of pipecolic acid may be generalized among other fungi as well as *R. leguminicola*.

Figure 2 visualizes that the initial steps of lysine catabolism in *R. leguminicola* are *via* D-lysine and D-N^6-acetyllysine, whereas L-lysine is utilized for the synthesis of L-pipecolate, which in turn serves as a precursor for the synthesis of piperidine alkaloids in this mold. Purification of the lysine racemase should permit a careful study of its properties and permit a clearer view of its physiological role in determining the flow of lysine metabolites relative to the pathways of Figure 2.

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Heterogeneity of the Outer Membrane of Mitochondria†

C. T. Huber and M. Morrison*

ABSTRACT: The arrangement of proteins in the outer membrane of rat liver mitochondria was explored. Exterior membrane proteins of the mitochondrion were labeled with ^{125}I using lactoperoxidase-catalyzed iodination. The iodine label thus served as a marker for outer membrane proteins. The specific activity of the outer membrane was 12-fold greater than that of the mitochondria from which it was isolated by digitonin treatment. Adenylate kinase, a soluble enzyme located between the outer and inner membranes, was assayed at various stages in the experiments to determine whether or not the mitochondria were intact. After iodination of the mitochondria the polypeptides of the organelles were separated into molecular weight classes by electrophoresis in a sodium dodecyl sulfate–acrylamide gel system. When intact mitochondria were iodinated, the label was distributed among polypeptides in 12 molecular weight classes. In microsomes the label was found in polypeptides of nine molecular weight classes. Although the iodinated polypeptides of both mitochondria and microsomes ranged from about 10,000 to 100,000 daltons, the radioactive gel patterns of the two organelles were quite different. About 70% of the label in the outer membrane isolated from the iodinated mitochondria was in polypeptides of the 14,000 molecular weight class. The distribution of the labeled polypeptides in the various molecular weight classes was different for proteins in isolated outer membrane fragments as compared to proteins remaining with the inner membrane matrix particles after the digitonin fractionation. This selective fractionation of outer membrane proteins suggested that the proteins were heterogeneously distributed in the plane of the membrane.

One of the most commonly used procedures for identification and determination of purity of subcellular components in cytochemical studies is the use of marker enzymes. Problems arise if the marker enzyme activity is found in more than one location. An example of this is the rotenone-insensitive NADH–cytochrome *c* reductase found in the outer membrane of mitochondria which is quite similar to the NADH–cytochrome *c* reductase system of microsomes (Raw and Mahler, 1959; Raw *et al.*, 1960; Sottocasa *et al.*, 1967a,b). Several other examples of enzymes in mitochondria with dual locations have been reported (Ernster and Kuylen-

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stierna, 1970; Brunner and Bygrave, 1969; Van Tol and Hulsmann, 1970; Landriscina *et al.*, 1970; Sarzala *et al.*, 1970). Historically there has sometimes been confusion and controversy concerning the location of marker enzymes in mitochondria (Sottocasa *et al.*, 1967a,b; Ernster and Kuylenstierna, 1970; Schnaitman *et al.*, 1967; Allmann *et al.*, 1968; Green *et al.*, 1968; Greenawalt and Schnaitman, 1970) due to the use of unproved fractionation methods.

The difficulties mentioned above as well as the instability of some enzyme activities have prompted the use of an alternative to marker enzymes as a method for the characterization of the outer membrane of mitochondria. Exposed proteins on the outer membrane were labeled with iodine-125 using lactoperoxidase-catalyzed iodination as was described previously for erythrocyte (Phillips and Morrison, 1970) and Ehrlich ascites (Morrison and Gates, 1972) plasma membranes. Introduction of the iodine label into the outer membrane proteins avoided some of the difficulties associated with the use of enzyme markers in identification and evaluation of membrane preparations. The iodination method permitted easy detection of selective fractionation of exposed membrane proteins, which was useful in assessment of the heterogeneity of the outer membrane.

Materials and Methods

Reagent grade chemicals were used in all experiments. Iodide ($Na^{125}I$) was purchased from New England Nuclear Corporation. Lactoperoxidase was isolated by the method of Morrison and Hultquist (1963).

Preparation of Mitochondria. Female Sprague-Dawley rats weighing 125–150 g were decapitated. Their livers were then excised and immediately minced into the isolation medium. The isolation medium contained 0.225 M mannitol, 0.075 M sucrose, 0.001 M EDTA, and 0.020 M morpholinopropanesulfonic acid at pH 7.4. All the solutions were kept at 4° during the course of the isolation of the mitochondria from the liver homogenate. The homogenate was centrifuged for 10 min at 830*g* and the supernatant was then centrifuged at 3300*g* for 10 min. The resulting pellet was washed by repeating the last step twice. In certian experiments the mitochondria were further purified after iodination by centrifugation at 55,000*g* for 3 hr through a linear sucrose gradient (1.0–1.8 M at 4°). The bands were removed from the gradient with a J pipet.

Preparation of Microsomes. The microsomal fraction was obtained from the supernatant of the 3300*g* 10-min centrifugation described above by the method of Remmer *et al.* (1967). The microsomes were resuspended in the isolation medium prior to use.

Digitonin Fractionation of Mitochondria. Outer membrane and the inner membrane–matrix fraction were obtained by the method of Schnaitman and Greenawalt (1968). The fractionation was carried out at 0–4°. In this procedure mitochondria were suspended to a concentration of 100 mg/ml of mitochondrial protein in the isolation medium containing 0.5 mg/ml of bovine serum albumin. To this suspension 1.2 mg of digitonin was added per 10 mg of mitochondrial protein, diluting the concentration of mitochondrial protein to 50 mg/ml. The mixture was stirred for 15 min and then was immediately diluted with three volumes of isolation medium containing bovine serum albumin and gently homogenized in a glass grinding vessel with two strokes of a Teflon plunger. The suspension was then centrifuged at 8000*g* for 10 min and the supernatant was carefully removed. The pellet was then resuspended in the same volume of isolation medium with bovine serum albumin and again centrifuged. The resulting pellet constituted the inner membrane matrix (IMM)[1] fraction, and the two supernatants were combined.

Iodination Procedure. Mitochondria, microsomes, and IMM particles were suspended in isolation medium to a protein concentration of 10 mg/ml. Lactoperoxidase was added to obtain a final concentration of 0.3 μM. A concentration of 1 mCi/ml of ^{125}I was obtained by addition of carrier-free stock. Each millicurie of the carrier free radionuclide was added to 1.7 μl of 1.0 mM sodium sulfite prior to use. The purpose of the sulfite is to reduce any I_2 present to I^-. This is important because I_2 is lipophilic and will readily penetrate membranes. It is an iodinating agent and is therefore capable of labeling proteins independent of their vectorial position in the membrane. Thus proteins on the inner surface of a membrane or even interior proteins could be labeled with I_2. It should also be pointed out that iodine will add to unsaturated lipids and thus label these components of the membrane as well.

Iodination was initiated by addition of H_2O_2. Each addition contained enough H_2O_2 to produce a final concentration of 10 μM in the iodination mixture. Hydrogen peroxide is a substrate for both glutathione peroxidase and catalase which are present in the mitochondrial preparation. These enzymes can compete with lactoperoxidase for the peroxide which is quickly destroyed. In a typical experiment, 30 aliquots of hydrogen peroxide were added to mitochondrial preparation at 30-sec intervals and 3–5 aliquots to a microsomal, IMM, or OM preparation at 60-sec intervals between additions. The reaction mixture was never diluted more than 5% by the peroxide addition. Control experiments contained all reagents except lactoperoxidase. The iodination procedure was performed at 0°.

After iodination the mitochondria or microsomes were centrifuged and the pellets were resuspended for 30 min at 0° in isolation medium containing 2.0 mM KI. Pellets obtained from subsequent centrifugation were then frozen or used immediately.

Lipid Extraction. Lipids were extracted from mitochondria and the extract washed to remove nonlipid contaminants as described by Rouser and Fleischer (1967).

Gel Electrophoresis. Samples containing about 300 μg of mitochondrial protein were incubated in 100–150 μl of 2–3% sodium dodecyl sulfate and 1% mercaptoethanol in a boiling water bath for 3 min. The sample was then subjected to electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate in 0.1 M phosphate, pH 7.2–7.4. After electrophoresis the polypeptides were visualized by staining with Coomassie Brilliant Blue according to a modification of the method of Weber and Osborn (1969). In the modified method 0.025% (w/v) Coomasie Brilliant Blue was used, and the gels were destained in a solution of 7.5% acetic acid–5.0% methanol.

The distribution of the iodine label was determined by cutting unstained gels into 2-mm slices and counting in a γ spectrometer. In order to correlate the positions of polypeptides in different gels, dansylated ovalbumin, carbonic anhydrase, and cytochrome *c* (Talbot and Yphantis, 1971) were employed as fluorescent markers. These proteins were

[1] Abbreviations used are: dansylated, 5-dimethylaminonaphthalene-1-sulfonyl derivative; IMM, inner membrane matrix; OM, outer membrane; and HSS, high-speed supernatant.

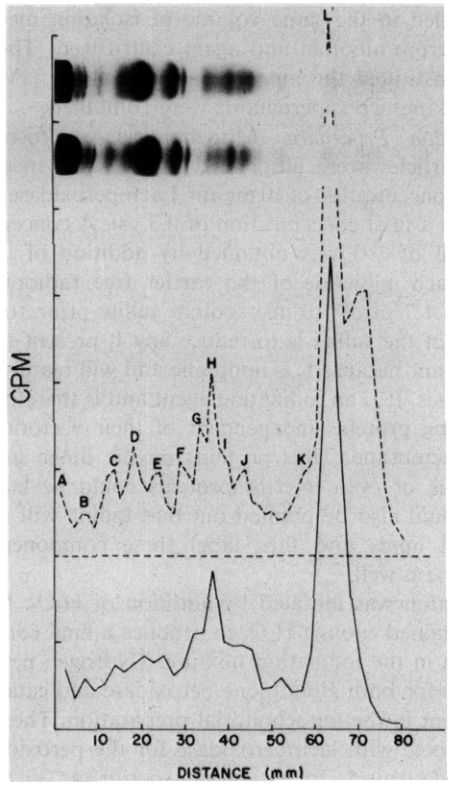


FIGURE 1: Acrylamide gel. The mitochondrial polypeptides separated into molecular weight classes by electrophoresis in sodium dodecyl sulfate–acrylamide gels. The upper gel contains protein of mitochondria obtained by differential centrifugation and iodinated using lactoperoxidase as described under Materials and Methods. The lower gel contains protein of iodinated mitochondria further purified by passage through a sucrose gradient. The gels were stained with Coomassie Blue in order to visualize the polypeptides. The distribution of iodine label in comparable gels is shown in the graphs: (- - - -) mitochondrial proteins of upper gel; (——) proteins of lower gel.

added to the sample before electrophoresis, and their position in the gel made possible a more accurate standardization of the sizes of the mitochondrial polypeptides. Phosphorylase *a* (mol wt 94,000), bovine serum albumin (mol wt 68,000), carbonic anhydrase (mol wt 29,000), and myoglobin (mol wt 17,200) were used as standards with each group of samples electrophoresed on the sodium dodecyl sulfate gels. The apparent molecular weights of various iodinated polypeptides were determined from a plot of log molecular weight *vs.* distance migrated.

The amount of label in each molecular weight class was estimated from the gel radioactivity profiles. The background counts were only a few per cent of the experimental counts but were subtracted in all of the data. Corrections for control experiments, which did not contain lactoperoxidase, were made when necessary but usually the controls did not make a significant contribution. The total radioactivity was the sum of the label incorporated into polypeptide molecular weight classes A–L.

Electrophoresis on basic gels was performed by the method of Davis (1964) except that the gels contained 1% Triton X-100.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951).

Adenylate Kinase Assay. Adenylate kinase was assayed at 25° by the method of Schnaitman and Greenawalt (1968) which involved adenylate kinase catalysis of ADP disproportionation to AMP and ATP. The ATP formed was coupled by hexokinase to the phosphorylation of glucose which was subsequently oxidized by glucose-6-phosphate dehydrogenase with the concomitant reduction of $NADP^+$. The $NADP^+$ reduction was monitored spectrophotometrically at 340 nm. The reaction mixture contained the following final concentrations in 1 ml of 70 mM glycylglycine buffer (final pH 8.0): 15 mM glucose, 5.0 mM $MgCl_2$, 0.75 mM $NADP^+$, 10 IU of hexokinase, 0.5 IU of glucose-6-phosphate dehydrogenase, 0.45 mM KCN, and 3.0 mM ADP. Particulate samples were incubated with Lubrol (0.3 mg of Lubrol/mg of protein) at 0° for at least 15 min prior to assay. ADP was incubated in the reaction mixture for 5 min prior to sample addition in order to consume trace amounts of ATP in the ADP.

Monoamine Oxidase Assay. Monoamine oxidase was assayed at 25° by the spectrophotometric method of Tabor *et al.* (1954). The production of benzaldehyde from benzylamine in 0.05 M sodium phosphate (pH 7.6) was monitored at 250 nm.

Results

Iodination of Mitochondria and Microsomes. The exterior proteins of the intact rat liver mitochondria were labeled using the lactoperoxidase macromolecular probe system. The mitochondrial proteins were solubilized in sodium dodecyl sulfate and the polypeptides separated on the basis of size by sodium dodecyl sulfate–acrylamide gel electrophoresis. As shown in Figure 1, a number of polypeptides of varying molecular size were labeled. The most highly labeled material was in the 14,000 molecular weight class. In order to determine whether or not there were any significant contributions by contaminating organelles, the iodinated mitochondrial preparation was further purified by centrifugation through a sucrose density gradient. Most of the material banded around a density of 1.19 at 4°.

The radioactivity profile from sodium dodecyl sulfate–acrylamide gel electrophoresis of iodinated mitochondria which had been purified by the gradient was compared with the profile from the preparation which had not been purified through the gradient. Figure 1 shows that the radioactivity pattern was not significantly different after the gradient purification except that much of the iodinated low molecular weight material which migrated ahead of the largest peak of radioactivity (peak L in Figure 1) seemed to be removed. This low molecular weight iodinated material often appeared as a shoulder or skewing of the lower part of peak L and seemed to be somewhat variable in amount from one preparation to another. In a comparison of the gels in Figure 1 stained for protein from the two preparations, the gels appeared to be identical. On the 10% acrylamide gels used in this work, about 25 polypeptides could be resolved. Although much of the radioactivity is associated with peak L, there is not much staining in this region of the gels.

The iodinated mitochondrial peptides in Figure 1 corresponded to the following molecular weights: (A) 100,000; (B) 86,000; (C) 71,000; (D) 63,000; (E) 53,000; (F) 42,000; (G) 38,000; (H) 33,000; (I) 29,000; (J) 26,000; (K) 16,000; and (L) 14,000. The labeled polypeptides A, I, and K are shoulders in Figure 1, but occasionally they have appeared as distinct peaks in other experiments.

The use of dansylated proteins as internal markers was helpful in establishing the molecular weight classes of the iodinated polypeptides. Peak H in Figure 1 migrated somewhat behind the dansylated carbonic anhydrase in the so-

dium dodecyl sulfate gels, while peak L migrated close to the dansylated cytochrome *c*. Although ideally the distance of migration into a sodium dodecyl sulfate gel is proportional to the log molecular weight (Shapiro *et al.*, 1967), there are instances in which this is not the case due to various complicating factors. Neville (1971) has reported that hemoglobin, cytochrome *c*, and other proteins do not migrate in his system strictly according to their size. Factors such as carbohydrate moieties of glycoproteins (Segrest *et al.*, 1971) and charge (Tung and Knight, 1971) have been reported to affect the apparent molecular weight of polypeptides determined in sodium dodecyl sulfate–acrylamide gel systems. Fish *et al.* (1970) have reasoned that polypeptides with molecular weights below 15,000 may display erratic properties in a sodium dodecyl sulfate solution because they may act as spheres rather than rods. Swank and Munkres (1971) have discussed the problems with polypeptides of molecular weight below 10,000. Discrepancies in molecular weights obtained with sodium dodecyl sulfate gels are possible, and anomalous results are expected in the lower molecular weight range.

Adenylate kinase, a soluble intermembrane enzyme (Schnaitman and Greenawalt, 1968; Sottocasa *et al.*, 1967a,b; Brdiczka *et al.*, 1968), was assayed in order to determine if the outer membranes were broken in the mitochondria preparations. When the outer membrane is broken, the adenylate kinase is lost and the preparation has a low specific activity. The specific activities obtained for adenylate kinase in our preparations were 670 ± 50 μmol of NADPH/min per mg of protein. These exceed the values of 420 and 308 μmol/min per mg reported by Schnaitman and Greenawalt (1968). It may also be useful to mention that no significant differences in the specific activities of adenylate kinase were observed with mitochondria prepared in isolation medium without EDTA compared to those prepared in medium with EDTA. Thus, it would appear that the outer membrane of the mitochondria used in these studies was intact.

The same procedure was used to determine whether or not the labeling procedure resulted in breaking the outer membrane. After iodination of the intact mitochondria no difference in the specific activity of adenylate kinase in the iodinated mitochondria from that in the untreated mitochondria was observed. As a more sensitive test of rupture of the outer membrane during the iodination procedure, the adenylate kinase activity of the iodination supernatant was compared to that of the supernatant from mitochondria which were not treated in any way. Both supernatants contained only 2.0 ± 0.5% of the adenylate kinase activity. The results showed no significant differences in activity due to the iodination. These results indicate that the mitochondria were intact before and after iodination.

It is evident that peak L in Figure 1 is the major molecular weight class of polypeptides iodinated in the intact mitochondria. Hemoglobin, a possible contaminant of the mitochondria, would be expected to be in or near peak L. When rat hemoglobin was compared to peak L material in basic and sodium dodecyl sulfate gel electrophoresis, it was found to migrate differently in both cases. It was also found that 0.1 M NaCl extracts of mitochondria contained iodinated material with a mol wt of about 14,000. These extracts, however, showed no spectral evidence of a hemoglobin component. It was concluded that hemoglobin contamination was not a serious problem in these experiments.

In order to clearly establish that the labeling pattern obtained was not attributable to contaminating microsomes, the microsomal fraction was prepared in the same isolation

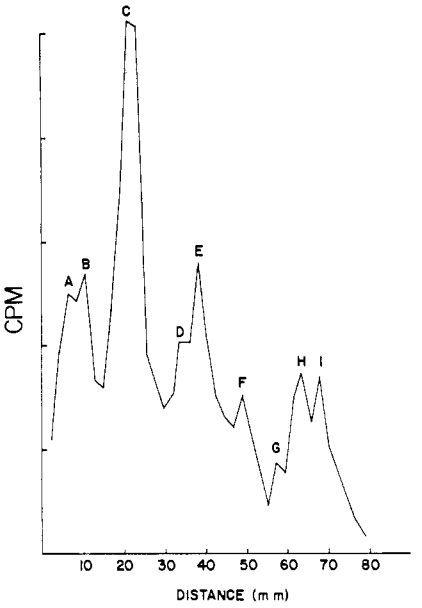


FIGURE 2: The distribution of iodine label in microsomal polypeptides separated into molecular weight classes by electrophoresis in sodium dodecyl sulfate–acrylamide gels. The microsomal fraction was iodinated using lactoperoxidase as described under Materials and Methods.

medium as was used for the mitochondria preparation. The iodinated microsomal polypeptides presented a radioactive labeling profile (Figure 2) which was quite different from that of iodinated mitochondria although there were some labeled polypeptides with similar apparent molecular weights in both of the organelles. The iodinated microsomal polypeptides corresponded to the following molecular weights: (A) 92,000; (B) 81,000; (C) 56,000; (D) 37,000; (E) 31,000; (F) 22,000; (G) 17,000; (H) 14,000; and (I) 12,000. The radioactive gel profile for iodinated microsomes showed eight peaks and several shoulders, indicating that polypeptides over a large molecular weight range were labeled, which was also true for iodinated mitochondria. Unlike mitochondria, however, the polypeptides at higher molecular weights were labeled as heavily or more heavily than those in the lower molecular weight classes.

When the microsomal proteins were visualized with Coomassie Brilliant Blue stain as is shown in Figure 3, about 19 polypeptide bands were resolved. These microsomal polypeptides were visible over a wide molecular weight range as was found with mitochondria, but microsomes and mitochondria could be readily distinguished. No stained band was observed which corresponded to peak I in Figure 2 for microsomal preparations.

Lipid Extraction of Iodinated Mitochondria. The lipids of iodinated mitochondria were extracted by the method of Rouser and Fleischer (1967). About 95% of the total protein was recovered after the extraction procedure. The radioactivity profile obtained after removal of the lipid from the mitochondria was similar to that of the original iodinated mitochondria before extraction. It was concluded that lipid made no significant contribution to the radioactivity profile.

Distribution of Iodinated Polypeptides in Inner and Outer Membrane Fractions. Intact iodinated mitochondria were treated with digitonin by the method of Schnaitman and Greenwalt (1968) and subsequently fractionated by differential centrifugation according to Scheme I. The low-speed pellet contained inner membrane matrix (IMM) particles,

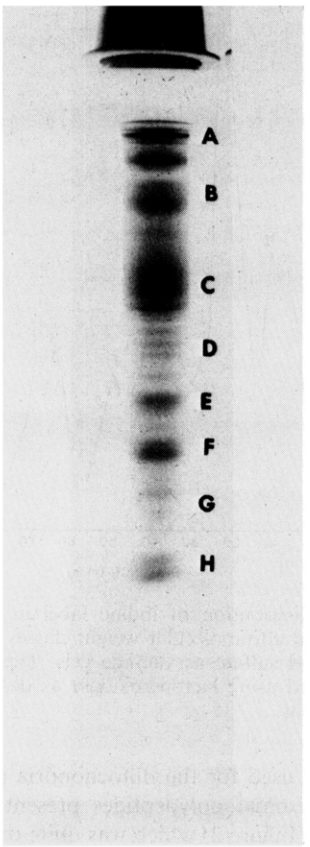
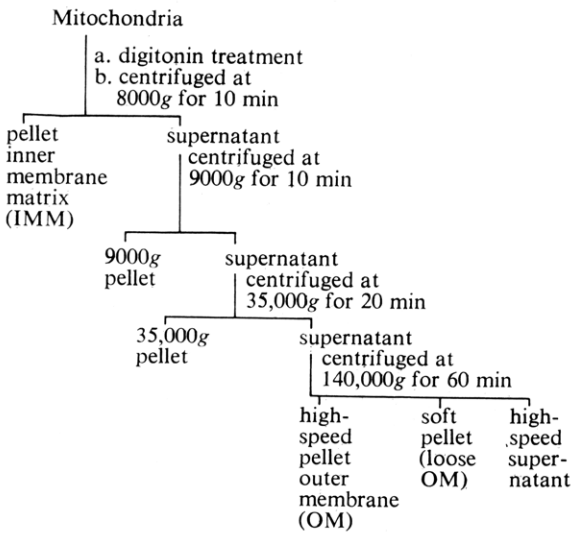


FIGURE 3: The microsomal polypeptides separated by electrophoresis in sodium dodecyl sulfate–acrylamide gels. The gel was stained with Coomassie Blue in order to visualize the polypeptides. A–H correspond to the peaks of radioactivity in Figure 2.

while the outer membrane was located in the high-speed pellet. Various intermediate fractions were also obtained. The soluble intermembrane material was in the high-speed supernatant.

Data on the fractionation of iodinated mitochondria are given in Table I which indicates that about 80% of the protein and counts were recovered in the various fractions. Most

SCHEME I: Mitochondrial Fractionation.[a]



[a] For details of procedures employed, see Materials and Methods.

TABLE I: Fractionation of Mitochondria.

Fraction	% of Total Protein	Rel Sp Act.[a]	Ratio of Counts in Peak L to Counts in Peak H[a]
Intact		1	3
IMM	63	0.8	2
9000*g* pellet	2	3	3
35,000*g* pellet	3	3	9
Soft OM	0.7	5	13
OM	0.2	12	16
HSS	13	1	5

[a] Estimated from gel data as described under Materials and Methods.

of the counts and protein were recovered in the IMM particle fraction.

Since the lactoperoxidase-catalyzed iodination labels only the outer membrane components, the specific activity of the outer membrane, *i.e.*, the amount of ^{125}I per milligram of protein, serves as an index of purification of this membrane. The relative specific radioactivities are given in column 2 of Table I. The outer membrane fraction had the highest specific radioactivity which was about 12 times that of the intact mitochondria. None of the other fractions had specific activities which approached that of the outer membrane fraction. In comparable experiments monoamine oxidase activity was also measured. A large percentage of the activity remained with the IMM fraction. The OM, however, had a tenfold higher specific activity than the IMM fraction.

The distribution of radioactivity obtained in sodium dodecyl sulfate gels is shown in Figure 4 for the intact iodinated mitochondria, the IMM particles, the OM pellet, and the high-speed supernatant (HSS). Peak L material is evident in all fractions, but it is apparent that iodinated polypeptides above 14,000 in the OM pellet contained a lower percentage of the label than they contained in the original intact mitochondria. In fact, peak L contains about 70% of the ^{125}I label in the outer membrane. In column 3 of Table I is given the ratio of the two largest peaks found in the original intact preparation, peaks L and H. A comparison of this ratio among the various fractions indicates that all fractions except the IMM fraction had a higher ratio of L to H than did the intact mitochondria. The experimental technique is sufficiently variable, however, that a ratio of 3 is perhaps not significantly different from a ratio of 2. Our data do indicate that the differences are indeed real, since in several experiments the L:H ratio of IMM particles was consistently less than the ratio for the intact mitochondria.

Figure 5 shows that about 16 and 18 molecular weight classes are present in the high-speed supernatant and outer membrane fractions, respectively. Each of the submitochondrial fractions give distinguishable patterns when stained for proteins except the IMM fraction, which is similar to the intact mitochondria.

Following isolation, IMM particles were labeled by lactoperoxidase-catalyzed iodination. The resulting radioactive gel pattern is shown in Figure 6 and should be compared with the pattern from IMM particles which had been isolated from labeled mitochondria (Figure 4b). The polypeptides of the

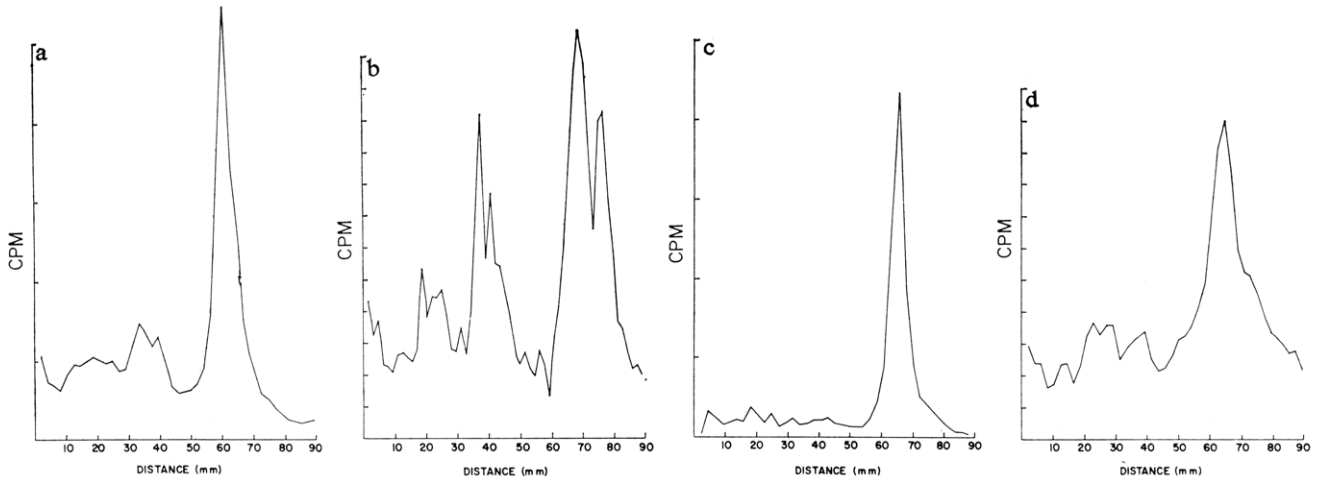


FIGURE 4: The distribution of iodine label in polypeptides of mitochondria and mitochondrial fractions separated by electrophoresis in sodium dodecyl sulfate–acrylamide gels. The fractions were isolated from iodinated mitochondria as outlined schematically in Scheme I: (a) intact mitochondria; (b) IMM particles; (c) outer membrane; and (d) high-speed supernatant.

IMM in the mol wt 30,000 class are not labeled. Otherwise, the two radioactivity patterns are quite similar.

Figure 7 shows the radioactive gel profile for OM which was labeled after isolation. This should be compared with results in Figure 5c of OM isolated from labeled intact mitochondria. The mol wt 14,000 class of polypeptides is the most prominent iodinated component in both instances. However, the OM iodinated after isolation has a much greater amount of label in the mol wt 30,000 and 60,000 classes.

Discussion

The membrane proteins iodinated by the lactoperoxidase procedure are limited to those which are exposed and capable of forming a complex with the enzyme (Morrison *et al.*, 1970). The outer membrane of mitochondria has been shown to be impermeable to cytochrome *c* and other proteins with a molecular weight greater than about 10,000 (Pfaff *et al.*, 1968; Wojtczak and Zaluska, 1969; Wojtczak and Sottocasa, 1972). Since lactoperoxidase has a mol wt of 77,500 (Rombauts *et al.*, 1967), the outer mitochondrial membrane should be impermeable to this enzyme. The proteins iodinated were therefore limited to those on the outer surface of the membrane. Electrophoresis of the iodinated material on sodium dodecyl sulfate–acrylamide gels (Figures 1 and 2) indicated that several molecular weight classes of polypeptides in mitochondria and microsomes were iodinated. However, the distribution of radioactive iodine in the gels was quite different indicating that contamination of the mitochondria by microsomes was not significant. The difference in iodine distribu-

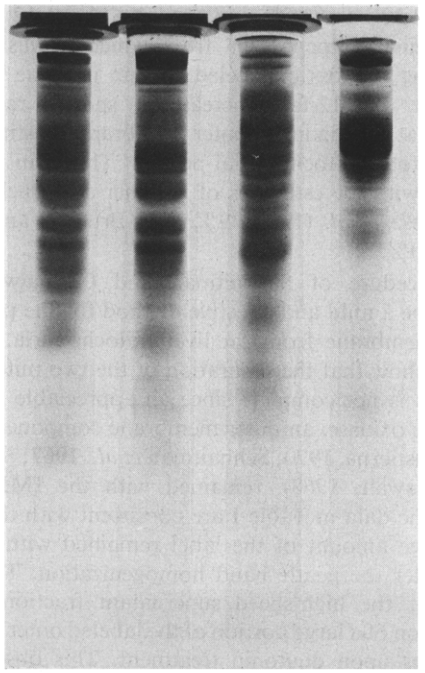


FIGURE 5: The polypeptides of the mitochondria and mitochondrial fractions listed in Figure 4 which were separated by electrophoresis in sodium dodecyl sulfate–acrylamide gels. The gels were stained with Coomassie Blue in order to visualize the polypeptides. Left to right: intact mitochondria, IMM particles, outer membrane, and high-speed supernatant.

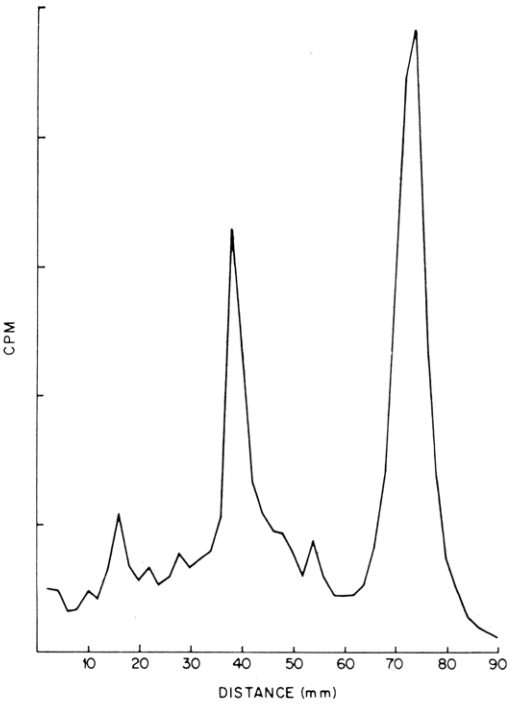


FIGURE 6: The distribution of iodine label among the polypeptides in the IMM fraction, which was labeled after isolation. The proteins were solubilized and separated as described under Materials and Methods.

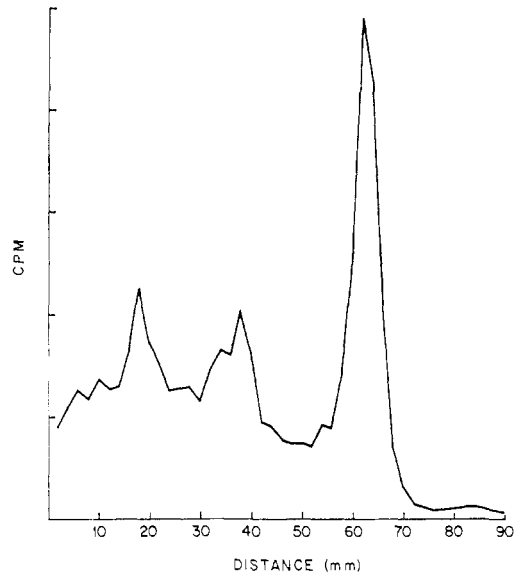


FIGURE 7: The distribution of iodine label among the polypeptides of the OM fraction, which was labeled after isolation. The proteins were solubilized and separated as described under Materials and Methods.

tion is interesting in light of the similarities in enzyme (Sottocasa *et al.*, 1967a,b; Brunner and Bygrave, 1969) and lipid composition (Parsons *et al.*, 1967; Parsons and Yano, 1967) for the two organelles.

The gels in Figures 1 and 3 which were stained for protein with Coomassie Blue were comparable to the results obtained by Neville and Glossman (1971) for mitochondria and smooth endoplasmic reticulum, respectively.

Although it is probable that there was some contamination of mitochondria by other organelles, this did not appear to seriously affect the iodination results. Further purification of the mitochondria did not alter the labeling pattern for polypeptides of 14,000 daltons or above (Figure 1).

The most striking aspect of the labeling pattern for intact mitochondria (Figure 1) is the prominent peak L corresponding to a mol wt of ~14,000. It is also evident that lower molecular weight polypeptides were iodinated to some extent (Figure 1). Swank *et al.* (1971) have reported evidence for the existence of low molecular weight polypeptides in various types of mitochondria. However, since most of the low molecular weight species iodinated in our experiments seemed to be removed by sucrose gradient centrifugation, it is uncertain whether these iodinated components were contaminants or small polypeptides loosely bound to the outer membrane.

If the outer membrane were ruptured in the course of the preparation of mitochondria or during the iodination procedure, both inner and outer membranes would have been iodinated. It was therefore important to establish that the outer membrane was intact. Adenylate kinase was assayed in our mitochondria preparations and the specific activities were determined. Since adenylate kinase is a soluble enzyme located in the membrane compartment between the outer and the innter membranes (Schnaitman and Greenawalt, 1968; Sottocasa *et al.*, 1967a,b; Brdiczka *et al.*, 1968), rupture of the outer membrane would result in a decrease in the specific activity of this enzyme in the mitochondria. The specific activities obtained for our preparations exceed those reported by Schnaitman and Greenawalt (1968), which is consistent with intact outer membranes. After iodination, the mitochondrial pellet and the supernatant were assayed for adenylate kinase activity in order to determine whether or not the outer membrane had been broken during the iodination procedure. The results indicated no breakage of the outer membrane during iodination. The small percentage of adenylate kinase present in the supernatant may be due to association of some of the cytoplasmic isozyme described by Criss (1970), inability to spin down some mitochondria in the supernatant, a small degree of rupture of the outer membrane, or a combination of these possibilities. However, breakage of the outer membrane does not seem a likely possibility since mitochondria which were not treated in any way showed the same amount of adenylate kinase in the supernatant. This was further supported by the isolation of the two types of cytochrome b_5 contained in mitochondria. According to Fukushima *et al.* (1972), there is a cytochrome b_5 located on the outer membrane. This cytochrome b_5 is tightly bound and can be released by trypsin or detergent treatment. A second cytochrome b_5 like pigment is present in the space between the inner and outer membranes. This interspace cytochrome b_5 is easily hypotonically extracted from mitochondria.

Intact mitochondria were labeled and the two cytochrome b_5 like pigments isolated, the outer membrane cytochrome by mild trypsin treatment and the interspace cytochrome by hypotonic treatment. The interspace cytochrome b_5 had a specific activity about 1–2% of that found for the outer membrane cytochrome (C. T. Huber and M. Morrison, manuscript in preparation).

Since the outer membrane envelopes the mitochondrion and since this membrane is not freely permeable to lactoperoxidase (mol wt 77,500), the iodination catalyzed by this enzyme occurs on the outer side of the outer membrane. The label therefore acts as a marker for the outer membrane. As the mitochondrial fractions become richer in outer membrane it would be expected that the specific radioactivity of these preparations would increase. The data in Table I indicate such an enrichment with a final outer membrane preparation that has a specific radioactivity about 12 times that of the original mitochondria from which it was prepared. Disregarding the loss of labeled protein into the high-speed supernatant, the 12-fold increase in specific radioactivity indicates that protein in the outer membrane constitutes about 8% of the total mitochondrial protein. This is in reasonable agreement with the estimates of Brunner and Bucher (1970), 6–10%, Liese *et al.* (1971), 9%, and Brunner and Bygrave (1969), 4–10%.

The procedure of Schnaitman and Greenawalt (1968) proved to be a mild and effective method for the preparation of outer membrane from rat liver mitochondria. However, their data show that the separation of the two mitochondrial membranes is not complete since an appreciable amount of monoamine oxidase, an outer membrane component (Ernster and Kuylenstierna, 1970; Schnaitman *et al.*, 1967; Schnaitman and Greenawalt, 1968), remained with the IMM particle fraction. The data in Table I are consistent with their results since a large amount of the label remained with the IMM fraction after the gentle hand homogenization. The data in Table I for the high-speed supernatant fraction indicated solubilization of a large portion of the labeled outer membrane polypeptides upon digitonin treatment. This observation is also consistent with the data of Schnaitman and Greenawalt (1968), which shows that an appreciable amount of monoamine oxidase was present in the high-speed supernatant.

It is apparent from a qualitative comparison of the labeling pattern of outer membrane and intact mitochondria in Figure

4 that there was a disproportionately high amount of the peak L component in the outer membrane. Approximately 70% of the iodine in the outer membrane fraction was estimated to be in the L component. The identity of the iodinated protein in peak L is currently under investigation.

A clearer picture is obtained from the ratio of L and H, the two major labeled components of the intact mitochondria. The data of Table I show that the amount of the H component relative to the L component is higher only in the IMM particle fraction. It appears that as the outer membrane is fragmented and separated upon digitonin treatment, parts of it selectively remain with the IMM particle. This is readily explainable if the outer membrane is heterogeneous with respect to protein components in the plane of the membrane. The fragments enriched with peak H and other higher molecular weight iodinatable polypeptides remain with the IMM particle to a greater extent than do peak L components of the outer membrane. The fragments which are ultimately isolated as outer membrane are rich in the iodinatable polypeptide components of peak L. Hackenbrock (1968) has demonstrated the existence of contact points between the outer and inner membranes of mitochondria. One model, then, explaining the apparent affinity of a certain portion of the outer membrane for the IMM particle might entail actual points of contact between the outer membrane and the IMM particle. The outer membrane is fractured in a manner such that fragments rich in high molecular weight iodinatable components, such as peak H polypeptides, remain with the IMM particle. These outer membrane fragments adhere to the IMM particle because they are at contact points where the inner and outer membranes converge. Those fragments of outer membrane which are dislodged from the mitochondrion by the digitonin treatment are rich in peak L polypeptides.

Another possibility is that outer membrane fragments which are rich in component H simply adhered to the IMM particles in a manner not involving contact points which may have existed in the intact mitochondria. Yet another alternative stems from the fact that many iodinated outer membrane proteins were solubilized during digitonin treatment. Component H and other higher molecular weight iodinated polypeptides that were solubilized may have selectively associated with the inner membrane.

The labeling patterns obtained when intact mitochondria and the IMM fraction were iodinated, although different, still contained many labeled proteins which occurred in the same molecular weight classes. This is attributable first to the fact that the inner membrane was not freed completely of outer membrane and secondly to the lack of discrimination of the technique employed to separate the membrane components. Thus, there are probably a number of different membrane polypeptides in any particular molecular weight class, and some may be localized on the outer membrane, while others may be on the inner membrane. This clearly points out the difficulties of identifying proteins with a single method of separation and emphasizes the need for other methods to distinguish the membrane polypeptides.

It is clear from the data that lactoperoxidase-catalyzed iodination of exterior membrane proteins provides a means of evaluation of membranes which is more useful and versatile than the marker enzyme method.

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Characterization of Microtubule Assembly in Porcine Brain Extracts by Viscometry†

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ABSTRACT: The assembly of microtubules in extracts of porcine brain tissue has been characterized by viscometry. The optimal conditions for preparing extracts have been defined in terms of the rate and extent of polymerization, and the stability of the maximal level. The reaction is endothermic, and maximal viscosity levels were attained within 15–20 min of incubation at 37°. No polymerization occurred at temperatures less than 15°, and the polymer was unstable at temperatures greater than 40°. The greatest polymerization occurred over a pH range of 6.7–6.9, and no increase in viscosity was observed at a pH of less than 5.8 or greater than 7.6. Guanosine triphosphate (GTP) augmented polymerization, producing a stable viscosity level at concentrations higher than 2.0 mM. However, at GTP concentrations greater than 3.0 mM, the initial rate of assembly was depressed. At a concentration of 2.5 mM, extracts prepared with other nucleoside triphosphates (ATP, CTP, UTP) or guanosine nucleotides (GDP, GMP) polymerized to a lesser degree than those extracts made with GTP. The extent of viscosity development was approximately proportional to the protein concentration of the extract over the range of 4–12 mg/ml; no polymerization occurred at lower protein concentrations. Viscosity development was completely inhibited by the addition of both monovalent and divalent cations at the following concentrations: Na^+ and K^+, 150 mM; Mg^{2+}, 10 mM; Ca^{2+}, 1 mM. Colchicine, which binds specifically to microtubule subunits, totally inhibited polymerization at a concentration of 1 μM. The conditions established in this paper will enable the use of optimum parameters for further investigations on microtubule assembly.

Microtubules are a common feature of eukaryotic cells, and several reviews on the cellular distribution, function, and biochemical composition of these organelles have appeared (Porter, 1966; Tilney, 1971; Olmsted and Borisy, 1973; Wilson and Bryan, 1973). Until recently, however, detailed information on the properties of microtubule assembly and disassembly was limited. Investigations on mitotic spindle formation and dissolution (Inoué and Sato, 1967) as well as other observations on cellular processes (see Tilney (1971) and Margulis (1973) for reviews) indicated that assembly *in vivo* was both endothermic and colchicine sensitive. *In vitro* studies on the association of tubule subunits derived from detergent-treated sea urchin sperm flagella demonstrated neither of these properties, although microtubules were observed (Stephens, 1968). In studies on the purified microtubule protein from porcine brain tissue, isolated subunits aggregated to form beaded and linear structures. This aggregation process was temperature dependent, colchicine inhibitable, and nucleotide and protein specific; however, the aggregates were not microtubules (Borisy *et al.*, 1972).

Recently, conditions were established for the temperature-induced formation of microtubules *in vitro* (Weisenberg, 1972; Borisy and Olmsted, 1972). As determined by electron microscopy, viscometry, and sedimentation, this reaction was sensitive to both low temperature and colchicine (J. B. Olmsted *et al.*, manuscript in preparation). This paper describes the use of viscometry to define the optimum conditions for preparing extracts of porcine brain tissue in which polymerization is rapid, and the formed tubules are stable. In addition, these experiments have broadly defined some of the basic characteristics of the *in vitro* assembly reaction.

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