Biochemical Pharmacology, Vol. 22, pp. 373–381. Pergamon Press, 1973. Printed in Great Britain.

INHIBITION OF HETEROLYSOSOME FORMATION AND FUNCTION IN MOUSE KIDNEYS BY INJECTION OF MERCURIC CHLORIDE

JOHN L. MEGO and JUDITH BARNES
Department of Biology, University of Alabama, University, Ala. 35486, U.S.A.

(*Received* 4 *April* 1972; *accepted* 25 *August* 1972)

Abstract—Intravenous or intraperitoneal injections of mercuric chloride (5 mg Hg/kg) into mice resulted in an inhibition of proteolytic activity in isolated kidney heterolysosomes containing intravenously injected denatured (formaldehyde) ^{125}I-albumin but not in these particles from the liver. The inhibition occurred if the mercuric chloride was injected up to about 17 hr before injection of labeled protein. The proportion of particle-bound radioactivity which could be released by osmotic shock was substantially decreased in subcellular suspensions from mercury-injected mice. In some experiments therefore, digestive rates were normal in mercury-injected mice if proteolysis was measured in terms of the osmotically releasable radioactivity rather than in terms of the total particle-bound material. This suggests that mercury inhibited either endocytosis of labeled protein or heterolysosome formation. However, in most experiments in which digestion was measured in the presence and absence of mercaptoethanol, stimulation of proteolysis by mercaptoethanol was significantly greater in heterolysosomes from mercury-injected animals than in the controls. This suggests that mercury affected proteolytic activity within the organelles as well as heterolysosome formation.

THERE is an increasing amount of evidence that hepatic lysosomes accumulate heavy metals. Koenig[1] found cytochemical evidence for iron, silver, copper, lead and mercury in liver lysosomes soon after injections of the salts of these metals into rats. Goldfischer[2] and Goldfischer and Moskal[3] found copper in the hepatic lysosomes of patients with Wilson's disease, a genetic defect of copper metabolism. Beryllium,[4] iron,[5,6] copper[7] and mercury[8] have been found by others to become localized in lysosomes, particularly in the liver. Tappel *et al.*[9] noted relatively high amounts of iron, copper, and zinc in lysosomes of normal (uninjected) animals, suggesting that lysosomes sequester dietary metallic cations.

It is well known that heavy metals are potent inhibitors of certain enzymes. Mercury, iron, zinc, silver, cobalt, nickel and copper ions are inhibitors of lysosomal proteolytic enzymes.[10] Furthermore, low concentrations of certain heavy metals such as silver, copper and mercury ions activate lysosomal enzymes in suspensions of intact rat liver particulate fractions,[11,12] suggesting interactions with lysosomal membranes. The toxic action of heavy metals in living animals, therefore, may to some extent be due to interactions with lysosomes.

A possible approach to the problem of interaction of heavy metals with lysosomal digestive activities may be to evaluate the effect of metals on the uptake and degradation of soluble, denatured, radio-iodinated serum albumin in the livers and kidneys of mice. The intravenously injected labeled protein becomes localized in heterolysosomes (phago-lysosomes) in cells of the liver and kidneys, and these osmotically

active organelles may be centrifuged intact from tissue homogenates. Degradation of the particle-bound labeled protein may then be studied *in vitro*.[13–15] Since proteolytic activity in these isolated heterolysosomes is dependent on uptake of the protein into cells by endocytosis, fusion of endocytic vesicles (phagosomes) with lysosomes, and normal rates of catalysis by cathepsins, an interference with any aspect of these processes should be reflected in the yield of osmotically active particles containing labeled protein and digestive activity in these organelles.[15] Mercuric chloride was chosen in the present studies because mercuric ion is an especially effective inhibitor of cathepsins[10] and little is known about the toxic action of this metal.

MATERIALS AND METHODS

Crystalline bovine serum albumin (Mann Research Laboratories, Inc., New York, N.Y., U.S.A.) was labeled with carrier-free ^{125}I-iodide according to the method of Bocci.[16] The preparation of formaldehyde-treated ^{125}I-albumin, injections into mice, and measurements of proteolytic activities in isolated subcellular fractions have previously been described.[14,15,17] However, 0·025 M Tris–acetate buffer, pH 5·0, was included in the incubation mixtures in the present experiments, since better rates of digestion were obtained in isolated kidney heterolysosomes, although there is little or no effect of this buffer on digestion in liver particles.[17] Quantitative estimates of heterolysosome formation in kidneys and livers of mice were obtained by measuring the relative amounts of labeled albumin which could be released from particulate suspensions by osmotic shock.[14,17] Samples of the suspension were diluted in 9 vol. of cold 0·05 M Tris–acetate buffer, pH 7·3, and in similarly buffered cold 0·025 M sucrose. These suspensions were allowed to set on ice for about 10 min and were then centrifuged to sediment all particulate material. The supernatant fractions were decanted and counted, and the radioactivity in the sample containing 0·25 M sucrose was subtracted from the corresponding dilution in buffer alone. The result was calculated as per cent of the total sample radioactivity.

Proteolytic activity and osmotically releasable radioactivity were measured in the 500–30,000 *g* subcellular fraction of tissue homogenates. Although the 0–500 *g* fraction contained a substantial proportion of the heterolysosomes, as measured by the presence of proteolytic activity in osmotically active particles, this fraction was discarded to insure that no whole cells were included in the suspensions. Furthermore, it was established that when the rate of proteolytic activity or the quantity of radioactivity in osmotically releasable form was decreased in the 30,000 *g* fraction, a similar decrease in these parameters was also present in the 500 *g* material.

Adult male or female albino Swiss–Webster mice were used in these studies. The animals were injected intravenously into the tail vein with labeled protein (1–2 mg and about 10^7 counts/min) mixed with the appropriate amount of mercuric chloride in 0·85% (w/v) sodium chloride, or the mercuric salt was injected intraperitoneally before injection of labeled protein. Radioactivity measurements were performed in a Packard spectrometer and well counter containing a thallium-activated 2-in. sodium iodide crystal. This system was about 50 per cent efficient with a window bracketing the two ^{125}I decay peaks. All counts were measured in volumes of 4 ml to minimize geometry effects.

RESULTS

The LD_{50} for mercuric ion in the mouse strain used in these experiments, calculated according to Weil,[18] was estimated to be 5·3 ± 1·1 (95 per cent confidence interval) mg/kg.

The effect of mercuric chloride, injected simultaneously with labeled albumin, on proteolytic activity in isolated heterolysosomes from mouse kidney and liver is shown in Fig. 1. Mercury had no effect on digestive activity in the particles from liver, but digestion was inhibited up to about 70 per cent in kidney heterolysosomes. These experiments were performed more than ten times, and the degree of inhibition in kidney heterolysosomes ranged from about 30 to as much as 73 per cent with a dose of 5 mg Hg/kg. The inhibition was less with smaller doses of mercury; the smallest quantity eliciting an effect was 2·5 mg/kg, which produced a 20 per cent inhibition, although the effect was not consistent at this dose level.

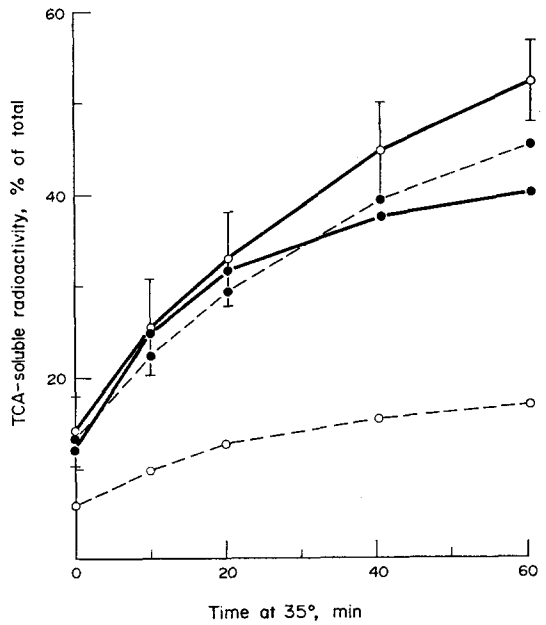

FIG. 1. Inhibition of proteolytic activity in isolated mouse kidney heterolysosomes containing intravenously injected ^{125}I-albumin by mercuric chloride. The dashed lines represent digestive rates in kidney (open circles) and liver (closed circles) heterolysosomes from a mouse injected intravenously with 5 mg Hg/kg at the time of ^{125}I-albumin injection. Solid lines represent average digestive rates in liver (closed circles; 7 experiments) and kidney (open circles; 12 experiments) heterolysosomes isolated from control animals which were not injected with mercuric chloride. The curve for the kidney controls is shown plus and minus the standard error.

Intraperitoneal injections of mercuric chloride produced the same effects as intravenous injections. Table 1 shows that the inhibitory effect of the metal on digestion was only temporary, and about 20 hr after injection of mercuric chloride kidney heterolysosomes showed a nearly normal rate of proteolytic activity. This interval perhaps may be the time required to excrete the greater proportion of mercuric ion from the kidneys.

TABLE 1. INHIBITION OF PROTEOLYTIC ACTIVITY IN ISOLATED MOUSE KIDNEY HETEROLYSOSOMES BY INJECTIONS OF MERCURIC CHLORIDE (5 mg Hg/kg)

Time (hr)	$HgCl_2$ injection	TCA-soluble radioactivity (% of total) (Increase in 40 min)	Inhibition %
Controls	None	30·3 ± 0·8	
0*	i.v.	15·2 ± 1·6	50
2–5	i.p.	20·6 ± 1·4	32
17–21	i.p.	25·0 ± 1·9	18
46	i.p.	31·2	0

* In these experiments, the mercuric chloride was injected simultaneously with labeled protein. In the remaining experiments, mercuric chloride was injected intraperitoneally (i.p.) at the times indicated before intravenous injections of labeled protein. Mice were sacrificed 30 min after injections of labeled protein, and digestion was measured in the 500–30,000 *g* subcellular particulate material as described in Materials and Methods. The data represent the means and standard errors of 12 experiments in the controls, 10 experiments in the animals injected with labeled albumin simultaneously with mercury, 6 experiments in the 2–5 hr group, 10 experiments in the 17–21 hr group and 3 experiments in the 46 hr group.

The actual rate of proteolysis, in terms of per cent increases in trichloroacetic acid-soluble radioactivity per unit time interval, did not appear to be affected in isolated heterolysosomes from mercury-injected mice. However, the initial trichloroacetic acid-soluble radioactivity was always considerably less in these heterolysosomes, resulting in the apparent inhibition noted in Fig. 1. If the mercury inhibited some aspect of heterolysosome formation so that fewer heterolysosomes were present in the particulate suspensions, this would result in an apparent inhibition of digestion when measured in terms of the total particle-bound radioactivity. Heterolysosomes are osmotically active, and the labeled protein may be released from these particles by suspension in dilute pH 7 buffer.[15,17,19] In order to determine if kidney particulate suspensions from mercury-injected mice contained fewer heterolysosomes relative to the total particle-bound radioactivity, the suspensions were subjected to osmotic shock by dilution in cold dilute Tris–acetate buffer, pH 7·3, as described in Materials and Methods. The per cent of the total radioactivity released into the soluble fraction by this technique in kidney heterolysosome suspensions from control mice was consistently about 70–80 per cent. In mercury-injected mice, the quantity released was significantly less, and in some experiments this decrease was approximately proportional to the degree of inhibition of digestion. Furthermore, no such effects were noted in heterolysosome suspensions from the livers of mercury-injected mice. These data are shown in Table 2. Figure 2 shows rates of digestion in terms of osmotically releasable radioactivity rather than as per cent of the total in an experiment in which the decrease in osmotically releasable radioactivity was approximately the same as the decrease in digestive activity. These results suggest that the effect of mercury was not on digestive activity within the heterolysosome, but that the metal appeared to inhibit heterolysosome formation in the kidney. This conclusion would also imply that mercury had little effect on binding of the protein to cell membranes

TABLE 2. OSMOTICALLY RELEASABLE RADIOACTIVITY AND PROTEOLYTIC ACTIVITY IN SUBCELLULAR PARTICULATE MATERIAL FROM MOUSE KIDNEY AND LIVER IN MERCURY-INJECTED AND CONTROL MICE

Experiment	% Total radioactivity released by osmotic shock		TCA-soluble radioactivity (% of total) (Increase in 40 min)*	
	Kidney	Liver	Kidney	Liver
Controls	72·4 ± 4·5	68·4 ± 6·6	30·3 ± 2·6	25·3 ± 4·0
1	44	66	18·2	25·0
2	37	71	9·6	26·0
3	46	79	14·8	21·7
4	40	69	11·2	28·4
5	31	81	11·0	31·0
6 (1 hr after injection)	31	80	12·2	26·5

* The TCA (trichloroacetic acid)-soluble radioactivity is a measure of protein digestion in heterolysosomes, and the figures in these columns represent the per cent of the total radioactivity solubilized in the acid during the first 40 min of incubation of the particulate fractions at 35°. Mercuric chloride (5 mg Hg/kg) was injected intravenously simultaneously with the labeled protein in all these experiments. The controls represent means and standard deviations of 12 experiments.

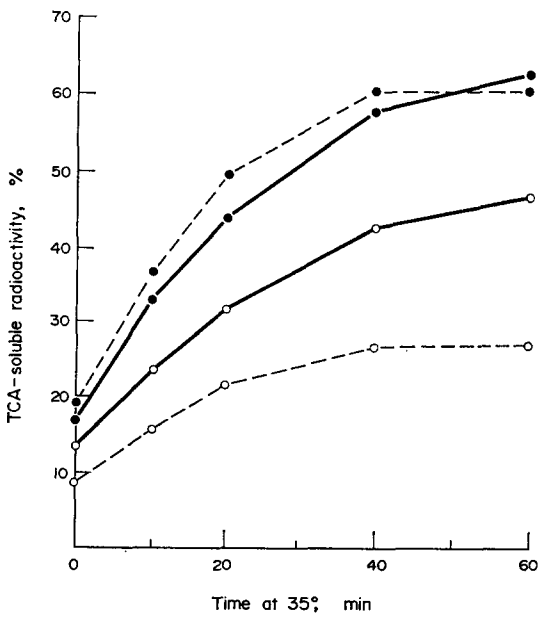

FIG. 2. Proteolytic activities in isolated mouse kidney heterolysosomes measured in terms of the osmotically releasable radioactivity (closed circles) and in terms of the total particle-bound radioactivity (open circles). Solid lines, control animal which did not receive mercuric chloride; dashed lines, mercuric chloride (5 mg Hg/kg) injected simultaneously with labeled protein.

prior to endocytotic uptake into the cell. Table 2 also shows the results of an experiment in which a mouse was sacrificed 1 hr after injection of mercury and labeled protein rather than 30 min after injection. This experiment was performed to determine if mercury slowed the rate of heterolysosome formation. The results indicate that the same degree of inhibition of digestion and of osmotically releasable radioactivity occurred.

The work of Misaka and Tappel[10] suggests that lysosomal proteolytic enzymes are sensitive to inhibition by mercury and other heavy metals. In most cases, however, a sulfhydryl reducing agent such as dithioerythritol reversed the inhibition. Since mercaptoethanol was routinely added to the incubation mixtures in which lysosomal digestive activities were measured in the present experiments, this substance may also have reversed any inhibition of proteolytic activity. Some experiments were performed, therefore, in which heterolysosomes from mercury-injected mice were incubated in the absence of mercaptoethanol. The results (Table 3) indicate that a

TABLE 3. STIMULATION BY MERCAPTOETHANOL OF PROTEOLYTIC ACTIVITY IN ISOLATED MOUSE KIDNEY HETEROLYSOSOMES FROM MERCURY-INJECTED AND CONTROL ANIMALS

Experiment	TCA-soluble radioactivity (% of Total) (Increase in 40 min)		% Stimulation
	With ME*	Without ME*	
5 mg Hg/kg	16·8 ± 1·1	4·8 ± 0·37	361
Controls	30·3 ± 0·8	13·6 ± 0·7	223

* ME = mercaptoethanol (4×10^{-2} M). These columns represent proteolytic activity in terms of the per cent of total radioactivity in the suspensions solubilized in 5% cold trichloroacetic acid during 40 min of incubation of subcellular kidney particles at 35°. Mercuric chloride (5 mg Hg/kg) was injected simultaneously with labeled protein, and the figures represent the mean and standard error of 5 experiments. The controls represent 12 experiments.

significantly greater degree of inhibition of proteolytic activity occurred in most of these experiments. In control heterolysosomes, mercaptoethanol stimulated digestion about 2¼ times. However, in kidney heterolysosomes from mercury-injected mice, the stimulation by mercaptoethanol was as high as 5 times. These results suggest that mercury may have been present in the heterolysosomes in sufficient quantity to inhibit digestion, but some of this inhibition was reversed by mercaptoethanol. Further support for this hypothesis is that digestive rates were not comparable to controls in all experiments when these rates were measured in terms of osmotically releasable radioactivity, suggesting that mercaptoethanol did not always completely reverse the inhibition. This may be seen in Table 2 in experiments 2, 3 and 4, in which the rates in kidney heterolysosomes were 26·0, 32·2 and 28·0 per cent, respectively, when measured in terms of osmotically releasable radioactivity (dividing by the per cent osmotically releasable radioactivity and multiplying the result by 100). In experiments 1, 5, and 1 hr after injection, the rates were 41·4, 35·5 and 38·8 per cent,

respectively, when measured in this manner. The controls averaged 41·9 per cent when measured in terms of osmotically releasable radioactivity.

The effects of relatively high doses of mercury on heterolysosome formation and function described thus far appeared to be due to the accumulation of the metal in the kidneys during excretion. It was of interest to determine if relatively small doses of mercury administered over prolonged periods might have a similar effect. Accordingly, experiments were performed in which two groups of 12 mice were injected daily intraperitoneally with 0·5 and 1·0 mg Hg/kg for 24 days. On days 9, 10, 12, 15 and 24, animals in both groups were injected intravenously with formaldehyde-treated labeled ^{125}I-albumin. Subcellular particulate fractions from kidneys and livers were analyzed with respect to radioactivity in osmotically active particles and digestive capacity. Comparison of the results (Table 4) with those of control animals (Table 2) shows that the injections had no effects on heterolysosome formation or on digestive activity.

TABLE 4. EFFECT OF DAILY INJECTIONS OF MERCURIC CHLORIDE ON HETEROLYSOSOME FORMATION AND PROTEOLYTIC ACTIVITY IN THESE ORGANELLES FROM MOUSE KIDNEYS*

Daily dose of Hg (mg/kg)	% Total radioactivity released by osmotic shock		TCA-soluble radioactivity (% of total) (Increase in 40 min)	
	Kidney	Liver	Kidney	Liver
0·5	72·9 ± 5·4	66·1 ± 3·7	30·4 ± 3·8	23·7 ± 1·7
1·0	69·9 ± 5·7	72·3 ± 1·0	31·0 ± 4·1	26·3 ± 2·9

* Mice were injected daily for 24 days with either 0·5 or 1·0 mg Hg/kg. On days 9, 10, 12, 15 and 24, animals from each group were injected with formaldehyde-treated ^{125}I-albumin and assayed for uptake into osmotically active particles and for digestion of the protein in isolated particulate suspensions. The results represent the means and standard deviations of all the animals sacrificed (five animals from each group).

DISCUSSION

An effect of mercury ions on heterolysosome formation and perhaps on digestion of injected labeled protein in the kidneys and not in the livers of mice suggests that these effects were a consequence of the accumulation of the metal in the kidneys during excretion. The LD_{50} for mercuric chloride injected intraperitoneally into the mice used in these experiments was 5·3 mg Hg/kg, and the quantity required to consistently elicit an effect on heterolysosome formation was about 5 mg/kg, very near the LD_{50}. None of the ten mice injected with 5 mg Hg/kg and sacrificed 17–21 hr after injection for lysosome studies died as a result of the mercury. Four of these animals showed rates of proteolytic activity in isolated heterolysosomes of 29·1–35·5 per cent degraded in 40 min of incubation, five showed rates of 20·2–23·9 per cent, and only one showed a decided inhibition (16·4 per cent degraded in 40 min). The four animals with normal rates obviously recovered from the mercury, since all animals injected with mercuric chloride simultaneously with labeled albumin exhibited abnormal digestive activities. The recovery rate therefore corresponded closely to the LD_{50}. However, only one of the mice died within 24 hr in LD_{50} studies,

and the remaining four of the ten animals injected with 5 mg Hg/kg succumbed within 48–72 hr. The results suggest that animals recovering from the effects of mercury also recovered their capacity for normal heterolysosome formation.

Endocytosis and the formation of heterolysosomes involve extensive membrane movements, and mercury clearly inhibited some aspects of the processes. The initial event in the uptake of macromolecules into cells most likely involves a binding to cell membranes. Subsequent steps involve invaginations of the membranes, aggregation of the resulting membrane-enclosed vesicles to form phagosomes, and the fusion of phagosomes with lysosomes to form osmotically active heterolysosomes in which hydrolytic activities take place.[20] The evidence presented in the present studies indicates that the proportion of osmotically active particles containing injected labeled protein was reduced with respect to the total particle-bound radioactivity in homogenates of the kidneys from mercury-injected mice. Since, in some experiments, rates of digestion of particle-bound labeled protein were normal when calculated in terms of the osmotically active material, this suggests that mercury reduced the quantity of heterolysosomes in the kidneys. If phagosomes are osmotically active, this inhibition of heterolysosome formation may perhaps be explained by the observations of Koenig[11] and Chvapil *et al.*[12] that mercury causes a labilization of lysosomal membranes. Interaction of the metal with lysosomal membranes *in vivo*, resulting in the release of acid hydrolases, or interference with the capacity of lysosomes to fuse with phagosomes would reduce the quantity of heterolysosomes in kidney homogenates. However, if phagosomes are osmotically active, then the inhibition of heterolysosome formation must have occurred at an earlier step, perhaps at the level of endocytosis, since the presence of osmotically active particles containing labeled protein and no lysosomal proteases, and therefore exhibiting no digestive capacity, would have produced results quite different from those obtained in the course of these investigations.

A greater degree of inhibition of proteolysis occurred in heterolysosomes isolated from mercury-injected mice incubated in the absence of mercaptoethanol than in these preparations from control mice (Table 3). This suggests that mercury may have been present in these heterolysosomes in sufficient quantity to inhibit proteolysis, since the inhibition of cathepsin C by mercury is reversed by sulfhydryl agents.[10] In view of the finding of Norseth and Brendeford[8] that mercury accumulates in lysosomes, it would appear likely that continuous exposure of animals to relatively low levels of mercury should produce an inhibition of digestive activities in heterolysosomes in the kidney and perhaps the liver. However, the experiments described in the present publication (Table 4) suggest either that mercury did not accumulate in quantities sufficient to inhibit lysosomal function or that the turnover of lysosomes is sufficiently rapid to prevent an accumulation in functional lysosomes.

Acknowledgement—Supported by Grant ES00591-02 from the National Institutes of Health.

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