HISTOCHEMICAL DEMONSTRATION OF MERCURY IN RENAL CELLS*

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A GREAT deal of effort has been expended, especially during the last decade, toward elucidation of the site and mechanism of action of mercurial diuretics. As a result of the contributions of many investigators, several concepts have developed and are generally held.

Histochemical studies dealing with renal protein-bound sulfhydryl groups[1,2] and stop-flow procedures[3,4] indicate that the site of action is the proximal tubule. That the loop of Henle and collecting duct may participate cannot be ruled out and, indeed, is actually implicated in the sulfhydryl studies and also by the fact the mercurials interfere with potassium transport.

Studies dealing with mechanism of action have centered around two focal points. The first involves the nature of the renal receptor and the second is concerned with the chemical nature of the reaction with receptor. A two-point receptor involving a sulfhydryl group has been suggested by Pitts *et al.*[5] and by Mudge and Weiner[6] but these investigators offer opposing views concerning the form of mercury which reacts with receptor. The former workers believe that mercury attaches by one valence to a point on the receptor. The second point of the receptor reacts with the organic fragment attached to the second valence of mercuric mercury; the latter have proposed that release of free mercuric ion by fracture of a carbon–mercury bond, and subsequent attachment of divalent mercury to the double receptor are necessary for inhibition of sodium reabsorption. This conclusion is well-supported by their demonstrations that inorganic mercury attached to a suitable carrier seems to be more active than any organic compound, that acidosis, which potentiates mercurial diuresis, speeds fracture of carbon–mercury bonds *in vitro*, and that stable mercury compounds are not diuretic.

The work described in this report is an outgrowth of all the valuable

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work reported above. It was undertaken with the purpose of testing and clarifying the concepts alluded to. It comprises an attempt to find mercury in renal cells attached to its receptors and to develop methods for characterizing the mercury-receptor bond.

METHODS

Adult mongrel dogs of both sexes were used in all experiments. In general two procedures were used.

Procedure A — Unanesthetized dogs were injected intravenously with doses of various mercurials ranging from 1 to 8 mg Hg/kg body weight. The animals were given lethal injections of pentobarbital at definite time intervals following mercury administration. Kidneys were removed prior to death. Control dogs were not given mercury injections.

Procedure B — Dogs lightly anesthetized with pentobarbital were given intravenous infusions of isotonic saline mixed with equal parts of isotonic NH_4Cl or $NaHCO_3$ a a rate of 0·5 ml/kg/min until urine became acid (pH 6·3 or less) or alkaline (pH 7·6 or more). When the desired urinary pH was achieved the dogs were given intravenous injections of 4 mg Hg/kg as chlormerodrin, $HgCl_2$, or *p*-chloromercuribenzoate. Urine flow, Na^+, K^+, and Cl^- excretion, and creatinine clearance were determined in each experiment using standard analytical methods. Kidneys were removed 1 hr after injection of mercury.

Histochemical procedure — Immediately on removal separate slices of kidney were placed in 10% trichloroacetic and in 10% formalin fixatives. Fixed tissue was dehydrated in alcohol, cleared in xylol, and embedded in paraffin. Paraffin sections 10 μ thick were cut using a rotary microtome and were then mounted on glass slides. Tissue sections were deparaffinized and rehydrated. Mercury present in tissue was stained by immersing sections for 20 min in a saturated solution of di-β-naphthylthiocarbazone (hereafter referred to as NTC) prepared by first dissolving the reagent in N,N-dimethylformamide before adding sufficient deionized water to bring about precipitation of a small quantity of NTC (approximately 45 parts of H_2O to 15 parts of solvent for 5 mg of NTC). After staining, sections were washed in H_2O, excess dye was decolorized in 0·1 N HCl, and tissues were covered with glass coverslips using glycerine jelly as mounting medium.

Relative concentration of mercury in renal cortex was determined using a microspectrophotometer similar to that employed in previous studies[7].

RESULTS

The reaction between NTC and mercury is well known. A colored complex with an absorption maximum of 515 mμ is formed[8]. Tissues

from control animals do not develop color since no interfering metal is normally present in sufficient concentration in kidney. When 10 μ sections of kidney from control animals are used as blanks for determination of absorption spectra the pattern illustrated in Fig. 1 is obtained. Note that the absorption characteristics of the mercury-NTC complex in tissue are indentical to those observed for chloroform solutions of the complex, and that the absorption maximum of 510 mμ is close to the reported value of 515 mμ (see above).

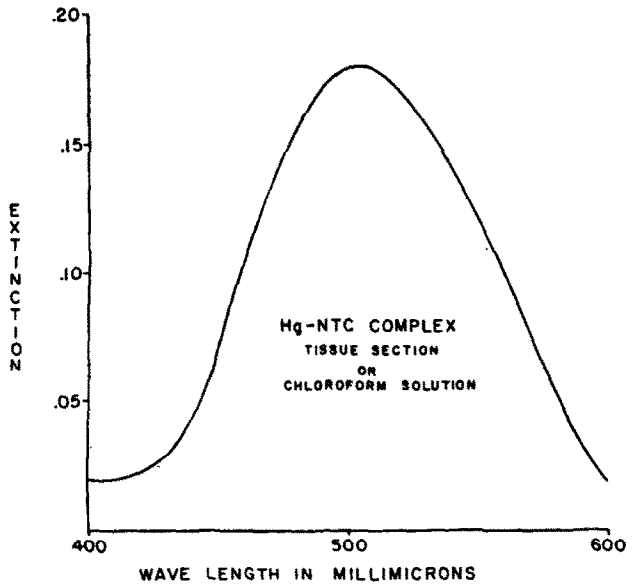

FIG. 1. Absorption spectrum of the mercury-di-β-naphthylthiocarbazone (NTC) complex. Readings were made through 10 μ sections of renal cortex from a dog treated with chlormerodrin (dose = 2 mg Hg/kg) and through a film of the complex dissolved in chloroform and spread on a microscopic slide. The extinction maximum was at 510 mμ.

The color reaction and distribution of mercury in renal cortex are depicted in Fig. 2. The photomicrograph in the left panel is of renal cortex from a control animal; the photomicrograph in the right panel is of cortex from a dog treated with 2 mg of chlormerodrin mercury/kg. Even though black and white photographs do not portray the actual differentiation as well as color photographs, some semblance of the pattern of distribution of mercury is retained. Mercury is present in cytoplasm of proximal tubule cells and is especially concentrated in both their apical and basal membranes. It can not be detected in glomeruli, nuclei, lumens of tubules, interstitial spaces, or distal tubule cells. A few empty distal tubule cells can be seen just below and to the right of the glomerulus in the right panel.

A photomicrograph taken at higher magnification is shown in Fig. 3. The animal from which the tissue in the right panel was taken received 4 mg Hg/kg as chlormerodrin. Monochromatic light of 510 mμ was used to photograph the sections. Mercury is scattered thoroughout the cytoplasm of the cells of the proximal tubule shown and is found also in the basal membrane. Fixation of the brush border is usually poor in dog kidney so that loss of some mercury in the apical portion probably occurred.

The distribution pattern noted for chlormerodrin was identical to that observed with other mercurials including the non-diuretic compound, *p*-chloromercuribenzoate. No mercury could be found in collecting ducts or in any part of renal medulla.

TABLE I

*Relative concentration of three mercurials in renal cortex of the dog**

Merculiar	Dose Hg mg/kg	Extinction†	
		Acidosis‡	Alkalosis‡
Chlormerodrin	4	0·30 0·23-0·24	0·13 0·11-0·16
$HgCl_2$	4	0·21 0·19-0·24	0·13 0·09-0·15
p-Chloromercuribenzoate	4	0·04 0·01-0·09	0·13 0·01-0·10

* Kidney tissue was removed one hour after intravenous administration of each drug.

† Upper value represents average extinction through 10 μ sections of renal cortex of 4 dogs; lower value indicates range of observations.

‡ Animals were made acidotic or alkalotic by sustained intravenous infusion of NH_4Cl or $NaHCO_3$.

The effects of acid-base balance on mercury concentration in renal cortex are shown in Table I. Acidosis enhanced diuretic activity and renal binding of chlormerodrin and $HgCl_2$, but did not alter binding of *p*-chloromercuribenzoate. Extinction values listed in the table merely provide a rough index of concentration since measurements were made over a wide area of cortex and not through individual cells. The values are presented in lieu of actual photographs. They correlate nicely with gross observation of sections and grading of tissue sections according to visible intensity of staining, but they do not provide accurate estimates of mercury levels in individual cells. Note that for a given dose,

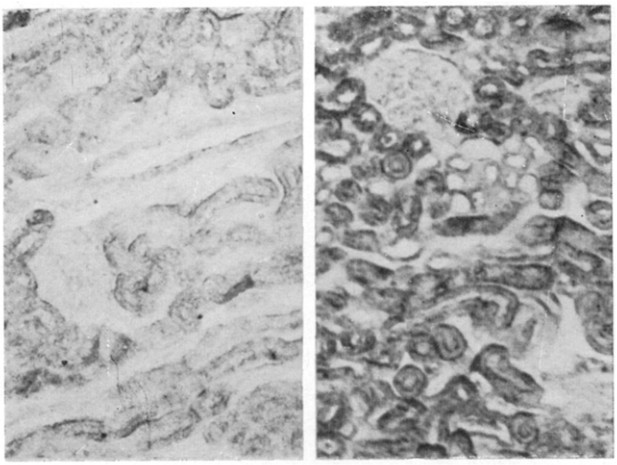

FIG. 2. Microphotograph of renal cortex of an untreated dog (left) and of a dog injected with chlormerodrin one hour before sacrifice (right). Dose of drug, 2 mg Hg/kg; Photographic procedure was the same for both panels.

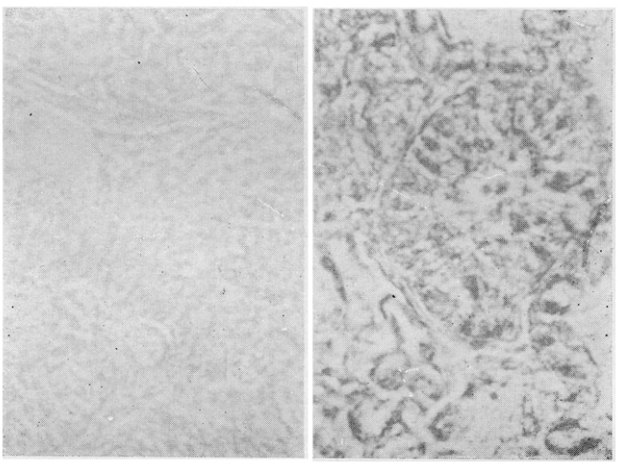

FIG. 3. Same as 2 except: dose of chlormerodrin, 4 mg Hg/kg, and photographs were made using monochromatic light at a length of 510 mμ.

mercury levels are highest when chlormerodrin is employed and lowest when *p*-chloromercuribenzoate is used.

DISCUSSION

Under conditions of these experiments, the color reagent, NTC, is specific for mercury. Color noted in renal tissue of dogs pre-treated with mercury truly represents the mercury-NTC complex, for tissue of untreated dogs does not develop color and the light absorption characteristics of the color in tissue sections of treated animals are identical to those of the pure complex (see Fig. 1).

Based on these studies, the major site of action of mercurial diuretics must be the proximal tubule, for it is here that large quantities of mercury are found and it is unlikely that mercury can be considered a "hit-and-run" drug. But the possibility exists that small, undectectable amounts of mercury in other cell types also contribute to diuresis. This must be considered seriously since mercury is known to inhibit potassium secretion[9], a function which does not appear to be proximally located, and since sulfhydryl concentrations in collecting ducts are affected by mercurials[1,2]. Of interest is the fact that mercury is present throughout the proximal tubule but sulfhydryl binding has never been demonstrated in convoluted portions of the proximal tubule. These divergent findings need to be explained. One possible answer is that most of the mercury present is concentrated near the periphery of the cells while measurements of sulfhydryl were made in cytoplasmic areas away from the periphery.

Results obtained in acid-base studies favor the concept that acidosis either increases reactivity of, or creates new receptors for, mercury. Otherwise, levels of mercury attained in cortex should not be increased in acidotic dogs injected with inorganic mercury (see Table I). On the other hand, inability to influence renal binding of mercury in acidotic dogs when *p*-chloromercuribenzoate was given fortifies the mercuric ion hypothesis. These findings can be resolved by combining the separate ideas and postulating that acidosis not only liberates more ion but also increases rate of reaction with or the number of receptors for mercuric ion. This "ion-receptor hypothesis" can probably be tested utilizing present histochemical methods.

Recently, White *et al.*[10] have shown that a single mercurial increases leakage of sodium through proximal cells into urine and have postulated that mercurials act on the cell membrane. Their results can be explained by accepting the idea that sodium flux is increased in both directions (a logical consequence of an increase in membrane permeability). With increased flux bi-directionally, inhibition of active tubular reabsorption is still necessary to account for greater sodium loss in the urine. A higher concentration of mercury in proximal cell membranes than in cytoplasm

was noted even when the non-diuretic mercurial, *p*-chloromercuribenzoate, was used.

Preliminary data obtained during the course of time-distribution studies with chlormerodrin offer an additional clue regarding mechanism of action. It was found that the peak level of bound mercury occurred between 0·5 and 1·0 hr following administration. Thereafter the concentration declined until, at 24 hr after injection, very little could be detected. The pattern for *p*-chloromercuribenzoate differed. Levels appeared to be constant over the first six hours following administration of the drug. These results indicate that removal of mercury from its binding site and not attachment to a receptor may be responsible for diuresis. This concept at present serves as one of the working hypotheses with which this laboratory struggles in the continued exploration of the pharmacology of mercurial diuretics.

These histochemical studies have not yet furnished specific information about the nature of the mercury-receptor bond, but they do offer a means for additional probing.

SUMMARY

Mercury was localized in renal cells of dogs injected with chlormerodrin, $HgCl_2$ or *p*-chloromercuribenzoate. It was found only in cytoplasm and membranes of proximal tubule cells. Distal tubules and medullary tissue did not appear to contain any mercury.

Renal concentration of mercury and diuresis are enhanced in acidotic animals injected with chlormerodrin or $HgCl_2$ but diuresis does not occur and renal mercury levels are not increased in acidotic dogs treated with *p*-chloromercuribenzoate.

The data warrant the conclusion that the proximal tubule is the major site of action of mercurials and indicate that acidosis either increases available receptors for mercuric ion or alters affinity of mercuric ion for its renal receptor.

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