HEAVY METALS IN THE TERRESTRIAL ISOPOD *PORCELLIO SCABER* LATREILLE. II. SUBCELLULAR FRACTIONATION OF METAL-ACCUMULATING LYSOSOMES FROM HEPATOPANCREAS

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Two populations of metal contaminated Porcellio scaber *Latreille were studied: one consisting of animals that had been fed heavy metals in the laboratory for several months, and one from a metal-polluted site in the field (Braubach, FRG). Density gradient centrifugation was performed on hepatopancreas homogenates in order to identify cellular fractions and their association with lead, copper and cadmium. Marker enzymes were used for localization of cellular fractions in the density gradient. Two lysosomal fractions, called the "light" and "heavy" fraction, were separated. They contained mainly lead, but also copper and some cadmium.*

INTRODUCTION

In the preceding report (Prosi and Dallinger, 1988), some ultrastructural and cytochemical properties of metal-containing vesicles from hepatopancreas of *Porcellio scaber* were presented. Due to their features the vesicles were identified as lysosomes. An additional approach for studying metal-containing vesicles is provided by methods of centrifugation. Subcellular fractionation of metal-containing lysosomes from invertebrate tissues is reported for *Mytilus edulis* by George et al. (1982) and for *Mytilus galloprovincialis* by Viarengo et al. (1985).

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2. Key words: acid phosphatase, heavy metal, isopod, lysosome, subcellular fractionation.

3. Abbreviations: AP, acid phosphatase; ER, endoplasmic reticulum; LDH, lactic dehydrogenase; MDH, malic dehydrogenase; NADH, nicotinamide adenine dinucleotide; *P. scaber, Porcellio scaber.*

In the present study we show that after density gradient centrifugation of metal-containing hepatopancreas from isopods, substantial amounts of heavy metals were found in fractions exhibiting an elevated activity of acid phosphatase. Due to their metal content, their enzyme activity and elevated density the fractions are characterized as belonging to the lysosomal system with the same origin as the vesicles shown by electron microscopy (Prosi and Dallinger, 1988).

METHODS

Animals. Two populations of metal-loaded and uncontaminated *Porcellio scaber* Latreille were used. One population was collected in the vicinity of Innsbruck. The animals were reared in plastic boxes with moist plaster of Paris as substrate at 18° with a photoperiod of 12 hr for several months before use. For two months the animals were fed dried birch leaf litter which had been enriched either with copper, lead, or cadmium by soaking for 1 hr in appropriate metal salt solutions: cadmium (chloride), 1 mg/liter; copper (chloride), 10 mg/liter; lead (nitrate), 1 mg/liter.

A second population of *P. scaber* was collected in an ancient lead mining area of Braubach, W. Germany. The individuals were transferred to the laboratory and reared for several weeks in plastic boxes as described above. The animals were fed contaminated litter from the mining area.

Density Gradient Centrifugation. The hepatopancreas tubules of 50 isopods were dissected and pooled in an ice-cold glass vessel. They were quickly homogenized without buffer by means of a small glass pestle. The osmolarity of the tissue sap, as determined by freezing point depression in a cryoscope (Knauer, FRG), proved to be 510–540 mosmol. These values are supported by measurements of other authors (Lindquist and Fitzgerald, 1976).

For density gradient centrifugation, the method described by Lackner (1986) was used and varied according to experimental necessities. The hepatopancreases of 70-100 animals were dissected under a microscope and pooled in 6-10 ml of an ice-cold iso-osmotic buffer solution (= buffer A, consisting of 100 mM triethanolamine-HCl with 180 mM KCl and 5% sucrose, pH 7.0) to which 2% serum albumin and two protease inhibitors had been added: 0.2 mM antipain and 0.05 mM pepstatin (Sigma). The time spent for preparation of all hepatopancreas tubules was about 3 hr. During this time the prepared organs were stored in a plastic tube on ice. Individual specimens of whole animals and dissected isopods without hepatopancreas were pooled separately, oven-dried, and stored at 60° for determination of heavy metals by atomic absorption spectrophotometry. Dissected hepatopancreas tubules were carefully homogenized three times at 550 rpm with a Potter-Elvehjem homogenizer. In an alternative approach, dissected hepatopancreatic tubules were immediately frozen in liquid propane and stored in a plastic tube under liquid nitrogen. Frozen tissues were ground in a nitrogen-cooled agate mortar and added to a volume of 10 ml ice-cold buffer A.

The homogenate or the suspension were centrifuged for 10 min at 650 × g on a Sorvall RC2-B centrifuge. 3 ml of the resulting supernatant were pipetted over a discontinuous sucrose gradient which consisted of 11 dilution grades (3 ml each) ranging from 60% to 10% sucrose in 100 mM triethanolamine-HCl with 180 mM KCl (pH 7.0), at 5% intervals. The gradient was prepared by pipetting the individual sucrose layers into a 36 ml centrifugation tube (polyallomer, DuPont). The sample was centrifuged overnight in a Sorvall OTD-2 ultra-centrifuge for 8 hr at an average centrifugal force of 53000 × g and a temperature of 4°. A Sorvall AH627 swinging bucket rotor was used. The next day the sample was fractionated into portions of 2 ml, in which specific gravity was determined. Aliquots of each fraction and of each pellet were prepared for metal analysis by atomic absorption spectrophotometry.

In a small aliquot of each fraction and in pellets the activities of the following marker, enzymes were recorded: lysosomes, acid phosphatase (AP), mitochondria and cytosol, malic dehydrogenase (MDH), cytosol, lactic dehydrogenase. All enzymes were measured according to Bergmeyer (1970). LDH and MDH were measured by recording the oxidation of NADH in the presence of pyruvate (LDH) and cisoxalacetate (MDH), respectively, at 340 nm in an LKB ultraspectrophotometer. AP was detected in the presence of 0.1% Triton X-100 (Sigma) by measuring the degradation of 4-nitrophenyl-phosphate at 25° after 30 min at a pH of 4.8 and a wavelength of 405 nm in an LKB Ultrospec photometer. Pellets were resuspended in 2 ml 100 mM triethanolamine-HCl and 180 mM KCl with 10% sucrose and 0.1% Triton X-100 (Sigma). The activities of enzymes were measured after filtering the pellet suspension through a syringe equipped with a 0.45 μm membrane filter. Three or four repeated fractionations were carried out for controls as well as each group of metal-exposed isopods.

Atomic absorption spectrophotometry. 1 ml of each fraction and an aliquot of pellet (5-10 mg dry wt.) were digested with 1 ml nitric acid (suprapur, Merck) in screw-capped 10 ml-polypropylene tubes (Greiner, Austria).

Whole isopods and carcasses of animals without hepatopancreas as well as dry leaves and litter were digested as described in Prosi and Dallinger (1988). All samples were analyzed for copper, cadmium, and lead in the flame of a Pye Unicam SP9 atomic absorption spectrophotometer with deuterium background correction or by graphite furnace atomic absorption spectrophotometry (Perkin Elmer 5000, HGA 500). NRC standard reference material (TORT-1 lobster hepatopancreas) was used in order to confirm reproducibility of the results (see Prosi and Dallinger, 1988).

RESULTS

The metal concentrations of control and enriched birch leaves, as well as of contaminated litter from Braubach on which laboratory-reared animals were fed, are given in Table 1.

TABLE 1
Metal Concentrations of Substrate[a]

Type of Feeding Substrate	Metal Concentration ($\mu g/g^{-1}$, dry wt.)		
	Cadmium	Copper	Lead
Control birch leaves (n = 12)	4.2 (± 1.5)	20.5 (± 5.5)	40.3 (± 5.4)
Birch leaves, metal-enriched (n = 6)	52.5 (± 11.2)	233.4 (± 31.3)	526.7 (± 125.1)
Contaminated litter from Braubach (n = 8)	41.9 (± 4.8)	628.4 (± 56.9)	1658.6 (± 300.3)

[a]Concentrations of heavy metals in uncontaminated and metal-enriched birch leaves as well as in litter substrate from the mining area in Braubach, on which laboratory animals were fed for several weeks. The concentrations of enriched birch litter were achieved by soaking leaves in metal solutions as described in the text.

Metal concentrations are expressed in $\mu g/g^{-1}$ dry weight; means and standard deviations (in brackets); n = number of measured samples derived from pooled individuals.

In Table 2, metal concentrations of contaminated isopods from Braubach, FRG, are compared with those of control and metal-fed animals from Innsbruck. Animals from Braubach had the highest concentrations of copper and lead, whereas metal-fed animals from Innsbruck showed the highest concentrations of cadmium.

In Table 3 the amounts of metals in the hepatopancreatic tubules are expressed as absolute concentrations and as a percentage of the metal content in whole individuals. The reported values demonstrate that in metal-loaded animals, metals were concentrated in the hepatopancreas compared to the remainder of the animals.

TABLE 2
Metal Concentrations in Isopods[a]

Animals	Feeding Conditions	Rearing Period	Metal concentration ($\mu g/g^{-1}$)		
			Cadmium	Copper	Lead
P. scaber, Innsbruck (n = 6)	control birch leaves	8 weeks	10.96 (± 5.61)	338.0 (± 85.4)	19.8 (± 6.1)
P. scaber, Innsbruck, metal-fed (n = 6)	metal-enriched birch leaves	8 weeks	238. 3 (± 54.0)	951.8 (± 12.3)	429.8 (± 46.3)
P. scaber, Braubach (n = 8)	contaminated litter from Braubach	4 weeks[b]	80.09 (± 13.53)	1205.8 (± 173.9)	1523.7 (± 177.9)

[a]Concentrations of heavy metals ($\mu g/g^{-1}$, dry wt.) in whole adult *Porcellio scaber* from Innsbruck and Braubach (n = number of measured samples). Means and standard deviations (in brackets) are shown. Both feeding conditions and rearing period in the laboratory are listed in the second and third column, respectively.

[b]Note that the individuals from Braubach were already exposed to metal-contaminated substrate under field conditions.

TABLE 3
Absolute Concentrations and Percent Amounts of Heavy Metals in Hepatopancreas of Isopods

Animals	Metal concentration in hepatopancreas (% of whole body load)		
	Cadmium	Copper	Lead
P. scaber, controls	8.82 ± 4.5	218.0 ± 55.9	3.92 ± 1.2
(Innsbruck)	(80.5)	(64.5)	(70.9)
P. scaber, metal-fed	162.7 ± 35.8	644.7 ± 8.7	358.9 ± 38.9
(Innsbruck)	(68.3)	(68.9)	(83.5)
P. scaber, contaminated	44.3 ± 8.1	986.3 ± 151.9	1100.1 ± 130.3
(Braubach)	(55.3)	(81.8)	(72.2)

[a]Concentrations of heavy metals in hepatopancreas of contaminated *Porcellio scaber* from Innsbruck and Braubach, expressed as μg metal/g dry weight of whole animal (with standard deviations), and relative amounts of heavy metals in the hepatopancreas expressed as % of whole body load (in brackets). Means of 6-8 measurements are shown. Both concentrations and percentage of heavy metals in hepatopancreas were calculated from the difference between metal contents of whole animals and dissected carcasses without hepatopancreas.

Figs. 1-3 show the results after density gradient centrifugation on hepatopancreas homogenates of animals from Innsbruck (Fig. 1, controls; Fig. 2, lead-fed animals) and on individuals from the ancient mining area in Braubach (Fig. 3).

In all animals the highest activities of AP appeared in the cytosol (fractions 17-20), indicating that a substantial amount of lysosomes has been broken up during the preparation. Nevertheless, marked activity of acid phosphatase was observed in the pellet, which we call the fraction of "heavy" lysosomes. Additionally, a broad peak of acid phosphatase was also seen at an equilibrium density of 1.14 to 1.22 (fractions 3-10), which we call the fractions of "light" lysosomes. In all cases the peaks of acid phosphatase coincided with pronounced elevations of metal contents, which was best seen for lead in lead-fed animals from Innsbruck (Fig. 2), and for lead and copper in contaminated isopods from Braubach (Fig. 3). The lysosomal cadmium peaks, however, were always less pronounced in comparison to the other metal peaks (Figs. 2 and 3). Patterns similar to those shown in Figs. 1-3 were observed in laboratory animals from Innsbruck fed with copper and cadmium (not shown). In control isopods, the metal peaks of lysosomal fractions were absent or less prominent, as shown in Fig. 1. Peaks for MDH appeared in the cytosol and at a density of about 1.19, indicating the fraction of mitochondria. No significant amounts of metals were associated with these organelles. LDH was present mainly in the cytosol and to a lesser extent throughout the whole density gradient.

In Table 4 the recovery of heavy metals after density gradient centrifugation is presented. The values are expressed as percentage of total metal content in the homogenate including the percentage of metal burden in "heavy" (pellet) and "light"

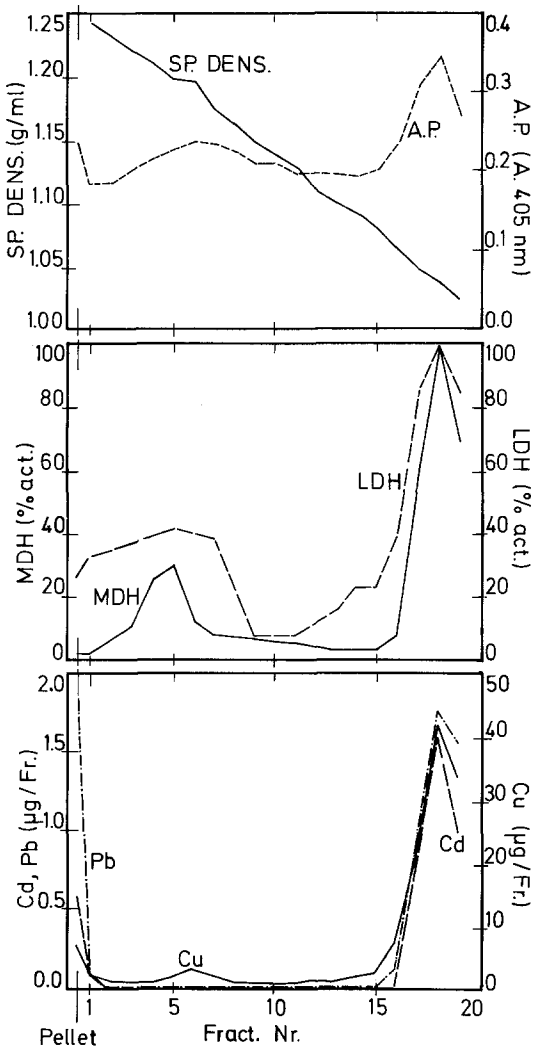

FIGURE 1. Density gradient centrifugation on hepatopancreas from control animals (Innsbruck). *Upper graph*: Specific density (left scale) and activity of acid phosphatase (right scale), expressed as absorption at 405 nm. *Middle graph*: Activities of malic dehydrogenase (left scale) and lactic dehydrogenase (right scale), both expressed as percentage of maximal activity. *Lower graph*: Contents of cadmium and lead (left scale) and of copper (right scale), expressed as microgram metal per fraction. Abbreviations: SP.DENS. specific density; A.P., acid phosphatase; MDH, malic dehydrogenase; LDH, lactic dehydrogenase.

lysosomal fractions (fractions 3-10), as calculated from metal contents in fractions with elevated activity of acid phosphatase (cytosolic fractions excluded). Since the "light" lysosomal fraction appeared as a broad shoulder rather than as a peak, metal

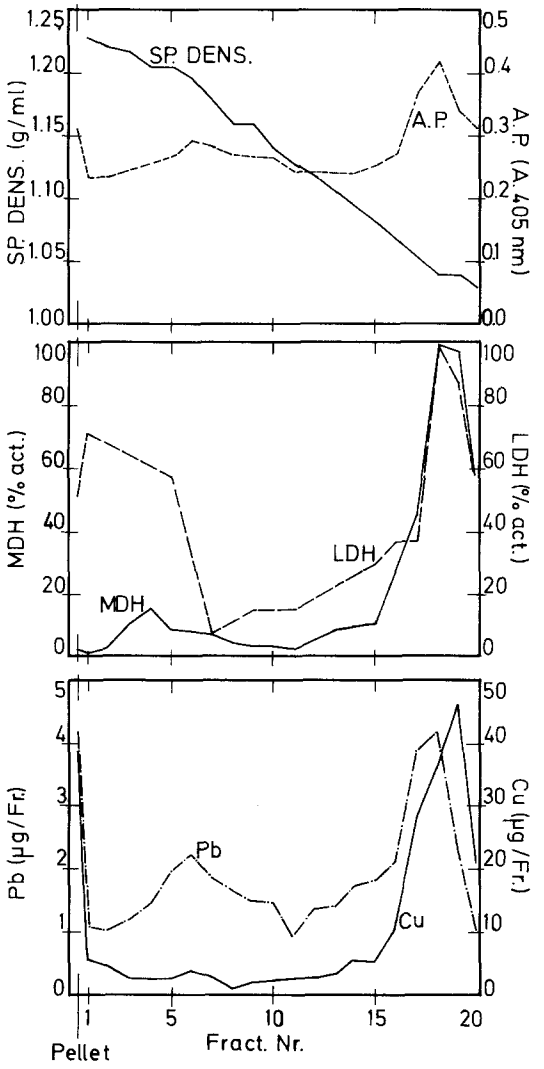

FIGURE 2. Density gradient centrifugation on hepatopancreas from lead-fed animals (Innsbruck). *Upper graph*: Specific density (left scale) and activity of acid phosphatase (right scale), expressed as absorption at 405 nm. *Middle graph*: Activities of malic dehydrogenase (left scale) and lactic dehydrogenase (right scale), both expressed as percentage of maximal activity. *Lower graph*: Contents of lead (left scale) and of copper (right scale), expressed as microgram metal per fraction. For abbreviations see Fig. 1.

contents of the whole shoulder were considered. The data indicate that lysosomes accumulated mainly lead and copper but only little cadmium. No significant amounts of metals seemed to be accumulated by mitochondria.

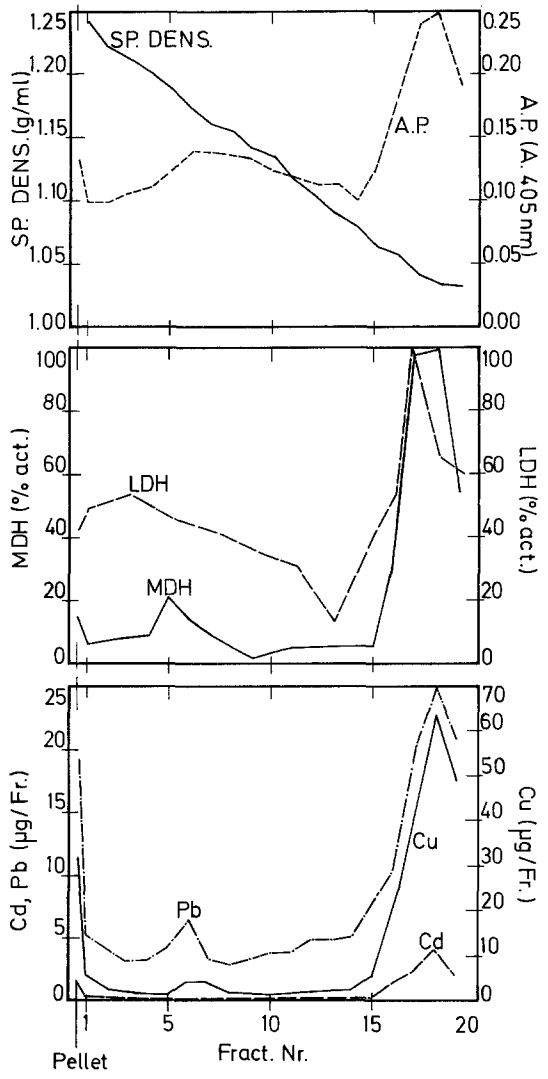

FIGURE 3. Density gradient centrifugation on hepatopancreas from contaminated isopods (mining area, Braubach). *Upper graph*: Specific density (left scale) and activity of acid phosphatase (right scale), expressed as absorption at 405 nm. *Middle graph*: Activities of malic dehydrogenase (left scale) and lactic dehydrogenase (right scale), both expressed as percentage of maximal activity. *Lower graph*: Contents of cadmium and lead (left scale) and of copper (right scale), expressed as microgram metal per fraction. For abbreviations see Fig. 1.

DISCUSSION

This study demonstrates that high concentrations of heavy metals accumulated in *Porcellio scaber* fed enriched birch leaves and litter containing a metal burden.

TABLE 4
Recovery of Heavy Metals and Percent Distribution in Lysosomal Fractions[a]

		Recovery (%) and relative amounts in lysosomal fractions		
Animals		**Cadmium**	**Copper**	**Lead**
Control, Innsbruck	R	113.3	94.4	65.9
	L	0-1%	3.6-4.8%	1.5-6.1%
	H	8.5-15.0%	9.6-17.4%	10.1-29.8%
	T	8-16%	12-20%	10-33%
Lead-fed, Innsbruck	R	nd	nd	86.1
	L	nd	nd	31.2-47.1%
	H	nd	nd	10.5-13.8%
	T	nd	nd	40-60%
Copper-fed, Innsbruck	R	nd	80.3	nd
	L	nd	15.7-18.2%	nd
	H	nd	10.0-14.2%	nd
	T	nd	25-30%	nd
Cadmium-fed, Innsbruck	R	125.2	nd	nd
	L	3.3-5.0%	nd	nd
	H	8.2-10.9%	nd	nd
	T	11-16%	nd	nd
Contaminated, Braubach	R	116.8	85.8	83.7
	L	3.5-5.7%	7.5-14.3%	22.8-36.7%
	H	9.8-11.9%	12.8-13.5%	10.3-15.8%
	T	13-16%	19-25%	32-50%

[a]Recovery of heavy metals after density gradient centrifugation and percent distribution of heavy metals in "light" and "heavy" lysosomal fractions of hepatopancreas from control, lead, cadmium, and copper fed animals (Innsbruck) as well as from contaminated isopods (Braubach). Recovery is expressed in percent of total metals in homogenate (mean of three experiments). Percent distributions of metals in lysosomal fractions are given in percent of total metals from whole fractions of density gradient centrifugation. Ranges of three replicate experiments are given. Abbreviations: R, recovery; L, "light" lysosomal fraction"; H, "heavy" lysosomal fraction (pellet); T, Total (sum of both); nd, no sufficient data available.

However, the concentration factors for cadmium and copper from food to isopods were different between animals from Innsbruck and those from Braubach. This may be due to the different availabilities of both metals in the food. A substantial amount of metals was concentrated in the hepatopancreas of isopods, as summarized in Table 3. These results corroborate the findings of Wieser (1961) and of Prosi et al. (1983). Hopkin and Martin (1982) documented that in contaminated animals, 75-95% of the total metal burden accumulates in the hepatopancreatic tubules. Our values are not quite as high, probably due to losses during preparation for density gradient centrifugation.

The results of density gradient centrifugation suggest that the so called "cuprosomes" (Wieser and Klima, 1969) belong to the lysosomal system. This conclusion is based on the assumption that the presence of AP activity in vesicles characterizes them as lysosomes (Barrett and Heath, 1977) and is supported by the results from electron microscopic observations (Prosi and Dallinger, 1988).

After fractionation on the density gradient the most prominent peak of AP appeared in the cytosol. On the condition that AP is a specific enzyme of lysosomes this is an indication that a considerable amount of these organelles was broken up during the preparation. Despite our efforts in optimizing the preparative approach we were not able to avoid this effect. Among various methods we tried homogenizing the hepatopancreas with buffers of different composition and osmolarity. An approach in which dissected tissue was frozen in liquid propane and ground in an agate mortar was also applied. The method yielding the best results, however, was that described above.

In addition to the cytosol, there were two distinct peaks of AP activity: one in the density range between 1.14 and 1.22; the other in the suspended pellet resulting from density gradient centrifugation (Figs. 1-3). We refer to the first peak as "light" lysosomes, whereas the pellet is called the fraction of "heavy" lysosomes. It must be noted, however, that due to the method of preparation this fraction may also contain other cellular debris. The characterization of these fractions as lysosomes is based on their elevated density, their metal content and on the presence of AP activity. Indeed, the results obtained by electron microscopy and histochemistry indicate that heavy metal storing vesicles do have lysosomal properties (Prosi and Dallinger, 1988).

The broad shoulder of AP as well as the activity of this enzyme in the pellet indicate that intact lysosomes may consist of variable specific density. This could be a result of different shape, size and metal content, as indicated also by the variable appearance of these vesicles on electron microphotographs (Prosi and Dallinger, 1988).

As to the metals, lysosomes undoubtedly provide a trap for lead. Up to 47% of lead can be found under the broad peak of AP from lead-fed animals (Table 4), indicating that in spite of losses a large amount of lead-containing lysosomes may have been preserved. If the lead content of the "heavy" lysosomal fraction is also taken into account, up to 60% of total lead was recovered in lysosomal fractions.

Whether the lysosomes are also a trap for copper is a point of discussion. It has been shown by several authors (Wieser and Klima, 1969; Brown, 1978; Prosi and Dallinger, 1983; Witkus et al., 1987) that in different species of isopods copper is a substantial component of the questionable vesicles from hepatopancreatic S-cells. Certainly the lysosomes isolated in our work play a role in deposition of this metal, since in copper-fed animals up to 30% of the metal could be found in both the "light" and "heavy" lysosomal fractions. Taking into account the possible losses during preparation, this value might even be higher.

Only small amounts of cadmium could be detected in "light" lysosomal fractions after

density gradient centrifugation; somewhat more appeared in the "heavy" lysosomal pellet, especially in control animals.

Variable but considerable amounts of heavy metals were always associated with the cytosol. This could partially be explained by the rupture of lysosomes during preparation. However, as shown in our first report (Prosi and Dallinger, 1988), X-ray microanalysis also revealed granular aggregates of lead, copper, and cadmium scattered throughout the cytoplasm. This indicates that a portion of heavy metals is associated with components in the cytoplasm. As far as cadmium and copper are concerned, one possible mechanism would be the complex of these metals with cytosolic metallothionein or related proteins. However, our efforts to isolate metal-binding proteins from cadmium-fed animals were unsuccessful, cadmium appearing to be associated with non-proteinaceous low molecular weight fractions (Dallinger and Prosi, 1986). Therefore, other metal-binding molecules such as peptides or amino acids should also be considered in this context.

The distribution patterns of heavy metals after density gradient centrifugation indicate that the mitochondria which are marked by the peak of MDH in fractions 4 or 5 do not accumulate considerable amounts of either lead, copper, or cadmium (Figs. 1-3). No clear indications are provided for distribution of metals in organelles other than lysosomes or mitochondria. However, the combined results from histochemistry and X-ray microanalysis (Prosi and Dallinger, 1988) indicate that significant concentrations of the three metals in other organelles such as nuclei or ER are unlikely. It thus seems that lysosomes are the only organelles in hepatopancreatic S-cells of *Porcellio scaber* in which metals are accumulated.

Metal concentrations in the lysosomes from tissues of other invertebrate species have been reported, for instance, in *Mytilus galloprovincialis* by Viarengo (Viarengo et al., 1981, 1985), in *Mytilus edulis* by (George et al., 1982), and in insects by Ballan-Dufrancais (Ballan-Dufrancais et al., 1980). However, most research on metal-concentrating lysosomes has been carried out on mammalian tissues. Copper associated with a metal-binding protein was found to be concentrated in hepatic lysosomes of humans and dogs (Hanaichi et al., 1984; Lerch et al., 1985); gold accumulates in lysosomes of human pulmonary macrophages (Paakko et al., 1984), and deposition of transuranic elements, such as 239-Pu or 241-Am, were reported from hepatic lysosomes of rats and hamsters (Sutterlin et al., 1984); chromium, aluminum, and gallium also concentrate in lysosomes of rat tissues (Berry et al., 1978, 1982, 1984). It thus appears that the accumulation of heavy metals and other inorganic elements in lysosomes is a feature of frequent occurrence.

A further point of discussion is whether the structures which we defined as lysosomes really are lysosomes of primary origin. They are likely to be secondary lysosomes, or even residual bodies. This is supported by the fact that the metal-containing vesicles shown by electron microscopy are bodies of varying size and shape, containing membrane fractions and other cellular debris (Schellens, 1977; Prosi and Dallinger, 1988).

In conclusion, we suggest that in the metal-polluted isopod species *Porcellio scaber*, mainly lead, but also copper and some cadmium accumulate in organelles which belong to the lysosomal system. This view is supported not only by cytochemical methods, but also by the results of density gradient centrifugation. More precise characterization of these organelles remains the aim of further studies.

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