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EFFECT OF ANTI-INFLAMMATORY DRUGS ON LYSOSOMES AND LYSOSOMAL ENZYMES FROM RAT LIVER*

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Abstract—Steroidal and nonsteroidal anti-inflammatory drugs were tested for their capacity to stabilize, *in vitro*, lysosomes and inhibit lysosomal enzymes. Lysosome membrane stability was measured by determining the effects of drugs on the release of aryl sulfatase and β-glucuronidase from lysosomes which were suspended in a hypo-osmotic sucrose buffer. Lysosomes obtained from a heavy mitochondrial (3500 *g*) rat liver fraction were found to be highly sensitive to membrane stabilization by naproxen, alclofenac, chloroquine, mefenamic acid, phenylbutazone, hydrocortisone, dexamethasone and methylprednisolone. Ibuprofen and flufenamic acid demonstrated moderate stabilizing activity, while indomethacin, aspirin and clonixin showed only weak activity. Imuran, as well as other anti-inflammatory drugs, was inactive. In addition to their membrane-stabilizing activity, chloroquine was found to be a potent inhibitor of aryl sulfatase and phenylbutazone an inhibitor of β-glucuronidase activity. Hydrocortisone, dexamethasone and paramethasone inhibited aryl sulfatase activity, while no steroid tested was effective as an inhibitor of β-glucuronidase. The data in this report support the hypothesis that anti-inflammatory drugs inhibit the release of enzymes from lysosomes. In addition, several of these drugs may act as inhibitors of lysosomal enzyme activity.

The role of lysosomal enzymes as mediators of the inflammatory process has been a subject of great discussion in recent years [1–8]. Indeed, elevated activity of lysosomal acid hydrolases has been detected in many inflammatory sites, including rheumatoid synovial membranes [9–12]. The contention that lysosomal enzymes do in fact contribute to tissue injury is supported by the knowledge that these enzymes possess the capacity to degrade the various components of connective tissue such as collagen [13, 14], glycoproteins [15], elastin [16] and protein–mucopolysaccharide complexes [17].

A function which has been attributed to many anti-inflammatory drugs, both steroidal and nonsterodial, is that of lysosome membrane stabilization. Implicit in this concept is the belief that these drugs in some way prevent the labilization of lysosomes and thus block the release of deleterious lysosomal enzymes. The lysosome-stabilizing effects of many anti-inflammatory drugs have appeared in the literature during the past decade. Although a number of these studies were performed *in vitro* [2, 5, 18–20], Ignarro, Weissmann *et al.* [7, 8, 21, 22] have demonstrated the capacity of such effective anti-inflammatory drugs as hydrocortisone, indomethacin and phenylbutazone to stabilize rat liver lysosomes *in vivo*. Ignarro and Columbo [23] also showed these and other drugs to be active stabilizers of lysosomal granules isolated from guinea pig peritoneal polymorphonuclear leukocytes.

In the studies reported here, several anti-inflammatory drugs were evaluated for their capacity to stabilize the membranes of lysosomes obtained from a rat liver fraction as well as inhibit the activity of lysosomal hydrolases. The observations were made that drugs which were previously thought to act via a stabilizing action on lysosome membranes also act as enzyme inhibitors. In addition, certain anti-inflammatory drugs, such as paramethasone, were found to act as enzyme inhibitors but not membrane stabilizers. Drugs in this class have always been characterized exclusively as lysosome membrane stabilizers with reference to their actions associated with the lysosome.

MATERIALS AND METHODS

Preparation of liver fraction. The two larger lobes of liver from a decapitated 250–300 g male Wistar/Lewis non-fasted rat (Charles River) were excised, weighed and placed in cold 0.25 M sucrose–0.02 M Tris acetate, pH 7.4. After mincing, a 10% homogenate was prepared in a cold portion of the same buffer. A 40-ml capacity Dounce tissue grinder (with the standard loose clearance pestle-A) was used with manual execution of ten to fourteen complete strokes of the pestle. The homogenate (40 ml) was then centrifuged at 600 *g* for 5 min at 4° in a Sorvall RC2-B centrifuge. Supernatants were removed, diluted with an equal volume of the same buffer, and centrifuged at 3500 *g* for 15 min at 4°. The intact pellets were resuspended in 4 ml of 0.45 M sucrose–0.04% glycogen–0.02 M Tris acetate, pH 7.4, by employing a gentle stroking action with a glass rod. Five sec of vortexing may or may not be necessary to achieve a homogeneous suspension free of clumping. The suspensions were kept at 4°C for 20 min prior to use.

Assay of lysosome membrane integrity. Labilization and stabilization of lysosomes were ascertained by

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determining the osmotic release of lysosomal marker enzymes by a modification of the method of Ignarro [19]. Briefly, a suspension of lysosome-containing crude liver fraction in 0.45 M sucrose 0.04% glycogen–0.02 M Tris acetate, pH 7.4, was warmed to 25° for 5 min; aliquots (0.2 ml) were added to glass tubes containing 2 ml of 0.18 M sucrose–0.04 M Tris acetate, pH 7.4, at 25°, with or without drug, and the tubes were incubated at 37° for 60 min in a Dubnoff metabolic shaker set at 75–80 agitation cycles/min. The incubation was terminated by high speed centrifugation (27,000 *g* for 15 min at 4°) after transfer of the samples to 15-ml polyethylene tubes. This high centrifugal force was employed in order to sediment much of the suspended particles, thereby rendering the clear supernatant fractions more suitable for subsequent measurements of enzyme activity. Supernatants were decanted into small plastic tubes and maintained at 4° until assayed for enzyme activity.

Assay of lysosomal enzyme inhibition. For these studies, aliquots (1.0 ml) of the high speed (after incubation of lysosomes, without drug) supernatant were incubated with various concentrations of drug at 37° for 20 min in the presence of the substrate (immersed in the appropiate buffer) specific for the lysosomal hydrolase under investigation.

Lysosomal enzyme assays. Acid phosphatase (EC 3.1.3.2; orthophosphoric monoester phosphohydrolase), aryl sulfatase (EC 3.1.6.1.; aryl-sulfate sulfohydrolase and β-glucuronidase (EC 3.2.1.31; β-D-glucuronide glucuronohydrolase) were employed as lysosomal marker enzymes. Acid phosphatase activity was determined by a modification of the method of Torriani [24]. The formation of *p*-nitrophenol from *p*-nitrophenylphosphate (CalBiochem) was measured. One ml of high speed supernatant (after incubation of lysosomes, with or without drug) was added to 2.0 ml of 0.1 M citrate buffer, pH 4.8. Incubations (at 37° for 22 min) were initiated by the addition of 50 μl of freshly prepared substrate (*p*-nitrophenyl phosphate disodium pentahydrate, 89.9 mg/ml in distilled water) and terminated by the addition of 0.2 ml of cold 4 N sodium hydroxide. Extinction values were determined at 405 nm and for all enzymes were measured with a Bausch & Lomb Spectronic 20 spectrophotometer.

Aryl sulfatase activity was determined by a modification of the method of Roy [25]. The formation of 4-nitrocatechol from *p*-nitrocatechol sulfate (Sigma) was measured. One ml of high speed supernatant (after incubation of lysosomes, with or without drug) was added to 2.0 ml of 0.2 M acetate, pH 5.8. Incubations (at 37° for 22 min) were initiated with 100 μl of freshly prepared substrate (*p*-nitrocatechol sulfate dipotassium salt, 25 mg/ml in distilled water) and terminated with 0.2 ml of cold 4 N sodium hydroxide. Extinction values were determined at 510 nm.

β-glucuronidase activity was determined by a modification of the method of Gianetto and DeDuve [26]. The formation of phenolphthalein from phenolphthalein glucuronic acid sodium salt (Sigma) was measured. One ml of high speed supernatant (after incubation of lysosomes, with or without drug) was added to 2.0 ml of 0.1 M citrate buffer, pH 4.8. Incubation (at 37° for 22 min) was initiated with 50 μl of freshly prepared substrate (phenolphthalein glucuronic acid, 63 mg/ml in distilled water) and terminated with 0.4 ml of 2.2 M glycine–10 N sodium hydroxide buffer, pH 12.5. Extinction values were determined at 540 nm.

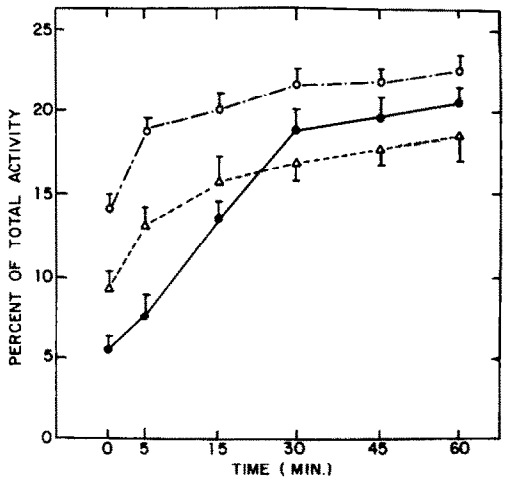

Fig. 1. Release of lysosomal enzymes from a rat liver lysosome suspension. Data are expressed as percentage of total enzyme activity released in 0.18 M sucrose 0.04 M Tris acetate, pH 7.4. Acid phosphatase (○ - - - - - ○), β-glucuronidase (△——△) and aryl sulfatase (●——●) were measured, and total activities yielded extinction values of 1.20 to 1.28 (405 nm), 0.58 to 0.60 (540 nm) and 0.84 to 0.95 (510 nm) respectively. Each value represents the mean ± S. E. of three to eight separate experiments.

Drugs. The drugs employed in this study were: acetylsalicylic acid (Sigma Chemical Co.); flufenamic acid, mefenamic acid and meclofenamic acid (Parke, Davis & Co.); phenylbutazone (Ciba-Geigy Corp.); indomethacin and dexamethasone dihydrogen phosphate (Merck, Sharp & Dohme); fenoprofen (Eli Lilly & Co), chloroquine phosphate (Sterling-Winthrop Research Institute); clonixin (Schering Corp.); naproxen and paramethasone acetate (Syntex Laboratories, Inc.); ibuprofen, methylprednisolone hemisuccinate and hydrocortisone hemisuccinate (The Upjohn Co.); fenbufen (Lederle Laboratories); furobufen (Ayerst Laboratories); flumizole (Pfizer Laboratories Division); alclofenac (Warner-Lambert Research Institute); and tolmetin (McNeil Laboratories, Inc).

All drugs tested were dissolved in dimethylsulfoxide (DMSO), except chloroquine which was dissolved in 0.18 M sucrose buffer. Sodium salts of acidic drugs could not be used since sodium ion interferes with the stability of lysosomes *in vitro* [19]. All the drugs were soluble under the defined incubation conditions and they produced no appreciable alteration of the pH of the incubation media. The small amounts of DMSO employed as a vehicle did not influence the stability of lysosomes *in vitro*.

RESULTS

Latency of acid phosphatase, aryl sulfatase and β-glucuronidase in a hypo-osmotic medium. The data in Fig. 1 illustrate the latency of three lysosomal enzymes during incubation of the 3500 *g* liver fraction in 0.18 M sucrose–0.04 M Tris acetate buffer, pH 7.4. It appears that acid phosphatase is more easily

Table 1. Release of enzymes from a rat liver lysosome fraction

Experimental condition	Release of enzymes from a 3500 *g* fraction of rat liver*	
	Aryl sulfatase	β-Glucuronidase
Total unsedimentable enzyme activity†	0.71 ± 0.04 (100)‡	0.49 ± 0.04 (100)
Zero-min unsedimentable enzyme activity§	0.28 ± 0.02 (10)	0.25 ± 0.02 (13)
Sixty-min unsedimentable enzyme activity§	0.65 ± 0.01 (23)	0.46 ± 0.03 (24)
Sixty-min sedimentable enzyme activity‖	0.48 ± 0.03 (68)	0.33 ± 0.04 (67)

* Data represent extinction values (510 nm for aryl sulfatase; 540 nm for β-glucuronidase) expressed as the mean ± S. E. (n = 4).

† An aliquot (0.05 ml) of liver fraction was added to 2 ml of 0.2% Triton X-100–0.04 M Tris acetate, pH 7.4, and incubated for 15 min at 37°. An aliquot of 1.0 ml was used in the enzyme assay.

‡ Numbers in parentheses represent per cent of total unsedimentable enzyme activity and were calculated from extinction values and appropriate dilution factors (aliquot of liver fraction and supernatants for enzyme assays).

§ An aliquot (0.2 ml) of liver fraction was added to 2 ml of 0.18 M sucrose–0.04 M Tris acetate, pH 7.4, and centrifuged either immediately or after a 60-min incubation period. An aliquot of 1.0 ml was used in the enzyme assay.

‖ After a 60-min incubation period (as in footnote §), the liver fraction was centrifuged and the resulting pellet disrupted in 0.2% Triton X-100–0.04 M Tris acetate, pH 7.4. An aliquot of 0.25 ml was used in the enzyme assay.

released or available than β-glucuronidase and that the latter, at least through 17 min of incubation, is more easily released than aryl sulfatase.

Release of lysosomal enzymes from a rat liver fraction. The data in Table 1 illustrate the release of aryl sulfatase and β-glucuronidase from lysosomes obtained from a heavy mitochondrial (3500 *g*) rat liver fraction. Incubation at 37° for 60 min results in the release of 23–24 per cent of total unsedimentable enzyme activity, while 10–13 per cent of the total is released immediately prior to any incubation (0 min unsedimentable enzyme activity).

In these and subsequent experiments, all determinations of total unsedimentable enzyme activities were conducted by incubation of appropriate aliquots of lysosome suspension in 0.2% (v/v) Triton X-100 in 0.04 M Tris acetate, pH 7.4, at 37° for specific time intervals ranging from 5 to 60 min. Under these conditions, complete solubilization of enzymes measured was obtained at the end of a given 15-min incubation period.

Effect of incubation media on latency of lysosomal enzymes. The data in Fig. 2 illustrate the latency of aryl sulfatase during incubation of the 3500 *g* liver fraction in various concentrations of sucrose, buffered with 0.04 M Tris acetate, pH 7.4. Lysosomes obtained from this heavy mitochondrial fraction possess minimum stability in a severe hypo-osmotic environment such as that afforded by 0.05 M and 0.10 M sucrose buffer. Osmotic protection is presented to lysosomes incubated in 0.18 M sucrose. However, when incubated in 0.27 M or 0.36 M sucrose, only 10 per cent of total unsedimentable aryl sulfatase activity was released after 60 min of incubation at 37°.

Effect of sucrose on stability of a rat liver fraction containing lysosomes. The data in Table 2 illustrate the marked capacity of buffers containing various concentrations of sucrose to influence the capacity of phenylbutazone to stabilize lysosomes and thus inhibit the release of aryl sulfatase. As demonstrated in Fig. 2, there is a loss of latency of aryl sulfatase when lysosomes are incubated in 0.05 M sucrose at 37°. Phenylbutazone (10^{-3} to 10^{-5} M) is significantly less effective in stabilizing lysosomes when tested in 0.05 M sucrose as compared to its capacity to inhibit

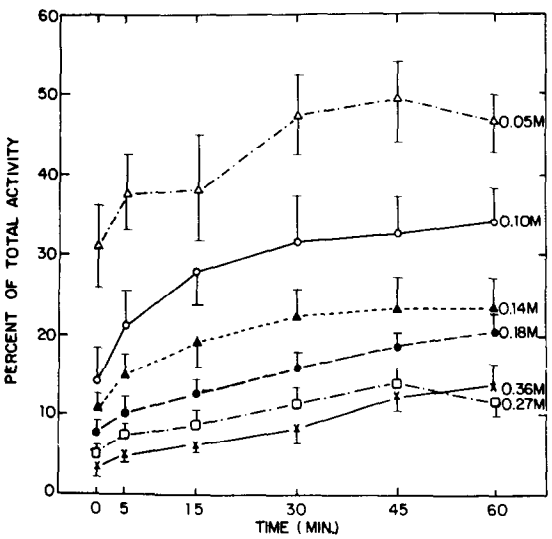

Fig. 2. Effect of sucrose concentration on stability of rat liver lysosomes *in vitro*. Data are expressed on the basis of total unsedimentable enzyme (aryl sulfatase) activity, which was determined by incubation of the appropriate aliquot (0.05 ml) of lysosomal suspension in 2 ml of 0.2% Triton X-100–0.04 M Tris acetate, pH 7.4, for 15 min. Incubation media contained various molar concentrations of sucrose in 0.04 M Tris acetate, pH 7.4. Values are expressed as the mean ± S. E. of four separate experiments.

Table 2. Effect of phenylbutazone on stability of rat liver lysosomes *in vitro*—effect of sucrose concentration

Concn (M)	Per cent inhibition of release of aryl sulfatase* 0.05 M Sucrose	0.18 M Sucrose	0.36 M Sucrose
10^{-3}	10.2 ± 5.1†	42.0 ± 6.0‡	33.8 ± 10.3‡
10^{-4}	2.8 ± 2.3	21.7 ± 4.5‡	22.2 ± 9.5
10^{-5}	1.3 ± 0.9	17.3 ± 5.5‡	4.5 ± 2.1

* Incubation media: various concentrations of sucrose were buffered in 0.04 M Tris acetate, pH 7.4. Incubations were conducted at 37° for 30 min as described previously.

† Each value represents the mean ± S. E. of six separate experiments. Actual extinction values (510 nm) for controls (incubations of aliquots of lysosome suspension without drug in various concentrations of sucrose) were: 1.52 ± 0.08 (0.05 M sucrose), 0.76 ± 0.11 (0.18 M sucrose), and 0.49 ± 0.07 (0.36 M sucrose) (mean ± S. E.) for the 3500 *g* fraction.

‡ Significantly different from control values ($p < 0.05$).

enzyme release in 0.18 M sucrose. In 0.36 M sucrose, phenylbutazone is less effective in stabilizing lysosomes at low (10^{-5} M) concentrations as compared to results obtained with 0.18 M sucrose.

Effect of nonsteroidal anti-inflammatory drugs on release and/or activity of lysosomal enzymes. The data in Table 3 indicate that certain nonsteroidal anti-inflammatory drugs prevent the release and/or activity of aryl sulfatase and β-glucuronidase from lysosomes present in the 3500 *g* rat liver fraction. Naproxen, alclofenac, chloroquine, mefenamic acid and phenylbutazone were among the more potent agents found, while ibuprofen, flufenamic acid and meclofenamic acid demonstrated moderate activity. Fenoprofen, indomethacin, clonixin, acetylsalicylic acid and tolmetin, as well as other anti-inflammatory drugs, did not stabilize lysosomes or inhibit lysosome enzyme activity.

Effect of nonsteroidal anti-inflammatory drugs on the activity of enzymes released from lysosomes in the 3500 g liver fraction. The data in Table 4 illustrate that chloroquine had a direct inhibitiory effect on aryl sulfatase activity, while naproxen, ibuprofen and other nonsteroidal anti-inflammatory drugs failed to inhibit the activity of this lysosomal hydrolase. β-Glucuronidase activity was significantly inhibited by phenylbutazone whereas chloroquine had no effect on the activity of this enzyme.

Effect of steroidal anti-inflammatory drugs on release and/or activity of lysosomal enzymes. The effect of steroidal anti-inflammatory drugs on the release and/or activity of enzymes from lysosomes (3500 *g* liver fraction) is illustrated in Table 5. Soluble forms of hydrocortisone, methylprednisolone and dexamethasone were extremely potent as inhibitors of the release and/or activity of the lysosomal enzymes, aryl sulfatase and β-glucuronidase. Paramethasone was unique among the steroids tested in that it had an effect on aryl sulfatase but not β-glucuronidase.

Effect of steroidal anti-inflammatory drugs on the activity of enzymes released from lysosomes in the 3500 g liver fraction. Figure 3 illustrates the effects of steroidal anti-inflammatory drugs on the activity of aryl sulfatase and β-glucuronidase. Hydrocortisone,

Table 3. Effect of nonsteroidal anti-inflammatory drugs on lysosomal enzymes *in vitro*

Drug	Marker enzyme	Per cent inhibition of release and/or activity of lysosomal enzyme* 10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M
Chloroquine	AS†	61	49	17	18
	β-G†	42	24	16	2
Flufenamic acid	AS	(315)‡	23	10	0
Mefenamic acid	AS	(106)	35	12	4
Meclofenamic acid	AS	9	22	23	2
Phenylbutazone	AS	43	34	21	9
	β-G	65	38	24	8
Acetylsalicylic acid	AS	13	7	6	7
Indomethacin	AS	(18)	11	8	1
Fenoprofen	AS	0	0	0	0
Clonixin	AS	6	9	10	7
Naproxen	AS	38	18	13	11
Ibuprofen	AS	23	12	1	0
Tolmetin	AS	(100)	1	1	0
Alclofenac	AS	39	26	11	10
Flumizole	AS	15	11	(107)	(102)
Fenbufen	AS	17	12	12	4
Furobufen	AS	(111)	(103)	(103)	1

* Aliquots (0.2 ml) of the 3500 *g* fraction and drugs were incubated in 2 ml of 0.18 M sucrose–0.04 M Tris acetate, pH 7.4, at 37° for 60 min as described previously. Each value represents the mean of five to eight separate experiments. Values varied by no more than 5–15 per cent of the corresponding mean in these experiments. Values of 15 per cent inhibition or greater were significantly ($P < 0.05$) different from control.

† AS, aryl suflatase; β-G, β-glucuronidase.

‡ Numbers in parentheses indicate per cent increase in release and/or activity of lysosomal enzyme. Values greater than 30 per cent were significantly ($P < 0.05$) different from control.

Table 4. Direct effect of nonsteroidal anti-inflammatory drugs on lysosomal enzyme activity

Drug	Concn (M)	Per cent inhibition of enzyme activity* Aryl sulfatase	β-Glucuronidase
Phenylbutazone	10^{-3}	5.4 ± 1.7†	72.3 ± 9.5‡
	10^{-4}	3.3 ± 1.2	25.1 ± 5.2‡
	10^{-5}	2.7 ± 0.9	13.7 ± 4.3
Chloroquine	10^{-4}	70.0 ± 9.7‡	0
	10^{-5}	23.0 ± 4.4‡	0
	10^{-6}	12.2 ± 0.6	0
Alclofenac	10^{-3}	0	
Ibuprofen	10^{-3}	0	
Naproxen	10^{-3}	3.2 ± 1.1	
Flumizole	10^{-4}	3.0 ± 0.7	
Flufenamic acid	10^{-4}	6.5 ± 0.5	
Mefenamic acid	10^{-4}	4.5 ± 1.6	
Meclofenamic acid	10^{-4}	7.0 ± 2.4	
Fenbufen	10^{-4}	4.4 ± 2.1	

* Incubation medium: the supernatant obtained from a lysosomal suspension (3500 *g* fraction) after a 60-min incubation at 37° in 0.18 M sucrose–0.04 M Tris acetate, pH 7.4, was employed as the enzyme preparation. Aliquots (1.0 ml) of enzyme were incubated with drug and substrate for 22 min at 37° in either 0.2 M acetate buffer, pH 5.8, or 0.1 M citrate buffer, pH 4.8, for determination of aryl sulfatase and β-glucuronidase activity respectively.

† Each value represents the mean ± S. E. of three to four separate experiments.

‡ Significantly ($P < 0.05$) different from control.

dexamethasone and paramethasone demonstrated a dose-dependent inhibitory effect on the activity of aryl sulfatase, while methylprednisolone was inactive. However, neither hydrocortisone, dexamethasone or paramethasone exhibited a capacity to inhibit the activity of β-glucuronidase.

DISCUSSION

Lysosomes are a distinct group of cytoplasmic organelles, first identified in rat liver but now known to occur in numerous animal tissues, and characterized by their content of a variety of acid hydrolases [27, 28]. Phagocytic cells such as polymorphonuclear leukocytes and macrophages, which accumulate at sites of tissue injury, are known to contain large numbers of granules (lysosomes) in their cytoplasm. The process of endocytosis, which is now well documented [29–31], is accompanied by the concomitant extrusion of lysosomal enzymes from these cells into the extracellular environment [32–37]. Once released, these enzymes have the capacity to destroy the various components of tissue and thus contribute to the inflammatory process and other pathological conditions.

One approach leading to the disruption of tissue injury caused by lysosomal enzymes would be to stabilize lysosomal membranes, thus preventing the release of the deleterious contents. The work of Ignarro and others clearly demonstrates that clinically active steroidal and nonsteroidal anti-inflammatory drugs inhibit the release of acid hydrolases *in vitro* [2, 5, 18–20] and *in vivo* [7, 8, 21, 22]. However, the systems employed in evaluating the lysosome membrane-stabilizing capacity *in vitro* by anti-inflammatory drugs do not in themselves answer the questions of the mechanism of action of the drug under investigation. It is possible that, although an agent seems to act by stabilizing lysosomal membranes because it is said that it inhibits enzyme release from organelles, it might in fact be blocking enzyme activity directly while exacting no influence on membranes.

The liver lysosome fractions employed in the present study and in those reported by other investigators are essentially heavy mitochondrial fractions. The finding of similar rates of release of three latent acid hydrolases argues in favor of the presence of lysosomes. Acid phosphatase was more readily available or released than other lysosomal marker enzymes in these experiments. This may be a reflection of the different capacities by which acid hydrolases are bound to the lysosome membrane [38]. An additional experiment in which β-galactosidase activity was measured revealed data similar to that reported for

Table 5. Effect of steroidal anti-inflammatory drugs on lysosomal enzymes *in vitro*

Drug	Marker enzyme	Per cent inhibition of release and/or activity of lysosomal enzyme* 10^{-3} M	10^{-4} M	5×10^{-5} M	10^{-5} M	10^{-6} M
Hydrocortisone	AS†	64	73		48	43
hemisuccinate	β-G†	45	28		12	4
Methylprednisolone	AS	64	20		7	3
hemisuccinate						
Dexamethasone	AS	100	41	28	14	2
dihydrogen phosphate	β-G	71	25		0	17
Paramethasone	AS	39	28	26	18	6
acetate	β-G	(4)‡	(21)		6	(16)

* Aliquots (0.2 ml) of the 3500 *g* fraction and drugs were incubated in 2 ml of 0.18 M sucrose–0.04 M Tris acetate, pH 7.4, at 37° for 60 min as described previously. Each value represents the mean of four to six separate experiments. Values varied by no more than 5–15 per cent of the corresponding mean in these experiments. Values of 15 per cent inhibition or greater were significantly ($P < 0.05$) different from control.

† AS, aryl sulfatase; β-G, β-glucuronidase.

‡ Numbers in parentheses indicate per cent increase in release and/or activity of lysosomal enzymes.

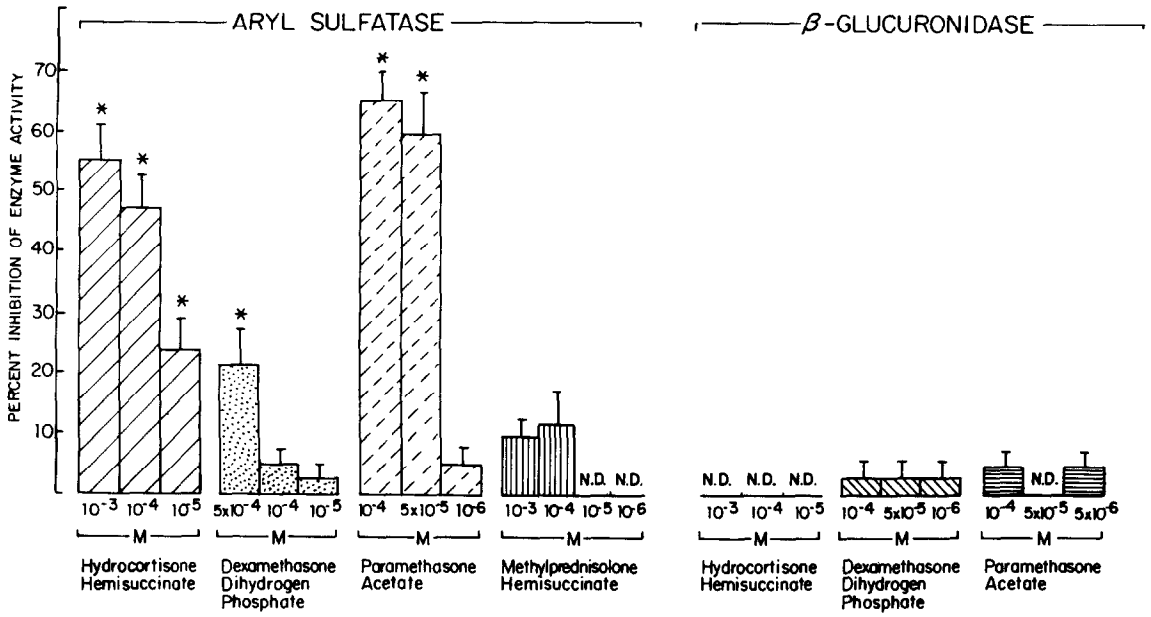

Fig. 3. Direct effect of steroidal anti-inflammatory drugs on lysosomal enzymes. Incubation medium: the supernatant obtained from a lysosomal suspension (3500 *g* fraction) after a 60-min incubation at 37° in 0.18 M sucrose–0.04 M Tris acetate, pH 7.4, was employed as the enzyme preparation. Aliquots (1.0 ml) of enzyme were incubated with drug and substrate for 22 min at 37° in either 0.2 M acetate buffer, pH 5.8, or 0.1 M citrate buffer, pH 4.8, for determination of aryl sulfatase and β-glucuronidase activity, respectively. Each bar represents the mean ± S. E. of two to four separate experiments. The asterisk (*) indicates significantly ($P < 0.05$) different from control. N.D. = no detectable inhibition of enzyme activity.

acid phosphatase. This finding is supported by the work of Ignarro [39] who found the rate of release of various lysosomal enzymes to vary depending upon the enzyme being studied. With reference to the 3500 *g* liver fraction employed in these studies, it is unlikely that the presence of mitochondria would interfere appreciably with the drug-induced effects of lysosomes, since the enzymes being measured are well known to be markers for lysosomes, i.e. acid phosphatase, aryl sulfatase and β-glucuronidase.

It was demonstrated previously that certain anti-inflammatory drugs inhibited the release of enzymes from liver lysosomes incubated in hypo-osmotic sucrose buffer. The data in this report illustrate that similar drug actions are obtained in addition to demonstrating membrane-stabilizing activity of several recently developed anti-inflammatory drugs. Naproxen, alclofenac, ibuprofen, phenylbutazone, chloroquine, flufenamic acid and mefenamic acid exhibited potent lysosome membrane-stabilizing activity, while indomethacin, acetylsalicylic acid, clonixin, fenoprofen, tolmetin and other drugs were inactive. At high concentrations flufenamic acid and mefenamic acid labilized lysosomes. Chlorpromazine has been reported to elicit biphasic effects on lysosomes in that it labilized or stabilized lysosomes *in vivo* at high or low concentrations respectively [40]. Steroids exhibiting potent anti-inflammatory activity, such as hydrocortisone, dexamethasone and methylprednisolone, all demonstrated a marked capacity to stabilize lysosomes.

We report here that, in addition to functioning as stabilizers of lysosome membranes, certain steroidal and non-steroidal anti-inflammatory drugs are very effective inhibitors of lysosomal enzyme activity. Chloroquine was found to block the activity of aryl sulfatase *in vitro*. Chloroquine has been reported to block the activity of rat skin collagenase and bacterial collagenase [41]. Phenylbutazone impaired the activity of β-glucuronidase, and this finding has been observed by other investigators [18, 19, 42]. Tanaka and Iizuka [18] also demonstrated that indomethacin interferes with β-glucuronidase activity. Naproxen, ibuprofen, clonixin and the other nonsteroidal anti-inflammatory agents were found to be inactive as inhibitors of the lysosomal marker enzymes employed in these studies. It was concluded, therefore, that they functioned as membrane stabilizers.

The role of steroids as enzyme inhibitors is well documented [43–47]. However, little is known concerning the effects of steroids on lysosomal enzymes. Data presented in this report clearly demonstrate hydrocortisone, dexamethasone and paramethasone to be inhibitors of aryl sulfatase activity. None of the steroids were effective blockers of β-glucuronidase activity and, therefore, appear to be somewhat selective as enzyme inhibitors. Hydrocortisone has been reported to block the activity of kallikrein, thereby interfering with bradykinin formation [48]. DeDuve *et al.* [49] have suggested that hydrocortisone inhibits acid phosphatase.

Ignarro [8, 19, 20] showed that paramethasone stabilized lysosomal membranes. Employing identical incubation conditions, we were unable to confirm this observation. However, lysosome membrane stabilization is a nonselective phenomenon. Therefore, if one enzyme is prevented from being released from lysosomes by a specific drug, the same would hold true

for any marker enzyme examined. We made the observation that, while paramethasone appeared to inhibit the release of aryl sulfatase, it did not impair the release of β-glucuronidase. Upon evaluating the direct effect of paramethasone on these two acid hydrolases, it was found that aryl sulfatase was inhibited, but not β-glucuronidase. This data would argue in favor of paramethasone acting as an enzyme inhibitor, while possessing no capacity to stabilize lysosomal membranes.

We have presented data illustrating a time-dependent release of lysosomal marker enzymes from rat liver lysosomes at 37° using a sucrose–Tris acetate buffer, pH 7.4. It was observed that varying the sucrose concentration had an effect on the rate of release of aryl sulfatase. When the effects of phenylbutazone, as a membrane stabilizer, were evaluated in 0.05 M, 0.18 M and 0.36 M sucrose media, the 0.05 M sucrose being very hypo-osmotic stressed the lysosomal membranes to such an extent that enzyme release was greatly accelerated. Consequently, large concentrations of drug had to be employed in order to obtain inhibition of enzyme release, while low concentrations of drug were inactive. The 0.36 M sucrose is more iso-osmotic to the lysosomes, and phenylbutazone was active at high concentration but no activity was observed at low concentrations. The 0.18 M sucrose buffer yielded more consistent results and was employed for all experiments involving the use of drugs.

In summary, various steroidal and nonsteroidal anti-inflammatory drugs were demonstrated to influence lysosomal enzymes by stabilizing lysosomal membranes and thus inhibiting enzyme release. In addition, several of these drugs were also found to exert a direct inhibitory effect on several lysosomal marker enzymes. Therefore, when evaluating the effects of drugs with established or potential anti-inflammatory activity, as lysosomal membrane stabilizers, it becomes necessary to test for direct inhibition of lysosomal hydrolase activity in order to fully understand the possible mechanisms of actions of drugs in inflammatory diseases.

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