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THE EFFECT OF MERCURIAL DIURETICS ON ADENOSINETRIPHOSPHATASE OF RABBIT KIDNEY *IN VITRO*

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Abstract—The effect of several mercurial compounds on ATPase activity of cell debris from rabbit kidney, has been examined in the light of recent evidence that certain ATPase systems are closely associated with sodium transport.

The ATPase of kidney cell debris was found to resemble that of erythrocyte and nerve in its cation requirements.

Organic mercurial compounds (Mersalyl, Merchloran, Esidrone and *p*-chloromercuribenzoate) preferentially inhibit the (Na^+ and K^+)-stimulated portion of kidney ATPase activity, unstimulated activity being only slightly inhibited. Mercuric chloride has a preferential effect on the stimulated activity but strongly inhibits the unstimulated portion as well.

Sulphydryl-reacting compounds iodoacetate and iodoacetamide do not inhibit ATPase nor do the diuretic compounds caffeine, theobromine, theophylline and chlorothiazide.

The possibility that inhibition of ATPase is the cause of mercurial diuresis is discussed.

INTRODUCTION

THERE is still considerable controversy on the mode of action of mercurial diuretics. The most widely accepted view is summarized in the statement by Pitts[1] that "they (mercurial diuretics) probably depress ion transport by forming inactive mercaptide complexes with sulfhydryl enzymes which supply energy to drive the reabsorptive machinery." On the other hand Kleinzeller and Cort[2] maintain that active sodium transport is itself unaffected by mercurials, and that the main action of these substances is to increase the permeability of the tubular cells, the sodium pump being rendered ineffective by reason of the increased passive movement of sodium, potassium and water across the cell membranes. Since the evidence for neither of these views is compelling it is reasonable to consider a third possibility, namely, the direct action of the mercurials on the mechanism for active sodium transport. A considerable volume of evidence has recently accumulated supporting the view that this mechanism is closely associated with that portion of the ATPase activity which is elicited by sodium and potassium.[3–8] This puts a new significance on the observation of Goth *et al.*[9], that kidney ATPase is inhibited to a mild degree by mersalyl and mercuric chloride. It seemed of interest, therefore, to examine the effect of mercurials on the ATPase of kidney, in the light of this new knowledge of the relationship between this enzyme system and sodium transport.

EXPERIMENTAL

Preparation of subcellular fractions

The kidney cell debris fraction was prepared by homogenizing 10 g of rabbit kidney cortex in 100 ml 0·25 M sucrose in a Waring blendor for 60 sec at maximum speed. The homogenate was filtered through fine nylon cloth and spun at 850 × *g* for 10 min. This and all other centrifugation was carried out in refrigerated centrifuges at 0–2 °C. The supernatant fluid was drained off and the sediment resuspended in 20 ml 0·25 M sucrose. This preparation, which is subsequently referred to as the stock cell debris fraction, contained 15–20 mg protein/ml, and was further diluted with 0·25 M sucrose according to the activity required. The supernatant fluid from this first centrifugation was used to prepare microsomes. Mitochondria were spun down at 10,000 × *g*, the pellet so formed being discarded. The supernatant fluid was then spun at 74,000 × *g* for 60 min and the microsomal pellet was resuspended in 15 ml 0·25 M sucrose. This microsomal fraction contained 10 mg protein/ml and was used without further dilution

Microsomes of rabbit brain were prepared from 10 g of whole brain in exactly the same way as that described above, except that the centrifugation at 850 × *g* was omitted, the cell debris being spun down with the mitochondria at 10,000 × *g*. Preparations of brain microsomes made in this way contained about 5 mg protein/ml. Protein was estimated by the method of Aldridge.[8]

Measurement of ATPase activity

ATPase activity was estimated in tubes (100 mm × 14 mm) containing 2·0 ml medium and 0·1 ml enzyme preparation. The medium always contained 30 mM tris: HCl buffer (pH 7·5) and 2·5 mM ATP, rendered sodium free by treatment with amberlite IR-120(H) and brought to pH 7·5 with tris. In addition to these constituents, $MgCl_2$, NaCl, KCl and choline chloride were present in varying concentrations according to the purpose of the experiment—these concentrations are given in the legends to the tables and figures.

In those experiments in which inhibition was to be measured, estimations of ATPase activity were carried out either (1) with 80 mM NaCl and 20 mM KCl added to the media, or (2) in the absence of sodium and potassium. No attempt was made in these experiments to raise the osmotic pressure of the sodium- and potassium-free solution since preliminary experiments had shown that making the second solution osmotically equal to the first by the addition of sucrose had no detectable effect on the results. In other experiments variations in osmotic pressure were controlled to a limited extent by the use of choline chloride, as noted in the legends. The tubes were incubated for 10 min in a water bath at 37 °C to allow temperature equilibration. The reaction was started by the addition of 0·1 ml of tissue preparation and stopped 10 min later by the addition of 2·0 ml trichloroacetic acid (10 g/100 ml). The inorganic phosphate liberated was estimated by the method of Fiske and SubbaRow.[10]

Manometry

Tissue respiration was measured by the conventional techniques of Warburg manometry. Tissue slices were prepared from rabbit kidney cortex by means of a slicer made to the specifications of Stadie and Riggs.[11] Two kinds of media, described by Whittam,[12] were used: (1) "sodium-rich", containing 150 mM NaCl, 5 mM KCl,

10 mM sodium phosphate (pH 7·4), 2 mM $MgCl_2$ and 10 mM glucose; and (2) "sodium-free", containing 150 mM choline chloride, 2·5 mM potassium phosphate (pH 7·4), 2 mM $MgCl_2$ and 10 mM glucose. The flasks had 3·0 ml medium in the main compartment and also a known weight of tissue slices—about 60 mg wet weight. The centre compartment contained 0·2 ml KOH (10 g/100 ml). The flasks were gassed with 100% oxygen and after 10 min equilibration at 37 °C respiration was measured for 60 min. The inhibitors were added, where appropriate, at the beginning of the experiment. The dry weight of the tissue in each flask was calculated from the wet weight by means of the wet:dry weight ratio determined on fresh samples of slices from the same experiment.

RESULTS

Cation requirements of the enzyme system

Under conditions which yield optimum activity (80 mM NaCl and 20 mM KCl) ATPase activity was found to be linear with time until 1·5 μmole of inorganic phosphate had been liberated. (Fig. 1). This is equivalent to 30 per cent of the terminal phosphate of the ATP present in each tube. In subsequent experiments the stock cell debris preparation was diluted so that with an incubation time of 10 min, less than 1·5 μmole inorganic phosphate was liberated.

In the absence of magnesium (Table 1 column 3), ATPase activity is low. The addition of either sodium (80 mM) or potassium (20 mM) causes some increase in activity, and when these two ions are present together there is a further rise. However, in absolute terms these increases in activity are very slight.

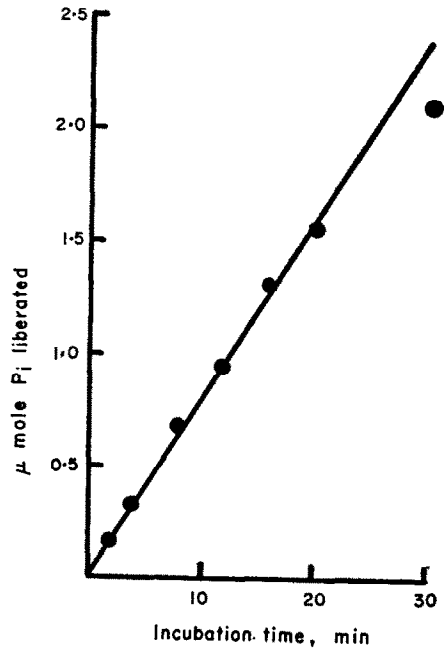

FIG. 1. The effect of incubation time on ATPase activity of rabbit kidney cell debris. The medium contained 30 mM tris: HCl buffer (pH 7·5), 2·5 mM ATP, 80 mM NaCl, 20 mM KCl and 5 mM $MgCl_2$.

In the presence of 5 mM $MgCl_2$ (Table 1 column 4) ATPase activity is considerable and this can be further stimulated by sodium and potassium. As in the absence of magnesium, stimulation by these ions when added separately is relatively small but when 80 mM NaCl and 20 mM KCl are added together activity is stimulated by

TABLE 1. THE EFFECT OF SODIUM AND POTASSIUM ON ATPASE ACTIVITY OF KIDNEY CELL DEBRIS IN THE PRESENCE AND ABSENCE OF MAGNESIUM

(The medium in each tube contained 2·5 mM ATP, 30 mM tris:HCl buffer (pH 7·5) and sufficient choline chloride to render it approximately 300m0sM).

NaCl Concentration (mM)	KCl Concentration (mM)	No $MgCl_2$	5 mM $MgCl_2$
		ATPase activity (μmole P_i/mg protein per hr)	
0	0	0·61	5·12
80	0	0·67	5·90
0	20	0·74	5·25
80	20	0·78	10·2

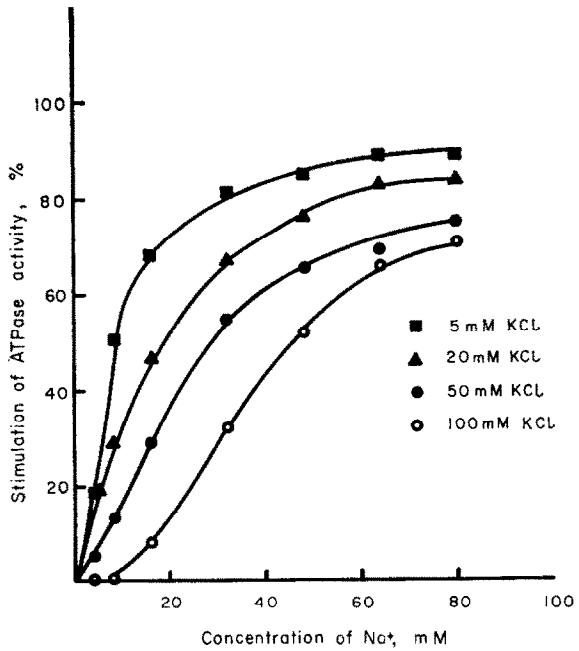

FIG. 2. The effect of sodium on ATPase activity of rabbit kidney cell debris, at four levels of potassium. The media in all tubes contained 30 mM tris: HCl buffer (pH 7·5), 2·5 mM ATP, and 5 mM $MgCl_2$ together with NaCl and KCl at the concentrations indicated. The total osmolarity of the media for any one curve was kept constant by the addition of choline chloride. The amount added to each tube was such that the final concentrations of choline chloride and sodium chloride were together equal to 100 mM. Similar results were obtained in the absence of choline chloride. The results are expressed as percentage stimulation—activity in the absence of added sodium being taken as 100.

100 per cent. These results are typical of those obtained with several preparations although the maximal activity, and the magnitude of the (Na^+ and K^+)-stimulation, varied considerably in different preparations.

Figure 2 shows the effect on ATPase activity of varying the sodium concentration at four different levels of potassium. The main points of interest are as follows:

(1) At low concentrations of potassium, ATPase activity increases with sodium concentration until a limiting velocity is reached—a saturation curve is obtained.

(2) The curve is shifted to the right by increasing the potassium concentration so that for any given velocity a higher sodium concentration is required.

(3) The sigmoidal character of the curves which is apparent even at 5 mM K^+ increases with concentration and is very marked at 150 mM K^+. It follows from this, that sodium-stimulation of activity does not conform to Michaelis–Menten kinetics, the plot of $1/V$ against $1/S$ being non-linear.

(4) The concentration of sodium required for half maximal activity increases with potassium concentration from about 10 mM Na^+ at 5 mM K^+ to about 55 mM Na^+ at 150 mM K^+.

The effect of varying potassium concentration at different levels of sodium is shown in Fig. 3. Although these results are from a different set of experiments they agree reasonably well with the results in Fig. 2. Here the main points of interest are:

(1) At any of the sodium concentrations used, activity increases with potassium

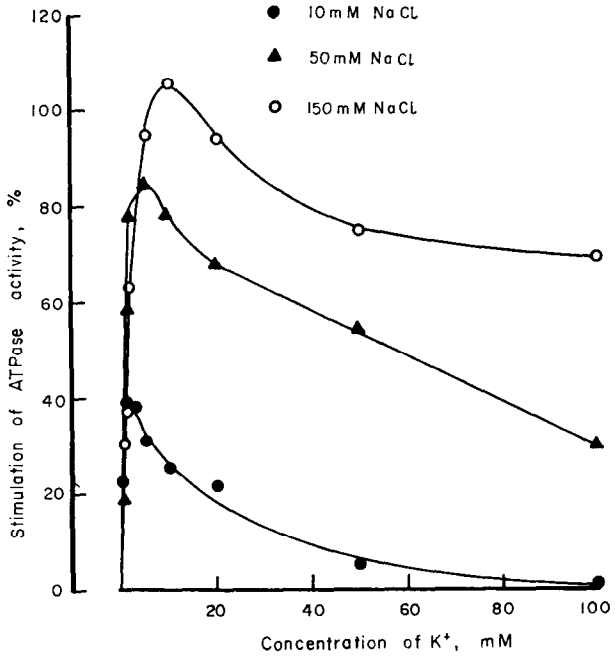

FIG. 3. The effect of potassium on ATPase activity of rabbit kidney cell debris at three levels of sodium. The experimental details were the same as described in Fig. 2 except that for each curve potassium concentration was varied at a constant sodium concentration and here the amount of choline chloride present in each tube was such that the final concentrations of choline chloride and KCl were together equal to 100 mM.

concentration to reach a maximum; thereafter, increasing the potassium concentration reduces activity.

(2) Half maximal activity is attained at low concentrations of potassium: 0·2 mM K^+ at 10 mM Na^+, 1·0 mM K^+ at 50 mM Na^+ and 1·5 mM K^+ at 150 mM Na^+.

(3) The lower the concentration of sodium the greater is the inhibition by high concentrations of potassium—with 150 mM Na^+, 100 mM potassium reduces maximal stimulation by only 25 per cent but with 10 mM Na^+ this concentration of potassium almost abolishes stimulation.

The data in Figs. 2 and 3 are presented, to show qualitatively, the pattern of ATPase stimulation by sodium and potassium. The accuracy of these experiments is limited by two factors. Firstly, there is a large blank value—unstimulated activity—and secondly, because of the large number of assays involved and the need to avoid delay in estimating phosphate, not all the assays in one experiment could be carried out in a single batch. The data are, therefore, considered insufficiently accurate for precise kinetic calculations and it is possible that minor quantitative discrepancies will be found in further studies.

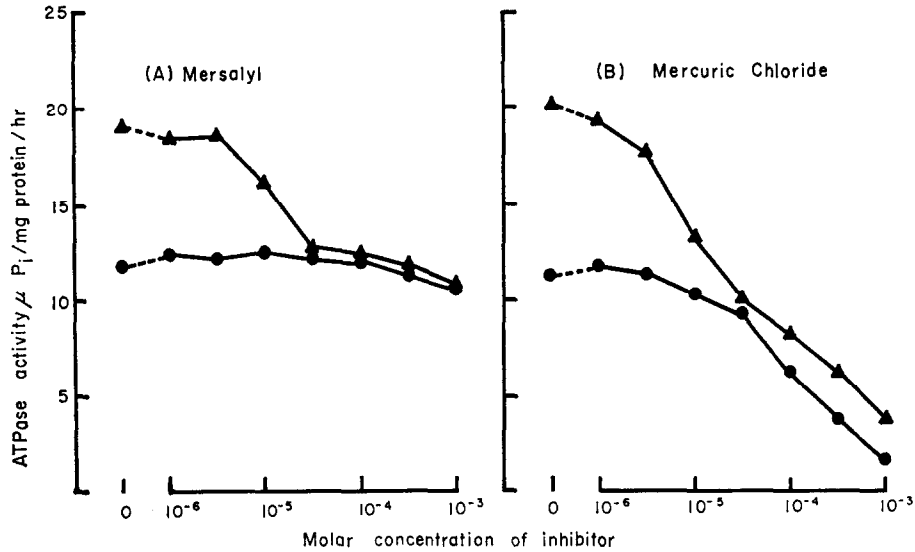

FIG. 4. The effect of Mersalyl and mercuric chloride on ATPase activity of rabbit kidney cell debris. All tubes contained 30 mM tris:HCl buffer (pH 7·5), 2·5 mM ATP and 5 mM $MgCl_2$. ▲—▲ 80 mM NaCl and 20 mM KCl; ●—● no NaCl or KCl present.

The effect of mercurial compounds on ATPase activity

In the following experiments a comparison is made of the effect of inhibitors on "unstimulated ATPase activity" (sodium and potassium absent) and on "($Na^+$and K^+)-stimulated ATPase activity" (80 mM NaCl and 20 mM KCl present).

The effect of mersalyl on the ATPase of kidney cell debris, under these conditions is seen in Fig. 4(a). In the absence of added sodium and potassium, concentrations of mersalyl up to 10^{-4} M have no detectable effect on activity and even at 10^{-3} M

inhibition is slight (15 per cent). In the presence of sodium and potassium, mersalyl causes considerable inhibition at 10^{-5} M and at concentrations of 10^{-4} M and above, activities are not significantly higher than those found in the absence of sodium and potassium. Mersalyl clearly has a preferential effect upon the (Na^+ and K^+)-stimulated portion of activity since stimulation is abolished at concentrations which have only a mild effect on unstimulated activity. Mercuric chloride also inhibits the ATPase of kidney cell debris but the pattern of inhibition is quite different (Fig. 4b). Although there is a preferential effect upon the stimulated portion of activity, as may be seen from the values for activity at 3×10^{-5} M mercuric chloride, unstimulated activity is also inhibited and at high concentrations this inhibition is severe.

The effect of two other mercurial diuretics, Esidrone (sodium salt of pyridine-dicarboxy-β-mercuri-ω-hydroxypropylamide-theophylline, Ciba) and Merchloran (3-chloromercuri-2-methoxypropylurea, Parke-Davis) has been determined in the same way (Fig. 5). The results are essentially similar to those found with mersalyl, the stimulation by sodium and potassium being virtually abolished at concentrations which have little or no effect upon unstimulated activity. The compound *p*-chloromercuribenzoate was tested at a single high concentration (10^{-3} M) and again stimulation is abolished with no detectable effect on unstimulated activity.

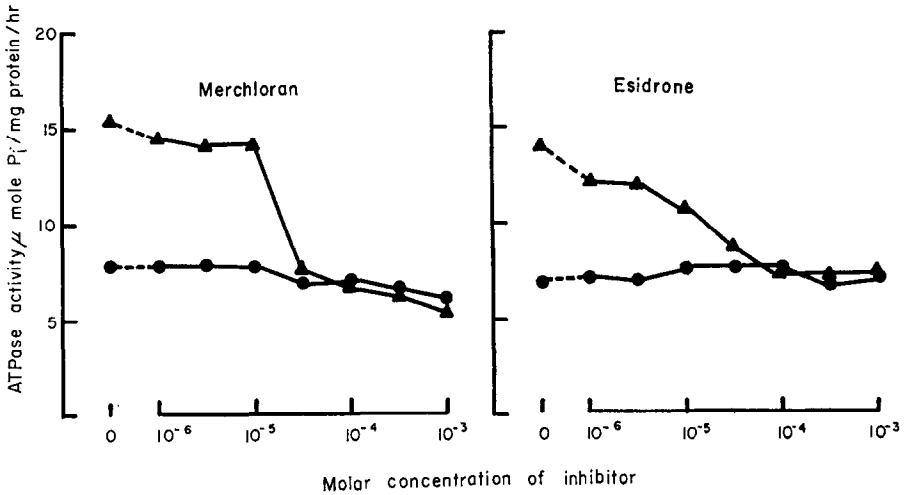

FIG. 5. The effect of Merchloran and Esidrone on ATPase of rabbit kidney cell debris. All tubes contained 30 mM tris:HCl buffer (pH 7·5), 2·5 mM ATP and 5 mM $MgCl_2$. ▲—▲ 80 mM NaCl and 20 mM KCl ; ●—● no NaCl or KCl present.

Mersalyl was tested on the ATPase of rabbit kidney microsomes, under conditions identical with those described for the experiments with cell debris. This preparation also exhibits (Na^+ and K^+)-stimulation, an activity of 13·6 μmole P_i/mg protein per hr being obtained with 80 mM NaCl and 20 mM KCl, and 9·9 μmole P_i/mg protein per hr in their absence. As with the cell debris preparation, unstimulated activity is only slightly inhibited by 10^{-3}M mersalyl but stimulation is strongly inhibited by concentrations of 10^{-5}M and above.

Figure 6 shows the inhibition of ATPase activity of rabbit brain microsomes by mersalyl and mercuric chloride. The inhibition by these substances is similar to that found with kidney preparations (Fig. 4) except that with brain microsomes the effective concentrations are slightly lower and mersalyl has a more pronounced effect on unstimulated activity.

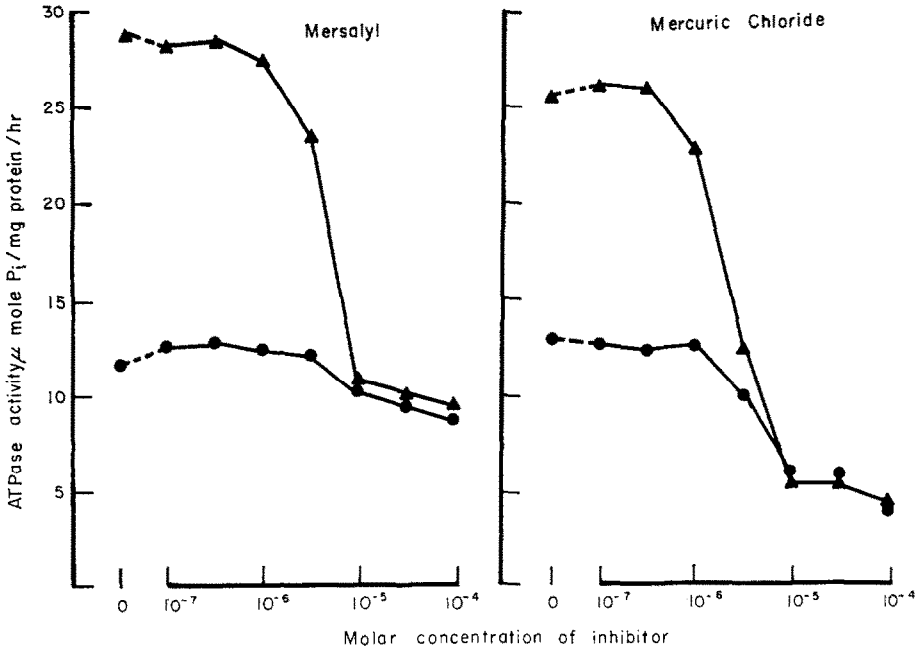

FIG. 6. The effect of Mersalyl and mercuric chloride on ATPase of rabbit brain microsomes. All tubes contained 30 mM tris:HCl buffer (pH 7·6), 2·5 mM ATP and 5 mM $MgCl_2$. ▲—▲ 80 mM NaCl and 20 mM KCl; ●—● no NaCl or KCl present.

Superficially, mersalyl presents a pattern of inhibition which, in the kidney at least, resembles that of ouabain. This similarity is more clearly demonstrated by the following experiments (Fig. 7) in which the effects of high concentrations of mersalyl (3×10^{-4}M) and ouabain (2×10^{-4}M) are compared.

(1) In the absence of sodium and potassium mersalyl causes about 10 per cent inhibition of activity and ouabain has no significant effect. When the two drugs are present together the activity obtained is the same as with mersalyl alone.

(2) In the presence of 80 mM NaCl and 20 mM KCl control activity is stimulated by 78 per cent. Under these conditions mersalyl and ouabain give the same activity as they do in the absence of sodium and potassium. When the two drugs are present together activity is reduced only to the level obtained with mersalyl alone, that is, ouabain has no effect on activity when the ATPase is already maximally inhibited by mersalyl.

These experiments confirm the similarity in the action of the mersalyl and ouabain. Both abolish that portion of the ATPase activity elicited by sodium and potassium but mersalyl causes in addition a slight inhibition of the residual ATPase activity.

This similarity between the effects of mersalyl and ouabain on ATPase made it of considerable interest to compare their effects on respiration of kidney slices since Willis and Whittam[13] demonstrated that ouabain only inhibits that part of the respiration dependent upon the presence of sodium. Table 2 shows the effect of ouabain and mersalyl on respiration in the presence and absence of sodium. Although the

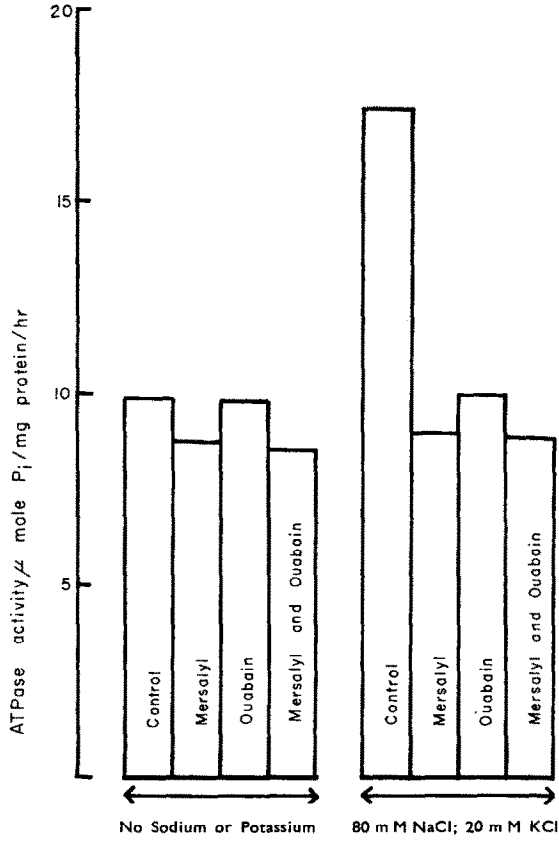

FIG. 7. Comparison of the effects of Mersalyl (3×10^{-4}M) and ouabain (2×10^{-4}M), separately and together, on ATPase activity of rabbit kidney cell debris. All tubes contained 30 mM tris:HCl buffer (pH 7·5), 2·5 mM ATP and 5 mM $MgCl_2$.

Q_{O_2} values found here are about 30 per cent lower than those reported by Willis and Whittam these experiments are in good agreement with their finding on the effect of sodium and ouabain on respiration. The presence of sodium in the medium stimulates respiration, and ouabain affects only that portion stimulated by sodium, abolishing the stimulation but having no effect on respiration in the absence of sodium. The action of mersalyl is different from that of ouabain. Both stimulated and unstimulated activity are inhibited and even at 10^{-3}M mersalyl, the Q_{O_2} in the presence of sodium is considerably greater than in its absence. These figures suggest that mersalyl has no specific effect upon sodium-stimulated respiration.

TABLE 2. THE EFFECT OF MERSALYL AND OUABAIN ON THE RESPIRATION OF RABBIT KIDNEY SLICES IN THE PRESENCE AND ABSENCE OF SODIUM

(The control values are the means from nine experiments. All other values are the means from three experiments. The composition of the media is given in the experimental section.)

Media	(1) "Sodium-free" Respiration Q_{O_2} (μl/mg dry wt. per hr)	(2) "Sodium-rich"	(3) Stimulation (%)
Control	5·59 ± s.e.m. 0·13	8·43 ± s.e.m. 0·21	51
Ouabain 5×10^{-5}M	5·6	5·7	2
Mersalyl 5×10^{-4}M	3·3	5·7	73
Mersalyl 10^{-3}M	2·7	4·4	63

The effect of iodoacetate, iodoacetamide and non-mercurial diuretics

Since frequent attempts have been made to explain the diuretic effect of mercurial compounds by their action on sulphydryl groups, two sulphydryl-reacting substances, iodoacetic acid and iodoacetamide have been tested for inhibition of ATPase. Neither iodoacetic acid (10^{-3}M) nor iodoacteamide (10^{-2}M) had any effect on ATPase activity in the presence of 80 mM NaCl and 20 mM KCl or in their absence.

Similarly, the diuretic compounds theophylline, theobromine, caffeine and chlorothiazide were tested and found to be without effect.

DISCUSSION

The experiments confirm the presence in cell debris of rabbit kidney of a magnesium-dependent ATPase which is stimulated by sodium and potassium. This has previously been reported by Wheeler and Whittam,[7] but their results differ in some respects from those described here. They found that in the presence of 50 mM potassium, the ATPase activity increased with sodium concentration up to about 50 mM, and that raising the sodium concentration beyond this severely reduced stimulation. Using potassium concentrations of 5, 20, 50 and 150 mM the author has failed to find any falling off in the stimulation curve with sodium concentrations up to 100 mM, although stimulation was reduced by very high concentrations of sodium (unpublished observation). It is possible that the discrepancy is due to a difference in pH of the media, since the experiments of Wheeler and Whittam were carried out at pH 8·0 and those of the author at pH 7·5.

The pattern of stimulation by sodium and potassium is qualitatively very similar to that found by Skou[3] for the ATPase of crab nerve and by Post *et al.*[4] for human erythrocyte ATPase. It seems reasonable, therefore, to assume that the relationship between (Na^+ and K^+)-stimulated ATPase and sodium transport which has been demonstrated in erythrocyte and nerve, probably holds for the kidney as well. There is a growing belief that ATPase may function as a part of the sodium transport mechanism; several schemes have been put forward to explain this in detail.[14, 15] It is of considerable interest, therefore, that organic mercurial compounds specifically

inhibit that portion of the ATPase activity which is thought to be responsible for sodium transport, namely, the (Na^+ and K^+)-stimulated activity. It would appear also that this specificity is principally a property of the organic mercurials since mercuric chloride inhibits unstimulated activity as well.

It is then, tempting to suggest that inhibition of ATPase is the underlying cause of mercurial diuresis. The concentrations which are shown here to be effective *in vitro* (10^{-5}M) are certainly within the pharmacologically relevant range; Borghgraef and Pitts[16] have shown that following an i.m. injection of chloromerodrin (Merchloran) at a dose of 1 mg Hg/kg 80 per cent of the drug is accumulated within the kidney, giving rise to a concentration of 100 μg Hg/g kidney, i.e. 5×10^{-4}M. This fifty-fold excess over the concentration effective *in vitro* does not prove that the enzyme would be inhibited *in vivo* under these conditions. However, there is some evidence that renal ATPase is inhibited *in vivo* by administration of mercurials. Goth *et al.*[9] observed inhibition of total ATPase activity in homogenates of kidney prepared from rats treated with high doses of mersalyl. A similar conclusion was drawn by de Groot *et al.*[17] from measurements of the changes in acid-labile phosphate content during incubation of kidney slices from rats treated with high doses of mersalyl. At the time these experiments were carried out the significance of sodium-stimulation was not known, so that they do not show whether the (Na^+ and K^+)-stimulated portion of ATPase activity is preferentially affected.

The inhibition of ATPase *in vitro* is not confined to the kidney, in fact the brain microsomes were rather more sensitive to mersalyl and mercuric chloride than kidney enzyme, and it is probable that the ATPase of other tissues will be inhibited as well. Despite this, *in vivo* specificity for kidney is very probable since it has been shown[17] that the concentration arising in the kidney, after administration of a therapeutic dose of chloromerodrin to dogs and rats, is at least fifty times greater than in any other tissue tested, and in several cases the ratio was very much higher than this. It is known also that after injection of mercuric chloride into rabbits, mercury is strongly concentrated in the kidney but only negligible amounts accumulate in the brain.[18]

Mersalyl, and presumably the other organic mercurials, are similar to ouabain in that they abolish (Na^+ and K^+)-stimulated ATPase activity, with little effect on unstimulated activity. The similarity does not, however, extend to their effects on respiration. Ouabain, as reported by Whittam and Willis,[13] abolishes sodium stimulated respiration in kidney slices but has no effect on unstimulated respiration. Mersalyl does not show this specificity. At 5×10^{-4}M mersalyl, unstimulated respiration is strongly inhibited while sodium-stimulation of respiration is certainly not abolished, in fact the percentage stimulation found, in the presence of either of the Mersalyl concentrations used, is greater than that in the control experiments. Mersalyl is known to affect enzymes of the citric acid cycle,[19] so that the effect of this drug on respiration may be expected to be more complex than that of ouabain. However, if it were subsequently shown that strong inhibition of sodium extrusion from kidney slices can be obtained without inhibition of sodium-stimulated respiration this would militate against the theory that inhibition of ATPase is the cause of reduced ion transport. Furthermore, in view of the postulated obligatory relationship between sodium transport and respiration such a result would be strong evidence in favour of Cort and Kleinzeller's theory that mercurials do not affect the active transport of sodium out of the cells, but only increase the permeability of the cells.

Failure of sulphydryl-reacting substances to inhibit an enzyme system cannot, by itself, be taken as proof that sulphydryl groups are inessential to that system.[20] However, the inability of iodoacetate and iodoacetamide to reduce ATPase activity lends no support to the view that sulphydryl groups are important in the ATPase system studied here. If in fact, mercurial inhibition of ATPase is due to mercaptide formation the sulphydryl groups involved must be concerned predominantly with the (Na^+ and K^+)-stimulation.

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