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PRESERVATION OF STRUCTURAL INTEGRITY OF LIVER LYSOSOMES AND MEMBRANE-STABILIZING ACTION OF ANTI-INFLAMMATORY DRUGS, CATECHOLAMINES AND CYCLIC ADENOSINE MONOPHOSPHATE IN ISOTONIC SALT MEDIA

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Abstract—Since the first description of lysosomes, most studies with these cytoplasmic organelles have been conducted in sucrose solutions containing little or no inorganic ions. Lysosomal enzymes have been characterized by their structure-linked latency in sucrose solutions of very low ionic strength. The data in this report illustrate that isotonic concentrations of certain inorganic ions can replace sucrose for the purpose of retarding osmotic lysis of liver lysosomes and thus preserving the latent properties of lysosomal enzymes. Acid phosphatase, β-glucuronidase, β-galactosidase and aryl sulfatase were the lysosomal marker enzymes measured. Iso-osmotic concentrations of potassium chloride and Hanks' Balanced Salt Solution were found to be suitable substitutes for sucrose in maintaining the structural integrity of lysosomes at neutral pH and 37°. Certain steroidal and non-steroidal anti-inflammatory drugs, such as hydrocortisone, chloroquine, acetylsalicylic acid, phenylbutazone, indomethacin and flufenamic acid, inhibited the release of several enzymes from lysosomes incubated in isotonic salt media. Further, the catecholamines, norepinephrine, epinephrine and isoproterenol, were found to inhibit lysosomal enzyme release in salt media. Similar lysosome membrane-stabilizing actions were obtained with cyclic 3′,5′-adenosine monophosphate and its N^6,$O^{2'}$-dibutyryl analog. The data reveal that sucrose can be replaced by certain salt solutions for the purpose of maintaining the integrity of lysosomes or demonstrating drug-induced lysosome membrane stabilization *in vitro*.

LYSOSOMAL enzymes possess the capacity to degrade the major components of connective tissue.[1–5] Thus, lysosomal enzyme release has been implicated in a variety of pathological conditions including rheumatoid arthritis and osteoarthritis.[6–12] Abundant evidence exists that clinically active steroidal and non-steroidal anti-inflammatory drugs inhibit the release of acid hydrolases from lysosomes *in vitro*[10,13–16] and *in vivo*.[9,12,17,18] The initial biochemical studies on lysosomes revealed that these cytoplasmic organelles retained partially their structural integrity in sucrose solutions containing little or no inorganic ions.[19–23] As a result of these early experiments, almost all subsequent studies on lysosomes have been conducted with sucrose solutions. Very little information is available with regard to the integrity of lysosomes in physiologic salt media containing little or no sucrose. Moreover, the interaction of anti-inflammatory drugs and lysosome fractions in salt media has not been reported.

Sucrose, due to its high content of hydroxyl groups, can easily form hydrogen bonds and thus might interfere with the interaction of lysosomes and drugs by competing for hydrogen bonding.[24] For example, isotonic sucrose was reported to

inhibit the disruption of lysosomes by monosodium urate crystals, which occurs when incubations are conducted in sucrose-free, phosphate-buffered saline.[24] The results of the present study indicate that, under certain experimental conditions, iso-osmotic concentrations of various salts can be employed as substitutes for sucrose in retarding the osmotic lysis of liver lysosomes, thereby preserving the latent properties of acid hydrolases. Steroidal and non-steroidal anti-inflammatory drugs, catecholamines, cyclic 3′,5′-adenosine monophosphate and N^6,$O^{2'}$-dibutyryl cyclic 3′,5′-adenosine monophosphate each possess the capacity to inhibit the release of enzymes from lysosomes during incubation in salt media at neutral pH and 37°.

MATERIALS AND METHODS

Drugs. Acetylsalicylic acid, chloroquine diphosphate, colchicine, hydrocortisone hemi-succinate sodium, *l*-norepinephrine bitartrate, *l*-epinephrine bitartrate, *l*-phenylephrine hydrochloride, cyclic 3′,5′-adenosine monophosphate and N^6,$O^{2'}$-dibutyryl cyclic 3′,5′-adenosine monophosphate were purchased from Sigma Chemical Company. Theophylline and *l*-isoproterenol hydrochloride were purchased from Mann Research and Aldrich Chemical Company respectively. Indomethacin and gold sodium thiomalate were supplied by Merck, Sharp & Dohme. Parke, Davis & Company provided flufenamic acid and Mead Johnson supplied cyclophosphamide. Dextropropoxyphene was provided by Lilly, and propranolol hydrochloride was supplied by Ayerst.

All drugs tested were dissolved in the appropriate incubation medium. Phenylbutazone, indomethacin, acetylsalicylic acid and flufenamic acid were first prepared as soluble sodium salts and tested immediately thereafter. Salts were prepared by dissolving the free acid in the exact equivalent of sodium hydroxide solution and diluting with the appropriate incubation medium. All compounds were soluble under the experimental conditions employed and they produced no appreciable alteration of the pH of the incubation media. Solutions of the catecholamines were used immediately, since decomposition and discoloration occurred rapidly in aqueous media.

Incubation media. The various incubation media used in this study are defined below. Sucrose (0·18 M) and KCl (0·0375–0·225 M) solutions were buffered with 0·05 M Tris–acetate, pH 7·4. Hanks' Balanced Salt Solution (Hanks' BSS, without phenol red; Microbiological Associates, Inc.) is buffered at pH 7·4. The composition of Hanks' BSS, based on anhydrous salts, is the following: NaCl, 137 mM; KCl, 5·4 mM; $CaCl_2$, 1·4 mM; $MgCl_2$, 0·50 mM; $MgSO_4$, 0·40 mM; Na_2HPO_4, 0·34 mM; KH_2PO_4, 0·44 mM; $NaHCO_3$, 4·2 mM; glucose, 5·6 mM. Physiologic medium, as used in this study, signifies Hanks' balanced salt solution (pH 7·4) containing 10% (v/v) isologous rat plasma. Plasma was obtained by drawing heparinized (50 units/ml) blood, by heart puncture, from lightly anesthetized (Nembutal) rats and centrifuging at 2000 *g* for 15 min at 4°. The clear plasma layers were removed and maintained at 4° until used.

Preparation of liver fractions. Liver lysosome fractions were prepared by a modification of the method described previously.[15] Briefly, the two larger lobes of liver from a decapitated and exsanguinated 275 g male Sprague–Dawley fasted (18 hr) rat (Carworth Farms) 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 per cent 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 twelve complete strokes of the pestle. The homogenate (40 ml) was 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°. Sediments were saved and the supernatants were centrifuged at 16,500 *g* for 30 min at 4°. Sediments from the 3500 *g* and 16,500 *g* centrifugations were individually rinsed twice in 0·45 M sucrose–0·04% glycogen–0·02 M Tris acetate, pH 7·4, and resuspended in 5 ml of the same buffer. The suspensions were maintained at 4° for the duration of the experiment. Both liver fractions represent crude preparations containing lysosomes as well as other organelles.[15]

Measurement of lysosome membrane integrity. Integrity of lysosomes was ascertained by measuring the release of lysosomal marker enzymes. Suspensions of the two different liver fractions containing lysosomes were warmed to 25° for 5 min, aliquots (0·2 ml) were added to glass tubes containing 2·0 ml of a given medium at 25°, with or without drug, and the tubes were incubated at 37° for a given time interval. All incubations were conducted in an Eberbach metabolic shaker set at 150 agitation cycles/min. Incubations were terminated by centrifugation (27,000 *g* for 15 min at 4°) after transfer of samples to 15-ml polyethylene tubes. Supernatants were maintained at 4° until assayed for lysosomal marker enzyme activity. Total enzyme activity was determined by incubation of 0·1-ml aliquots of liver lysosome fractions in 2·0 ml of 0·1% Triton X-100–0·05 M Tris–acetate, pH 7·4, at 37° for 30 min. Samples were then agitated (Vortex mixer) at high speed for 10 min and centrifuged at 27,000 *g* for 15 min at 4°. Aliquots of 1·0 ml were used in the enzyme assays.

Lysosomal enzyme assays. Aryl sulfatase (EC 3.1.6.1, aryl sulfate sulfohydrolase) was measured, using 2-hydroxy-5-nitrophenyl sulfate as substrate, according to the method described by Roy.[25] β-Glucuronidase (EC 3.2.1.31, β-D-glucuronide glucuronohydrolase) was measured, using phenolphthalein glucuronide as substrate, by the method of Gianetto and DeDuve.[22] β-Galactosidase (EC 3.2.1.23, β-D-galactoside galactohydrolase) was measured, using *p*-nitrophenyl-β-D-galactopyranoside as substrate, according to Patel and Tappel.[26] Acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase) was measured, using β-glycerophosphate as substrate, by the method of Gianetto and DeDuve.[22] Extinction values were measured with a Bausch & Lomb Spectonic 20 colorimeter.

Under the experimental conditions employed (i.e. 3- to 4-fold dilution of post-incubation lysosome-drug supernatant), none of the drugs tested significantly inhibited any of the lysosomal marker enzymes.[15]

RESULTS

Effect of various incubation media on latency of lysosomal enzymes. The data in Fig. 1 illustrate the latency of β-glucuronidase during incubation of the 3500 *g* or 16,500 *g* liver fraction in various concentrations of KCl, buffered with 0·05 M Tris acetate, pH 7·4. Iso-osmotic (0·15 M) and hyper-osmotic (0·225 M) concentrations of KCl afford considerable protection of lysosomes against labilization at 37°. Latency of β-glucuronidase can be demonstrated also in other types of salt media (Fig. 2). Although lysosomes appear to be slightly more fragile in 0·15 M KCl than in 0·18 M sucrose, they are more stable in Hanks' BSS than in either of the two former media,

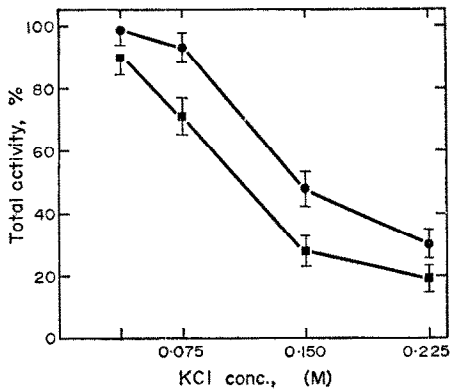

FIG. 1. Latency of β-glucuronidase in KCl solution, buffered at pH 7·4. Aliquots (0·2 ml) of the 3500 *g* or 16,500 *g* liver fraction were incubated in 2·0 ml of 0·05 M Tris acetate, pH 7·4, containing 0·0375 M to 0·225 M KCl, at 37° for 30 min and centrifuged as described previously. Supernatants (1·0-ml aliquots) were assayed for β-glucuronidase activity. Each value represents the mean ± S.E. of three separate experiments. Symbols: ●, 3500 *g* fraction; ■, 16,500 *g* fraction.

at pH 7·4. Each of the points corresponding to the 30- 60- and 90-min incubation periods, with regard to both the 3500 *g* and 16,500 *g* liver fractions, for the Hanks' BSS medium (Fig. 2C) was significantly different ($P < 0·05$) than the respective points for either the isotonic sucrose (Fig. 2a) or the isotonic KCl (Fig. 2b) medium. Inclusion of 10% isologous rat plasma in Hanks' BSS greatly facilitates release of

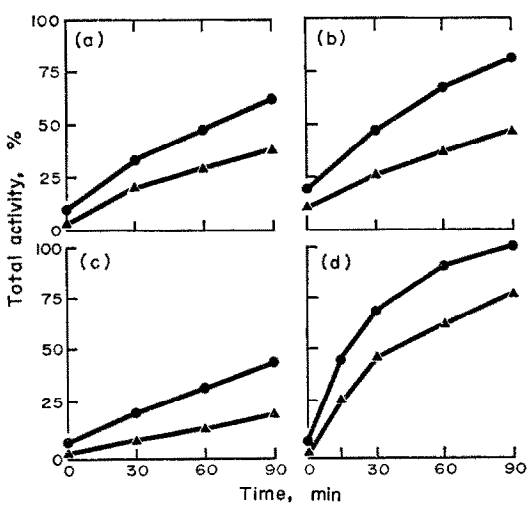

FIG. 2. Latency of β-glucuronidase in various media, buffered at pH 7·4. Aliquots (0·2 ml) of the 3500 *g* or 16,500 *g* liver fractions were incubated in 2·0 ml of medium at 37° for various time intervals and centrifuged as described previously. Supernatants (1·0-ml aliquots) were assayed for β-glucuronidase activity. Each value represents the mean of three separate experiments. Individual values varied by no more than 10 per cent of the corresponding mean. Symbols: ●, 3500 *g* fraction; ▲, 16,500 *g* fraction; (a) 0·18 M sucrose–0·05 M Tris acetate, pH 7·4; (b) 0·15 M KCl–0·05 M Tris acetate, pH 7·4; (c) Hanks' Balanced Salt Solution, pH 7·4; (d) Hanks' Balanced Salt Solution, pH 7·4; containing 10% (v/v) isologous rat plasma.

β-glucuronidase from both liver lysosome fractions. Each of the points corresponding to the 30-, 60- and 90-min incubation periods, with regard to both the 3500 *g* and 16,500 *g* liver fractions, for the Hanks' BSS medium with plasma (Fig. 2d) was significantly different ($P < 0.001$) than the respective points for the Hanks' BSS medium (Fig. 2C). Similar data were obtained when aryl sulfatase and β-galactosidase were the marker enzymes measured. Addition of bovine serum albumin or ovalbumin at 0·1 mg/ml, instead of plasma, to Hanks' BSS yielded data similar to those illustrated for β-glucuronidase. The data in Fig. 3 demonstrate the latent properties of β-galactosidase, acid phosphatase and aryl sulfatase during incubation of the 3500 *g* or 16,500 *g* liver fraction in Hanks' BSS. It appears that β-galactosidase is more easily released or readily available than acid phosphatase and that the latter is more easily released than aryl sulfatase. This relationship holds true for both liver lysosome fractions.

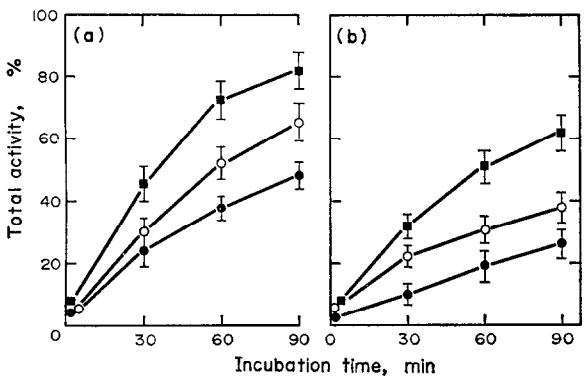

FIG. 3. Latency of β-galactosidase, acid phosphatase and aryl sulfatase in Hanks' Balanced Salt Solution, pH 7·4. Aliquots (0·2 ml) of the 3500 *g* or 16,500 *g* liver fraction were incubated in 2·0 ml of medium at 37° for various time intervals and centrifuged as described previously. Supernatants (1·0-ml aliquots) were assayed for lysosomal marker enzyme activity. Each value represents the mean ± S. E. of three separate experiments. Symbols: ■, β-galactosidase; ○, acid phosphatase; ●, aryl sulfatase; (a) 3500 *g* fraction; (b) 16,500 *g* fraction.

Incubation of lysosome lysates (37°) for 90 min in distilled water or 0·18 M sucrose resulted in a small (10–15 per cent) deterioration of β-glucuronidase and β-galactosidase but not aryl sulfatase activity. This small decrease in enzyme activity was not observed when incubations were conducted either for 60 min in water or sucrose solution, or in isotonic KCl or Hanks' BSS for up to 90 min.

In this study, lysosome fractions are suspended in 0·45 M sucrose–0·04% glycogen, buffered at pH 7·4, as described previously. Therefore, addition of 0·2 ml of this suspension to 2·0 ml of sucrose-free media yields a final sucrose concentration of 0·041 M. The data in Fig. 4 illustrate that this small concentration of sucrose contributes no appreciable osmotic protection to lysosomes when the 3500 *g* liver fraction is incubated in either distilled water or 0·05 M Tris acetate, pH 7·4. Osmotic protection is afforded to lysosomes incubated in 0·18 M sucrose, buffered at pH 7·4. Experiments in which Tris acetate was replaced with 0·05 M potassium acetate, pH 7·4, both in the preparation and incubation of lysosomes yielded data similar to those illustrated in Fig. 4.

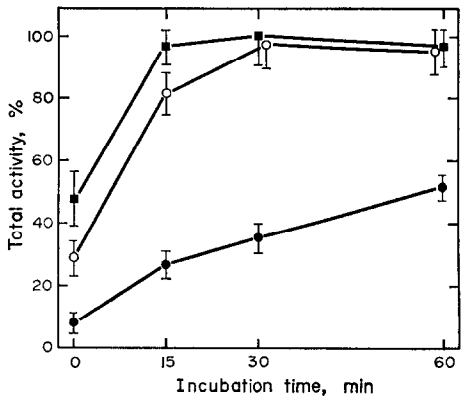

FIG. 4. Loss of latency of aryl sulfatase in hypotonic buffer, pH 7·4. Aliquots (0·2 ml) of 3500 *g* liver fraction were incubated in 2·0 ml of medium at 37° for various time intervals and centrifuged as described previously. Supernatants (1·0-ml aliquots) were assayed for aryl sulfatase activity. Each value represents the mean ± S. E. of four separate experiments. Symbols: ■, distilled water; ○, 0·05 M Tris acetate, pH 7·4; ●, 0·18 M sucrose–0·05 M Tris acetate, pH 7·4.

Effect of heparin on latency of lysosomal enzymes. As indicated previously, the inclusion of 10% rat plasma, prepared from heparinized blood, in Hanks' BSS sharply increased the fragility of lysosomes incubated in this medium. The data in Table 1 indicate that heparin, at a concentration of 25 units/ml, did not alter the integrity of lysosomes from either the 3500 *g* or 16,500 *g* liver fraction. At 100 U/ml, heparin significantly ($P < 0·05$) stabilized lysosomes.

TABLE 1. EFFECT OF HEPARIN ON INTEGRITY OF LYSOSOMES *in vitro*

Incubation medium*	β-Galactosidase activity†	
	3500 *g*	16,500 *g*
Hanks' BSS	0·58 ± 0·039 (48)	0·53 ± 0·031 (33)
Hanks' BSS + heparin (25 units/ml)	0·57 ± 0·027 (48)	0·55 ± 0·038 (34)
Hanks' BSS + heparin (100 units/ml)	0·46 ± 0·041 (38)	0·43 ± 0·030 (27)

* Incubation medium, Hanks' Balanced Salt Solution, pH 7·4. Heparin, 100 units equal 0·63 mg. Heparin did not inhibit β-galactosidase activity directly.

† Aliquots (0·2 ml) of the 3500 *g* or 16,500 *g* liver fraction were incubated in 2·0 ml of medium at 37° for 30 min. After centrifugation (27,000 *g* for 15 min), 1·0-ml aliquots of supernatants were assayed for β-galactosidase activity. Data represent extinction values (405 nm) expressed as the mean ±S. E. ($n = 3$). Numbers in parentheses signify per cent of total enzyme activity.

Effect of heat denaturation on the facilitation of lysomal enzyme release by plasma and albumin. Inclusion of 10% isologous rat plasma, 0·01% bovine serum albumin or 0·01% ovalbumin in Hanks' BSS facilitates significantly the release of aryl sulfatase and β-galactosidase from liver lysosomes *in vitro* (Table 2). Pretreatment of Hanks'

TABLE 2. EFFECT OF HEAT DENATURATION ON THE FACILITATION OF LYSOSOMAL ENZYME RELEASE BY PLASMA AND ALBUMIN

Experimental condition*	Marker† enzyme	Acid hydrolase activity‡ BSS†	BSS + 10% plasma	BSS + 0·01% BSA†	BSS + 0·01% OVA†
Unaltered	AS	0·19 ± 0·008	0·39 ± 0·024§	0·42 ± 0·018§	0·36 ± 0·022§
	β-gal	0·36 ± 0·026	0·70 ± 0·042§	0·67 ± 0·036§	0·64 ± 0·040§
63° for 60 min	AS	0·18 ± 0·010	0·22 ± 0·016	0·19 ± 0·008	0·22 ± 0·014
	β-gal	0·37 ± 0·030	0·39 ± 0·022	0·36 ± 0·020	0·40 ± 0·028

* Each of the four different media was used unaltered or heated at 63°; plasma, BSA or OVA were heated directly in the BSS medium.

† AS, aryl sulfatase; β-gal, β-galactosidase; BSS, Hanks' Balanced Salt Solution (pH 7·4); plasma, isologous plasma; BSA, bovine serum albumin; OVA, ovalbumin.

‡ Aliquots (0·2 ml) of 3500 *g* liver fraction were incubated in 2·0 ml of appropriate medium at 37° for 15 min and centrifuged as described previously. Supernatants (1·0 ml) were assayed for acid hydrolase activity. Data represent extinction values (510 mμ, AS; 405 mμ, β-gal) expressed as the mean ± S. E. ($n = 4$). Total acid hydrolase activity, determined by incubation of lysosome fractions in appropriate media containing 0·1% Triton X-100 at 37° for 15 min, was 0·96 ± 0·058 (AS) and 1·36 ± 0·074 (β-gal).

§ Significantly different ($P < 0·05$) than corresponding values in the BSS column; all other values are not different ($P > 0·05$).

TABLE 3. EFFECT OF ANTI-INFLAMMATORY AND OTHER DRUGS ON RELEASE OF ENZYMES FROM LYSOSOMES INCUBATED IN 0·15 M KCl, BUFFERED AT pH 7·4

Drug	Marker enzyme	Per cent inhibition of release of marker enzyme* Molar concn of drug 10^{-3}	10^{-4}	10^{-5}	10^{-6}
Chloroquine	β-G†	100	65	36	8
	AS†	100	88	49	21
Hydrocortisone	β-G	100	67	40	17
	AS	100	73	48	19
Phenylbutazone	AS	100	63	29	11
Indomethacin	AS	55	27	0	0
Acetylsalicylic acid	β-G	86	47	24	8
	AS	78	44	21	0
Flufenamic acid	AS	(84)‡	(21)‡	33	8
Gold sodium thiomalate	β-G	0	0	0	0
Cyclophosphamide	AS	0	0	0	0
Dextropropoxyphene	AS	0	0	0	0
Colchicine	β-G	8	0	0	0

* Aliquots (0·2 ml) of 3500 *g* liver fraction were incubated in 2·0 ml of 0·15 M KCl–0·05 M Tris acetate, pH 7·4, at 37° for 15 min and centrifuged as described previously. Supernatants (1·0 ml) were assayed for lysosomal marker enzyme activity. Each value represents the mean of four separate experiments. Individual values varied by no more than 10 per cent of the corresponding mean. Actual extinction values for controls (incubations of liver fraction without drugs) were 0·46 ± 0·032 (mean ± S. E.) for β-glucuronidase and 0·30 ± 0·022 for aryl sulfatase.

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

‡ Numbers in parentheses signify per cent increase in release of aryl sulfatase.

BSS containing plasma or albumin by heating at 63° for 60 min results in abolition of their facilitating effect on lysosomal enzyme release.

Effect of anti-inflammatory drugs on release of enzymes from lysosomes incubated in various media. The effect of various drugs on the release of enzymes from lysosomes (3500 *g* liver fraction) incubated in various salt media is illustrated in Tables 3 and 4. Soluble sodium salts of phenylbutazone, indomethacin, acetylsalicylic acid and flufenamic acid were prepared and tested. The other drugs were soluble in aqueous media. Chloroquine and hydrocortisone were the most potent lysosome membrane-stabilizing agents tested in 0·15 M KCl–0·05 M Tris–acetate, pH 7·4 (Table 3). Phenylbutazone and acetylsalicylic acid showed good activity while indomethacin was relatively weak. Flufenamic acid demonstrated a biphasic effect; labilization at high and stabilization at lower concentrations. In other experiments, lysosomes and drugs were incubated in 0·15 M KCl buffered at pH 7·4 with either 0·05 M potassium–acetate or 0·2 M Tris–acetate. Under these conditions the drugs tested, chloroquine, hydrocortisone, phenylbutazone and acetylsalicylic acid, yielded data similar to those illustrated in Table 3.

TABLE 4. EFFECT OF ANTI-INFLAMMATORY DRUGS ON RELEASE OF ARYL SULFATASE FROM LYSOSOMES INCUBATED IN HANKS' BALANCED SALT SOLUTION, pH 7·4, WITH OR WITHOUT 10% PLASMA

Drug	Incubation medium*	Per cent inhibition of release of aryl sulfatase† Molar conc of drug 10^{-3}	10^{-4}	10^{-5}	10^{-6}
Chloroquine	− Plasma	(53)‡	(12)‡	44	21
	+ Plasma	(64)‡	(21)‡	39	13
Hydrocortisone	− Plasma	100	81	47	23
	+ Plasma	72	43	30	0
Phenylbutazone	− Plasma	100	60	38	17
	+ Plasma	100	58	34	17
Indomethacin	− Plasma	67	36	28	10
	+ Plasma	69	39	28	7
Acetylsalicylic acid	− Plasma	100	52	36	12
	+ Plasma	100	62	28	6
Flufenamic acid	− Plasma	(95)‡	(37)‡	44	18
	+ Plasma	(63)‡	(30)‡	38	12

* Hanks' Balanced Salt Solution without (−) or with (+) 10% (v/v) isologous plasma.

† Aliquots (0·2 ml) of 3500 *g* liver fraction were incubated in 2·0 ml of Hanks' Balanced Salt Solution, pH 7·4 (with or without plasma) at 37° for 15 min and centrifuged as described previously. Supernatants (1·0 ml) were assayed for aryl sulfatase activity. Each value represents the mean of four to five separate experiments. Individual values varied by no more than 12 per cent of the corresponding mean. Actual extinction values (mean ± S. E.) for controls (incubations of liver fraction without drugs) were 0·18 ± 0·009 without plasma and 0·38 ± 0·027 with plasma.

‡ Numbers in parentheses signify per cent increase in release of aryl sulfatase.

Incubation of lysosomes and drugs in Hanks' BSS, pH 7·4, yielded similar results except that chloroquine now demonstrated a biphasic effect like that of flufenamic acid (Table 4). Inclusion of 10% isologous rat plasma in Hanks' BSS did not alter the action of the drugs for the most part (Table 4). Chloroquine showed a biphasic

effect and hydrocortisone was slightly less active in the medium containing plasma.

Gold sodium thiomalate, cyclophosphamide, dextropropoxyphene and colchicine elicited no appreciable effect on enzyme release from lysosomes incubated in any of the media employed.

TABLE 5. EFFECT OF CATECHOLAMINES, CYCLIC ADENOSINE MONOPHOSPHATE AND OTHER AGENTS ON RELEASE OF ARYL SULFATASE FROM LYSOSOMES INCUBATED IN SALT MEDIA, BUFFERED AT pH 7·4

Agent(s) tested	Incubation medium*	Per cent inhibition of release of aryl sulfatase† Molar concn of agent 10^{-4}	10^{-5}	10^{-6}	10^{-7}
Epinephrine (E)	KCl	44	21	12	0
	BSS	59	36	20	0
E + theophylline (10^{-3} M)‡	KCl	91	66	42	27
	BSS	100	76	48	32
E + propranolol (10^{-4} M)‡	KCl	11	0	0	0
	BSS	15	0	0	0
Norepinephrine	KCl	36	19	10	0
	BSS	48	28	9	0
Isoproterenol	KCl	46	29	22	0
	BSS	67	46	30	11
Phenylephrine	KCl	0	0	0	0
	BSS	0	0	0	0
Cyclic AMP§	KCl	43	11	0	0
	BSS	52	24	10	0
Cyclic AMP§ + theophylline (10^{-3} M)‡	KCl	65	38	26	12
	BSS	71	49	34	18
Dibutyryl cyclic AMP§	KCl	68	33	20	0
	BSS	66	36	21	9

* KCl, 0·15 M KCl–0·05 M Tris acetate, pH 7·4; BSS, Hanks' Balanced Salt Solution, pH 7·4.

† Aliquots (0·2 ml) of 3500 *g* liver fraction were incubated in 2·0 ml of appropriate salt medium at 37° for 15 min and centrifuged as described previously. Supernatants (1·0 ml) were assayed for aryl sulfatase activity. Each value represents the mean of three to five separate experiments. Individual values varied by less than 15 per cent of the corresponding mean. Actual extinction values (mean ± S. E.) for controls (incubations of liver fraction without drugs) were 0·30 ± 0·022 for the KCl medium and 0·20 ± 0·008 for the BSS medium.

‡ Theophylline (10^{-3} M) or propranolol (10^{-4} M) alone did not inhibit enzyme release.

§ Cyclic AMP, cyclic 3′,5′-adenosine monophosphate; dibutyryl cyclic AMP, N^6,$O^{2'}$-dibutyryl cyclic 3′,5′-adenosine monophosphate.

Effect of catecholamines and cyclic nucleotides on release of enzymes from lysosomes incubated in various media. The data in Table 5 illustrate that epinephrine, norepinephrine, isoproterenol, cyclic AMP and dibutyryl cyclic AMP each inhibited the release of aryl sulfatase from lysosomes incubated in 0·15 M KCl–0·05 M Tris–acetate, pH 7·4, or Hanks' BSS, pH 7·4. Phenylephrine elicited no effect. Theophylline enhanced the effect of epinephrine and cyclic AMP. Propranolol inhibited the action of epinephrine. Slightly greater effects were observed when incubations were conducted in the Hanks' BSS. Inclusion of 10% plasma or 0·01% bovine serum albumin markedly reduced the activity of the catecholamines that was observed in

Hanks' BSS alone. This might be attributed to the strong capacity by which catecholamines bind to proteins.

Effect of agents on release of enzymes from lysosomes in the 16,500 g *liver fraction.* Anti-inflammatory drugs inhibited the release of β-galactosidase from lysosomes contained in the 16,500 *g* liver fraction (Table 6). Incubations were conducted in 0·15 M KCl–0·05 M Tris–acetate, pH 7·4. Magnitudes of drug effects were less with the 16,500 *g* than with the 3500 *g* liver fraction. Epinephrine, cyclic AMP and dibutyryl cyclic AMP also inhibited enzyme release from the 16,500 *g* lysosome fraction, although such effects were of slightly lower magnitudes than those observed with the 3500 *g* fraction.

TABLE 6. EFFECT OF VARIOUS AGENTS ON RELEASE OF β-GALACTOSIDASE FROM 16,500 *g* LIVER LYSOSOME FRACTION INCUBATED IN 0·15 M KCl, BUFFERED AT pH 7·4

Agent tested	Per cent inhibition of release of β-galactosidase* Molar concn of agent 10^{-3}	10^{-4}	10^{-5}	10^{-6}
Chloroquine	88	32	14	0
Hydrocortisone	71	29	16	0
Phenylbutazone	77	23	10	0
Acetylsalicylic acid	90	38	19	0
Epinephrine (E)		46	24	0
E + theophylline (10^{-3} M)†		74	51	30
Cyclic AMP‡	60	37	10	0
Dibutyryl cyclic AMP‡	78	49	21	0

* Aliquots (0·2 ml) of 16,500 *g* liver fraction were incubated in 2·0 ml of 0·15 M KCl–0·05 M Tris acetate, pH 7·4, at 37° for 15 min and centrifuged as described previously. Supernatants (1·0 ml) were assayed for β-galactosidase activity. Each value represents the mean of four separate experiments. Individual values varied by less than 15 per cent of the corresponding mean. Actual extinction values for controls (incubations of liver fraction without drugs) were 0·39 ± 0·026 (mean ± S. E.).

† Theophylline (10^{-3} M) alone did not inhibit enzyme release.

‡ Cyclic AMP, cyclic 3′,5′-adenosine monophosphate; dibutyryl cyclic AMP, N^6,$O^{2'}$-dibutyryl cyclic 3′,5′-adenosine monophosphate.

DISCUSSION

Liver lysosome granules were first discovered by the observation that acid phosphatase was bound to cytoplasmic granules recovered in the mitochondrial fraction.[19] Further studies revealed that acid hydrolases in general were associated with a distinct group of cytoplasmic organelles,[20–23] later termed lysosomes. These acid hydrolases were characterized uniquely by their structure-linked latency in sucrose suspension. That is, the structural integrity of lysosomes was preserved in solutions of sucrose that were nearly iso-osmotic and relatively free of inorganic ions. Ever since these early studies, with very few exceptions, most of the experiments dealing with lysosomes, including isolation, characterization and interactions with drugs, have been conducted in sucrose solutions containing little or no inorganic ions. The finding that sucrose, by competing for hydrogen bonding, interferes with the

interaction of lysosomes and certain agents such as monosodium urate crystals,[24] suggests that a similar interference of interaction between lysosomes and drugs is possible. Therefore, studies were conducted to determine the effects of salt media on the integrity of lysosomes *in vitro*. The data presented in this report illustrate that, under specific experimental conditions *in vitro*, isotonic concentrations of certain inorganic ions can replace sucrose for the purpose of retarding osmotic lysis of liver lysosomes and preserving the latent properties of acid hydrolases. Iso-osmotic salt media such as 0·15 M KCl or Hanks' Balanced Salt Solution (Hanks' BSS) were just as effective as sucrose in retarding the labilization of lysosomes during incubation of different liver lysosome fractions at pH 7·4 and 37°. Four different lysosomal marker enzymes, β-glucuronidase, β-galactosidase, aryl sulfatase and acid phosphatase, retained their characteristic property of latency during incubation of lysosomes in salt media. The inclusion of 10% isologous rat plasma in Hanks' BSS significantly reduced the capacity of the latter to protect liver lysosomes at 37°. Further, anti-inflammatory drugs, catecholamines and cyclic AMP inhibited the release of enzymes from lysosomes during incubation in various salt media at neutral pH and 37°. These results are similar to those reported previously where incubations of lysosomes and drugs were conducted in sucrose solutions free of inorganic ions.[15,16,27,28]

Appelmans and DeDuve[21] reported that isotonic concentrations of either KCl or NaCl were inadequate in protecting liver lysosomes against lysis at acid pH (5–6) and 0° unless 0·25 M sucrose was present. The acidic conditions could have accounted for such marked instability of lysosomes *in vitro* since even 0·25 M sucrose alone was incapable of preserving the integrity of lysosomes that were incubated under conditions of acid pH and 37°, i.e. 90 per cent of the total acid phosphatase was released after 1 hr. Similar experiments at pH 7·4 and 37° were not reported by these investigators. Gianetto and DeDuve[22] reported that liver lysosomes, incubated at pH 5 and 0° for 3 hr, were more fragile in 0·15 M NaCl than in 0·25 M sucrose. Similarly, hypo-osmotic concentrations of NaCl were shown to decrease the stability of liver lysosomes incubated in sucrose solution at pH 7·4 and 37°.[15] Further, anti-inflammatory drugs that stabilize lysosomes when incubated in sucrose solution were reported to labilize lysosomes when incubated in sucrose-containing, hypo-osmotic concentrations of sodium acetate.[15,29]

Bowers *et al.*[30] demonstrated that isotonic KCl solution could replace hypotonic sucrose solution for the purpose of preserving the integrity of lysosomes during homogenization of spleen tissue. Isotonic KCl solution,[18] buffered at pH 7·4, was also shown to be suitable for retarding the disruption of liver lysosomes at 0° and 37°, and for detecting small changes in lysosome stability elicited by drugs. Rosenberg and Janoff[31] reported that iso-osmotic Ringer–Locke-sucrose media protected rat liver lysosomes against lysis *in vitro*. For example, about 90 per cent of the total β-glucuronidase activity remained granule-bound after incubation at pH 7·4 and 0° for 1 hr.

An iso-osmotic concentration of KCl was found to be a suitable substitute for hypo-osmotic solutions of sucrose in decreasing the rate of labilization of liver lysosomes during incubation at pH 7·4 and 37° for periods of up to 90 min. Similar results were obtained when Hanks' BSS (pH 7·4, iso-osmotic) was employed as the incubation medium (Fig. 2). β-Galactosidase was more readily available or released than other lysosomal marker enzymes in these experiments (Fig. 3). This may be a

reflection of the different capacities by which acid hydrolases are bound to the lysosome membrane.[32] Lysosomes were more stable in Hanks' BSS than in sucrose solution. However, when lysosomes were incubated in Hanks' BSS containing 10% isologous heparinized rat plasma, a significant decrease in stability was observed (Fig. 2). Similarly, lysosomes incubated in Hanks' BSS containing small amounts of bovine serum albumin or ovalbumin lost their integrity more rapidly than in the salt medium alone (Table 2). The similarity in actions of plasma and two different purified albumin preparations suggests that the facilitation of lysosomal enzyme release by plasma is due to an effect by albumin or some other plasma protein. Moreover, the protein must be in its native configuration as heat denaturation abolishes the action of albumin and plasma on lysosomes. Heparin, at concentrations of 25–100 units/ml, did not facilitate the labilization of lysosomes (Table 1). In fact, at 100 units/ml, heparin inhibited lysosomal enzyme release. Thus, heparin cannot account for the increased fragility of lysosomes incubated in salt medium containing heparinized plasma.

All experiments were conducted with lysosomes that were suspended in buffered 0·45 M sucrose–0·04% glycogen. Routinely, 0·2-ml aliquots of lysosome suspensions were added to 2·0 ml of incubation media. Therefore, incubation mixtures of lysosomes, media and drugs contained 0·041 M sucrose–0·004% glycogen as well. Such low concentrations of sucrose and glycogen did not contribute significantly to the protection of lysosomes against hypo-osmotic lysis in either distilled water or 0·05 M Tris acetate, pH 7·4. Lysosomes were not fractionated and resuspended in sucrose-free medium because under the experimental manipulations of mincing, homogenization, centrifugation, and consecutive washing and resuspending of liver fractions, each of these procedures being conducted at 0–4°, lysosomes were disrupted to a greater extent.

It was demonstrated previously that certain anti-inflammatory drugs inhibited the release of enzymes from liver lysosomes incubated in buffered hypo-osmotic sucrose, free of inorganic ions.[15–16] The data in this report illustrate that similar drug actions are obtained when incubations are conducted in salt media. Chloroquine, hydrocortisone, phenylbutazone, indomethacin, acetylsalicylic acid and flufenamic acid stabilized lysosomes in 0·15 M KCl, buffered at pH 7·4 (Table 3). At high concentrations flufenamic acid actually labilized lysosomes. A similar biphasic effect was observed in sucrose solution.[15,16] Chlorpromazine also appears to elicit biphasic effects on lysosomes. Chlorpromazine was reported to labilize or stabilize lysosomes *in vivo* at high or low concentrations respectively.[33] Some investigators have reported that chlorpromazine stabilizes[33] and others have indicated that the drug labilizes[34] liver lysosomes *in vitro*. Incubation of lysosomes and drugs in Hanks' BSS (Table 4) yielded data similar to that obtained with 0·15 M KCl except that chloroquine elicited a biphasic effect like that of flufenamic acid. The observation that high concentrations of chloroquine labilized liver lysosomes *in vitro* in a balanced salt solution, but not in sucrose or KCl solution, may be of significance in view of the report by Filkins[35] that chloroquine labilized liver lysosomes *in vivo* and in the isolated liver perfused with a balanced salt solution but did not labilize lysosomes *in vitro* in sucrose solution. Although the inclusion of 10% isologous rat plasma in Hanks' BSS significantly increased the fragility of lysosomes during incubation at 37°, this modification of medium did not alter appreciably the action of drugs on lysosomal enzyme release

(Table 4). It was reported that Tris can interfere with the interaction between lysosomes and monosodium urate crystals by virtue of its capacity to compete for hydrogen bonding.[24] Replacement of 0·05 M Tris acetate, in the KCl medium, with either 0·2 M Tris acetate or 0·05 M potassium acetate did not influence the interaction of lysosomes and anti-inflammatory drugs.

In previous reports it was shown that epinephrine, norepinephrine, isoproterenol, cyclic AMP and dibutyryl cyclic AMP each inhibited the release of enzymes from lysosomes incubated in hypo-osmotic sucrose, buffered at pH 7·4 and free of inorganic ions.[27,28] The action of the catecholamines was enhanced by phosphodiesterase inhibitors such as theophylline, whereas such action was blocked by β-adrenergic receptor antagonists such as propranolol. Drug actions similar to those described previously were obtained when incubations were conducted in salt media, buffered at pH 7·4 (Table 5).

Previous reports indicated that lysosomes in the 3500 *g* liver fraction were more sensitive or responsive than lysosomes in the more conventional light fractions (16,000–25,000 *g*) to the membrane-stabilizing action of anti-inflammatory drugs.[15] Such experiments were performed with hypo-osmotic sucrose solution. This relationship was observed with 0·15 M KCl, buffered at pH 7·4, for anti-inflammatory drugs, epinephrine, cyclic AMP and dibutyryl cyclic AMP (Table 6).

In conclusion, isotonic concentrations of inorganic salts, buffered at pH 7·4, were found to be suitable substitutes for sucrose in retarding the labilization of liver lysosomes during short periods of incubation at 37°. Certain anti-inflammatory drugs, catecholamines and cyclic AMP were found to inhibit the release of enzymes from lysosomes incubated in isotonic salt media. Thus, it appears that the presence of sucrose is not a prerequisite either for preserving the integrity of lysosomes or for the stabilization of lysosomes by membrane-active agents.

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REFERENCES

1. A. Janoff and J. D. Zeligs, *Science, N.Y.* **161,** 702 (1968).
2. G. S. Lazarus, R. S. Brown, J. R. Daniels and H. M. Fullmer, *Science, N.Y.* **159,** 1483 (1968).
3. G. Weissmann and I. Spilberg, *Arthritis Rheum.* **11,** 162 (1968).
4. A. J. Anderson, *Biochem. J.* **113,** 457 (1969).
5. S. Mahadevan, C. J. Dillard and A. L. Tappel, *Archs Biochem. Biophys.* **129,** 525 (1969).
6. J. T. Dingle, *Biochem. J.* **79,** 509 (1961).
7. J. T. Dingle, J. A. Lucy and H. B. Fell, *Biochem. J.* **79,** 497 (1961).
8. H. B. Fell and L. Thomas, *J. exp. Med.* **114,** 343 (1961).
9. G. Weissmann and J. T. Dingle, *Expl Cell Res.* **25,** 207 (1961).
10. G. Weissmann and L. Thomas, *J. exp. Med.* **116,** 433 (1962).
11. A. Janoff and B. W. Zweifach, *J. exp. Med.* **120,** 747 (1964).
12. L. J. Ignarro and J. Slywka, *Biochem. Pharmac.* **21,** 875 (1972).
13. G. Weissmann, *Fedn Proc.* **23,** 1038 (1964).
14. K. Tanaka and Y. Iizuka, *Biochem. Pharmac.* **17,** 2023 (1968).
15. L. J. Ignarro, *Biochem. Pharmac.* **20,** 2847 (1971).
16. L. J. Ignarro, *Biochem. Pharmac.* **20,** 2861 (1971).
17. G. Weissmann and L. Thomas, *J. clin. Invest.* **42,** 661 (1963).
18. L. J. Ignarro, *J. Pharmac. exp. Ther.* **182,** 179 (1972).
19. J. Berthet and C. DeDuve, *Biochem. J.* **50,** 174 (1951).
20. C. DeDuve, R. Gianetto, F. Appelmans and R. Wattiaux, *Nature, Lond.* **172,** 1143 (1953).
21. F. Appelmans and C. DeDuve, *Biochem. J.* **59,** 426 (1955).
22. R. Gianetto and C. DeDuve, *Biochem. J.* **59,** 433 (1955).

23. F. APPELMANS, R. WATTIAUX and C. DEDUVE, *Biochem. J.* **59,** 438 (1955).
24. G. WEISSMANN and G. A. RITA, *New Biology* **240,** 167 (1972).
25. A. B. ROY, *Biochem. J.* **53,** 12 (1953).
26. V. PATEL and A. L. TAPPEL, *Biochim. biophys. Acta* **191,** 86 (1969).
27. L. J. IGNARRO, J. SLYWKA and N. KRASSIKOFF, *Life Sci.* **10,** Part I, 1309 (1971).
28. L. J. IGNARRO, N. KRASSIKOFF and J. SLYWKA, *Life Sci.* **11,** Part I, 317 (1972).
29. J. BROWN and N. SCHWARTZ, *Proc. Soc. exp. Biol. Med.* **131,** 614 (1969).
30. W. E. BOWERS, J. T. FINKENSTAEDT and C. DEDUVE, *J. Cell Biol.* **32,** 325 (1967).
31. M. ROSENBERG and A. JANOFF, *Biochem. J.* **108,** 889 (1968).
32. F. M. BACCINO, G. A. RITA and M. F. ZURETTI, *Biochem. J.* **122,** 363 (1971).
33. P. S. GUTH, JOSÉ AMARO, O. Z. SELLINGER and LLOYD ELMER, *Biochem. Pharmac.* **14,** 769 (1965).
34. C. S. POPOV, *Biochem. Pharmac.* **18,** 1778 (1969).
35. J. P. FILKINS, *Biochem. Pharmac.* **18,** 2655 (1969).