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RENAL AND HEPATIC MITOCHONDRIAL EFFECTS OF DIURETICS IN THE RAT*

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Abstract—The effect of diuretics on oxygen consumption rate (Qo_2) and swelling of mitochondria isolated from rat kidney cortex (C), outer medulla (OM) and liver was measured. In addition, the effect of isolated cell membrane fraction (ER) on the diuretic-induced changes in mitochondrial oxygen utilization was determined. Qo_2 was measured in a Gilson oxygraph utilizing either glutamate-malate or succinate as substrate. Qo_2, expressed in natoms O_2/mg of protein/min, was always higher in C than OM: 159.8 ± 8.3 vs 119.8 ± 11.4 ($P < 0.001$) with glutamate-malate, and 199.8 ± 11.4 vs 149.9 ± 9.3 ($P < 0.005$) with succinate. The Qo_2 of liver mitochondria was lower: 102.6 ± 7.1 with glutamate-malate, and 125.5 ± 9.6 with succinate. A dose–response curve was constructed for each of the following: sodium ethacrynate (EA), furosemide (F), chlorothiazide (CTZ), acetazolamide (ACTZ) and chlormerodrin (CH). All diuretics exerted an equal inhibitory effect on C, OM and liver mitochondrial Qo_2. CH was the most potent inhibitor, followed by EA, F, CTZ and ACTZ. The concentration of diuretic which inhibited Qo_2 by 50 per cent was utilized to examine mitochondrial swelling. At these doses, F and ACTZ had only a slight effect on liver mitochondria. EA had the greatest effect, followed by CH and CTZ. By contrast, CH had the greatest effect on C and OM, followed by EA and F, while CTZ and ACTZ had only a slight effect. Cysteine and dithiothreitol prevented the effect of CH on Qo_2 and swelling. The effect of EA on swelling, but not on Qo_2, was blocked by cysteine. The cell membrane had its greatest protective effect with CH, had only a modest effect with EA, and exerted no protective effect on F, CTZ and ACTZ.

The nephronal site of action of most diuretic agents has been well localized by clearance and micropuncture techniques [1]. The mechanism by which diuretic agents exert their effects, however, has not been clearly delineated. Evidence has been advanced that diuretics may exert their effects by interfering with cellular metabolism [1–9]. Other workers, however, have suggested that diuretics act by altering the permeability of cell membrane rather than by a direct interference with cell metabolism [10, 11]. The cellular and subcellular mechanism of action of diuretics, therefore, remains undefined. Isolated mitochondria have been examined as a possible locus of the action of these agents [12–18]. An effect *in vitro* on isolated kidney mitochondrial oxidative phosphorylation has been reported [12–18]. The present studies were designed to examine the effect of several diuretic agents on the oxygen consumption rate and rate of swelling on mitochondria obtained from the kidney and liver of the rat. In addition, because of the close association of renal mitochondria with tubular cell membrane and its protective effect *in vitro* on diuretic action [17], the effect of isolated cell membrane fraction on the diuretic-induced changes *in vitro* in mitochondrial oxygen utilization was also determined.

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MATERIALS AND METHODS

Experiments were performed on normal albino Sprague–Dawley rats of either sex weighing 250–350 g. The effects of varying doses of diuretics on mitochondrial respiration and swelling were measured, utilizing mitochondria isolated from liver, renal cortex and outer medulla.

Rats were killed by a blow to the head, and the kidneys and liver immediately removed. Kidney tissue from ten to thirteen rats was pooled for each study in order to obtain adequate amounts of renal medullary tissue for mitochondrial isolation. The kidneys were placed in an iced solution of KEA medium containing 0.18 M KCl, 10 mM EDTA and 0.5% bovine serum albumin-Sigma Fraction V, adjusted to pH 7.4 with Tris buffer, with a final osmolality of 258 mOsmoles/kg. The kidneys were hemisected longitudinally and the cortex and outer medulla separated by sharp dissection. The renal mitochondrial isolation technique has been previously described in detail [12]. The liver was placed in a beaker of ice-cold isolation medium (0.25 M sucrose, 1 mM EDTA and 1 mM Tris buffer, pH 7.4), after which it was blotted, weighed and placed in a fresh beaker of isolation medium. The tissue was then minced with scissors, the isolation medium decanted off and the minced tissue homogenized and centrifuged by the same technique used for renal mitochondrial isolation.

The final pellet from kidney or liver was then resuspended in isolation medium to a concentration of 25–35 mg of mitochondrial protein/ml, and its protein

content determined by the method of Lowry *et al.* [19]. The mitochondrial suspension from each tissue was then diluted with isolation medium to a final concentration of 20 mg/ml. The protein concentration of the kidney mitochondrial preparation was adjusted for the presence of albumin in the isolation KEA medium.

Cortical and outer medullary cell membrane fractions were prepared by the method of Landon and Norris [20].

Measurement of mitochondrial respiration. Mitochondrial respiratory activity was measured in a Gilson Oxygraph (Gilson Medical Electronics, Middleton, Wisc.) as previously described [12].

For examination of renal mitochondrial respiratory activity using NADH-linked substrate, the reaction mixture consisted of 1.5 ml of assay medium (10 mM glutamate, 10 mM K_2HPO_4 and 0.25 M sucrose adjusted with 10 mM Tris buffer to pH 7.4), 40 μl of 0.5 M K_2HPO_4, 25 μl of 0.5 M glutamate, 15 μl of 0.5 M malate and 100 μl of ADP (about 500 nmoles). For examination of flavoprotein-linked respiration, 25 μl of 0.5 M succinate was added as substrate instead of glutamate and malate, and 5 μl of rotenone (1000 μg/ml) added to the chamber to block NADH-linked respiration.

For examination of hepatic mitochondrial respiratory activity, the reaction mixture consisted of 1.5 ml of assay medium [0.3 M KCl, 0.2 M EDTA, 1 M Tris–acetate, 0.1 M $MgCl_2$ and 0.5 M K_2HPO_4 (pH 7.4)], 0.1 ml of either 0.1 M glutamate-malate or 0.1 M succinate, and 0.1 ml of ADP.

To the reaction mixture, 200 μl of mitochondrial preparation (equivalent to 4.0 mg of mitochondrial protein) was added, and State 4 respiration recorded. One hundred μl ADP was then added and the reaction allowed to run until State 3 respiratory activity was completed.

In studies of the effect of membrane fraction on mitochondrial oxygen consumption, the reaction mixture consisted of 1.5 ml of a medium containing 77 mM KCl, 17 mM Na_3PO_4 (adjusted with Tris–HCl to pH 7.4), 3 mM $MgCl_2$ and 75 mM sucrose, to which was added the appropriate substrate in the same concentrations as above. Control measurements were obtained after the addition of 2.5 mg of mitochondrial protein (200 μl). Cell membrane fraction (2.0 mg in a volume of 200 μl) was then added and the measurements were repeated.

Measurement of mitochondrial swelling. The swelling experiments were performed by a modification of the method of Lehninger [21] in matched 12 × 75 mm tubes containing 1.5 ml of 0.25 M KCl, buffered to a pH of 7.4 with 40 mM Tris buffer, and brought to a final volume of 2.9 ml by the addition of water. At zero time, 0.1 ml of the mitochondrial suspension (equivalent to 2.0 mg of mitochondrial protein) was added and mixed gently by inversion using a small square of parafilm to close the tube. Absorbency measurements were made at 520 nm in a Coleman Jr. spectrophotometer (Coleman Instruments, Maywood, Ill.) against a water blank at specified time intervals up to 45 min. Addition of 0.1 ml mitochondrial suspension (2 mg of mitochondrial protein) yielded an initial absorbency of 0.35 to 0.45 for renal cortical and outer medullary mitochondria and 0.6 to 0.7 for liver mitochondria. All solutions and the mitochondrial suspension were always kept ice-cold.

In studies of drug action, after the control measurements were obtained, the diuretics were added and the measurements repeated. Matched control and experimental measurements were obtained simultaneously. The molar concentration of the test diuretic was gradually increased until a dose–response curve was established for renal cortical, outer medullary and hepatic mitochondria. In studies of drug action in the presence of cell membrane, the diuretics were added either directly to the reaction chamber after addition of cell membrane or incubated with the cell membrane for 30–45 min prior to addition.

The diuretics studied were sodium ethacrynate, furosemide, chlorothiazide, acetazolamide and chlormerodrin. The molar concentrations of the diuretics were expressed as the final concentrations in the reaction chamber in the respiratory activity studies and in the cuvette in the swelling studies.

Data are presented as mean ± the standard error of the mean. The differences between the means were tested for statistical significance by the Student's *t*-test [22].

RESULTS

Hepatic and renal cortical and outer medullary mitochondrial oxygen consumption. In control experiments, the mean oxygen consumption rate for cortical mitochondria was significantly higher than that of medullary mitochondria (Table 1). A significant difference in the respiratory control index between cortical and outer medullary mitochondria was also present with both substrates examined. The difference in ADP:O ratio, however, was significantly different only when succinate was utilized as substrate.

The oxygen consumption rate of liver mitochondria was lower than that of renal mitochondria and averaged 102.6 ± 7.1 natoms O_2/mg of protein/min with glutamate-malate as the substrate, and 125.5 ± 9.6 with succinate as the substrate.

Effect of diuretics on mitochondrial oxygen consumption rate. All diuretics examined exerted an inhibitory effect on renal and liver mitochondrial function. A dose–response curve for each agent on mitochondrial respiration was established. The 50 per cent inhibitory dose of each agent is shown in Table 2. It is apparent from this table that acetazolamide had the weakest inhibitory effect on the mitochondrial respiration, while chlormerodrin exerted the strongest effect, followed in potency by ethacrynic acid, furosemide and chlorothiazide. Despite the difference in baseline oxygen consumption rate of the mitochondrial populations (Table 1), the 50 per cent inhibitory dose of each diuretic was not different for all three mitochondrial populations.

Effect of diuretics on mitochondrial swelling. In preliminary studies all diuretics induced swelling of hepatic and renal mitochondria of a degree which varied directly with the concentration of the diuretic agent added. To determine the relative potency of these agents on mitochondrial swelling and allow a correlation between the degree of swelling and effects on the oxygen consumption rate, the concentration of diuretics which inhibited mitochondrial respiration by

Table 1. Hepatic and renal cortical and outer medullary mitochondrial respiratory function

	N*	Qo_2†	RCI‡	ADP:O§
Substrate: glutamate-malate				
Liver	14	102.6 ± 7.1	6.17 ± 0.50	3.19 ± 0.09
Cortex	14	159.8 ± 8.3	6.48 ± 0.79	3.39 ± 0.14
Outer medulla	14	119.7 ± 6.2	4.45 ± 0.37	3.18 ± 0.17
Statistical comparison, P between cortex and outer medulla		< 0.001	< 0.05	NS‖
Substrate: succinate				
Liver	11	125.5 ± 9.6	4.33 ± 0.29	1.99 ± 0.05
Cortex	11	199.8 ± 11.4	3.37 ± 0.23	2.06 ± 0.20
Outer medulla	11	149.9 ± 9.3	2.27 ± 0.11	1.81 ± 0.09
Statistical comparison, P between cortex and outer medulla		< 0.005	< 0.001	< 0.05

* Number of experiments.

† Rate of oxygen consumption expressed as natoms O_2/mg of protein/min.

‡ Respiratory control index, ratio of that amount of oxygen consumed during State 3 to that of State 4 respiration.

§ Amount of ADP esterified/nmole of oxygen consumed during State 3 respiration.

‖ Not significant.

50 per cent was utilized to examine mitochondrial swelling (Fig. 1). At these doses, furosemide and acetazolamide had only a slight effect on liver mitochondria, while ethacrynic acid had the greatest effect. By contrast, chlormerodrin had the greatest effect on renal cortical and outer medullary mitochondria, followed in descending order by ethacrynic acid and furosemide. Chlorothiazide and acetazolamide, however, had only a slight effect at these concentrations.

Effect of sulfhydryl group binding. The effect of cysteine, a monothiol inhibitor of sulfhydryl binding, and of dithiothreitol, a sulfhydryl protector agent, on the mitochondrial effects of diuretics was examined. As can be seen in Fig. 2 for chlormerodrin, the addition of cysteine (1.6×10^{-4} M) or dithiothreitol (1.7×10^{-4} M) restored respiration virtually to the control level. Qualitatively similar results were obtained in the mitochondrial swelling studies. Preincubation of mitochondria with either cysteine or dithiothreitol completely prevented chlormerodrin-induced swelling of mitochondria. When these agents were added to the reaction mixture after the initiation of chlormerodrin-induced mitochondrial swelling, further swelling was inhibited but existing swelling was not reversed. It has been proposed that ethacrynic acid may also exert its effects by binding of sulfhydryl groups. In the current studies, however, neither cysteine nor dithiothreitol could reverse the inhibitory effect of ethacrynic acid on mitochondrial respiratory activity. On the other hand, in the study of mitochondrial swelling, the addition of cysteine but not that of dithiothreitol could prevent the further swelling of mitochondria induced by ethacrynic acid.

Effect of cell membrane fraction. The addition of cell membrane fraction resulted in no significant change in mitochondrial oxygen consumption rate (Table 3). These results are in contrast to those of Landon [23], who showed that the membrane fraction stimulated mitochondrial respiration when measured manometrically. Results similar to those of Landon have been obtained in our laboratory using the Gilson differential respirometer (Gilson Medical Electronics, Middleton, Wisc.), but not when mitochondrial respiratory activity was measured polarographically. It should be noted, however, that the respiratory control index (RCI) and ADP:O were signifi-

Table 2. Concentration of diuretics producing 50 per cent inhibition of rat renal and hepatic oxygen consumption rate*

	Cortex		Medulla		Liver	
	Concn (M)	RP†	Concn (M)	RP	Concn (M)	RP
Substrate: glutamate-malate						
Acetazolamide	10.8×10^{-3}	0.68	12.4×10^{-3}	0.65	10.7×10^{-3}	1.08
Chlorothiazide	7.4×10^{-3}	1.0	8.1×10^{-3},	1.0	11.6×10^{-3}	1.0
Furosemide	1.5×10^{-3}	4.9	1.8×10^{-3}	4.5	1.7×10^{-3}	6.82
Ethacrynic acid	4.2×10^{-4}	17.6	4.6×10^{-4}	17.6	4.6×10^{-4}	25.2
Chlormerodrin	3.5×10^{-5}	211.4	5.2×10^{-5}	155.7	3.0×10^{-5}	386.6

* The molar concentration at which chlorothiazide inhibited the mitochondrial oxygen consumption rate by 50 per cent has been considered as unity. The relative potency of each diuretic has then been calculated from the 50% respiratory inhibitory concentration of that diuretic compared to that of the concentration of chlorothiazide. The results obtained with succinate as the substrate were identical.

† Relative potency.

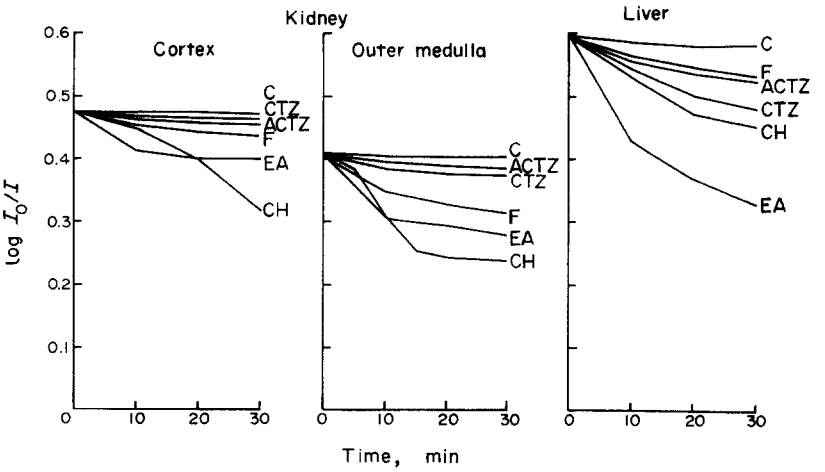

Fig. 1. Effects of chlorothiazide (CTZ), acetazolamide (ACTZ), furosemide (F), ethacrynic acid (EA) and chlormerodrin (CH) on the rate of mitochondrial swelling. C = control mitochondria with no diuretic added.

cantly reduced by addition of cell membrane fraction, indicating the stimulation of State 4 respiration and the continuous availability of ADP for State 3 mitochondrial respiration consequent to the enhanced availability of ADP in the presence of an ATPase system in the cell membrane fraction, as previously suggested by Landon.

The addition of cell membrane did not alter the effect of furosemide, chlorothiazide and acetazolamide on mitochondrial respiration. The addition of cell membrane fraction partially abolished the effect of chlormerodrin (Fig. 3). This effect was more apparent when chlormerodrin was pre-incubated *in vitro* with cell membrane fraction. The addition of the cell membrane fraction did not prevent the effect of ethacrynic acid on mitochondrial respiration (Fig. 4). However, with ethacrynic acid in the presence of cell membrane, the oxygen consumption rate was slightly but significantly higher ($P < 0.02$) than with ethacrynic acid alone utilizing succinate as substrate.

DISCUSSION

The results of the present study on mitochondria from rat kidney confirm our previous observations on the dog and indicate the presence of a difference in the oxygen consumption rate between the mitochondrial population of the cortex and that of the outer medulla [12]. The existence of two functionally different mitochondrial populations in the kidney is also supported by previous morphological and biological studies [24, 25]. As in the dog, all diuretics examined inhibited cortical and medullary oxygen consumption rate equally. The concentration of diuretic used to induce these changes is higher than those attained *in vivo* and the same concentration of diuretics exerted equal inhibitory effects on hepatic mitochondrial oxygen consumption rate (Table 2). This may be construed as evidence for a non-specific effect of diuretics on isolated mitochondria. Inhibition of oxygen consumption of liver mitochondria by diuretic agents is not a surprising finding in and of itself, since most cells of the body actively extrude sodium from the cell contents. The relative specificity of the diuretics for kidney tissue as compared to other tissue may derive from the greater concentration of the drugs attained in renal tissue, from differences in cell binding, or in the intracellular metabolism of the drug. Another source of relative specificity of diuretics on the kidney may derive from the secretion of these drugs by renal tubular cells and by virtue of the fact that these agents also exert their effects, when presented, to renal cells from the luminal side. This, coupled with the fact that the tubular fluid is reduced in volume as it traverses the nephron, would result in the delivery of higher concentrations of the diuretic to the site of action of these agents. When diuretics were administered to animals *in vivo* and the kidneys removed at the peak of diuresis, however, the isolated

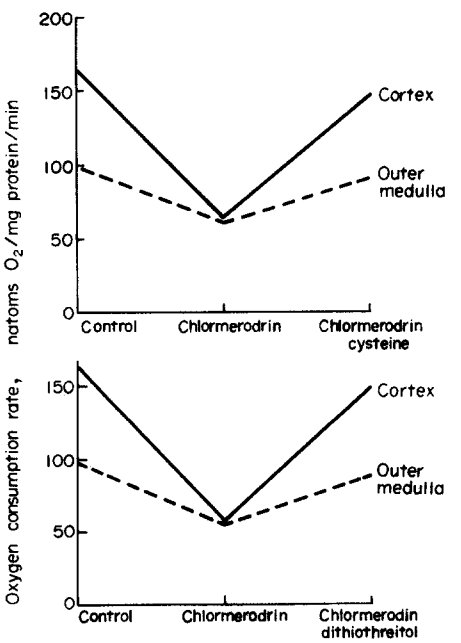

Fig. 2. Effects of chlormerodrin, chlormerodrin plus cysteine (upper panel) and chlormerodrin plus dithiothreitol (lower panel) on oxygen consumption rate.

Table 3. Effect of cell membrane fraction (ER) on renal mitochondrial function

		Control	ER membrane	P
Substrate: glutamate-malate (13)*				
Cortex	Qo_2†	189.61 ± 7.15	173.77 ± 5.91	NS‡
	RCI§	3.25 ± 0.24	1.78 ± 0.09	< 0.001
	ADP:O‖	2.91 ± 0.10	2.50 ± 0.15	< 0.05
Outer medulla	Qo_2	136.83 ± 5.61	127.75 ± 4.82	NS
	RCI	1.82 ± 0.10	1.30 ± 0.03	< 0.001
	ADP:O	2.09 ± 0.11	1.73 ± 0.09	< 0.02
Substrate: succinate (12)*				
Cortex	Qo_2	261.0 ± 10.63	235.47 ± 14.15	NS
	RCI	2.98 ± 0.27	1.58 ± 0.07	< 0.001
	ADP:O	1.70 ± 0.06	1.42 ± 0.05	< 0.05
Outer medulla	Qo_2	197.11 ± 8.32	190.38 ± 8.16	NS
	RCI	1.81 ± 0.12	1.27 ± 0.03	< 0.001
	ADP:O	1.27 ± 0.06	1.07 ± 0.06	< 0.025

* Number of experiments.
† Rate of oxygen consumption expressed as natoms O_2/mg of protein/min.
‡ Not significant.
§ Respiratory control index, ratio of that amount of oxygen consumed during State 3 to that of State 4 respiration.
‖ Amount of ADP esterified/nmole of oxygen consumed during State 3 respiration.

mitochondria from these organs show no difference in respiratory activity when compared to mitochondria from non-treated rats [13, 17]. Unpublished observations from this laboratory confirm these findings. When the intact kidneys were examined by electron microscopy, however, there were definite morphological changes of "swelling" that were apparent in the mitochondria of the kidney from the treated versus the non-treated animals (Fig. 5). Similar changes in morphologic appearance of mitochondria have been described by Trump [26] when renal sodium reabsorption was inhibited. The inability to show the changes on the isolated mitochondria of animals which had received diuretics prior to the isolation of mitochondria may be attributed to the fact that these agents are washed off during the isolation procedure [13, 17, 27]. Obviously, independent of the

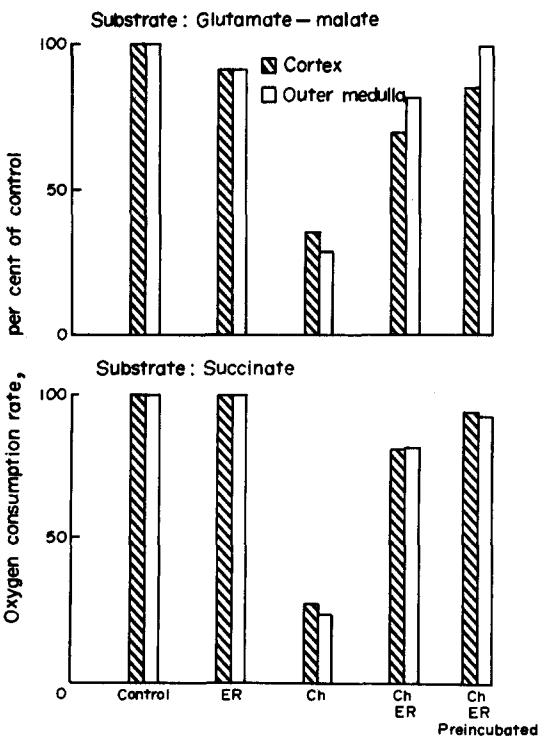

Fig. 3. Effects of chlormerodrin (CH), cell membrane fraction (ER), cell membrane fraction plus chlormerodrin (CH/ER), and pre-incubation (30–45 min) or chlormerodrin with cell membrane fraction (CH/ER pre-incubated for 30–45 min) on the change in oxygen consumption expressed as per cent of control values.

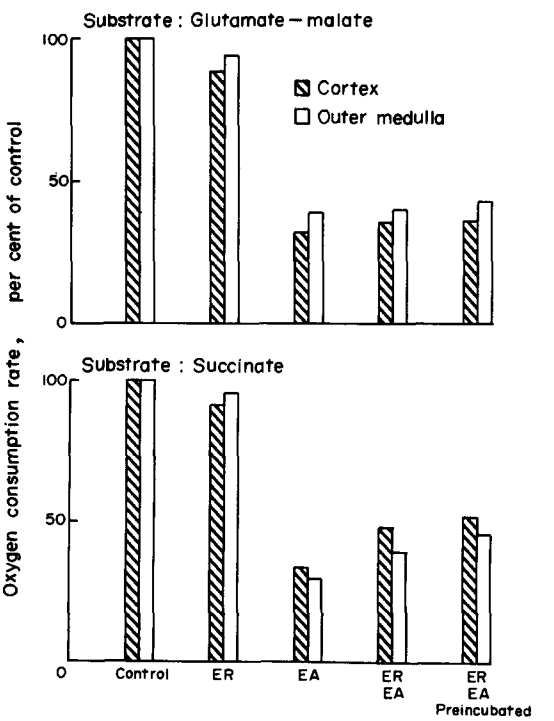

Fig. 4. Effects of ethacrynic acid (EA) and cell membrane fraction (ER) on oxygen consumption rate expressed as per cent of control values.

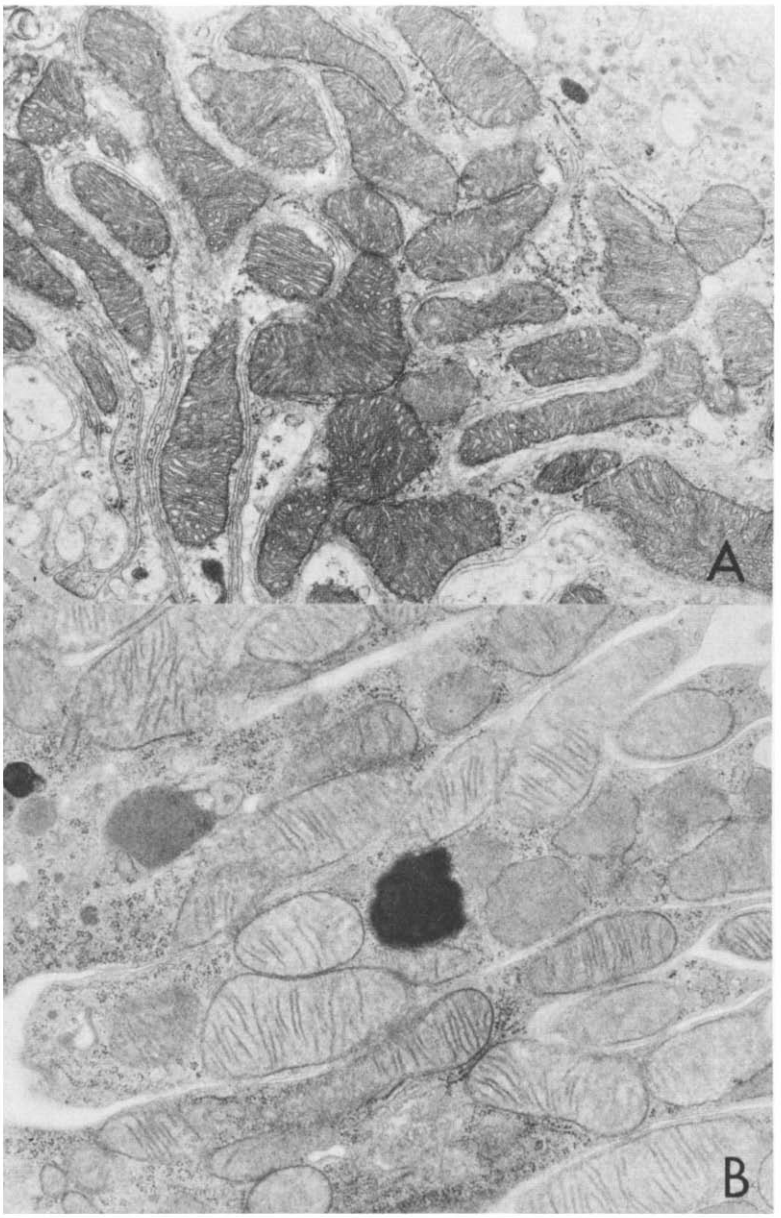

Fig. 5. Typical appearance of mitochondria in the intact kidney of a control rat (panel A) and the swelling of the mitochondria in kidneys removed from the animal during peak diuresis after the administration of furosemide intravenously (panel B). Magnification 37,500.

observed morphological changes, a direct causal effect of the diuretics on mitochondria cannot be established from morphologic changes alone since the observed alterations *in vivo* may have been secondary to an effect of diuretics on the cell membrane or cytoplasm.

Studies on mitochondrial swelling–shrinkage may be relevant to changes in mitochondrial electron transport and membrane permeability [28]. The stability and integrity of the mitochondrial membrane under various conditions are of interest because the respiratory carrier proteins are intimately associated with membrane permeability [29], and changes in the electron transfer chain may affect mitochondrial structure [30]. In this study, all diuretics examined induced changes of swelling of renal cortical and outer medullary and hepatic mitochondria. When the concentrations of diuretic agents which inhibited mitochondrial respiration by 50 per cent were employed to examine mitochondrial swelling, there was a difference in the responses observed. Utilizing hepatic mitochondria, ethacrynic acid was the most potent, followed closely by chlormerodrin, while furosemide and acetazolamide were the least potent (see Fig. 1). The response of renal mitochondria, however, was parallel to the changes observed on oxygen consumption rate with chlormerodrin exerting the greatest change, followed by ethacrynic acid, furosemide, chlorothiazide and acetazolamide. The difference in the effect of diuretics on hepatic and renal mitochon-

drial function is not, therefore, as non-specific as was suggested from the oxygen consumption studies.

The studies with cell membrane fraction revealed an additional difference on the effects of diuretics on mitochondrial function. The cell membrane had its greatest protective effect with chlormerodrin but had only a modest effect with ethacrynic acid (see Figs. 3 and 4), and exerted no protective effect on the mitochondrial respiratory inhibition by furosemide, chlorothiazide and acetazolamide (see Table 3). The results obtained with chlormerodrin and ethacrynic acid are similar to those reported by Landon and Fitzpatrick [17, 31]. Taken together with the results on hepatic mitochondrial swelling (see Fig. 1), the observed effects may be attributed to the structural characteristics of the agents examined, specifically the presence of a sulfamyl group in furosemide, chlorothiazide and acetazolamide; on one hand, mercurials and ethacrynic acid have no sulfamyl group and may act by binding to sulfhydryl groups. Indeed, both cysteine and dithiothreitol prevent the effect of chlormerodrin on mitochondrial respiration and swelling (see Fig. 4). Cysteine and dithiothreitol have also been shown to block the effect of ethacrynic acid on cell membrane and cell-free preparations [6, 10]. We were unable to show such an effect on mitochondrial respiration with these agents, but could block mitochondrial swelling with cysteine, indicating that ethacrynic acid may indeed exert its effect, in part at least, by inhibiting the sulhydryl group.

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