; DPN, 1 μmole, EDTA; 4 μmole; DNP, 0.24 μmole.

‡ R.C., respiratory control ratio.

was observed with pyruvate in the absence of malate. The addition of DPN to the system oxidizing pyruvate + malate produced a 30 per cent increase in the rate of oxygen utilization and a small diminution of the P/O ratio.

The addition of DNP prior to the addition of substrate (pyruvate + malate or succinate) or cofactors produced no effect on the rate of oxygen utilization. Stimulation of the rate could only be demonstrated by adding DNP after the addition of ADP.

The results obtained here by measuring oxidative rates both manometrically and polarographically are at variance with the results of Bellamy and Bartley (48) who found that brain mitochondria exhibited rates measured manometrically

which were four to five times as great as those obtained polarographically. They found that this was reversed with liver mitochondria, and that pigeon breast muscle mitochondria exhibited nearly identical rates measured by either method.

If the rates of oxidation obtained polarographically as shown in Table II are corrected from 25 to 30°C (assuming a temperature coefficient, Q_5 of 1.4) and compared to those obtained manometrically (Table I), it can be seen that the ratio of the rate obtained manometrically to that obtained polarographically is close to one (0.8 to 1.5).

Sacktor *et al.* (49) reported that rat brain mitochondria oxidize α-glycerophosphate at a faster rate than succinate or any of the other substrates tested. In their system, the α-glycerophosphate oxidation was inhibited 75 per cent by 2 mM EDTA and this inhibition was completely reversed by 10 mM $MgCl_2$. These experiments were carried out in the presence of inorganic phosphate but in the absence of exogenous ADP. As shown in Table II, the effects of EDTA and $MgCl_2$ on the oxidation of α-glycerophosphate by beef brain mitochondria (Method II) were the reverse of those reported by Sacktor *et al.* (49). In the absence of ADP, 2 mM EDTA produced a slight *stimulation* in the rate of oxygen utilization and 10 mM $MgCl_2$ *decreased* the rate by 50 per cent. In the presence of ADP, 2 mM EDTA inhibited oxidation slightly (8 per cent) and this inhibition was reversed by 10 mM $MgCl_2$. Phosphorylation accompanying the oxidation of α-glycerophosphate was completely inhibited by EDTA and restored by $MgCl_2$. Since Sacktor *et al.* did not study phosphorylation with their mitochondrial fraction, it is difficult to compare the two results. It is possible, however, that the rat brain mitochondria studied by these investigators were more tightly coupled and contained more endogenous ADP than the beef brain mitochondria reported here.

There are two pieces of evidence germane to this point, one of which argues for, the other against, this possibility: (*a*) under conditions which gave the highest phosphorylative efficiency (Table II), the oxidation rate with α-glycerophosphate was higher than that with succinate as substrate, in agreement with Sacktor *et al.* Under all other conditions tested, the oxidation rate with succinate was greater than with α-glycerophosphate. On the other hand, (*b*) the rate of oxidation of exogenous DPNH reported by Sacktor *et al.* (0.35 μM O_2/second = 0.034 μatom O/minute/mg protein) was twice that exhibited by our mitochondrial preparation (Table II). The oxidation of exogenous DPNH is usually interpreted to mean some degree of alteration of the permeability of the mitochondrial membrane with a decline in phosphorylative efficiency and respiratory control; thus it would seem that the rat brain mitochondria used by Sacktor *et al.* were not more tightly coupled than the beef brain mitochondria. The differences seen in the effects of EDTA and $MgCl_2$ on the oxidation of α-glycerophosphate may be inherent differences between beef and rat brain mitochondria.

CONTAMINATION: The total contamination by non-mitochondrial particles in the mitochondrial fractions will be the sum of that derived from the less dense particles (microsomes and fluff) and fragments of denser particles which appear in the debris (R_1).

Since electron micrographs allow only a qualitative estimate of the degree of contamination, more accurate means of assessing the purity of the preparation were sought. Glucose-6-phosphatase activity, often used as a microsomal marker (50), cannot be used with brain tissue due to the low activity of the enzyme in this organ (51).

Although a number of previous workers have shown that the brain mitochondrial preparations used in their studies contain large amounts of cholinesterase (ChE), the highest specific activity was found in the microsomal fraction (52–54). De Robertis *et al.* (23) located ChE in the mitochondrial fraction (M) of rat brain homogenates prepared in 0.32 M sucrose; but fraction M could be separated into five distinct fractions by density gradient centrifugation. The ChE activity was found to be highest in fractions containing mainly pinched-off nerve endings with synaptic vesicles and lowest in the fraction with high succinic dehydrogenase activity (mainly mitochondria). Preliminary results of the distribution of ChE in the various fractions of bovine brain homogenates indicated that ChE activity was low in the mitochondrial fraction. It was decided, therefore, to explore further the possibility of using ChE activity as a measure of contamination of the mitochondrial fraction by the microsomes, (R_6) and fluff (R_7). The results of such determinations on fractions prepared by Method I are shown in Table III. These data indicate that the specific activity of ChE in R_6 and R_7 was more than 20-fold higher than the activity in R_4. In the initial

separation of mitochondria, the material superincumbent to the mitochondrial pellet, R_2, was composed of fluff with entrained microsomes. Two fractions of this contaminating particulate material were prepared. One, designated fluff, R_6, still retained a large proportion of the microsomal material; the other, microsomes, R_7, was considerably contaminated with fluff. The impurities in the mitochondrial preparation included both these components. While the ChE is concentrated in the microsomal fraction (52–54) the ChE activity of highly purified microsomes is not suitable for calculation of contamination by both fluff and microsomes. Table III presents the calculated

TABLE III

Cholinesterase Activity of Subcellular Particles from Bovine Brain

Type of preparation	Mitochondria (R_4) specific activity*	"Fluff" (R_6) specific activity*	$\frac{R_4}{R_6} \times 100$	"Microsomes" (R_7) specific activity*	$\frac{R_4}{R_7} \times 100$
Method I	0.21	6.4	3.3	4.45	4.7
	0.29	8.0	3.6	6.88	4.2
0.25 M Sucrose	0.52	1.94	27	2.79	18.7

* μmoles of acetylcholine hydrolyzed per mg protein per hour.

contamination using the specific ChE activity of both R_6 and R_7. The results are similar in each case, lying between 3 and 5 per cent.

The ChE activities for mitochondrial, microsomal, and fluff fractions prepared in 0.25 M sucrose are also shown in Table III. These data would indicate a contamination of 18 to 27 per cent. No hydrolysis of acetylcholine (ACh) was observed in preparations treated with Nagarse (Method II), probably due to proteolysis of the ChE. The assumption that ChE is not an integral constituent of the mitochondria would seem to be a logical inference, since the specific activity of ChE decreases with increasing purity of the mitochondrial preparation, as judged from both electron microscopy and the increase in the rate of oxidation of substrate, as well as from the evidence presented by De Robertis *et al.* (23).

The amount of contamination due to fragments of particles denser than the mitochondrial fraction has been calculated from the DNA content of R_1 and R_4. The results of these determinations are shown in Table IV. It can be seen that the contamination from this source is of the same order as that from R_6 and R_7. Thus, the total contamination from both sources is approximately 10 per cent.

Similar studies were undertaken using RNA as an indicator of microsomal contamination of R_4. For example, for fractionation Method II, the RNA content of the mitochondrial fraction was 13.72 ± 3.6 μg RNA/mg protein, and of fluff and microsomes, R_5, 23.58 ± 2.8 μg RNA/mg protein. Calculation of the contamination on this

TABLE IV

DNA Content of Subcellular Particles from Bovine Brain

Type of preparation	Mitochondria (R_4) DNA content*	Debris (R_1) DNA content*	$\frac{R_4}{R_1} \times 100$
Method I	0.67	12.9	5.2
Method II	0.29	10.8	2.7
	0.30	9.0	3.3

* μg per mg protein of extracted residue.

basis gave values much higher than those obtained from ChE data. Truman and Korner (55) have recently shown a unique RNA fraction in rat liver mitochondria.

Lipid analyses of both the fluff and mitochondrial fractions, when available, may give a better indication of contamination by myelin fragments.

DISCUSSION

The isolation of mitochondria from neural tissue, particularly brain, has been fraught with difficulties. Petrushka and Giuditta (18) and De Robertis *et al.* (23) have urged the use of electron microscopy for evaluation of the quality of brain mitochondrial preparations, and McIlwain and Rodnight (56) have advised caution in interpreting data based on brain mitochondria prepared according to methods developed for other tissues and not further characterized.

Løvtrup and Zelander (27), after examining various methods of preparing mitochondrial fractions from rat and rabbit brain, have concluded

that mitochondria isolated using 0.44 M sucrose exhibit the most satisfactory morphological and enzymic characteristics. The rates of oxidation of various citric acid cycle substrates by their mitochondrial fraction are roughly equivalent to those reported here (35 to 90 μatoms O/hour/mg N = 0.047 to 0.122 μatoms O/minute/mg protein), as are the P/O ratios and respiratory control values. From the electron micrograph shown by Løvtrup and Zelander, it would appear that their mitochondrial fraction is somewhat more contaminated with non-mitochondrial particulate material (myelin for example) than is the mitochondrial fraction prepared by Method II.

Separation of brain homogenates into characterized subfractions has been accomplished by density gradient centrifugation (23, 57–60); however, these studies have been concerned mainly with obtaining preparations of microsomes, nerve endings, synaptic vesicles, etc., rather than with obtaining mitochondrial fractions *per se*. To the authors' knowledge, only Gray and Whittaker (21), Whittaker (59), and De Robertis *et al.* (23) have published electron micrographs of mitochondrial fractions from brain obtained in this manner. No data were given by these authors on the phosphorylative capacity of the mitochondrial subfractions. The electron micrograph shown by De Robertis *et al.* appears comparable to Fig. 3 with respect to contamination, although most of the mitochondria appear swollen; the micrograph by Whittaker indicates a higher degree of contamination.

The density gradient studies have employed sucrose concentrations from 0.80 to 1.4 M. From the work of Brody and Bain (1) and our own experience,[6] markedly hypertonic sucrose depresses oxidative phosphorylation by brain mitochondria. It is probable that density gradient centrifugation in media composed of higher molecular weight compounds (such as Ficoll used in this study), as suggested by McIlwain and Rodnight (56), would be advantageous; however, the amount of mitochondria which can be isolated with currently available high speed swinging-bucket rotors is limited.

Jordan and Darwin (61) have published a preliminary study on the isolation of subcellular particles from brain tissue by filtration through millipore filters. Although a mitochondrial fraction from rat brain has been obtained, the authors state that at the present time the equipment size does not permit the separation of very large quantities of mitochondria.

The combination of parts of previous methods (12, 28, 29) for isolation of mitochondria, which has resulted in Method II, permits the separation of a relatively pure (*ca.* 90 per cent) mitochondrial fraction from beef cortex, using conventional preparative high speed rotors. The use of Nagarse has the disadvantage of destroying some enzymic activities which might be used to determine the amount of contamination by non-mitochondrial particles, but it has no adverse effect on oxidative phosphorylation. Although the yield of mitochondria per gram of tissue is low, the amount of mitochondria which can be obtained is only limited by the amount of starting material and equipment. Manipulations may be reduced by the use of continuous-flow centrifugation.[7]

Many variations in the method of isolation, which are not described here, were tried before Method II was developed. The large variation in cell type and the high lipid content of brain account for the failure of methods developed for other tissues. The use of mainly cortical tissue minimizes some of the problems related to these factors. The addition of PES (or heparin) to the medium minimizes agglutination, and the addition of Ficoll provides the necessary density, without untoward osmotic effects, to permit separation of mitochondria from other particles rich in lipid.

Johnson (12) has used media of 0.30 M sucrose with the addition of 15 to 22 per cent Ficoll for the purification of a rat brain mitochondrial fraction. The activities of succinic dehydrogenase and glycolytic enzymes were measured but no data were reported on oxidative phosphorylation. We found that Ficoll concentrations of greater than 8 per cent yielded mitochondrial fractions which exhibited greatly diminished P/O ratios.[8]

The criteria which have been used to test the enzymic and morphological integrity of mitochondrial preparations include: (*a*) the degree to which theoretical P/O ratios are approached upon oxidation of substrate, (*b*) the extent of respiratory control, (*c*) the lack of requirement for exogenous DPN and cytochrome *c* for oxidation of substrate, (*d*) the degree to which exogenous DPNH is oxidized, and (*e*) the appearance of the isolated

[6] W. L. Stahl, unpublished data.

[7] Unpublished observations using the Sorvall Szent-Gyorgi–Blum continuous flow apparatus.

[8] W. L. Stahl, unpublished experiments.

mitochondria compared with that of mitochondria *in situ* as observed in electron micrographs.

The brain mitochondrial fraction (Method II) satisfies these criteria reasonably well, although the data relevant to criteria *b*, *c*, and *d* indicate a slight degree of alteration of membrane permeability, and some alteration of the mitochondrial structure is clearly indicated by the electron micrographs.

The data presented here indicate that a brain mitochondrial fraction can be prepared which is comparable to those prepared from other tissues. The quality of the preparation with respect to both contamination and enzymic integrity is considered by the authors to be sufficiently high to permit a comparison of the enzymic and chemical properties of mitochondria from brain with the properties of mitochondria of other tissues.

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