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THE MECHANISM OF ACTION OF MERCURIAL DIURETICS IN RATS; THE RENAL METABOLISM OF *p*-CHLOROMERCURIBENZOATE AND ITS EFFECTS ON URINARY EXCRETION

BY

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Weiner, Levy & Mudge (1962) observed that organic mercurials which produce diuresis in dogs released mercuric ions *in vitro*. This observation supports the old belief that mercurial diuretics act through release of mercury. Recently Clarkson, Rothstein & Sutherland (1965) detected mercuric ion in rat tissues after injection of an organic mercurial diuretic. The mercuric ion levels in kidney and their persistence were related to the duration of diuresis.

The work to be reported here was undertaken to see if *p*-chloromercuribenzoate released mercuric ion in rats. *p*-Chloromercuribenzoate does not release mercuric ion *in vitro* (Weiner *et al.*, 1962) and consequently may be stable *in vivo*. Since this compound produces diuresis in rats (Mussini, 1958), it may furnish the first example of a mercurial acting as the intact molecule.

METHODS

Experimental animals. These were male white rats of the Porton strain (180 to 220 g) and were housed, in pairs, in metabolism cages (Gage, 1961). Food and water were *ad libitum* in all experiments. The room temperature was in the range 18 to 20° C.

Measurement of the metabolism of p-chloromercuribenzoate. Rats were injected intramuscularly with *p*-chloromercuribenzoate (3.7 mg of mercury per kg body weight) or with mercuric ion complexed with cysteine (1.25 mg of mercury per kg). The volume injected was always 0.1 ml. The mercury was labelled with the ^{203}Hg isotope to give a radioactivity dose between 1 and 10 μc per rat. The mercuric-cysteine was prepared as described by Clarkson *et al.* (1965). *p*-Chloromercuribenzoate was dissolved in 0.1 N-sodium hydroxide and the *p*H was adjusted to approximately 9.0 with 0.1 N-nitric acid. Labelled *p*-chloromercuribenzoate was supplied as a solid by the Radiochemical Centre (Amersham) at a specific activity of approximately 1 mc per 100 mg of *p*-chloromercuribenzoate.

The nonradioactive *p*-chloromercuribenzoate was checked for contamination with mercuric ion by iodometric titration (Boyer, 1954) and by amperometric titration against glutathione (Stricks & Kolthoff, 1953). Within the limits of error of each technique (1%), no free mercuric ion could be detected. The radiochemical purity of the labelled *p*-chloromercuribenzoate was checked by the exchange technique described below (Table 1).

After killing the rats by intraperitoneal injection of pentobarbitone sodium at various times after injection of mercury, their kidneys were removed, chopped, placed in ice-cold phosphate buffer, *p*H 7.4, and homogenized as described by Clarkson *et al.* (1965). The homogenate was diluted with buffer so that 20 ml. of

the suspension contained 1 g wet weight of the original kidney tissue. Kidneys from untreated animals were homogenized in buffer containing *p*-chloromercuribenzoate or mercuric-cysteine added in amounts equivalent to the mercury found in the kidneys of injected animals. All homogenates were stored at −10 to −20° C before analysis on the following day.

The homogenates were analysed for mercuric ion or intact molecules of *p*-chloromercuribenzoate as follows: 1 ml. of homogenate was added to 1 ml. of 0.3 M-cysteine-hydrochloride followed by 1 ml. of 1.0 N-trichloroacetic acid. The mixture was centrifuged to remove the precipitate and 1 ml. of the supernatant solution was added to a Conway microdiffusion unit. 99% of the *p*-chloromercuribenzoate and 90% of the mercuric ion were extractable from the homogenate (see column headed " TCA recovery " in Table 1). The Conway microdiffusion unit, containing metallic mercury in the central well, was used to determine the exchangeability of the ^{203}Hg in the trichloroacetic acid extract as described by Clarkson *et al.* (1965).

Urinary excretion. This was measured in four groups of six rats. The first 24-hr urine samples were rejected since these samples had a larger volume than samples collected on subsequent days. After collection of the second 24-hr sample, three groups of rats were injected with *p*-chloromercuribenzoate (1.9, 3.7 or 7.5 mg/kg of mercury) and the fourth group (controls) received a solution of identical volume (0.1 ml.) and composition to that in which the *p*-chloromercuribenzoate was dissolved. A 24-hr urine sample was collected on the day after injection.

The recoveries of urinary volume and solutes from the metabolism chambers were measured as follows. Known volumes (from 10 to 30 ml.) of urine samples, previously analysed for sodium, potassium, chloride and total solutes, were poured into the animal compartment of the chambers in small successive volumes over a period of 24 hr. The chambers had each contained two rats for the previous 24 hr and were not cleaned when the rats were removed. The average amounts recovered (from six experiments) in the urine collectors were: volume 80%, sodium 89%, chloride 90%, potassium 95% and total solutes 93%. The standard deviations did not exceed 5%. The low volume recovery was due to evaporation.

Urinary excretion of any substance is expressed as the " excretion ratio " which is the amount excreted 24 hr after injection divided by the amount excreted during the 24 hr just before the injection. The use of excretion ratios has two advantages: it is economical since excretion of different substances may be expressed in the same units and thus presented in the same figure (Fig. 2), and it reduces the variability of the results since the urinary excretion from any given cage if high at the start tends to remain high throughout the experiment.

To allow conversion of the ratios to absolute values, the excretion rates per 24 hr before injection are given in the legend of Fig. 2. The sodium and chloride excretion rates of 2.5 and 4.1 mequiv per 200 g rat per day are higher than values calculated from Brunner (1959) of 1.37 and 1.54 mequiv/200 g/day respectively. The higher values in our experiments probably resulted from the presence of 1% sodium chloride in the animal feed (Bruce & Parks, 1949). Salt excretion is greatly influenced by diet. Rats starved for 20 hr before the measurement period had excretion rates of sodium chloride five times lower than those quoted above (Light, 1959). The volume and potassium excretion of 9.0 ml. and 1.5 mequiv/200 g/day are close to those calculated from Brunner's data of 9.1 ml. and 1.3 mequiv/200 g/day respectively. Brunner's measurements were over 16 hr with food and water *ad libitum.*

Analysis of urinary content of sodium and potassium. This was made by flame photometry (SP 900 flame photometer, Unicam Instruments, Cambridge) and urinary chloride by electrometric titration (Cotlove, Trantham & Bowman, 1958) using an EEL Chloride Meter (Evan Electroselenium Ltd. Halstead, Essex). The specificity of the chloride meter for urinary chloride was checked by comparing the results in six urine samples with those obtained by the method of Schales & Schales (1941). Both sets of analyses agreed within 5%. The total urinary solutes were calculated from the freezing point depression as measured by a Fiske Osometer (Fiske Associated, Bethel, Connecticut, U.S.A.). The instrument was standardized with known sodium chloride solutions so that the freezing point depression observed on any urine sample could be expressed as the equivalent in mosmoles of sodium chloride.

RESULTS

The amount of mercury (total ^{203}Hg activity) in the kidney rose rapidly during the first hour after injection of *p*-chloromercuribenzoate (Fig. 1). During the following 24 hr, the

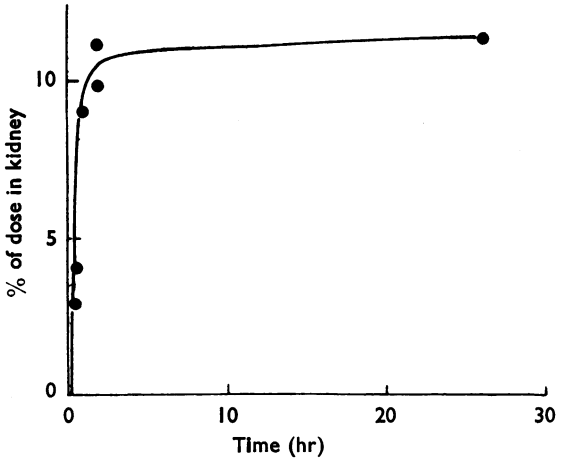

Fig. 1. The total mercury in kidneys of rats at various times after injection of 3.7 mg of mercury per kg body weight as *p*-chloromercuribenzoate labelled with ^{203}Hg.

mercury level remained steady at approximately 10% of the injected dose. Kessler, Lozano & Pitts (1957) observed a similar curve in dogs.

The ^{203}Hg label of *p*-chloromercuribenzoate in the injection solution and of *p*-chloromercuribenzoate added *in vitro* to the homogenate exchanged with a half time of 3,000 min or more (Table 1). In contrast the ^{203}Hg exchanged with half times of 220 min or less from

TABLE 1

THE METABOLISM OF *p*-CHLOROMERCURIBENZOATE

Rats were injected with *p*-chloromercuribenzoate (3.7 mg of mercury per kg body weight) or mercuric cysteine (1.25 mg/kg mercury), and killed at times indicated in column 2. Both mercury compounds were labelled with ^{203}Hg. The values at zero time are from measurements either on the injection solution (inj. sol.) or on the homogenates containing added mercury (*in vitro*). "TCA recovery," column 3, is the total ^{203}Hg in the trichloroacetic acid (TCA) extract expressed as the percentage of ^{203}Hg in the homogenate. The last two columns are from homogenates suspended in 0.1 N-cysteine-sodium hydroxide as described by Clarkson *et al.* (1965). All figures are single observations except the *in vitro* results which are means of seven experiments

			Exchangeable ^{203}Hg			
			TCA extract		0.1 M-Cysteine-NaOH	
Compound	Time in rat	TCA recovery (%)	Half-time (min)	Amount (%)	Half-time (min)	Amount (%)
p-Chloromer-	0 (Inj. sol.)	100	3,750	100		
curibenzoate	0 (Inj. sol.)	98	6,500	100	150	100
	0 (*In vitro*)	99	3,000	100		
	15 min	83	140	94		
	30 min	88	160	96	58	85
	60 min	87	220	85		
	120 min	83	175	100	34	95
	120 min	100	150	90		
	26 hr	89	155	100		
Mercuric-	0 (Inj. sol.)	95	220	100	40	93
cysteine	0 (Inj. sol.)	100	157	95		
	0 (*In vitro*)	90	216	90		
	15 min	91	140	99	40	95
	120 min	90	180	95	36	95
	120 min	97	140	89		
	26 hr	85	160	94		

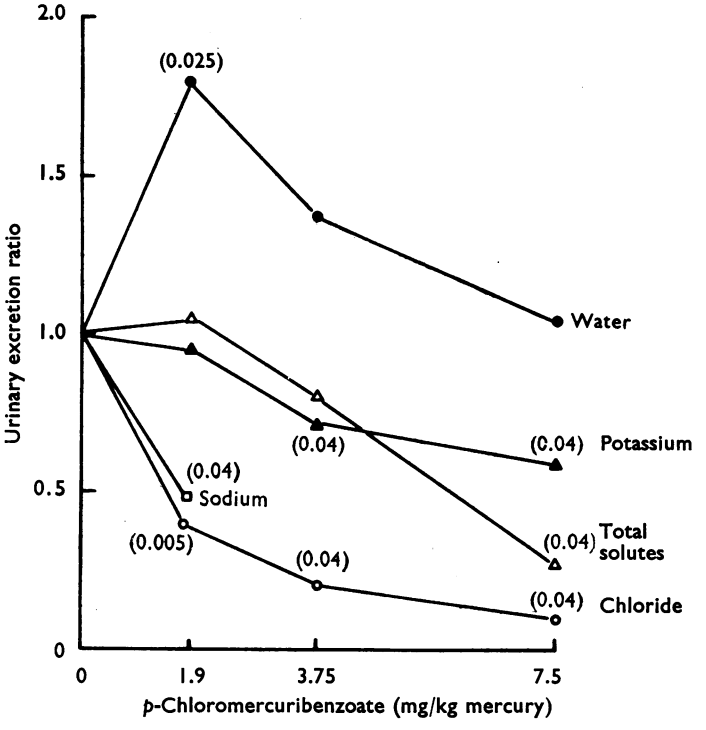

Fig. 2. The effect of *p*-chloromercuribenzoate on urinary excretion. The urinary excretion ratio is plotted on the ordinate as the ratio of the 24-hr excretion after injection to the 24-hr excretion just before injection. Control urines (points corresponding to zero on the abscissa) were from animals injected with a solution of identical composition to the solution containing *p*-chloromercuribenzoate. *P* values are given in parentheses where they are less than 0.1 and give the degree of significant difference between experimental and control values. They were calculated by the nonparametric method of Mann-Whitney (Auble, 1953). The mean absolute excretion rates per 200-g rat for the 24 hr before injection calculated from both control and experimental observations were as follows: water, 9 ml.; chloride, 4.1 mequiv; sodium, 2.5 mequiv; potassium, 2.5 mequiv; total solutes, 16 mosmoles.

kidneys of rats injected with *p*-chloromercuribenzoate. The latter half times were identical with those observed from kidney homogenates of rats injected with mercuric-cysteine. Whether rats were given *p*-chloromercuribenzoate or mercuric-cysteine, the exchange half times from homogenates suspended in 0.1 M-cysteine-sodium hydroxide (last two columns, Table 1) were similar to those reported by Clarkson *et al.* (1965) for mercuric ion in kidney tissue. Thus the evidence indicates that *p*-chloromercuribenzoate breaks down rapidly in rats so that within 15 min of injection all the mercury in kidney tissue is in the form of mercuric ion.

p-Chloromercuribenzoate increased urine flow by a factor of 1.8 at a dose of 1.9 mg/kg of mercury (Fig. 2). This diuresis was significant ($P \simeq 0.025$) according to the nonparametric statistic of Mann-Whitney (Auble, 1953). Higher doses of *p*-chloromercuribenzoate were less effective. Chloride excretion was depressed by more than 50% of control values at all doses. Sodium excretion, measured only at the lowest dose, was also depressed. The excretions of potassium and total solutes were depressed only at the two higher doses.

DISCUSSION

The results indicate that *p*-chloromercuribenzoate produces diuresis through release of mercuric ion. First all the mercury in the kidney was in the form of mercuric ion during the period over which diuresis was observed (first 24 hr after injection). The observations of Farah & Kruse (1960) suggest that the mercuric ion is bound to tissue sulphydryl groups. Secondly the mercuric ion levels which are associated with diuresis following injection of *p*-chloromercuribenzoate are very close to those eliciting diuresis after a dose of chlormerodrin. Diuresis was initiated when the mercuric ion in kidney attained a threshold level of approximately 7% of the 2.5 mg/kg mercury injected dose of chlormerodrin (Clarkson *et al.*, 1965). This corresponds to approximately 35 μg of mercuric ion in the kidneys of 200 g rats. In comparison the diuretic dose of *p*-chloromercuribenzoate (1.9 mg/kg mercury) corresponds to 38 μg in the kidney as calculated from Fig. 2.

The fact that doses of *p*-chloromercuribenzoate higher than 1.9 mg/kg mercury become increasingly less effective until at 7.5 mg/kg mercury no diuresis was observed (Fig. 2) may be best explained in terms of mercuric ion levels. Thus Kessler *et al.*, (1957) have observed in dogs that, whereas low doses of mercuric chloride (1 and 2 mg/kg mercury) produces diuresis, a higher dose (4 mg/kg mercury) actually inhibits urine flow. The inhibition of urine flow in dogs was associated with a collapse in the glomerular filtration rate.

Our finding that *p*-chloromercuribenzoate caused an increase in urine flow confirms the original observation of Mussini (1958). Unfortunately there is no published information of the effect of *p*-chloromercuribenzoate on electrolyte excretion. In general *p*-chloromercuribenzoate has similar effects to those elicited by well-known mercurial diuretics: first, the limited dose range over which *p*-chloromercuribenzoate is diuretic has also been observed with chlormerodrin (Light, 1959) and mersalyl (McColl, Parker & Ferguson, 1956); secondly, the maximum increase in urine flow (approximately 1.8 times the control value in Fig. 2) is similar in magnitude to that observed in rats injected with chlormerodrin (Brunner, 1959; Cummings, Haynes, Lipchuck & Ronsberg, 1960); and thirdly, the depression in chloride excretion has also been observed in rats injected with chlormerodrin (Light, 1959) and mersalyl (Dicker, 1948).

p-Chloromercuribenzoate apparently differs from chlormerodrin in that it depresses sodium excretion in the first 24 hr after injection. However, this effect of chlormerodrin is time-dependent, sodium excretion being high in the first 4 to 8 hr after injection but falling to control values or less in the next 16 hr. A careful study of the effect of *p*-chloromercuribenzoate on sodium excretion over short time intervals is required before concluding that our results differ significantly from those reported for chlormerodrin.

In comparison of the effects of *p*-chloromercuribenzoate in other species with the results obtained from rats, the most significant point is that this mercurial is nondiuretic in dogs in all doses studied (1 to 4 mg/kg mercury, Kessler *et al.*, 1957; Weiner *et al.*, 1962; Miller & Farah, 1962). The studies of Weiner *et al.* (1962) strongly suggest that *p*-chloromercuribenzoate is nondiuretic because it does not release mercuric ion in the dogs.

SUMMARY

1. This work is a further application of a new technique which distinguishes between different chemical forms of mercury in animal tissues. Previous results indicated that the

organic mercurial diuretic, chlormerodrin, produced diuresis in rats by release of mercuric ion.

2. The technique was modified to distinguish between *p*-chloromercuribenzoate and mercuric ion in kidney homogenates.

3. The results indicated that *p*-chloromercuribenzoate rapidly released mercuric ion in rat tissue.

4. Approximately the same kidney levels of mercuric ion (35 μg of mercury per g wet weight) produced diuresis in rats whether released from *p*-chloromercuribenzoate or chlormerodrin.

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