Effect of ATP on Cysteine and Other Thiol-Induced Labilization of Rat Liver Lysosomes (35743)

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Cysteine was previously reported by Keiser *et al.* (1) to labilize rabbit liver lysosomal β-gluronidase. However, the effect of adenosine triphosphate (ATP) on cysteine-induced lysosomal labilization was not tested, although the same authors reported that in sucrose solutions, ATP increased the solubilization of lysosomal β-glucuronidase attained with streptolysin S.

Much of the current emphasis pertaining to thiols has been on the role of anti-inflammatory agents (AIA) on disulfide–sulfhydryl interchange reactions (SS–SH) in serum proteins (2, 3). Whatever roles cysteine and other thiols play in structural rearrangements of lysosomal membranes through disulfide interchange remains obscure.

In view of the possible role of lysosomes in the inflammatory process, it was of interest to establish the effects of various AIA and ATP on cysteine-induced lysosomal labilization. In a prior study, ATP was shown to stabilize lysosomal fractions (4). The present report provides evidence for the stabilizing effect of ATP on lysosomal fractions exposed to cysteine and other thiols. In conjunction with the above study, cysteine also enhanced lysosomal fraction adenosine triphosphatase (ATPase).

Materials and Methods. All reagents used were of analytical grade. The sucrose used in the preparation of lysosomal fractions was of ultrapure grade, free of ribonuclease, obtained from the Mann Research Laboratories. Vitamin A alcohol and chloroquine (diphosphate salt) were obtained from Sigma Chemical Co.; phenylbutazone from Geigy Pharmaceutical Co.; hydrocortisone from C. Pfizer & Co.; indomethacin from Merck, Sharpe & Dohme; and mefenamic acid and flufenamic acid from Park, Davis & Co.

SPF, male Sprague-Dawley rats, weighing 150–200 g, were maintained on Purina Chow and water *ad libitum*. All livers were obtained from rats which were exsanguinated by decapitation.

Preparation of rat liver M-L fractions was as described in a previous report (4). A 10% (w/v) rat liver homogenate was freed of high density particles by low spin centrifugation (755*g* and 1935*g*). The final lysosomal enriched supernatant was spun at 27,000*g* for 15 min. The resulting pellet was washed and resuspended in 0.25 *M* sucrose to give a final suspension containing 5.5 to 6.5 mg of protein/ml.

In the isolation of microsomal membranes and mitochondria, the liver was rapidly removed and immediately placed in an ice slurry of 0.25 *M* sucrose. The liver was weighed and macerated and the mitochondria were extracted from a 10% (w/v) homogenate as previously described (5). Microsomal membranes were isolated from a 10% (w/v) rat liver homogenate according to Siekevitz (6).

The incubation mixture contained the following: lysosomal fraction (M-L), 1.2–2.0 mg of protein; sucrose–Tris acetate buffer (0.25 *M* sucrose in 0.04 *M* Tris acetate) pH 7.4; 5×10^{-3} *M* $MgCl_2$ whenever ATP was used, and test compounds as indicated. The final volume of the incubation mixture was 4.0 ml and incubation was for 60 min at 37°. Addition of ethanolic solutions of various anti-inflammatory agents (AIA) were such that the final concentration of ethanol did not exceed 1% (v/v). At the end of the incubation period, the entire mixture was spun at 15,000*g* for 15 min at 0°. The resulting clear supernatant containing free acid hydrolases (nonsedimentable activity) was assayed. All values, unless otherwise indicated, are ex-

TABLE I. Labilization of M-L Fractions by Various Thiols as Compared to Ascorbate.[a]

Compound ($\times 10^{-4}$ M)	% of control[b]	
	Acid phosphatase	β-Glucuronidase
GSH, 1	137 ± 10	165 ± 29
Dithioerythritol, 1	260 ± 36	206 ± 23
Cysteine, 6	990 ± 145	850 ± 248
Ascorbate, 1	1163 ± 205	700 ± 137

[a] ± SD from 3 rats.

[b] The percentage values are based on the following control values expressed as units of enzyme released per milligram of N (total no. of rats, 3). Definitions of units and enzyme assays are described in a previous report (4). Acid phosphatase: 1.45 ± 0.09 units/mg of N; β-glucuronidase: 1.81 ± 0.10 units/mg of N.

pressed as percentage of control (absence of test compound).

Acid phosphatase (EC 3.1.3.2) and β-glucuronidase (EC 3.2.1.31) were assayed according to previously described methods (4).

ATPase (adenosine triphosphatase) activity was measured in a mixture containing: 230 mM sucrose, in 0.04 M Tris acetate buffer, pH 7.4; ATP (disodium salt) 3×10^{-3} M; $MgCl_2$, 5 mM; cysteine, 6×10^{-4} M and subcellular fraction (0.3–0.6 mg of N) in a final volume of 4.0 ml. The mixture was incubated for 60 min at 37° and the reaction was stopped with an equal volume of 20% (w/v) trichloroacetic acid. The protein was separated by centrifugation at 3000 rpm and a suitable aliquot was removed from the supernatant. Net phosphate release was interpreted as a measure of ATPase activity. Inorganic phosphate (P_i) was determined by the method of Gomori (7) and P_i release expressed as micromoles per milligram of nitrogen. The latter was determined by a micro-Kjeldahl technique (4).

Results. The earlier work of Keiser *et al.* (1) did not show if thiols other than cysteine also labilized lysosomal fractions. For this study, the concentration of cysteine and ascorbate was fivefold less than those used by the above authors. The thiol data presented in Table I are compared with that of ascorbate since it labilizes lysosomes in a manner reminiscent of vitamin A (1, 8). As shown in Table I, cysteine at 6×10^{-4} M will produce an effect similar to that observed with ascorbate at 1×10^{-4} M.

Cysteine, when tested at 1×10^{-4} M showed no significant effect in labilizing lysosomes. The lack of labilizing effects at the lower concentration may be attributed to the crude M-L preparation's ability to rapidly oxidize cysteine. Both reduced glutathione (GSH) and dithioerythritol labilized M-L fractions.

In preliminary studies, the disulfides, cystine and oxidized glutathione (GSSG) at equivalent concentrations were ineffective.

The release of lysosomal acid phosphatase as induced by the test thiol compounds can be blocked by the addition of the nucleotide ATP but not by GTP, IMP, ADP, or AMP. As shown in Table II, ATP in the absence of added thiol, reduces acid phosphatase and β-glucuronidase release to 28 and 55% of the control value, respectively, during the 60-min incubation period. However, the stabilizing effect on acid phosphatase release of ATP plus thiol is not as complete as that observed with ATP alone with the exception of glutathione (GSH). This incomplete blocking action of ATP is particularly noticeable when cysteine is present in the incubation mixture. On the other hand, ATP appears to have little effect on blocking β-glucuronidase release in the presence of thiol.

ATP also blocked the labilizing effect of ascorbate and vitamin A on M-L lysosomal fractions as shown in Table III. The effect of either vitamin on β-glucuronidase in the presence of ATP was similar to that shown for cysteine above.

In view of the above data, it was of interest to determine if cysteine affected ATPase activity present in M-L fractions. The ques-

TABLE II. ATP and Thiol-Induced Labilization.[a]

Additions (M)	% of control[b]	
	Acid phosphatase	β-Glucuronidase
ATP, 3×10^{-3}	28 ± 2	55 ± 5
Dithioerythritol, 6×10^{-4}	408 ± 59	274 ± 49
+ ATP, 3×10^{-3}	56 ± 18	129 ± 35
GSH, 6×10^{-4}	144 ± 8	147 ± 9
+ ATP, 3×10^{-3}	34 ± 10	132 ± 12
Cysteine, 6×10^{-4}	1000 ± 130	863 ± 238
+ ATP, 3×10^{-3}	282 ± 97	740 ± 226

[a] ± SD, 3 rats.

[b] See Table I footnote *b*. Acid phosphatase: 1.49 ± 0.08 units/mg of N; β-glucuronidase: 1.72 ± 0.08 units/mg of N.

tion of a lysosomal membrane bound ATPase was speculated on by Duncan (9). However, Thinés-Sempoux (10) presented evidence indicating that the membranes of lysosomes isolated from animals injected with Triton WR-1339 or Dextran 500 resembled the plasma membrane in their phospholipid or cholesterol composition. Lysosomal ATPase activity might be derived from a part of the ATPase-containing cell membrane formerly limiting a phagosome and later forming—after a phagosome–lysosome fusion—part of the lysosomal membrane. This hypothesis seems to be supported by the findings of Allison (11) and Straus (12), who found ATPase activity in highly puried kidney lysosomes and by those of Wachstein and Besen (13), who demonstrated ATPase activity in kidney epithelial cell lysosomes by electron microscopy.

The M-L preparations used for these studies are contaminated with ATPase-containing mitochondria, microsomes, and other cell particulates according to earlier electron micrographs (4).

An attempt was made to localize the ATPase activity by separate cellular fractionations of rat liver microsomal, mitochondrial, and lysosomal fractions. All cellular fractions were incubated under conditions identical to that described for the M-L fraction in the Materials and Methods section above. The data obtained are summarized in Table IV, where the effect of cysteine on the above cellular fractions is compared. Table IV shows that cysteine stimulates an ATPase associated with the M-L fraction. Since this fraction is rich in lysosomes that are readily stabilized by ATP (4), it is possible that the cysteine-sensitive ATPase activity observed may be of lysosomal nature. In preliminary experiments, neither GSH nor dithioerythritol stimulated M-L fraction ATPase.

The possibility that anti-inflammatory agents may affect cysteine-induced labilization was also investigated in the hope of

TABLE III. Effect of ATP on Ascorbate and Vitamin A-Induced Lysosomal Labilization.[a]

Additions (M)	Enzyme release (% of control)[b]	
	Acid phosphatase	β-Glucuronidase
Ascorbate, 1×10^{-4}	1139 ± 165	707 ± 134
+ ATP, 3×10^{-3}	275 ± 46	515 ± 41
Vitamin A, 1×10^{-4}	662 ± 96	412 ± 6
+ ATP, 3×10^{-3}	267 ± 14	341 ± 20

[a] ± SD, 3 rats.

[b] See Table I footnote *b*. Acid phosphatase: 1:55 ± 0.06 units/mg of N; β-glucuronidase: 1.78 ± 0.08 units/mg of N.

TABLE IV. Effect of Cysteine on the ATPase Activity of Various Cellular Components.[a]

Fraction source	P_i released (μmoles/mg of N)		
	Control	Control + cysteine	% Change
Microsomal	44.8	40.8	— 8.9
membrane (2)	34.0	36.1	+ 1.2
Mitochondrial	29.0	31.3	+ 7.9
(2)	28.6	27.8	— 1.4
Lysosomal (M-L)	24.3	36.2	+ 49
(3)	9.8	34.0	+248
	20.4	55.0	+170

[a] Method for determining ATPase activity described in the Materials and Methods section. Number of rats used is shown in parentheses.

obtaining some clue as to a common mode of action of these structurally diverse compounds.

None of the nonsteroidal agents tested affected cysteine-induced labilization except phenylbutazone which gave a small decrease in acid phosphatase ($p<0.05$) release. On the other hand, hydrocortisone enhanced cysteine-induced labilization of lysosomes as evidenced by an increase in both acid phosphatase and β-glucuronidase release ($p<0.05$). The above data are presented in Table V. In preliminary studies, neither ATP block of cysteine-induced labilization nor M-L ATPase activity was affected by any of the anti-inflammatory agents tested.

Discussion. It was previously suggested that lysosomal acid phosphatase and β-glucuronidase release sites differ (4). The finding that ATP in the presence of a test thiol compound is more effective in inhibiting acid phosphatase than β-glucuronidase release lends further support to the above contention. The stimulation of ATPase activity by cysteine occurred only with M-L fractions (Table IV). ATP, in the absence of cysteine exerted a positive stabilizing effect on both acid phosphatase and β-glucuronidase release. From these data, it would appear that lysosomal acid hydrolase activity may be controlled to some extent by a membrane bound ATPase system since neither microsomal nor mitochondrial enriched fractions containing ATPase were stimulated by cysteine.

The coenzyme, NADPH, required for glutathione reductase participation in sulfhydryl–disulfide interchange reactions has been found to be as effective as cysteine, at equimolar concentrations, in labilizing our M-L fractions. The possible relationship between anti-inflammatory agents and a

TABLE V. Effect of Various Anti-inflammatory Agents on Cysteine-Induced Labilization.[a]

AIA (1×10^{-4} M)	% of Cysteine control[b]	
	Acid phosphatase release	β-Glucuronidase release
Chloroquine	110 ± 10	102 ± 12
Phenylbutazone	82 ± 6	88 ± 7
Flufenamic acid	97 ± 9	96 ± 6
Mefenamic acid	98 ± 5	98 ± 8
Indomethacin	95 ± 9	108 ± 5
Hydrocortisone	132 ± 6	151 ± 23

[a] ± SD, minimum of 3 rats.

[b] 6×10^{-4} M cysteine was present in each tube containing the incubation mixture as described in the Materials and Methods section. See Table I footnote *b*. Acid phosphatase: 14.8 ± 1.1 units/mg of N; β-glucuronidase: 13.9 ± 0.9 units/mg of N.

NADPH generating system is currently being studied.

The present data point to the possible significance of ATP as a lysosomal stabilizer.

Summary. Evidence is presented indicating that lysosomal release sites for acid phosphatase and β-glucuronidase differ. ATP stabilized the release of both acid hydrolases. However, ATP in the presence of cysteine stabilized the release of acid phosphatase but not β-glucuronidase. Some of the data obtained suggest the possible involvement of a lysosomal membrane bound ATPase in controlling acid hydrolase release.

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