Biochemical and Morphological Aspects of Mitochondrial Swelling in Ammonia Toxicity[1] (36098)

KYE-WING CHOW AND WILSON G. POND

Department of Animal Science, and the Graduate School of Nutrition, Cornell University, Ithaca, New York 14850

Small concentrations of ammonium salts have been shown to inhibit respiration of phosphorylating rat liver and cat brain mitochondria (1–3). Explanation for this effect had been: uncoupling (1), competition between glutamate dehydrogenase and the electron transport chain (2), and suppression of keto-acid decarboxylation (3) by ammonia. In those studies, incubations were carried in phosphate buffers containing magnesium ions along with the usual substrates and phosphate acceptor systems necessary for oxidative phosphorylation to occur.

Ammonia readily forms the highly insoluble (and hence, stable) complex salt, magnesium ammonium phosphate, with magnesium and phosphate ions at neutral or alkaline pH (4–7). This depletion of magnesium and phosphate ions might possibly have accounted for ammonia's effect on respiration (5).

In the context of complex salt formation by ammonia, a previous study by us showed significant impairment of phosphate metabolism in the mildly hyperammonemic chick and rat (8). The present study was aimed at determining the ramifications of complex salt formation by ammonia at the molecular level.

Materials and Methods. Normal 200–300 g male rats of the Sprague–Dawley strain fed commercial rat pellets were used throughout. Liver mitochondria for swelling studies were isolated by the method of Schneider (9) employing 0.25 *M* sucrose with no EDTA added. Standard test systems and procedures for mitochondrial swelling were those of Lehninger *et al.* (10), except that 0.25 *M* rather than 0.30 *M* sucrose solution was used, and incubations carried out at 26°. Total incubation volumes in all studies were exactly 10 ml.

A. Mitochondrial swelling profile. Optical density (OD) readings were made every 3 min for 48 min. Swelling profile was illustrated as a plot of per cent OD extinction divided by time lapse against time lapse. Ammonium chloride treated tubes contained 6 m*M* of the salt. Approximately 50 mg tissue equivalent of mitochondria were used.

B. Ammonia toxicity and ^{45}Ca labeling by isolated rat liver mitochondria. Mitochondria (approx. 0.8 mg of protein) were allowed to swell in 50 ml plastic centrifuge tubes for exactly 20 min in media as in A but containing in addition 0.5 microcuries of ^{45}Ca (New England Nuclear, specific activity 14.1 mCi/mg $CaCl_2$). Swelling was terminated by quick immersion of the tubes into an ice bath with gentle swirling. The mitochondria were at once separated by centrifugation at 15,000*g* in a refrigerated Sorvall superspeed centrifuge, Model RC2-B. The supernatant was decanted into glass vials and 1 ml aliquots transferrd into glass scintillation counting vials. Ten milliliters of Bray's counting solution (11) were added to each vial and radioactivity determined in a Nuclear Chicago Mark II Model Liquid Scintillation Counter. Labeling was expressed as per cent of dose. One tube with only mitochondria omitted served as the dose standard. Magnesium in the supernatant (counting) fraction was measured with a Perkin–Elmer Atomic Absorption Spectrophotometer Model 303 and inorganic phosphate by the method of Fiske and Subbarow (12).

C. Ammonia toxicity and ^{45}Ca loss from labeled isolated rat liver mitochondria. One

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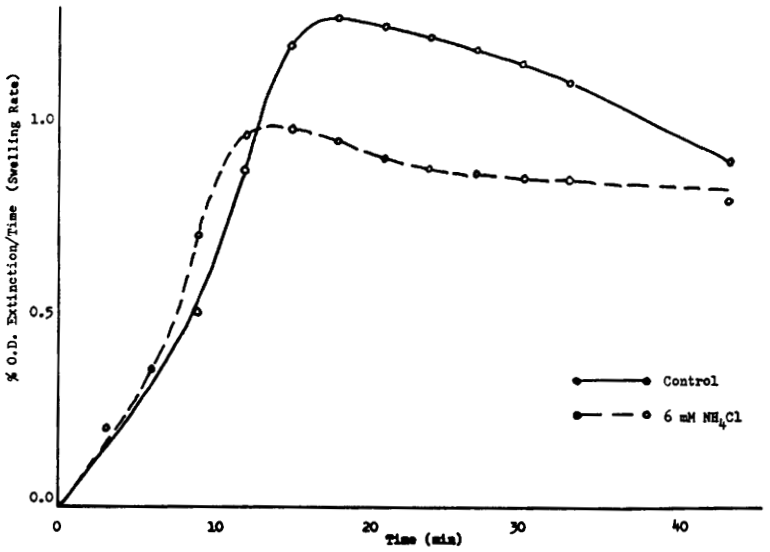

FIG. 1. Effect of NH_4Cl on the swelling profile of rat liver mitochondria.

rat was dosed with approximately 50 μCi of ^{45}Ca intraperitoneally and sacrificed by decapitation 30 min later. The liver was carefully checked for absence of gross defects at necropsy and mitochondria obtained as described. The mitochondria (approx. 0.5 mg protein) were allowed to swell spontaneously as in B and the loss of radioactivity from the mitochondria after 20 min was expressed as a percentage of radioactivity originally present in the mitochondria. Magnesium and inorganic phosphate in the counting fraction were measured as described in B.

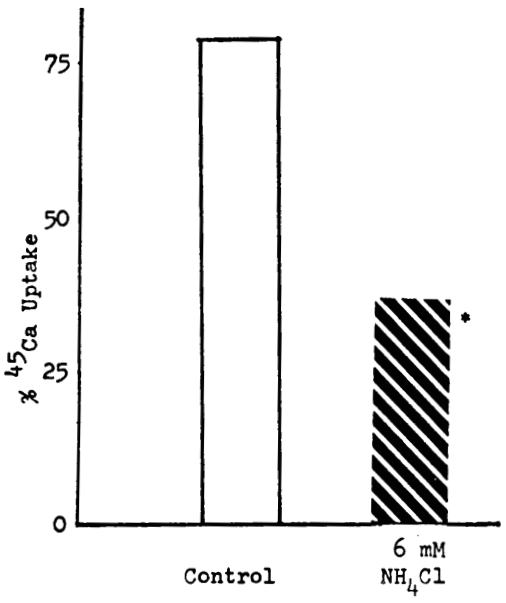

FIG. 2. Effect of NH_4Cl on ^{45}Ca labeling of rat liver mitochondria 20 min after onset of spontaneous swelling: $*p<.0005$.

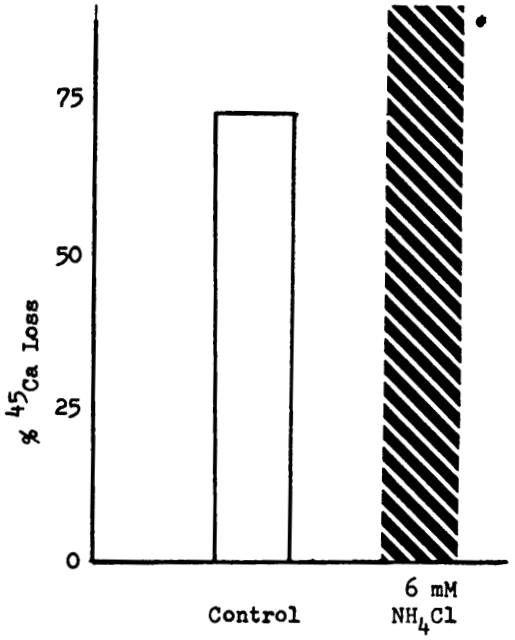

FIG. 3. Effect of NH_4Cl on loss of ^{45}Ca label from rat liver mitochondria 20 min after onset of spontaneous swelling: $*p<.0005$.

TABLE I. Effect of NH_4Cl on Ion Efflux from Swelling Rat Liver Mitochondria.

	Label-uptake study[a]		Label-loss study[b]		
	Control	NH_4Cl	Control	NH_4Cl	p value
Magnesium[c]	2.82 ± 0.07	5.70 ± 0.07	3.84 ± 0.21	5.07 ± 0.12	.0005
Phosphate[c]	16.74 ± 0.72	20.42 ± 0.29	19.57 ± 1.97	22.80 ± 0.60	.0005

[a] Values representing Means ± SEM of 3 replicates.
[b] Values representing Means ± SEM of 4 replicates.
[c] Mitochondrial protein (μ/mg) in 20 min swelling time.

D. Effects of added Mg, PO_4, and NH_4 ions on ^{45}Ca labeling by isolated rat liver mitochondria. Unlabeled mitochondria (approx. 1.9 mg protein) were allowed to swell in media as in B but treatment included 4 m*M* NH_4Cl, phosphate (K salts), or $MgCl_2$, respectively. One additional treatment contained all three ions. Radiocalcium was determined and expressed as in B. The precipitate which formed in the supernatant after high speed separation of the mitochondria incubated with media containing all three ions was isolated and subjected variously to X-ray diffraction (Debye–Scherrer powder method using a Siemens camera) and infrared analyses (potassium bromide pellet method using a Perkin–Elmer Model) for chemical characterization.

E. ^{45}Ca labeling profile in spontaneous swelling of isolated rat liver mitochondria. Mitochondria (approx. 0.7 mg of protein) were allowed to swell for 5, 10, 15, and 20 min in media with or without 4 m*M* NH_4Cl added, and the uptake of label was determined and expressed as in B.

F. Effect of acute ammonia intoxication on rat liver mitochondria. Rats were given intraperitoneal doses of 20% ammonium carbonate solution at the rate of 2 ml/kg of body weight. Control animals received

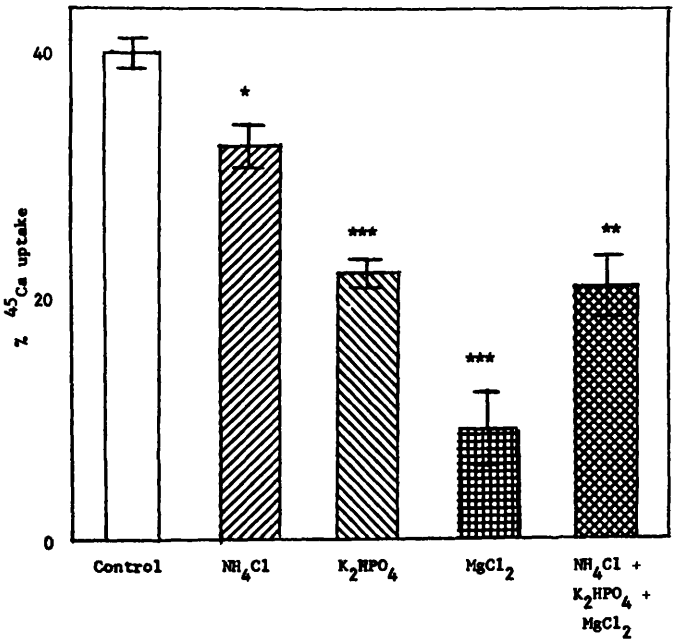

FIG. 4. Variable effects of ions on ^{45}Ca labeling of rat liver mitochondria 20 min after onset of spontaneous swelling: equimolar (4 m*M*) salt concentrations; * $p<.01$; ** $p<.001$; *** $p<.0005$.

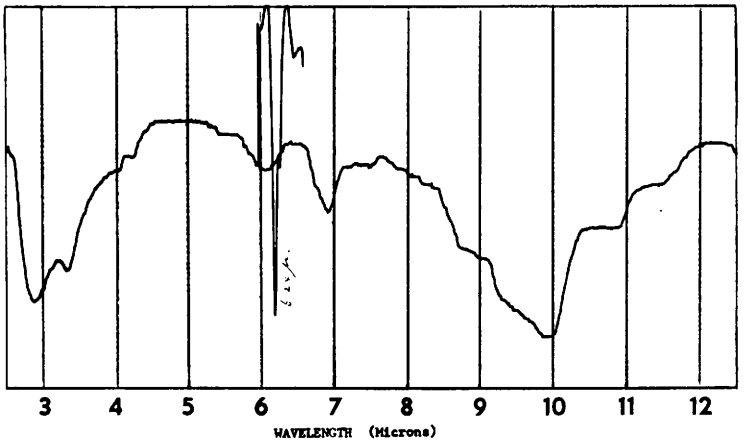

FIG. 5. Infrared spectrogram of residue isolated from supernatant fraction (15,000*g*) of mitochondrial system containing added NH_4, Mg and PO_4 ions (representative of three replicates). Spectrogram identified it as magnesium ammonium phosphate. Spectrogram of polyethylene superimposed for calibration purposes.

physiological saline. The animals were anesthetized with ether 40 min after the administration of ammonia and the livers quickly removed and prepared for electron microscopy according to the method of Legg and Wood (13).

Results and Discussion. The profiles of spontaneously swelling rat liver mitochondria in buffered sucrose solution as influenced by time and 6 m*M* NH_4Cl are shown in Fig. 1. Ammonia caused an initial increase in swelling but this higher rate of swelling was not sustained. Slightly lower concentrations (4 m*M*) of the ammonium salt resulted in a marked decrease in labeling of the mitochondria (Fig. 2) and at the same time caused a greater loss of the label (Fig. 3) 20 min after the onset of swelling. These events were accompanied by increased losses of magnesium and phosphate ions from the mitochondria (Table I).

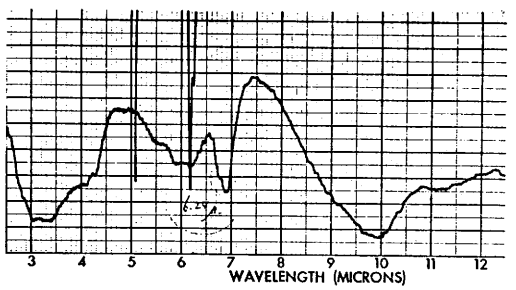

FIG. 6. Infrared spectrogram of magnesium ammonium phosphate, $MgNH_4PO_4 \cdot 6H_2O$, prepared with NH_4Cl, $MgCl_2$, and K_2HPO_4. Spectrogram of polyethylene superimposed for calibration purposes.

Ammonium, phosphate or magnesium variably reduced ^{45}Ca labeling of isolated mitochondria in that order. However, when all three ions were present together, the depressive effect on labeling was not additive but approached that for treatment with phosphate alone (Fig. 4). Also, uneven crystalline forms were shown to settle out from the counting fraction upon refrigerated storage for about two weeks. The amount of precipitate in each case was too small to be of use for X-ray diffraction analysis, although infrared spectrograms revealed these to be struvite, or magnesium ammonium phosphate hexahydrate. A representative spectrogram from this precipitate is shown in Fig. 5 and the standard spectogram for $MgNH_4PO_4 \cdot 6H_2O$ is in Fig. 6.

Despite the very minute quantities of Ca added (for labeling purposes), the uptake of the divalent cation was rapid and almost complete within 5 min in both ammonium treated and control mitochondria (Fig. 7). The level of labeling at 5 min after the onset of swelling in both treatments was not different, but beyond 10 min, treatment differences were consistent with those found in the 20-min studies.

Acute ammonia intoxication resulted in

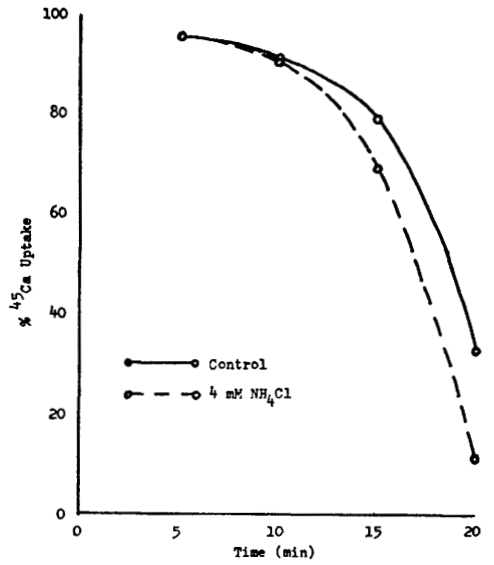

FIG. 7. Effect of NH_4Cl on ^{45}Ca labeling of spontaneously swelling rat liver mitochondria.

spectacular changes to rat liver mitochondria and endoplasmic reticulum (ER) (Fig. 8a, b). The mitochondria appeared rounded and swollen to many times normal size. Much of the mitochondrial matrix was also lost.

Swelling of mitochondria in sucrose solution generally does not occur unless coupled to energy-linked functions over the pH range of 7.0–8.0 (14, 15). Mitochondria contain bound magnesium which is released after some minutes of incubation in isotonic sucrose solution (16, 17). Ultrastructural changes through rearrangement of the inner membrane may be central to the problem of swelling (15) and although mitochondria do not require magnesium in media for maximum P/O values, the sustained loss of magnesium causes significant morphological changes in the particles which would adversely affect respiration (18). The early stimulation of swelling of mitochondria observed in the present study may be related possibly to unsustained stimulation of respiration resulting from such morphological changes, or to the postulated intramitochondrial pH changes which must take place as a result of spontaneous movement of free ammonia into the mitochondria (19). The differences in the pattern of labeling by ^{45}Ca in both the label-uptake and label-loss studies probably reflect increased loss of the label in the ammonium treated groups in both instances, perhaps as a result of irreversible changes to the mitochondrial membrane stemming from the loss of magnesium from the system through complex formation. This irretrievable loss of magnesium is compared with the normal internal release of the ion from bound sites within the mitochondria as described by Cereijo-Santalo (14).

Earlier studies of phosphorylating rat liver mitochondria showed depressed respiration by ammonia (1, 2). While the latter workers attributed this to increased oxidation of NADH by glutamic dehydrogenase, the data of Okada (20) suggested the reverse effect, *i.e.*, depressed respiration preceded reduction in levels of pyridine nucleotides. Cereijo-Santalo (21) had shown that magnesium strongly was not affected by the absence of magnesium ions in the incubating media. The obvious role of phosphate in respiratory processes needs no further comment. The loss of both magnesium and phosphate from the system through complex formation with ammonia would produce results consistent with those observed in studies with phosphorylating liver mitochondria mentioned earlier: reduction in NADH levels and reduced respiratory rate without an effect on P/O values.

Morphological changes to isolated rat liver mitochondria by ammonia as judged by increased "leakiness" to endogenous ions are underscored by results with ammonia intoxicated rats showing fine structural changes to livers. Such changes may be related to magnesium loss.

Summary. Molecular aspects of ammonia intoxication were studied with rat liver mitochondria swelling spontaneously in buffered isotonic sucrose solution containing ^{45}C label. Results were compared with electron microscopic study of livers of ammonia intoxicated rats.

Small concentrations of NH_4Cl (4 and 6 m*M*) added to the swelling media markedly decreased the labeling of mitochondria. This decreased labeling was found to be due to increased efflux of the label. Ammonia also caused increased loss of intramitochondrial magnesium and phosphate.

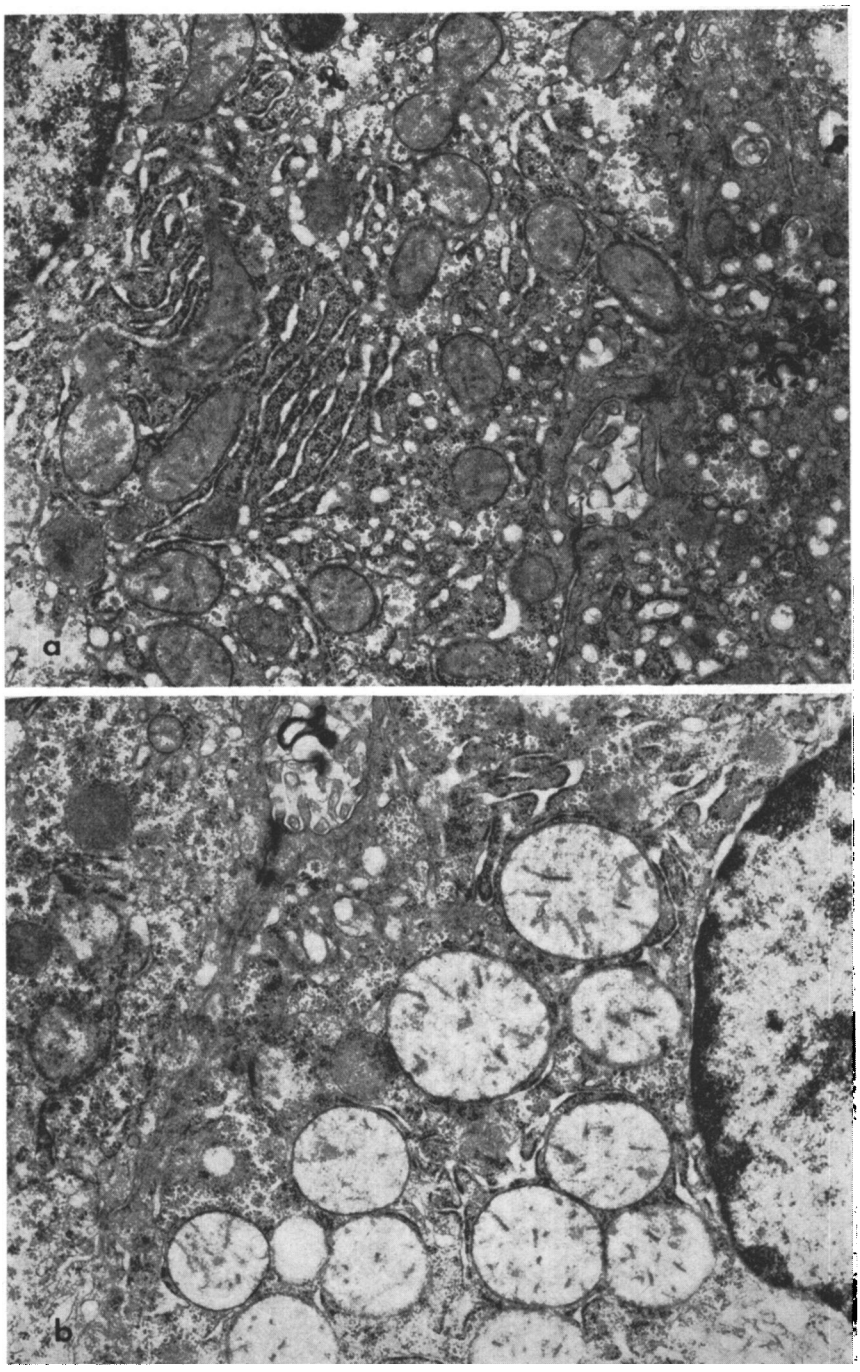

FIG. 8a. Electron micrograph of normal rat liver: Animal received an intraperitoneal dose of physiological saline prior to sacrifice under anesthesia 40 min later; glutaraldehyde fixation; ×17,250. (b). Electron micrograph of liver of hyperammonemic rat: Animal received an intraperitoneal dose of 20% ammonium carbonate solution (1.5 ml/kg of body wt) prior to sacrifice under anesthesia 40 min later; glutaraldehyde fixation; ×17,250.

Ammonium, phosphate and magnesium variably and increasingly reduced ^{45}Ca labeling of swelling mitochondria, but when all three ions were present in the medium, the reduction in labeling was not additive. A crystalline material was isolated from the supernatant fraction after high speed centrifugal separation of the mitochondria previously swelling in the medium containing added Mg, PO_4 and NH_4 ions. This material was identified as magnesium ammonium phosphate by infrared spectrography.

Electron micrographs of liver of the hyperammonemic rat revealed radical changes to fine structure of the liver cell. Mitochondria were swollen to many times normal size, with considerable loss of mitochondrial matrix. Disorganization of the endoplasmic reticulum was also apparent.

The effect of ammonia on respiring mitochondria may not only be substrate and cofactor depletion but may also be due to morphological changes to mitochondrial membrane structure through magnesium loss, although the mechanism by which this occurs would be similar.

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