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Two-dimensional gel electrophoretic resolution of the polypeptides of rat liver mitochondria and the outer membrane

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The proteins of highly purified rat liver mitochondria were resolved by two-dimensional polyacrylamide gel electrophoresis, and detected by staining with either Coomassie blue or silver. Approximately 250 polypeptides were detected with silver staining which is 2- to 3-times that observed with Coomassie blue. Silver staining was especially more effective than Coomassie blue for detecting polypeptides of less than 50 000 daltons. A two-dimensional gel pattern of rat liver microsomes was distinct from that of the mitochondria. The mitochondrial outer membrane was prepared from purified mitochondria either with digitonin or by swelling in a hypotonic medium. As assessed by marker enzymes, the latter method yielded a considerably purer outer membrane preparation (20-fold purification) than the former (2.6-fold purification). Approximately 50 polypeptides were observed in a two-dimensional gel (pH 3–10) of the higly purified outer membrane fraction. Three isoelectric forms of the pore (VDAC) protein were observed with p*I* values of 8.2, 7.8 and 7.1. Monoamine oxidase was identified as a polypeptide of M_r 60 000. About 50 polypeptides were also resolved in a reverse polarity non-equilibrium pH gradient electrophoresis gel of the outer membrane, pH 3–10, with at least six isoelectric forms of the VDAC protein observed under these conditions. The six isoforms of the VDAC protein were also observed in a non-equilibrium gel with 2 μg of the purified protein.

Introduction

The mitochondrial outer membrane of rat liver was initially isolated in 1966 [1,2]. In recent years there has been an increased interest in studying this mitochondrial subfraction. An important function of the outer membrane is to form a barrier through which substances of molecular weight higher than 10 000 [3] must penetrate in order to gain entrance into the other mitochondrial compartments. Evidence indicates that outer membrane proteins apparently mediate interactions of mitochondria with the cytoskeleton, nucleus or other mitochondria [4–6]. Also, the fate of fatty acids is largely determined at the level of the outer membrane. The long chain fatty acyl-CoA's synthesized by the outer membrane ligase can be used either catabolically in β-oxidation to yield energy or anabolically as a substrate in the biosynthesis of phospholipids.

Since the preponderance of mitochondrial proteins are coded by nuclear genes, and synthesized extramitochondrially, these proteins must be imported into the mitochondrion. It has been postulated that there are receptors on the outer membrane which are essential for the passage of the cytoplasmically synthesized mitochondrial proteins or their precursors into the organelle [4,5]. Small hydrophilic molecules pass freely into the mitochondrion through the outer membrane [7,8]. Apparently these molecules can permeate the outer membrane bilayer by passage through a channel formed by the pore or VDAC protein [9,10]. This major outer membrane protein has been investigated in mitochondria from mung beans [11], *Neurospora crassa* [12], *Saccharomyces cerevisiae* [13], and rat liver [14,15], and shown by electrophoresis to have a molecular weight of 30–33 kDa [9]. Examination of purified VDAC protein

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Abbreviations: CPS, carbamyl phosphate synthetase; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; MAO, monoamine oxidase; MOM, mitochondrial outer membrane; NEPHGE, non-equilibrium pH gradient electrophoresis; VDAC, voltage-dependent anionic channel.

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from the outer membrane of rat liver mitochondria by two-dimensional gel electrophoresis has shown it to consist of three isoelectric forms [14].

The majority of electrophoretic studies on the protein composition of mitochondrial outer membrane from various sources or on whole mitochondria from rat liver have employed one-dimensional SDS-polyacrylamide gel electrophoresis with detection by Coomassie blue staining [16–24]. The vastly improved resolution of total cell protein by the two-dimensional gel technique [25,26] in combination with the increased sensitivity of silver staining [27] presents an opportunity to examine the protein components of the whole mitochondrion and its subcompartments in greater detail. With respect to the outer membrane, this type of analysis is important for laying the groundwork for the future investigation of the protein topography, function and assembly of this membrane.

Experimental procedures

Materials. Ampholytes were obtained from LKB. SDS was purchased from Gallard-Schlesinger. Acrylamide electrophoresis grade, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine (TMED), and Hypo Clearing Agent were all purchased from Eastman Kodak. Ultra-pure grade of urea, and glycine were purchased from Schwarz/Mann. β-Mercaptoethanol was purchased from Fisher Scientific; ammonium persulfate, Bromothymol blue and Coomassie blue were purchased from Bio-Rad. Triton X-100, agarose and Tris-Base were bought from Sigma Chemical Co. and silver nitrate from Aldrich Chemical Co. Protein molecular weight standards for two-dimensional gel electrophoresis were purchased from Pharmacia. Male Sprague-Dawley rats were obtained from Taconic Farms, Germantown, New York.

Preparation of mitochondria and microsomes. Liver mitochondria and microsomes were isolated from 250–300 g male Sprague-Dawley rats essentially as described by Monroy et al. [28]. The cells were homogenized and centrifuged in a medium consisting of 0.25 M sucrose (pH 7.2), adjusted by the addition of 1 M Tris-HCl buffer. The mitochondrial fraction was purified further in a 0.88 to 2.2 M sucrose density gradient containing 1.0 mM EDTA by centrifugation at 50 000 × *g* for 3 h. The purified mitochondria were recovered from the middle portion of a visibly turbid band at a density of approximately 1.36 M sucrose.

Preparation of the outer membrane and intermembrane proteins. The outer membrane fraction of the mitochondrion was prepared either by digitonin fractionation [29] or by swelling in a hypotonic medium followed by sucrose gradient centrifugation [30]. In either method, upon the initial loss of mitochondrial integrity, the supernatant fluid served as the source of the intermembrane proteins.

Preparation of the inner membrane and matrix protein fractions. The mitoplasts derived from the digitonin or swelling procedures served as the source of the inner membrane and matrix protein fractions. The digitonin mitoplasts (10 mM Tris, pH 7.2) were sonicated for 2 min at 30-s intervals on a Megason Ultrasonic Disintegrator at 0.9 maximum output. The sonicated suspension was centrifuged at 80 000 × *g* for 1 h; the supernatant fluid contained the matrix proteins and the pellet contained the inner membrane which was resuspended in 0.3 M sucrose, pH 7.2.

The mitoplasts from the swelling procedure were suspended in 0.25 M sucrose (pH 7.2), sonicated as above, and the sonically disrupted material was centrifuged at 140 000 × *g* for 45 min. The supernatant fluid contained the matrix proteins and the pellet contained the inner membrane fraction which was resuspended in 0.3 M sucrose (pH 7.2).

Protein determination. Protein was determined by the method of Lowry et al. [31] using crystalline BSA as a standard.

Marker enzymes. Marker enzymes for mitochondrial subfractions, microsomes or purified whole mitochondria are as follows. Monoamine oxidase (outer membrane) was assayed by the procedure of Tabor et al. [32]. Adenylate kinase (intermembrane space) was measured by the method of Schnaitman and Greenawalt [33]. Cytochrome oxidase (inner membrane) was determined by the procedure of Wjotczak et al. [34], and malate dehydrogenase (matrix) was assayed by the method of Freeman et al. [35]. Cytochrome-*c* reductase was measured according to Sottocosa et al. [36] with either 1.0 mM NADH, 0.1 M sodium succinate or 1.0 mM NADPH as the substrate to assay the rotenone-insensitive NADH–cytochrome-*c* reductase (outer membrane), or the reductase activity in inner membrane and microsomes respectively. Rotenone was used at a concentration of 5 μM. Glucose-6-phosphatase, assayed according to Swanson [37], was used as a specific marker for microsomes. Glycerophosphate acyltransferase assayed in the presence and absence of *N*-ethylmaleimide [38] was also used to assess the purity of whole mitochondria. The specific activity of each marker enzyme was expressed as nmol/min per mg protein.

Gel electrophoresis. Two-dimensional (2D) gel electrophoresis was run according to O'Farrell [25] with the following modifications. The width of the second dimension plates was 16 cm which permitted the use of a 13 cm gel in the first dimension. The thickness of the second dimension gel was 0.75 mm and it was 11.5% with respect to acrylamide concentration. All protein samples (40 μg in all experiments unless mentioned otherwise) were prepared for application to the isoelectric focusing gels by the method of Cabral and Schatz [39]. The pH in the isoelectric focusing gel was determined with the use of a micro combination pH

probe, Microelectrodes, Inc., Londonberry, N.H. by probing the extruded gel at 0.5-cm intervals. Six standard proteins (Pharmacia) were used as molecular weight markers for the second dimension: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21 kDa; and α-lactalbumin, 14 kDa. These proteins (2 μg of each) were mixed and allowed to polymerize with an IEF gel which lacked ampholytes. A gel with the molecular weight standards was run in parallel with each experimental sample. Non-equilibrium pH gradient electrophoresis (NEPHGE) gels were run according to the procedure of O'Farrell et al. [26].

Staining of proteins on gels. For staining with Coomassie blue, the gels were shaken for 24 h with a solution consisting of 0.05% Coomassie blue R-250, 1% trichloroacetic acid, 7.5% (v/v) glacial acetic acid, and 50% ethanol. This was followed by at least a 1 h wash in the same solution without ethanol. A solution of 5% methanol and 10% glacial acetic acid (v/v) was used for destaining. Silver staining was done by the method of Wray et al. [27].

Results

Isoelectric focusing gel pH measurements

To measure the pH in the isoelectric focusing gel, two methods, a microelectrode probe technique, and an aqueous extraction technique [25] were compared (Fig. 1). With the microelectrode technique, consistent linear readings were obtained between pH 5.0 and 7.0. In the extraction method the pH values were always lower at the basic end of the gel in comparison to the microelectrode probe technique. Since the probe technique was the faster, the more convenient, and the more accurate of the two, it was used whenever necessary.

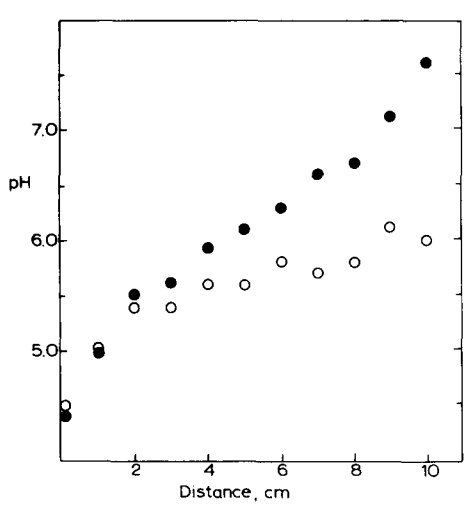

Fig. 1. pH gradient of the first dimension. The pH of the gel was measured either according to O'Farrell [25] (open circles) or with a microelectrode (closed circles) as described in the text.

TABLE I

Purity of the mitochondrial fraction: assay of marker enzymes

The rat liver mitochondria were purified through a sucrose density gradient and marker enzymes were assayed. The results are expressed as nmol/min per mg ± S.D.

Enzyme	Specific activity (mean ± S.D.)		% contamination
	mitochondria	microsomes	
NADPH–cytochrome-*c* reductase (nmol/min per mg)	2.1 ±0.5 ($n = 5$)	55.8 ±3.3 ($n = 5$)	3.8
Glucose-6-phosphatase nmol/20 min per mg)	0.24 ± 0.03 ($n = 8$)	5.23 ± 0.2 ($n = 8$)	4.6
Glycerophosphate acyltransferase (nmol/min per mg)			
control	4.78 ± 0.33 ($n = 3$)		
control + NEM	4.65 ± 0.11 ($n = 4$)		2.7

Two-dimensional gel profile of highly purified mitochondria

The purity of the whole mitochondria was demonstrated by assaying glucose-6-phosphatase [37], and NADPH–cytochrome-*c* reductase [36] which are marker enzymes for microsomes (Table I). Since microsomal, but not mitochondrial glycerophosphate acyltransferase (GAT) activity is inhibited by *N*-ethylmaleimide [38], this enzyme was also used to assess the microsomal contamination of the mitochondrial preparation. On the basis of these three assays the microsomal contamination was estimated to be only 3 to 4%.

The polypeptides of the purified whole mitochondria were resolved by 2D gel electrophoresis and detected by either Coomassie blue or silver staining, (Fig. 2a and b). Approximately 250 polypeptides were detected by silver staining which was 2–3-times the number of spots observed with Coomassie blue staining. The difference in detection was most notable for polypeptides with a molecular weight of less than 50 kDa. For detection by silver staining, protein loads of 10, 15, 20, 30, 40, 60 and 80 μg per gel of purified mitochondrial protein were tested. A 20–40 μg load appeared to be the best range for detecting all of the spots by silver staining without severe overloading of the major polypeptides.

In Fig. 2b, the polypeptides labeled 1 through 18 appeared as major spots in all whole mitochondrial 2D gels. Spot 1 represents carbamyl phosphate synthetase (CPS), a protein which comprises roughly 20% of the matrix protein fraction, which is equivalent to about 12% of the total mitochondrial protein [40]. According to our results, CPS has a molecular mass of 125 kDa in

close agreement with Raijman and Jones [41], and Henslee and Srere [42], and a p*I* of 6.5 which is similar to the p*I* of 6.4 reported previously [42]. Based on its migration in the 2D gel, spot 2 should represent pyruvate carboxylase. Spots 8 and 9 were the major spots obtained in a 2D gel of a crude inner membrane preparation (results not shown), and are attributable, respectively, to the α- and β-subunits of F_1-ATPase [42]. Spot 10 represents the position of purified MAO, when it is resolved by 2D electrophoresis (see Fig. 5a and Discussion). Spot 15 has been tentatively identified as the pore protein as judged by M_r, p*I* and also selective gel code staining (not shown). The identity of the other spots are unknown.

A 2D gel of purified microsomes (Fig. 3) showed a pattern which was distinct from that of the purified mitochondria. Thus, the purity of the mitochondria was confirmed not only by marker enzyme analysis (Table I), but also by the 2D gel analysis of each fraction.

Two dimensional gel analysis of the mitochondrial outer membrane. To analyze the 2D gel pattern of the mitochondrial outer membrane (MOM), this subfraction was prepared by two procedures, digitonin [29], and swelling [30]. The analyses of marker enzymes in this subfraction and other mitochondrial subcompartments are presented in Tables II and III. The primary marker enzyme used to determine the purification of the MOM was MAO. In the digitonin procedure (Table II), the specific activity of MAO in the MOM fraction is only 2.6-times that of the whole mitochondria; the specific activity of this enzyme in the inner membrane fraction was increased only 1.8-fold over that of the whole mitochondria indicating a poor separation of the outer and inner membranes of the mitochondrion by this procedure. There was also significant contamination of the outer membrane fraction by cytochrome oxidase which is an inner membrane marker.

In contrast, by using the swelling procedure to procure MOM, the specific activity of MAO in this subfraction was 20-fold higher than that of the whole mitochondrion. The specific activity of MAO in the inner membrane fraction was less than one fourth in comparison to that of the whole mitochondria. In addition the rotenone insensitive NADH–cytochrome-*c* reductase, another MOM enzyme, showed a distinct increase in specific activity in this fraction (Table III,

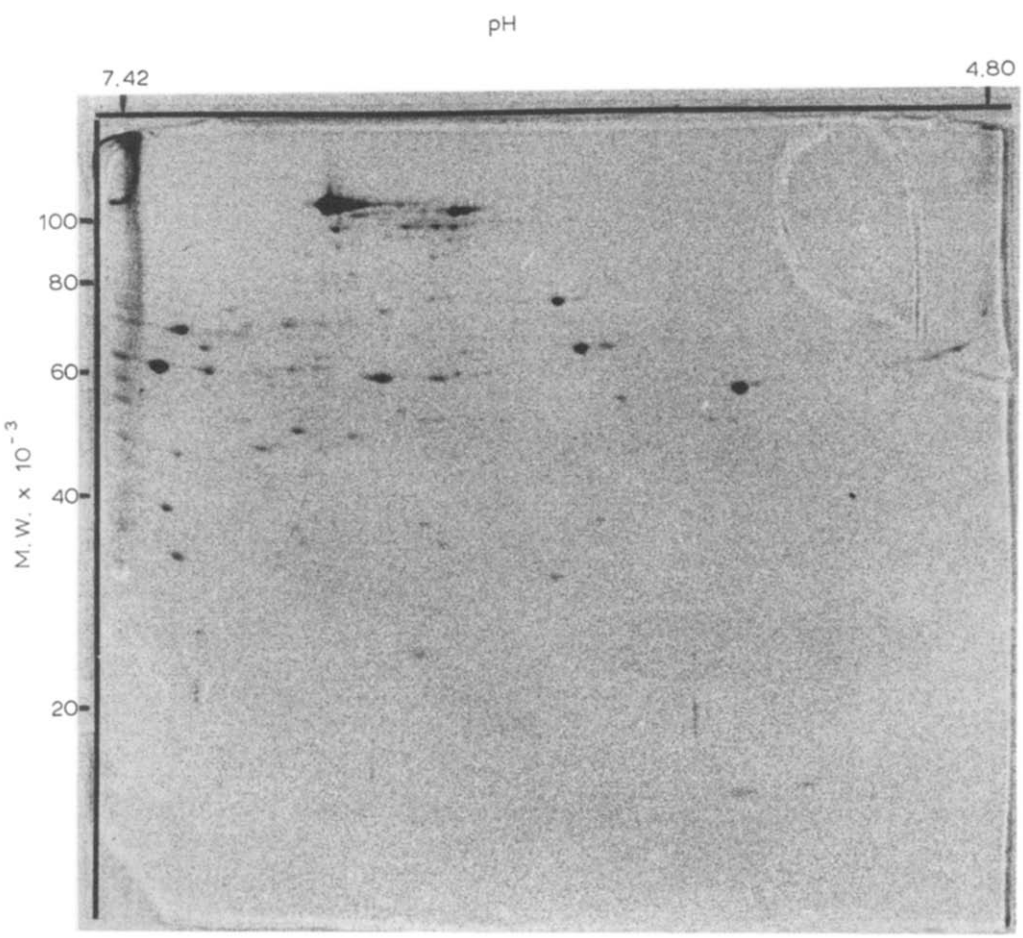

Fig. 2.(a and b) 2D gel profile of liver mitochondria. Input: 40 μg protein. Stained by either Coomassie blue (a) or silver (b). The identity of some of the spots are as described in the text.

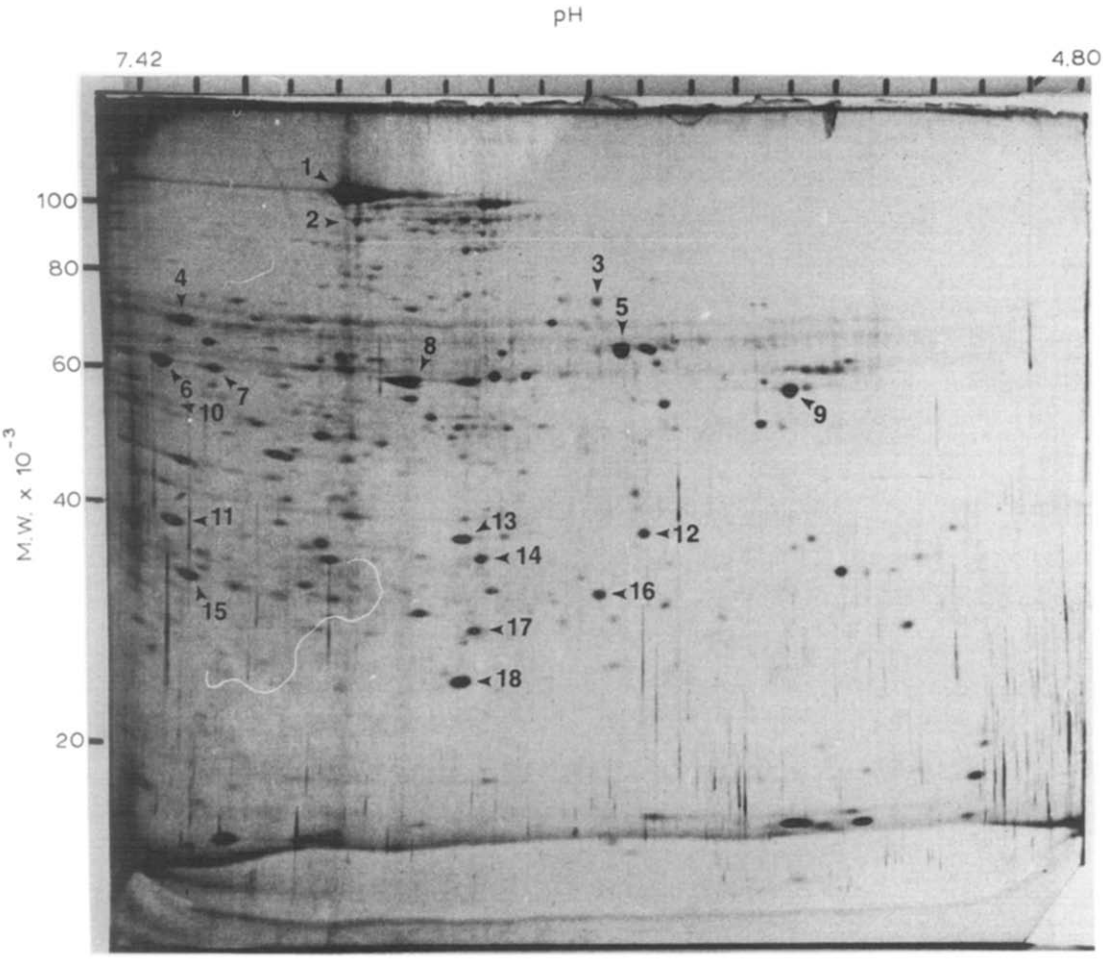

Fig. 2 (continued).

column 4 compared to column 3). There was insignificant contamination of the MOM fraction by microsomal or matrix marker enzymes or by the inner membrane marker succinate–cytochrome-*c* reductase. There was still some contamination with the inner membrane marker cytochrome oxidase, but the amount of contamination (3% recovery in the MOM) was less than that observed with the digitonin procedure (13% recovery).

The protein components of the MOM prepared by the swelling technique were analyzed first by single-dimensional SDS gel electrophoresis and then by 2D gel

TABLE II

Specific activities of marker enzymes in the mitochondrial subfractions prepared by the digitonin method

Purity of the outer membrane prepared by the digitoning method [29]. The mitochondrial outer membrane preparation and other subfractions of rat liver mitochondria were assayed for different marker enzymes as described in the text.

	Specific activity (nmol/min per mg protein)						
	monoamine oxidase	adenylate kinase	NADPH–cytochrome-*c* reductase	glucose-6-phosphatase	malate dehydrogenase	cytochrome oxidase	succinate–cytochrome-*c* reductase
Whole mitochondria	9.3	52.2	3.2	0	212.1	377.0	24.9
Outer membrane	24.1	5.0	0.1	0	0	277.1	14.0
Inter-membrane	0.0	150.0	1.3	0	0	46.3	1.5
Inner membrane	17.2	135.1	1.1	0	178.2	93.2	173.0
Matrix	7.1	8.0	1.6	0	315.0	162.0	3.8
Microsomes			55.1	7.0			

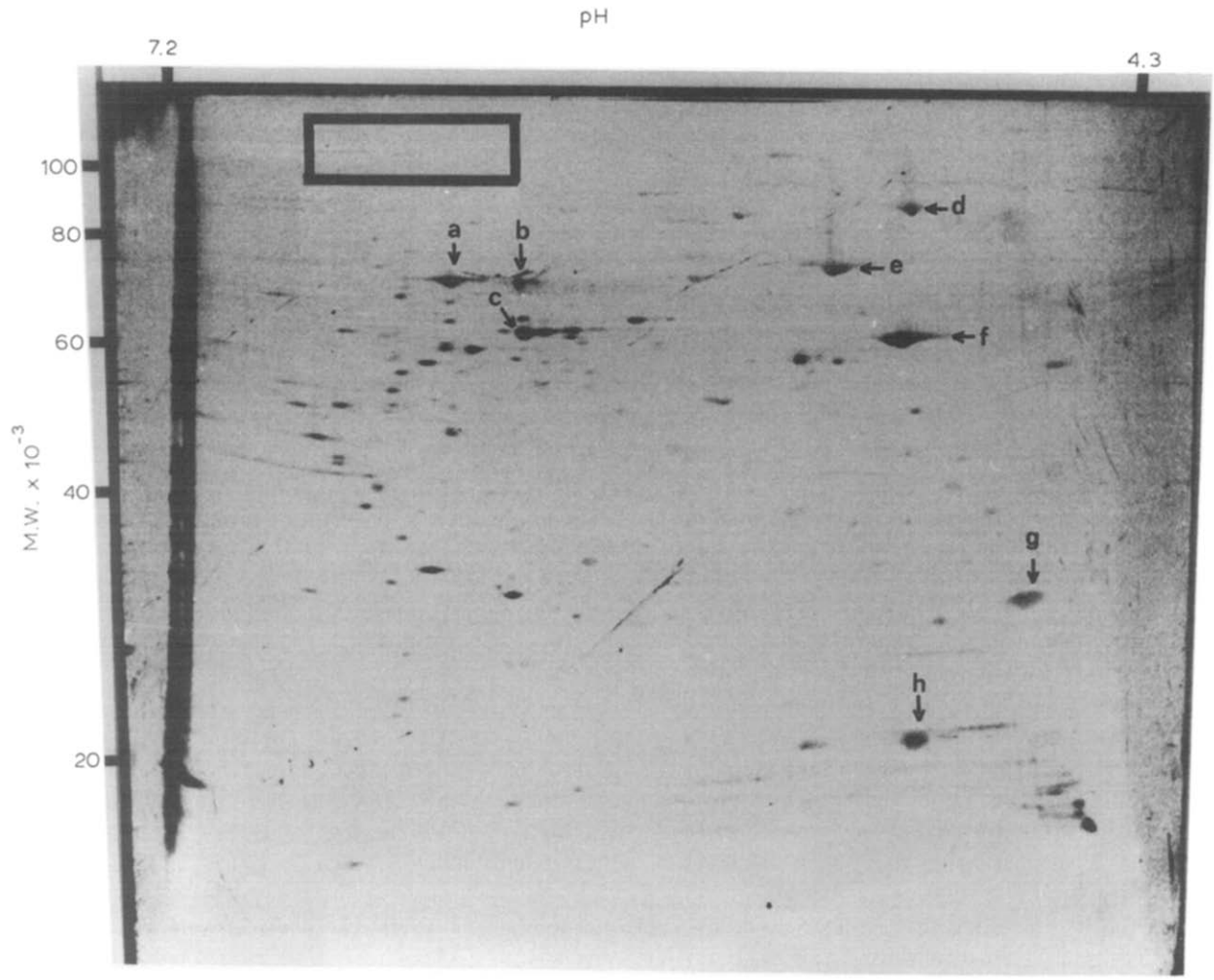

Fig. 3. 2D gel profile of rat liver microsomes. Silver stained. pH 5–7. Input 40 μg. Spots a through h are most intensively stained in the gel. The absence of mitochondrial spot 1 (Fig. 2b) within the box is noteworthy.

TABLE III

Specific activities of marker enzymes in the mitochondrial subfractions prepared by the swelling method

Purity of the mitochondrial outer membrane prepared by the swelling method [30]. The different mitrochondrial subfractions were assayed for marker enzymes.

	Specific activity (nmol/min per mg protein)								
	monoamie oxidase	adenylate kinase	NADH-cytochrome-*c* reductase − rotenone	NADH-cytochrome-*c* reductase + rotenone	NADPH-cytochrome-*c* reductase	glucose 6-phosphatase	malate dehydrogenase	cytochrome oxidase	succinate-cytochrome-*c* reductase
Whole mitochondria	14.9	15.1	2.5	1.9	2.1	0	280.0	306.1	37.6
Outer membrane	303	1.2	15.3	16.2	0	0	0	141.2	1.3
Inter-membrane	0.1	139	2.1	1.9	0.9	0	0	23.1	1.8
Inner membrane	3.2	3.2	2.5	1.0	0.4	0	208.9	707.8	126.6
Matrix	1.8	1.1	10	0.4	0.4	0	397.1	175.4	4.3
Microsomes	–	–	–	–	61.3	8.3	–	–	–

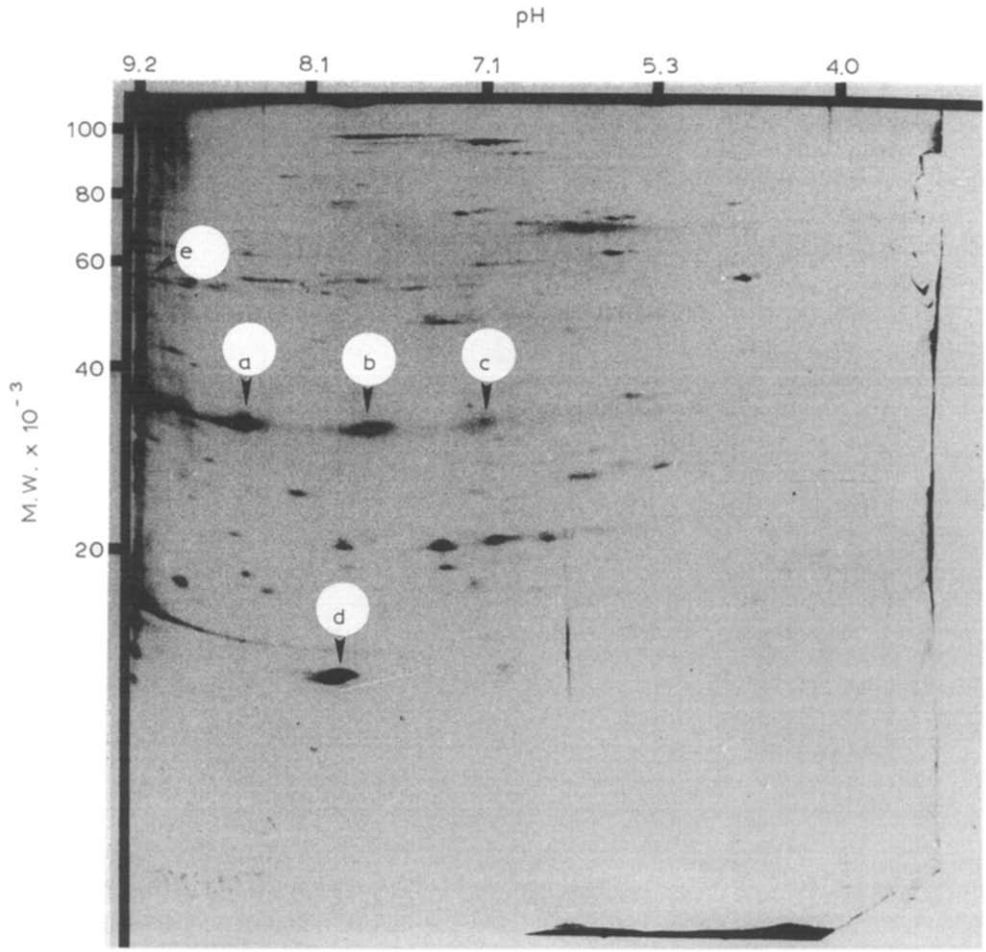

Fig. 4. 2D gel profile of the mitochondrial outer membrane. 40 μg of outer membrane was resolved by two-dimensional electrophoresis and stained by silver. The VDAC protein (a, b and c), 15 kDa protein (d) and MAO (e) are identified.

electrophoresis. The first dimensional gel pattern showed remarkable similarity with the pattern obtained by Noel et al. [24]. The major species consisted of polypeptides of 15 kDa, 32 kDa, 35 kDa, five bands between 40 and 70 kDa although stained with different intensities, and a band at about 90 kDa (results not shown). The 2D gel

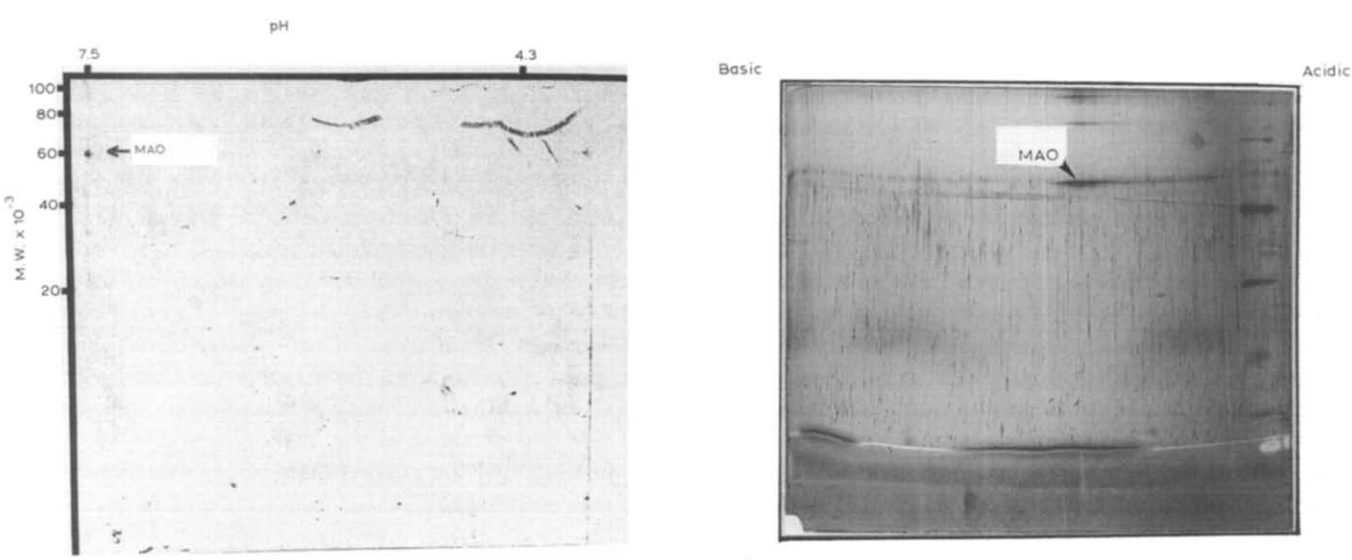

Fig. 5.(a and b) 2D gel electrophoresis of MAO resolved in the first dimension by normal polarity (a) or non-equilibrium reverse polarity (b). Only 2 μg of MAO was loaded per gel.

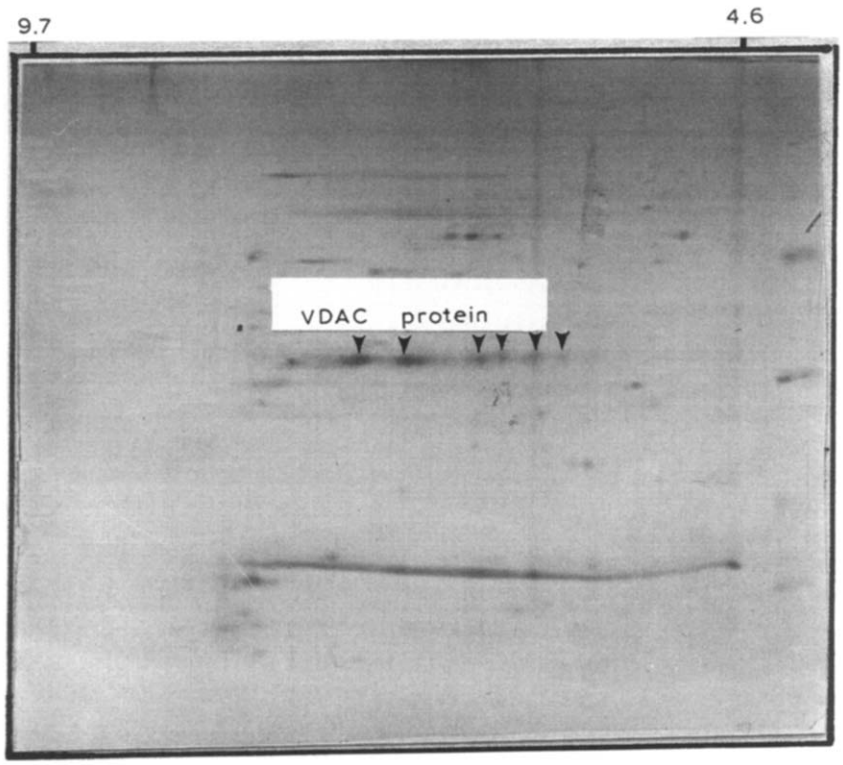

Fig. 6. Non-equilibrium reverse polarity two-dimensional gel electrophoresis of outer membrane. 40 μg of protein was loaded.

pattern of the MOM was much more revealing. This gel (Fig. 4) differed from the previous 2D gels (Fig. 2a and b) in that the pH gradient in the first dimension was 3–10 instead of 4.5 to 7.5. Approximately 50 polypeptides of the MOM were observed upon silver staining although some of these are apparently isoforms of the same protein. The absence of CPS, which was the major polypeptide in the 2D gel of whole mitochondria, attests further to the purity of the MOM fraction obtained by the swelling technique. A major protein which apparently exists in three isoelectric forms (a–c) is the pore (VDAC) protein with an apparent molecular mass of 32 kDa. The p*I* values of the three forms are a, 8.2; b, 7.8; and c, 7.1. Spot d represents a major polypeptide estimated to have a molecular mass of 14 kDa. In a one-dimensional SDS analysis of the proteins of the MOM from rat hepatocytes, the two major bands were reported to have a molecular mass of 31 and 15 kDa, respectively [43]. Spot e represents MAO based on the 2D gel electrophoresis of 2 μg of purified MAO (Fig. 5a) which showed this protein to have a molecular mass of 60 kDa. However, as this protein penetrated poorly into the first dimensional gel, it was difficult to determine its p*I*. Thus, whereas with normal polarity during electrofocusing this protein remained in the basic end of the gel, with reverse polarity it remained mostly in the acidic side with some portion trailing continuously up to pH 7.3 and with two spots between 6.3 and 7.3. (Fig. 5b).

In a NEPHGE gel of the MOM fraction (pH gradient 3–10), the pore protein was found in a parallel band composed of six isoelectric forms as shown in Fig. 6. A NEPHGE gel of 2 μg of purified pore protein (Fig. 7) produced the same six isoforms seen in the NEPHGE gel of the MOM.

Discussion

Earlier studies aimed at the separation and identification of mitochondrial polypeptides have approached this task primarily via one-dimensional SDS PAGE [16–24]. In only a few studies was the more powerful resolving power of two dimensional gel electrophoresis employed to analyze either whole mitochondria [44–46], the mitochondrial matrix from rat liver [42], yeast mitochondrial inner membrane [39] or the purified pore protein from rat liver mitochondria [14]. A limitation in most of the above studies was the sensitivity of detection of the proteins due to the usage of Coomassie blue staining. In the analysis of the whole mitochondria or the yeast mitochondrial inner membrane, only the polypeptides synthesized within the mitochondria were labeled and detected by autoradiography [44,45].

In analyzing the polypeptides of the mitochondria or its subcompartments three factors are of prime importance. Firstly, they must be prepared with minimal contamination, otherwise an excessive number of polypeptides will be attributed to any given subcompart-

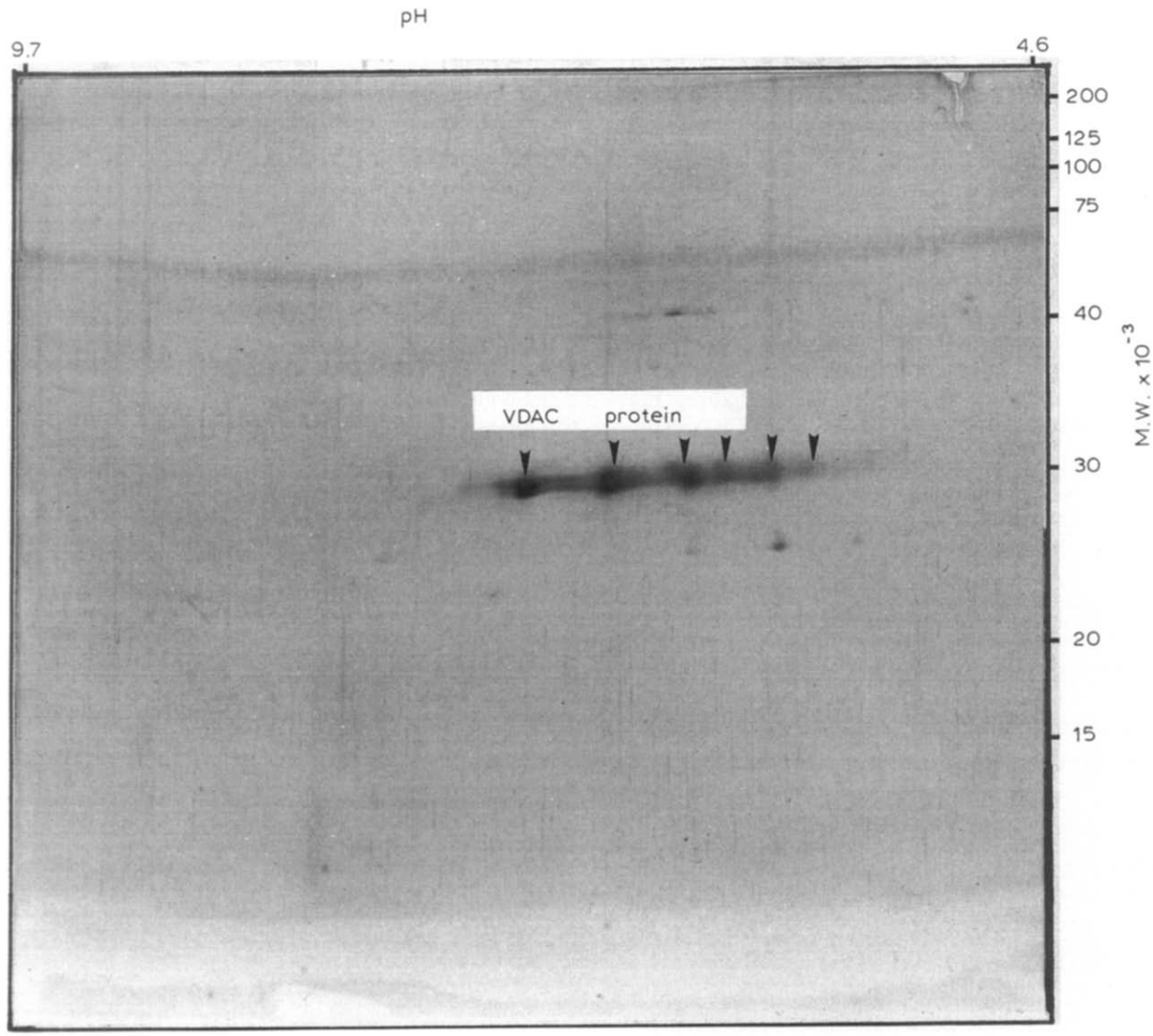

Fig. 7. 2 μg of the VDAC protein was resolved by non-equilibrium reverse polarity gel electrophoresis.

ment or to the whole mitochondrion. Secondly, there is a need to resolve the polypeptides by the most powerful means at our disposal, and last is the need for sensitive detection. In this work the latter two objectives were met by the use of 2D gels and silver staining [25,27]. The results showed that approximately 250 polypeptides could be detected in whole mitochondria by silver staining which is 2–3-times the number observed with Coomassie blue. The greatest difference in sensitivity between the two staining methods is for polypeptides having a molecular mass under 50 kDa. These low molecular weight proteins migrate a longer distance in the gel and might diffuse considerably more than the high molecular weight proteins which travel a comparatively shorter distance. Consequently, the concentration of the low molecular weight polypeptides at the center of the spots could be low and can be detected more sensitively by silver staining than by Coomassie blue.

Do lipids or phospholipids interfere with the gel electrophoretic resolution of the polypeptides? It does not seem so because, the proteins which are run through the two-dimensional gel procedure are denatured with urea, non-ionic detergents and SDS. Therefore, it is reasonable to assume that if lipids or phospholipids were bound to a protein that they would either dissociate from the protein upon denaturation or be displaced, especially by SDS. Nevertheless, should some lipids remain bound to the membrane proteins, it is likely that this would be a stochastic process. This would result in a population of protein molecules with varying amounts of lipid bound to them. The electrophoresis of such a population should result in both a smearing of the proteins on the gel, as well as irreproducible results. This is in contrast to what we observed – highly reproducible results with discrete polypeptide spots. However, if a phospholipid remained stoichiometrically

bound to a protein, this could potentially result in isoforms with a different net charge. The VDAC protein showed six isoforms upon electrophoresis of the outer membrane (Fig. 6); but so did highly purified VDAC protein (Fig. 7) indicating that the isoforms are likely due to modifications of the protein [14] other than phospholipid binding.

The purifity of the outer membrane fraction obtained by the swelling procedure was excellent, and compatible with the purification of this fraction from rat hepatocytes by Gellerfors and Linden using the same procedure [43]. Microsomal contamination of the purified MOM as judged by marker enzyme analysis was nil (Table III), hence the number of contaminating polypeptides in the 2D gel of this fraction should be minimal. Likewise the purity of the whole mitochondria and its distinct 2D gel pattern from the microsomes indicates minimal contamination of the whole mitochondrial gel. In contrast, a mitochondrial preparation not purified through a gradient and, with 7% microsomal contamination, had 600 polypeptide spots (see also Ref. 46) upon 2D gel electrophoresis in comparison to the 250 spots observed with the purified mitochondrial preparation (data not shown).

A protein component of the MOM which has attracted considerable attention is the VDAC protein. This protein is postulated to form channels which permit the entrance of low molecular weight solutes into the mitochondrion. Studies on the mitochondrial outer membrane from *Neurospora crassa* [12], yeast [13], and rat hepatocytes [43] employing one-dimensional SDS-PAGE have shown the pore protein to have a molecular mass in the range of 30 to 33 kDa. This protein from rat liver has been shown to be identical to the hexose-binding protein [47], and NEPHGE two-dimensional electrophoresis of purified rat liver pore protein with detection by Coomassie blue indicated the presence of three isoforms with p*I* values of 7.9, 7.6 and 7.2 [14].

In this work the VDAC protein from intact MOM as well as the purified pore protein were analyzed by 2D gel electrophoresis. Using a pH 3 to 10 gradient, three isoelectric forms of pore protein (M_r 32000) were observed, but with the NEPHGE gels (also pH 3 to 10), up to six isoforms were observed from the intact membrane fraction, and six isoforms were also seen with 2 μg of purified VDAC protein (Fig. 7). The greater number of VDAC protein isoforms observed in this work can be explained, at least in part, by the greater sensitivity of detection by silver compared to Coomassie blue staining. It is difficult to compare the p*I* values found for the isoforms of VDAC protein found here to those obtained by Linden et al. [14] because these values can vary with ampholytes from different sources, and even from batch to batch from the same source [48].

Because of its poor penetration into the first dimensional gel, and incomplete focusing, the p*I* of MAO could not be accurately determined. However, the molecular mass of 60 kDa is compatible with that reported by other investigators using different methodologies [49–51], as well as by Dr. James Salach (personal communication) who supplied the purified protein. Therefore, on the basis of the latter, and its position at the basic end of the 2D gel, the spot at M_r 60000 (Fig. 2b, spot 10) is tentatively identified as MAO.

These data represent the first two dimensional gel analysis of both highly purified mitochondrial outer membrane and whole mitochondria. Analysis of the protein components of the mitochondrion, and its subcompartments is an important step toward understanding the structure, function and biogenesis of this organelle, and by identifying the various mitochondrial polypeptides it will become possible to study the regulation of each in greater detail.

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