Studies on the Binding of Mercury in Tissue Homogenates

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1. This paper describes an attempt to learn more about the binding of Hg^{2+} to tissues at pharmacological concentrations of this metal. Other methods were not applicable to such low concentrations of mercury. 2. The method involved equilibrium dialysis of Hg^{2+} against 1% homogenates of rat kidney or liver in the presence of penicillamine. Two classes of mercury-binding sites were observed, one class having a chemical affinity for mercury 100-fold greater than the other class. The binding capacities of the class of higher and lower affinity were respectively 1.0×10^{-7} and 30×10^{-7} mole of mercury/g. wet wt. of tissue. The same classes of binding sites were found in both liver and kidney homogenates. 3. The binding sites of both classes reacted with only one valency of Hg^{2+} , the other valency forming a bond with penicillamine. Thus the total binding capacities of both classes are equivalent to 50% of the total reactive protein-bound thiol groups in the homogenate. 4. The results eliminate three possible mechanisms for the preferential accumulation of mercury by kidney. They support the idea that the permeability changes in kidney cells resulting in diuresis are similar to the permeability changes produced on the membranes of other mammalian cell species by mercury.

As part of a study of the behaviour of mercury as a diuretic and nephrotoxic agent, an attempt has been made to learn more about the sites at which mercury is bound. This was regarded as a first step in understanding the biochemical mechanisms underlying the preferential accumulation of this metal by kidney (Rothstein & Hayes, 1960) and its effects on urine flow. The latter depend on the concentration of mercury in the kidney, low concentrations being associated with diuresis (Clarkson, Rothstein & Sutherland, 1965) and higher concentrations with a profound depression in glomerular filtration rate (Flanigan & Oken, 1965).

When the binding of Hg^{2+} by tissue homogenates was studied by conventional techniques (in this case equilibrium dialysis), the results were obtained as given in Fig. 1, plotted according to Scatchard (1949). These results indicate two major difficulties with the method. First, at the low concentrations of bound mercury which just produce diuresis, approx. 2×10^{-7} mole of mercury/g. wet wt. of kidney, the data of Fig. 1 give no reliable information on the binding sites: the Scatchard curves vary from one homogenate preparation to another and

the slopes of the curves are anomalous. Scatchard curves should always have a negative gradient whereas their initial slopes in Fig. 1 are positive. The variability of the data and the anomalous slopes are due to the low concentrations of free (diffusible) mercury. Such concentrations are greatly affected by traces of diffusible mercury-binding substances released from the homogenate so that Hg^{2+} may be present in a variety of chemical states of combination. Weed, Eber & Rothstein (1962) have observed similar anomalous Scatchard curves in studies of the binding of Hg^{2+} to dilute suspensions of erythrocytes and suggested the release of mercury-binding materials as the cause. Secondly, the data in Fig. 1 give no information on the stoichiometry of the reaction between Hg^{2+} and the tissue. For example, at the higher concentrations of bound mercury, where the difficulties mentioned above are not so important, the Scatchard curves indicate an intercept on the abscissa at approx. 3×10^{-6} mole of bound mercury/g. wet wt. Assuming that each Hg^{2+} ion reacts with two thiol groups, the intercept corresponds to the total number of protein-bound thiol groups in the dialysed homogenate, 6×10^{-6} mole/g. wet wt. However, Hughes (1949) has shown that, in the presence of excess of chloride, as obtained in the present experiments, one Hg^{2+} ion reacts with only

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Fig. 1. Binding of mercury to 1% homogenate plotted according to the Scatchard equation. The abscissa is the bound mercury in $\mu\text{moles/l.}$ of homogenate, and the ordinate is the ratio of the bound mercury to the unbound metal. The four curves represent four different experiments.

one thiol group of serum albumin, the other valency of the Hg^{2+} ion forming a bond with chloride. Thus the intercept of Fig. 1 may correspond to only 50% of the total protein-bound thiol groups.

The method described below is a modification of the equilibrium-dialysis technique which ensures that all the diffusible mercury is present as one chemical species and which gives information on the stoichiometry of the reaction.

METHODS

Outline of method

The difficulties discussed above are avoided by carrying out the dialysis in the presence of penicillamine, which forms a diffusible highly undissociated complex with mercury. By choosing an appropriate concentration of penicillamine, it may be arranged that all the diffusible mercury is present as a single chemical species, namely $\text{Hg}(\text{Pen})_2$, and that sufficient mercury is in the diffusible form to allow accurate measurement. Since the data are obtained at equilibrium, equations based on the Law of Mass Action may be applied. A modified form of the Scatchard equation (see the Appendix) allows calculation of the different classes of binding sites distinguishable by their chemical affinity for mercury. Moreover, both this equation and that described by Schubert, Russell & Myers (1950) indicate whether one or two penicillamine molecules are displaced from the complex $\text{Hg}(\text{Pen})_2$ when mercury is attached to the tissue. In this way the stoichiometry of the reaction becomes known.

Solution and reagents

5,5'-Dithiobis-(2-nitrobenzoic acid) reagent. This was prepared by dissolving 40 mg. of 5,5'-dithiobis-(2-nitrobenzoic acid) (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) in 100 ml. of tris buffer.

Mercury solutions. The ^{203}Hg (The Radiochemical Centre, Amersham, Bucks.) was supplied as a mercuric acetate solution, pH 2.8, specific activity 1 mc/mg. of Hg in 0.5 ml. of solution. Suitable dilutions of the isotope were made into stock solutions of HgCl_2 dissolved in 0.1 N- HNO_3 ; from these further dilutions were made in sucrose-EDTA solution and the pH was adjusted to 7.4 with 0.1 N- NaOH .

Metaphosphoric acid solution. This was prepared by dissolving 1.67 g. of metaphosphoric acid, 0.2 g. of EDTA (disodium salt) and 30 g. of NaCl in 100 ml. of water.

p-Chloromercuribenzoic acid solution. This was prepared by dissolving 50 μmoles of p-chloromercuribenzoic acid (reagent grade; British Drug Houses Ltd., Poole, Dorset) in 50 ml. of tris buffer.

Penicillamine solution. This was prepared by dissolving 0.1 m-mole of DL-penicillamine (British Drug Houses Ltd.) in 80 ml. of tris buffer, adjusting the pH to 7.4 with 0.1 N- NaOH and making up the volume to 100 ml. with tris buffer. Further dilutions were also made with tris buffer. The penicillamine solutions were prepared immediately before use.

Sucrose-EDTA solution. This contained sucrose (A.R. grade) (0.3 M) and EDTA (A.R. grade) (1.0 mM).

Tris buffer. This contained tris (L. Light and Co. Ltd., Colnbrook, Bucks.) (15 mM), KCl (A.R. grade) (125 mM) and EDTA (1 mM). The pH was adjusted to 7.4 with N-HCl, which was prepared from conc. HCl (A.R. grade). Commercially available standard solutions of HCl could not be used because of their high mercury content.

Tissue preparation and dialysis

Homogenization. Kidneys or livers were taken from decapitated 300–350 g. white male rats of the Porton strain. The chopped tissue was added to sucrose-EDTA solution at approx. 4° in the proportion 1 g. of tissue to 8 ml. of solution. Homogenization was carried out with the apparatus described by Aldridge, Emery & Street (1960), with 0.02 in. clearance between the mortar and pestle. The homogenate was centrifuged for 10 min. at 800g to remove debris and diluted with sucrose-EDTA solution to give a final homogenate concentration of 10% (wet wt. of tissue to final volume).

Dialysis. The 10% homogenate was mixed with an equal volume of sucrose-EDTA solution containing $^{203}\text{HgCl}_2$. After 30 min. at 4° , 2 ml. of the suspension was added to 8 ml. of tris buffer containing penicillamine, giving a final homogenate suspension of 10 mg. wet wt. of tissue/ml. After 30 min., 10 ml. samples of the suspension were bubbled for 1 min. with N_2 (O_2 -free) and placed in cellophane sacs (Visking tubing, inflated diam. 20/32 in.), the free space being filled with N_2 before closing the sac. The sac was placed in a 50 ml. Pyrex test tube coated with silicone (Repelcote; Hopkin and Williams Ltd., Chadwell Heath, Essex) and containing 10 ml. of the mercury-free penicillamine solution of composition identical with that of the solution in which the homogenate was suspended. The test tube was filled with N_2 , sealed and shaken for the desired period, usually 4 hr. All the above operations were carried out at 4° .

After dialysis the concentration of ^{203}Hg was measured in the solutions inside and outside the sac. Since the volumes of the two solutions were the same the quantities of diffusible mercury at equilibrium were equal inside and

outside. The diffusible mercury remaining inside the sac at equilibrium (P_D) expressed as a percentage of the total mercury inside the sac is given by the relation:

$$P_D = 100C_o/C_i$$

where C_o and C_i are the final counts in the outside and inside solutions respectively. In most experiments at least one control was also run which was identical in every respect with the experimental except that homogenate was absent. In all control experiments 95–100% of the mercury was diffusible (see below). The percentage diffusible in the homogenate was corrected for the control.

Validation of equilibrium distribution. Fig. 2 illustrates three important aspects of the dialysis technique. First, the mercury–penicillamine complex diffused rapidly and closely approached its equilibrium distribution within 4 hr.,

Fig. 2. Rate of diffusion of mercury from tris buffer (outside the sac) to homogenate (inside the sac) (▲) and from homogenate (inside the sac) to tris buffer (outside the sac) (●). The concentration of penicillamine was 10 mM both inside and outside and the initial HgCl_2 concentration was $1 \mu\text{M}$. When mercury was added to the tris buffer the mercury remaining outside (▲) approached the asymptote (----) of 41%. The outside mercury minus the asymptote (Δ) gave a linear semi-logarithmic plot against time with a half-time of 39 min. Thus at the end of 4 hr. dialysis the mercury–penicillamine complex had diffused to within 2% of the equilibrium distribution. When mercury was initially present in the homogenate, the mercury remaining inside (●) approached the asymptote (----) of 58%. The inside mercury minus the asymptote gave a linear semi-logarithmic plot (○) against time with a half-time of 39 min.

whether initially present outside or inside the sac. When initially present outside, the mercury remaining outside had fallen to 42% of its initial value in 4 hr. as compared with the equilibrium value (asymptote) or 41%, i.e. $C_o = 41$, $C_i = 59$ and $P_D = 70\%$. When mercury was initially present in the homogenate the percentage mercury remaining inside was 59% and the asymptote 58%, i.e. $C_o = 42$, $C_i = 58$ and $P_D = 72\%$. Thus the equilibrium point was the same whether the mercury was initially wholly inside or outside the sac (i.e. in the homogenate or out of the homogenate). Secondly, the semi-logarithmic plot of the remaining mercury minus the asymptote gave a straight line, indicating that mercury diffused as a single component with a half-time of approx. 40 min. Similar half-times were observed at other concentrations of penicillamine and in the absence of homogenate.

Choice of complex-forming agent

Fig. 3 is typical of data obtained after 4 hr. dialysis and illustrates two advantages of using penicillamine as a complex-forming agent. The mercury–penicillamine complex is completely diffusible (95–100%) and the affinity of penicillamine for Hg^{2+} is such that altering the concentration of the complex-forming agent produces large changes in the amounts of mercury bound to the homogenate, i.e. penicillamine can compete with the complex-forming group in the homogenate for the mercury. For example, nearly all the mercury is bound to homogenate at 0.5 mM-penicillamine, whereas less than 30% is bound at 10 mM-penicillamine.

Very little penicillamine was destroyed during dialysis.

Fig. 3. Equilibrium dialysis of mercury from homogenates containing HgCl_2 ($1 \mu\text{M}$) and different concentrations of penicillamine. ○, Control curve obtained with no homogenate; ●, experimental curves with 1% kidney homogenate (—) and with 1% liver homogenate (---); Δ, sac binding expressed as a percentage of the total mercury initially present in homogenate.

Table 1. *Stability of penicillamine during equilibrium dialysis for 4 hr. at 4°*

The final concentrations of penicillamine given are the results of single observations. The values in parentheses after the estimated losses represent the experimental error and were calculated as follows: The standard error of the technique was calculated for eight analyses of standard penicillamine solutions over the range 0.5–10 mM. The initial (added) penicillamine was assumed to be the correct concentration. The error of the final concentration was taken as twice the standard deviation.

Initial concn. of penicill- amine (mM)	Concn. of penicillamine in diffusate (mM)		Concn. of penicillamine in homogenate (mM)	
	Final	Loss	Final	Loss
10	9.8	0.2 (0.3)	9.3	0.7 (0.6)
5	4.6	0.4 (0.2)	4.4	0.6 (0.25)
1	0.9	0.05 (0.02)	0.77	0.2 (0.05)
0.5	0.4	0.11 (0.01)	0.34	0.16 (0.02)

Table 2. *Protein and reactive thiol groups in kidney homogenate*

Centrifugation was at 800g for 10 min. at 4°. All values are means of three observations and refer to concentrations in 1% homogenates. The values in parentheses were calculated from the data of Surtain & Yagi (1958).

	Concn. of protein (g./l.)	Concn. of reactive thiol groups (μM)	
		Total	Free
Before centrifugation	1.65 (1.52)	150 (169)	48.3
After centrifugation	1.11 (1.20)	127 (138)	40.7 (37.4)

The destruction of penicillamine, measured as change in non-protein bound thiol groups, corresponds to an absolute loss of approx. 0.2 mM or less during the 4 hr. dialysis (Table 1). Thus the change in the penicillamine concentration at 10 mM and 5 mM inside the dialysis sac (homogenate) or outside the sac (diffusate) was close to the analytical error (Table 1). However, the destruction of thiols at 0.5 mM-penicillamine corresponds to a 20–30% change in the penicillamine concentration. Other evidence is presented in the Results section confirming that a small amount of penicillamine was destroyed.

The presence of penicillamine does not affect the concentration of protein-bound thiol groups in 1% homogenate. After 4 hr. dialysis in the presence of 10 mM-penicillamine the concentration of protein-bound thiol groups was 66.4 μM and in the absence of penicillamine it was 58.7 μM. Each value is a mean of three determinations.

Other complex-forming agents were tried and found to be unsuitable because: (a) of inability to remove mercury from homogenate, e.g. acetonecyanhydrin and EDTA; (b) less than 100% of the mercury complex was diffusible, e.g. cysteine; (c) of chemical instability, e.g. 2,3-dimercapto-propanol; (d) of large changes in pH, e.g. KCN.

Comparison with other homogenate preparations

Protein and thiol concentrations in the 1% homogenates prepared as above but not containing HgCl₂ or penicillamine are given in Table 2. The values indicate that

protein and thiol concentrations before and after centrifugation agree with those reported in the literature for similar homogenates (Surtain & Yagi, 1958).

The present results were obtained from homogenates after centrifugation at 800g (see above), since a more homogeneous suspension was obtained free from incompletely homogenized tissue, and, as Table 2 indicates, the concentrations of the total and the protein-bound thiol groups (unmasked) were also decreased. However, the mercury-binding sites were unaffected, first because the decrease in thiol was not very great (10–20%) and secondly because the concentrations of mercury used were very much less than the total thiol concentration (see the Results section.)

Radioisotopic and chemical analysis

²⁰³Hg activity. This was measured by an automatic scintillation spectrometer, model 450A (Packard Instrument Co., La Grange, Ill., U.S.A.); the counting efficiency was 40%.

Chemical concentration of mercury. Mercury in the stock solutions and the mercury contamination of the diluents were determined by the method of Clarkson, Gatzky & Dalton (1961).

Protein concentrations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Total reactive thiol groups. A 0.2 or 0.4 ml. portion of 10% homogenate was diluted with water to 1.0 ml. The homogenate was treated with *p*-chloromercuribenzoic acid

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(1 mm) and metaphosphoric acid according to the method of Isles & Jocelyn (1963) for blood proteins. After precipitation the unbound *p*-chloromercuribenzoic acid was treated with penicillamine and the excess of penicillamine was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) reagent as in the determination of non-protein thiol groups. The concentration of total reactive thiol groups was calculated from the difference between the moles of *p*-chloromercuribenzoic acid added plus the moles of penicillamine that had reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) minus the moles of penicillamine added.

Non-protein thiol groups. These were determined by the method of Beutler, Duran & Kelly (1963), with 0.4 or 0.6 ml. of 10% homogenate instead of 0.2 ml. of blood. The same colour reaction was also used to check the concentration of thiol compounds used as complex-forming agents.

Administration of mercury in vivo

Mercuric acetate (0.13 g.) and cysteine hydrochloride (0.240 g.) were dissolved in 50 ml. of 0.1 M-carbonate buffer, pH 9.0. The mercury was labelled with $^{203}\text{Hg}^{2+}$, giving a final specific activity of $3 \mu\text{C}/\text{mg}$. The ^{203}Hg -cysteine complex was administered intramuscularly to three 300–350 g. male rats at a dose of 0.8 mg. of mercury/kg. body wt. At 17 hr. after the injection the animals were decapitated and their organs were homogenized. Six 260 g. male rats were given intramuscularly ^{203}Hg -penicillamine complex (prepared as the Hg-cysteine complex) at a dose of 1.25 mg. of mercury/kg. body wt. At intervals the animals were killed for ^{203}Hg determinations in their kidneys and livers.

RESULTS

Since the observed distribution of mercury between the homogenate and penicillamine was very close to the equilibrium distribution, equations based on the Mass Law Principle could be applied.

In general, experiments were carried out in which the penicillamine concentration was varied and the Hg^{2+} concentration kept constant or in which the Hg^{2+} concentration was varied and the

penicillamine concentration was unchanged. From the results it can be discovered whether there is only one binding site or more, and how many penicillamine moieties are lost from a mercury atom when it is bound to a site. As the penicillamine-mercury complex contains two molecules of penicillamine (Table 3), *a priori* either one or two might be lost:

or

At low mercury concentrations, when only a small fraction of the sites is affected, the Schubert equation (Schubert *et al.* 1950) can be applied. If *y* is the number of molecules of penicillamine displaced, the equation is:

$$1/K_d = [\text{P}]^y/K_d + 1/K_d^0 \quad (1)$$

where $[\text{P}]$ is the concentration of the penicillamine,

$$K_d = \frac{\% \text{ of Hg bound to homogenate}}{\% \text{ of Hg bound to penicillamine}} \quad (2)$$

and $K_d^0 = K_d$ when $[\text{P}] = 0$. A graph of $1/K_d$ against $[\text{P}]$ was a straight line (Fig. 4). Thus $y = 1$, i.e. one penicillamine molecule only is displaced.

Fig. 4. Equilibration of mercury between homogenate and various concentrations of penicillamine plotted, according to Schubert *et al.* (1950); $1 \mu\text{M-HgCl}_2$ was present in the homogenate before dialysis. ● (with vertical lines), Means ± 2 s.e.m. of ten experiments, when mercury was added to the homogenate; these points give the straight line. ○, Means of three experiments, when mercury was deposited in the kidney *in vivo*. The mercury concentration in the homogenate after deposition *in vivo* was equivalent to approx. $1 \mu\text{M-HgCl}_2$.

Table 3. Combination of Hg^{2+} with penicillamine

The experiments were carried out as follows: HgCl_2 was added to 3.0 ml. of 0.1 M-penicillamine; the volume was made up to 10 ml. with tris buffer and the penicillamine not combined with mercury was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) reagent. The results are the means of two analyses.

HgCl ₂ added (μmole)	Penicillamine remaining (μmole)	Penicillamine combining with Hg (moles/mole of Hg)
0.025	0.248	2.1
0.050	0.190	2.2
0.075	0.154	2.0
0.100	0.101	2.0
0.125	0.056	2.0

This intercept gives $1/K_d^0$ negative, which is absurd. But direct measurement of K_d^0 showed that it was large (all Hg^{2+} bound to homogenate in absence of penicillamine), so that $1/K_d^0$ should be zero. The discrepancy is not very great, and could be accounted for by the destruction of penicillamine known to occur during dialysis: 0.2 mM was destroyed (see the Methods section). The intercept corresponds to 0.7 mM (S.E.M. ± 0.2 mM).

The symbols $\circ$ in Fig. 4 represent the means of three observations on homogenates where the Hg^{2+} was added *in vivo*. The concentration of mercury in the 1% homogenate was equivalent to 0.8–1.2 μM -mercuric chloride. The results of these experiments *in vivo* did not differ significantly from the results obtained (symbols $\bullet$) when the mercury was added *in vitro*.

To analyse results at higher mercury concentrations the Scatchard (1949) equation was used, modified for the presence of a complex-forming agent (see the Appendix).

For one binding site, the equation is:

$$\bar{v}/[\text{AP}_2] = (n - \bar{v})k'/[\text{P}]^y \quad (3)$$

where $\bar{v}$ is the mercury bound to homogenate, $[\text{AP}_2]$ is the mercury bound to penicillamine, n is the concentration of binding site, k' is the equili-

brum constant for the relevant chemical reaction (A or B), and $[\text{P}]$ and y are as defined above; n and $\bar{v}$ are expressed at m-moles of mercury/l. of 1% homogenate (i.e. 10g. wet wt. of kidney), and $[\text{P}]$ as mM.

In Figs. 5 and 6 the left-hand side of eqn. (3) was plotted against $\bar{v}$ from data obtained by varying the initial concentration of mercuric chloride in the homogenate from 1 μM and 0.1 mM and maintaining the penicillamine concentration constant ($[\text{P}] = 0.5$ mM in Fig. 5 and 10 mM in Fig. 6). If only one class of binding sites were involved, a straight line should have been obtained. In fact, each curve of Figs. 5 and 6 approximates to two straight lines, one at a low and the other at a high concentration of bound mercury.

This suggests that there are two binding sites with different properties, and the results can be analysed by the Scatchard equation for two sites present in concentrations n_1 and n_2 :

$$\frac{\bar{v}}{[\text{AP}_2]} = \frac{n_1 X_1}{1 + [\text{AP}]X_1} + \frac{n_2 X_2}{1 + [\text{AP}_{2-y}]X_2} \quad (4)$$

where $X_1 = k'_1/[\text{P}]^y$, and $X_2 = k'_2/[\text{P}]^y$. In this equation the first term is that for the more powerfully binding site, for which $y=1$. In the second term y is the number of penicillamine molecules displaced when mercury is bound to the site with

Fig. 5. Distribution of mercury between homogenate and 0.5 mM-penicillamine. The points are plotted according to the modified Scatchard equation (Appendix eqn. 6). The curve has been calculated according to eqn. (5) assuming two classes of binding sites, $n_1=2$, and $n_2=23 \mu\text{M}$ in 1% homogenate, and that $k'_1[\text{P}]^y=40$ and $k'_2[\text{P}]^y=8$. $\bullet$, Centrifuged homogenate; $\circ$, uncentrifuged homogenate (see the Methods section). The curve represents the centrifuged homogenate.

Fig. 6. Distribution of mercury between homogenate and 10 mM-penicillamine. The points are plotted according to eqn. (6). The curve has been calculated according to eqn. (5) assuming two classes of binding sites, $n_1=1$ and $n_2=30 \mu\text{M}$ in 1% homogenate, and that $k'_1[\text{P}]^y=0.36$ and $k'_2[\text{P}]^y=0.0047$. $\bullet$, Centrifuged homogenate; $\circ$, uncentrifuged homogenate (see the Methods section). The curve represents the centrifuged homogenate.

less affinity. By using eqn. (4) values of n_1 , n_2 , X_1 and X_2 were chosen to give the best fit (least-square analysis) to the experimental results.

The curves at high penicillamine concentrations gave n_1 and X_1 fairly precisely, but little information on n_2 and X_2 , as very little mercury was bound to the second site under these conditions. Conversely, those at low penicillamine concentration gave n_2 and X_2 , but little information on n_1 and X_1 . For the experiments in Figs. 5 and 6 and for similar experiments at 1mM- and 5mM-penicillamine the values of n_1 lay between 1mM- and 2mM-mercury and those of n_2 between 20mM- and 30mM-mercury. The values of X_1 and X_2 are functions of the penicillamine concentration.

Graphs of $1/X_1$ and $1/X_2$ against $[P]$, i.e. of

Fig. 7. Data on binding of mercury plotted according to Appendix eqn. (8). $[P]^w/k_1''$ (●, left-hand ordinate) and $[P]^w/k_2''$ (○, right-hand ordinate) were calculated as described in the legends of Figs. 5 and 6. The data were obtained from Figs. 5 and 6 and from similar experiments at 1mM- and 10mM-penicillamine.

$[P]^w/k_1''$ and $[P]^w/k_2''$ against $[P]$, gave straight lines (Fig. 7), showing that $y=1$ in both cases. Thus at the second site also one penicillamine molecule only was lost in the process of binding. The gradients of the lines give $1/k_1''$. It is found that $k_1''=100k_2''$, i.e. that equilibrium at the site with more affinity favours binding of mercury by about 100-fold. Again the graphs did not pass through the origin (cf. Fig. 4), probably for the reason given above.

Experiments were also carried out with uncentrifuged homogenates (Figs. 5 and 6) with the same results.

Fig. 3 indicates that kidney and liver homogenates bind mercury with approximately equal affinity. Scatchard analysis of the data for liver homogenate gives values of n_1 and n_2 and k_1'' and k_2'' not significantly different from those calculated for kidney homogenate. These observations may be compared with the binding of the metal *in vivo* (Table 4), where the mercury bound per unit weight of kidney tissue was approx. 50-fold that bound to liver.

DISCUSSION

This method achieves its principal purpose in allowing the study of mercury-binding sites at pharmacological concentrations of the metal. Diuresis in rats is elicited by 2×10^{-7} moles of mercury/g. wet wt. of kidney (Clarkson *et al.* 1965). By comparison, the two classes of binding sites described here have maximal mercury-binding capacities 1×10^{-7} (the high-affinity class) and 30×10^{-7} moles of mercury/g. wet wt., and together constitute approx. 50% of the total reaction protein-bound thiol groups of kidney, 6.0×10^{-6} moles/g. wet wt.

Strictly speaking, any conclusions from our results apply only to homogenates. The extent to which the mercury-binding sites, measured here, relate to the behaviour of mercury in the animal depends first on the assumption that no chemical

Table 4. Deposition of mercury in kidney and liver after intramuscular administration of 1.25 mg. of Hg^{2+} /kg. body wt. as the penicillamine complex

Time after Hg administration (hr.)	Wt. of kidneys (g.)	Deposition of Hg in kidneys		Wt. of liver (g.)	Deposition of Hg in liver	
		(% of the administered dose)	(μ g./g. wet wt.)		(% of the administered dose)	(μ g./g. wet wt.)
1	1.83	53.7	95	11.95	8.0	2.2
2	1.70	54.8	104	9.72	5.0	1.6
3	1.73	73.2	138	9.76	7.7	2.2
4	1.81	60.8	109	10.6	8.6	2.6
5	1.75	51.0	95	8.34	4.2	1.6
6	1.77	49.6	91	9.27	8.4	2.9

changes take place during homogenization, and secondly on the assumption that mercury is bound to the same sites *in vivo* as *in vitro*.

The first assumption is probably correct. First of all, after homogenization carried out under mild conditions (at 4° and at pH 7.4), the mitochondria are known to be preserved in a viable state (Aldridge & Street, 1964). Secondly, the thiol group content of our homogenates is similar to that reported by others (Table 2). As the possibility of chemical change is unlikely, our results are relevant to the problem of preferential accumulation of mercury by kidney (cf. Table 4).

Previous reports eliminate glomerular filtration as an important mechanism in the uptake of mercury by kidney (Passow, Rothstein & Clarkson, 1961; Berlin & Gibson, 1963). Our results constitute evidence against three other possible mechanisms of preferential kidney uptake: (1) That kidney possesses binding sites of uniquely high affinity. Our results indicate that liver and kidney have identical binding sites. (2) That mercury, carried to the tissue as a soluble complex (cf. Weiner, Levy & Mudge, 1962), is precipitated in the tissue. This mechanism would predict that mercury deposited *in vivo* would behave differently on dialysis against penicillamine from mercury added *in vitro*. Our results contradict this (Fig. 5). (3) That kidney binding sites of uniquely high affinity exist *in vivo* but are constantly breaking down and being regenerated (cf. Weiner *et al.* 1962). Such sites may disappear on homogenization at 4°. The mechanism would also predict that mercury deposited *in vivo* would behave differently from that added *in vitro* and the same objection would apply as in (2).

Thus our results and those referred to above eliminate practically all the mechanisms previously considered in the literature. The possibility remains that the preferential uptake by kidney may be coupled to metabolism. Certain organic mercurial compounds are known to be actively secreted by the cells in the distal third of the proximal convoluted tubule (Campbell, 1959). This mechanism could be tested by studying the effects of various metabolic inhibitors on mercury accumulation.

Until more is known about the mechanism of renal accumulation, the assumption that the binding sites observed in homogenates also bind mercury *in vivo* must remain in doubt. Nevertheless, the limited information in the literature supports this assumption. The latter will be valid provided that the mercury reaches equilibrium with all binding sites shortly after deposition in the kidney. Mercury is known to equilibrate with all thiol groups of erythrocytes in less than 5 min. (Weed *et al.* 1962) and to penetrate muscle cells (Demis & Rothstein, 1955) and the epithelial cells of the small intestine (Clarkson & Cross, 1961) in approx. 20 min. By

analogy it seems likely that mercury deposited in kidney 4 hr. after injection (Table 4) is close to equilibrium with cellular ligands.

Thus it seems reasonable to compare the properties of the sites measured by this method *in vitro* with what is already known about the sites *in vivo*. First, the fact that mercury produces the same functional changes in kidney cells as other mammalian cells suggests that the kidney binding sites are not unique. White, Rolf, Bisno, Kasser & Tosteson (1961) demonstrated that mercury produces diuresis by increasing the permeability of the proximal tubular cells to Na⁺. Mercury has a similar effect on the membranes of erythrocytes (Willbrandt, 1941; Joyce, Moore & Weatherall, 1954; Vincent, 1958), of muscle cells (Ohr, 1961) and of intestinal cells (Clarkson *et al.* 1961). Secondly, Mudge & Weiner (1958) have deduced from observations *in vivo* that Hg²⁺ initiates diuresis by combining with a receptor site having two ligands, one a thiol group and the other a group of lower affinity, presumably not a thiol group. One would expect that only the thiol group of this receptor would be able to displace penicillamine from the mercury-penicillamine complex, as was observed in our experiments.

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APPENDIX

A Modified Scatchard Equation

The binding of a ligand A by a macromolecule R may be described by the equation (Edsall & Wyman, 1958):

$$\bar{\nu} = \sum_{i=1}^m \frac{n_i k_i [A]}{1 + k_i [A]} \quad (1)$$

where $\bar{\nu}$ is the average number of moles of A bound by one mole of R, n_i is the number of binding sites of class i , and k_i is the association constant of the i th class of sites as defined by:

$$k_i = [RA_i]/[R][A] \quad (2)$$

where $[RA_i]$ is the moles of A bound to the i th class of sites. For one class of binding sites, Scatchard (1949) has shown that:

$$\bar{\nu}/[A] = k(n - \bar{\nu}) \quad (3)$$

In applying eqn. (1) to the data reported above, the homogenate is regarded as the macromolecule R and Hg^{2+} as the ligand A. AP_x , the mercury-penicillamine complex, is assumed to release A to the homogenate according to the reaction:

Reaction (a) may be formally regarded as the sum of two equilibria:

having equilibrium constants k_b and k_c respectively. Reaction (b) may be regarded as the binding of AP_{x-y} to the i th class of binding sites on the homogenate R. Thus k_b is equivalent to k_i of eqn. (2) and $[AP_{x-y}]$ is equivalent to $[A]$. But the Mass Law equation for reaction (c) gives:

$$[AP_{x-y}] = [AP_x]/k_c[P]^y \quad (4)$$

Substitution for k_i and $[A]$ in eqn. (1) gives:

$$\bar{\nu} = \sum_{i=1}^m \frac{n_i k'_i [AP_x]/[P]^y}{1 + k'_i [AP_x]/[P]^y} \quad (5)$$

where $k'_i = k_b/k_c$.

In practical terms $[P]$ is equal to the concentration of penicillamine initially present since this compound is present in high excess over Hg^{2+} ; $[AP_x]$ is equal to the diffusible mercury and $\bar{\nu}$ is equated to the amount of non-diffusible mercury since the concentration of the homogenate (1%, w/v) is constant in these experiments.

The 'Scatchard equation' derived from eqn. (5) for one class of binding sites is:

$$\bar{\nu}/[AP_x] = (n - \bar{\nu}) k'/[P]^y \quad (6)$$

At a constant concentration of penicillamine eqn. (6) will give a straight line of intercept n (abscissa) and gradient $k'/[P]^y$. By making a series of graphs each at a constant concentration of penicillamine, it follows from eqn. (6) that:

$$\text{observed gradient} = X = k'/[P]^y \quad (7)$$

or:

$$1/X = [P]^y/k' \quad (8)$$

By plotting $1/X$ against $[P]$, it should be possible to determine the value of y .

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