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EFFECTS OF ANTI-INFLAMMATORY DRUGS ON THE STABILITY OF RAT LIVER LYSOSOMES *IN VITRO*

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Abstract—Certain steroidal and nonsteroidal anti-inflammatory drugs were found to stabilize *in vitro* lysosomes obtained from a heavy mitochondrial rat liver fraction. Membrane stabilization was measured by determining the effects of drugs on the release of acid phosphatase and β-glucuronidase from lysosomes. Specific experimental conditions were necessary for the measurement of drug-induced stabilization. The 3500 *g* liver fraction, prepared in a Ten Broeck tissue grinder and incubated at 37° in sucrose–tris acetate buffer (pH 7·4) free of sodium ion, was found to be the most suitable source of lysosomes in these studies. Chloroquine, hydrocortisone, acetylsalicylic acid, phenylbutazone, flufenamic acid and niflumic acid exhibited good lysosome membrane-stabilizing activity, while indomethacin showed only moderate activity. Mefenamic acid, ibufenac, gold, Imuran and Cytoxan as well as dozens of drugs from other therapeutic classes were inactive. When lysosomes were incubated in a buffer containing sodium acetate rather than tris acetate or at 45° rather than 37°, anti-inflammatory drugs no longer stabilized but actually enhanced the labilization of lysosomes. The results of these studies indicate that considerable caution must be exercised in the preparation, handling and incubation of lysosomes in order to reveal the stabilizing capacity *in vitro* of anti-inflammatory drugs.

THE DISCOVERY of lysosomes as subcellular organelles containing acid hydrolases capable of degrading many different types of macromolecules[1] has led to the formulation of concepts implicating lysosomes in a variety of pathological conditions. One particular manifestation of the inflammatory process is the accumulation and labilization of lysosomes at sites of injury[2–7] and evidence exists for the possible key role of lysosomal enzymes as mediators of this process.[4–6] It is well known, for example, that lysosomal enzymes possess the capacity to degrade completely the components of connective tissue such as collagen,[8,9] protein–mucopolysaccharide complexes,[10] mucopolysaccharides,[11] glycoproteins[11] and elastin.[12]

It appears, therefore, that one approach to interrupting the inflammatory process might be to block lysosome labilization by the use of agents which stabilize the lysosomal membrane. Although many studies have been reported on this approach, the current status on lysosome membrane stabilization *in vitro* by anti-inflammatory drugs is quite unclear. For example, Tanaka and Iizuka[13] reported that high concentrations of some anti-inflammatory drugs stabilize rat liver lysosomes, but only to a very limited extent. Brown and Schwartz[14] reported that such drugs actually labilize rather than stabilize rat liver lysosomes. Other investigators have reported that only steroidal anti-inflammatory drugs stabilize rat[5] and rabbit[6,15] liver lysosomes *in vitro* against labilization induced by artificial means, such as ultraviolet irradiation, endotoxin and vitamin A.

The results of extensive investigation in our laboratory indicate that not all rat liver lysosome fractions are suitable for measurement of drug-induced membrane stabilization and that the experimental manipulations in the preparation, handling and incubation of lysosomes are critical. One rat liver fraction, sedimenting between 600 and 3500 *g*, appears to be suitable for the detection of lysosome membrane-stabilizing agents. A preliminary report of these findings has appeared earlier.[16]

MATERIALS AND METHODS

Drugs. Acetylsalicylic acid, sodium salicylate and hydrocortisone were obtained from Sigma. Chloroquine (phosphate) and hydroxychloroquine (phosphate) were purchased from Winthrop Research Laboratories. Flufenamic acid, mefenamic acid and meclofenamic acid were generously supplied by Parke, Davis & Company. Merck. Sharp & Dohme provided indomethacin and gold sodium thiomalate, and Boots Pure Drug Company Ltd. supplied ibufenac (4-isobutylphenylacetic acid). Paramethasone acetate was provided by Syntex Laboratories.

All drugs tested were dissolved in dimethylsulfoxide (DMSO), except chloroquine and hydroxychloroquine which were dissolved in 0·18 M sucrose solution. Concentrations of all stock drug solutions were 0·2 M and the solutions were prepared just prior to use. Sodium salts of acidic drugs could not be employed reliably, since sodium ion interfered with the stability of lysosomes *in vitro* (see Results). All the drugs were soluble under the incubation conditions used 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*. Further, DMSO did not modify the membrane-stabilizing effects of water-soluble drugs (chloroquine and paramethasone).

Preparation of liver fractions. The two larger lobes of liver from a decapitated and exsanguinated 250 g male Sprague–Dawley nonfasted rat (Charles River) were excised, weighed and placed in cold 0·25 M sucrose–0·02 M Tris acetate, pH 7·4. After rinsing and mincing, a 10% homogenate was prepared in a cold portion of the same buffer. A 40-ml capacity Ten Broeck tissue grinder (with a standard loose clearance pestle) was used with manual execution of ten complete strokes of the pestle. The homogenate (40 ml) was then centrifuged at 600 *g* for 5 min at 4° in a Sorvall RC 2-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 rinsed gently twice in 0·45 M sucrose–0·04% glycogen–0·02 M Tris acetate, pH 7·4, and resuspended in 4 ml of the same buffer. A light liver fraction was prepared by centrifuging the 3500 *g* supernatant fraction at 25,000 *g* for 15 min at 4°. The suspensions were kept at 4° for the duration of the experiment.

Although no ultrastructural examinations were made, the heavy mitochondrial fraction (3500 *g*) as described above and by other investigators[13] is a crude liver preparation containing lysosomes as well as other organelles. Microscopic examination revealed absence, for the most part, of contaminating nuclei and cellular membrane debris. Considerable numbers of mitochondria were present and this was substantiated by the detection of significant activity of cytochrome c oxidase. Similar observations were made and results obtained for the 25,000 *g* liver fraction. Thus, it is clear that the liver fractions employed in these studies do not represent isolated or

purified lysosomes and may, in fact, represent mitochondrial fractions contaminated with lysosomes and perhaps peroxisomes.

Assays of lysosome membrane stability. Labilization or stabilization of lysosomes was ascertained by determining the release of lysosomal marker enzymes. The suspension of crude liver fraction containing lysosomes was warmed to 25° for 5 min and 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 added drug, and the tubes were incubated at 37° for 15 min in an Eberbach metabolic shaker set at 75 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 glass tubes and maintained at 4° until assayed for lysosomal enzyme activity.

Lysosomal enzyme assays. Acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase) 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.[17] The formation of *p*-nitrophenol from *p*-nitrophenyl phosphate (Calbiochem) was measured. One ml of high speed supernatant (after incubation of lysosomes, with or without drug) and 1·0 ml of distilled water were added to 1·0 ml of 0·3 M citrate buffer, pH 4·8. Incubation (at 37° for 20 min) was initiated by the addition of 0·04 ml of freshly prepared substrate (*p*-nitrophenyl phosphate, 79 mg/ml in distilled water) and terminated by the addition of 0·2 ml of 4 N sodium hydroxide. Extinctions, at 405 mμ, were measured with a Zeiss spectrophotometer.

Beta-glucuronidase activity was determined by a modification of the method of Gianetto and DeDuve.[18] The formation of phenolphthalein from phenolphthalein glucuronide (Calbiochem) was measured. One ml of high speed supernatant (after incubation of lysosomes, with or without drug) and 1·0 ml of distilled water were added to 1·0 ml of 0·3 M citrate buffer, pH 4·8. Incubation (at 37° for 20 min) was initiated with 0·04 ml of freshly prepared substrate (phenolphthalein glucuronide, 63 mg/ml in distilled water) and terminated with 0·4 ml of 2·2 M glycine–10 N sodium hydroxide buffer, pH 12. Extinctions, at 540 mμ, were measured with a Zeiss spectrophotometer.

Under these experimental conditions (i.e. 3-fold dilution of post-incubation lysosome-drug supernatant), none of the drugs tested significantly inhibited either acid phosphatase or β-glucuronidase. Phenylbutazone inhibited β-glucuronidase (35 per cent) only at concentrations (10^{-3} M) which were never attained in the final enzyme reaction mixtures.

RESULTS

Release of lysosomal enzymes from two rat liver fractions. The data in Table 1 illustrate the release of acid phosphatase and β-glucuronidase from the 3500 *g* and 25,000 *g* rat liver lysosome fractions. Incubation at 37° for 15 min results in the release of 17–27 per cent of total unsedimentable enzyme activity, while only 6–8 per cent of the total is released spontaneously prior to any incubation (0 min unsedimentable enzyme activity). Three additional experiments yielded data similar to those reported

TABLE 1. RELEASE OF ACID PHOSPHATASE AND BETA-GLUCURONIDASE FROM TWO RAT LIVER LYSOSOME FRACTIONS

Experimental condition	Release of enzymes from two fractions of rat liver*			
	Acid phosphatase		Beta-glucuronidase	
	3500 *g*	25,000 *g*	3500 *g*	25,000 *g*
Total unsedimentable enzyme activity†	1·28 ± 0·11 (100)‡	1·42 ± 0·14 (100)	0·22 ± 0·02 (100)	0·31 ± 0·02 (100)
0 min unsedimentable enzyme activity§	0·38 ± 0·02 (7)‖	0·30 ± 0·02 (6)	0·08 ± 0·01 (8)	0·09 ± 0·01 (7)
15 min unsedimentable enzyme activity§	1·36 ± 0·12 (27)	0·94 ± 0·10 (17)	0·24 ± 0·02 (27)	0·27 ± 0·03 (22)
15 min sedimentable enzyme activity‖	0·92 ± 0·11 (72)	1·21 ± 0·15 (85)	0·15 ± 0·02 (70)	0·24 ± 0·03 (78)

* Data represent extinction values (405 mμ for acid phosphatase; 540 mμ for beta-glucuronidase) expressed as the mean ± S.E. ($n = 4$).

† Aliquot (0·05 ml) of liver fraction added to 2 ml of 0·2% Triton X-100 and incubated for 15 min at 37°. An aliquot of 1·0 ml was used in the enzyme assay. Aliquots (0·05 ml) of 3500 *g* and 25,000 *g* fractions contained 0·74 mg and 0·64 mg, respectively, of protein.[19]

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

§ Aliquot (0·2 ml) of liver fraction added to 2 ml of 0·18 M sucrose–0·04 M tris acetate, pH 7·4, and centrifuged either immediately or after 15-min incubation period. An aliquot of 1·0 ml was used in the enzyme assay.

‖ After 15-min incubation period (as in §), the liver fraction was centrifuged and the resulting pellet was disrupted in 2 ml of 0·2% Triton X-100. An aliquot of 0·25 ml was used in the enzyme assay.

in Table 1. An additional experiment in which aryl sulfatase activity[20] (using nitrocatechol sulfate as substrate) was measured revealed data similar to those presented in Table 1.

Note that in these and subsequent experiments all determinations of total unsedimentable enzyme activities were conducted by incubation of appropriate aliquots of crude lysosome suspension in 0·2% (v/v) Triton X-100 in 0·04 M tris acetate, pH 7·4, at 37° (or at 45°) for specific time intervals ranging from 15 to 60 min. Under these conditions, virtually complete solubilization of enzymes measured was obtained at the end of a given 15-min incubation period. Further solubilization of enzymes could not be achieved when incubations were conducted at 37° for 60 min or at 25° or 4° for up to 8 hr.

Effect of incubation temperature and time on stability of rat liver fractions containing lysosomes. This experiment was conducted to obtain data on rates of release of enzymes from two different rat liver fractions. The data (Table 2) illustrate that, for a given liver fraction at a given incubation temperature, acid phosphatase and β-glucuronidase are released at similar rates. Release of enzymes at 37° using the 25,000 *g* liver fraction was less than with the 3500 *g* liver fraction. Elevation of the temperature (45°) promoted a marked increase in the release of both enzymes from each of the two liver fractions studied. An additional experiment in which acid phosphatase activity was

TABLE 2. EFFECT OF INCUBATION TEMPERATURE AND TIME ON STABILITY OF RAT LIVER LYSOSOMES *IN VITRO*

Incubation time† (min)	Release of enzyme as per cent of total enzyme activity*			
	3500 *g*		25,000 *g*	
	AP‡	βG‡	AP	βG
At 37°				
0	7§	8	5	7
5	14	12	9	10
15	28	27	16	21
30	32	32	17	23
45	34	37	18	24
60	41	43	24	26
At 45°				
0	7	8	5	7
5	26	26	26	26
15	46	51	50	50
30	49	55	53	57
45	54‖	59	56	61
60	65	65	64	67

* Total unsedimentable enzyme activity was determined by incubation of appropriate aliquots of liver fraction in 2 ml of 0·2% Triton X-100.

† Incubation medium: 0·18 M sucrose–0·04 M tris acetate, pH 7·4.

‡ AP, acid phosphatase; βG, beta-glucuronidase.

§ Each value represents the mean of three separate experiments. Values varied by no more than 10 per cent of the corresponding mean. Estimates of actual extinction values can be obtained from the data in Table 1.

‖ Data are corrected for slight deterioration of total unsedimentable enzyme activity (less than 15 per cent) which occurred at incubation times exceeding 30 min at 45°.

measured by a modification of the method of Gianetto and DeDuve,[18] using β-glycerophosphate (Sigma) rather than *p*-nitrophenylphosphate as substrate, yielded data similar to those in Table 2.

Effect of sodium ion on stability of rat liver fractions containing lysosomes. The experiments reported by Brown and Schwartz[14] reveal that in the presence of sodium acetate buffer drugs no longer stabilize, but in fact labilize lysosomes *in vitro*. In view of these findings, it was decided to study the effects of sodium acetate and sodium phosphate on lysosome stability. The data in Table 3 illustrate the marked capacity of buffers containing sodium to enhance the release of acid phosphatase from both the 3500 *g* and 25,000 *g* rat liver lysosome fractions. Comparable elevations of the concentration of Tris acetate have no marked effect on the release of acid phosphatase. Similar data were obtained when β-glucuronidase was employed as the marker enzyme.

Effect of pH on stability of rat liver fractions containing lysosomes. The data in Table 4 indicate that rat liver fractions (3500 *g* and 25,000 *g*) incubated at 37° for 15 min possess optimal stability in the range of pH 6·4. Once again the release of acid phosphatase and β-glucuronidase are similar for a given liver fraction.

Effect of experimental conditions on anti-inflammatory drug-induced lysosome membrane stabilization. Anti-inflammatory drugs were tested for their capacity to stabilize lysosomes obtained from two different liver fractions. The data presented in

TABLE 3. EFFECT OF SODIUM ON STABILITY OF RAT LIVER LYSOSOMES *IN VITRO*

Buffer concn* (M)	Release of acid phosphatase† 3500 *g*	25,000 *g*
Sodium acetate (pH 7·4)		
0·002	23‡	10
0·006	41	21
0·020	63	38
0·060	88	68
0·100	99	88
Sodium phosphate (pH 7·4)		
0·002	29	16
0·006	45	25
0·020	68	44
0·060	80	67
0·100	94	84
Tris acetate (pH 7·4)		
0·002	21	5
0·006	25	8
0·020	30	10
0·060	30	11
0·100	38	15

* Incubation medium: 0·18 M sucrose in indicated buffer.

† Expressed as per cent of total unsedimentable enzyme activity, which was determined by incubation of appropriate aliquots of liver fraction in 2 ml of 0·2% Triton X-100 for 15 min.

‡ Each value represents the mean of three separate experiments. Values varied by no more than 10 per cent of the corresponding mean. Estimates of actual extinction values can be obtained from the data in Table 1.

TABLE 4. EFFECT OF pH ON STABILITY OF RAT LIVER LYSOSOMES *IN VITRO*

pH	Release of enzyme as per cent of total enzyme activity* 3500 *g* AP†	3500 *g* βG†	25,000 *g* AP	25,000 *g* βG
4·0	64‡	70	22	26
5·0	28	32	5	8
6·4	9	12	4	6
7·4	26	30	18	21
8·0	54	58	37	40

* Aliquots (0·05 ml) of liver fraction were added to 2·0 ml of 0·18 M sucrose–0·04 M tris acetate at varying pH values and incubated at 37° for 15 min. Total enzyme activity was determined by incubating 0·05-ml aliquots of liver fraction in 2 ml of 0·2% Triton X-100 at 37° for 15 min.

† AP, acid phosphatase; βG, beta-glucuronidase.

‡ Each value represents the mean of three separate experiments. Values varied by no more than 15 per cent of the corresponding mean.

Table 5 indicate that the 3500 *g* liver fraction is more susceptible to drug-induced membrane stabilization than is the 25,000 *g* liver fraction. Although not shown in Table 5, use of acid phosphatase as the lysosomal marker enzyme gave results very similar to those of β-glucuronidase.

TABLE 5. EFFECT OF ANTI-INFLAMMATORY DRUGS ON STABILITY OF LYSOSOMES *IN VITRO*—COMPARISON OF SEVERAL DIFFERENT RAT LIVER FRACTIONS

Drug	Concn (M)	Per cent inhibition of release of β-glucuronidase* 3500 *g*	25,000 *g*
Phenylbutazone	10^{-3}	100†	42
	10^{-4}	52	8
	10^{-5}	37	0
Paramethasone	10^{-3}	78	22
	10^{-4}	56	10
	10^{-5}	29	0
Acetylsalicylic acid	10^{-3}	77	26
	10^{-4}	41	0
	10^{-5}	25	0
Chloroquine	10^{-3}	100	41
	10^{-4}	71	7
	10^{-5}	35	0
Flufenamic acid	10^{-3}	(50)‡	0
	10^{-4}	50	10
	10^{-5}	36	5
Indomethacin	10^{-3}	58	8
	10^{-4}	33	0
	10^{-5}	17	0

* Incubation medium: 0·18 M sucrose–0·04 M tris acetate, pH 7·4. Incubations were conducted at 37° for 15 min as described previously. Each value represents the mean of three separate experiments. Values varied by no more than 10 per cent of the corresponding mean.

† Actual extinction values (540 mμ) for controls (incubations of aliquots of liver fraction without drugs) were 0·26 ± 0·03 (mean ± S.E.) for the 3500 *g* fraction and 0·28 ± 0·02 for the 25,000 *g* fraction.

‡ Numbers in parentheses signify per cent increase in release of β-glucuronidase.

Brown and Schwartz[14] reported that when lysosomes were incubated at 45° drugs failed to stabilize their membranes. The data in Table 6 illustrate the marked labilization of lysosomes effected by incubation at 45°. At this temperature anti-inflammatory drugs no longer stabilize but rather potentiate the labilization of lysosome membranes that normally occurs at such elevated temperature.

The data presented in Table 7 reveal that considerable caution must be exercised in the selection of an appropriate buffer for lysosome suspensions and incubations with drugs. As indicated earlier (Table 3), sodium ion markedly decreases lysosome stability *in vitro*. In the presence of more fragile lysosomes, induced by sodium acetate

TABLE 6. EFFECT OF ANTI-INFLAMMATORY DRUGS ON STABILITY OF LYSOSOMES *IN VITRO*—EFFECTS OF ELEVATED TEMPERATURE (45°)

Drug	Concn (M)	Per cent inhibition (stimulation) of release of β-glucuronidase* 3500 *g*	25,000 *g*
Phenylbutazone	10^{-3}	0 (70)†	0 (48)
	10^{-4}	0 (40)	0 (29)
	10^{-5}	0	0
Paramethasone	10^{-3}	10	0
	10^{-4}	0 (36)	0 (24)
	10^{-5}	0	0
Acetylsalicylic acid	10^{-3}	0 (61)	0 (39)
	10^{-4}	0 (36)	0 (18)
	10^{-5}	0	0
Chloroquine	10^{-3}	0 (45)	0 (25)
	10^{-4}	40	25
	10^{-5}	0	0
Flufenamic acid	10^{-3}	0 (100)	0 (81)
	10^{-4}	0 (91)	0 (50)
	10^{-5}	0 (33)	0 (14)
Indomethacin	10^{-3}	0 (55)	0 (45)
	10^{-4}	0 (27)	0 (21)
	10^{-5}	0	0

* Incubation medium: 0·18 M sucrose–0·04 M tris acetate, pH 7·4. Incubations were conducted at 37° for 15 min as described previously. Each value represents the mean of three separate experiments. Values varied by no more than 10 per cent of the corresponding mean.

† Numbers in parentheses signify per cent increase in release of β-glucuronidase. Actual extinction values for controls (incubations of aliquots of liver fraction without drugs) were 0·47 ± 0·05 (mean ± S.E.) for the 3500 *g* fraction and 0·59 ± 0·05 for the 25,000 *g* fraction.

buffer, drugs which otherwise stabilize lysosomes actually potentiate sodium-induced lysosome labilization.

The data illustrated in Table 8 reveal that anti-inflammatory drugs possess a much lower capacity to stabilize lysosomes (from 3500 *g* fraction) at pH 5·4 than at 7·4.

Effect of anti-inflammatory drugs on lysosome membrane stability. The data presented thus far reveal the suitability of the 3500 *g* liver fraction, incubation temperatures of 37°, and Tris acetate buffer (pH 7·4) for the measurement of drug-induced lysosome membrane stabilization. Under these conditions *in vitro*, steroidal and nonsteroidal anti-inflammatory drugs were tested for their capacity to stabilize lysosomes contained within the heavy mitochondrial fraction (Table 9). Chloroquine, phenylbutazone and flufenamic acid were among the more potent agents found. Paramethasone, hydrocortisone, acetylsalicylic acid, niflumic acid and indomethacin were also active. Sodium salicylate, mefenamic acid, meclofenamic acid, ibufenac and gold sodium thiomalate were inactive. The immunosuppressive drugs, Imuran and Cytoxan, also displayed negligible activity.

TABLE 7. EFFECT OF ANTI-INFLAMMATORY DRUGS ON STABILITY OF LYSOSOMES *IN VITRO*—EFFECTS OF SODIUM ION

Drug	Concn (M)	Per cent inhibition (stimulation) of release of β-glucuronidase*	
		3500 *g*	25,000 *g*
Phenylbutazone	10^{-3}	0 (46)†	0 (43)
	10^{-4}	22	10
	10^{-5}	0	0
Paramethasone	10^{-3}	0 (31)	0 (13)
	10^{-4}	21	11
	10^{-5}	0	0
Acetylsalicylic acid	10^{-3}	0 (55)	0 (36)
	10^{-4}	19	10
	10^{-5}	0	0
Chloroquine	10^{-3}	0 (22)	0
	10^{-4}	41	29
	10^{-5}	14	0
Flufenamic acid	10^{-3}	0 (60)	0 (44)
	10^{-4}	0 (54)	0 (35)
	10^{-5}	0 (10)	0
Indomethacin	10^{-3}	0 (44)	0 (31)
	10^{-4}	0 (12)	0
	10^{-5}	0	0

* Incubation medium: 0·18 M sucrose–0·04 M sodium acetate, pH 7·4. Incubations were conducted at 37° for 15 min as described previously. Each value represents the mean of three separate experiments. Values varied by no more than 10 per cent of the corresponding mean.

† Numbers in parentheses signify per cent increase in release of β-glucuronidase. Actual extinction values for controls (incubations of aliquots of liver fraction without drugs) were 0·57 ± 0·06 (mean ± S.E.) for the 3500 *g* fraction and 0·54 ± 0·04 for the 25,000 *g* fraction.

When plotted in the form of dose–response curves, phenylbutazone, oxyphenbutazone, chloroquine, hydrocortisone (data for these four drugs are illustrated in Fig. 1), hydroxychloroquine, paramethasone and indomethacin gave linear regression lines. The slopes of the regression lines for the nonsteroidal drugs were all equal (parallel dose–response curves). Although the regression lines for the steroidal drugs (i.e. hydrocortisone, Fig. 1) were linear, the slopes of these lines were significantly different ($P < 0.05$) from the slopes of the lines for nonsteroidal agents.

Many drugs from other therapeutic classes were tested and found to possess no capacity to stabilize lysosomes *in vitro* (Table 10) under the experimental conditions described.

DISCUSSION

The data presented in this report illustrate that, under specific experimental conditions, certain clinically useful steroidal and nonsteroidal anti-inflammatory drugs possess the capacity to stabilize *in vitro* lysosomes contained within a crude heavy

TABLE 8. EFFECT OF ANTI-INFLAMMATORY DRUGS ON STABILITY OF LYSOSOMES *IN VITRO* AT pH 5·4

Drug	Concn (M)	Per cent inhibition of release of β-glucuronidase* 3500 *g*
Phenylbutazone	10^{-3}	56†
	10^{-4}	31
	10^{-5}	0
Paramethasone	10^{-3}	22
	10^{-4}	38
	10^{-5}	16
Acetylsalicylic acid	10^{-3}	39
	10^{-4}	17
	10^{-5}	0
Chloroquine	10^{-3}	45
	10^{-4}	28
	10^{-5}	0
Flufenamic acid	10^{-3}	0‡
	10^{-4}	32
	10^{-5}	12
Indomethacin	10^{-3}	42
	10^{-4}	16
	10^{-5}	0

* Incubation medium: 0·18 M sucrose–0·04 M tris acetate, pH 5·4. Incubations were conducted at 37° for 15 min as described previously. Each value represents the mean of two separate experiments. Values varied by no more than 10 per cent of the corresponding mean.

† Actual extinction values (540 mμ) for controls (incubations of aliquots of liver fraction without drugs) were 0·23–0·25 for the 3500 *g* fraction.

‡ At 10^{-3} M, flufenamic acid elicited a 47 per cent increase in release of enzyme.

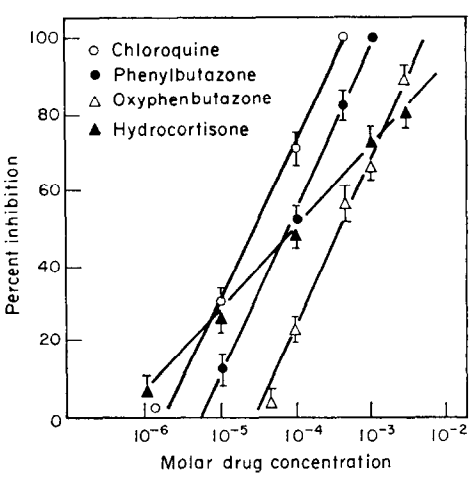

FIG. 1. Effect of anti-inflammatory drugs on lysosome membrane stability *in vitro*. Aliquots (0·2 ml) of 3500 *g* liver fraction and drugs were incubated in 0·18 M sucrose–0·04 M tris acetate, pH 7·4, at 37° for 15 min. The ordinate signifies the per cent inhibition of release of β-glucuronidase. Each value represents the mean ± S.E.M. obtained from three to six separate experiments.

TABLE 9. EFFECT OF ANTI-INFLAMMATORY DRUGS ON LYSOSOME MEMBRANE STABILIZATION *IN VITRO*

Drug	Marker enzyme	Per cent inhibition of release of Lysosomal enzyme*				
		10^{-3} M	5×10^{-4} M	10^{-4} M	10^{-5} M	10^{-6} M
Phenylbutazone	βG†	100	82	52	37	0
	AP†	100		58	36	
Oxyphenbutazone	βG	67	56	23	0	0
Paramethasone	βG	78	70	56	29	10
	AP	74		56	26	
Hydrocortisone	βG	70		60	25	
	AP	63		50	27	
Chloroquine	βG	100	100	71	30	0
	AP	100		68	40	
Hydroxychloroquine	βG	90	70	35	0	0
Acetylsalicylic acid	βG	77	64	41	25	0
	AP	78		40	22	
Sodium salicylate	βG	0	0	0	0	0
Flufenamic acid	βG	0‡	0	50	36	30
	AP	0‡		61	40	
Mefenamic acid	βG	8	0	0	0	0
Meclofenamic acid	βG	12	0	0	0	0
Niflumic acid	βG	84	84	67	17	0
Indomethacin	βG	58	42	33	17	0
	AP	54		34	11	
Ibufenac	βG	10	0	0	0	0
Gold sodium thiomalate	βG	0	0	0	0	0
Imuran	βG	26	16	5	0	0
Cytoxan	βG	0	0	0	0	0

* Aliquots (0·2 ml) of 3500 *g* liver fraction and drugs were incubated in 2 ml of 0·18 M sucrose–0·04 M Tris acetate, pH 7·4 at 37° for 15 min as described previously. Each value represents the mean of three to six separate experiments. Values varied by no more than 5–10 per cent of the corresponding mean in this series of experiments. Values of 20 per cent inhibition or greater were significant ($P < 0·05$).

† βG, beta-glucuronidase; AP, acid phosphatase.

‡ Flufenamic acid (10^{-3} M) increased release of both enzymes by 50 per cent ($P < 0·01$).

mitochondrial rat liver preparation. Lysosomes obtained from this heavy mitochondrial fraction are much more susceptible to the membrane-stabilizing actions of drugs than are lysosomes from a lighter mitochondrial fraction. These findings are similar to those reported previously[13] and suggest that rat liver lysosomes are heterogeneous pharmacologically with respect to the biochemical effects of drugs. Conclusions regarding morphologic heterogeneity cannot be made on the basis of our experiments, since no ultrastructural studies were made. However, further studies are in progress to

TABLE 10. DRUGS WHICH *IN VITRO* LACK LYSOSOME MEMBRANE-STABILIZING ACTIVITY

I.	Analgesics	VII.	Cancer chemotherapeutics
	Acetophenetidin		Chlorambucil
	Aminopyrine		Methotrexate
	Antipyrine	VIII.	Cardiac glycosides
	Dextropropoxyphene		Acetyl digitoxin
	Salicylamide		Digitoxin
II.	Anesthetics	IX.	Diuretics
	Cocaine HCl		Aminophylline
	Dibucaine HCl		Chlorothiazide
	Lidocaine HCl		Chlorthalidone
	Pentobarbital sodium	X.	Immunosuppressants
	Phenobarbital sodium		Cyclophosphamide
	Thiopental sodium		6-Mercaptopurine
III.	Antidepressants	XI.	Tranquilizers
	Desmethylimipramine HCl		Chlorpromazine HCl
	Imipramine HCl		Prochlorperazine HCl
IV.	Antihistamines		Promazine HCl
	Chlorpheniramine malcate		Reserpine
	Diphenhydramine HCl		Trifluprazine HCl
	Tripellenamine	XII.	Miscellaneous
V.	Antimalarials		Alloxan
	Chlorguanide		Bishydroxycoumarin
	Pyrimethamine		Caffeine
VI.	Anti-thyroidals		Colchicine
	Propylthiouracil		Ergotamine tartrate
	Thiouracil		Phentolamine HCl
			Zoxazolamine

characterize the properties of lysosomes contained within the heavy mitochondrial fraction of rat liver.

Tanaka and Iizuka[13] reported that some nonsteroidal anti-inflammatory drugs exhibited a limited capacity to stabilize lysosomes obtained from a similar heavy mitochondrial rat liver fraction. In our studies, both the maximum attainable degree of membrane stabilization and potency of a given drug were considerably higher than those reported by these investigators. For example, while the reported values (per cent protection against lysis) for 10^{-3} M phenylbutazone, 10^{-3} M acetylsalicylic acid and 10^{-4} M flufenamic acid were 28, 42 and 13 per cent, respectively, we obtained values of 100, 78 and 61 per cent respectively. Although these investigators also employed the 3500 *g* rat liver fraction, the partial discrepancy in results probably stems from the differences in methodology employed. The inclusion of a buffer (0·02 M tris acetate, pH 7·4) in the 0·25 M sucrose medium, which was omitted by Tanaka and Iizuka,[13] was found necessary to maintain the integrity of lysosomes during homogenization. Use of 0·18 M sucrose buffer in the incubation medium was found to be essential in order to obtain an optimal rate of enzyme release (28–30 per cent) from lysosomes *in vitro*. As a result of the employment of 0·25 M sucrose buffer, Tanaka and Iizuka[13] obtained a much lower rate of enzyme release (2–10 per cent). It would appear that

with such low rates of enzyme release, one might find greater difficulty quantitating the degrees of drug-induced lysosome membrane stabilization.

It was found necessary to employ glass tubes, instead of plastic, and mild agitation procedures for the incubation of lysosomes with drugs. The combination of the glass surface and agitation contributed to the labilization of lysosomes, a procedure which could be effectively inhibited by anti-inflammatory drugs.

Brown and Schwartz[14] reported that anti-inflammatory drugs actually labilized rather than stabilized rat liver lysosomes *in vitro*. However, it appears that these investigators may have employed lysosomes which became much too fragile as a result of their subjection to more strenuous experimental conditions. For example, we found that homogenization in a Potter-type tissue grinder with a glass or Teflon pestle (which was employed by these other investigators) resulted in the preparation of liver fractions containing damaged and fragile lysosomes. Incubation of such lysosomes in 0·18 or 0·25 M sucrose buffer at 37° or 45° resulted in extensive labilization, which was further potentiated by anti-inflammatory drugs. In addition, as illustrated in the present report, sodium acetate buffer and incubation temperatures of 45°, both of which were employed by Brown and Schwartz,[14] were found to be unsuitable for the measurement of drug-induced lysosome membrane stabilization. Although the buffering capacity of sodium acetate at pH 7·4 is considerably less than that of Tris acetate, the pH of these liver suspensions with or without drugs does not change appreciably during incubation. Therefore, it is unlikely that alterations in pH accounted for the results reported by these earlier investigators.[14] Since similar effects, that is drug-induced lysosome labilization, were obtained with sodium phosphate buffer, it appears likely that the presence of sodium ion alone is sufficient to account for the labilizing effects observed.

Not all procedures for rendering lysosomes more fragile are alike with regard to the subsequent actions of drugs on lysosome membrane stabilization. Preliminary experiments from our laboratory indicate that, while pretreatment of liver lysosome fractions with sodium ion or elevated temperature results in fragile organelles which are further labilized by drugs, lysosomes made more fragile by incubation with vitamin A alcohol or by fasting the rats for 16–24 hr can be effectively stabilized by drugs. Cortisol has been reported to protect isolated liver lysosomes against labilization induced by vitamin A,[21] streptolysin O,[21] etiocholanolone[22] or progesterone.[22] Paramethasone, phenylbutazone and indomethacin were reported to reduce, *in vivo*, the liver lysosome fragility which results during the development of adjuvant-induced polyarthritis in the rat.[23] Thus, the method of inducing lysosome fragility becomes quite important when one is measuring the membrane-stabilizing or labilizing capacity of drugs.

The liver lysosome fractions employed in the present study and in those reported by other investigators are all contaminated with mitochondria. In fact, these fractions might be considered as primarily mitochondrial preparations contaminated with lysosomes and other organelles. The finding of closely similar rates of release of three latent acid hydrolases argues strongly in favor of the presence of lysosomes, even though they are contaminated with other organelles. However, it is unlikely that the presence of mitochondria would interfere appreciably with the drug-induced effects on lysosomes, since the enzymes being measured are well known to be markers for lysosomes, i.e. acid phosphatase, β-glucuronidase and aryl sulfatase. The possibility exists that mitochondria might contribute to an apparently lower degree of drug-induced

lysosome stabilization, which could be attributed to nonspecific protein binding of such drugs. Such binding of drug to mitochondrial protein could result in a lower effective drug concentration at the site of the lysosomal membrane.

The data presented here indicate that a heavy liver mitochondrial fraction (3500 *g*) of the rat contains lysosomes which, under specific experimental conditions, are highly susceptible to anti-inflammatory drug-induced membrane stabilization. The lack of activity of non-anti-inflammatory drugs in this model suggests that lysosome membrane stabilization may be a specific mechanism of drug action limited to some clinically useful anti-inflammatory drugs.

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