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INFLUENCE OF SUBSTANCES WITH THIOL FUNCTIONS AND OF THEIR REAGENTS ON THE FRAGILITY LYSOSOMES

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Abstract—The influence of different sulphydryl substances and their differences on the fragilization of the lysosomes *in vitro* was studied by measuring the cathepsin liberation at pH 7·4. The thiol substances were found to inhibit the cathepsic activity and stabilize the lysosomes. Amongst the substances containing a disulphide group, only those with two cyclic groups connected by a disulphide bridge fragilize the lysosomes. The thiol-oprive substances including azoic agents which oxydize the thiol group, inhibit the cathepsic activity and increase the fragility of the lysosomes very much. The stabilizing properties of sulphydryl substances on the lysosomes *in vitro* do not seem to be in relation with their radioprotective properties in mammals. Cystamine, inactive *in vitro*, provokes *in vivo* modifications of the stability and the cathepsic activity of the lysosomes, but of a non specific nature, due to its shock provoking power.

DURING the shock provoked by administration of certain substances with thiol functions and by some kinds of radiation, the lysosomes can be hurt and induced to liberate the enzymes they contain.[2–5] Furthermore, since the integrity of the —SH functions of biological membranes is of capital importance in the upkeep of their normal permeability,[6] we have studied the *in vitro* action on the lysosomes of different substances with thiol functions, of some of their disulphides, of substances reacting with the thiol group, and of related substances. Some experiments have also been executed *in vivo*.

MATERIALS AND METHODS

Materials. The lysosomes are collected from the livers of male Wistar rats weighing about 200 g, killed by cervical fracture and bled immediately afterwards. For the *in vivo* experiments the livers are taken after intraperitoneal injection of a substance.

The livers are homogenized in a Potter homogenizer with Teflon pestle in 4 vol. of sucrose 0·25 M.[7] According to a technique described by Weissmann and Thomas,[8] the homogenate is first centrifuged at 800 *g* during 20 min, then the supernatant is again centrifuged at 15,000 *g* during 20 min.

Labilization of the lysosomes. For our *in vitro* experiments we have applied a technique derived from those of Weissmann and Thomas[8] and of Hyttel and Jørgensen,[9] described in detail before.[10] The pellet of centrifugation is suspended in a Sørensen phosphate buffer pH 7·4. The substance to be studied, dissolved in the same buffer, is added in proportions to reach a final sulphur concentration of $2{\cdot}5 \times 10^{-3}$ M. In the case of a substance without sulphur, the final chosen concentration is still $2{\cdot}5 \times 10^{-3}$ M, except when stated otherwise. The solution is then divided into two test

tubes: To the first Triton X-100 is added to a final concentration of 0·1%, to the other a corresponding volume of phosphate buffer. The tubes are incubated during 30 min at 37°. Then they are centrifuged at 25,000 *g* during 20 min. The supernatant is taken for dosing. For the experiments *in vivo*, the pellet of each rat liver is suspended not in 20 ml of phosphate buffer, but in 20 ml of acetate buffer pH 5·1, and incubated during 40 min. This pH was chosen according to Hyttel and Jørgensen,[9] the true labilization taking place in the liver at physiological pH. But apart from this modification all steps are the same as for the experiments *in vitro*.

Dosing of the cathepsins. The cathepsins released by the lysosomes during incubation are dosed in the supernatant by a polarographic method described by Homolka and Soušek[11] slightly modified.[10,12] Bovine albumin is used as a substrate. The activity is measured at pH 3·8 in arbitrary units. The activity measured in the supernatant in the presence of Triton X-100 is considered as representing the total cathepsic activity (= 100 per cent). It is compared with the activity in the sample incubated without Triton and this latter expressed in per cent of the total activity. The influence of a substance on the fragilization of the lysosomes is estimated by comparison of the percentages obtained in the presence and absence of the substance in the same liver homogenate.

In certain control experiments we have applied a different method of determining the proteolytic activity, described by Rinderknecht *et al.*,[13] the substrate being prepared according to the manner described by these authors from hide powder on which remazol brilliant blue (Hoechst) had been fixed. Since this method is less sensitive than the polarographic method, the pellet containing the lysosomes has not been dissolved in phosphate buffer but in distilled water at a ratio of 6 ml for 10 g of liver, in order to obtain a more concentrated cathepsin solution after centrifugation. For each test 10 mg of powdered hide, 3 ml of citrate buffer pH 3·8 and 0·2 ml of supernatant containing the cathepsins are taken and incubated together during 3 hr at 37°. Then the extinction at 595 nm is measured; it is proportional to the quantity of liberated dye. In the supernatant the phenol functions were dosed with the help of the Folin reagent.[14] After precipitation of the proteins the positivity of this reaction is in connection with the enzymatic activity.

Statistical analysis. The results represented on the tables are means. The number of results they cover is indicated (n). As a statistical test we have applied the paired series test,[15] in which the enzymatic activity or fragilization in the presence and absence of a substance are compared. The difference between the control activities without a substance and the activities with a substance of the same series is considered statistically significant, if P is inferior to 0·05. The values $P<0{\cdot}05$, $<0{\cdot}01$ and $<0{\cdot}001$ are marked respectively with one, two or three asterisks after the mean of the activities measured after exposure to the substance.

Since the results of the cathepsic activity and of the fragility of the lysosomes may vary from one preparation and one experiment to the other (for 25 control preparations we find $93{\cdot}04 \pm 4{\cdot}51$ per cent for the total activity, and $48{\cdot}36 \pm 2{\cdot}80$ per cent for the fragilization value), the results should be compared by pairs, which means horizontally, on the first three tables.

Substances. The substances we have used are generally p.a. purchased commercially, except the following; their origin is indicated on Tables 1–3 unless they figure below.

TABLE 1. INFLUENCE OF VARIOUS THIOLS AND DISULPHIDES ON THE FRAGILIZATION OF LYSOSOMES *in vitro* AT THE SULPHUR CONCENTRATION OF $2{\cdot}5 \times 10^{-3}$ M

Substances	n	P	Controls		Treated	
			Total activity	Fragilization (%)	Total activity	Fragilization (%)
Mercaptoethanol[3]	5	−	88·8	37·2	69·2*	32·2
Cysteamine	5	+	92·0	36·8	85·6	32·6
Cystamine	5	+	92·0	36·8	78·4	36·2
L 6188	5	+	93·6	41·4	92·0	40·4
Selenocystamine	5	−	93·6	41·4	86·0*	84·2**
Cystein[5]	5	+	92·0	36·8	83·6	34·6
Penicillamine[5]	7	−	94·3	43·1	75·4**	32·4*
Glutathione (SH)[4]	7	+	102·3	43·3	92·3*	35·5*
Glutathione (SS)[3]	7		102·3	43·3	94·6	45·0
4-Mercaptopyridoxine[5]	5	−	102·8	31·4	82·0*	27·0
5-Mercaptopyridoxine[5]	5	+	102·8	31·4	82·4*	28·2
Thioglycolic acid[3]	5	−	75·6	60·2	72·4	40·4**
Dithiothreitol[3]	5		104·8	32·4	64·0*	30·4
UCB 3983	5	−	100·8	38·8	98·0	29·0
DTNB[1]	5		102·8	33·8	112·4	76·0***
Dithio α-dipyridine	6		83·3	58·3	61·8**	88·7***
Dithio β-dipyridine	7		84·6	57·4	71·4**	64·9*
Dithio γ-dipyridine	6		83·3	58·3	66·3*	82·7**
Dithio δ-dipyridine	6		83·3	58·3	65·7*	80·5**

Number of experiments, P– the radioprotective power in mammals, if known (+ or −). For the abbreviations see paragraph *Substances*.

Sources: [1] Aldrich, [2] BDH, [3] Calbiochem, [4] Fluka, [5] Merck, [6] Sigma.

TABLE 2. INFLUENCE OF SUBSTANCES REACTING WITH THIOL GROUPS ON THE FRAGILIZATION OF LYSOSOMES *in vitro* AT THE CONCENTRATION OF $2{\cdot}5 \times 10^{-3}$ M

Substances	n	Controls		Treated	
		Total activity	Fragilization (%)	Total activity	Fragilization (%)
Iodoacetic acid[5]	5	92·0	31·8	66·4*	31·8
N-Ethyl-maleimide[2]	5	92·0	31·8	68·8**	62·6**
Na *p*-chloromercuribenzoate[3]	5	106·0	40·0	94·8	90·2**
Iodoacetamide[3]	5	67·2	44·0	55·6*	43·4
Na *m*-arsenite[5]	5	93·6	45·6	84·0	48·4
K tellurite (Sat)[2]	6	93·3	46·7	80·0*	58·3
Methylphenylazoformate[3]	5	98·4	35·4	72·6	92·6**
Diamide[3]	5	98·0	35·0	82·8**	57·0*
Alloxane[5]	5	90·4	36·2	87·6	49·2*
$AgNO_3$ (1/10) (Sat)[5]	6	84·0	59·3	79·3	96·7***
$CuSO_4$ (1/10)[5]	5	70·0	53·6	66·8	96·2**

(Sat), substance is not completely soluble at the given concentration; 1/10, the concentration is $2{\cdot}5 \times 10^{-4}$ instead of $2{\cdot}5 \times 10^{-3}$ M.

See legend of Table 1.

TABLE 3. INFLUENCE OF VARIOUS SUBSTANCES ON THE FRAGILIZATION OF LYSOSOMES AT THE CONCENTRATION OF $2{\cdot}5 \times 10^{-3}$ M, UNLESS OTHERWISE STATED

Substances	n	Controls		Treated	
		Total activity	Fragilization (%)	Total activity	Fragilization (%)
$CaCl_2$[5]	6	91·7	54·0	79·0	30·5**
$MgCl_2$[5]	5	74·0	54·2	74·4	50·6
$FeCl_2$ (1/10) (Sat)[5]	5	61·6	53·8	63·6	57·8
$FeCl_3$ (1/10)[5]	5	61·6	53·8	66·0	56·8
$CrCl_3$ (Sat)[5]	5	96·8	37·2	91·6	26·4
$CoCl_2$ (Sat)[5]	5	90·8	42·2	90·4	24·4*
$Co(NH_3)_6Cl_3$[5]	5	90·8	42·2	82·0	26·8
$ZnSO_4$ (1/10)[5]	5	77·2	60·0	80·0	41·8**
EDTA (1/10)[5]	5	80·8	59·4	76·8*	37·4*
Na_2SO_4[5]	5	69·2	59·6	68·8	61·0
Na_2SO_3[5]	5	108·0	37·4	114·8	48·0*
Na_2SeO_3[5]	5	96·4	54·6	74·0**	74·4**
$Na_2S_2O_3$[5]	5	110·0	42·0	128·0**	62·6*
$Na_2S_2O_4$[5]	5	110·0	42·0	127·6	47·2
KCN[5]	5	105·6	34·4	88·8	42·8
ATP[6]	5	102·8	45·0	91·2	27·2*
Urea[5]	5	73·6	51·4	75·6	53·6
Thiourea[5]	5	73·6	51·4	72·4	50·4
Glyvenol	5	66·8	59·2	72·8	60·4

See legends to Tables 1 and 2.

Cystamine 2 HCl and its ureic derivative, bisureidoethyl disulphide (NH_2–CO–NH–CH_2–CH_2–S–)$_2$ (L 6188) were gifts from Labaz, Brussels. Cysteamine HCl was a gift from Braco, Milan. Sodium thioethane sulphonate ($NaSO_4$–CH_2–CH_2–SH) (UCB 3983) was a gift from Union Chimique, Brussels. Selenocystamine (NH_2–CH_2–CH_2–Se–)$_2$ 2 HCl was synthesized by Dr. M. Renson and Dr. C. Draguet of the Laboratory of Organic Chemistry, University of Liège. 6,6′ dithio α, β, γ and δ-dipyridine carboxylic acids were synthesized for us by Dr. J. Delarge of the Pharmaceutical Institute, University of Liège. Glyvenol was kindly put at our disposal by Dr. J. Druey, Ciba, Basle. Remazol brilliant blue was a gift from Hoeschst, Frankfurt a/M.

In addition to the abbreviations already mentioned we have used: DTNB for 5,5′-dithiobis-2-nitrobenzoic acid, EDTA for disodium salt of ethylendiaminetetraacetic acid, and ATP for adenosine triphosphate.

RESULTS

Experiments in vitro. The results of the experiments on lysosome fragilization are given on three tables. On the first we have assembled the substances containing a thiol group, some disulfides and the selenic derivative of cystamine, on the second the substances without —SS— or —SH which react to different degrees with thiol groups, and on the third the substances which can influence the stability of the lysosomes, or more generally the tissular permeability. The incubation time has been chosen so as to obtain a fragilization of the control organites not far from the value of 50 per cent. For each substance the total activities (that is in the presence of Triton)

for the same amount of enzyme in the presence and absence of the substance are compared, as well as the fragilization. This comparison between the total activities on one hand and the fragilization on the other allows to show a possible action (on the lysosomes or cathepsins) other than a fragilization.

Table 1 shows that certain substances containing thiol groups stabilize the lysosomes, but they also diminish the total cathepsic activity. Selenocystamine, contrary to cystamine, increases the permeability of the lysosomes. DTNB and the four derivatives of pyridine carboxylic acid exercise a fragilizaing influence on the lysosomes, the other disulphides appear to be inactive. Contrary to DTNB, the derivatives of pyridine carboxylic acid diminish also the total cathepsic activity. On this table indications on radioprotective power in mammals are based on published data[16] and on personal research (for L 6188, UCB 3983 and selenocystamine); possible sensitizations are not taken into account.

Table 2 shows that most substances combining with —SH groups, several of which are used either as blocking agent or as reagent of the —SH group, increase the permeability of the lysosomes on one hand, but diminish the total activity of the cathepsins.

On Table 3 we see that a substitution of sulphur in sodium sulphite by selenium renders the molecule more active, as we have already seen for cystamine. Ca^{2+}, Zn^{2+}, Co^{2+}, ATP and rutin[10] plainly diminish the lysosomal permeability, glyvenol on the contrary, active *in vivo* on the vascular permeability, is inactive in our experimental conditions.

The decrease of the total cathepsin activity in the presence of certain thiol substances, derivatives of pyridine carboxylic acid (Table 1), and of thioloprive substances (Table 2) is due to an inhibiting effect on the cathepsic activity, since it appears as well, when these substances are added to the cathepsins liberated either by Triton X-100 or distilled water before incubation with the substrate.

The substances modifying the total activity have also been tried on cathepsic solutions incubated in presence of powdered hide. They inhibit the cathepsic activity. The only exception we have found is the case of DTNB, which does not inhibit the cathepsic activity in the polarographic method but well in the colorimetric method.

Experiments in vivo. Cystamine administered by parenteral injection provokes in the animal a prolonged hypotension,[17] accompanied by a series of blood modifications which we find also in other forms of shock.[18] We also know,[19] that the lysosomes are rendered more fragile by the shock. We have seen above that cystamine is inactive *in vitro*. On Table 4 we find, 3 hr after i.p. injection of 150 mg of cystamine 2 HCl, that is at the peak of the shock, a decrease of the total quantity of cathepsins

TABLE 4. INFLUENCE OF A i.p. INJECTION OF CYSTAMINE 2 HCl (150 mg/kg) ON THE LABILIZATION OF LYSOSOMES IN THE RAT

Delay	n	Total activity	Labilization (%)
0	10	40·9 ± 4·9	13·2 ± 1·8
25 min	6	45·0 ± 4·8	16·5 ± 1·7
3 hr	10	29·3 ± 4·7	19·5 ± 1·7

The results are means followed by their standard error.

as well as a fragilization. These modifications are not surprising since injection of cystamine, as other forms of shock, provokes a liberation of enzymes of lysosomal origin.[3,12]

DISCUSSION

Our experiments are confined to the study of the influence of various substances on the labilization of lysosomes at a given concentration and pH (7·4). Furthermore the measuring of enzymatic activity is restricted to the proteolytic activity at one pH only (3·8). We know that there exist several cathepsins, and that their optimal pH's differ. Though in the labilization tests pH 7·4 may not be the optimum pH for some of the substances studied, we have chosen it, because it may be considered as being the most physiological one.

Certain sulphydryl substances exercise a stabilizing action on the lysosomes *in vitro* (see f.inst. Weissmann[19]). There is no connection between this action and the radioprotective power of these substances in mammals, since it is found in substances with radioprotective power as well as without (Table 1). These substances inhibit moreover the total cathepsic activity in our experimental conditions, while substances containing —SH groups activate certain cathepsins,[20] and substances reacting with thiols inactivate them.[21] It seems that the activation or inhibition at pH 3·8 depend on the concentration of the added —SH, in our experiments with bovine albumine as well as with powdered hide as a substrate (Table 5). It is also possible, that the effect

TABLE 5. CATHEPSIC ACTIVITY EXPRESSED IN OPTICAL DENSITY (E) IN THE PRESENCE OF GROWING DOSES OF PENICILLAMINE WITH POWDERED HIDE AS A SUBSTRATE

Penicillamine (in added μM)	E
0	0·462
0·005	0·508
0·025	0·508
0·05	0·495
0·25	0·346
0·5	0·307
2·5	0·350

of the thiols, at least regarding cathepsin D, may not be due to an action on the cathepsin itself, but to an indirect effect on exopeptidases present in the preparation and acting on the peptides liberated by the cathepsic activity.[22]

The permeability of biological membranes depends partly on the —SH groups it contains (see f.inst. Knauf and Rothstein[23]). Merishi and Grassetti[24] and Grassetti and Murray[25] have shown that a disulphide derivative of pyridine carboxylic acid combines with —SH groups of the cellular membrane. This property of substances with this structure explains on one hand the increase of lysosomal fragility, and on the other the inhibition of cathepsic activity in our experimental conditions. DTNB which acts in the same way on the —SH groups,[26] has some influence on the permeability of the lysosomes, but it does not diminish the total cathepsic activity measured

by the polarographic method, but with powdered hide as a substrate. This discrepancy does not seem to be due to a modification in the repartition of cathepsins between the soluble and the particulate phase[27,28] under the influence of the substance. It may be due to the fact that the quantity of cathepsins needed with the hide method is more important than with the polarographic method. Indeed the result depends on the relation of the quantities of supernatant (containing the cathepsins) and of DTNB (Table 6). This discrepancy may therefore be explained by the presence of activators (or other proteolytic enzymes) in the supernatant, and of inhibitors to which the affinity of DTNB is not the same.

TABLE 6

DTNB (ml)	Cathepsic activity (%)	Supernatant (ml)	Cathepsic activity (%)
0·01*	104	0·01	139
0·05	86	0·05	136
0·10	88	0·10*	108
0·20	74	0·20	98
0·30	65	0·30	94
0·40	59	0·40	84

Cathepsic activity in the presence of increasing quantities of DTNB (12·1 mg/ml) for a constant quantity of supernatant (0·1 ml) (left), and for increasing quantities of supernatant (containing the cathepsins) in the presence of a constant quantity of DTNB (0·1 ml) (right). The activities have been measured by polarography. The results are expressed in percentage of the activity without DTNB.
* Quantities of DTNB and supernatant used in the experiments of Table 1.

The other disulphides (cystamine, L 6188, glutathion SS), the formulas of which contain no hexagonal cycles, are inactive. The substitution of sulphur by selenium in cystamine and in sodium sulphite (selenocystamine and Na_2SeO_3) renders the molecule active or more active on the fragilization of the lysosomes.

The substances reacting with the —SH groups inhibit the cathepsic activity and fragilize the lysosomes. Diamide, and above all methylphenylazoformate, azoic agents, oxydize the reduced glutathion (2GSH → GSSG),[1,29] fragilize the lysosomes and inhibit the cathepsins. This fragilization by diamide has recently been demonstrated by Malbica.[30] The fact that mono-iodo-acetic acid does not fragilize the lysosomes, while *N*-ethylmaleimide is active, may be due to a factor of pH and of penetration.[31] ATP has a stabilizing effect, as shown by Markley and Smallman[32] *in vivo* and by Malbica and Hart[33] *in vitro*. The ions combining with —SH fragilize the lysosomes, others as Ca^{2+}, Zn^{2+} and Co^{2+} have on the contrary a stabilizing effect. According to Ignarro *et al.*[34] it would appear that Zn^{2+} inhibits the phosphodiesterase, an enzyme inactivating cyclic AMP.

The injection of cystamine *in vivo*, provoking a strong shock at the dose used in radioprotection,[18] diminishes after an adequate delay the total quantity of particulate cathepsins, while it enhances the fragility of the lysosomes. These modifications at the lysosomal level correspond with those observed in other forms of shock.[2,19] They are accompanied by an increase of the cathepsic activity in the blood.[12] It is therefore a non specific action.

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