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EFFECTS OF SELECTED CHELATING AGENTS AND METALS ON THE STABILITY OF LIVER LYSOSOMES

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Abstract—Diethyldithiocarbamate (DDC), penicillamine and sodium ethyldiaminetetraacetate (Na-EDTA) in 1 mM concentrations significantly stabilized the membrane of isolated liver lysosomes, as shown by a decreased release of β-glucuronidase upon incubation at 37° and pH 5·0. Thiol compounds which are readily auto-oxidized (cysteine, glutathione and dithiothreitol) did not affect lysosomal fragility when subjected to 1 mM concentration; at a higher concentration (5 mM), these substances increased the release of β-glucuronidase. Stabilization by DDC and penicillamine, both of which contain sulphydryl-like groups, may be related to their nonauto-oxidizability, although their chelation effect cannot be excluded. Various chelating agents relatively specific for Fe^{2+} or Fe^{3+}, such as 1,10-phenanthroline, 5,6-dimethyl-1,10-phenanthroline, 2-2′-dipyridyl, Desferal, and pyrocatechol-3,5-disulfonic acid (Tiron), had no effect on lysosomal stability. Zinc ions (0·05–2·5 mM) significantly stabilized lysosomal membranes; this stabilization was concentration dependent, was not affected by thiol compounds, and was potentiated by equimolar complex with 8-hydroxyquinoline (8-HQ); 8-HQ alone (1 mM) labilized lysosomes. Calcium ions had no effect, whereas Cu^{2+} and Hg^{2+} labilized the lysosomes. Labilization by Cu^{2+} was reversed by chelating agents and decreased by thiol compounds. Incubation of lysosomes in nitrogen and dark prevented labilization by copper, and did not affect stabilization by zinc. The effects of certain chelating agents (8-HQ, DDC, penicillamine, EDTA) or metals (Zn^{2+}, Cu^{2+}, Hg^{2+}) on the stability of the lysosomal membranes are discussed in terms of the reactivity of these agents with components of the membrane, or interference with metal-catalyzed lipid peroxidation.

TISSUE injury induced by certain toxic agents (CCl_4, dimethylnitrosamine or silica particles), biological stimuli (endotoxin), or physical factors (irradiation) is associated with increased fragility of various biological membranes including the lysosomal membrane.[1] The general damage caused by some of these agents can be significantly reduced by simultaneous treatment with chelating agents such as 1,10-phenanthroline or 2,2′-dipyridyl.[2]

The mechanism(s) by which each of these toxic agents damages the membrane is not known, nor is the mechanism by which chelating agents protect the animal against their toxic effects. However, peroxidative decomposition of the structural lipids of biological membranes after exposure to some of the toxins may be a key reaction leading to subsequent cell necrosis.[3] Alternatively, membrane stability might also be impaired by destroying thiol groups, since they are thought to be necessary for maintaining integrity and controlling permeability of the membrane.[4]

Metallic cations such as ferrous iron are capable of catalyzing the oxidation of unsaturated lipids[3] or converting sulfhydryl groups to disulfide bonds[4] *in vitro*, and of impairing the structural and functional integrity of various cell membranes. We

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felt that chelating agents might exert their protective effects by binding such metals, thus reducing the extent of membrane damage.

In order to investigate this possibility, we tested the effect of chelating agents, metals and thiol compounds on the stability of the lysosomal membrane by measuring the release of β-glucuronidase upon incubation *in vitro*.

MATERIALS AND METHODS

Sprague–Dawley rats, male and female, 3–4 months old, were used in all experiments. Rats were sacrificed by decapitation. The liver was perfused *in situ* with ice-cold saline, weighed and homogenized in cold 0·25 M sucrose–0·01 M phosphate buffer (pH 7·4), 1 : 7 or 1 : 10 (w/v), which contained no EDTA. Special care was used to follow the same homogenization procedure and to use the same set of all-glass homogenizers for each sample and experiment in order to reduce variations in the degree of lysosome disruption. The homogenate was fractionated by the method of Weissmann and Thomas.[5] The material sedimenting between 800 and 15,000 *g* was assayed in the presence of 0·1% Triton X-100 for the bound lysosomal enzymes, β-glucuronidase[6] and acid phosphatase.[7] Since similar results were obtained with both enzymes in our early experiments, we later discontinued assaying acid phosphatase and we present only the data on β-glucuronidase in this paper.

In the lysosome fragility test *in vitro*, a lysosome-rich fraction was prepared from rat livers as described above. The material sedimenting between 800 and 15,000 *g* was carefully resuspended in 0·25 M sucrose (1 : 1·5, w/v) by gentle homogenization. The effect of chelating agents (see below), metals (Ca, Cu, Hg, Zn) and some thiol compounds (cysteine, CySH; cysteine, CySS; glutathione, GSH; and dithiothreitol, DTT) on the release of lysosomal enzymes was determined as follows: aliquots (1 mM) of the suspension were adjusted to pH 5·0 with 2 vol. of 0·25 M sucrose–0·01 M acetate buffer which contained the agents being tested at a final concentration of 1 mM. After incubation at 37° for 30 min, the suspensions were centrifuged at 15,000 *g* and the supernatants were assayed for released β-glucuronidase.

The ability of agents to interact with lysosomes at neutral pH and subsequently to affect lysosomal stability at pH 5 was also determined. Aliquots of the lysosomal suspension were exposed to the agents for 30 min at room temperature in the presence of 0·25 M sucrose and 0·01 M phosphate buffer, pH 7·4. After centrifugation at 15,000 *g*, the supernatants were removed and the pellets were carefully resuspended in 0·25 M sucrose. The lysosomes were then incubated in the presence of 0·01 M acetate buffer, pH 5·0 as described above, with the exception that further amounts of the tested substances were not added. Details on the procedure used in each experiment are given in the appropriate tables.

In experiments testing lysosomal fragility in the absence of oxygen or light, the 0·25 M sucrose–acetate buffer (pH 5·0) was bubbled with nitrogen, then lysosomes were added, the tubes were gassed again with nitrogen, capped and incubated in the dark as above.

The effect of the above-mentioned compounds on the activity of partially purified β-glucuronidase was also determined. The enzyme was prepared from sheep liver by the method of Fishman and Bernfield,[8] and assayed in the presence of a 1-mM concentration of each agent.

The agents tested were diethyldithiocarbamate (DDC), 1,7-phenanthroline (City

Chemical Corp., New York); 1,10-phenanthroline, 8-hydroxyquinoline (8-HQ), pyrocatechol-3,5-disulfonic acid (Tiron), 2,2′-dipyridyl (Fisher Scientific Company); 5,6-dimethyl-1,10-phenanthroline, 2,9-dimethyl-1,10-phenanthroline (G. Frederick Smith Chemical Company, Columbus, Ohio); ethylenediaminetetraacetate, disodium salt (Na-EDTA, Methason, Coleman & Bell) and dicalcium salt (Ca-EDTA, Eastman Organic Chemicals); DL-penicillamine, glutathione, cysteine, cystine, (Sigma Chemical Company); and dithiothreitol (K & K Laboratories, Plainview, N. Y.).

Variability in the data is given by standard error of the mean. Student's *t*-test was used to calculate the significance of the results.

RESULTS

Effect of chelating agents on the fragility of liver lysosomes and the activity of purified β-glucuronidase. The individual chelating agents tested were selected according to their preferential metal binding affinity.[9] Three main groups of chelating agents were studied: those binding preferentially (1) ferrous iron (1,10-phenanthroline, 5,6-dimethyl-1,10-phenanthroline, 2,2′-dipyridyl); (2) ferric iron (Desferal, Tiron, sodium-EDTA); and (3) cupric ion (diethyldithiocarbamate, penicillamine, 8-hydroxyquinoline, and 2,9-dimethyl-1,10-phenanthroline). 1,7-Phenanthroline, which has no chelating capacity, was also tested. The results (Table 1). showed no effect of divalent or trivalent iron chelators other than sodium-EDTA on the fragility of lysosomes. However, 1,7-phenanthroline significantly increased the release of β-glucuronidase from lysosomes. Both of the copper chelators (DDC, penicillamine) significantly

TABLE 1. EFFECT OF SOME CHELATING AGENTS ON THE FRAGILITY OF LIVER LYSOSOMES AND ON THE ACTIVITY OF PURIFIED β-GLUCURONIDASE

Chelating agent*	Free β-glucuronidase† (% control)	*t*-test‡	Purified β-glucuronidase (% control)
Control	100 ± 0·80 (17)		100
1,10-Phenanthroline	95·6 ± 1·79 (13)	2·22	106
5,6-Dimethyl-1,10-phenanthroline	103·0 ± 2·28 (8)	1·26	96
2,2′-Dipyridyl	97·0 ± 1·53 (8)	1·70	108
1,7-Phenanthroline	121·7 ± 2·98 (11)	7·07§	113
Desferal	94·9 ± 3·44 (10)	1·42	105
Tiron	93·7 ± 2·58 (4)	2·30	114
8-Hydroxyquinoline	132·2 ± 3·27 (5)	9·56§	109
2,9-Dimethyl-1,10-phenanthroline	98·6 ± 2·19 (7)	0·58	102
Diethyldithiocarbamate, sodium	70·6 ± 2·39 (11)	11·67§	101
Penicillamine	86·4 ± 1·51 (7)	7·95§	103
Na-EDTA	86·2 ± 0·59 (10)	14·02§	107
Ca-EDTA	99·8 ± 0·96 (5)	0·11	100

* All chelating agents were tested at 1 mM concentration in a 0·25 M sucrose solution or in sucrose with 10% ethanol. Control samples were treated in the same way, but without any chelating agent. Lysosomes were incubated with the tested substance for 30 min at pH 5·0, centrifuged and then the supernatant was assayed.

† Refers to the enzyme released from lysosomal particles during 30 min of incubation at pH 5·0 in the presence of the substance tested.

‡ The values of Student's *t*-test refer to the data on release of β-glucuronidase from treated lysosomes and the control.

§ Results significantly different from the control ($P < 0·01$).

stabilized lysosomes. A very selective copper chelator, 2,9-dimethyl-1,10-phenanthroline, was without any effect, however; 8-HQ at 1 mM concentration significantly labilized lysosomes. Sodium-EDTA decreased the release of β-glucuronidase, whereas its calcium salt was without effect.

Purified β-glucuronidase was assayed in the presence of chelating agents to see whether they interfered with the determination of the enzyme. None of the agents studied influenced the assay of pure β-glucuronidase (Table 1). The increased or decreased activity of β-glucuronidase in the lysosome fragility test is therefore related directly to labilization or stabilization of the cell structures containing this enzyme.

Effect of some metals and chelating agents on the stability of liver lysosomes and on the assay of β-glucuronidase. The group of chelating agents which stabilize lysosomes (Table 1) is rather nonhomogeneous as far as their structure and their affinity for a certain metal are concerned; some function other than chelation might be related to their stabilizing effect. Accordingly, we tested two possible hypotheses: (1) that the agents acted solely by chelating trace metals which otherwise would destabilize the membranes; and (2) that in the case of DDC and penicillamine the stabilizing effect was related to the presence of sulfhydryl or similar groups.

One millimolar concentrations of calcium, copper and zinc ions were tested for their effect on the release of β-glucuronidase from lysosomal particles (Table 2). Calcium was without effect, while copper labilized the membranes. Zinc was consistently found to stabilize lysosomes. This effect was further studied at various

TABLE 2. EFFECT OF SOME METALS AND CHELATING AGENTS ON THE STABILITY OF LIVER LYSOSOMES AND PURIFIED β-GLUCURONIDASE

Sample*	β-Glucuronidase released (% control)	Purified β-glucuronidase (% control)
Control	100	100
Triton X-100	315	
Ca^{2+}	102	106
Cu^{2+}	167	100
Zn^{2+}	64	97
$Zn^{2+} + Cu^{2+}$	71	
DDC	61	
DDC + Cu^{2+}	95	
DDC + Ca^{2+}	56	
DDC + Zn^{2+}	61	
8-HQ	60	
8-HQ + Cu^{2+}	104	
8-HQ + Ca^{2+}	70	
8-HQ + Zn^{2+}	41	
Na-EDTA	86	
Na-EDTA + Cu^{2+}	101	
Na-EDTA + Zn^{2+}	103	

* All substances were tested in 1 mM final concentration and were incubated with isolated lysosomes at pH 5·0 in sucrose–acetate buffer for 30 min.

concentrations of zinc (Fig. 1); the degree of stabilization was concentration-related from 0·05 to 2·5 mM zinc. In the presence of both zinc and cupric ions, the effect of zinc prevailed (Table 2).

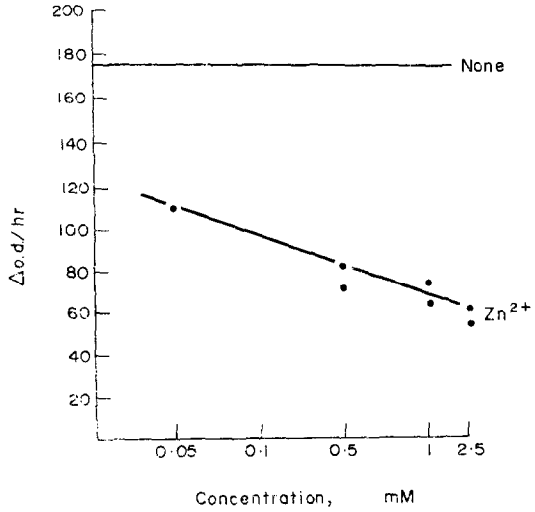

FIG. 1. Effect of various concentrations of zinc on the stability of isolated lysosomes incubated at 37° and pH 5. The individual results of duplicate incubations are plotted as the amount of released β-glucuronidase (absorbancy/hr) vs. zinc concentration (mM). The amount of enzyme released in the absence of zinc is shown.

DDC, 8-HQ and Na-EDTA all blocked the labilizing effect of copper. However, each of these three chelating agents had a different effect on the stabilization by zinc. Incubation of the lysosomal fraction with both zinc and 8-HQ present resulted in a markedly greater stabilizing effect than when each agent was used separately. However, the effect of zinc was not modified by the presence of DDC and was completely reversed by sodium-EDTA. Here again, none of these substances at 1 mM concentration affected the assay of pure β-glucuronidase (Table 3).

TABLE 3. EFFECT OF THE PRETREATMENT AT NEUTRAL pH WITH SOME CHELATING AGENTS AND METALS ON THE RELEASE OF β-GLUCURONIDASE FROM LYSOSOMES

	Activity of the released β-glucuronidase* (% of enzyme released from control sample)		
Sample	Preincubation (pH 7·4, 20°)	Second incubation (pH 5·0, 37°)	Direct incubation (pH 5·0, 37°)
Control	100	100	100
1,10-Phenanthroline	102	105	95·6
DDC	75	83	70·6
Zn^{2+}	69	90	75·0
Cu^{2+}	300	380	162
Hg^{2+}	346	305	

* Each value represents the average of two independent liver lysosome samples, analyzed in duplicate. The experimental design is presented in the text.

Effect of pretreating lysosomes at neutral pH. All of the above experiments were carried out with lysosomes incubated at pH 5·0 in the presence of the tested substance. However, it is well known that pH affects the degree of interaction and the stability of the complex formed between metals and chelating agents and/or biological substrates. For this reason we studied the stability of lysosomes which were first exposed to certain chelating agents or metals at 20° for 30 min at pH 7·4 (Table 3). After centrifugation, the pellets were then resuspended in sucrose–acetate buffer, pH 5·0, and incubated another 30 min at 37° without the addition of further amounts of the metals or chelating agents. The supernatants from the first (neutral) and second (acid) incubations were assayed for the activity of β-glucuronidase; these values were corrected for the total enzyme activity present in the lysosomes.

1,10-Phenanthroline was inactive at both pH levels, while cupric and mercuric ions caused an intense release of β-glucuronidase when the lysosomes were incubated directly with the metals at neutral pH or after pretreatment at pH 7·4 and subsequent incubation at pH 5·0. Although DDC and zinc were stabilizing at pH 7·4, this effect was less obvious at pH 5·0 after preincubation than when lysosomes were incubated directly with the agents. In control samples, the amount of β-glucuronidase released during the 30 min of preincubation at neutral pH represented 5 per cent and at pH 5·0 almost 10 per cent of the total lysosomal activity of this enzyme.

Effect of nitrogen and dark on lysosomal fragility. If the hypothesis is correct that substances which undergo oxidation and/or auto-oxidation[10] or which stimulate lipid peroxidation labilize membranes, then this phenomenon should be diminished when lysosomes are incubated under nitrogen in the dark. Such an experiment is described and presented in Table 4. The presence of air and light contributes to the labilization of lysosomal vacuoles at pH 5·0. In nitrogen and in the dark, the labilization of lysosomes by cupric ions at pH 5·0 is completely prevented, but there is no effect on stabilization by zinc.

TABLE 4. EFFECT OF NITROGEN + DARK ON LYSOSOME STABILITY AT pH 5 IN THE PRESENCE OF CUPRIC AND ZINC IONS*

	N_2 + dark		Air + light	
	(O.D.$_{550}$)	(% control)	(O.D.$_{550}$)	(% control)
Control	0·307	100	0·379	100
Cu^{2+}	0·293	95	0·780	206
Zn^{2+}	0·138	45	0·223	56

* Results are the averages of duplicate determinations. All agents tested were at a final concentration of 1·0 mM.

DISCUSSION

Effect of chelating agents and metals on lysosomal fragility. The role of metal ions in catalyzing oxidation of free sulfhydryl groups and unsaturated fatty acids is becoming more evident as a causative factor in membrane damage.[4] This paper presents a study of the protection by several chelating and/or sulfhydryl agents of the lysosomal membrane against such damage.

We have shown that a variety of chelating agents, which for the most part are rather selective for copper, stabilize liver lysosomes *in vitro*. One notable exception to this specificity for copper is Na-EDTA, a well known stabilizer of lysosomes,[11] which preferentially binds ferric iron. However, Na-EDTA was less effective than DDC or penicillamine.

In order to see whether agents might be acting by chelating trace metals present in the medium, we tested their ability to affect lysosomal stability when certain metal ions were present in millimolar concentrations. As was expected from earlier studies,[5] both cupric and mercuric ions labilized the membrane. Calcium had no effect on the stability.

A rather intriguing finding was the pronounced stabilization in the presence of zinc ions. This was observed at a concentration as low as 0·05 mM zinc, and the degree of stabilization was concentration dependent from 0·05 to 2·5 mM. Furthermore, at 1 mM zinc, stabilization was consistently increased by the presence of an equimolar amount of 8-HQ. Both DDC and 8-HQ prevented the labilization due to cupricions, suggesting that they may indeed stabilize by chelating trace metals.

There are two indications that the metals as well as the chelating agents studied will be efficient at concentrations lower than 1 mM. The first refers to zinc which stabilized at 0·05 mM concentration; the second evidence is derived from the experiment on the pretreatment of lysosomes, where the final fragility test was carried out without the addition of further substances.

Effect of thiol compounds on lysosomal fragility. Although DDC and penicillamine are strong chelating agents, it is possible that these compounds might stabilize lysosomes by virtue of their sulfhydryl-like groups. Several other thiol compounds were tested. At a concentration of 1 mM they did not affect membrane stability themselves, but decreased the labilization induced by cupric ions by 20 per cent. All of the sulfur-containing agents tested (DDC, CySH, GSH, DTT, CySS) had little or no effect on the stabilization by zinc. As was reported by Desai *et al.*,[10] at a higher concentration (5 mM) CySH, GSH and DTT are labilizing (data not presented).

Possible mechanisms of action. A discussion of the mechanisms by which chelating agents, metals, and thiol compounds affect membrane stability should include at least two major types of chemical reactions which control the stability of lysosomal membranes. One is the auto-oxidation of both lipids and thiols. Auto-oxidizable compounds such as cysteine and glutathione labilize lysosomes when present at a concentration of 5 mM.[10] Metal redox systems, especially ferrous ferric ions, are known to stimulate lipid peroxidation.[12] Since copper and mercury are also redox systems, they may labilize by a similar mechanism. The damaging effect of lipid peroxides on the integrity of biological membranes has been commonly agreed.[13] The effect of DDC and penicillamine may be due to chelation, as both agents have low reactivity and do not auto-oxidize.[14] Our finding that thiol compounds at 1 mM concentration decrease the labilizing effect of copper may be related to chelation by thiols or to the fact that some thiols keep metal ions in the reduced state.[14]

The importance of metal redox systems in catalyzing peroxidation of unsaturated lipid constituents of the lysosomal membrane (probably by a free radical mechanism) is suggested by the fact that the labilization by copper is prevented when the incubations are done under a nitrogen atmosphere in the dark.

The second possible mechanism responsible for the effect of metals and chelating

agents on labilization is the activation of enzymes controlling membrane structure and function. Stabilization by sodium-EDTA may be due to chelation of Ca^{2+}, which is required for lysosomal phospholipase A activity.[15,16] This would explain the lack of effect of calcium-EDTA.

At present we do not have a definite explanation for the striking stabilization of lysosomes by zinc. The presence of thiol and disulfide groups in biological membranes is well established and it may be assumed that they play a role in the integrity and permeability of the membrane.[17] In the case of mercuric ions, the suggested mechanism was the formation of mercaptides by interaction with thiol groups.[17] This paper does not present any direct evidence on the formation of zinc or copper mercaptides with membrane proteins. However, it has been shown that a sulfhydryl group is involved in binding zinc to carboxypeptidase B,[18] and reference is made to Vallee's finding[19] that zinc is bound to the sulfhydryl function of the sole cysteine residue. The possibility of the formation of zinc (and other metal) complexes with higher fatty acids of phosphoric acid residues should also be taken into consideration.

Our finding that in the presence of both cupric and zinc ions the lysosomal membrane is not labilized is in agreement with the established order of preference of metals for bidentate nitrogen–sulfur ligands such as cysteine or 2-mercaptoethylamine which follow the order $Hg^{2+} \gg Cd^{2+} > Zn^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$.[19] It may be assumed, therefore, that in such cases thiol groups have greater avidity for zinc ions than for copper. Displacement of copper from sulfhydryl binding sites on metallothionein by zinc has been shown recently by Evans *et al.*[20] Of the chelating agents and thiols tested, 8-HQ was unique in that it enhanced the stabilizing action of zinc. Albert[9] classified 8-HQ as a cooperative chelating agent, which means that a metal may become much more chemically active after chelation. Thus 8-HQ does not remove the metal essential for a certain function, but forms a complex with the metal present in the structure.

The fact that the unsaturated 1 : 1 complex of Zn–8-HQ, which does not permeate membranes,[9] promotes the stabilizing effect of zinc supports the view that zinc is acting at the surface of the lysosomal membrane.

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