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STUDIES ON MITOCHONDRIAL PROTEINS

I. SEPARATION AND CHARACTERIZATION BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

Rat liver mitochondria were fractionated into inner and outer membranes and soluble intermembrane space and matrix. The protein components of these fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Mitochondria contained at least 20 components ranging in molecular weights from 10 000 to 140 000. Inner membranes differed markedly from outer membranes both in number of components and size distribution. The intermembrane space contained a few polypeptide species. These were of low molecular weight. The matrix was characterized by a high molecular weight component (130 000) which comprised 30% of this fraction. A major carbohydrate-containing polypeptide with an approximate molecular weight of 93 000 was detected in outer membrane preparations.

INTRODUCTION

Membrane function is determined both by the nature of components involved and the manner in which these components are arranged within the membrane. For example, mitochondrial electron transfer and energy coupling apparently requires specific spatial arrangements between respiratory components. Although some progress has been made in the topographical analysis of specific mitochondrial inner membrane components (*e.g.*, cytochrome *c*, ATPase)[1], the architecture of the membrane as a whole remains to be established. The present study is the first in a series dealing with the composition and disposition of rat liver mitochondrial membrane proteins in preparations placed under various metabolic and functional states. In the present study we have characterized mitochondrial proteins employing polyacrylamide gel electrophoresis, a technique which has proved useful in establishing the protein composition of various membrane systems[2–4] and isolated membrane components (respiratory chain complexes[5], cytochrome oxidase[6,7], ATPase[8]).

Although these studies will emphasize the two membrane systems of the mi-

Abbreviation: TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

tochondrion, the analysis of matrix and intermembrane space compartments is included in the present study. Since the mitochondrial subfractions (inner membrane, outer membrane, matrix, and intermembrane space) embody a unique set of functions, we considered it important to compare the protein composition of these fractions. The results obtained in the present study indicate wide heterogeneity in the protein makeup of mitochondrial subfractions.

MATERIALS AND METHODS

Isolation of mitochondrial subfractions

Mitochondria were isolated from rat liver as previously described[9]. Outer membranes and mitoplasts (inner membranes *plus* matrix) were prepared by the method of osmotic lysis described by Parsons *et al.*[10]. The intermembrane space was collected from the supernatant fraction after mitochondria were swollen in 0.02 M phosphate buffer (pH 7.2) and sedimented at 35 000 $\times g$ for 20 min. This fraction was concentrated by placing the solution in dialysis tubing, applying dry Sephadex G-200 over the bag and placing the bag in the cold (4 °C) overnight. Outer membranes and mitoplasts were also prepared by the digitonin procedure of Schnaitman and Greenawalt[11]. After removal of the outer membrane with digitonin, the intermembrane space was collected in the 140 000 $\times g$ (45 min) supernatant. The matrix was separated from the inner membrane by sonicating the the mitoplasts at 10 mg protein/ml (15 ml total volume) in 0.25 M sucrose on ice for 2 min (4 times for 30 s) at 5 A with a Branson Sonifier. Unbroken mitoplasts were sedimented at 12 000 $\times g$ for 15 min and inner membranes were subsequently pelleted at 140 000 $\times g$ for 45 min. The remaining supernatant was the matrix fraction. Lubrol particles and microsomes were prepared as described by Schnaitman and Greenawalt[11].

Enzyme determinations

Marker enzymes used to characterize the purity of each fraction were monoamine oxidase[12] for the outer membrane, adenylate kinase[13] for the intermembrane space, cytochrome oxidase[14] for the inner membrane and glutamate dehydrogenase[13] for the matrix. NADPH cytochrome *c* reductase[15] was measured to assess microsomal contamination. All enzymes were assayed on freshly isolated fractions according to the procedure cited. Triton X-100 was employed to lyse the membrane fractions prior to assay. Protein was determined by the method of Lowry *et al.*[16] using bovine serum albumin as a standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Electrophoresis was performed in gels containing 6% acrylamide, 0.16% bisacrylamide, 0.1% sodium dodecyl sulfate, 0.05% N,N,N′,N′-tetramethylethylenediamine (TEMED), 0.1% ammonium persulfate, 0.04 M Tris, 0.02 M sodium acetate, and 0.002 M EDTA (pH 7.4). The gel solution was poured into glass tubes (5 mm $\times$ 100 mm) to a height of 85–90 mm and overlayerd with water. After gelation (60 min at room temperature), the water was replaced by electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.4) and the gels were allowed to sit overnight before being used.

Samples (2.0 mg protein/ml) were solubilized by mixing three parts suspen-

sion with one part of solubilization mixture to afford the following concentrations: sodium dodecyl sulfate (1%), mercaptoethanol (1%), glycerol (5%), pyronin Y (12.5 μg/ml) in electrophoresis buffer. The solubilized samples (20–50 μl, 30–75 μg protein) were layered beneath the buffer onto the gel surface. A current of 1 mA/gel was administered until the pyronin Y (tracking dye) entered the gel. The current was then increased to 8 mA/gel until the tracking dye migrated to within 2–3 mm of the end of the gel (approximately 90 min). After the position of the tracking dye was marked with India ink, the gels were fixed and stained for protein with Coomassie brilliant blue according to the method of Fairbanks *et al.*[2]. Carbohydrate was localized by the periodic acid–Schiff procedure[2]. Fixed gels were stained with metachromatic toluidine blue as described by Sottocasa *et al.*[17]. Protein and carbohydrate were localized on the same gel using procedures described by Fairbanks *et al.*[2]. Periodic acid–Schiff-stained gels were stained for protein by soaking in 0.01% Amido Black in 10% acetic acid for 5 h, followed by destaining in 10% acetic acid. Dual scanning of doubly-stained gels was carried out at 525 nm and 620 nm (ref. 2).

The gel system was calibrated for molecular weight determination by measuring the migration of several standard proteins (thyroglobulin, 165 000; β-galactosidase, 130 000; phosphorylase *a*, 94 000; serum albumin, 68 000; ovalbumin, 43 000; D-amino acid oxidase, 37 000; chymotrypsinogen A, 25 700; myoglobin, 17 200; cytochrome *c*, 11 700). Molecular weight standards were solubilized as described above. Commercial samples of a few of the standards contained significant amounts of salt. These were dialyzed overnight against water (4 °C) prior to solubilization. Absolute mobilities were normalized to the migration of the tracking dye. Some deviation in the calibration curve occurred at low molecular weight (less than 18 000) resulting in overestimation of values. Replicate determinations of mobilities indicated a maximum uncertainty in reported molecular weights of ±10%. Densitometry of stained gels was carried out using a Gilford spectrophotometer equipped with a linear transport accessory. Percent distribution of components was estimated by cutting out peaks and weighing them to the nearest mg. It has been shown that Coomassie blue stain intensity increases linearly with protein levels up to 10 μg per band[18].

Neuraminidase treatment

The reaction mixture contained mitochondrial fractions (2.0 mg protein/ml) in 0.05 M sodium phosphate buffer (pH 6.0) and neuraminidase (0.17 mg/ml, 0.35 units/ml). After incubation at 37 °C for 60 min, the samples were treated with sodium dodecyl sulfate and mercaptoethanol and analyzed by gel electrophoresis as described above.

Materials

Acrylamide, bisacrylamide, TEMED, and pyronin Y were obtained from Eastman Organic Chem. Co. (Rochester, N.Y.). Coomassie brilliant blue R, neuraminidase (Type VI), thyroglobulin, β-galactosidase, phosphorylase *a*, and D-amino acid oxidase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sodium dodecyl sulfate, serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome *c* were supplied by Mann Research Laboratories (New York, N.Y.). Digitonin and Amido Black 10B were obtained from Calbiochem (La Jolla, Calif.).

RESULTS

Enzyme characterization of mitochondrial subfractions

Mitochondria are composed of two membrane systems and enclosed compartments. To obtain information concerning the location of the various polypeptide components within the mitochondrion, the following subfractions were prepared: (a) mitoplasts; (b) inner membrane; (c) matrix; (d) intermembrane space; and (e) outer membrane.

Prior to electrophoretic analysis, one must obtain highly purified fractions. Therefore, we have employed several methods to isolate mitochondrial subfractions and have selected those preparations displaying the least contamination for further study. The specific activity of marker enzymes in the various mitochondrial subfractions is shown in Table I. The use of digitonin to fractionate mitochondria resulted in the solubilization of outer membrane protein as ascertained by detection of monoamine oxidase activity in the intermembrane space fraction. Outer membranes prepared by digitonin treatment contained significant cytochrome oxidase activity suggesting that this procedure results in some degree of alteration of mitoplasts. In addition, intermembrane space material obtained after digitonin treatment contained glutamate dehydrogenase activity. Leakage of matrix material into the intermembrane space also occurred during osmotic lysis of mitochondria; however, this leakage was not as great as that caused by digitonin treatment. Furthermore, mitoplasts prepared by digitonin treatment exhibited less outer membrane contamination than those prepared by osmotic lysis. Our data on cytochrome oxidase distribution indicate that some inner membrane components do not sediment with the inner membrane fraction after sonication. Colbeau *et al.*[19] have reported similar purities of mitochondrial membrane fractions obtained by digitonin treatment and osmotic lysis.

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN MITOCHONDRIAL SUBFRACTIONS

Preparations were prepared either by osmotic lysis (A) or digitonin treatment (B). Results are expressed as nmoles/min per mg protein.

Fraction	*Monoamine oxidase*	*Glutamate dehydrogenase*	*Adenylate kinase*	*Cytochrome oxidase*
Mitochondria	7.8	200	660	730
A. Mitoplasts	6.4	83	6.5	850
Outer membrane	140	4.9	67	0
Intermembrane space	0	42.0	7900	0
B. Mitoplasts	0.1	160	11	1200
Inner membrane	0.6	7.0	2.3	1500
Outer membrane	64	9.0	111	850
Intermembrane space	33	100	2600	0
Matrix	0	310	0.5	470

Therefore, on the basis of marker enzyme activities, the following were selected for electrophoretic analysis: (A) outer membranes and intermembrane space prepared by osmotic lysis and (B) mitoplasts, inner membranes, and matrix obtained by digitonin treatment.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of rat liver mitochondria resulted in the complex pattern shown in Fig. 1A. Each band is assumed to represent single polypeptide chains because, under the conditions of solubilization, depolymerization of oligomeric proteins occurs. Rigorous calibration of the gel system with standard monomeric proteins made possible the assignment of molecular weights to the various components visible on the electrophorogram.

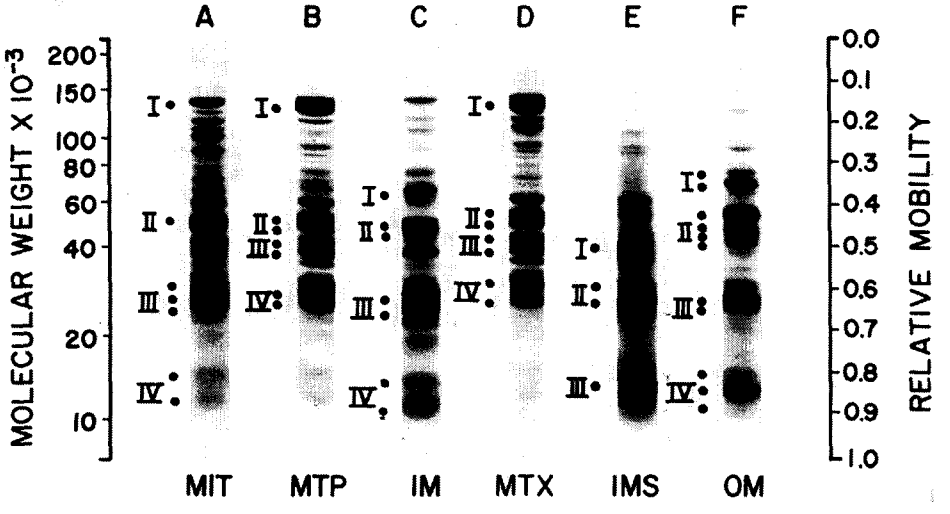

Fig. 1. Electrophoretic analysis of rat liver mitochondrial subfractions. The various preparations (2 mg protein/ml) were solubilized with sodium dodecyl sulfate (1%) and mercaptoethanol (1%), subjected to electrophoresis in 6% polyacrylamide gels, and stained with Coomassie blue as described in Methods and Materials. Each gel contained 30 μg protein. The gel origin is at the top. The Roman numerals and dots denote major bands. Abbreviations: MIT, mitochondria; MTP, mitoplasts; IM, inner membrane; MTX, matrix; IMS, intermembrane space; OM, outer membrane.

The per cent composition of the various mitochondrial polypeptides as determined by quantitative densitometry of stained gels is given in Table II. Although mitochondria possess nearly 20 polypeptides ranging in molecular weight from 10000 to 140000, eight of these comprise greater than 60% of the protein of the organelle.

The contribution of the various mitochondrial subfractions to the intact organelle pattern was assessed by examining each fraction individually. Mitoplasts were characterized by a major component of mol. wt 127000 (Fig. 1B). This component, which comprised nearly 30% of the mitoplast protein, was released almost completely into the matrix fraction during the production of inner membranes by sonication (Figs 1C and 1D). Inner membranes also contained a high molecular weight

TABLE II

MOLECULAR WEIGHT AND RELATIVE ABUNDANCE OF MAJOR MITOCHONDRIAL POLYPEPTIDES

The per cent composition values were obtained from densitometer tracings of the gels shown in Fig. 1. The gels were scanned at 545 nm and the peaks cut out and weighed to the nearest mg. Replicate determinations of mobilities of mitochondrial polypeptides and standard proteins indicate a maximum uncertainty of ±10% in the assignments of molecular weights. The Roman numerals refer to those shown on Fig. 1. A range of molecular weights is given when the degree of resolution obtained precluded determination of the per cent composition of individual bands. These clustered bands are marked by dots on Fig. 1.

Component	*Mol. wt*	*Composition (%)*
Mitochondria		
I	130 000	12.8
II	51 000–49 000	19.2
III	34 000–26 000	24.9
IV	15 000–13 000	9.2
Mitoplasts		
I	130 000	29.8
II	52 000–48 000	16.7
III	44 000–37 000	12.1
IV	29 000	19.7
Inner membrane		
I	66 000	11.6
II	52 000–49 000	17.6
III	29 000	32.0
IV	16 000–13 000	19.5
Matrix		
I	130 000	29.9
II	52 000–50 000	20.3
III	44 000–40 000	11.8
IV	30 000–26 000	13.9
Intermembrane space		
I	44 000–36 000	12.7
II	27 000	31.5
III	14 000	40.0
Outer membrane		
I	71 000–64 000	15.8
II	49 000–41 000	35.4
III	29 000–27 000	22.6
IV	14 000–10 000	20.7

component which accounted for 5% of the total material. Although it is possible that this component represents residual matrix material, it is a reproducible characteristic of the inner membrane pattern. Inner membranes prepared by Lubrol treatment exhibited patterns essentially identical to those prepared by sonication. The patterns obtained in the present study differ somewhat from those reported by

Schnaitman[3] for Lubrol particles, which may be explained in part by the use of different electrophoretic methods.

Intermembrane space preparations contained low amounts of high molecular weight components (Fig. 1E). A broad low molecular weight species (14000) represented 40% of the intermembrane space material. High molecular weight material was essentially absent from electrophorograms of outer membrane (Fig. 1F). A major band of mol. wt 49000 was noted on these gels. The patterns of outer membrane presented here are similar to those reported by Schnaitman[3] for rat liver mitochondria but differ somewhat from those derived from beef heart mitochondria[20]. However, some similarity did exist between rat liver and beef heart preparations, namely the low percentage of high molecular weight material.

Carbohydrate-containing components were localized on the gels using the periodic acid–Schiff stain. At the level of protein examined (75–150 μg), mitochondria, mitoplasts, and inner membranes displayed only one prominent periodic acid–Schiff-positive band (relative mobility, 0.9), which did not stain with Coomassie blue and may represent inositol-containing phospholipid. This fast-moving periodic acid–Schiff-positive band was the only component present on gels of matrix and was barely detectable. No periodic acid–Schiff-positive material was observed on gels of intermembrane space preparations. The low levels of glycolipid present in both matrix and intermembrane space preparations suggest a low level of membrane contamination in these samples.

Several periodic acid–Schiff-positive bands were observed on electrophorograms of outer membrane material (Fig. 2). The most prominent band (relative mobility, 0.9) corresponds to glycolipid, while those of lower mobility are assumed to represent glycoproteins. At least three outer membrane glycoprotein bands were detected with a major component (periodic acid–Schiff-positive outer membrane component, relative mobility 0.23–0.25) comprising approximately 80% of the

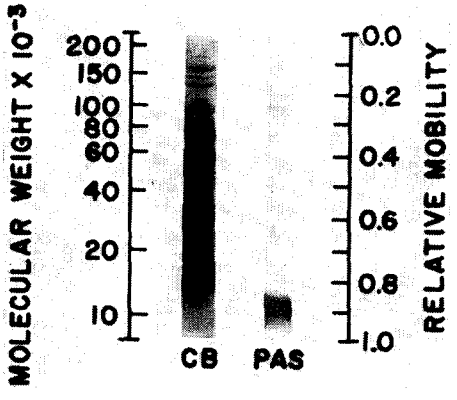

Fig. 2. Electrophoretic analysis of rat liver outer membranes. Outer membranes (2 mg protein/ml) were solubilized with sodium dodecyl sulfate (1%) and mercaptoethanol (1%) and subjected to electrophoresis in 6% acrylamide gels as described in Methods and Materials. Duplicate gels (containing 75 μg protein) were stained for protein (CB, Coomassie blue) and carbohydrate (PAS, periodic acid–Schiff stain). The gel origin is at the top. The major periodic acid–Schiff-positive band (relative mobility, 0.23–0.25) is arbitrarily designated as the periodic acid–Schiff-positive outer membrane component.

total non-lipid periodic acid–Schiff reactivity. As outer membranes contained significant microsomal contamination, we considered the possibility that the periodic acid–Schiff-positive outer membrane component represented a contaminant. This proved not to be the case, for although electrophoretic analysis of microsomal samples revealed the existence of several periodic acid–Schiff-positive bands, none of these possessed mobilities similar to that of the periodic acid–Schiff-positive outer membrane component. The periodic acid–Schiff-positive outer membrane component, which possessed an apparent mol. wt of 93000, did not stain with toluidine blue while the lipid zone showed intense staining.

To ascertain the nature of the periodic acid–Schiff-positive components, outer membrane preparations were treated with neuraminidase prior to examintion by sodium dodecyl sulfate–gel electrophoresis. This treatment resulted in a 40% decrease in staining intensity of the periodic acid–Schiff-positive outer membrane component, while leaving the minor components unchanged. These results agree with those of Bosmann *et al.*[21] who found that neuraminidase treatment of isolated outer membranes resulted in a similar decrease in sialic acid content. Neuraminidase treatment, while reducing the intensity of the periodic acid–Schiff-positive outer membrane component, did not affect the distribution of Coomassie blue staining. However, samples which were incubated both with and without neuraminidase afforded electrophorograms having diffuse bands and persistent background. The reasons for this are unclear. As sufficient neuraminidase was present in our reaction mixture to insure removal of all the outer membrane sialic acid (based on sialic acid content reported in ref. 22), we suggest that either (a) a large proportion of the sialic acid is unavailable to the enzyme or that (b) some of the periodic acid–Schiff reactivity is due to groups other than sialic acid. Although we cannot distinguish between these possibilities at this time, our results do indicate the sialoglycoprotein nature of periodic acid–Schiff-positive outer membrane component. Furthermore, the paucity of periodic acid–Schiff-positive outer membrane species suggests that the sialic acid present in this membrane system may reside almost totally in the 93 000 mol. wt glycoprotein.

Although the periodic acid–Schiff-positive outer membrane component was located near a Coomassie blue stained band (Fig. 2), closer examination revealed the lack of coincidence of these components. When the location of the periodic acid–Schiff-positive outer membrane component was marked with India ink and the gel stained with Coomassie blue, the ink mark resided above the nearby blue band. That the periodic acid–Schiff-positive outer membrane component did not bind protein stains was also demonstrated by examination of single gels stained both for carbohydrate (periodic acid–Schiff) and protein (Amido Black) (Fig. 3). The difference profile obtained by dual scanning of these gels at 525 and 620 nm (see ref. 2) indicated that the periodic acid–Schiff-positive outer membrane component did not coincide with any band stained by Amido Black. Although the periodic acid–Schiff-positive outer membrane component did not bind conventional stains, the large size of this component suggests that it is indeed a glycoprotein. The inability to stain erythrocyte membrane sialoglycoproteins with protein dyes has also been reported[2]. Our results indicate the importance of verifying the correspondence of components on replicate gels.

The periodic acid–Schiff-positive outer membrane component was present

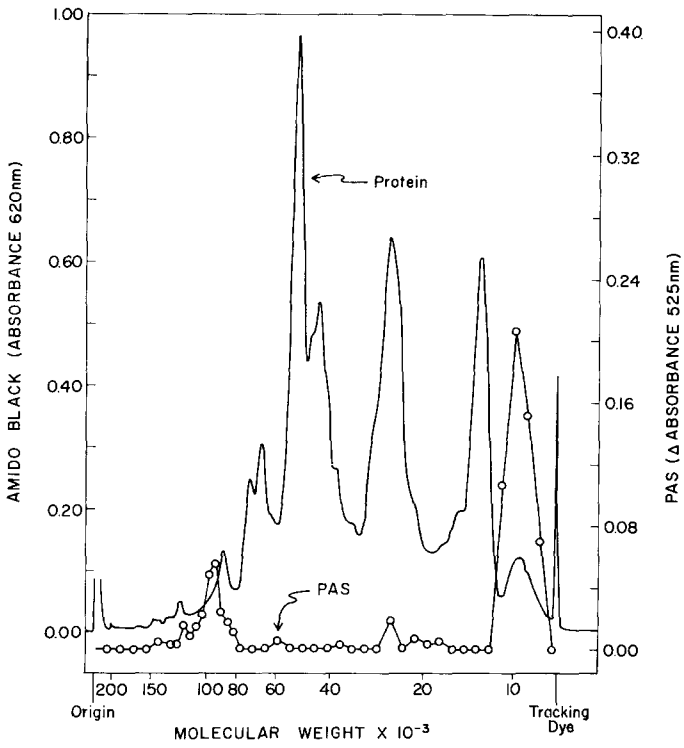

Fig. 3. Electrophoretic analysis of rat liver outer membranes. Outer membranes (2 mg protein/ml) were solubilized with sodium dodecyl sulfate (1%) and mercaptoethanol (1%) and subjected to electrophoresis in 6% acrylamide gels as described in Methods and Materials. Single gels (containing 45 μg protein) were doubly stained with periodic acid–Schiff and Amido Black as described in Methods and Materials and analyzed by the dual scanning procedure of Fairbanks *et al.*[2]. The periodic acid–Schiff (PAS) trace (○—○) was obtained by subtracting the Amido Black contribution at 525 nm from the total absorbance at this wavelength. The Amido Black trace (———) was obtained by scanning at 620 nm.

in reduced amounts in outer membranes prepared by digitonin treatment. Furthermore, intermembrane space material obtained by this method displayed a periodic acid–Schiff-positive component possessing a relative mobility similar to that of the periodic acid–Schiff-positive outer membrane component. Apparently, digitonin treatment caused the release of outer membrane material into the intermembrane space fraction. This conclusion is supported by the observation that intermembrane space preparations obtained using digitonin contained significant monoamine oxidase activity.

DISCUSSION

The present study has clearly demonstrated the marked heterogeneity of mitochondrial proteins. Utilizing the powerful resolving capacity of polyacrylamide gel electrophoresis, we have separated mitochondrial proteins into about 20 distinct and reproducible components.

It has been shown that the various mitochondrial subfractions (inner and outer membranes, intermembrane space, and matrix) embody unique functional activities. This functional divergence is paralleled by a diversity in protein composition. It must be noted that fractionation of mitochondria results in concentration of the proteins of the isolated subfractions. The outer membrane is concentrated 10–15 times while the matrix is concentrated about 1.5 times relative to whole mitochondria. Most proteins of the outer membrane (unless greater than 10% of this fraction) are hardly seen in the gels of whole mitochondria, while the matrix and inner membrane protein make the major contributions. Inner membranes exhibit a more complex protein pattern than outer membranes, an observation also reported by Schnaitman[3]. However, our electrophoretic patterns differed somewhat from those described by Schnaitman[3]. The employment of different electrophoretic systems may be responsible in part for these differences. In the present study, outer membranes were characterized by a low concentration of high molecular weight polypeptides. This property of outer membranes has also been demonstrated for preparations isolated from rat liver[3] and bovine heart mitochondria[20]. Employing a different electrophoretic method than that used in this study, Smoly *et al.*[23] reported that outer membranes of beef heart mitochondria possessed a major protein component, which accounted for a large part of the total protein of the preparation. We could find no evidence for such a protein in our preparations.

Several studies have reported the existence of carbohydrate in the various fractions of the mitochondrion. It has been shown that outer membranes contain 4–5 times as much sialic acid as inner membranes[19,21,22]. Furthermore, it was concluded that a portion of the carbohydrate was bound to macromolecules of undetermined nature[21]. Sottocasa *et al.*[17] have demonstrated the existence of a glycoprotein (mol. wt 51 000) in the intermembrane space of rat liver mitochondria[17]. In the present study, glycoproteins present in the inner membrane and intermembrane space apparently existed at levels too low to be detected by our staining methods. In order to visualize the glycoprotein of the intermembrane space, Sottocasa *et al.*[17] loaded 30 times as much protein onto gels as we did. A major sialoglycoprotein, the periodic acid–Schiff-positive outer membrane component (mol. wt 93 000), was detected in our outer membrane preparations. However, some caution must be exercised when assigning molecular weight values to glycoproteins as these components may display atypical behavior in the sodium dodecyl sulfate–gel system[24]. Our results suggest that the periodic acid–Schiff-positive outer membrane component may contain nearly all the outer membrane sialic acid. Hayashi and Capaldi[20] concluded that bovine heart mitochondrial outer membrane contained no glycoprotein. This conclusion was based on the absence of gel components staining with toluidine blue. It was of interest to note that, in the present sudy, the periodic acid–Schiff-positive outer membrane component failed to stain with toluidine blue. It has been reported that this stain exhibits low affinity for certain glycoproteins[25]. At present, it is not possible to assign functional significance to the outer membrane sialoglycoprotein.

The present study also demonstrates that electrophoretic analysis can be useful in assessing the integrity of a particular isolated preparation. For example, outer membranes prepared by digitonin and osmotic lysis differed not only in enzymic makeup but also in sodium dodecyl sulfate–gel electrophoretic profiles. The

periodic acid–Schiff-positive outer membrane component was eluted from the membrane by digitonin treatment and apparently appeared in the intermembrane space preparation. It is conceivable that this glycoprotein may serve as a marker for the outer membrane, which could be monitored electrophoretically.

The indentification of components on the electrophorograms is made difficult because under the conditions of solubilization, native proteins are reduced to single polypeptides. Therefore, sodium dodecyl sulfate–electrophoresis affords little information concerning the oligomeric associations existent in mitochondrial proteins *in situ*. Some progress has been made in the investigation of oligomeric proteins of the erythrocyte membrane by employing controlled fixation in conjunction with sodium dodecyl sulfate–electrophoresis[26]. We are currently utilizing such approaches to examine the native associations between proteins in the mitochondrion.

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