The Metabolism of Rat Brain Mitochondria

PREPARATION AND CHARACTERIZATION*

JOHN B. CLARK‡ and WILLIAM J. NICKLAS§

From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104

SUMMARY

Since most previous studies on brain mitochondria have used a relatively crude preparation, a method was developed for preparing a purified mitochondrial fraction from rat brain cerebral cortex utilizing a rapid simple discontinuous Ficoll density gradient procedure. This mitochondrial preparation was shown by electron microscopy and enzymatic assay to be (a) free from contamination by synaptosomes or other membranous fragments and (b) predominantly of neocortical origin. These mitochondria exhibited good respiratory control and respiratory rates with various substrates (pyruvate > succinate > glutamate > citrate, acetylcarnitine, isocitrate, α-ketoglutarate, α-glycerophosphate), comparable with other mammalian mitochondria. Pyruvate plus malate was oxidized at rates higher than any of the other substrates tested and consistently showed respiratory control ratios greater than seven. The mitochondria were further characterized with respect to nicotinamide and adenine nucleotide content, and cytochrome complements and contents. The ratios of cytochrome b:ci:c1:aa3 were 0.6:0.9:1.7:1:0.6, similar to those observed in other mammalian mitochondria. As has been noted previously with brain preparations, increasing the K+ in the incubation medium caused an increase in the oxygen uptake with all the above substrates (except α-glycerophosphate). This effect was studied in more detail with pyruvate plus malate as substrate. Half-maximal respiration rates were obtained with 10 to 20 mM K+ in both ADP and valinomycin-stimulated mitochondria. The effect of K+ was not directly associated with an activation of electron transport per se, but rather with an increase in the availability of substrate for oxidation.

The literature is replete with reports regarding the preparation of mammalian brain mitochondria, e.g. Jöbsis (2) cites 19 and Ozawa et al. (3) cite 26 different preparative procedures, but the resulting data have often been contradictory, misleading, or incomplete. Before engaging in an extensive study of the metabolism of brain mitochondria, therefore, the development of a method for the routine preparation of relatively uncontaminated, metabolically active brain mitochondria was of primary importance. Problems associated with the preparation of brain mitochondria, as compared with other tissues, are often the result of the high lipid content of the brain. This causes the release of membranous fragments on homogenization which undergo spontaneous vesiculation, leading to the formation, of among other artifacts, synaptosomes (4). There is little doubt that the crude mitochondrial preparations used by some workers (e.g. 2, 3, 5, and 6) are contaminated with such material, since this is the starting point for the preparation of synaptosomes (4, 7). This may be responsible for the misleading reports of mitochondrial function.

The mitochondrial preparation described here utilizes a density gradient separation procedure with Ficoll which is specifically designed to remove synaptosomes and other nonmitochondrial material. The method has been developed from existing procedures for the preparation of mitochondria and synaptosomes, particularly those of Stahl et al. (8) and Basford (9), Kurokawa, Sakamoto, and Kato (10), Moore (11), and Salganicoff.1 From the results it may be seen that this preparation exhibits good respiratory quotients and control with no requirement for bovine serum albumin (cf. 7, 8) together with properties which suggest that it is exhibiting a purely mitochondrial function, free from synaptosomal contamination.

METHODS

Preparation of Mitochondria—Male Sprague-Dawley rats (weight 160 to 180 g) fed ad libitum on laboratory chow were decapitated and the cerebral hemispheres rapidly removed into ice-cold isolation medium (0.25 M sucrose-10 mM Tris-0.5 mM K+-EDTA, pH 7.4). The tissue was chopped finely with scissors while being washed frequently with ice-cold isolation medium. The material from eight rats was placed in a Dounce homogenizer (Blaessig Glass, Rochester, New York 14609) together with 40 ml of cold isolation medium, and manually homogenized by eight up and down strokes with a glass pestle (total clearance 1 L. Salganicoff, private communication.

‡ Medical Research Council Traveling Fellow on leave of absence from Biochemistry Department, St. Bartholomew’s Hospital Medical College, University of London, Charterhouse Square, London, E. C. 1, England.
§ National Institutes of Health Postdoctoral Fellow, 1968 to 1970. Present address, Department of Neurology, College of Physicians and Surgeons of Columbia University, New York, New York 10032. To whom inquiries regarding this paper should be sent.

Received for publication, March 10, 1970
0.002 inches). A further 20 ml of ice-cold isolation medium were added and the total homogenate centrifuged at 2° for 3 min at 2,000 × g. The supernatant from this spin was then centrifuged for 5 min at 12,500 × g. The crude mitochondrial pellet was resuspended to a final volume of 10 ml in a 3% Ficoll medium (3% Ficoll:0.12 M mannitol:0.03 M sucrose:25 μM K+-EDTA, pH 7.4). This suspension was carefully layered onto 20 ml of a 6% Ficoll medium (6% Ficoll:0.24 M mannitol:0.06 M sucrose:50 μM K+-EDTA, pH 7.4) and centrifuged for 30 min at 11,500 × g. The supernatant from this spin was decanted and the slight fluffy layer removed from the pellet. The mitochondrial pellet was resuspended in isolation medium and recenterfuged for 10 min at 12,500 × g. The mitochondria were made up to a concentration of 20 mg of protein per ml in the isolation medium. The average yield per rat brain was between 3 and 4 mg of mitochondrial protein. Synaptosomes were prepared essentially according to the method of Bradford (12).

Incubation Conditions—Oxygen uptakes were measured polarographically with a Clark-type microelectrode (Yellow Springs Instrument Company, Yellow Springs, Ohio). The media used routinely contained either 5 or 150 mM K+ and consisted of 5 mM K+ medium: 225 mM mannitol-75 sucrose-5 mM Tris phosphate, pH 7.2-10 mM Tris-Cl, pH 7.4-0.05 mM EDTA-5 mM KCl; all adjusted to pH 7.4 with 2 M Tris; 150 mM K+ medium: 5 mM Tris phosphate, pH 7.2-10 mM Tris-Cl, pH 7.4-0.05 mM EDTA-150 mM KCl; all adjusted to pH 7.4 with 2 M Tris. In those cases where the K+ concentration was varied the mannitol-sucrose concentrations were adjusted to maintain the same osmolality. The incubation temperature in all cases was 28°.

Fluorometric Procedures—Pyridine nucleotide reduction was followed with a modified Eppendorf fluorometer (13) and in some cases, samples were removed for pyridine and adenine nucleotide estimation (14).

Cytochrome Measurements—Low temperature spectra of the cytochromes of mitochondria and synaptosomes were obtained with a split beam scanning spectrophotometer (15, 16). Samples were prepared in cells with a path length of 2 mm by the trapped steady state technique (16). Total cytochrome content was estimated from both the low temperature spectra with the appropriate intensification factors (16) and by the use of the Amino-Chance dual wave length spectrophotometer. The wave lengths used in the latter method were derived from room temperature split beam spectra and the extinction coefficient for each cytochrome was taken from the work of Wilson and Epel (17).

Lactate dehydrogenase activity in subcellular fractions was estimated by following NADH oxidation at 340 nm in a medium containing 50 mM potassium phosphate, pH 7.4, 1 mM pyruvate, 0.2 mM NADH, and 0.3% Triton X-100. Succinate dehydrogenase was measured by the method of Pennington (18). Proteins were measured by the biuret method (19).

Chemicals—Ficoll was obtained from Pharmacia, Uppsala, Sweden, and purified by dialysis before use. Monooxaloacetic acid was a gift of Dr. E. Kun to Dr. J. R. Williamson. Pyruvic acid was twice distilled under vacuum and stored at 2°. All other substrates were commercial preparations of the highest purity available. Enzymes used for fluorometric analyses of pyridine and adenine nucleotides were purchased from Boehringer Mannheim.

Electron Microscopy—This was kindly performed by Dr. Caroline Domsky of the Biology Department, University of Pennsylvania.

The mitochondria were pelleted in 0.15 M phosphate buffer and fixed in 3% glutaraldehyde. After rinsing, postfixation was carried out in 2% osmium tetroxide. The preparations were then dehydrated in alcohol, embedded in Epon and sectioned with a Porter-Blum MT-2 ultramicrotome. Staining was carried out with uranyl acetate in 50% alcohol and lead citrate. The sections were examined with an AEI EM6B microscope (Associated Electric Industries).

RESULTS

Electron micrographs of Fraction G (Table I) indicated a preparation from rat brain which was predominantly mitochondrial and was contaminated only slightly with other subcellular structures and nonspecific membranous material (Fig. 1). The few intact synaptosomes that were present contained mitochondria much different from the bulk of the preparation which suggested that this preparation of brain mitochondria was mainly of non-nervous ending origin.

Further confirmation of the low synaptosomal contamination of this preparation is evident from Table I where the succinate and lactate dehydrogenase activities of each of the fractions of the separation procedure are reported. The crude mitochondrial fraction (Fraction D) possessed a succinate to lactate dehydrogenase activity ratio only slightly higher than the original homogenate (3.8 : 5.6). Since succinate dehydrogenase may be considered as a mitochondrial marker and lactate dehydrogenase as a cytoplasmic marker, this fraction (D) was still substantially contaminated with cytoplasmic inclusions. These inclusions were almost certainly in the form of synaptosomes since such a crude fraction forms the starting point for a synaptosomal preparation (4). It is also worth noting that this fraction often has been used as the final mitochondrial preparation by other workers (e.g., 2, 3, 5, and 6).

However, after passing such a fraction through a discontinuous Ficoll gradient a 6-fold increase in the succinate to lactate dehydrogenase ratio occurred together with the removal of almost 75% of the protein. A further washing removed more protein and increased the succinate to lactate dehydrogenase ratio still further; the final mitochondrial pellet (G) had a ratio of succinate to lactate dehydrogenase 11-fold greater than the original homogenate. The yield, in terms of mitochondrial protein, was on an average 3 to 4 mg of protein per rat brain.

As this preparation of brain mitochondria had a large proportion of the nonmitochondrial contaminants removed, it was felt desirable to characterize and compare this preparation with other mitochondrial preparations. Table II lists the cytochrome content of a crude mitochondrial preparation, the purified preparation as described here, and a synaptosomal preparation (12), as estimated by two different techniques. It may be seen from the low temperature spectra of a fully reduced sample versus a fully oxidized sample (Figs. 2 and 3) that both the mitochondrial and synaptosomal preparation contained the normal complement of mammalian cytochromes absorbing at wave lengths which are similar to those of cytochromes in other tissues. The alpha region of the spectra revealed a reduced cytochrome a and a1 absorption with a maximum at 601 nm for the mitochondria and 599 nm for the synaptosomes, a reduced cytochrome b absorption apparent as a shoulder at 563 nm in both preparations and a reduced cytochrome c peak with a sharp absorption maximum at 548 nm in the mitochondria and 549 nm in the synaptosomes. Cytochrome c1, which can only be visualized clearly in low temperature spectra appeared as a slight shoulder at 556 nm in the
crystalline cytochrome c and dual wave length spectroscopy using
wave length pairs derived from spectra obtained from a split
using an intensification factor derived from control studies on
chromes in the mitochondria were the low temperature spectra
together with the cytochrome b and c shoulders at 430 and 415
nm which were only visible in the mitochondrial preparation.

Washed mitochondria

sgnaptosomal preparation, whereas in the mitochondria it ap-
peared as a split, but distinct, shoulder at 556 and 554 nm. The
Soret region of the spectra showed the typical intense absorption
of reduced cytochrome a and a3 at 443 nm in both preparations
and a total adenine nucleotide content twice that of nicotinamide
nucleotides (Table III). These data confirm the observations of
Klingenberg, Slenczka, and Ritt (5) using a crude mitochondrial
preparation, in which the total nucleotide content was consider-
ably less than that found in mitochondria of other rat tissues
(liver, heart, and kidney).

Additional experiments were carried out to estimate the total
nicotinamide and adenine nucleotide content of these mitochond-
ria. There was approximately 5 times as much NAD as NADP
and a total adenine nucleotide content twice that of nicotinamide
nucleotides (Table III). These data confirm the observations of
Klingenberg, Slenczka, and Ritt (5) using a crude mitochondrial
preparation, in which the total nucleotide content was consid-
ernably less than that found in mitochondria of other rat tissues
(liver, heart, and kidney).

Previous workers (5) had indicated some difficulty in observing
NAD(P) reduction on the addition of substrate (e.g. pyruvate) to
their brain mitochondrial preparations. This function of these
mitochondria was therefore investigated more closely by direct
fluorometric read out and by extraction and enzymatic estima-
tion of the nicotinamide nucleotides. Fig. 4 shows the degree of
reduction of NAD(P) in various metabolic states of brain mito-
chondria oxidizing pyruvate and malate; samples were removed at
various points and enzymatically assayed for the nicotinamide
nucleotides in order to provide confirmatory evidence of the
fluorescence changes. Initially both NAD and NADP were
greater than 90% oxidized. On addition of 2 mM malate, there
was an increase in fluorescence which was not changed on adding
0.2 mM ADP. However, assay at this stage revealed that all the
increase in fluorescence must be attributable to NADP reduction
(83 % reduced) since there was no change in the NAD oxidation-
reduction status. When 1 mM pyruvate was added there was a
sharp increase in fluorescence followed by a slower increase which
late cytochrome concentrations from dual wavelength measure-
ments were oxidized by adding 10 PM rotenone and the measure-
ments were: cytochrome c1 (a1 = 556 nm) was assumed to have parameters equal to those used were the same as above. Cytochrome b was also reduced and estimated by adding 10 µg of antimycin per ml and 5 mM succinate. The low temperature spectra were run in the same medium at protein concentration of approximately 5 mg per ml. All cases the reference (oxidized) sample contained 10 µm rotenone and the measure (reduced) sample 10 µm rotenone plus dithionite. Cytochrome content was calculated assuming an intensification factor equal to that of cytochrome c (la), and the extinction coefficients used were the same as above. Cytochrome c1 (a1 = 556 nm) was assumed to have parameters equal to those of cytochrome c (17). Each value is average of at least three separate estimations.

<table>
<thead>
<tr>
<th>Preparation and Method of Estimation</th>
<th>Cytochrome Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td>Crude mitochondria</td>
<td></td>
</tr>
<tr>
<td>Dual wavelength spectrophotometer</td>
<td>0.025</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.86</td>
</tr>
<tr>
<td>Low temperature spectra</td>
<td>0.029</td>
</tr>
<tr>
<td>Ratio</td>
<td>1</td>
</tr>
<tr>
<td>Purified mitochondria</td>
<td></td>
</tr>
<tr>
<td>Dual wavelength spectrophotometer</td>
<td>0.14</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.88</td>
</tr>
<tr>
<td>Low temperature spectra</td>
<td>0.11</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.61</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td></td>
</tr>
<tr>
<td>Low temperature spectra</td>
<td>0.033</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.59</td>
</tr>
</tbody>
</table>

leveled off at a new steady state (State 4). There was a small increase in NADP reduction but most of the fluorescence was the result of a 5-fold increase in NAD reduction. On the addition of 1 mM ADP (State 3) there was a decrease in fluorescence attributable to a decrease in the reduction of NAD⁺ to one-third of its State 4 value and a slight oxidation of NADP. When rotenone was added all the NADP was reduced but only 70% of the NAD. From these results, therefore, it was concluded that these mitochondria function in a qualitatively similar way to heart mitochondria (21).

Respiration Rates—Table IV shows the respiratory activities of these brain mitochondria in States 3 and 4 in media containing either 5 or 150 mM K⁺. The respiration rates were assessed in both these media since it has been known for some time that K⁺ has a stimulatory effect on the respiration of brain preparations (22) and, more recently, on that of mitochondrial preparations (23). As an indication of their integrity, these mitochondria routinely showed respiratory control quotients of 7 to 10 with pyruvate and malate and 3 to 4 with succinate. Assessment of the actual rates of oxygen uptake with each substrate was complicated by the presence of a small endogenous respiration present when malate and ADP were added.

Although pyruvate alone was not oxidized appreciably, in the presence of malate it was oxidized rapidly in both State 3 and the uncoupled state with carbonylcyanide-p-trifluoromethoxyphenylhydrazone. This is appropriate since pyruvate formed via glycolysis is thought to provide the main substrate for energy metabolism in brain (24). Succinate was oxidized at rates comparable to pyruvate, and was inhibited by malonate, an inhibition which could be 50% reversed by the addition of concentrations of malate equimolar with malonate. Citrate, isocitrate, α-ketoglutarate, and glutamate were all oxidized in the presence of malate at rates about 50% that of pyruvate-malate or succinate. The oxidation of citrate is of interest since it has been reported that citrate permease activity is low in brain mitochondria (25). When the malate-dependent transport of citrate was in-
Table III

Nicotinamide and adenine nucleotide content of rat brain mitochondria

The nicotinamide nucleotide levels are the average values ± S.E.M. for the experiment shown in Fig. 4. The adenine nucleotides were also measured enzymatically in the same 5 mM K+ buffer with the mitochondria respiring in State 4.

<table>
<thead>
<tr>
<th>Total nucleotide</th>
<th>nmoles/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Σ(NAD + NADH)</td>
<td>2.48 ± 0.07</td>
</tr>
<tr>
<td>Σ(NADP + NADPH)</td>
<td>0.52 ± 0.06</td>
</tr>
<tr>
<td>Σ(ATP + ADP + AMP)</td>
<td>5.92 ± 0.1</td>
</tr>
</tbody>
</table>

Addition of 5 mM butylmalonate inhibited oxygen uptake, whereas addition of 5 mM malate partially restored it (50%). Although acetylcarnitine was oxidized at rates comparable to isocitrate or glutamate, acetate itself was not oxidized significantly even in the presence of malate and carnitine. Likewise NADH, octanoate, and β-hydroxybutyrate did not stimulate the endogenous rate of oxygen uptake even in the presence of malate. Monofluoroxalacetate, a malate dehydrogenase inhibitor (27), added in the presence of malate, inhibited the oxidation of endogenous substrate in these mitochondria but pyruvate, added subsequently, was oxidized at a rate which was 40% of the normal pyruvate plus malate rate.

Table IV

Effect of K+ on oxidation of substrates by rat brain mitochondria

The media used contained either 5 or 150 mM K+, and oxygen uptake followed polarographically as described under "Methods." Mitochondrial protein (10 mg) was suspended in a final volume of 3 ml in each case. State 3 conditions were initiated by the addition of 0.5 mM ADP. In each case, the endogenous State 3 rate was measured prior to addition of substrate.

<table>
<thead>
<tr>
<th>Added substrate</th>
<th>State 4</th>
<th>State 3</th>
<th>State 4</th>
<th>State 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>+ 2.5 mM malate</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>+ 2.5 mM pyruvate</td>
<td>10</td>
<td>10</td>
<td>14.5</td>
<td>14.5</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 10 μM FCCP</td>
<td>17</td>
<td>106</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 10 μM FCCP</td>
<td>17</td>
<td>106</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>+ 1 mM pyruvate</td>
<td>39</td>
<td>39</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 10 μM acetylcarnitine</td>
<td>59</td>
<td>27</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>+ 2.5 mM malate</td>
<td>17</td>
<td>66</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 5 mM butylmalonate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ 5 mM malate</td>
<td>17</td>
<td>66</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>+ 5 mM citrate</td>
<td>20</td>
<td>56</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>+ 5 mM malate</td>
<td>39</td>
<td>39</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>+ 1 mM pyruvate</td>
<td>110</td>
<td>47</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 5 mM α-ketoglutarate</td>
<td>16</td>
<td>46</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 5 mM α-ketoglutarate</td>
<td>16</td>
<td>46</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 2 mM glutamate</td>
<td>11</td>
<td>65</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 10 mM acetate</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>+ 1 mM carnitine</td>
<td>34</td>
<td>122</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td>10 mM succinate</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>+ 5 mM malonate</td>
<td>20</td>
<td>0</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>+ 5 mM malate</td>
<td>66</td>
<td>83</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>+ 25 mM malate + 1 mM octanoate</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 1 mM β-hydroxybutyrate</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>0.5 mM NADH</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ 2.5 mM malate</td>
<td>18</td>
<td>18</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>+ 2.5 mM malate + 10 μM rotenone</td>
<td>56</td>
<td>66</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>+ 10 μM rotenone</td>
<td>48</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>+ 10 μM D,L-α-glycerophosphate</td>
<td>52</td>
<td>52</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

* The abbreviations used are: FCCP, carboxylic anhydride-p-trifluoromethoxyphenylhydrazone; F6OAA, monofluoroxalacetate.

This confirms the findings of Kun and Volfin (27). α-Glycerophosphate was also oxidized by these mitochondria (28).

With the exception of α-glycerophosphate, the respiratory rates in the 150 mM K+ medium were greater than those found in the 5 mM K+ buffer with all the above substrates. With either pyruvate-malate (Fig. 5) or succinate (Fig. 6) as substrates, there was a half-maximal stimulation of oxygen uptake in State 3 at approximately 20 mM K+. Ozawa et al. (23) previously reported a half-maximal stimulation of glutamate and succinate oxidation with 40 mM K+ in their brain mitochondrial preparation. The rates in State 4 with both substrates also doubled on increasing the K+ concentration but more gradually. The addition of valinomycin, an antibiotic which stimulates the K+-accumulating mechanism of mitochondria (29), caused a stimulation of the State 4 respiration to a rate approaching that of State
FIG. 5. Effect of K+ on oxygen uptake of rat brain mitochondria incubated with 1 mM pyruvate-2.5 mM malate in presence and absence of ADP or 5 ng per ml of valinomycin (Val). The media contained varying amounts of KCl; sucrose and mannitol were correspondingly adjusted to maintain isotonicity with the media described under "Methods." Other conditions are the same as those in Table IV. St, state.

FIG. 6. Effect of K+ on oxygen uptake of rat brain mitochondria incubated with 5 mM succinate in the presence and absence of ADP. The endogenous oxygen uptake of these mitochondria was inhibited by addition of 10 μM rotenone, otherwise, the experimental conditions are those in Fig. 5. St, state.

FIG. 7. Effect of valinomycin on the oxygen uptake of rat brain mitochondria incubated with 1 mM pyruvate plus 5 mM malate in media containing either 5 or 150 mM K+. Conditions are those described in Table IV. Val, valinomycin.

3 at that particular concentration of K+ (Fig. 5). The concentration of valinomycin used to achieve maximal stimulation was the same (4 to 5 ng per mg of protein) for both the 150 and 5 mM K+ media (Fig. 7).

To examine the hypothesis that K+ exerts a direct effect on the cytochrome system in brain mitochondria (23), low temperature cytochrome spectra were made in the presence and absence of ADP with pyruvate-malate as substrate (Table V). In media containing either 5 or 150 mM K+, the addition of ADP caused the oxidation of the cytochromes. In the case of cytochrome b, approximately half that reduced in State 4 became reoxidized in State 3. However, in both States 3 and 4 twice the amount of cytochrome b was reduced in the 150 mM K+ medium as in the 5 mM K+ medium (Table V).

DISCUSSION

It has been known for some time that brain mitochondria can be prepared relatively free from contaminating subcellular particles by density gradient separation procedures (7, 30, and 31). However, in these techniques the mitochondria are centrifuged at very high speeds in extremely hypertonic sucrose concentrations for long periods of time. Thus, the metabolic activities of the preparation are limited and not easily correlated with mitochondria from other tissues which are not subjected to such rigorous preparative procedures. Because of these drawbacks, many workers have preferred to prepare brain mitochondria with various adaptations of the Schneider and Hogeboom (32) procedure for liver mitochondria (e.g. 2, 3, 5, 6, 33, and 34). This crude brain mitochondrial fraction has been shown to be grossly contaminated with other subcellular particles and fragments such as myelin (8) and synaptosomes (4, 30). Indeed, the latter authors use this crude fraction as the starting point in their separation of synaptosomes. The resulting contamination of the crude fraction by nonmitochondrial protein and enzymes could well explain the inconsistencies in the metabolic properties attributed to brain mitochondria in some of the above reports, e.g. the data of Klingenberg et al. (5) indicated that NAD was not reduced by brain mitochondria oxidizing pyruvate and malate. This observation can be explained by the presence of lactate dehydrogenase in synaptosomal inclusions in the preparation. Other reports of lactate utilization by brain mitochondria (35) can be similarly explained. Once this contamination is removed, as in the present work (Table I), NAD is rapidly reduced by pyruvate and malate (Fig. 4).

Thus, to study the metabolism of isolated brain mitochondria, it is necessary to use a procedure which avoids the rigors of the sucrose density gradient separation method (hypertonicity and time) but succeeds in purifying the mitochondrial fraction. This paper reports a simple, rapid, discontinuous gradient method...
using Ficoll for the purification of a crude mitochondrial fraction which provides brain mitochondria suitable for metabolic investigation. The method described here differs from other published methods utilizing Ficoll in several important respects. Kurokawa et al. (10) developed a rapid method for isolating nerve ending particles with a gradient consisting of 0.32 M sucrose, 3% Ficoll, and 13% Ficoll. Although this procedure has no known deleterious effects on synaptosomal metabolism, Stahl et al. (8) have shown that Ficoll solutions in excess of 8% yield mitochondria with poor respiratory activity. Stahl et al. (8) did not use a discontinuous Ficoll gradient but suspended the crude mitochondrial pellet in an 8% Ficoll medium and then centrifuged. The resulting mitochondria have an absolute requirement for bovine serum albumin to show respiratory activity and control and, in addition, are able to oxidize exogenous NADH and cytochrome c. The mitochondria described here did not have these limitations.

Electron microscopy (Fig. 1) showed little contamination of this preparation by synaptosomes; the lactate dehydrogenase distribution (Table I) indicated that this synaptosomal contaminant was less than 2%. Salganicoff and Koeppe (36) have reported that 75% of the succinate dehydrogenase activity in brain is associated with nerve ending mitochondria. The results in Table I, therefore, are consistent with there being little contamination of this preparation by nerve ending particles or mitochondria derived from them; this suggests the mitochondria described here are primarily of glial origin. Further inspection of the electron micrographs reveals only slight contamination with mitochondrial or other membranous fragments such as myelin.

Since this brain mitochondrial preparation is different from those described previously, the mitochondria were characterized with respect to cytochrome content, nucleotide content, and substrate utilization prior to metabolic investigations. The cytochromes found in these mitochondria were similar in their spectral properties, in total content on a per mg of protein basis and in their relative concentrations (Table II) as those found in other mammalian mitochondria (20, 37). The results are consistent with the data found on brain mitochondria by Backtor and Packer (28) and Klingenberg et al. (5) if allowance is made for the relative protein contents of the preparation but are inconsistent with the values reported by Williams (38) who used a different method of extraction and estimation. This is further emphasized by the observation that, apart from cytochrome c, the cytochrome contents of the crude and purified mitochondria and synaptosomes have similar relationships to each other and only differ when they are related to the protein content of the preparation. The additional cytochrome c in the crude mitochondrial fraction is probably of extramitochondrial origin.

The total content of pyridine nucleotide was 2- to 3-fold greater on a per mg of protein basis (Table III) than those published by others (5, 28), and the ratio of NADP: NAD in these mitochondria was 30% greater than that reported by Klingenberg et al. (5). This latter difference may be caused by the greater purity of the preparation or because of more efficient techniques of extraction and estimation.

The brain mitochondria consistently showed a small, but significant, oxygen uptake on addition of malate and ADP (Table IV). This oxidation of malate was accompanied by the formation of citrate (1 to 2 nmoles min⁻¹ mg of protein⁻¹) indicating that acetyl-CoA from endogenous sources was also being utilized. The presence of this malate-stimulated endogenous respiration accentuates the importance of the order of addition of substrates and ADP to the mitochondrial suspension. Indeed, one could observe an apparent oxidation of an added substrate when, in fact, this was the result of the endogenous respiration. Since in much of the earlier literature, there was no recognition of this fact, the resulting data concerning respiration rates are often uninterpretable (e.g., 3, 23, 28). It has been suggested that glutamate is the chief endogenous substrate for isolated brain mitochondria (39, 40). However, the mitochondria used in the latter studies correspond to the crude mitochondrial fraction described in this paper, and conclusions were based on measured amino acid contents of the preparation. We have observed that, while there is a significant amount of glutamate in this crude fraction, the purified mitochondria contain less than 5 nmoles per mg of protein. This is hardly sufficient to maintain the observed endogenous rates. It is more likely that fatty acids are the endogenous fuels utilized by isolated brain mitochondria.

The highest respiration rate and respiratory control of all the substrates tested was shown by pyruvate plus malate (Table IV). This is entirely consistent with current models of brain metabolism in which 90% of respiration is accounted for by the products of glycolysis (24). The absolute requirement of malate for pyruvate oxidation could have several explanations. The isolated mitochondria may be extremely depleted of oxalacetate and malate serves as a source of this compound. In view of the observed enhancement of respiration by K⁺, perhaps the malate acts to facilitate the transport of K⁺ into the mitochondria. Harris, Cockrell, and Pressman (29) have suggested that K⁺ may penetrate the mitochondria as salts of substrate anions such as malate.

These mitochondria utilized a complement of substrates normal for mammalian mitochondria. It is of interest to note that citrate and isocitrate were oxidized at rates comparable to that of α-ketoglutarate or glutamate. These observations are inconsistent with the reports by Chappell (25) that the tricarboxylic acids are not utilized very well by brain mitochondria. The relative intactness of the mitochondria was indicated by the fact that exogenous NADH was not oxidized.

The oxidation of 5 mM L-α-glycerophosphate, which was very much insensitive, was unique among the substrates tested in that the respiration was lower in the 150 mM K⁺ than in the 5 mM K⁺ at the same α-glycerophosphate concentration. Preliminary experiments indicate that this may be caused by a change in the Kₐ₅ for α-glycerophosphate concentration. Measurements of the state of reduction of cytochromes (Table V) by trapped steady state technique (15) indicated, at least in the

1 J. B. Clark and W. J. Nicklas, unpublished observations.
2 J. B. Clark, W. J. Nicklas, and A. Azzi, unpublished observations.
presence of ADP, no forward crossovers between O₂ and cytochrome b that would support such a conclusion. In State 4, there is a reverse crossover between cytochromes c, c₁, and b when K⁺ is increased. This could be caused by an inhibition at this site by K⁺ or K²⁺ might activate at some site between oxygen and cytochromes c₁ and c. However, in both States 3 and 4, there was a forward crossover between cytochrome b and substrate oxidation. Thus, the principal effect of increasing K⁺ in the medium is to enhance the availability of substrate for oxidation (Figs. 5 and 6). Valinomycin, which enhances the transport of K⁺ into mitochondria (29), when titrated with brain mitochondria respiring in State 4 in medium with 5 or 150 mM K⁺, yielded maximal rates identical with the rates found with mitochondrial K⁺ sensitivity of liver mitochondria proposed that K⁺ sensitivity resides in reactions closely allied with phosphate esterification. Therefore, the rate of transport of K⁺ into the mitochondria would appear to be intimately associated with the achievement of maximal respiratory rates. This stimulation may well be associated with the classic observation that K⁺ stimulates respiration and glycolysis in other brain preparations (22), as well as altering the metabolism of amino acids in brain slices (43, 44). Pressman and Lardy (45) in their studies on the potassium requirements of liver mitochondria proposed that K⁺ sensitivity resides in reactions closely allied with phosphate esterification. The activity of the multi-enzyme pyruvate dehydrogenase complex isolated from mitochondria of bovine kidney and heart and pig liver has been shown recently to be regulated by phosphorylation and dephosphorylation reactions catalyzed by an ATP-dependent kinase and a phosphatase (46, 47). Thus, the K⁺-stimulated oxidation of pyruvate by brain mitochondria may be a reflection of the state of activation of the pyruvate dehydrogenase complex. It is interesting to conjecture whether the analogous α-ketoglutarate dehydrogenase is similarly regulated. The more detailed mechanism by which the stimulation occurs with brain mitochondria is the subject of a forthcoming publication.

This report has concerned itself with the preparation and some of the properties characterizing rat brain mitochondria. This preparation has been shown to be metabolically active and substantially free from nonmitochondrial contamination. It is hoped that the use of this preparation will help solve some of the perplexities which too often have been associated with the study of the metabolism of the central nervous system.

Acknowledgments—We wish to thank Dr. J. R. Williamson for his constant encouragement and advice during the course of this work. We are further indebted to Professor Britton Chance for fruitful discussion and criticism and to Dr. D. F. Wilson for his help and advice on the cytochrome studies.

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The Metabolism of Rat Brain Mitochondria: PREPARATION AND CHARACTERIZATION

John B. Clark and William J. Nicklas


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