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EFFECT OF ADENOSINE TRIPHOSPHATE AND SOME ANTI-INFLAMMATORY AGENTS ON A PURIFIED LYSOSOMAL FRACTION HAVING HIGH ACID PHOSPHATASE AND LABILE β-GLUCURONIDASE ACTIVITY

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Abstract—A lysosomal fraction (P-2), with 6- to 7-fold increase in acid phosphatase and a 2-fold decrease in β-glucuronidase content has been isolated and partially purified from rat liver homogenates. This fraction showed an excessive loss of β-glucuronidase but not acid phosphatase in the presence of ATP, which stabilized crude mitochondrial-lysosomal fractions. The anti-inflammatory agents, hydrocortisone (10^{-4} M), chloroquine (10^{-4} M) and phenylbutazone (10^{-4} M) showed no lysosomal membrane stabilizing activity when tested at pH 7·4 and pH 5·0. Instead, hydrocortisone and chloroquine enhanced the rate of release of acid phosphatase whereas the latter drug also enhanced the release of β-glucuronidase.

THE THEORETICAL role of lysosomes in the pathogenesis of inflammation has been and continues to be a subject of great controversy. There has been increasing evidence in the last few years that the inflammatory manifestations observed in connective tissue diseases may be mediated by hydrolytic enzymes released from lysosomes.[1–3] Experimental data presented within the above framework have placed much of the emphasis on the role of anti-inflammatory agents as stabilizers of lysosomal membranes, notably the work of Weissmann *et al.*[2,3] However, of late, a number of reports have questioned the conclusions of some of the earlier work regarding the role of anti-inflammatory agents as lysosomal membrane stabilizers.[4–6]

Tanaka and Iizuka[4] found a lack of response of anti-inflammatory agents on a so-called "light" lysosomal fraction. The authors, however, failed to show what the state of purity of this fraction was. Their report implied that lysosomal heterogeneity is a factor in determining sensitivity to nonsteroidal anti-inflammatory drugs. The present study was therefore undertaken in order to determine if a partially purified hepatic lysosomal fraction (P-2 fraction), having excessive β-glucuronidase lability, could be stabilized in the presence of various anti-inflammatory agents. Further, the work described was part of a program designed to investigate the importance of drug interaction with lysosomal membranes in anti-inflammatory activity.

At slightly alkaline and acid pH, the P-2 fraction was not stabilized by hydrocortisone, chloroquine or phenylbutazone. Instead, hydrocortisone and chloroquine had a labilizing effect at pH 7·4 whereas phenylbutazone inhibited β-glucuronidase directly. The morphologic appearance of lysosomes in the P-2 fraction was similar to those appearing in a crude mitochondrial-lysosomal fraction (M–L fraction) as revealed by electron microscopy.

EXPERIMENTAL

Materials

All reagents used were of analytical grade. The sucrose used in the preparation of lysosomes was of ultrapure grade, free of ribonuclease, obtained from the Mann Research Laboratories. Cytochrome *c*, type VI, and chloroquine (diphosphate salt) were obtained from the Sigma Chemical Co. Hydrocortisone was obtained from C. Pfizer & Co. and phenylbutazone from Geigy Pharmaceutical Co.

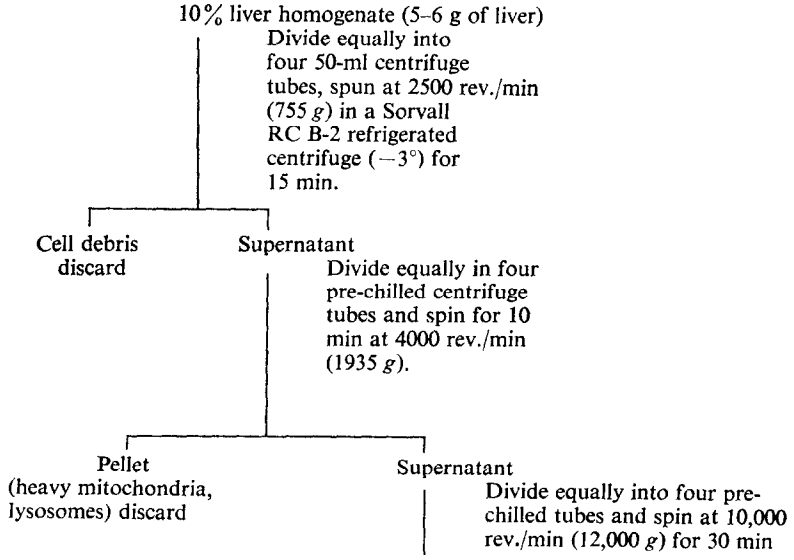
Animals

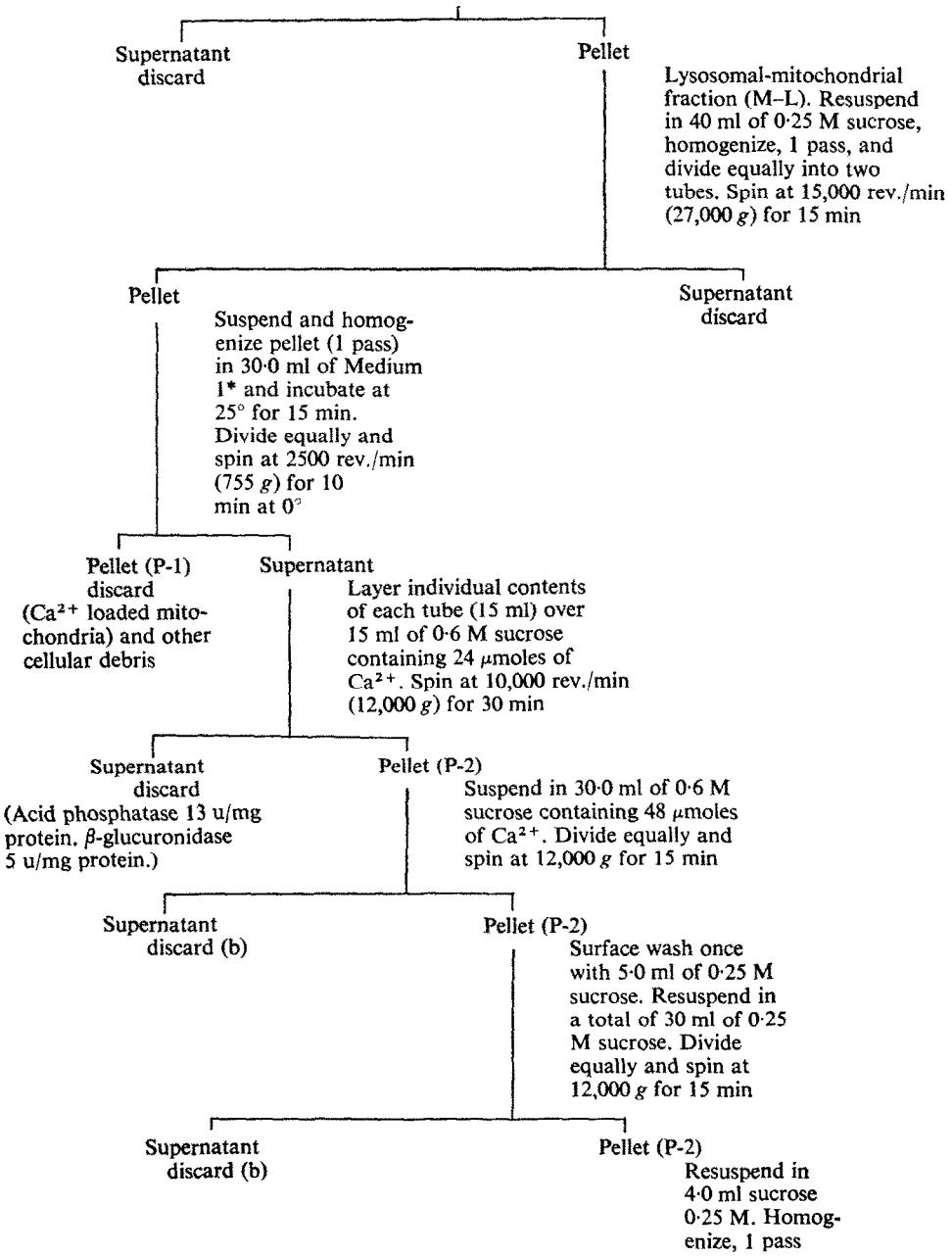
SPF, male Sprague–Dawley rats, weighing 200–300 g, were maintained on Purina Chow and water *ad lib.* All livers were obtained from rats which were exsanguinated by decapitation.

Preparation of a partially purified lysosomal fraction (P-2 Fraction)

An enriched lysosomal fraction was obtained by moderate loading of mitochondria with calcium phosphate (400–450 m-μmoles Ca^{2+}/mg protein). According to Greenawalt *et al.*[7] the active accumulation of Ca^{2+} and P_i would result in an increase of specific gravity of the mitochondria. However, not all mitochondria accumulate Ca^{2+} and inorganic phosphate (P_i) equally. Nevertheless, approximately 36 per cent of the M–L fraction protein nitrogen was removed as P-1 fraction at low speed after incubating the lysosomal-mitochondrial fraction (see below) in a medium which supports accumulation of Ca^{2+} and P_i. Considerable cytochrome oxidase activity was found in this fraction and although the mitochondria were respiring, the response obtained when ADP was added indicated that the mitochondria were uncoupled.

The excised rat liver was quickly chilled in ice-cold normal saline and rinsed until free of blood. The liver was then minced and transferred to a glass homogenizer containing enough 0·25 M sucrose to give a 10% homogenate (w/v). The preparation was homogenized with a Teflon pestle attached to a motor drive set at 1700 rev./min. The isolation procedure is shown in the following flow sheet.





* Medium 1 consists of 90 mM tris–HCl, 75 mM NaCl, 10 mM Na succinate, 10 mM $MgCl_2$, 10 mM KH_2PO_4, pH 7·4, 3 mM Na_2 ATP, 0·8 mM $CaCl_2$ and 60 mM sucrose.

(b) Concentration of free acid hydrolases present were too low to measure.

Incubation of lysosomal fractions

The incubation mixture for studies at pH 7·4 contained the following: lysosomal fraction suspension, 1·2–2·0 mg protein; sucrose–tris acetate (0·25 M sucrose in 0·04 M tris acetate), pH 7·4; and the test compounds as indicated. For studies at pH 5·0, 0·15 M sodium acetate buffer was used in lieu of sucrose–tris. The final volume of the incubation mixture was 4·0 ml and incubation was for 60 min at 37°, unless otherwise specified. Additions of alcoholic solutions of cortisol and phenylbutazone were such that the final concentration of ethyl alcohol in the incubation mixture did not exceed 1%, v/v. At the end of the incubation period, the entire mixture was spun at 15,000 *g* for 15 min at 0°. The resulting clear supernatants containing free acid hydrolases were stored at − 10° in screw-capped vials until ready for analysis (within 48 hr).

Enzyme assays

Acid phosphatase (EC 3.1.3.2) was assayed by using *p*-nitrophenylphosphate as the substrate. To each tube was added 2 mM substrate, 75 mM acetate buffer (pH 5·0) and enzyme solution containing 0·03–0·05 mg N in a total volume of 1·0 ml. Incubation was for 15 min at 37°. The reaction was stopped by addition of 4·0 ml of 0·1 N NaOH. Color density was measured at 410 mμ using a Beckman Model DU spectrophotometer with a Gilford 2000 automatic cuvette changer attachment. A unit of acid phosphatase activity is arbitrarily defined as μmoles of *p*-nitrophenol released per 15 min/mg of protein N.

β-Glucuronidase (EC 3.2.1.31) was assayed by the method of Fishman[8] with some modification. To each tube was added 0·5 mM phenolphthalein glucuronidate, 60 mM acetate buffer, pH 4·5 and enzyme solution (0·03–0·05 mg N) in a final volume of 1·0 ml. Incubation was for 30 min at 37°. The reaction was stopped by addition of 5·0 ml of 0·2 M glycine buffer, pH 10·4, in 0·2 M NaCl. The color developed was read at 540 mμ. A unit of β-glucuronidase activity is arbitrarily defined as μmoles of phenolphthalein released per 30 min/mg protein N.

Cytochrome oxidase (EC 1.9.3.1) was assayed by the method of Wharton *et al.*[9] Mitochondrial respiration was determined polarographically using a Clark oxygen electrode (Yellow Springs Instruments). The test medium consisted of 90 mM tris–HCl buffer, pH 7·4, 75 mM NaCl, 10 mM sodium succinate, 10 mM $MgCl_2$, 10 mM KH_2PO_4, pH 7·4 and 60 mM sucrose in a final volume of 3·0 ml. Oxygen uptake was measured at 30°.

Total enzyme activity

Total enzyme activity was determined with the aid of a sonifier (Heat Systems, Inc.). A lysosomal suspension (1·2–1·4 mg N/ml in 10·0 ml of 0·25 ml sucrose) was sonified for 30 sec in a 10·0 ml rosette cell, at 0° and 60 W input. The mixture was then centrifuged at 90,000 *g* for 30 min, using a No. 40 rotor (Spinco, Model L). The resulting clear supernatant was used for assays. The protein nitrogen of the lysosomal fractions tested was determined by micro-Kjeldahl digestion. The nitrogen content of the digest was determined colorimetrically with Nessler's reagent.

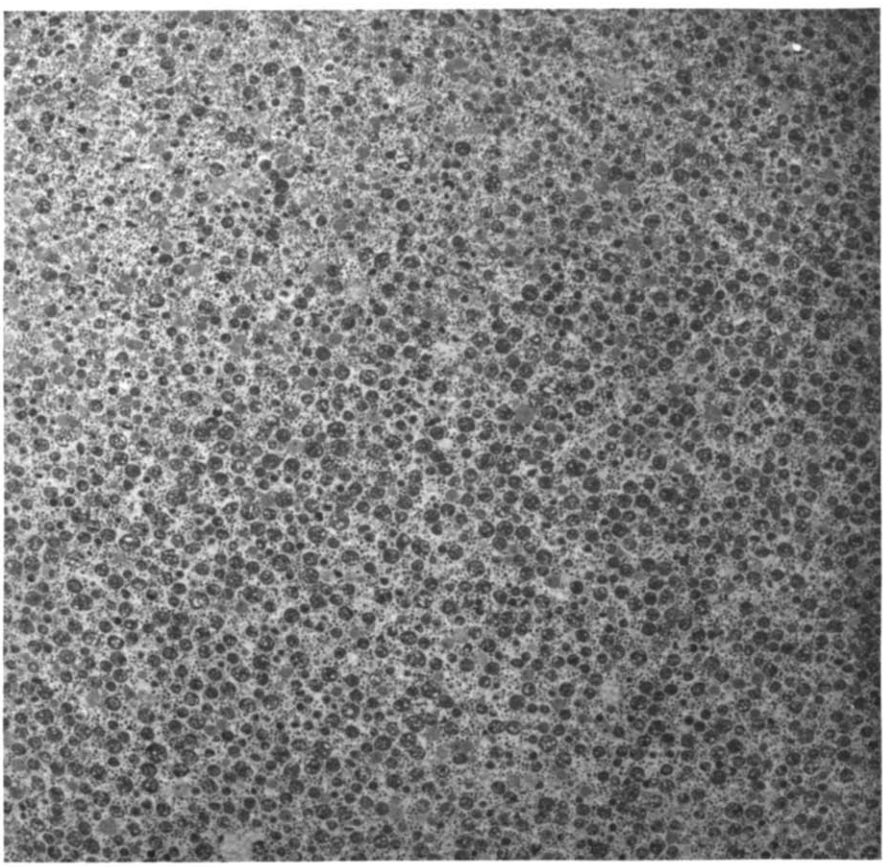


FIG. 1. Section through M-L fraction. Field is dominated by mitochondria in the condensed state. (× 4400.)

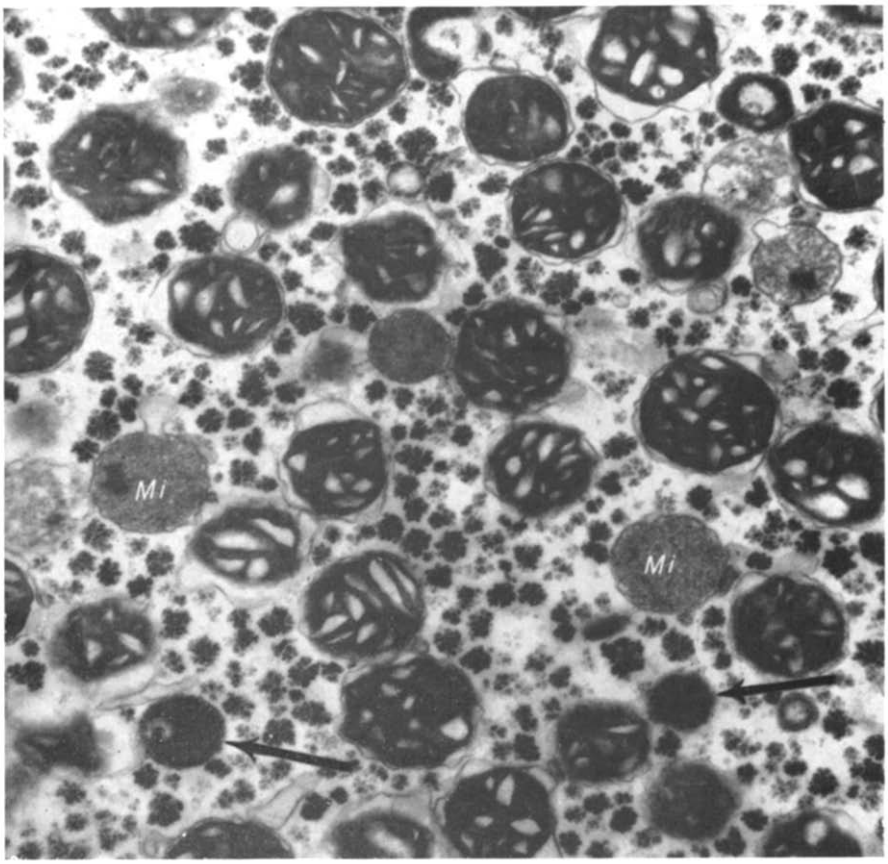


FIG. 2. Section through M–L fraction. Condensed mitochondria are the major organelle present. ↑ = lysosomes; Mi = microbodies. (× 37,600.)

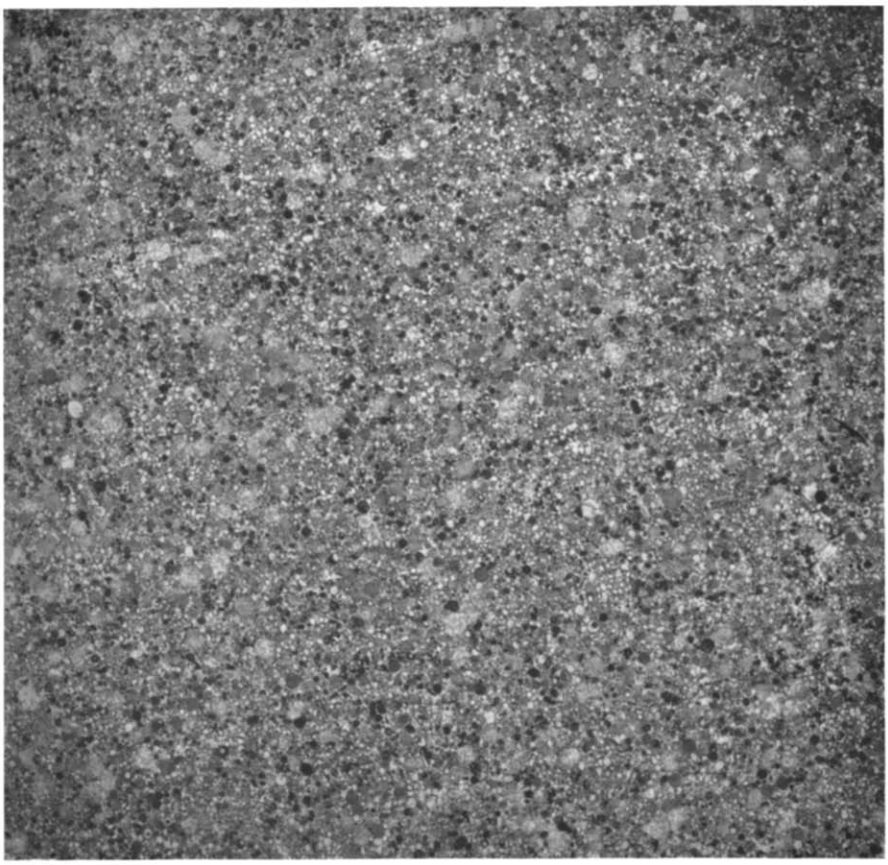


FIG. 3. Section through P-2 fraction. Lysosomes appear as dark staining spherical to ovoid bodies. (× 4400.)

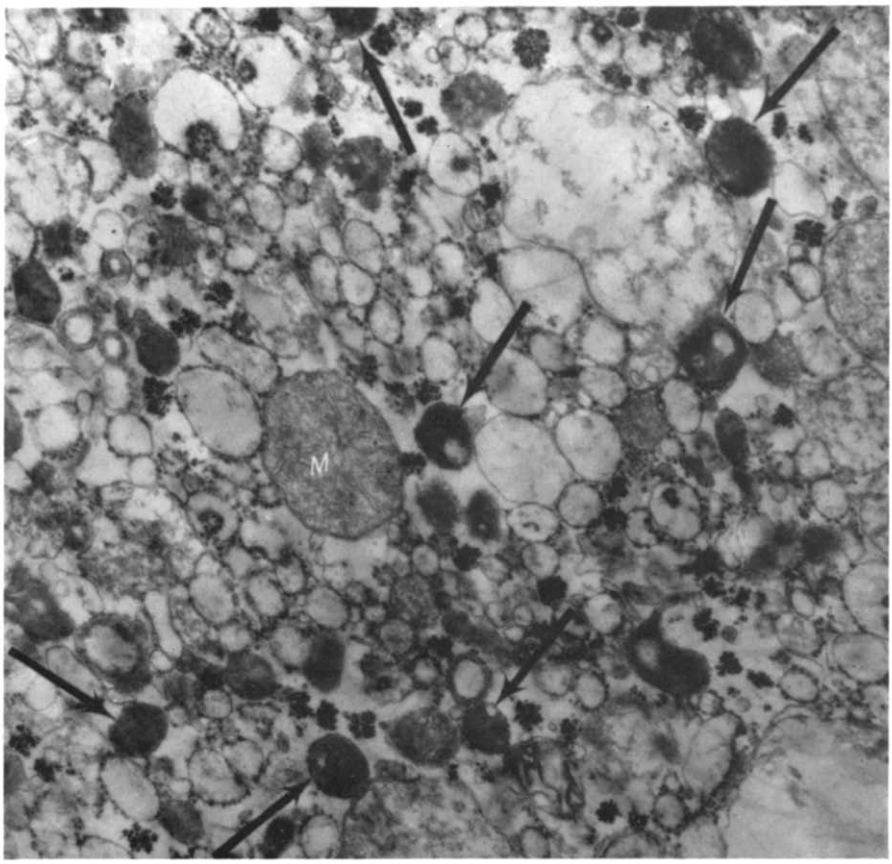


FIG. 4. Section through P-2 fraction. Lysosomes (↑) appear normal. M = swollen mitochondria with poorly defined cristae. (× 37,600.)

Tissue fixation

Initial fixation was with 2·5% phosphate-buffered glutaraldehyde after which the fixed tissues were rinsed with phosphate buffer containing sucrose. Post-fixation was with 1·0% osmic acid in 0·1 M phosphate buffer for 1 hr at 4°.

Electron micrographs were taken at magnifications of 4400 and 37,700 with an RCA EMU 3-M electron microscope using a low-range projector pole piece and a high contrast-type specimen holder at an accelerating voltage of 50 kV.

RESULTS

Comparison of M–L and P-2 fractions

Initial studies indicated that the P-2 lysosomal fraction was devoid of respiratory activity as compared to the mitochondrial lysosomal fraction (M–L). Correspondingly, the cytochrome oxidase activity of P-2 was about 1/10 that of the M–L fraction. The results of a typical experiment are shown in Table 1.

TABLE 1. RESPIRATORY ACTIVITY OF M–L AND P-2 LYSOSOMAL FRACTIONS

Fraction	mμ atoms 0 (min^{-1} mg^{-1} Protein)	Cytochrome oxidase Specific activity
M—L	56	336
P-2	0	36

Assay as described in Experimental section.

The appearance of the two fractions, under high and low magnification is shown in Figs. 1–4. A comparison of the M–L and P-2 fractions at the higher magnification (Figs. 2 and 4) shows that the lysosomes are morphologically similar. Relatively few mitochondria were seen in the P-2 fraction and almost all of those seen were swollen with poorly defined cristae. Amorphous material and mitochondrial remnants as well as cross-sections of smooth and rough endoplasmic reticulum were also observed in the P-2 specimens.

The specific activities of acid phosphatase and β-glucuronidase for both the M–L and P-2 fractions is shown in Table 2. There is a 6- to 7-fold increase in the specific

TABLE 2. SPECIFIC ACTIVITIES OF ACID PHOSPHATASE AND β-GLUCURONIDASE IN M–L AND P-2 FRACTIONS

	Specific activity	
Fraction	Acid phosphatase	β-Glucuronidase
M–L	18 ± 5	8·5 ± 0·5
P-2	123 ± 45	4·3 ± 0·9

Total enzyme activity was determined as described in Experimental.

activity of acid phosphatase in the P-2 fraction over the M–L. In contrast, there is a 2-fold decrease in the specific activity of β-glucuronidase in the P-2 fraction.

Previous studies showed that Ca^{2+} (1·6 mM) in 0·6 or 0·25 M sucrose had no effect on M–L or P-2 lysosomal release of acid phosphatase or β-glucuronidase.

Effect of ATP on lysosomal membranes

The reported presence of an ATPase system in pig brain and rat liver lysosomes[10] as well as Popov's finding that 5′-ATP protected lysosomes against the harmful effects of chlorpromazine[11] prompted us to study the effect of ATP on our M–L and P-2 fractions.

ATP in the presence of Mg^{2+} and P_i markedly reduced the release of acid phosphatase during a 60-min incubation period (pH 7·4) in both the M–L and P-2 fractions

TABLE 3. EFFECT OF ATP ON RELEASE OF LYSOSOMAL ACID HYDROLASES

		Acid phosphatase additions		β-Glucuronidase additions	
		Units released/mg protein			
Fraction	Exp.	None	ATP*	None	ATP*
M–L	1	1·58	0·227	1·92	0·260
	2	1·37	0·286	1·47	0·130
	3	1·53	0·222	1·70	0·300
P-2	1	4·95	1·260	0·90	0·67
	2	2·90	1·490	1·36	1·84
	3	11·40	1·750	2·70	1·85

* Incubation conditions as described in Experimental section except 3 mM ATP, 5 mM $MgCl_2$ and 10 mM KH_2PO_4, pH 7·4 were also added.

as shown in Table 3. However, although ATP decreased the release of β-glucuronidase in the M–L fraction, it had variable effects on the release of P-2 β-glucuronidase.

On the other hand, the high concentration of free β-glucuronidase along with its very low specific activity suggests an increased permeability for this enzyme. Considerable variation is also seen in the release of acid phosphatase from the P-2 fraction in the absence of ATP.

A comparison of the P-2 and M–L fractions for acid hydrolase leakage in the presence and absence of ATP (pH 7·4) is presented in Table 4. The values shown are for a typical experiment and expressed as per cent of total lysosomal enzyme activity. It is readily seen that the amount of free acid hydrolases released by the M–L and P-2 fractions is small except for P-2 β-glucuronidase. Here we see an excessive leakage, even when ATP is present. In these experiments, ADP could not be substituted for ATP.

TABLE 4. LYSOSOMAL HYDROLASE LEAKAGE AFTER 1-hr INCUBATION AT 37°

Fraction	Additions	% of Total activity	
		Acid phosphatase	β-Glucuronidase
M–L	None	6·5	12·0
	ATP*	1·5	6·6
P-2	None	11·4	89·0
	ATP*	1·8	61·0

*Conditions of incubation as described in Experimental section except that 3 mM ATP, 5 mM $MgCl_2$ and 10 mM KH_2PO_4, pH 7·4 were also added.

The effect of various anti-inflammatory agents on P-2 and M–L lysosomal fraction at pH 7·4 and pH 5·0

Most of the previous work reported in lysosomal stabilization by anti-inflammatory drugs was either at pH 5·0 or at a slightly alkaline pH. For this study we chose to investigate the effect of a few known anti-inflammatory agents at both pH levels using our P-2 and M–L fraction in well-buffered solutions.

None of the anti-inflammatory agents tested at pH 7·4 indicated stabilization of lysosomal membranes. Instead, hydrocortisone (10^{-4} M) and chloroquine (10^{-4} M)

TABLE 5. EFFECT OF VARIOUS ANTI-INFLAMMATORY AGENTS ON M–L AND P-2 LYSOSOMAL FRACTIONS, pH 7·4

Additions	Enzyme released expressed as % of control			
	Acid phosphatase		β-Glucuronidase	
1×10^{-4}M	P-2	M–L	P-2	M–L
Hydrocortisone	128 ± 7*	144 ± 14*	108 ± 10	135 ± 14*
Phenylbutazone	110 ± 4	106 ± 4	62 ± 8	81 ± 5
Chloroquine	169 ± 29*	129 ± 12*	129 ± 10*	145 ± 13*

± S.D., four rats.
* $P < 0{\cdot}05$ as compared to control (Student's *t*-test employing a 95 per cent level of confidence).

showed significant labilizing activity ($P < 0{\cdot}05$, Table 5). Neither of these compounds affect free acid phosphatase or β-glucuronidase at the concentrations employed. On the other hand, the response obtained with phenylbutazone (10^{-4} M) was due to a direct inhibition of free β-glucuronidase in agreement with previously reported data.[4,5]

The results of a number of experiments conducted at pH 5·0 are shown in Table 6. No stabilizing effect of the test anti-inflammatory agents employed could be demonstrated. The apparent stabilizing action seen with phenylbutazone is due to inhibition of free enzyme, as mentioned earlier.

The labilizing effects of hydrocortisone and chloroquine observed at pH 7·4 could not be demonstrated at pH 5·0 since acid pH's are known to induce considerable labilization of lysosomes. However, under our experimental conditions, there was no indication that the above anti-inflammatory drugs accelerated the release of free hydrolases.

Similar results were obtained with M–L fractions as shown in Tables 5 and 6. The only apparent difference between these two fractions is in regard to their respective specific activities of acid phosphatase and β-glucuronidase as shown earlier.

TABLE 6. EFFECT OF VARIOUS ANTI-INFLAMMATORY AGENTS ON M–L AND P-2 FRACTIONS, pH 5·0

	Enzyme released expressed as % of control			
Additions	Acid phosphatase		β-Glucuronidase	
1×10^{-4} M	P-2	M–L	P-2	M–L
Hydrocortisone	104 ± 9	106 ± 7	107 ± 4	109 ± 11
Phenylbutazone	97 ± 8	103 ± 8	58 ± 10	53 ± 10
Chloroquine	107 ± 7	94 ± 3	110 ± 10	109 ± 10

± S.D., four rats.

The effect of lowering the concentration of hydrocortisone and chloroquine is summarized in Table 7. It is readily seen that neither compound exhibits a biphasic response. P-2 lysosomal acid phosphatase and M–L β-glucuronidase appeared to be more sensitive to the labilizing effect of hydrocortisone. On the other hand, both the M–L and P-2 β-glucuronidase are more sensitive to the action of chloroquine. In no instance, however, did we observe a stabilizing effect of either chloroquine or hydrocortisone at 10^{-6} M and 10^{-7} M respectively.

TABLE 7. LABILIZING EFFECT OF HYDROCORTISONE AND CHLOROQUINE WITH DECREASING CONCENTRATIONS, pH 7·4

	Enzyme released expressed as % of control			
	Acid phosphatase		β-Glucuronidase	
Additions	P-2	M–L	P-2	M–L
Hydrocortisone				
$\times 10^{-5}$ M	140 ± 11	115 ± 8	109 ± 4	143 ± 11
$\times 10^{-6}$ M	150 ± 10	114 ± 7	109 ± 4	145 ± 5
$\times 10^{-7}$ M	116 ± 2	114 ± 7	103 ± 5	121 ± 8
Chloroquine				
$\times 10^{-5}$ M	112 ± 4	106 ± 6	131 ± 6	142 ± 7
$\times 10^{-6}$ M	114 ± 2	102 ± 4	128 ± 5	152 ± 6

± S.D., three rats.

DISCUSSION

The P-2 lysosomal preparation described above is unusual in that it is acid phosphatase enriched but poor in β-glucuronidase as compared to the M–L fraction. In electron micrographs of both the M–L and P-2 fractions, the lysosomes appeared to be morphologically similar in every detail.

In some of our preliminary experiments, it was found that the nucleotide ATP, but not ADP, in the presence of Mg^{2+}, stabilized the M–L lysosomal fractions during incubation periods up to 90 min. During this interval, acid hydrolase leakage was negligible. However, P-2 fractions incubated under similar conditions (Table 3) demonstrated considerable leakage of β-glucuronidase but not acid phosphatase. The effect of ATP on lysosomal membrane stability supports the hypothesis of an ATPase mechano-chemical complex governing lysosomal permeability,[12] at least in so far as the M–L fraction and the two enzyme markers used are concerned. However, the exceptional behavior of the P-2 fraction with respect to β-glucuronidase seems to suggest biochemical differences among the various lysosomes.

It may be that the P-2 lysosomal fraction represents a more selective group out of a heterogeneous lysosomal preparation (M–L fraction). In other words, the P-2 fraction may represent a more homogeneous group of lysosomes having certain biochemical features not found in other lysosomes. The labilization of β-glucuronidase by hydrocortisone at 10^{-6} M on M–L but not P-2 fractions (Table 7) add further support to our contention that these two fractions are not biochemically equivalent. The evidence presented also suggests that the release of acid phosphatase is independent of β-glucuronidase. Recently, Brown and Schwartz[5] showed that some drugs enhance thermal labilization of acid phosphatase but not β-glucuronidase (pH 6·0). We have obtained similar results with hydrocortisone using the P-2 fraction (Tables 5 and 7). Chloroquine, on the other hand, continued to labilize β-glucuronidase but not acid phosphatase release when the concentration was lowered to 10^{-6} M (Table 7).

The anti-inflammatory drugs had no effect on the release of acid hydrolases when the pH was lowered to 5·0. The data presented in Tables 5 and 6 are consistent with the fact that pH affects membrane permeability as well as accessibility of a given drug to a reactive site. Whether or not the known anti-inflammatory drugs exert a direct or indirect influence on ATP mediated stabilization of lysosomal membranes is currently under study.

The fact that we were unable to demonstrate a stabilizing effect of the various anti-inflammatory agents tested on lysosomal fractions *in vitro* does not necessarily mean that this is the case *in vivo*. Membranes, being dynamic structures, may in their natural environment undergo conformational changes amenable to anti-inflammatory agent-protein interactions. However, it should also be pointed out, using the same line of reasoning, that since labilization and not stabilization of lysosomes was observed, the same may also be true *in vivo*. Perhaps lysosomes are influenced by anti-inflammatory drugs indirectly during an inflammatory process. The anti-inflammatory drug would have more of a regulatory role, where depending upon the immediate environment, the drug could either help labilize or stabilize the lysosomal membrane and its release of acid hydrolases. (This need not be an all or none type but a matter of degree, much as in the control of flow in a river by a flood gate.) According to a recent postulation by Stone,[13] "as cellular debris accumulates outside the cells, it is no longer isolated from the immune system and antibody formation occurs". It would

therefore seem desirable, in this instance, to labilize lysosomes in a controlled fashion to help clear the inflamed area of cellular debris.

The evidence presented herein does not refute the role of lysosomes in the inflammatory process but it does lead one to question the current concept that direct stabilization of lysosomes by certain drugs is a part of the anti-inflammatory process.

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