

MITOCHONDRIAL AUTONOMY

Sialic Acid Residues on the Surface of Isolated Rat Cerebral Cortex and Liver Mitochondria

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ABSTRACT

N-acetylneuraminic acid at the surfaces of rat cerebral cortex and liver mitochondria and derived mitoplasts (inner membrane plus matrix particles) was studied biochemically and electrokinetically. Rat cerebral cortex mitochondria in 0.0145 M NaCl, 4.5% sorbitol, pH 7.2 ± 0.1 , 0.6 mM NaHCO₃, had an electrophoretic mobility of -2.88 ± 0.01 μ /sec per v per cm. In the same solution the electrophoretic mobility of rat liver mitochondria was -2.01 ± 0.02 , of rat liver mitoplasts was -1.22 ± 0.07 , and of rat cerebral cortex mitoplasts -0.91 ± 0.04 μ /sec per v per cm. Treatment of these particles with 50 μ g neuraminidase/mg particle protein resulted in the following electrophoretic mobilities in μ /sec per v per cm: rat cerebral cortex mitochondria, -2.27 ; rat liver mitochondria, -1.40 ; rat cerebral cortex mitoplasts, -0.78 ; and rat liver mitoplasts, -1.10 . Rat liver mitochondria, mitoplasts, and outer mitochondrial membranes contained 2.0, 1.1, and 4.1 nmoles of sialic acid/mg protein, respectively. 10% of the liver mitochondrial protein and 27.5% of the sialic acid was solubilized in the mitoplast and outer membrane isolation procedure. Rat cerebral cortex mitochondria, mitoplasts, and outer mitochondrial membranes contained 3.1, 0.8, and 6.2 nmoles sialic acid/mg protein, respectively; 10% of the brain mitochondrial protein and 49% of the sialic acid was solubilized in the mitoplast and outer membrane isolation solution procedure. Treatment of both the rat liver and cerebral cortex mitochondria with 50 μ g neuraminidase (dry weight)/mg protein resulted in the release of about 50% of the available outer membrane sialic acid residues. Treatment of all of the particles with trypsin caused release of sialic acid but did not greatly affect the particle electrophoretic mobility. In each instance, curves of pH vs. electrophoretic mobility indicated that the particle surface contained an acid dissociable group, most likely a carboxyl group of sialic acid with $pK_a \sim 2.7$. Treatment of either the rat liver or the cerebral cortex mitochondria with trypsinized concanavalin A did not affect the particle electrophoretic mobility but did cause a decrease in the electrophoretic mobility of L5178Y mouse leukemic cells.

INTRODUCTION

Sialic acid is a known constituent of plasma membrane surface glycolipids and glycoproteins (1) and as a terminal carbohydrate residue contributes greatly to the net cell surface charge and electrophoretic mobility (2). Sialic acid is also found in internal cell membranes such as the smooth endo-

plasmic reticulum (3) and the rough endoplasmic reticulum (3). Recently, the presence of sialic acid has been demonstrated in mitochondria (4) of rabbit livers; isolated mitochondria have been shown to autonomously synthesize the carbohydrate portion of glycoproteins and glycolipids (5-7) and the glycoprotein nature of mitochondrial structural protein (8) and the products of autonomous protein synthesis (9, 10) have been described. The role of the glycoproteins and sialic acid in the mitochondrion is unknown.

Mitochondria have for some time been recognized as being capable of autonomous synthesis of limited amounts of certain types of macromolecules including DNA, RNA, protein, lipid, glycoprotein, and glycolipid (11). This partial autonomy, the size of the mitochondrion, and other factors have led to the speculation that mitochondria may be descendants from an earlier evolutionary invasion of cells by bacteria and the subsequent formation of a symbiotic relationship between the mitochondria (bacteria) and the cell. The question remains as to how much of the mitochondrion is synthesized by the mitochondrion and how much is synthesized in the cell nucleus and extramitochondrial cytoplasm. The control mechanisms regulating mitochondrial synthesis and the correct integration of mitochondrial and extramitochondrial macromolecules to form the organelle are unknown (12). The present study describes some parameters relating to molecules on the surface of the mitochondrion and mitoplast and discusses whether these molecules are synthesized by the mitochondrion or extramitochondrially.

Electrophoretic mobility of biological cells or subcellular fractions is thought to be primarily due to the presence of ionogenic groups. Changes in electrophoretic mobility upon treatment with group specific reagents, selective degradative enzymes, or selective adsorptive agents (e.g., concanavalin A) provide information about the nature of these ionogenic groups at the cell or organelle periphery. In the present set of experiments this technique has been exploited to determine the nature of the surfaces of isolated rat cerebral cortex and liver mitochondria and submitochondrial particles, the "mitoplasts".

MATERIALS AND METHODS

Solutions

All solutions were prepared in glass distilled water. The solution utilized in most of the studies was termed

saline sorbitol and contained 0.0145 M NaCl, 0.6 mM NaHCO₃, pH 7.2 ± 0.1, 4.5% sorbitol. This solution and isosmotic solutions of NaOH and HCl for adjusting solution pH were prepared as described by Heard and Seaman (13). All percentages used in the text are w/v.

Materials

Neuraminidase (EC 3.2.1.18) (purified, proteolytic activity free [14, 15], with an activity of 0.7 units/mg where one unit of activity equals 1 μmole of *N*-acetylneuraminic acid released per min at 37°C at pH 5) and purified trypsin (EC 3.4.4.1) were purchased from Worthington Biochemical Corp., Freehold, N. J. Concanavalin A and albumin (crystalline, bovine serum) were purchased from Calbiochem, LaJolla, Calif. Density gradient grade sucrose was purchased from Mann Research Labs., Inc., New York. Other biochemicals utilized were purchased from Sigma Chemical Corp., St. Louis, Mo.

Mitochondrial Isolation

Essentially pure rat liver (6-9) and rat cerebral cortex (reference 16, fraction WPP5) mitochondria were isolated as previously described from 100-200 g male rats that had been starved for 16 hr before sacrifice. Mitochondria were utilized immediately after preparation.

Preparation of Mitoplasts

In order to determine properties of the inner mitochondrial membrane, mitochondria stripped of the outer mitochondrial membrane (the so-called inner membrane plus matrix particles or mitoplasts) were obtained by the procedure of Schnaitman and Greenawalt (17), the procedure of Wojtczak et al. (18), or that of Sottocasa et al. (19). The three preparations gave essentially similar results, and the data reported herein are for the digitonin inner membrane-matrix fraction of Schnaitman and Greenawalt (17); this preparation is referred to herein as mitoplasts.

Growth of L5178Y Mouse Leukemic Cells

L5178Y cells were grown in Fischer media (20) in suspension culture as described previously (21). Cells were utilized for experiments in the midlogarithmic phase of growth.

Electrophoretic Mobilities

Measurements were made at 25°C ± 0.1°C in a horizontal cylindrical chamber of 5 ml volume equipped with reversible, blacked platinum electrodes (23, 24). The chamber was viewed by transillumination in the apparatus obtained from Rank Brothers,

Bottisham, England. The mobilities of the particles were calculated in μ/sec per v per cm ; each value was obtained by timing the movement of at least 20 particles with reversal of polarity after each measurement. The alignment of the apparatus was checked by the method of Heard and Seaman (13). Determinations of the mobility of washed human erythrocytes were made in 0.0145 M NaCl, 4.5% sorbitol made 0.6 mM with respect to NaHCO_3 . Normal blood for this purpose was obtained from healthy donors of the phenotype A RH⁺, taken into EDTA, immediately washed, and electrophoretic mobilities were determined. Heard and Seaman (13) reported a value for the electrophoretic mobility of human erythrocytes of $-2.78 \pm 0.08 \mu \text{ sec}^{-1} v^{-1} \text{ cm}^{-1}$ while in the present experiments we found a value of $-2.79 \pm 0.04 \mu \text{ sec}^{-1} v^{-1} \text{ cm}^{-1}$ in saline sorbitol. A minimum of three independent experiments were performed for each electrophoretic mobility determination, and the values are the means of at least 80 readings \pm SEM or \pm SD.

Protein Determination

Protein was determined by the method of Lowry et al. (25), using bovine serum albumin as standard.

Neuraminidase or Trypsin Treatment of Mitochondria or Mitoplasts

The procedure used (22, 24) was to treat mitochondria or mitoplasts with neuraminidase or trypsin and to measure the electrophoretic mobility of a portion of the treated mitochondria or mitoplasts. Mitochondria or mitoplasts removed with as little excess sucrose solution as possible, corresponding to 0.1–1 mg (as protein), were treated with 1 ml of various concentrations of neuraminidase or trypsin dissolved in physiological saline. The pH was adjusted to 6.5–7.0 and the tubes were incubated in a gently rocking water bath at 37°C for 30 min. The treated mitochondria or mitoplasts were centrifuged at 7000 g for 10 min, washed three times with physiological saline, centrifuged for 10 min at 2500 g , and finally washed in saline-sorbitol. The washed mitochondria or mitoplasts were resuspended in 1 ml of saline-sorbitol, and four drops of this solution were added to 30 ml saline-sorbitol to obtain a dilute mixture for observation of electrophoretic mobility. The particles were timed successively in both directions at 60 v (interelectrode distance: 8.596 cm) for two grids (166 μ). The chamber was routinely washed with $\text{K}_2\text{Cr}_2\text{O}_7/\text{H}_2\text{SO}_4$, distilled water, and saline-sorbitol between runs. The pH of the solution was kept at 7.2 ± 0.1 .

Treatment with Trypsinized Concanavalin A

Concanavalin A was trypsinized by a modified method of Burger and Noonan (26). 25 mg of con-

canavalin A was dissolved in 2.4 ml of 0.2 M phosphate, pH 7.0, 0.1 ml of 2.5% trypsin was added, and the solution was incubated at 37°C for 5 hr. The trypsinization was stopped by adding 2.5 ml of 1% trypsin inhibitor. 0.5 ml of mitochondria, mitoplasts or, in some instances, L5178Y cell suspension were incubated with 0.1 ml trypsinized concanavalin A solution or control solution plus 0.4 ml of physiological saline for 30 min. The particles were centrifuged at 7000 g for 10 min and washed twice with physiological saline. Suspensions for the observation of electrophoretic mobility were made and measurements were taken as outlined above.

Sialic Acid

Released or free sialic acid was measured by the Warren (27) procedure while total sialic acid was determined by the Svennerholm (28) procedure.

Calculation of Parameters

Conversions of electrophoretic mobilities to other electrokinetic parameters were made by the Helmholtz-Smoluchowski equation and the generalized Gouy equation for a uni-divalent ionic system, as described by Abramson (29) and Heard and Seaman (13). The following diameters as approximations derived from electron micrographs were used in these calculations: rat liver and cerebral cortex mitochondria, $d = 0.7 \times 10^{-6}$ m; rat liver and cerebral cortex mitoplasts, $d = 0.5 \times 10^{-6}$ m. The equation for surface area of a sphere was used to calculate the following surface areas, realizing that the particles in fact may not be spherical and that the invaginations and folding of the surfaces, depending upon the suspending media, might cause the area calculation to be off by an order of magnitude: rat liver and cerebral cortex mitochondria, $SA = 1.5 \times 10^{-12}$ m², and rat liver and cerebral cortex mitoplasts, 0.8×10^{-12} m².

RESULTS

Electrophoretic Mobilities of Isolated Rat Liver and Cerebral Cortex Mitochondria and Mitoplasts and Electrokinetic Parameters

The data given in Table I summarize the electrophoretic mobilities and electrokinetic parameters for rat cerebral cortex mitochondria and mitoplasts and rat liver mitochondria and mitoplasts. Determinants of these electrophoretic mobilities are given in the studies described below, and conversion of other mobilities to other electrokinetic parameters can be made using the equations given by Heard and Seaman (13). The rat cerebral cortex mitochondria had a higher

TABLE I
Electrophoretic Mobility of Rat Cerebral Cortex and Liver Mitochondria and Derivative Mitoplasts and Conversion of Mobilities to Other Electrokinetic Parameters

Particle	Mobility	Zeta potential	Surface charge density	No. of electrons
	$\mu/\text{sec}/\text{v}/\text{cm}$	<i>mv</i>	<i>e.s.u./cm²</i>	<i>millions/particle surface</i>
Liver mitochondria	-2.01 ± 0.02	26.4	2.24×10^3	0.067
Cerebral cortex mitochondria	-2.88 ± 0.01	37.8	3.41×10^3	0.102
Liver mitoplasts	-1.22 ± 0.07	16.0	1.35×10^3	0.022
Cerebral cortex mitoplasts	-0.91 ± 0.04	12.0	0.97×10^3	0.015

Data for electrophoretic mobilities are means ± 1 SD (see text for explanation of statistic; the total N in each instance was from 200 to 600). All data are for saline-sorbitol ionic strength of 0.0145 at 25°C at 0.6 mM NaHCO₃, pH 7.2 \pm 0.1. Experiments were performed as given in Materials and Methods.

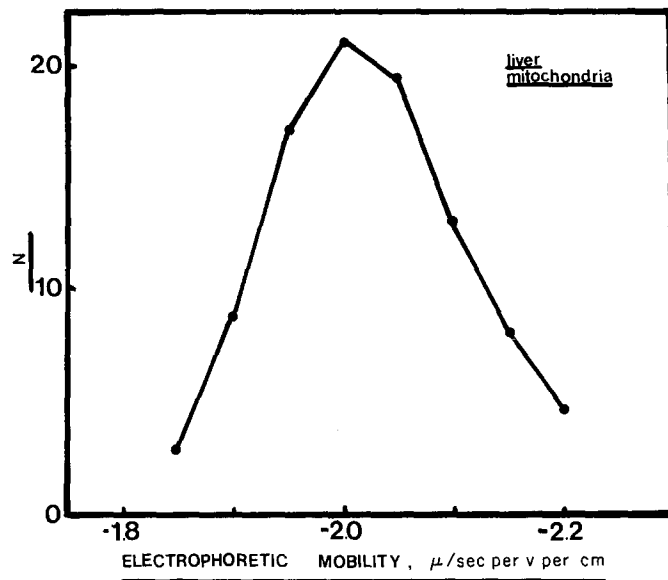


FIGURE 1 Distribution of electrophoretic mobilities of isolated rat liver mitochondria. 94 observations from four independent experiments are plotted where N equals the number of observations having the indicated electrophoretic mobility. All electrophoretic mobilities were rounded up to the nearest 0.05; i.e., -1.81 , -1.83 , and -1.85 would be plotted as -1.85 while -1.98 , -1.99 , and -1.96 would be plotted as -2.00 . Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaHCO₃, pH 7.2 \pm 0.1, 4.5% sorbitol, 25°C.

electrophoretic mobility than the rat liver mitochondria, indicating that the surfaces of the two particles are probably somewhat different. The liver mitochondria were calculated to have 67,000 electrons/mitochondrion surface while the cerebral cortex mitochondria were calculated to have 102,000 electrons/mitochondrion surface, assuming an identical surface area. The electrophoretic mobilities of the mitoplasts were: (a) much lower than those of the respective mitochondria from which they were derived, (b)

different for the rat liver and brain, and (c) different in the opposite sense than their parent mitochondrial mobilities, i.e., the cerebral cortex mitochondria had a higher electrophoretic mobility while the cerebral cortex mitoplasts had a lower electrophoretic mobility when compared to their liver counterparts. The liver mitoplasts were calculated to have 22,000 electrons/mitoplast surface while the cerebral cortex mitoplasts were calculated to have 13,000 electrons/mitoplast. In Fig. 1, data are presented showing the distribution

of electrophoretic mobilities for a group of readings on individual rat liver mitochondria. It is evident that the electrophoretic mobilities have a rather normal distribution, and there is no evidence of multimodes. These data indicate the homogeneity of the mitochondrial preparation and more importantly the homogeneity of the electrophoretic mobilities of the particles. Similar distributions obtained for all the other mobility measurements described herein; i.e., in no instances were bi- or trimodal distributions of mobility encountered and in all cases the mobilities measured behaved like those derived from one homogeneous population of particles.

Effect of Neuraminidase Treatment on the Electrophoretic Mobility of Isolated Rat Liver Mitochondria and Release of Sialic Acid by Neuraminidase Treatment

The curve in Fig. 2 indicates that treatment with neuraminidase decreased the electrophoretic mobility of rat liver mitochondria. The decrease was

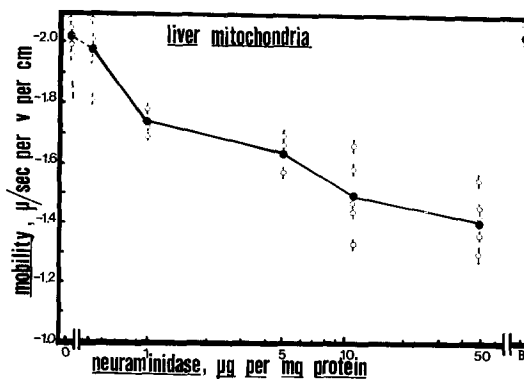


FIGURE 2 Electrophoretic mobilities of neuraminidase treated rat liver mitochondria. Open circles give the mean value ± 1 sd for each experiment (minimum of 20 measured particles) while the closed circles give the mean value for the various experiments. B refers to neuraminidase (50 $\mu\text{g}/\text{mg}$ mitochondrial protein) which was boiled for 5 min before use in the experiment. Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaHCO₃, pH 7.2 \pm 0.1, 4.5% sorbitol, 25°C.

TABLE II
Sialic Acid of Rat Cerebral Cortex and Liver Mitochondria and Derivative Mitoplasts and Amount of Sialic Acid Released by Treatment with Enzymes

Particle	Total	Sialic Acid nmoles/mg particle protein					
		Released upon treatment with neuraminidase			Released upon treatment with trypsin		
		boiled (50 $\mu\text{g}/\text{mg}$)	10 $\mu\text{g}/\text{mg}$	50 $\mu\text{g}/\text{mg}$	boiled (50 $\mu\text{g}/\text{mg}$)	0.01%	1%
Liver mitochondria	2.0 (200)*	0‡	0.20	0.31	0	0.12	0.19
Liver mitoplasts	1.1 (83)	0	0.06	0.07	0	0.08	0.08
Liver outer membrane	4.1 (62)	—§	—	—	—	—	—
Cerebral cortex mitochondria	3.1 (310)	0	0.36	0.48	0	0.14	0.22
Cerebral cortex mitoplasts	0.8 (60)	0	0.03	0.04	0	0.04	0.04
Cerebral cortex outer membrane	6.2 (93)	—	—	—	—	—	—

Experiments were performed as described in Materials and Methods, and treatment with neuraminidase or trypsin was as given in Materials and Methods.

* Number in parenthesis denotes relative recoveries of sialic acid based on recovery of protein in the sub-mitochondrial fraction; the whole mitochondrion is arbitrarily set at 100. Of this 100 mg of protein, 15 mg of protein are found in the outer mitochondrial membrane and 75 mg in the mitoplasts or inner membrane and matrix fraction, and 10 mg are solubilized.

‡ 0 denotes not measurable.

§ — denotes experiment not performed.

dependent on the amount of neuraminidase utilized to treat the mitochondria; treatment with 50 μg neuraminidase/mg mitochondrial protein decreased the mobility from -2.01 to -1.40 $\mu/\text{sec per v per cm}$, a decrease of 0.61 units or 30%. That the decrease was not due to adsorption of the neuraminidase on the mitochondrial surface is supported by the fact that boiled neuraminidase (50 $\mu\text{g}/\text{mg}$ mitochondrial protein) did not alter mobility. The data in Table II indicate that treatment with 10 μg neuraminidase/mg mitochondrial protein released 0.20 nmoles of sialic acid/mg rat liver mitochondria while treatment with 50 μg neuraminidase/mg rat liver mitochondrial protein released 0.31 nmoles/mg mitochondrial protein. Higher amounts of neuraminidase did not cause release of more sialic acid. It is interesting to note that the liver outer membrane contains 62 nmoles sialic acid/100 mg of original liver mitochondria or 0.62 nmoles of sialic acid/mg protein; exactly one-half or 0.31 nmoles/mg mitochondrial protein is released by neuraminidase treatment. This 0.31 nmoles/mg mitochondrial protein represents 15% of the 2.0 nmoles/mg protein present in the intact liver mitochondrion (Table II). Thus, sialic acid is a terminal constituent of the liver mitochondrial outer membrane, is released upon neuraminidase treatment of the

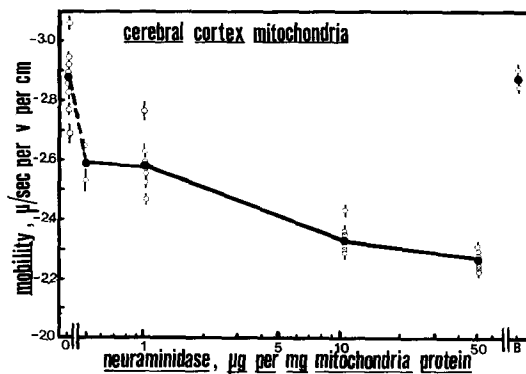


FIGURE 3 Electrophoretic mobilities of neuraminidase treated rat cerebral cortex mitochondria. Open circles give the mean value ± 1 sd for each experiment (minimum of 20 measured particles) while the closed circles give the mean value for the various experiments. *B* refers to neuraminidase (50 $\mu\text{g}/\text{mg}$ mitochondria protein) which was boiled for 5 min before use in the experiment. Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaHCO_3 , pH 7.2 ± 0.1 , 4.5% sorbitol, 25°C.

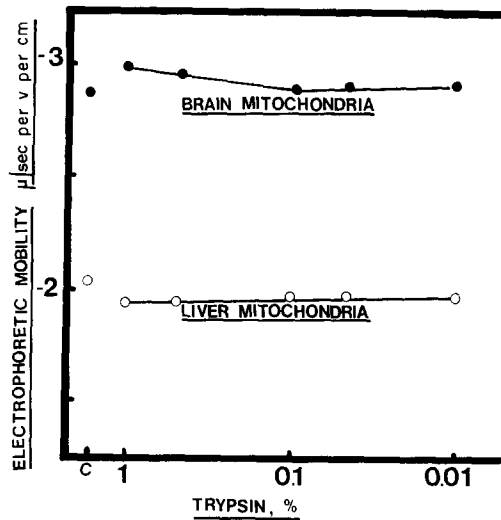


FIGURE 4 Electrophoretic mobilities of trypsin treated rat liver or cerebral cortex mitochondria. *C* represents control or nontreated mitochondria. Data are means from four independent experiments. Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaHCO_3 , pH 7.2 ± 0.1 , 4.5% sorbitol, 25°C.

liver mitochondrion, and the electrophoretic mobility of the mitochondrion decreases with neuraminidase treatment.

Effect of Neuraminidase Treatment on the Electrophoretic Mobility of Isolated Rat Brain Mitochondria and Release of Sialic Acid by Neuraminidase Treatment

The data of Fig. 3 demonstrate that the electrophoretic mobility of isolated rat cerebral cortex mitochondria also decreased upon treatment with neuraminidase. The decrease was not due to adsorption of the neuraminidase on the mitochondrial surface, as is shown by the boiled control. At 50 $\mu\text{g}/\text{ml}$ of neuraminidase per mg mitochondrial protein the electrophoretic mobility decreased from -2.88 to -2.27 $\mu/\text{sec per v per cm}$, a decrease of 0.61 or 21%. Thus even though the net decrease in electrophoretic mobility was essentially the same between the rat liver (0.61) and rat cerebral cortex (0.61) mitochondria, the percent decrease was not (liver, 30%; brain, 21%). This points up a further difference between the liver and cerebral cortex mitochondria. Treatment of the cerebral cortex mitochondria with 50 μg of neuraminidase/mg mitochondrial protein resulted

in the release of 0.48 nmoles of *N*-acetylneuraminic acid/mg mitochondrial protein. The cerebral cortex mitochondria outer membrane contains 93 nmoles of sialic acid/100 mg mitochondrial protein or 0.93 nmoles/mg protein. Again, the amount of *N*-acetylneuraminic acid released by the maximum concentration of neuraminidase equals roughly 50% of the available *N*-acetylneuraminic acid associated with the outer membranes.

Sialic Acid Content of Mitochondria

The data of Table II also provide some information about the location of mitochondrial sialic acid. The liver mitochondrion contains 2.0 nmoles of sialic acid/mg protein or 200 nmoles/100 mg of liver mitochondria. This means that since the liver mitochondrion outer membrane contains 62 nmoles/100 mg protein and the mitoplasts 83 nmoles/100 mg protein, about 55 nmoles/100 mg protein were "solubilized" or "lost" in the separation procedure. Similarly, the cerebral cortex contains 310 nmoles/100 mg protein (much higher than the liver mitochondria), of which 60 nmoles/100 mg protein were associated with the mitoplasts and 93 nmoles/100 mg protein were associated with the cerebral cortex mito-

chondrial membrane, giving a result that 157 nmoles/100 mg protein (much higher than the liver mitochondria) were released during the separation procedure.

Electrophoretic Mobilities of Isolated Rat Liver and Cerebral Cortex Mitochondria Treated with Trypsin

The data in Fig. 4 illustrate that treatment of either the liver or cerebral cortex mitochondria with the proteolytic enzyme trypsin did not greatly affect the electrophoretic mobility of the particles. This result was obtained in spite of the fact that trypsin caused release of proteins (measured by the Lowry et al. method [25]) from the mitochondrial surfaces, and, as shown in Table II, in spite of the fact that large amounts of sialic acid were liberated from the mitochondria. It is evident (Table II) that treatment of the liver mitochondria with 1% trypsin releases 0.19 nmoles of sialic acid/mg particle protein while 10 μ g neuraminidase releases 0.20 nmoles/mg particle protein; the former treatment causes a slight decrease in electrophoretic mobility to -1.91μ /sec/v/cm (Fig. 4) while the latter causes a significant decrease in electrophoretic mobility to

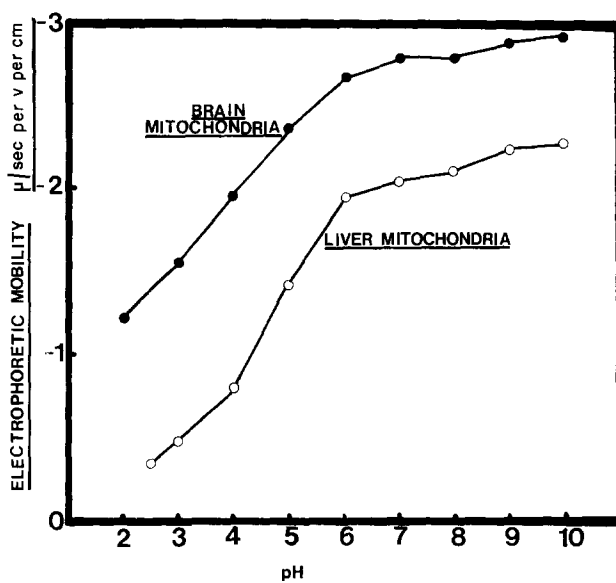


FIGURE 5 Electrophoretic mobility of isolated rat liver or cerebral cortex mitochondria as a function of pH. Experiments were performed as given in Materials and Methods and points are means from four independent experiments. The solution for measurement was 0.0145 M NaCl, 4.5% sorbitol, 25°C, pH adjustments were made with isosmotic NaOH or HCl.

– 1.50 μ /sec/v/cm (Fig. 2). Similar results are obtained for the brain mitochondria, i.e., even though relatively large amounts of sialic acid are released upon trypsin treatment (Table II), little change in electrophoretic mobility (indeed with the 1% trypsin treatment a slight increase was observed) was found (Fig. 4). These results are not unique, however, since Seaman and Uhlenbruck (30) have found that chicken erythrocyte electrophoretic mobility increased after trypsin treatment even though sialic acid was also released by the proteolytic action; similar results have also been obtained for guinea pig cerebral cortex synaptosomes (24).

Effect of pH on the Electrophoretic Mobility of Isolated Rat Cerebral Cortex or Liver Mitochondria

The curves of Fig. 5 give the pH vs. mobility relationships for the isolated rat cerebral cortex or liver mitochondria. The curves are quite similar, with a steep rise in mobility between pH 2 and pH 6 with a relatively stationary level of electrophoretic mobility maintained between pH 6 and

pH 10. These data are consistent with the existence of a dissociable acid function, probably a carboxyl group of sialic acid with pK_a of approximately pH 2.7.

Electrophoretic Mobilities of Rat Liver or Cerebral Cortex Mitochondria as a Function of Ionic Strength

The mobility: ionic strength relationships for the isolated rat cerebral cortex or liver mitochondria are given in Fig. 6. It is of interest to note that at ionic strengths greater than 0.12 the electrophoretic mobilities of the liver and brain mitochondria were similar (at a mobility of – 1.00 μ /sec per v per cm).

Electrophoretic Mobilities of Rat Liver or Cerebral Cortex Mitochondria Treated with Concanavalin A

Treatment of the mitochondria with concanavalin A (up to 20 μ g/mg mitochondrial protein), as given in Materials and Methods, did not affect their electrophoretic mobility

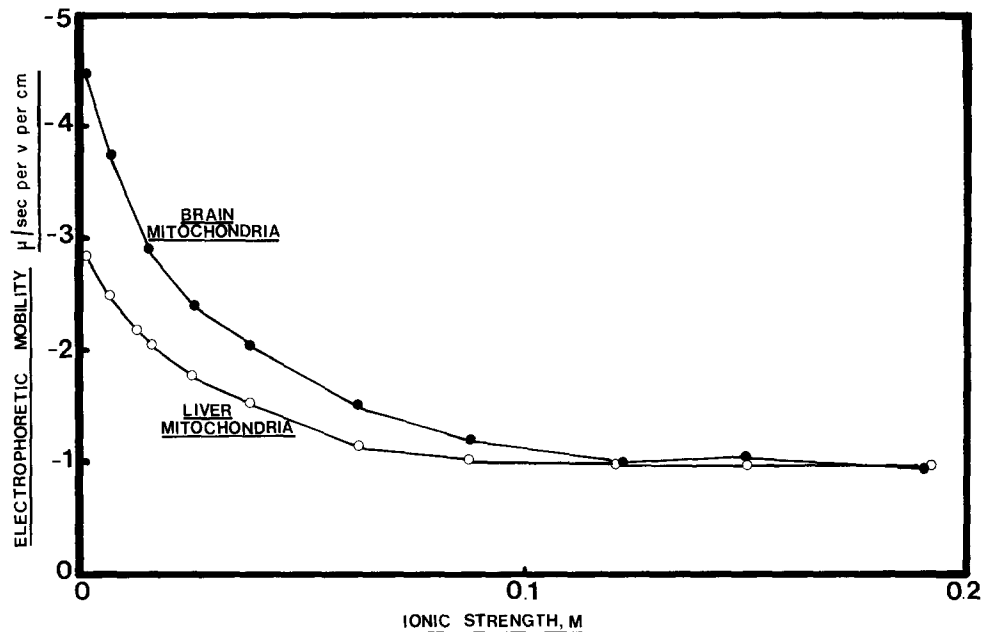


FIGURE 6 Electrophoretic mobility of isolated rat liver or cerebral cortex mitochondria as a function of ionic strength. Experiments were performed as given in Materials and Methods and points are means from four independent experiments. The solution for measurement was 4.5% sorbitol, 0.6 mM NaHCO_3 , pH 7.2 ± 0.1 , 25°C ; ionic strength adjustments were made with NaCl and for very low ionic strengths the NaHCO_3 was omitted and the measurements were made in distilled water containing 4.5% sorbitol.

at all. This was not the case with the leukemic cells L5178Y which showed a decrease in the net negative electrophoretic mobility with increasing concentrations of trypsinized concanavalin A. At 200 μg concanavalin A/mg L5178Y protein the electrophoretic mobility was reduced from a control of $-1.28 \mu\text{/sec per v per cm}$ to $0.96 \mu\text{/sec per v per cm}$. Treatment of isolated guinea pig synaptosomes with concanavalin A also results in a decrease in the net negative electrophoretic mobility (24).

Electrophoretic Mobilities of Isolated Rat Liver or Cerebral Cortex Mitoplasts Treated with Neuraminidase

The data in Fig. 7 demonstrate that both the rat liver and cerebral cortex mitoplasts had decreased

mobilities after neuraminidase treatment; however, the decreases found were not as great as for the whole mitochondrion (i.e., decreases in electrophoretic mobility caused by action of the neuraminidase on the outer mitochondrial membrane). Treatment with 50 μg of neuraminidase/mg mitochondrial protein decreased the brain mitoplast electrophoretic mobility from -0.91 to $0.78 \mu\text{/sec per v per cm}$ (a 14% decrease compared to a 21% decrease for the brain mitochondria treated with 50 μg neuraminidase/mg protein) and decreased the liver mitoplast electrophoretic mobility from -1.22 to $1.10 \mu\text{/sec per v per cm}$ (a 10% decrease compared to a 30% decrease for the liver mitochondria treated with 50 μg neuraminidase/mg protein). The data of Table II illustrate that the mitoplasts contained much less sialic acid/mg protein than the respective mito-

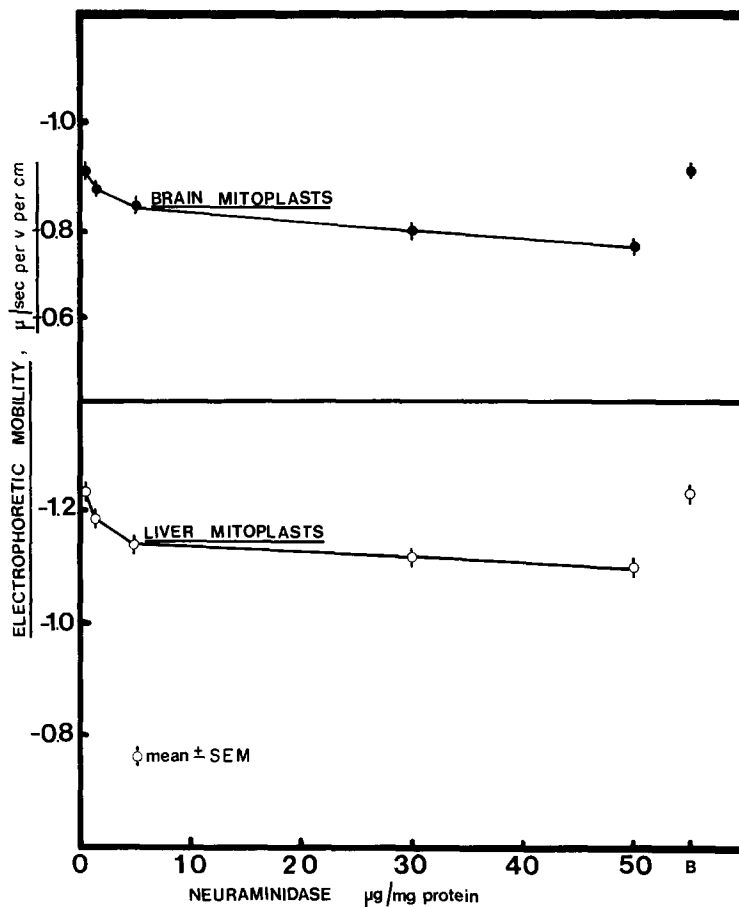


FIGURE 7 Electrophoretic mobilities of isolated rat liver or cerebral cortex mitoplasts treated with neuraminidase. Experiments were performed as given in Materials and Methods and are means \pm SEM for three experiments. B refers to neuraminidase which was boiled for 5 min before use in the experiment. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaCO₃, pH 7.2 \pm 0.1, 25°C, 4.5% sorbitol.

chondrial outer membranes and that much less sialic acid was released upon neuraminidase treatment from the mitoplasts than from the mitochondria from which they were derived. For example, treatment with 50 μg neuraminidase/mg particle protein released 0.31 nmoles/mg protein from the intact liver mitochondrion and only 0.07 nmoles/mg protein from the liver mitoplasts. This 0.07 nmoles/mg protein represents 7 nmoles/100 mg protein, and therefore only 7 out of the 83 nmoles present per 100 mg protein or 8.5% of the available sialic acid was released from the liver mitoplasts. Similarly, only 6.7% of the available sialic acid was released by the brain mitoplasts upon treatment with 50 μg neuraminidase/mg cerebral cortex mitoplast protein.

Effects of Trypsin Treatment on the Electrophoretic Mobility of Isolated Mitoplasts of Rat Cerebral Cortex or Liver

The data of Fig. 8 indicate that treatment of the liver and brain mitoplasts with trypsin slightly increased their electrophoretic mobility. These data point up a major characteristic of the mitoplast surface. The differences in electrophoretic mobility after protease treatment were slight even though substantial amounts of sialic acid (Table II) were released. As much sialic acid was released with the trypsin treatment in the liver mitoplasts as with the high level of neuraminidase, but the former treatment *increased* the electro-

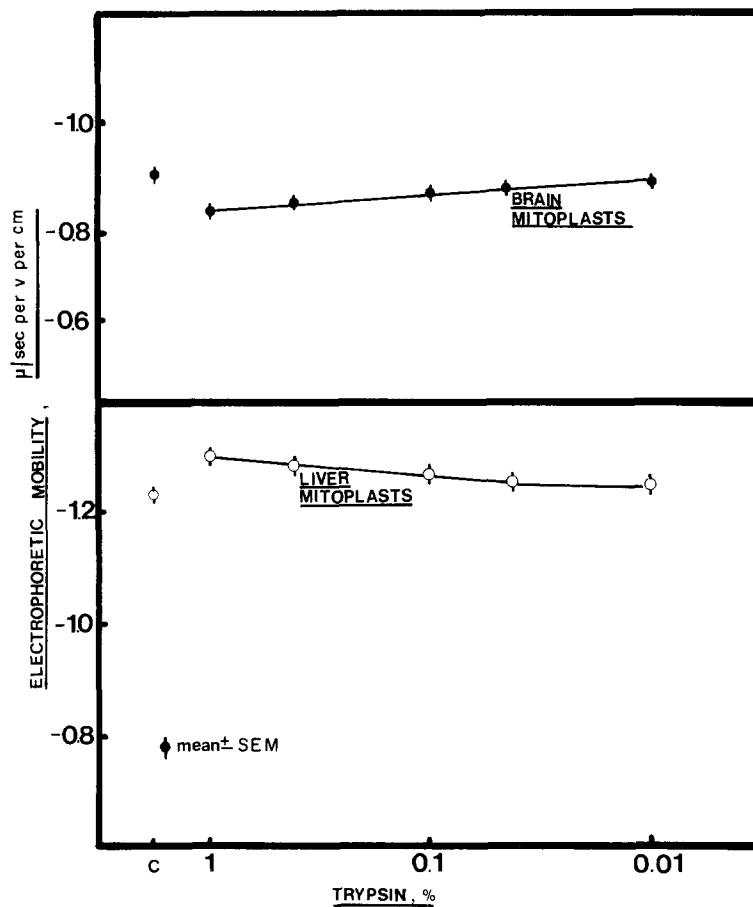


FIGURE 8 Electrophoretic mobilities of trypsin treated rat liver or cerebral cortex mitoplasts. *C* refers to control or nontreated mitoplasts. Experiments were performed as given in Materials and Methods and the data are means \pm SEM for three independent experiments. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaHCO₃, pH 7.2 \pm 0.1, 25°C, 4.5% sorbitol.

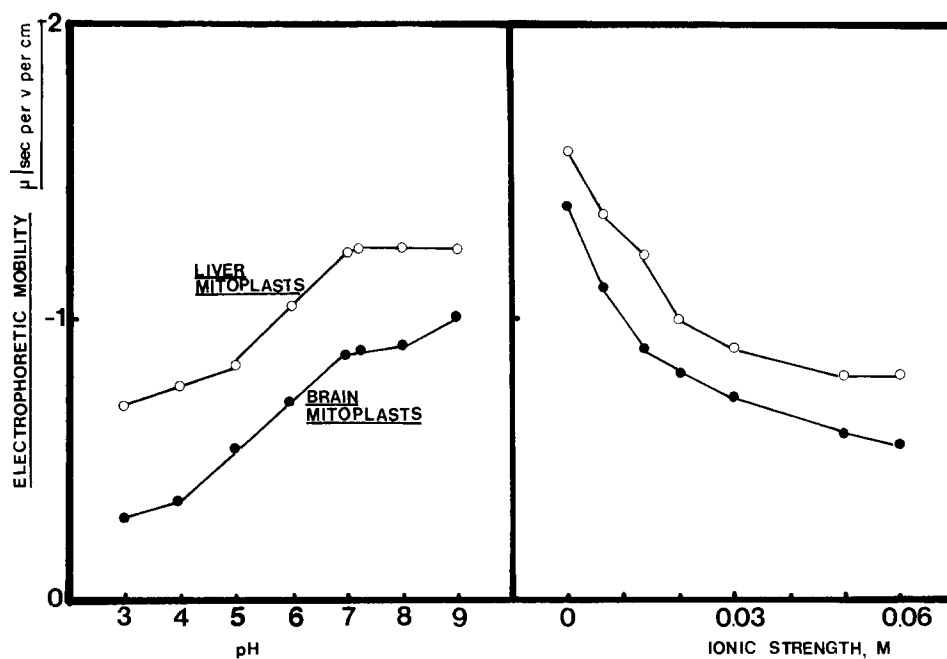


FIGURE 9 Electrophoretic mobilities of isolated rat cerebral cortex or liver mitoplasts as a function of pH or ionic strength. Experiments were performed as given in Materials and Methods and the data points are means from three independent experiments. For the pH:mobility curves the measuring solution was 0.0145 M NaCl, 25°C, 4.5% sorbitol, and pH was adjusted with isosmotic NaOH or HCl. For the ionic strength:mobility curves the measuring solution was 25°C, 0.6 mM NaHCO₃, pH 7.2 ± 0.1, 4.5% sorbitol except at low ionic strengths when the NaHCO₃ was omitted.

phoretic mobility whereas the latter *decreased* the mobility.

Effects of pH and Ionic Strength on the Electrophoretic Mobilities of Isolated Cerebral Cortex or Liver Mitoplasts

The data of Fig. 9 indicate that the electrophoretic mobility of the brain mitoplasts rose rather continuously from pH 3 to pH 9 whereas the mobility of the liver mitoplasts rose between pH 3 and pH 7 and was constant at -1.22 from pH 7 to pH 9. The electrophoretic mobilities of the mitoplasts decreased with increasing ionic strength and leveled off above an ionic strength of 0.05.

DISCUSSION

The data presented herein clearly show that sialic acid residues are present on the outer surface of the mitochondrion and, to some extent, on the outer surfaces of mitoplasts. It is of interest to speculate about the function of this electronegative group on

the organelle surface and indeed on the mitochondrial inner membrane, outer surface. In whole cells, sialic acid residues have been implicated in cell adhesiveness, communication, contact inhibition, permeability, recognition, antigenic sites, etc. In the mitochondrion, it is possible that surface sialic acid-containing macromolecules also function as recognition or regulatory macromolecules. In this regard the differences in the electrophoretic mobility and sialic acid content between the liver and cerebral cortex mitochondria, which are statistically significant, may indicate fundamental differences in the properties and functions of these mitochondria from two different tissue sources. Indeed, just as various cell types are characterized by varying electrophoretic mobilities, their derived mitochondria may also exhibit varying electrophoretic mobilities.

Heidrick et al. (31) have demonstrated by preparative free-flow electrophoresis that the mitochondrion's outer limiting membrane is the sole determinant of mitochondrial surface charge and that the mitochondrial inner membrane-matrix fraction exhibited a markedly different

electrophoretic mobility. The results presented herein are in agreement with these observations, and in particular the differences between the mitochondrial outer membrane surface and the mitoplast outer membrane surface were shown to be quite great with respect to electrophoretic mobility and sialic acid content. The only previous determination of mitochondrial electrophoretic mobility was done by Nakamura et al. (32), who reported a value for mouse liver mitochondria in glucose:potassium phosphate buffer (ionic strength, 0.167; pH, 7.30) of -0.60 to -0.65 μ /sec per v per cm while rat erythrocytes, as standard material under the same experimental conditions, showed the value of -1.100 ± 0.040 μ /sec per v per cm; no evaluation of the determinants of the observed mitochondrial electrophoretic mobility was given (32).

The point must be made that the results of electrophoretic mobilities and electrokinetic behavior of cellular organelles suspended in relatively simple electrolyte should be interpreted with caution when attempts are made to relate the findings to the *in vivo* cellular situation. The mitochondria described herein are present *in vivo* in a highly complex cytoplasmic milieu quite unrelated to the simple electrolyte solutions used for measurements. Furthermore, the mitochondria themselves would be expected to be in various stages of maturity or, put in another way, at various stages of the "cell mitochondrial cycle" (see below and 12). Finally, the mitoplast is a rather unnatural particle and certainly the suspending medium used in the present measurements is an unnatural milieu for this particle created through biochemical manipulation. Nonetheless, the fact that bimodal or multimodal distribution of the mobility measurements did not occur for any of the particles examined indicates that the results were probably not complicated by adsorption of cellular debris to the particles and that homogeneous populations (at least with respect to surface phenomena) were being investigated. It is suggested that the present findings are true evidence of surface phenomena of the particles and that the changes brought about by the enzymic treatment and environmental alterations represent definite changes in the physical-chemical properties of the particle surfaces.

The data in this report clearly show that the outer membranes of the rat liver and cerebral cortex mitochondria are different and that the surfaces of the mitoplasts of the rat liver and

cerebral cortex are also different. Finally, it can be unequivocally concluded that the exposed surfaces of the mitochondrion and its derived submitochondrial particle, the mitoplast, are dissimilar.

Treatment of the mitochondria and, to a lesser degree, the mitoplasts with neuraminidase decreased the electrophoretic mobilities of the particles, presumably due to the removal from the surface of the carboxyl groups of sialic acid since it was demonstrated biochemically that sialic acid was indeed released. It seems unlikely, since neuraminidase is an endo-glycosidase, that the action of the enzyme would generate cationic groups. Furthermore, it seems unlikely that the enzyme binds nonspecifically to the particle surfaces since boiled neuraminidase did not alter the electrophoretic mobility of the mitochondria or mitoplasts and did not result in the release of sialic acid. Although the neuraminidase utilized in these experiments has an acid pH optimum, Kraemer (33) has shown that at pH 7.0 the neuraminidase activity was such that 91% of the available sialic acid residues were removed from Chinese hamster cell surfaces upon neuraminidase treatment. The fact that at least some of the mitochondrial sialic acid is released from the outer membranes by trypsin indicates that this sialic acid is present in glycoproteins and not in glycolipid. The reason why trypsin causes release of sialic acid but no decrease in electrophoretic mobility can probably be explained in a manner analogous to that used by Seaman and Uhlenbruck (reference 30 and personal communication) to explain the same effect with certain erythrocytes (30): the action of trypsin on surface glycoproteins generates terminal amino acid carboxyl groups in the cleaved glycoproteins; thus, although sialic acid (and its carboxyl group) is removed, a carboxyl with a similar pK is generated in the carboxy-terminus by trypsin, the net result of which is a similar electrophoretic mobility.

It is interesting to note that, in both the rat liver and cerebral cortex mitochondria, almost exactly one-half of the sialic acid content was the maximum released with the neuraminidase. This means that 50% of the residues were not neuraminidase susceptible; it is tempting to speculate that the mitochondrial membrane is symmetrical with respect to sialic acid. The inner surface of the outer membrane might be a mirror image of the outer surface, with 50% of the sialic acid residues on this inner surface of the outer mitochondrial

membrane and 50% on the outer surface. Perhaps some of the sialic acid released by trypsin is not identical with that released by neuraminidase, such that the decrease in mobility produced by neuraminidase reflects loss of surface sialic acid residues while trypsin releases sialic acid from internal protein sites.

In the present work, in both the liver and brain mitochondria about 10% of the protein was released or solubilized in the isolation procedure for the mitoplasts and outer membrane. In the liver mitochondria, however, 27.5% of the sialic acid was solubilized while in the cerebral cortex mitochondria 49% of the sialic acid was lost (Table II). These data, in some respects, support the work of the Italian workers (34, 35), who have reported that 80% of the total sialic acid and 77% of the hexosamines of rat liver mitochondria are not an integral part of membrane structure or are very loosely bound to it (35). These workers (35) have reported the presence of a glycoprotein located in the intermembrane space of rat mitochondria. The present results on the amount of sialic acid released from the mitochondrial outer or inner membrane by neuraminidase treatment allow for accountability of only a small amount of the mitochondrial sialic acid. The data are consistent with the presence of an "intermembrane space glycoprotein" containing sialic acid. Furthermore, this laboratory has reported (7) that glycoproteins containing neutral sugars and hexosamines are present in the so-called "structural protein" fraction of the inner membrane even though this fraction is more correctly referred to as an insoluble protein fraction since it is heterogeneous and probably contains denatured ATPase among its several components (7). In any event, glycoproteins other than surface glycoproteins and soluble glycoproteins exist and probably account for a good deal of the mitochondrial sialic acid.

The mitochondrial electrophoretic mobilities were unaffected by incubation with concanavalin A while the L5178Y cell electrophoretic mobility and isolated synaptosome electrophoretic mobility (24) was decreased in the presence of trypsinized concanavalin A. These data are of interest since this plant lectin is thought to selectively agglutinate tissue culture cells transformed by oncogenic viruses or chemical carcinogens (36), although recently (37, 38) the amount of bound concanavalin A has been shown not to be the determinant of agglutination (i.e., "normal" and "transformed" cells bind about the same amount

of concanavalin A). The present results could be explained by several alternatives: (a) The mitochondria bind no concanavalin A, (b) although the mitochondria bind concanavalin A it generates no cationic groups nor blocks any anionic groups, or (c) the ionic groups generated or blocked are balanced by equal generations or blockage of ionic groups of opposite charge. Since mitochondrial binding of labeled concanavalin A has recently been reported (39, 40), alternatives (b) or (c) must obtain.

Nicolson et al. (39) have reported that other terminal sugar residues also exist on mitochondrial outer membrane surfaces, using specific agglutinins to identify these terminal residues. Thus, it may be that outer membranes of the mitochondria are quite similar, in their terminal monosaccharide units, to the cell plasma membrane.

Experiments have been performed to determine the origin of the sialic acid residues on mitochondrial membranes. Although incorporation of labeled uridine diphosphate (UDP)-glucose, guanosine diphosphate (GDP)-mannose, and UDP-galactose into glycolipid and glycoprotein occurred autonomously into both rat liver and cerebral mitochondria, no incorporation of label from cytidine monophosphate (CMP)-sialic acid occurred in the same system (Myers, M. W., and H. B. Bosmann, unpublished observations).

These data mean that one of two conclusions obtains: (a) Sialic acid-containing macromolecules of the mitochondria are synthesized extramitochondrially and subsequently integrated into the mitochondrial membranes, or (b) autonomous mitochondrial synthesis of sialic acid-containing macromolecules occurs by pathways other than those by which other monosaccharide-containing macromolecules are autonomously synthesized by the mitochondrion (e.g., by a precursor other than CMP-sialic acid). This problem is being further studied.

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