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Organic Mercurial Activation of Renal Ammonia Production (40580)

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The availability of glutamine within the matrix space determines the activity of the mitochondrial glutaminase I pathway (1–4). Glutamine's inability to penetrate the inner membrane is altered during metabolic acidosis (2, 3, 5) through a response mediated by adrenocorticoids (6–8). This is associated with a 100-fold increase in membrane permeability to glutamine (2, 5) providing the necessary substrate for a 20-fold activation in the mitochondrial pathway (2, 9–12). Since glucocorticoids (13, 14) and metabolic acidosis (15) accelerate mitochondrial swelling and ammonia production, it seemed likely that other agents having a similar effect might also activate ammonia production. The organic mercurial, *p*-chloromercuribenzoate, is a potent mitochondrial swelling agent (16) raising the possibility that, if swelling is associated with a permeability increase, then it might activate ammonia production. The results to follow show that *p*-CMB acts directly upon the kidney to accelerate ammonia production via the mitochondrial pathway and induces in situ enlargement of proximal tubule mitochondria.

Materials and methods. Male Sprague–Dawley rats weighing between 350 and 450 g were employed in this study. Each was housed in specially designed metabolic cage for 24-hr urine collections. Urine samples were preserved, collected and analyzed for sodium and ammonium as described (8). Blood acid base balance was determined from samples obtained by cardiac puncture during light ether anesthesia; they were then monitored on a Radiometer pH–pCO_2 analyzer. *p*-Chloromercuribenzoate (Calbiochem) was freshly prepared in alkalanized 0.01 *N* NaOH, isotonic saline and administered as a single injection, 1.9 mg Hg kg^{-1} intramuscularly; controls received the isotonic saline and collections were made over a 3-day period.

Perfused kidneys. Nontreated rats were anesthetized with sodium pentobarbital, 30 mg kg^{-1}, and their kidneys isolated (17) and perfused with an artificial plasma solution containing either 1 m*M* L-glutamine or 1 m*M* L-glutamine plus 0.025 m*M* *p*-CMB. For tracer experiments, aliquots of ^{203}Hg *p*-chloromercuribenzoate, 100 mCi/g (Amersham Searle) were added to the perfusate and samples drawn at 15-min intervals. After 60 min of perfusion, kidneys were either homogenized for subcellular fractionation or perfused with fixative for electron microscopy depending upon the presence or absence of ^{203}Hg. For homogenization, the procedure, briefly, was to dice the kidney in ice-cold 0.44 *M* sucrose, pH 7.2, with Hepes, 2 m*M*, and to homogenize using a Potter–Elvehjem hand-driven homogenizer. The homogenate was then centrifuged in a Sorvall refrigerated centrifuge at 450*g* for 10 min to give the "nuclear fraction," followed by centrifugation of the supernatant at 13,000*g* for 10 min to yield the mitochondrial fraction. The resulting supernatant was transferred to a Beckman L50 ultracentrifuge 105,000*g* for 1 hr to sediment the microsomal fraction and to obtain the soluble fraction. Sucrose aliquots of each fraction were monitored for ^{203}Hg activity, using beta emissions, in a Beckman liquid scintillation spectrometer with correction for quenching by the channel ratio method; fractions were counted in duplicate and the results expressed as the percentage of total kidney ^{203}Hg. For fine structure studies, the perfusion solution was switched at 60 min by means of a three-way stopcock to ice cold 1% glutaraldehyde in isotonic Clark's solution (18) and cortical slices of the fixed kidney were cut, processed, and postfixed in 1% osmium tetroxide; thin sections were cut on a microtome and viewed under an electron microscope. Mitochondrial size changes were quantitated from pictures, magnified 28,000 ×, of proximal tubule cells; particle area was traced using a calibrated planimeter.

Isolated mitochondria. Nontreated rats

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were anesthetized as above and their kidneys quickly removed to ice cold 0.88 *M* sucrose, pH 7.2. After decapsulating, cortical cubes were cut and homogenized in 0.44 *M* sucrose, 2 m*M* Hepes, and 1 m*M* EGTA, pH 7.2 (1: 10 w/v). The mitochondrial fraction was obtained as above after clearing the nuclear fraction and debris followed by centrifugation of the supernatant at 8000*g* × 10 min. The mitochondria were washed three times and resuspended in isolating medium by gently drawing up and down in a Pasteur pipette. Suitable aliquots were transferred to 2 ml of 300 m*M* sucrose, pH 7.2, with 1 m*M* Hepes containing 1 m*M* L-glutamine or 1 m*M* L-glutamine plus 0.025 m*M* *p*-CMB. The samples were incubated at 23°C for 15 min with swelling monitored on a Gilford 240 spectrophotometer at 510 mμ; ammonia production was determined in a duplicate cell after 10 min of incubation.

Analysis. Perfusate concentrations of ammonium and glutamine were enzymatically determined as previously described (17) except for the initial sample in which the presence of the mercurial inhibited the enzymes; in this instance glutamine was deamidated by acid hydrolysis with ammonia measured directly (4). Ammonia production by isolated mitochondria was determined by microdiffusions (19) and the protein content by the biuret method (20) using bovine albumin as the standard.

Results are considered significantly different from the controls at the 0.05 percentile level using the Student *t* test analysis.

Results. Figure 1 presents the effect of a single *p*-CMB injection upon 24-hr sodium and ammonium excretion. Base-line sodium excretion, 1.08 ± 0.33 meq day^{-1}, rose significantly during the first 24 hr, a finding in accord with other reports (21) and then returned to control values. Ammonium excretion, on the other hand, reached its peak of 230% above the base line, 850 ± 95 μmole day^{-1}, during the second day. This inappropriate ammonium excretion, from the point of view of acid–base balance, lead to a significant rise in plasma HCO_3^- concentration, 29.6 ± 1.3 vs 25.4 ± 0.9 m*M*. Isolated kidneys from nontreated rats avidly extract *p*-CMB from the perfusate (Fig. 2) and accumulate it in association with the mitochondrial, 34%

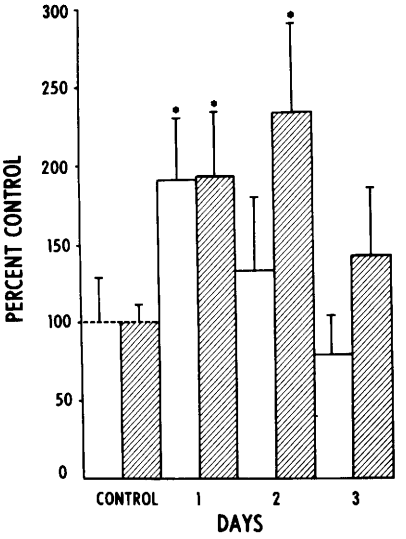

FIG. 1. Influence of single *p*-CMB injection, 1.9 mg Hg kg^{-1}, on 24-hr sodium □ and ammonium ▨ excretion; results are mean ± SEM from six rats.

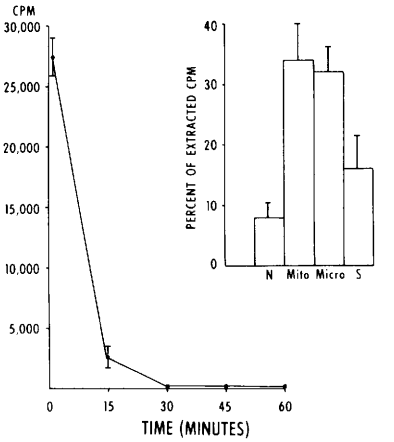

FIG. 2. Renal uptake and subcellular distribution of ^{203}Hg–*p*-CMB. Four kidneys were perfused for 60 min followed by differential fractionation. Insert shows percent of ^{203}Hg taken up localized in the nuclear (N), mitochondrial (Mito), microsomal (Micro), and soluble (S) fraction.

and microsomal fraction, 31%. Within 15 min of perfusion onset, 90% of the mercurial had been taken up while the extracted ^{203}Hg could be quantitatively recovered at the termination of the experiment. The effect of this uptake on ammonia production is seen in Table I. Production rates, linear during the 60-min perfusion period, were 2.6 times greater in *p*-CMB perfused kidneys; glutamine uptake, in

comparison to ammonia production, rose disproportionately from 0.44 ± 0.06 to 0.71 ± 0.06 μmole min^{-1} kg^{-1}. Consequently the ammonia/glutamine ratio jumped from 1.25 to 1.97 indicative of mitochondrial glutaminase 1–glutamate dehydrogenase pathway activation (2, 11, 12). Figure 3 shows the effect of *p*-CMB upon proximal tubule fine structure. Kidneys perfused with *p*-CMB, 3b, display enlarged, "giant," mitochondria also seen in glucocorticoid-treated animals (13, 14, 22); this is accompanied by increase in vacuolization in the apical regions. The size distribution of proximal tubule cell mitochondria is shown in 3C. Although there is extensive overlapping, mitochondria in the *p*-CMB-treated kidney cells had an average enlargement of 38%. To determine whether a similar biochemical–morphological correlate was observable in the isolated organelles, mitochondria from nontreated rats were incubated in 1 m*M* L-glutamine plus 0.025 m*M* *p*-CMB. As shown in Figure 4, after a 2-min latency period, mitochondria suspended in *p*-CMB undergo drastic swelling while controls maintain their original volume; swelling occurs most rapidly during the 2- to 10-min period and thereafter remains stable. Ammonia production during the period of maximum swelling increased some threefold, 3.2 ± 0.1 to 11.5 ± 2.3 nmole min mg^{-1} ($P < 0.05$).

Discussion. Administering *p*-CMB to rats stimulated renal ammonia production (Table I) and excretion (Fig. 1) as a consequence of glutaminase 1 pathway activation (Table I, Fig. 4). Activation, in situ as well as with the isolated organelle, correlated with a definite morphological alteration: enlargement (Figs. 3, 4). This phenomena reflects a change in membrane permeability characteristics (23) and apparently facilitates glutamine entry into the matrix charging the glutaminase 1–glutamate dehydrogenase ammonia-liberating reactions. Numerous studies have shown a relationship between mitochondrial swelling and glutaminase activation (11, 15, 24). Nevertheless, swelling per se may not be the precondition for activation but rather reflects the incipient subtle membrane alteration which ultimately leads to enlarged mitochondria.

TABLE I. INFLUENCE OF *p*-CMB ON RENAL GLUTAMINE UPTAKE AND AMMONIA PRODUCTION[a]

	Ammonia production	Glutamine uptake	Ammonia / Glutamine
Control	0.548[b] ±0.056	0.438 ±0.056	1.25
p-CMB	1.418 ±0.110	0.710 ±0.063	1.97

[a] Nontreated kidneys perfused with 1 m*M* L-glutamine and 0.025 m*M* p-CMB.

[b] Mean ± SD from four kidneys, μmole min^{-1} g^{-1}

Our observation that *p*-CMB stimulates ammonia production by isolated mitochondria is in agreement with the intact functioning organ's response (Fig. 1, Table I) but at variance with two other reports dealing with isolated mitochondria (3, 25). Both of these studies observed, upon exposure to 1 m*M* *p*-CMB, an inhibition of glutamine uptake and ammonia production. Adam and Simpson (3) employed *p*-CMB as an inhibitor of glutaminase 1, a fact established in studies with purified glutaminase 1 (26), and determined matrix space glutamine content. Since there was none detected, they concluded, in the absence of glutamine diffusion equilibrium between the outer and inner compartments, that glutamine uptake was carrier mediated. Goldstein (25) observed a marked fall in glutamine uptake and concluded *p*-CMB binds to the glutamine carrier and renders it inoperative. In neither study, in which *p*-CMB was variously interpreted as acting on the enzyme directly (3) or its carrier within the membrane (25), was morphologic correlates provided. In our studies 0.025 m*M* *p*-CMB was used for two reasons: (1) This concentration stimulated the mitochondrial pathway in the perfused kidney and (2) higher concentrations virtually disrupted the mitochondria.

Consistent with this interpretation is the dose–response results shown in Table II. At 0.025 m*M* *p*-CMB ammonia production is

FIG. 3. (A) Proximal tubule of kidney perfused for 60 min and fixed as described under Methods; magnification 28,000×. (B) Proximal tubule or kidney perfused with *p*-CMB and fixed at 60 min; same magnification as A. (C) Size-frequency distribution for proximal tubule mitochondrial population from control and *p*-CMB perfused kidneys. A total of 69 organelles were counted in each group; the difference between control and *p*-CMB treated average mitochondrial size, 0.060 vs 0.084, is statistically significant ($P < 0.01$).

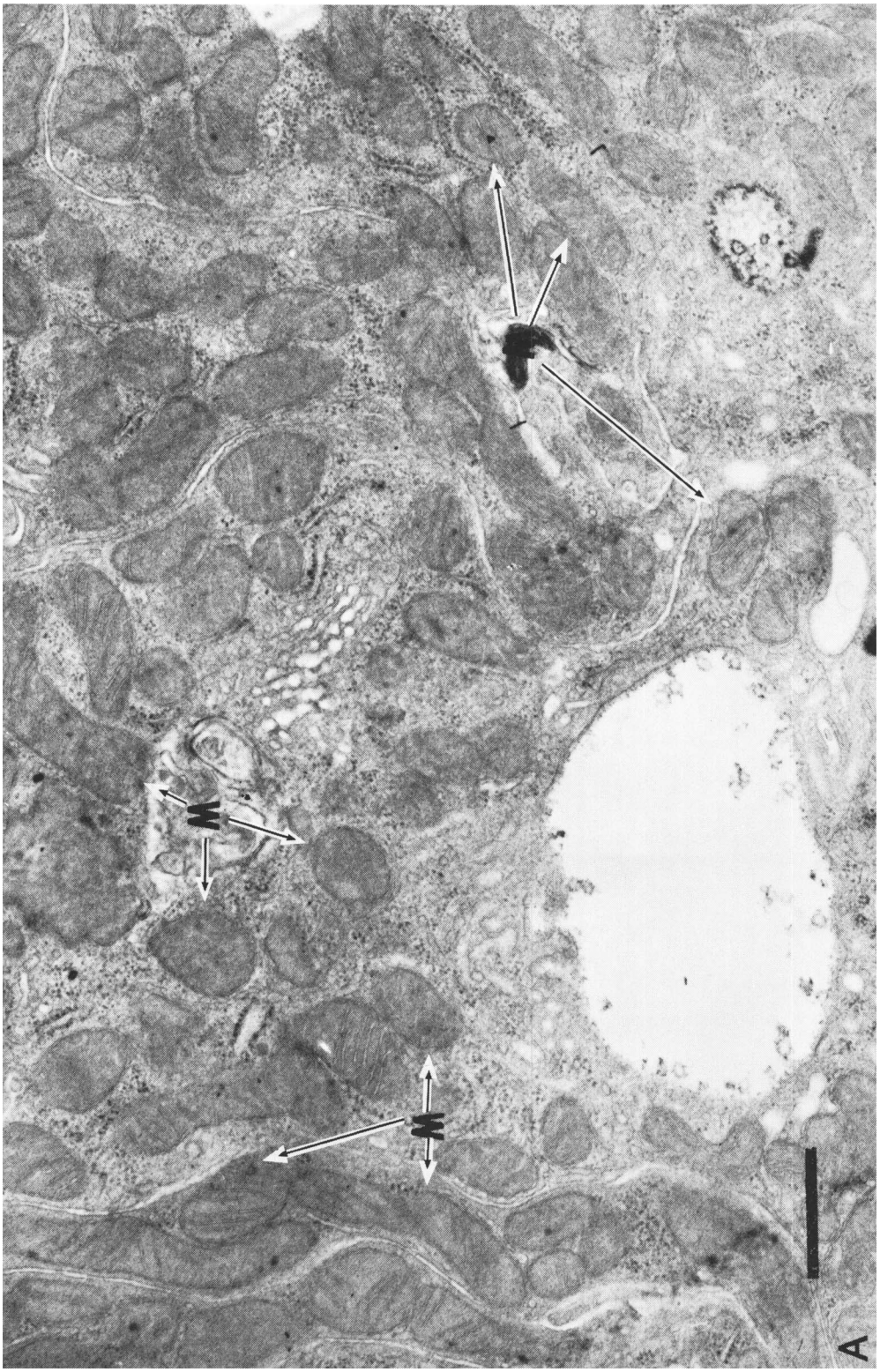

FIG. 3A.

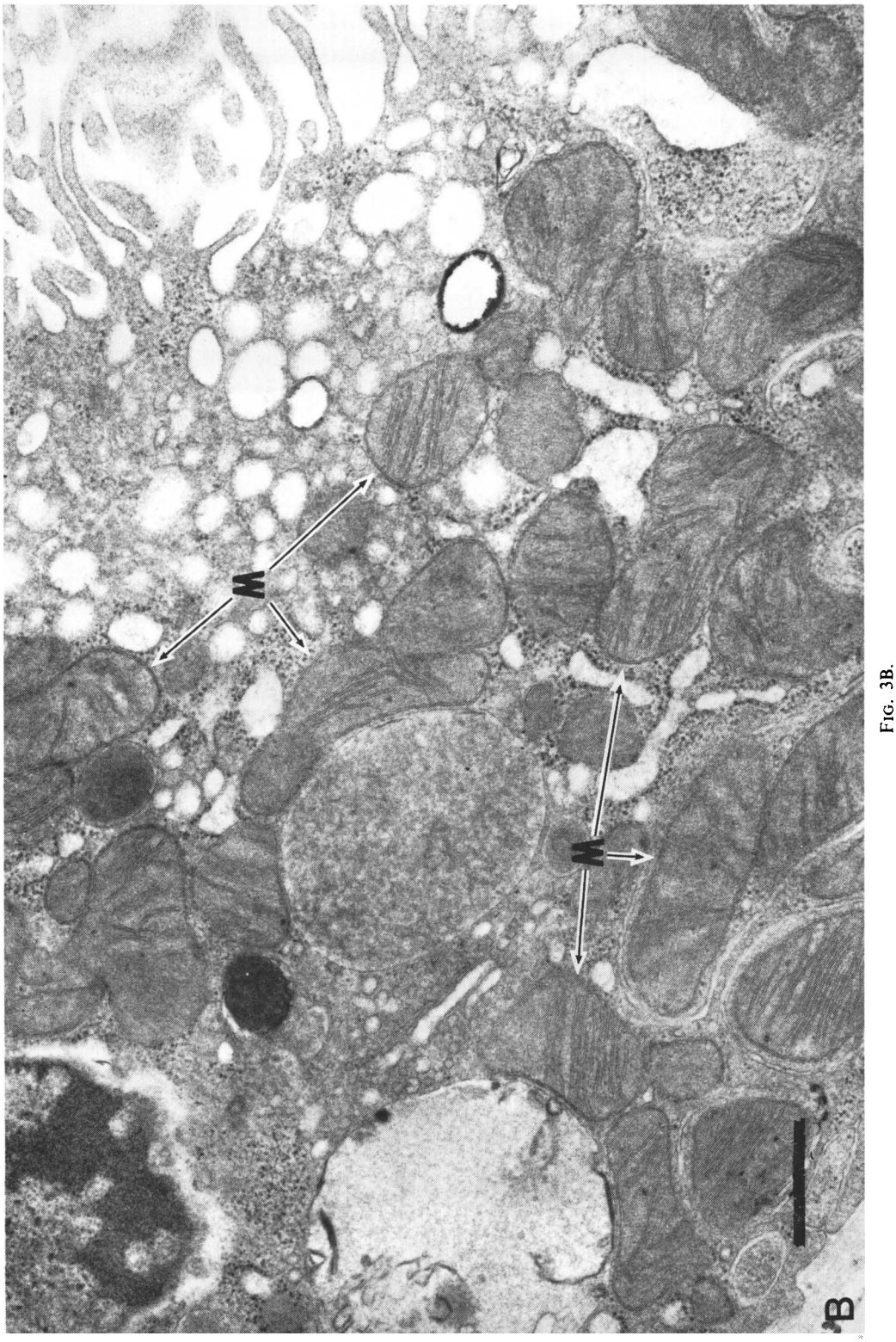

FIG. 3B.

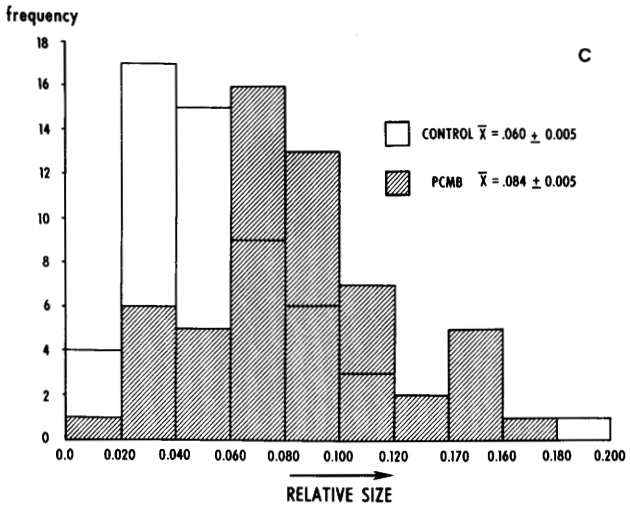

FIG. 3C.

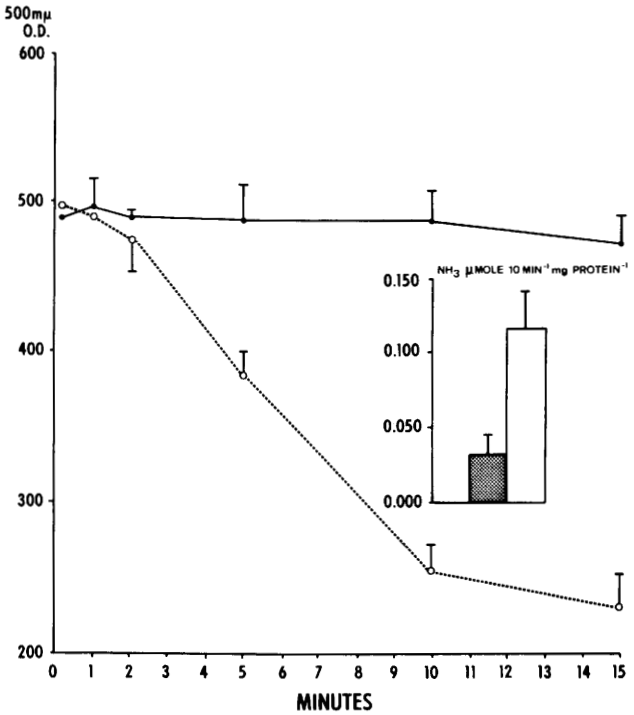

FIG. 4. Correlation between *in vitro* mitochondrial swelling and ammonia production (insert). Mitochondria were suspended in incubating media plus 1 m*M* L-glutamine (●) or 1 m*M* L-glutamine plus 0.025 m*M* *p*-CMB (○). Ammonia production control ▨ and *p*-CMB-treated □ mitochondria were measured over the initial 10-min period of rapid swelling.

maximal while increasing the levels results in a decline in the rates and gross kidney swelling. In fact, 0.025 m*M* or one-fourtieth of that used in the above-cited studies, is probably a very high exposure level relative to the level causing mitochondrial enlargement in the perfused kidney since only 33% of the extracted mercury is associated with the mitochondria (Fig. 2).

Interestingly, the urinary sodium and ammonium excretion response to metabolic acidosis (27), triamcinolone (8), and *p*-CMB

TABLE II. EFFECT OF PERFUSATE *p*-CMB CONCENTRATION ON AMMONIA PRODUCTION AND KIDNEY SWELLING[a]

p-CMB m*M*	Ammonia production (μmole h^{-1} g^{-1} dry wt)	Wet/Dry[b]
0 (4)[c]	89 ± 9	4.73
0.025 (4)	227 ± 19	5.05
0.038 (3)	156	6.45
0.075 (3)	108	7.00

[a] Kidneys perfused for 1 h^{-1} with 1 m*M* L-glutamine.
[b] Wet wt determined at end of perfusion; dry wt measured after being dried to a constant wt at 70°C.
[c] Number of kidneys perfused.

(Fig. 1) are remarkably similar. The pattern in each instance is an initial peak in sodium followed on the down swing by a rise in ammonium excretion. This phenomena has never been adequately explained although it's plausible to consider an alteration in mitochondrial function. For example, both mercury and triamcinolone (14) lead to enlarged mitochondria similar to those seen in Figure 3B. Unfortunately, the effects of *in vivo* metabolic acidosis on renal mitochondria fine structure have not been published although swollen mitochondria reportedly (15) appear with chronic metabolic acidosis. Regardless, it seems possible that ammonium and sodium excretion patterns in these instances may be coupled reflections of a subtle mitochondrial alteration.

Summary. *p*-Chloromercuribenzoate (*p*-CMB) was employed to determine whether mitochondrial swelling correlates with glutaminase 1 activation. Diuretic dosage of *p*-CMB produced swelling of in situ as well as isolated mitochondria. Ammonia production from glutamine rose two- to threefold with a rise in the ammonia produced per glutamine-utilized ratio from 1.25 to 1.97. The results are consistent with mitochondrial glutaminase 1 activity dependent upon mitochondrial permeability.

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