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EFFECTS OF FENBUFEN AND OTHER ANTI-INFLAMMATORY DRUGS ON RAT LIVER LYSOSOMES

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Abstract—Fenbufen, biphenylacetic acid and other non-steroidal anti-inflammatory drugs were found to labilize the lysosomal membrane of liver lysosomes *in vitro*. The extent of labilization was pH dependent. All the non-steroidal drugs labilized to a much greater extent at pH 5.0 than at pH 7.4. The two steroids tested, cholesterol and cortisone, stabilized the lysosomes at both pH 5.0 and 7.4. Oral administration of fenbufen and indomethacin caused no change in lysosomal membrane stability when assayed *in vitro*. Hydrocortisone showed a small degree of stabilization under these conditions. It is concluded that lysosomal membrane stabilization cannot account for the anti-inflammatory activity of fenbufen, biphenylacetic acid or other non-steroidal anti-inflammatory drugs.

The specific release of lysosomal enzymes from leukocytes and other cells has been postulated as part of the inflammatory response [1]. Lysosomes, especially of leukocytes, contain several basic proteins which can induce an inflammation. One of these basic proteins apparently acts by disrupting mast cells and might be involved in histamine release [2]. In addition, the hydrolytic enzymes that are contained within the lysosomes of all cells are potentially harmful under abnormal conditions where they may hydrolyze and destroy different tissues [3].

It has been proposed that a number of pharmacological agents, toxins, physical procedures, etc. which render lysosomes more permeable *in vitro* may do so *in vivo*. Examples are X- and ultraviolet irradiation [4, 5], streptolysins O and S [6, 7], staphylococcal alpha toxin [8], and carbon tetrachloride [9]. Cortisone, cortisol and their synthetic analogues protect lysosomes against damage by these and other agents [10–12].

Since anti-inflammatory steroids are membrane-stabilizing agents, experiments were carried out to see whether the new non-steroidal anti-inflammatory drug, fenbufen,* and its active metabolite, biphenylacetic acid, have a similar mechanism of action.

MATERIALS AND METHODS

Preparation of lysosome-rich fraction. Male Sprague–Dawley rats, weighing approximately 200 g, were used in all preparations. Animals were killed by decapitation, and the liver was quickly removed and dropped into a beaker containing ice-cold 0.25 M sucrose. The liver was removed, blotted on a paper towel and transferred to a second tared beaker containing approximately 10 ml of ice-cold 0.25 M sucrose. After weighing, the liver was cut into small pieces with a pair of scissors and the volume of sucrose adjusted to give 0.5 g liver/ml. Homogenization was carried out in a glass tube fitted with a Teflon pestle (manufactured by A. H. Thomas, Philadelphia, Pa.). The pestle was driven at about 2200 rev/min by a fixed hand drill operated by a foot pedal. The tube, maintained in crushed ice, was raised and lowered once. The homogenate was then transferred to a 50-ml glass conical centrifuge tube and the volume brought to 40 ml with 0.25 M sucrose. The homogenate was centrifuged for 10 min at 1700 rev/min (580 *g*). After centrifugation, the supernatant was carefully removed, transferred to an Erlenmeyer flask and maintained at 0° until further fractionation. The pellet was redispersed after the addition of 15 ml of 0.25 M sucrose and transferred to the glass homogenizer. It was homogenized and centrifuged as above. The supernatant from the second homogenization was combined with the first supernatant and transferred to a 50-ml polycarbonate tube. Volumes were adjusted to 40 ml in each tube. The supernatants were centrifuged at 12,500 rev/min for 20 min in the Servall model SS-3 using the SS-34 rotor (R_{max} = 10.8 cm, R_{min} = 5.0 cm; *g* (av) = 18,800). After centrifugation, the supernatant was removed and discarded. Ice-cold 0.25 M sucrose was added to give a concentration of 1 g wet weight liver/ml and the pellet gently dislodged with a glass rod. The lumpy suspension was transferred to a 15-ml Dounce homogenizer and the pellet resuspended by two strokes with the loose fitting pestle and two strokes with the tight fitting pestle. The suspension was transferred to a 50-ml polycarbonate centrifuge tube, the volume was brought to 40 ml, and the contents were centrifuged as above. This procedure was repeated once again so that the lysosome fraction was washed twice. After the final centrifugation, the pellet was resuspended to a concentration representing 0.125 g wet weight liver/ml.

Membrane stabilization assay. The lysosome suspension (1 ml) was mixed with 10 μl of the concentrated drug dissolved in either dimethylsulfoxide or dioxane. Solvent alone was added to control tubes. After the addition of 1 ml of 0.1 M sodium acetate

* Fenbufen is the U.S.A.N.-approved generic name for 3-(4-biphenylylcarbonyl)propionic acid and biphenylacetic acid is one of its several metabolites *in vivo*. A clinical report on fenbufen's activity in rheumatoid arthritis has recently appeared [*Curr. Ther. Res.* **18**, 295 (1975)].

buffer, pH 5.0, made up in 0.25 M sucrose, these suspensions were incubated at pH 5.0 and 37°. During this incubation, the concentration of acetate plus acetic acid was 0.05 M, that of the solvent 0.5% (v/v).

Acid phosphatase determination. To measure free acid phosphatase, sodium β-glycerophosphate was added to the lysosome suspension to a final concentration of 0.05 M, as a solution in 0.25 M sucrose containing 0.05 M sodium acetate buffer, pH 5.0, in order not to modify the composition of the medium. The incubation was carried out for 10 min. The reaction was terminated by the addition of ice-cold trichloroacetic acid to a final concentration of 6%. An aliquot of the phosphate set free was then determined by a colorimetric assay [13]. Total enzyme activity was determined by disrupting lysosomes with 0.1% Triton X-100.

Experiments in vivo. Male Sprague–Dawley rats weighing between 175 and 200 g were used. Animals were given, by gavage, the following doses of anti-inflammatory drugs suspended in 0.067 M phosphate buffer containing 1% starch: 2 mg/kg of indomethacin, 100 mg/kg of hydrocortisone, 400 mg/kg of aspirin, and 50 mg/kg of fenbufen. These doses could be considered the maximum tolerated for chronic adminstration. The drugs were given at 3:00 p.m. for 3 consecutive days and the animals sacrificed the morning of day 4. Livers were removed, prepared and assayed for membrane stabilization as described above.

Reagents. Cholesterol, hydrocortisone and cortisone were purchased from CalBiochem. Aspirin was purchased from Penick & Co. Fenbufen, *p*-biphenylacetic acid, and phenylbutazone were generously supplied by the Process and Analytical Section of Lederle Laboratories. We gratefully acknowledge the gift of indomethacin from Merck & Co.

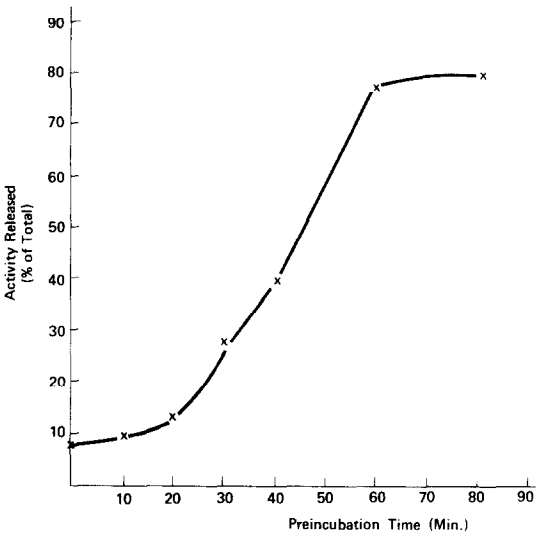

Fig. 1. Release of acid phosphatase from lysosomes *in vitro*. Lysosomal fraction, representing 125 mg wet weight liver, was suspended in 0.05 M sodium acetate, pH 5.0, containing 0.25 M sucrose and incubated at 37°.

RESULTS

Latency of acid phosphatase. Figure 1 illustrates a typical experiment showing the magnitude of acid phosphatase release from rat liver lysosomes with time of incubation. By 60 min, greater than 80 per

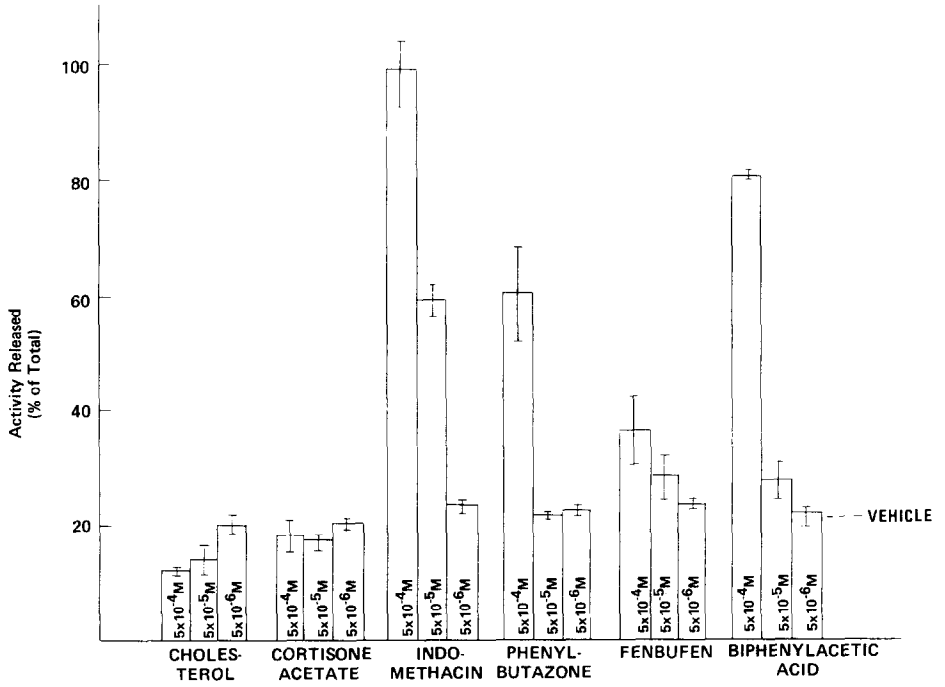

Fig. 2. Effect of fenbufen, cholesterol and selected anti-inflammatory drugs, at concentrations 5×10^{-6} to 5×10^{-4} M, on acid phosphatase release. Lysosomal fraction was suspended in 0.05 M sodium acetate, pH 5.0, containing 0.25 M sucrose, and incubated at 37° for 40 min. Each bar represents the mean of three experiments. Verticle lines are ± S. E. M.

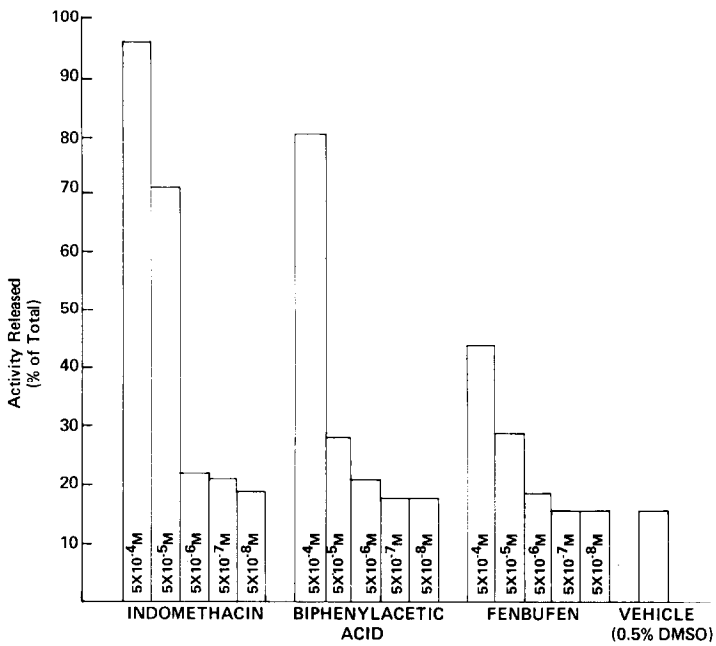

Fig. 3. Effect of fenbufen, biphenylacetic acid and indomethacin, at concentrations 5×10^{-8} to 5×10^{-4} M, on acid phosphatase release. Lysosomal fraction was suspended in 0.05 M sodium acetate, pH 5.0, containing 0.25 M sucrose, and incubated at 37° for 40 min. Each bar represents the mean of duplicate determinations from a single experiment.

cent of the total acid phosphatase has been released into the medium, after which time there are small increases. At zero time, approximately 8 per cent of the total activity is already soluble. This zero time value varies between 0 and 10 per cent and is an indication of the state of the preparation. Preparations giving greater than 10 per cent zero time values were discarded since the lysosomes were assumed to have been labilized by the preparative procedure.

Effect in vitro *of drugs.* Figure 2 shows the effect of fenbufen and other anti-inflammatory drugs on lysosomal membrane stability. Cholesterol and cortisone acetate are known lysosomal stabilizers [11, 14]. Both cholesterol and cortisone, used in these experiments as standards, do function as stabilizers whereas fenbufen and the non-steroidal anti-inflammatory drugs are labilizers. Indomethacin is the most potent non-steroidal anti-inflammatory drug in accelerating the release of acid phosphatase from the lysosome fraction, whereas fenbufen shows the smallest effect. In one experiment, as shown in Fig. 3, the concentration range for indomethacin, biphenylacetic acid and fenbufen was extended to 5×10^{-8} M to determine whether there is stabilization at lower concentrations. Some investigators have observed such a biphasic reaction for some anti-inflammatory drugs [15]. In the experimental system reported in this paper, no such biphasic reaction was seen.

It has been reported [16] that the experimental conditions that are selected can determine whether drugs will act as stabilizers or labilizers. Therefore, the effect of pH, osmotic conditions and the nature of the subcellular populations were examined. A population of particles containing only larger lysosomes (SS-34 head; Servall model SS-3; $R_{max} = 10.8$ cm, $R_{min} = 5.0$ cm; g (av) = 3640) was isolated and the effect of fenbufen and the other anti-inflammatory drugs was tested as described above. No differences were found between this subcellular

Table 1. Effect of anti-inflammatory drugs on release of acid phosphatase from rat liver lysosomes at pH 7.4 and 0.18 M sucrose*

Drug	Concn (M)	N	% Release of acid phosphatase (mean ± 1 S.E.M.)
Indomethacin	5×10^{-4}	2	14.5 ± 1.8†
	5×10^{-5}	2	10.3 ± 1.3
	5×10^{-6}	2	9.4 ± 1.0
Phenylbutazone	5×10^{-4}	2	7.4 ± 0.7†
	5×10^{-5}	2	7.9 ± 0.7
	5×10^{-6}	2	8.6 ± 1.0
Biphenylacetic acid	5×10^{-4}	2	9.7 ± 0.9
	5×10^{-5}	2	9.2 ± 0.9
	5×10^{-6}	2	9.4 ± 1.0
Fenbufen	5×10^{-4}	2	10.5 ± 0.8
	5×10^{-5}	2	9.4 ± 0.8
	5×10^{-6}	2	9.0 ± 1.0
Cholesterol	5×10^{-4}	2	4.9 ± 0.5†
	5×10^{-5}	2	6.7 ± 0.6‡
	5×10^{-6}	2	8.2 ± 0.8
Cortisone	5×10^{-4}	2	7.6 ± 0.7
	5×10^{-5}	2	8.4 ± 0.7
	5×10^{-6}	2	8.4 ± 0.7
Control (vehicle)		2	9.0 ± 0.5

* An aliquot (0.2 ml) of liver fraction was added to 2.0 ml of 0.18 M sucrose–0.04 M Tris–acetate, pH 7.4, and centrifuged either immediately or after a 15-min incubation period. An aliquot (0.5 ml) was used in the enzyme assay.

† Statistically significant differences from control group with $P < 0.05$.

‡ Statistically significant differences from control group with $P < 0.01$.

Table 2. Effect of oral administration of drugs on the stability of rat liver lysosomes*

Drug	% Change in body weight	% Release of acid phosphatase *in vitro*†
None	+ 11.6 ± 0.7‡	36.3 ± 2.5
Hydrocortisone (100 mg/kg)	+ 5.7 ± 0.8	27.8 ± 3.8 ($P < 0.10$)
Fenbufen (50 mg/kg)	+ 11.5 ± 1.1	33.8 ± 3.7 (NS)
Indomethacin (2 mg/kg)	+ 12.0 ± 1.2	32.7 ± 3.8 (NS)

* Male Sprague–Dawley rats, weighing 175–200 g, were given, by gavage, the indicated dose of drugs in phosphate buffer containing 1% starch for 3 consecutive days.

† An aliquot (1.0 ml) of lysosome suspension was added to 1.0 ml of 0.1 M sodium acetate buffer, pH 5.0, made up in 0.25 M sucrose and incubated at 37° for 40 min. Sodium β-glycerophosphate was then added (0.5 ml) to a final concentration of 0.05 M as a solution in 0.25 M sucrose containing 0.05 M sodium buffer, pH 5.0. The reaction was terminated by the addition of 0.4 ml of trichloroacetic acid (35%, w/v). An aliquot was assayed for the free phosphate [13]. NS = not significant.

‡ Values represent the mean of eight determinations ± 1 S.E.M.

population and the subcellular population containing a more complete lysosomal population. The method of Ignarro [16] was used to examine the effect of pH and osmotic medium (Table 1). In contrast to Ignarro's results, fenbufen, biphenylacetic acid and indomethacin labilize the lysosomes under these conditions at all concentrations. They are, however, much weaker labilizers than they are at pH 5.0. Phenylbutazone, in this assay system, acts as a stabilizer.

Effect in vivo *of drugs.* Table 2 shows the results of stabilization assays of lysosomes from rats treated with fenbufen, indomethacin and hydrocortisone for 3 days. The experiments were designed to see whether the membrane properties of lysosomes were altered by administration *in vivo* as tested under conditions *in vitro*. The results show that neither fenbufen nor indomethacin is significantly different from untreated controls in affecting membrane stability. In contrast, the anti-inflammatory steroid, hydrocortisone, did show a small amount of stabilization.

DISCUSSION

The experiments described in this report demonstrate that fenbufen and biphenylacetic acid are labilizers of the lysosomal membrane. These two drugs have the same effect on the lysosomal membrane as do the other non-steroidal anti-inflammatory drugs tested in these experiments. All the non-steroidal anti-inflammatory drugs showed a potent capacity to disrupt the lysosomal membrane at pH 5 and a moderate ability to do so at pH 7.4. On the other hand, under the same experimental conditions, both cholesterol and cortisone were membrane stabilizers. Others have reported similar findings with other non-steroidal anti-inflammatory drugs. Weissman [17] found that neither salicylates, indomethacin nor flufenamic acid had the capacity to stabilize lysosomes. He found these compounds to be labilizers. Similarly, different investigators have not demonstrated stabilizing properties for selected non-steroidal anti-inflammatory drugs [18–22]. Fenbufen and biphenylacetic acid, therefore, show a similar property as other members of this class of drugs.

As shown in Table 1, stabilization by non-steroidal anti-inflammatory drugs could not be demonstrated by incubating the lysosomes in a Tris–acetate buffer at pH 7.4. This is in contrast to Ignarro [16], who found non-steroidal anti-inflammatory drugs to stabilize the lysosomal membrane under these conditions. In addition, the use of a "large lysosomal" fraction (sedimenting at 3500 *g* range), under these experimental conditions, did not result in stabilization. Lysosomes are more stable at pH 7.4 in Tris–acetate buffer compared to sodium acetate buffer at pH 5.0 as shown by Ignarro (see also Table 1). However, except for phenylbutazone, fenbufen and the other non-steroidal anti-inflammatory drugs were labilizers under both conditions but to different degrees. The differences between the results shown in this report and those of Ignarro [16] and Tanaka and Iizuka [23] could be due to subtle differences in the preparation procedures.

The mechanisms whereby non-steroidal anti-inflammatory drugs affect lysosomes are not known. As discussed by deDuve *et al.* [11], lysosome disruption may involve an initial enzymatic reaction which may serve to initiate a series of events which include changes in permeability, diffusion of solutes and an osmotic type of swelling before leading to disruption. A test compound might interact with the membrane of the particles and either influence its susceptibility to the attacking enzymes or modify the way in which its physical properties are affected by a given degree of enzymatic degradation. The known ability of non-steroidal anti-inflammatory drugs to bind to serum proteins [24] might be an indication of their ability to bind to plasma membrane proteins and change the properties of the lysosomal membrane. This mechanism probably accounts for the effects of steroids which bind to membrane phospholipids [25].

This study reveals that indomethacin is the most potent labilizer and fenbufen the mildest at pH 5. It is possible that these findings could be related to the ulcerogenic effect of these two drugs. Lewis *et al.* [15] studied the effect of phenylbutazone on isolated stomachs and found an increased rate of release of *p*-nitrophenylphosphatase which correlated with the ulcerogenic action of phenylbutazone. In addition, Lewis [26] has found that non-steroidal drugs accelerate the thermal denaturation of albumin at high concentrations (10^{-3} M). From these observations, along with the results presented in this report, it could be inferred that indomethacin should be the most ulcerogenic and fenbufen the least based on the ability to release lysosomal enzymes at an acid pH.

In the experiments *in vivo*, hydrocortisone showed a small degree of stabilization while indomethacin and fenbufen showed no differences from untreated controls. The results with hydrocortisone are consistent with the experiments *in vitro*. However, the two non-steroidal anti-inflammatory drugs tested do not cause an increased fragility of the isolated lysosomes. Indomethacin and fenbufen at these doses, therefore,

most likely do not bind to lysosomal membranes *in vivo* and render them more susceptible to challenging agents as occurs *in vitro*.

It appears, therefore, that fenbufen and biphenylacetic acid fall into the same class as the other non-steroidal anti-inflammatory drugs with respect to their ability to affect the lysosomal membrane. Since labilization of lysosomal membranes will result in cell and tissue destruction, this effect cannot be used to explain the anti-inflammatory mechanism of fenbufen or other non-steroidal drugs.

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