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COMPARISON OF *IN-VIVO* AND *IN-VITRO* TOXIC EFFECTS OF
MICROCYSTIN-LR IN FASTED RATS

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G.A. MIURA, N.A. ROBINSON, T.W. GEISBERT, K.A. BOSTIAN, J.D. WHITE and J.G. PACE. Comparison of *in-vivo* and *in-vitro* toxic effects of microcystin-LR in fasted rats, *Toxicol* , - , 19 . The toxic effects of microcystin-LR, a cyclic heptapeptide isolated from the blue-green alga *Microcystis aeruginosa*, were studied in the fasted rat model and in sub-cellular fractions from fasted, toxin-treated and control rats. Hepatotoxic effects of a lethal dose (100 μ g/kg) were examined 15 to 90 min post-injection. Elevations of serum enzymes, particularly sorbitol dehydrogenase, specific for liver mitochondria, correlated with hepatic damage. Electron micrographs showed progressive cellular disruption, including dilation of rough endoplasmic reticulum, incorporation of cellular components into cytolysosomes, hydropic mitochondria devoid of electron-opaque deposits, loss of desmosome-associated intermediate filaments, disruption of sinusoidal architecture, and ultimately lysis of hepatocytes. Appearance of hydropic mitochondria correlated with loss of coupled electron transport. Changes in plasma membrane-associated cytoskeletal filaments correlated with loss of desmosome tonofilaments. In contrast to *in-vivo* exposure to microcystin-LR, *in-vitro* exposure to toxin had no effect on mitochondria or cytoskeletal filaments, suggesting that the toxic effects observed *in-vivo* were indirect and may be dependent on bioactivation of the toxin or a cascade of events not supported in *in-vitro* models. (A 11)

INTRODUCTION

MICROCYSTIN-LR (MCYST-LR) (CARMICHAEL *et al.*, 1988), or cyclo-D-Ala-L-Leu-erythro-8-methyl-D-isoAsp-L-Arg-Adda-D-isoGlu-N-methyldehydroAla, where Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (SLATKIN *et al.*, 1983; HOTES *et al.*, 1985), isolated from the blue-green alga *Microcystis aeruginosa* strain 7280 (DABHOLKAR and CARMICHAEL, 1987) is one of several cyclic peptide toxins known to be hepatotoxic. Toxins from algal blooms, which contaminate water supplies, represent a potential health hazard to livestock (CARMICHAEL *et al.*, 1985) and human populations (FALCONER *et al.*, 1983).

In mice, highly purified toxin or crude cell extracts produce thrombocytopenia, microscopic pulmonary thrombi (ADAMS *et al.*, 1985; SIEGELMAN *et al.*, 1984), liver damage (RUNNEGAR and FALCONER, 1981; DABHOLKAR and CARMICHAEL, 1987) and death 50-70 min post-injection. Vesiculation and whorls of rough endoplasmic reticulum (ER), swollen mitochondria, and cytoplasmic vacuoles are observed in livers of intoxicated mice (DABHOLKAR and CARMICHAEL, 1987) and rats (DABHOLKAR *et al.*, 1987).

The *Microcystis* toxins have pronounced ultrastructural effects on isolated primary hepatocytes as both crude algal extracts (AUNE and BERG, 1986) and as purified toxin (RUNNEGAR and FALCONER, 1986; THOMPSON *et al.*, 1987; ERIKSSON *et al.*, 1987). Morphological effects appear to be specific for primary hepatocytes. Human erythrocytes, mouse fibroblasts (ERIKSSON *et al.*, 1987), VERO and Chinese hamster ovary cells (THOMPSON *et al.*, 1987), and established hepatoma cell lines (personal communication, W.L. Thompson) are unaffected by these toxins. Similar morphological changes have been reported with known effectors of cellular cytoskeleton, leading many investigators to

propose that the *Microcystis* toxins' mechanism of action involves cytoskeletal alterations (RUNNEGAR and FALCONER, 1986; ERIKSSON *et al.*, 1987; ROBINSON *et al.*, 1988).

We have initiated *in-vivo* studies in the rat model as the basis for future investigations, on the mechanism of action of the toxin, with the perfused rat liver and primary rat hepatocyte culture. We report here (a) ultrastructural effects of MCYST-LR on fasted rat liver and (b) correlation of those effects with biochemical changes *in-vivo* and *in-vitro*, with emphasis on toxin-induced cytoskeletal and mitochondrial changes.

MATERIALS AND METHODS

MCYST-LR (MW 994), isolated from *M. aeruginosa* strain 7820, (purity >95% by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) analyses (HARADA *et al.*, 1988)) was purchased from Dr. Wayne W. Carmichael (Wright State University, Dayton, OH). Stock solutions of MCYST-LR (1 mg/ml in water or saline) were stored at -10°C. HPLC performed according to the methods of KRISHNAMURTHY *et al.* (1986) showed that the toxin was stable under these conditions for at least 6 months.

Male Fischer 344 rats (Charles River, Wilmington, MA) weighing 200-250 g were maintained on a 12-hr light/dark cycle and allowed food and water *ad libitum*. Rats were fasted the night before the studies, but water was available.

ANOVA and Duncan's Multiple Range test were used to determine statistical significance (Table 2, Fig. 4 and Fig. 5). The Student's t-test was used for data analysis in Table 1.

IN-VIVO EXPERIMENTS

Sample collection

Fasted rats were injected i.p. with MCVST-LR (100 µg/kg; LD₇₅) in normal saline and killed 15, 30, 60 or 90 min later. Controls received normal saline. Livers were excised and processed for electron microscopy or fractionated for mitochondria or plasma membranes. Blood for serum chemistries was collected by cardiac puncture.

Electron microscopy

Sections of liver from the central region of the right lateral lobe were removed at each time point, cut into small cubes (ca. 1 mm³) and fixed in 2.0% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (2 hr). The tissue was washed in buffer overnight at 4°C, post-fixed in 1% osmium tetroxide (1 hr), followed by blocked staining in 0.5% uranyl acetate (1 hr). Tissue was dehydrated in 10%, 50%, 80%, 95% and 100% ethanol and propylene oxide and embedded in Epon 812. Ultrathin sections were cut with a diamond knife on an Om U3 ultramicrotome (Reichert Jung, Vienna, Austria), stained with 5% uranyl acetate and lead citrate, and examined with a 100CX electron microscope (JEOL, Tokyo, Japan). Micrographs were made from each sample at a plate magnification of 10,000X, enlarged 2.75 times photographically, and used for stereological analysis with a Videoplan 2 (Carl Zeiss, Thornwood, NY).

Preparation of isolated mitochondria from treated rats

The liver was minced and homogenized with a Potter-Elvehjem tissue homogenizer in ice-cold, 220 mM mannitol and 75 mM sucrose (pH 7.4) (DIMARCO and HOPPEL, 1975). After centrifugation (700X g, 10 min), the pellet was discarded, and the supernatant was centrifuged (8000X g, 10 min). The pellet

containing mitochondria was resuspended in 2.5 ml of the homogenizing solution. Respiratory rates were determined at 28°C with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH). The assay mixture (3 ml) contained mitochondria (2-4 mg protein), 150 mM KCl, 10 mM potassium phosphate, 20 mM Tris, and 3 mM MgCl₂ (pH 7.4). ADP (1 μmol) and substrate (30 μmol malate plus 30 μmol pyruvate, 10 μmol glutamate or 25 μmol succinate) were added.

Analysis of plasma membrane associated filaments

Liver plasma membranes were isolated as described by HUBBARD *et al.* (1983). Purified plasma membranes were pelleted at 5,000 g in an Eppendorf microfuge (Brinkmann Instruments Co., Westbury, NY). The supernatant was completely removed and membrane proteins extracted with 2% Triton X-100 in 0.15 M NaCl, 20 mM phosphate buffer, pH 7.4 and 5 mM EDTA. Samples were centrifuged, and the pellets were dissolved in 30 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2.5% SDS, 5% 2-mercaptoethanol, 10 mM potassium phosphate, pH 7.0, 0.02% bromophenol blue, and 20% glycerol). Electrophoresis of 1 μl samples and Coomassie blue staining of the gel were performed with a Phastgel system (Pharmacia, Piscataway, NJ) according to manufacturer's directions. The stained gels (9-15% acrylamide) were scanned with a thin layer chromatography plate scanner (Camag, Muttenz, Switzerland), and areas under the peaks were integrated.

Chemical analyses

Protein determinations (Lowry *et al.*, 1951) and serum chemistries were done on the COBAS BIO Centrifugal Analyzer (Roche Analytical Instruments, Nutley, NJ). Kits for glucose, blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase were obtained from Roche. Sorbitol dehydrogenase (SDH), 5'-nucleotidase (5'N) and Lowry total protein kits were purchased from Sigma Chemical Company (St. Louis, MO).

IN-VITRO EXPERIMENTS

In-vitro toxin treatment of mitochondria from control rats

Mitochondria from control, fasted rats were isolated as described above. Respiratory rates were measured with a Clark-type electrode (28°C). Mitochondria (2-4 mg protein) suspended in assay medium (3 ml) containing 2 mM MgCl₂, 80 mM KCl, 50 mM 3-(n-morpholino)propane sulfonic acid (MOPS), 1 mM EGTA and 5 mM potassium phosphate (pH 7.4) were treated with MCYST-LR (0.01 to 100 µg/ml) for 2 min before the addition of substrates.

Mitochondrial swelling experiments were performed by a modified method of LEHNINGER (1959). Control, fasted-rat liver was homogenized in cold 0.25 M sucrose (pH 7.4). Mitochondria were isolated as described above. The pellet containing mitochondria was washed three times with 0.25 M sucrose and resuspended in 125 mM KCl and 20 mM Tris (pH 7.4) to achieve an absorption of 0.5 to 0.7 at 520 nm. Swelling of mitochondria was monitored spectrophotometrically (520 nm) at 20°C after the addition of MCYST-LR (0.01 to 50 µg/ml) or other compounds described in Results.

In-vitro toxin treatment of plasma membranes

To determine whether MCYST-LR could destabilize intermediate filaments *in-vitro*, plasma membranes (2 mg/ml protein, final concentration) were incubated at 30°C with MCYST-LR (0.1, 1, 10 or 100 µg/ml) in a total volume of 0.5 ml of 0.25 M sucrose, 5 mM Tris, pH. 7.6, and 1 mM MgCl₂. At 15, 30, 60 and 90 min, 0.10 ml portions were removed, centrifuged, and the pellets were treated with Triton X-100 solution. Samples were centrifuged, and pellets were analyzed as described above. To determine if MCYST-LR could stabilize actin filaments, plasma membranes (2 mg/ml protein) were incubated at 22°C with 0.1 to 100 µg/ml MCYST-LR for 30 min. The pH was adjusted to 10, and samples were incubated an additional 60 min, followed by centrifugation. Pellets were treated with Triton X-100 solution and analyzed as above. In a control experiment, membranes were incubated with 0.1 to 100 µg/ml phalloidin.

RESULTS

IN-VIVO EXPERIMENTS

Liver response/serum analyses

In fasted rats administered 100 µg/kg MCYST-LR (IP), livers became engorged with blood. At 60 min, liver increased from 3% (controls) to 5% of the body weight, serum levels of 5'N, BUN and creatinine increased significantly, and serum glucose levels declined. At 90 min, serum levels of SDH, ALT, AST and LDH rose significantly. Serum alkaline phosphatase levels remained unchanged. Death occurred at 2 hr.

Electron microscopy

Examination of livers from control-fasted rats by transmission electron microscopy showed glycogen stores depleted, rough ER arranged in parallel layers or in association with mitochondria and mitochondria with dense matrices (Fig. 1a). Sinusoids (Fig. 2a), desmosomes and bile canaliculi were normal (Fig. 3a).

The following sequence of events was determined from electron micrographs of livers from fasted rats administered 100 µg/kg MCVYST-LR (IP): (a) 15 min post-injection - the presence of hydropic mitochondria with pale matrices (Table 1); (b) 30 min post-injection - dilation and vesiculation of smooth and rough ER and widening of space of Disse; (c) 60 min post-injection - formation of rough ER whorls, further widening of the space of Disse with leakage of red cells into the space and loss of sinusoid architecture (Fig. 2b); (d) 90 min post-injection - incorporation of mitochondria and associated rough ER into cytolysosomes, necrosis of hepatic parenchyma cells, absence of desmosome filaments (Fig. 3b), disrupted canaliculi, further disruption of sinusoids, and partitioned mitochondria (Fig. 1b). Hydropic mitochondria, devoid of electron dense deposits, were found at all time points (Table 1; Fig. 1b and Fig. 2b).

Electron transport

Mitochondria isolated from intoxicated-fasted rats (100 µg/kg MCVYST-LR) showed increased oxygen uptake during state 3 respiration with the three substrates used (Fig. 4). Fifteen min after toxin administration, ADP/O ratios and respiratory control indices (state 3 respiration/state 4 respiration) decreased (Fig. 5), typical of altered respiration by hydropic mitochondria (LEHNINGER, 1962). Sixty min after administration, state 3 respiration was completely inhibited.

Plasma membrane-associated filaments

An IP injection of 100 µg/kg MCVYST-LR had a two fold effect on hepatocyte plasma membrane isolation. At 90 min, protein content of the homogenate increased ($p < 0.05$) from 1408 ± 80 mg to 2532 ± 426 mg and hydrodynamic properties of the plasma membrane changed, resulting in a three fold decrease in recovery. Upon ultracentrifugation of the preparation from MCVYST-LR-treated rats, $25 \pm 3\%$ of the plasma membrane was located at the sucrose step gradient interface compared to $47 \pm 5\%$ in preparations from control rats ($p < 0.05$). At 60 min, there was a significant increase in protein content of the homogenate (2263 ± 376 mg), but hydrodynamic properties and recovery of plasma membranes were not altered. We noted no differences 15 or 30 min post-injection.

The effect of toxin on plasma membrane associated cytoskeletal filaments was determined by SDS-PAGE analysis of Triton X-100 extraction-resistant plasma membrane protein (HUBBARD and MA, 1983). A typical scan of the SDS gels is shown in Fig.6. Areas beneath the three main protein bands, peak 1 [unknown(s)], cytokeratin components A and D, and actin were quantitated and presented in Table 2 as a ratio of toxin-treated area to control area. There was a loss of desmosome-associated intermediate filaments (composed of cytokeratin A and D) 30 to 90 min post-injection and an increase in plasma membrane-associated actin 60 to 90 min post-injection. Peak 1, an unidentified-high molecular weight-Triton X-100-resistant protein(s), appeared to increase with toxin treatment.

IN-VITRO EXPERIMENTS

Electron transport

In contrast to mitochondria from livers of intoxicated animals, liver mitochondria isolated from control-fasted rats treated *in-vitro* with MCYST-LR (0.01 to 100 $\mu\text{g/ml}$) showed unchanged ADP/O ratios and respiratory control indices (data not shown).

Mitochondria swelling

Mitochondria isolated from control-fasted rat liver and treated with MCYST-LR (0.01 to 50 $\mu\text{g/ml}$) did not swell under conditions where known swelling agents such as 1 mM calcium chloride, 10 mM phosphate, 3 μM oleate, and 1 mM phloridzin were effective. Swelling promoted by 50 μM calcium chloride was not affected by the addition of 10 $\mu\text{g/ml}$ toxin (data not shown).

Plasma membrane-associated cytoskeletal filaments

In contrast to *in-vivo* results, intermediate filament destabilization of MCYST-LR could not be demonstrated *in-vitro*. Isolated plasma membranes were incubated with 0.1 to 100 $\mu\text{g/ml}$ of toxin for up to 90 min at 30°C without loss of cytokeratin A and D (data not shown). MCYST-LR had no effect on basic pH-destabilization of actin filaments, while phalloidin, a known stabilizer of actin filaments, tripled the amount of plasma membrane-associated actin after treatment at pH 10.

DISCUSSION

In the fasted rat model, a lethal dose of MCYST-LR (100 µg/kg) produced progressive hepatic disruption including the destruction of sinusoids, incorporation of cellular components into cytolysosomes, swollen mitochondria, dilation of ER, formation of ER whorls, partitioned mitochondria, and loss of desmosomes. Results of biochemical analyses correlated with the ultrastructural damage: (a) SDH, a specific liver enzyme, rose in sera, (b) tight coupling in the electron transport chain was lost, and (c) desmosome tonofilaments associated with the plasma membrane fraction disappeared.

Calcium ion flux across damaged plasma membrane has been proposed as a common pathway for a variety of toxins that kill primary cultures of rat hepatocytes (SCHIANNE *et al.*, 1979). Our findings suggest that MCYST-LR was not a calcium ionophore. Electron micrographs of hepatocytes revealed hydropic mitochondria with diffuse matrices but did not demonstrate the electron-dense calcium deposits that were found in rat liver mitochondria after carbon tetrachloride poisoning (REYNOLDS, 1965), in mitochondria from whole cells of toad urinary bladder, and in isolated mitochondria from rat kidney when calcium was present in the bathing medium (PEACHEY, 1964). Also, *in-vivo* effects of MCYST-LR on electron transport of mitochondria were distinct from effects due to calcium loading. Whereas calcium uptake into mitochondria does not damage oxidative phosphorylation once external calcium is depleted or removed (CARAFOLI and LEHNINGER, 1967), ADP/O ratios and respiratory control indices were decreased in mitochondria from MCYST-intoxicated rats. In addition, the presence of EGTA or the lack of calcium ions did not prevent MCYST-YA (a related *Microcystis* toxin)-induced blebbing of hepatocytes (RUNNEGAR *et al.*, 1986) or toxin-induced phosphorylase a activity (RUNNEGAR *et al.*, 1987).

We investigated the *in-vivo* effect of MCYST-LR on two of the three main components of the cytoskeleton, microfilaments and intermediate filaments. Short oligomers of actin are part of the structure of the cytoplasmic surfaces of cell membranes and, as such, are part of the membrane skeleton that regulates overall cell shape. Intermediate filaments are found in association with desmosomes and tube-like ensleavement of bile canaliculi. Both actin filaments and intermediate filaments were affected by the toxin. We were especially interested in the intermediate filaments associated with desmosomes (tonofilaments) as light microscope studies have shown that cyanobacterial toxins alter intercellular attachment (THEISS and CARMICHAEL, 1986). Electron microscopy and SDS-PAGE analysis of plasma membrane-associated filaments showed that intermediate filaments were lost from desmosomes. This report is the first to demonstrate a toxic effect on this class of cytoskeletal elements. In addition, plasma membrane-associated actin increased with toxin treatment. RUNNEGAR and FALCONER (1986) found that, unlike phalloidin, MCYST-LR did not change the ratio of g-actin to f-actin in hepatocyte suspensions. These results, together with our findings, indicate that there was a toxin-induced redistribution of actin filaments rather than a change in the overall amount of filamentous actin.

Three *in-vivo* effects of MCYST-LR, inhibition of respiration, promotion of hydropic mitochondria, and changes in the plasma membrane-associated cytoskeletal filament, could not be reproduced *in-vitro*. At least two explanations are possible: (a) a MCYST-LR metabolite was the active compound *in-vivo* and (b) *in-vitro* conditions did not support the cascade of events that precede toxic effects. The bioactivation hypothesis is supported by the lack

of toxicity of MCVST-LR in livers that were chemically damaged and livers from young mice which had not fully developed their complement of hepatic enzymes (ADAMS *et al.*, 1985). No information is available at this time on the metabolic fate of MCVST-LR.

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TABLE 1. MORPHOMETRIC MEASUREMENTS OF MITOCHONDRIAL PERIMETERS

Time Post-Injection (min)	Increase of Mean Mitochondrial Perimeter (% of control)
15	16.7 ^a
30	17.4 ^b
60	23.3 ^b
90	19.5 ^b

At least 101 mitochondria in the electron micrographs of livers were measured for each time point. Results are expressed as percent increase over mitochondrial perimeters of control rats injected with normal saline. a: $p < 0.005$ toxin-treated compared to controls. b: $p < 0.001$ toxin-treated compared to controls.

TABLE 2. EFFECT OF MCYST-LR ON TRITON X-100-RESISTANT
PLASMA MEMBRANE PROTEINS

Time post-injection (min)	Area toxin-treated/Area control		
	Cytokeratins A & D	Actin	Peak 1
15	1.03 ± 0.16	1.13 ± 0.18	1.57 ± 0.27
30	0.66 ± 0.04 ^a	1.14 ± 0.06	1.22 ± 0.19
60	0.20 ± 0.03 ^a	1.77 ± 0.28 ^b	3.52 ± 1.18
90	0.32 ± 0.05 ^a	1.86 ± 0.20 ^b	3.76 ± 0.69

Values are mean ± SEM, n = 4. a: p<0.01 b: p<0.05

Fig. 1. ELECTRON MICROGRAPHS OF CONTROL AND TOXIN-TREATED RAT LIVER.

- a. Control fasted rat liver. Note depleted glycogen, endoplasmic reticulum (ER) in parallel layers, mitochondria (M) with dense matrices surrounded by ER and nucleus (N). Bar = 1 μ m.
- b. Fasted rat liver 90 min after injection of MCVST-LR (100 μ g/kg). Note dilated ER, hydropic mitochondria (M) with pale matrices lacking electron dense deposits, partial loops of ER associated with mitochondria, and partitioned mitochondria (\uparrow). Bar = 1 μ m.

Fig. 2 a. CONTROL FASTED RAT LIVER.

Normal sinusoids (Sn), space of Disse (*) and erythrocyte (E).
Bar = 1 μ m.

- b. Fasted rat liver 60 min after injection of MCVST-LR (100 μ g/kg). Note the widening of the space of Disse (*) and loss of sinusoidal architecture. Bar = 1 μ m.

Fig. 3 a. CONTROL FASTED RAT LIVER.

Note the presence of desmosomes (D). BC = bile canaliculi. Bar = 1 μ m.

- b. Fasted rat liver 90 min after injection of MCVST-LR (100 μ g/kg). Note the absence of desmosomes in the area of the bile canaliculi. Bar = 1 μ m.

Fig. 4 STATE 3 OXYGEN CONSUMPTION OF LIVER MITOCHONDRIA ISOLATED 0 (\square),

15 (\boxtimes) and 30 (\boxdot) MIN AFTER RATS WERE INJECTED WITH

100 μ g/kg MCVST-LR (i.p.).

Oxygen consumption was inhibited in mitochondria isolated 60 min after toxin exposure. * $p < 0.01$ compared to control value. † $p < 0.05$ compared to control value. n = 3.

Fig. 5 ADP/O RATIOS AND RESPIRATORY CONTROL INDICES OF LIVER
MITOCHONDRIA ISOLATED 0 (□), 15 (■) AND 30 (▨) MIN AFTER
RATS WERE INJECTED WITH 100 µg/kg MCYST-LR (I.P.).

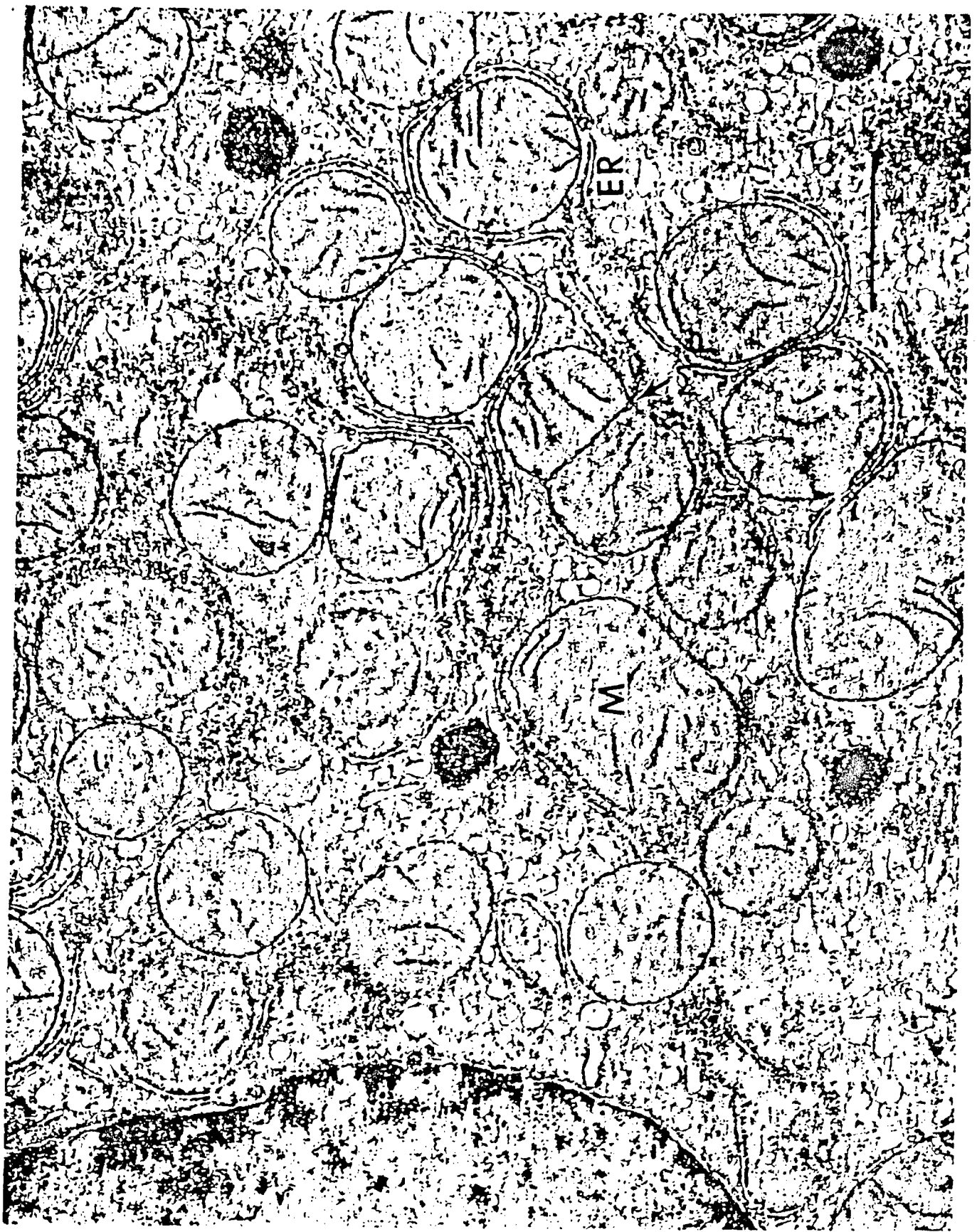
At 60 min post-injection, state 3 respiration was completely
inhibited. * p<0.01 compared to control value. # p<0.05 compared
to control value.

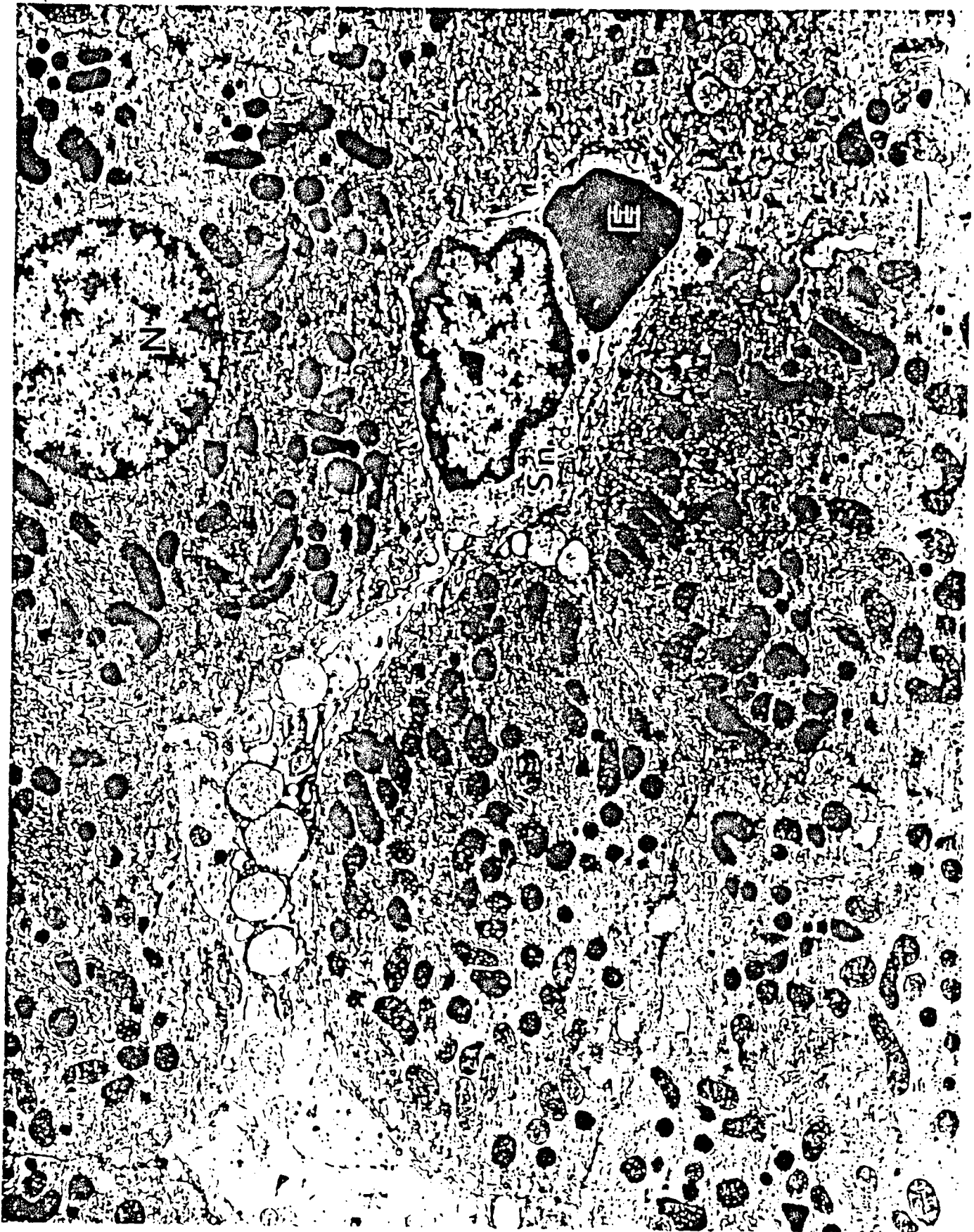
n = 3.

Fig. 6 SDS GEL SCAN OF TRITON X-100 EXTRACTION-RESISTANT
PLASMA MEMBRANE PROTEINS.

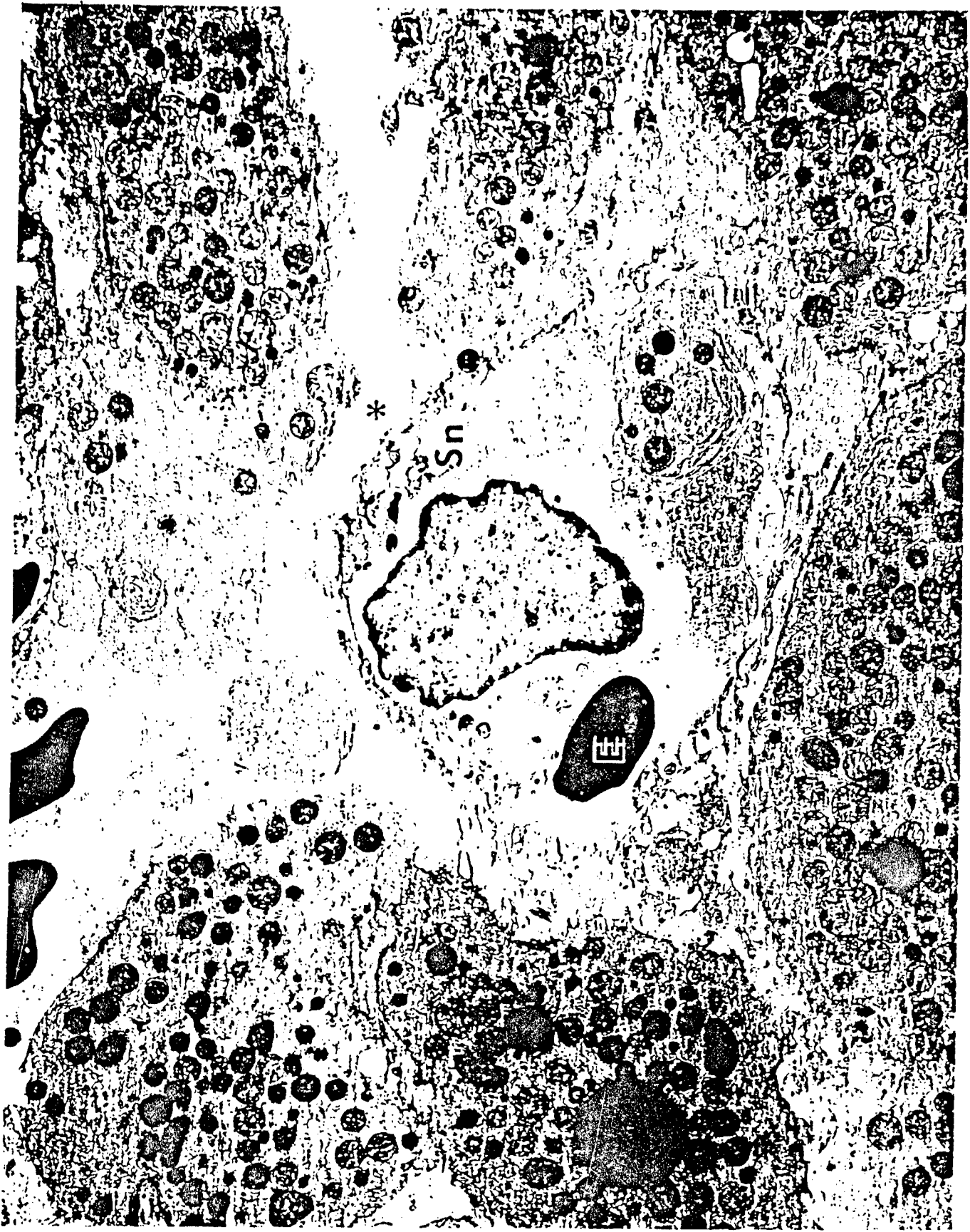
Samples of plasma membrane (0.052 U 5'N) isolated from liver of
control or 100 µg/kg MCYST-LR-treated rats, were extracted with
Triton X-100 solution and analyzed on a 9-15% SDS PAGE. The
Coomassie blue stained gel was scanned with a Camag scanner.
Pharmacia SDS high and low molecular weight standards were used to
calibrate the gel. The molecular weights of the three major
protein bands were 150,000 ± 2,000 for peak 1, 58,000 ± 1,000 for
cytokeratin A, 55,000 ± 1,000 for cytokeratin D, and 45,000 ±
1,000 for g-actin.





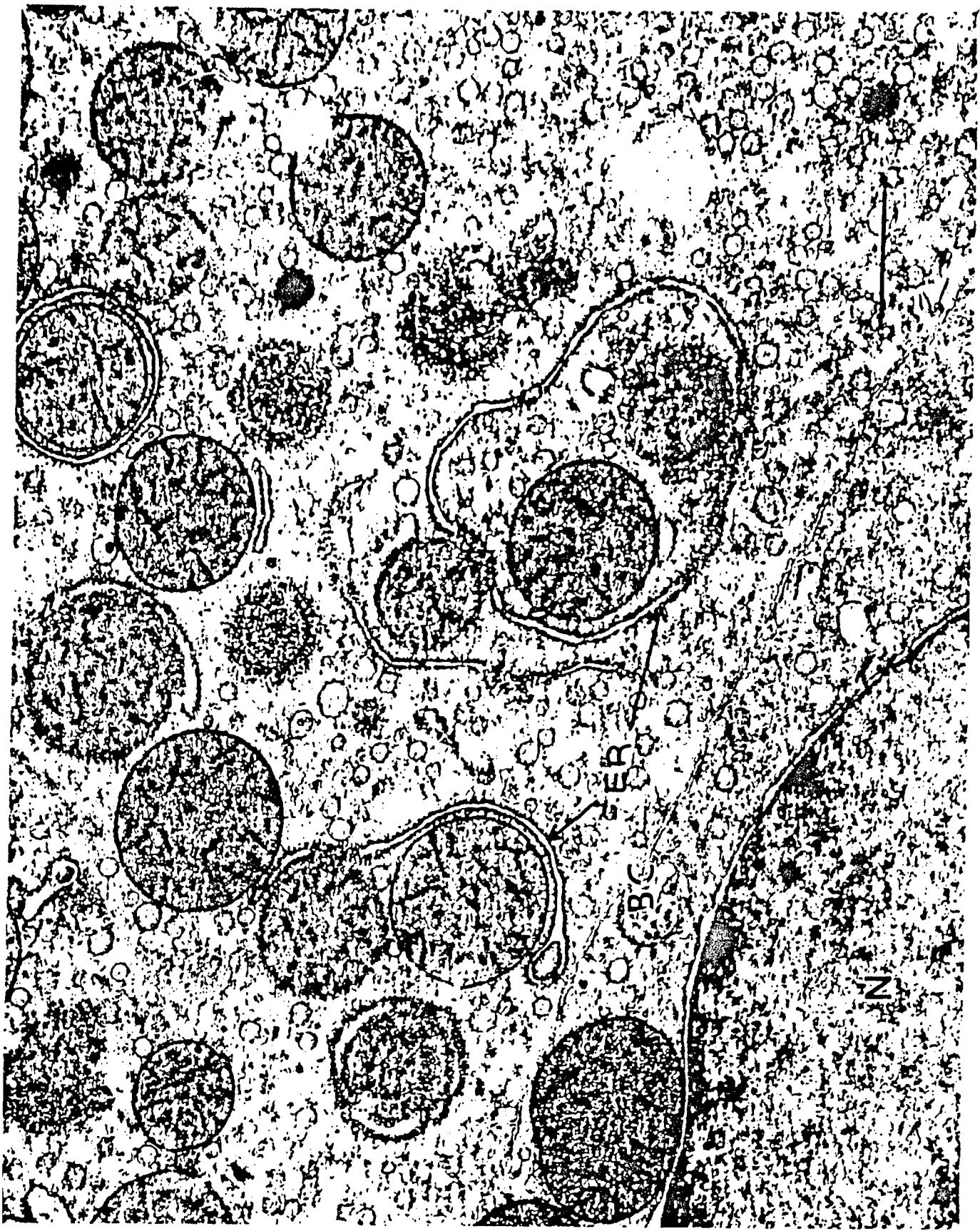


2a



2b





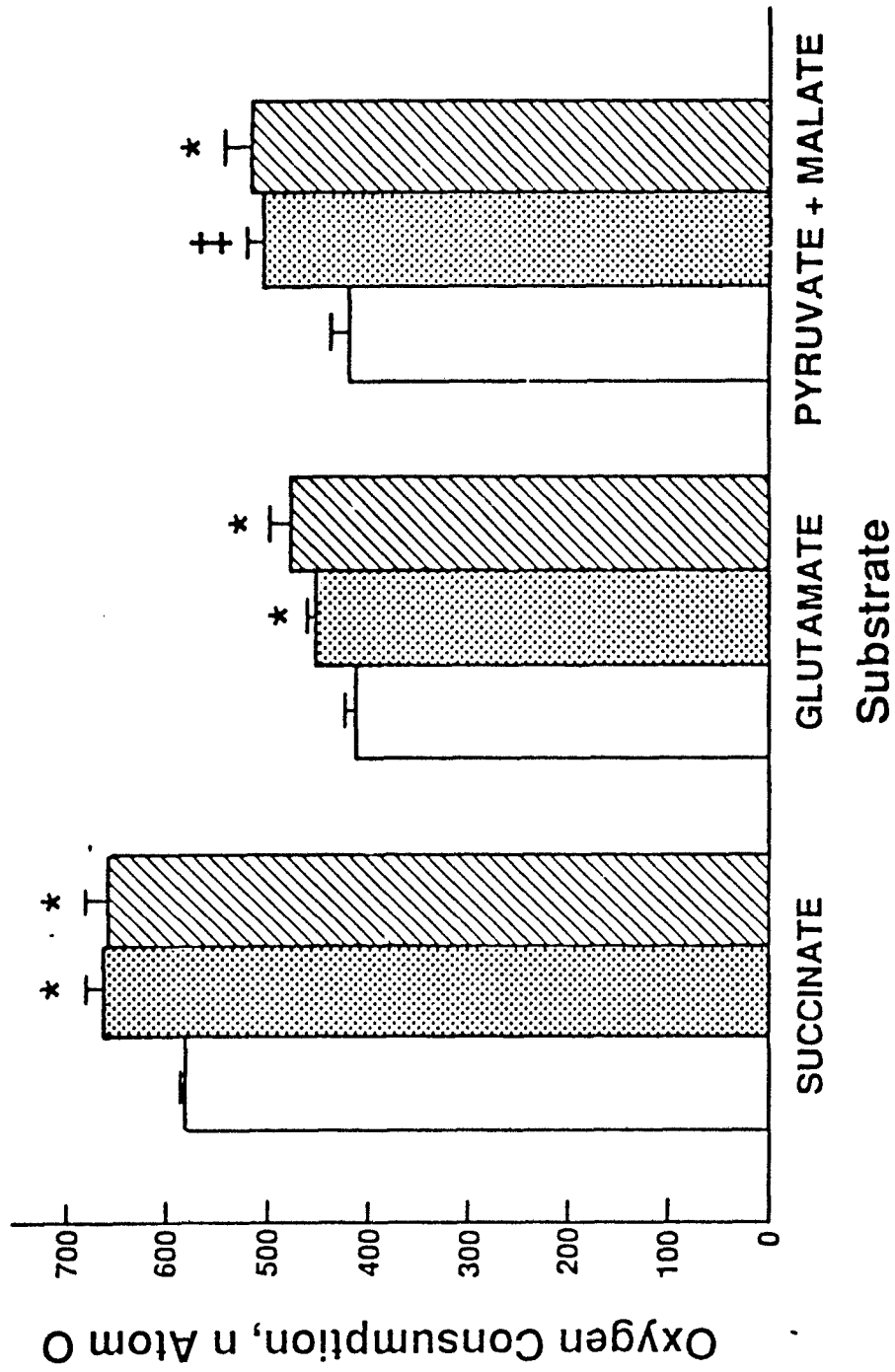


Fig 4

FIG. 4

Fig. 5

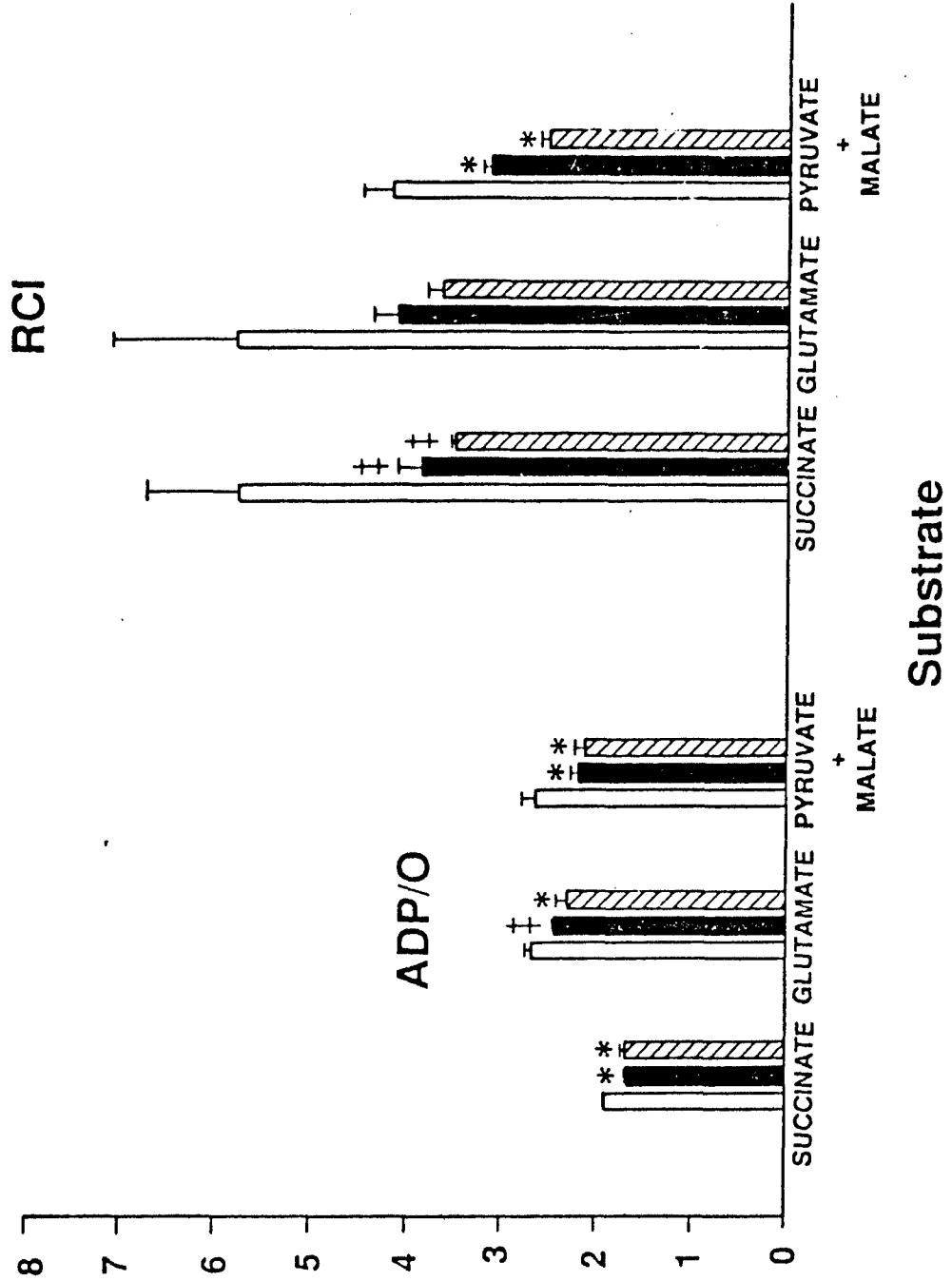


Fig. 6

