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Interaction of Lysosomes and Anti-inflammatory Drugs (33938)

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Recent experiments have resulted in a system for the detection of anti-inflammatory drugs based on the stabilization of cellular membranes (1, 2). Lysosomes have not been investigated in depth in this respect. The present study was conducted to establish optimal thermal-labilizing conditions for lysosomes, including pH studies and subsequently, the effect of anti-inflammatory drugs on lysosomal stability and lysosomal enzyme activity per se *in vitro*.

Methods. Preparation of lysosome-rich fractions. Modifications of methods described by Weissmann and Thomas (3) and by Dingle (4) were used for isolating lysosomes. Male, fasted (18 hr) rats (Harlan, Wistar strain, 140–200 g) were sacrificed by dislocation of cervical vertebrae. Livers from two animals were immediately removed, minced, and washed in cold (2°) 0.25 *M* sucrose until free of blood. A 10% (w/v) homogenate (H) was prepared in a Potter-type homogenizer (suspended in a 2° bath) fitted with a motor-driven Teflon pestle and centrifuged (1000*g*, 4°, 10 min) to remove unbroken cells and nuclear debris (N). The supernate (C) from (N) was decanted and recentrifuged (25,000*g*, 4°, 10 min) to obtain a lysosome-rich fraction (L). Aliquots of all fractions were maintained at 2° until assayed.

Initial labilization of lysosomes. In preliminary studies aliquots of (L) resuspended in 0.25 *M* sucrose to volume of (S),[2] were incubated at 45° for 0, 30, 60, 90, and 120 min to effect thermal enzyme release. Maximal release was determined by submitting a duplicate aliquot of (L) to hypotonic media (suspension in distilled water) or Triton ×-100 (0.2%) for 30 min at room temperature. Centrifugation (25,000*g*, 10 min, 4°) yielded two fractions, supernate and residue (containing undisrupted lysosomes); the former aliquots exposed to hypotonic media were also incubated at 45° in 0.25 *M* sucrose for 0, 30, 60, 90, and 120 min to assess stability of the released enzymes.

Enzyme activity. Aliquots of each liver fraction (usually 0.1 ml) were assayed directly for acid phosphatase and β-glucuronidase activities. Enzyme activity represents increase in absorbance (OD) per milliliter after 30-min incubation at 37°. Acid phosphatase activity was measured in all studies as outlined in Sigma Technical Bulletin No. 104. The reaction mixture contained 2.0 mg of *p*-nitrophenyl phosphate, 0.5 ml of distilled water, 0.5 ml of 0.09 *M* citric acid, pH 4.8, and 0.1 ml of liver fraction. The β-glucuronidase activity was measured according to the procedure outlined in Sigma Technical Bulletin No. 105. The reaction mixture consisted of 0.1 ml of 0.01 *M* phenolphthalein glucuronic acid, 1.3 ml of 0.075 *M* phosphate buffer, pH 4.5, and 0.1-ml aliquot of liver fraction. Commercially available acid phosphatase (Worthington) and β-glucuronidase (Worthington) were assayed at three different levels in each study involving lysosomes. Results of 31 experiments with 0.125, 0.250, and 0.500 mg/ml acid phosphatase yielded absorbance values (± SE), respectively, of 0.151 ± 0.002, 0.279 ± 0.005, and 0.512 ± 0.009; 0.1 ml of standard enzyme solutions was used per assay. Results of 31 experi-

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[2] Supernate of (L).

TABLE I. Enzyme Activities of Tissue Fractions Isolated in 0.25 *M* Sucrose.

Enzyme	H	N	C	S	L	S_2	S_{45}
Acid phosphatase[a]	69.24	23.12	41.89	27.05	16.07	1.30	7.92
β-Glucuronidase[a]	16.95	7.65	11.62	5.19	7.39	0.32	2.54

[a] Mean of three experiments; liver fractions were isolated as outlined in "Methods." Values of standard errors ranged from 3 to 10%.

ments with 0.50, 1.50, and 4.50 mg/ml of β-glucuronidase gave absorbance values (±SE), respectively, of 0.068 ± 0.001, 0.207 ± 0.004, and 0.610 ± 0.010; 0.1 ml of standard enzyme solution was again utilized.

Drug studies. Fraction (C) from 7.0 g of liver (two or more pooled livers) homogenized in 70 ml of 0.25 *M* sucrose was divided into 8.0-ml aliquots and centrifuged (25,000*g*, 4°, 10 min) to yield eight lysosome-rich fractions (L), six of which were resuspended in cold (2°) 0.25 *M* sucrose containing 0.005 *M* sodium acetate buffer, pH 6.0, and drug (10^{-4} or 10^{-5} *M*) to a final volume of 8.0 ml, and subsequently divided into two 4.0-ml aliquots for incubation at either 2° or 45°. Control suspensions were treated similarly but did not contain drug.

"Free" enzyme solutions were obtained by suspending the two remaining lysosome-rich fractions in 8.0 ml of distilled water with sequent incubation for 30 min at room temperature, removing 6.0 ml of supernate from each after centrifugation (25,000*g*, 4°, 10 min), combining the two supernates, and adding 6.0 ml of distilled water to a final volume of 18.0 ml. To 3.0 ml of the resulting "free" enzyme solution, 3.0 ml of drug solutions (2.0×10^{-4} or 10^{-5} *M*) were added. and the tubes incubated for 90 min at 2 or 45°. Drug or control solutions contained twice the normal concentration of buffer (0.01 *M*) and sucrose (0.50 *M*) yielding final concentrations as those used in intact lysosome studies.

Results. Increasing amounts of enzyme activities were released at 45° from the lysosome-rich fraction (L) after 0–120 min incubation. Because "free" acid phosphatase activity was reduced after incubation for 120 min, a 90-min incubation interval was selected for subsequent drug studies. Reduction of β-glucuronidase activity was not apparent. Subsequent experiments showed that incubation at 37° (Table II) for 120 min released much less activity; in preliminary ex-

TABLE II. Comparison of Labilizing Methods on Lysosomal Enzyme Release.

	(OD/ml)[a]			
	Acid phosphatase		β-Glucuronidase	
Treatment	2°	45°	2°	45°
Thermal labilization	1.49[b]	7.10	0.31	1.91
Hypotonic labilization	24.69	27.98	6.95	6.90
Triton	42.33	37.79	9.49	9.84
		37°		37°
Thermal labilization[c]	1.09	1.83	0.18	0.54

[a] Lysosome fractions (4.0 ml) incubated for 90 min in 0.25 *M* sucrose containing sodium acetate buffer (0.005 *M*), pH 6.0, at 2 or 45°, and centrifuged (25,000*g*, 4°, 10 min); 0.1 ml of supernate was then removed for enzyme assay; mean values are given.

[b] Values of standard errors ranged from 2 to 9% based on 4–10 replicate determinations.

[c] Lysosome fractions (4.0 ml) incubated for 120 min at 2 or 37° in 0.25 *M* sucrose containing 0.005 *M* sodium acetate buffer, pH 6.0, and centrifuged (25,000*g*, 4°, 10 min); 0.1 and 0.3 ml of the supernate were then removed for assay of acid phosphatase and β-glucuronidase, respectively.

TABLE III. Effect of pH on Thermal Labilization of Lysosomes.

Lysosomes	Temp (°)	(OD/ml)[c] Control	pH 4.0[a]	pH 5.0[a]	pH 6.0[a]	pH 7.0[b]	pH 8.0[b]
Acid phosphatase	2	1.41[d]	0.70	0.72	1.44	1.80	1.84
	45	7.18	17.03	17.93	6.66	5.09	7.27
β-Glucuronidase	2	0.40	0.12	0.07	0.29	0.53	0.65
	45	2.00	2.53	4.67	1.72	2.19	4.23

[a] Incubated for 90 min, 45°, in 0.005 *M* sodium acetate buffer and 0.25 *M* sucrose; controls contained only 0.25 *M* sucrose.

[b] Incubated for 90 min, 45°, in 0.002 *M* sodium phosphate buffer and 0.25 *M* sucrose.

[c] Lysosome fractions (4.0 ml) were incubated at 2 or 45° for 90 min, and centrifuged (25,000 *g*, 4°, 10 min); 0.1 ml of supernate was then removed for enzyme assay; mean values are given.

[d] Values of standard errors based on 6 replicate determinations ranged from 4 to 11%.

periments, incubation at 50° for 120 min produced greater loss of "free" enzyme activities.

Three complete liver fractionations were performed involving the simultaneous determination of acid phosphatase and β-glucuronidase in each fraction as outlined in Table I. Higher enzymic activities of statistical significance were obtained in supernates from heated (S_{45}) compared with nonheated (S_2) lysosomes.

Incubation of lysosomes in distilled water for 90 min at 2° or 45° resulted in release of similar amounts of enzyme activities on comparing the activity of each enzyme in the supernates (2 vs. 45°), indicating lysosome rupture prior to thermal labilization and absence, therefore, of heat-induced release of enzymes. In a separate experiment, fractionation of liver in distilled water gave analogous results on incubation of (L) at 2 or 45° for 90 min. Acid phosphatase and β-glucuronidase were previously shown to be localized rather exclusively in lysosomes (5, 6). Thus these data satisfy the criterion for stating that fraction (L) does indeed contain lysosomes.

The effect of various labilizing techniques on enzyme release is listed in Table II. Disruption of the lysosome-rich fraction by incubation in distilled water results in approximately 74 and 70% release of acid phosphatase and β-glucuronidase, respectively, into the supernate, by comparison with Triton ×-100 which was employed to ascertain maximal release of enzymes. Thermal labilization at 45° effects 19 and 19% release of the latter substances, respectively, compared with Triton ×-100, while labilization at 37° educes only 5 and 5% release of these entities. Thus much less enzymic activity is released at 37 than 45°.

Data listed in Table III indicate that maximal thermal (45°) discharge of acid phosphatase occurs at pH 5.0, *viz.* 45.4% compared with Triton ×-100. β-Glucuronidase is maximally released at pH 5.0 or 8.0, *viz.* 45.4% at pH 5.0 and 42.9% at pH 8.0. At 2°, a trend of incremental release of acid phosphatase and β-glucuronidase occured after lysosomes were incubated progressivlely at pH 4.0 through 8.0. At pH 4.0 or 5.0, however, lysosomes settled out of suspension as did the "free" enzymes (Table IV) when incubated at 2 or 45°. Such lysosomes were not disrupted when this phenomenon occured because they could be disrupted maximally with distilled water. Because of this "settling out" phenomenon and the possibility, therefore, of inadequate mixing on incubation with drugs in subsequent experiments, and also the possibility of labilizing lysosomes further by mechanical shaking, pH 4.0 (or 5.0) was not employed for studying drug effects.

At pH 4.0, 5.0, 6.0, 7.0, no activating or inhibitory actions were seen on "free" enzymes Table IV, except for slight inhibition (14–25%) of acid phosphatase (45° compared with 2°). At pH 8.0, however, acid phosphatase activity (at 45°) is reduced by ap-

TABLE IV. Effect of pH on Free Enzyme Activity.[a]

	Enzyme (OD/ml)			
	Acid phosphatase[b]		β-Glucuronidase	
pH	2°	45°	2°	45°
Control	7.41	6.04	2.44	2.30
4.0[c]	7.33	5.67	2.42	2.30
5.0[c]	7.27	6.33	2.36	2.09
6.0[c]	7.31	6.21	2.42	2.33
7.0[d]	7.09	5.31	2.49	2.41
8.0[d]	7.42	2.92	2.48	2.40

[a] Values are listed as the means of 6 replicate determinations; SE ranged from <1 to 2%.

[b] Free enzyme fractions (4.0 ml) incubated for 90 min at 2 or 45°; 0.1-ml aliquot was then removed for enzyme assay.

[c] Incubated for 90 min, 2 or 45°, in 0.005 *M* sodium acetate buffer and 0.25 *M* sucrose; controls contained only 0.25 *M* sucrose.

[d] Incubated for 90 min, 2 or 45°, in 0.002 *M* sodium phosphate buffer and 0.25 *M* sucrose.

proximately 60%. Such reduction was not observed with "free" β-glucuronidase.

As shown in Table V, neither steroidal nor nonsteroidal drugs prevented thermally-induced release of acid phosphatase from lysosomes. To the contrary, acetylsalicylic acid, phenylbutazone, indomethacin, dexamethasone, and dimethylsulfoxide (DMSO), enhanced labilization at 45°. Mefenamic acid and cortisone[3] had no effect at 10^{-4} or 10^{-5} *M* concentrations (45°).

Data in Table VI show that dexamethasone and dimethylsulfoxide also significantly, but slightly, enhanced thermally-induced labilization of β-glucuronidase. Mefenamic acid, cortisone,[3] acetylsalicylic acid and indomethacin were ineffective. Phenylbutazone was the only compound found to prevent release of β-glucuronidase. On the basis of four to eight replicate studies, none of these drugs at the concentrations studied, inhibited "free" acid phosphatase activity, while only DMSO (5%) reduced the "free" β-glucuronidase activity. Phenylbutazone, however, at the 10^{-3} *M* level, significantly inhibits "free" β-glucuronidase by 59% ($n = 6$, $p < .005$) but still does not affect acid phosphatase. Indomethacin at the 10^{-3} *M* level likewise inhibits β-glucuronidase (12%, $n = 6$, $p < .005$) while acid phosphatase is unaffected.

When individual drug experiments were compared statistically with 46 cumulative controls obtained after completion of all studies, instead of the "paired" controls which were determined in each drug study on a given day, similar results were obtained with four exceptions. First, phenylbutazone showed no enhancement of thermally-labilized acid phosphatase. Second, phenylbutazone significantly attenuated labilization of β-glucuronidase at both the 10^{-4} and 10^{-5} *M* levels. Third, indomethacin blocked release of β-glucuronidase at both the 10^{-4} and 10^{-5} *M* levels. Fourth, mefenamic acid enhanced thermal labilization of both acid

TABLE V. Effect of Drugs on Release of Acid Phosphatase.

Compound	Conc (*M*)	(OD/ml)	% of control
Acetylsalicylic acid	C[a]	5.47[b]	—
	10^{-4}	9.12	167[c]
	10^{-5}	5.89	108
Phenylbutazone	C	4.62	—
	10^{-4}	6.88	149[d]
	10^{-5}	5.83	126[c]
Indomethacin	C	4.86	—
	10^{-4}	9.63	198[d]
	10^{-5}	5.26	108
Dexamethasone + 1% DMSO	C	8.51	—
	10^{-4}	10.51	124[e]
	10^{-5}	8.78	103
DMSO	C	6.50	—
	5%	10.34	159[d]
	1%	7.08	109

[a] C = controls without drug. Lysosomal fractions (4.0 ml) were incubated for 90 min at 45° in 0.005 *M* sodium acetate buffer, pH 6.0, containing 0.25 *M* sucrose then centrifuged (25,000*g*, 4°, 10 min); 0.1 ml of supernate was then removed for enzyme assay.

[b] Values given as means based on 4–10 replicate determinations; SE varied from 4 to 16%.

[c] $p < 0.05$.

[d] $p < 0.01$.

[e] $p < 0.02$.

[3] Suspended in 1% DMSO.

TABLE VI. Effect of Drugs on Release of β-Glucuronidase.

Compound	Conc (*M*)	(OD/ml)	% of control
Acetylsalicylic acid	C[a]	1.55[b]	—
	10^{-4}	1.98	128
	10^{-5}	1.60	103
Phenylbutazone	C	1.30	—
	10^{-4}	0.84	65[c]
	10^{-5}	1.35	104
Indomethacin	C	1.47	—
	10^{-4}	1.07	73
	10^{-5}	1.37	93
Dexamethasone + 1% DMSO	C	2.06	—
	10^{-4}	2.48	120[c]
	10^{-5}	2.31	112
DMSO	C	1.60	—
	5%	1.97	123[c]
	1%	1.68	105

[a] C = controls without drug: Lysosomal fractions (4.0 ml) were incubated for 90 min at 45° in 0.005 *M* sodium acetate buffer, pH 6.0, containing 0.25 *M* sucrose then centrifuged (25,000*g*, 4°, 10 min); 0.1 ml of supernate was then removed for enzyme assay.

[b] Values given as the means based on 3–10 replicate determinations; SE varied from 2 to 13% except for indomethacin controls (25%).

[c] $p < 0.01$.

phosphatase and β-glucuronidase at the 10^{-4} *M* level. Drug effects using liver slices were similar to those utilizing isolated lysosomes, Table VII.

Discussion. Results demonstrate that a lysosome-rich fraction can be isolated routinely from rat liver and by employing thermal labilization at pH 6.0, release of large amounts of acid phosphatase and β-glucuronidase can be induced with little loss of "free" enzyme activity. The significant difference in lysosomal enzyme release between the S_2 and S_{45} fractions facilitates studying the influence of agents or procedures on the release of enzymes from lysosomes. Disruption of lysosomes with distilled water or Triton ×-100 yields some indication of the maximal amounts of releasable enzyme activity and also provides a source of "free" enzymes.

Why certain drugs enhance thermal labilization of acid phosphatase but not β-glucuronidase cannot be stated with certainty. Since acid phosphatase, in part, is located in the lysosomal membrane (7, 8), it is possible that certain drugs could alter the membrane in such fashion that acid phosphatase is released from the external surface of the membrane, but concomitantly these drugs could stabilize the membrane as a whole to disruption, thereby preventing release of β-glucuronidase from the interior. However, initial studies in this laboratory indicate that β-glucuronidase is also associated in part, with the lysosomal membrane fragments.

In studying the thermal labilization of lysosomes, most investigators have relied solely on incubations for varying periods of time up to 3 hr at 37°. Weissmann stated (9, 10) that only slight amounts of total acid phosphatase or β-glucuronidase activity (of rabbit liver lysosomes) are released after 60- to 90-min incubation at 37°. Alpers and Isselbacher (11) have also shown that incubation of rat liver lysosomes at 37° for up to 120 min releases little or no acid phosphatase or β-glucuronidase.

We have studied lysosomes under conditions related to one of the cardinal signs of inflammation, *viz*, abnormal temperature, and found a rather large release of lysosomal enzymes. Under these conditions only phenylbutazone, of the anti-inflammatory drugs studied, antagonized release of β-glucuronidase but not acid phosphatase. That anti-inflammatory drugs exert their prime pharmacologic action at the level of the lysosome is thus very doubtful. In previous studies by Weissmann (9, 10), retardation of acid hydrolase release from rabbit liver lysosomes during incubation at 37° for 60 min by cortisome, cortisol, or their acetates was apparently not significant. Moreover, De-Duve (6) has shown that cholesterol inhibits release of certain enzymes from lysosomes and this compound certainly does not produce therapeutic effects in human arthritides. Other investigators (12) stated that acetylsalicylic acid, hydrocortisone, and chloroquine at 10^{-3} and 10^{-4} *M* concentrations inhibit release of acid phosphatase and β-glu-

TABLE VII. Effect of Phenylbutazone and Cortisone on Release of Lysosomal Enzymes from Liver Slices.

Treatment	Conc (M)	Tissue wt (mg)	Incubation temp (°)	Absorbance/ml of filtrate[a]			
				Acid phosphatase	% of control	β-Glucuronidase	% of control
Sucrose	0.25	100	2	0.56	—	0.06	—
Sucrose + phenylbutazone	$0.25 + 10^{-4}$	100	2	0.55	98	0.04	67
Sucrose	0.25	100	45	1.10	—	0.27	—
Sucrose + phenylbutazone	$0.25 + 10^{-4}$	100	45	1.73	157	0.21	78
Sucrose + cortisone (in 1% DMSO)	$0.25 + 10^{-4}$	100	45	1.37	124	0.25	93
Sucrose	0.25	200	45	1.68	—	0.31	—
Sucrose + cortisone (in 1% DMSO)	$0.25 + 10^{-4}$	200	45	2.52	150	0.48	155

[a] Liver slices, obtained by sectioning with a Stadie-Riggs microtome, were suspended for 90 min in 4.0 ml of 0.25 M sucrose containing 0.005 M sodium acetate buffer, pH 6.0, with or without drug. After incubation, solutions were filtered with Whatman no. 1 paper; 0.1 ml and 0.3 ml of filtrate were assayed for acid phosphatase and β-glucuronidase activities, respectively.

curonidase from rat liver lysosomes. However, since no data were given concerning absolute amounts of enzyme activity released, it may be assumed that release of a very small percentage of the total available enzymes at 37° was being effected. Nonetheless, those studies with acetylsalicylic acid are not in agreement with data in this report and the difference could be due to the apparent absence of buffer in the cited incubation medium. The pH values of our lysosome suspensions in 0.25 M sucrose were about 5.6–6.0, but addition of acetylsalicylic acid to 10^{-3} M lowers pH considerably, and incubation at pH 4.0 or 5.0 causes lysosomes or "free enzymes" to settle out of suspension or solution. We therefore employed buffers at pH 6.0 in all drug studies, readjusting pH, if necessary, before adding drug solutions to lysosomes.

Although Weissman (9) showed that 5×10^{-4} M concentrations of certain steroids (*e.g.*, progesterone, etiocholanolone, lithocholic acid) cause considerable release of enzymes from lysosomes (labilization), and we confirmed this for etiocholanolone (at 37°), many nonanti-inflammatory steroids and nonsteroidal anti-inflammatory drugs stabilize erythrocyte suspensions to heat-induced (53°) hemolysis (2). This suggests that either lysosomal membranes differ from erythrocyte membranes in composition, thus explaining different drug actions in the two systems, or mechanisms for drug action differ, *per se,* in the two systems.

Data reported herein do not deny the theoretical role of lysosomes in the pathogenesis of inflammation but do indicate that under conditions of one of the cardinal signs of inflammation, augmented temperature, anti-inflammatory drugs, in general, do not stabilize lysosomes and furthermore, at certain concentrations, actually labilize these subcellular entities. The latter drug-induced labilization could relate to analgesic-induced renal necrosis (13, 14), ulcerogenic properties of these anti-inflammatory drugs (15), aseptic bone necrosis sequent to corticosteroid therapy (16), and inflammation induced by dimethylsulfoxide in rats (17) or humans (18). Moreover, stabilization becomes less important if anti-inflammatory drugs inhibit the released lysosomal enzymes, *per se,* as demonstrated with phenylbutazone and indomethacin. Whether these drugs inhibit other lysosomal enzymes remains to be determined. Anti-inflammatory drugs may subsequently be found to exert a protective effect on lysosomes when nondisease-related labilizing methods are employed for lysosomal disruption.

Work of Frimmer *et al.* (19) indicates that the mechanism for lysosomal enzyme release may be more complex than simple membrane rupture. Lysosomal enzymes were rapidly released when phalloidin was added to isolated, perfused rat liver, but had no effect on isolated lysosomes from bovine leukocytes. Potassium was released from liver just before lysosomal enzymes were liberated and this corresponded in time with the destruction of liver cell membranes as seen in the electron microscope (19). Increases in the number of lysosomes and cytolysomes in synovial cells of membranes collected from rheumatoid arthritics may therefore be part of a pathologic change occurring after some form of injury to the whole cell (20, 21). Moreover, such changes are certainly not specific for rheumatoid arthritis (20).

Summary. Optimal conditions for thermal labilization (45°), *in vitro*, of isolated, hepatic, lysosomal suspensions were defined with respect to the release of acid phosphatase and β-glucuronidase, and the stability of these enzymes obtained by hyptonic disruption of lysosomes. Neither steroidal nor non-steroidal anti-inflammatory drugs, in general, stabilized lysosomes with respect to release of both enzymes when the lysosomes were labilized thermally; some drugs enhanced labilization. Indomethacin (10^{-3} *M*) and phenylbutazone (10^{-3} *M*) inhibited β-glucuronidase obtained from lysosomes. Results are discussed.

Addendum. Tanaka and Iizuka (22) have recently published on work similar to that reported herein. Our results are essentially in agreement with theirs and both conflict with an earlier report by Miller and Smith (12). In particular, our results confirm the labilization of lysosomes by acetylsalicylic acid in acidic sucrose medium and inhibition of "free" *beta*-glucuronidase activity by phenylbutazone and indomethacin. We, however, did not incubate lysosomes with mild shaking as indicated in the above work (22). Agitation of the pastic tubes due to the swirling water of the bath plus thermal agitation at 45° seemed sufficient for our purposes.

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