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SUPPRESSION OF ENZYME RELEASE FROM ISOLATED RAT LIVER LYSOSOMES BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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Abstract—The stabilizing effect of non-steroidal anti-inflammatory drugs on the isolated rat liver lysosomes was investigated by determining the enzyme activity released from lysosomes and appearing in the supernatant. Acetylsalicylic acid, phenylbutazone and flufenamic acid suppressed the release of acid phosphatase and β-glucuronidase significantly in neutral buffer (pH 7·4)-sucrose medium at 37°. In acidic buffer (pH 5·0)-sucrose medium, acetylsalicylic acid accelerated the release of acid phosphatase slightly. The lysosome-stabilizing effect of drugs shown at neutral pH was more prominent when the heavy lysosomal fraction (precipitated between 700 and 3500 *g*) was used, instead of the whole lysosomal fraction. On the other hand, stabilization was not demonstrated significantly by drugs when the light lysosomal fraction (precipitated between 3500 and 18,000 *g*) was used. Thus, the rat liver lysosome seems to to be heterogeneous, pharmacologically. The dose-response relationship for enzyme release from the heavy lysosomes, was studied. For acetylsalicylic acid, the effect increased with increasing concentrations of the drug while for the other drugs, an optimum concentration for the maximum response was observed. The minimum concentrations causing a 30 per cent inhibition of the enzyme release were, 8, 170 and 500 μM for flufenamic acid, phenylbutazone and acetylsalicylic acid, respectively. Indomethacin was almost inactive in a wide range of concentrations.

SINCE de Duve and his co-workers[1] reported the lysosomes as subcellular bags of acid hydrolases exhibiting structure-linked latency, further biochemical and morphological evidences have been accumulated and argued on their physiological or pathological significance.

In relation to the etiology of inflammation, some hypotheses have been proposed,[2–4] in which it is postulated that lysosomal contents such as hydrolytic enzymes or cationic proteins play an important role for the initiation of inflammation. In this connection, it is interesting that some anti-inflammatory drugs were reported to stabilize the isolated lysosomes,[5–7] although many data for this have not yet been accumulated so far.

In this report, possible stabilizing effect of some typical non-steroidal anti-inflammatory drugs was studied on the isolated rat liver lysosomes, by means of determination of the released enzyme activity.

Our attention was focussed on the following two points:

(a) The pH of medium in which lysosomes were incubated with drug solution, and (b) possible heterogeneity of rat liver lysosomes with respect to their sensitivity to anti-inflammatory drugs.

At neutral pH, the lysosomes, precipitated in the so-called heavy mitochondrial fraction, were revealed to be stabilized significantly by acetylsalicylic acid, phenylbutazone and flufenamic acid at the respective concentrations comparable to those required for their *in vivo* effect.

EXPERIMENTAL

Materials. Cortisone was purchased from Roussel Uclaf. Phenylbutazone, indomethacin and flufenamic acid were kindly supplied by Dr. Y. Yoshida of these Laboratories. Acetylsalicylic acid was of Pharmacopoeia Japonica grade and the other chemicals were of reagent grade.

Preparation of lysosomal fractions. Sprague–Dawley female rats, weighing between 280 g and 380 g, were used. Three to six animals were anesthetized with ether. After decapitation and bleeding, the liver was perfused *in situ* with 30 ml of 0·25 M ice-cold sucrose medium via portal vein at a rate of approximately 15 ml/min, and then was removed quickly. After weighing, the tissue was cut into small pieces and dispersed in medium placed in a Dounce-type homogenizer. Homogenization was carried out by 5 strokes of the teflone pestle, and the volume was adjusted to give 17 per cent (w/v) homogenate. After filtration through three sheets of gauze, the homogenate was centrifuged at 2700 rpm for 15 min (minimum and maximum radii were 6·6 and 10·4 cm, respectively). The sediment was washed under the same conditions, then the first supernatant and the wash were combined (Supernatant I).

The lysosomal preparations used were three kinds of centrifugal pellets, each of which was corresponding to the whole mitochondrial, the heavy mitochondrial and the light mitochondrial fraction, respectively.

In this paper, we designated the just mentioned three fractions as the whole, the heavy and the light lysosomal fraction, respectively.

The whole lysosomal fraction was prepared by centrifuging Supernatant I at 14,000 rpm for 15 min. After washing once, the sediment was resuspended in the sucrose medium to give 1·25 g of liver equivalent per ml as the final concentration (the whole lysosomal fraction).

In order to prepare the heavy and the light lysosomal fractions, Supernatant I was centrifuged at 6000 rpm for 15 min. The sediment was washed once and then resuspended in medium to give 1·25 g of liver equivalent per ml (the heavy lysosomal fraction). The resulted supernatant was further centrifuged at 14,000 rpm for 15 min and the washed sediment was resuspended in sucrose medium to give 2·0 g of liver equivalent per ml (the light lysosomal fraction).

Throughout procedures, pipetting required for resuspending was carried out most carefully to keep gentle.

Incubation of lysosomes with drug solution. Incubation mixture consists of 3 ml of the lysosomal preparation, 10·5 ml of buffer-sucrose medium (usually 0·25 M sucrose in 0·04 M Tris-acetate buffer of pH 7·4 was used, unless otherwise specified), and 1·5 ml of drug solution in buffer. The drug was, at first, dissolved in ethanol, the concentration of which was adjusted to become 0·5 per cent in the final incubation mixture.

In order to avoid rupture of lysosome caused by sudden elevation of the temperature, the ice-cold lysosomal preparation was warmed up to 25° previously 2 min before the incubation at 37°. After the mixture was made up in a shaking flask (this moment is

designated as the *zero time* of incubation), flask was incubated at 37° under the reciprocating shakes (46 strokes/min and 5 cm amplitude). At definite time intervals, aliquotes (usually 2 ml) were sampled and centrifuged at 13,200 rpm for 15 min (average radius was 8·0 cm). The resulted supernatant was then subjected to enzyme assay to determine the free activity. For the determination of total activity of lysosomal preparation, the freezing-thawing pretreatment was recycled twice in acetate buffer (pH 5·0) containing Triton X 100 as 0·2 per cent. Then, after centrifugation, the resulted supernatant was subjected to the enzyme assay.

In experiments to check the direct effect of drugs on enzyme activity, the enzyme sample was prepared at first, as follows.

The lysosomes were incubated at 37° for 90 min in buffer-sucrose medium (pH 7·4) without drug. The concentration of lysosomes were twice as much as in the usual case. After incubation, the suspension was centrifuged and the resulted supernatant was used as the enzyme sample. To an aliquot of this enzyme sample, an equal volume of drug solution (in Tris-acetate buffer pH 7·4 containing 1% of ethanol) was added, and the incubation was carried out at 37°. With definite time intervals, aliquots were sampled and immediately subjected to enzyme assay.

Enzyme assay. Activity of acid phosphatase (EC 3.1.3.2, Orthophosphoric monoester phosphohydrolase), was measured using sodium p-nitrophenyl phosphate as substrate. Total volume was 3·0 ml. The concentrations of substrate and buffer (pH 4·8), were 5·8 and 67 mM, respectively. Enzymation was carried out for 15 min at 37° and the reaction was terminated by addition of 1 ml of 1 N sodium hydroxide solution. After adequate dilution with distilled water, the extinction at 410 mμ was measured using Hitachi, Perkin–Elmer Spectrophotometer UV-VIS No. 139.

Activity of β-glucuronidase (EC 3.2.1.31, β-D-glucuronide glucuronohydrolase), was measured according to the conditions reported by Gianetto and de Duve[8] with slight modifications. Total volume was 2 ml, and the concentrations of substrate (*p*-nitrophenyl glucuronide) and acetate buffer (pH 5·2), were 1·25 and 80 mM, respectively. After enzymation for 30 min at 37°, the reaction was stopped by addition of an equal volume of 0·1 N sodium hydroxide solution. In this case, the extinction at 410 mμ was measured between 10 and 20 min after addition of alkaline solution, because a slight decomposition of unreacted substrate occurred after a prolonged standing.

RESULTS AND DISCUSSION

Effect of cortisone and acetylsalicylic acid at acidic pH

In an attempt to reconfirm the effect of anti-inflammatory drugs on the stability of isolated rat liver lysosomes,[5, 6] time course of the acid phosphatase release from lysosomes was followed in the presence of cortisone or acetylsalicylic acid. In these experiments, acetate buffer (pH 5·0)-sucrose (0·25 M) medium was used, and the whole lysosomal fraction was used as lysosomal preparation. Results of a typical experiment are illustrated in Fig. 1. The initial free activity was not affected by the presence of cortisone at $3{\cdot}5 \times 10^{-4}$ M. However, the free activity increment during the first 60 min of incubation was significantly suppressed by this drug, in agreement with the data reported by de Duve *et al.*[5]

Acetylsalicylic acid, on the other hand, did not suppress the enzyme release. Furthermore, when non-bufferized sucrose solution was used as the incubation

medium, a drastic acceleration of the enzyme release was observed by addition of acetylsalicylic acid within the first 60 min of incubation, contrary to the data by Miller *et al.*[6]

In these experiments, severe drop in pH of the medium was observed, for instance from 7·2 to 5·95 and to 4·5, by the addition of acetylsalicylic acid of 3×10^{-4} M and 10^{-3} M, respectively.

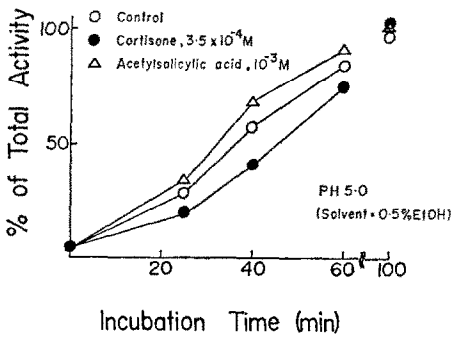

FIG. 1. Effect of cortisone and acetylsalicylic acid on release of acid phosphatase from lysosomes at acidic pH. Ordinate: free activity of acid phosphatase detectable in the supernatant. Incubation mixture: 0·04 M acetate buffer (pH 5·0), 0·25 M sucrose, 0·5% ethanol, and lysosomes at 0·25 g of liver equivalent/ml. The whole lysosomal fraction was used as lysosome preparation. Incubation was carried out at 37°.

The lability of rat liver lysosomes was studied earlier by Berthet *et al.*[9] and recently by Sawant *et al.*,[10] as a function of pH. In the latter, they reported that the instability of lysosomes was shown in the acidic pH range below 6·4. Consequently, in order to reproduce such a possible effect of acetylsalicylic acid as the stabilizer on lysosomes suggested by Miller, some other experimental conditions should be searched for.

Direct effect of drugs on enzyme activity

In order to know the stabilizing effect of drugs on lysosomes by means of determination of the activity of enzymes which released from lysosomes during the incubation, the direct effect of drugs on the enzyme itself have to be checked at first.

As shown in Table 1, acid phosphatase activity was affected neither by acetylsalicylic acid nor by flufenamic acid at the concentrations used in the succeeding experiments. Also, acid phosphatase activity was not practically influenced by phenylbutazone or indomethacin at 3×10^{-4} M, although only a slight loss of activity was observed after a prolonged incubation of 90 min, for instance, at 37°.

On the other hand, β-glucuronidase activity was impaired considerably by phenylbutazone and indomethacin at the concentrations more than 10^{-4} M and 3×10^{-4} M, respectively, while this enzyme was not affected by acetylsalicylic acid or flufenamic acid.

Consequently, acid phosphatase was an adequate enzyme as a marker of the lysosomal contents, whereas β-glucuronidase was only available in the experiments, in which the effect of acetylsalicylic acid or flufenamic acid was examined.

In addition, the loss of β-glucuronidase activity due to the action of either phenylbutazone or flufenamic acid, might support, at least partly, their pharmacological validity as anti-inflammatory drugs *in vivo*.

TABLE 1. DIRECT EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON LYSOSOMAL ENZYME ACTIVITY

Drug	Concn.* (M)	Enzyme activity			
		Acid phosphatase		β-glucuronidase	
		10†	90†	10†	90†
		(% of corresponding control ± S.E.)			
Acetylsalicylic acid	10^{-3}	102·2 ± 1·5	102·4 ± 1·0	102·0 ± 4·1	99·4 ± 0·5
Phenylbutazone	10^{-3}	100·3	96·0	66·5 ± 2·1	65·4 ± 5·3
	3×10^{-4}	98·2 ± 0·7	94·1 ± 1·1	85·9 ± 4·6	75·5 ± 1·6
	10^{-4}			96·3 ± 6·0	82·7 ± 3·6
Flufenamic acid	10^{-4}	98·3 ± 1·5	99·4 ± 0·7	98·3 ± 1·2	100·8 ± 2·1
Indomethacin	3×10^{-4}	96·5 ± 1·9	93·8 ± 1·1	91·1 ± 2·2	78·6 ± 3·9

* indicates the concentration at the preincubation stage.

† indicates preincubation time (min).

Enzyme preparation used, was the supernatant obtained from lysosomal suspension (the heavy lysosomal fraction) after an incubation for 90 min at 37° in buffer (pH 7·4)-sucrose (0·25 M) medium. Preincubation of enzyme with drug solution was carried out for 10 and 90 min at 37° in buffer-sucrose containing ethanol as 0·5 per cent.

Effect of anti-inflammatory drugs on release of lysosomal enzymes at neutral pH

In the preliminary experiments, acetylsalicylic acid and phenylbutazone exhibited suppression of the enzyme release from lysosomes in neutral buffer-sucrose (0·25 M) medium, but data obtained are lacking in reproducibility. In these experiments, relatively minute amounts of inorganic salts such as sodium or potassium chloride were found to affect the results,[11] as those observed in the leucocyte lysosomes.[12]

Thus, in order to avoid such hazardous effect of inorganic salts, Tris-acetate buffer (pH 7·4)-sucrose (0·25 M) solution was selected as one of the adequate media for lysosome incubation, and drugs were dissolved in ethanol to solubilize them, instead of neutralization with alkaline solution. In addition, mild shaking conditions were introduced in the incubating procedures. The details of these conditions were described in Experimental.

Under these experimental conditions, the stabilizing effect of acetylsalicylic acid, phenylbutazone and flufenamic acid on rat liver lysosomes was ascertained after the repeated trials. Some typical results are illustrated in Figs. 2 and 3, and summarized data are shown in Fig. 4.

As shown in Fig. 2, acetylsalicylic acid of 10^{-3} M lowered the enzyme activity of acid phosphatase and β-glucuronidase detectable in the supernatant after 90 min incubation. As already shown in the preceding section, acetylsalicylic acid did not affect the activity of these enzymes themselves. Thus, the data obtained suggest directly that acetylsalicylic acid suppressed the enzyme release from lysosomes as a result of stabilization of lysosomes.

With respect to the effect of 10^{-5} M of flufenamic acid, it was also the case as shown in Fig. 3.

Phenylbutazone was more inhibitory on β-glucuronidase than on acid phosphatase at the concentration of 3×10^{-4} M. Preseumably, this may reflect the difference in the inhibitory effect on these two enzymes as shown in Table 1.

From the summarized data in Fig. 4, it is clear that none of the tested drugs affected the initial free activity, whereas the suppression of enzyme release became more

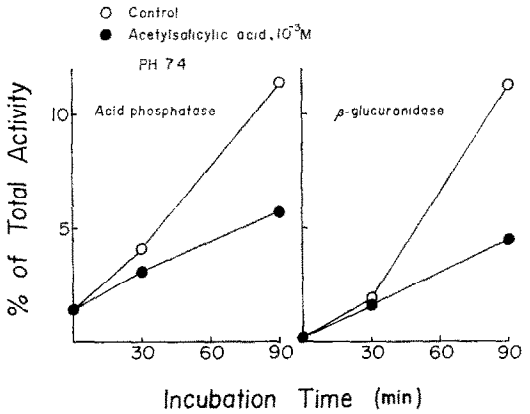

FIG. 2. Effect of acetylsalicylic acid on release of acid phosphatase and β-glucuronidase from lysosomes at neutral pH. Ordinate: free activity of enzymes detectable in the supernatant. Incubation mixture: 0·04 M Tris-acetate buffer (pH 7·4), 0·25 M sucrose, 0·5% of ethanol, and lysosomes at 0·25 g of liver equivalent/ml. The whole lysosomal fraction was used as lysosome preparation. Incubation was carried out at 37°.

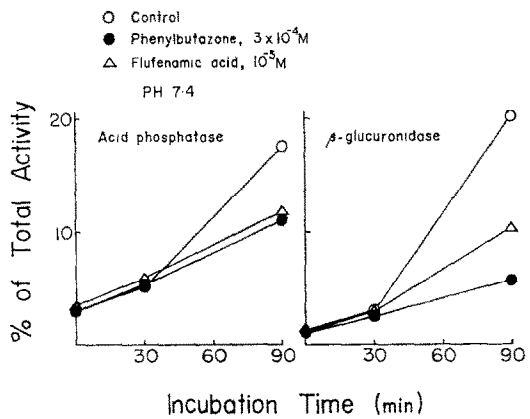

FIG. 3. Effect of phenylbutazone and flufenamic acid on release of acid phosphatase and β-glucuronidase from lysosomes at neutral pH. Ordinate: free activity of enzymes detectable in the supernatant. Experimental conditions were the same as in Fig. 2.

remarkable with time lapse until 90 min. Moreover, after a prolonged incubation such as more than 2 hr, the stabilizing effect of the three drugs were still observed. But accurate data were difficult to obtain, because the degree of inactivation of enzyme became sometimes significant in the control run.

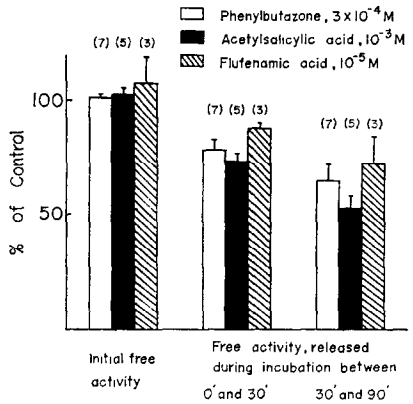

FIG. 4. Effect of non-steroidal anti-inflammatory drugs on release of acid phosphatase from lysosomes at neutral pH. Experimental conditions were the same as in Fig. 2. Figures in parentheses indicate the number of experiments.

Heterogeneity of rat liver lysosomes in drug sensitivity

In the light of the definition of lysosomes, it is obvious that they are heterogeneous in nature. On the recent knowledge of lysosomes, they are classified into several sub-types with regard to their functional aspects.[13]

From a preparative point of view, the rat liver lysosomes were fractionated into at least two parts.[1]

Then, attempts were made to investigate the possible heterogeneity of rat liver lysosomes with special reference to their sensitivity to non-steroidal anti-inflammatory drugs.

As is apparent from Tables 2, 3 and 4, the light lysosomes being present in the light mitochondrial fraction were not affected practically by none of the drugs tested. In

TABLE 2. EFFECT OF ACETYLSALICYLIC ACID ON RELEASE OF ACID PHOSPHATASE FROM HEAVY AND LIGHT LYSOSOMES

Lysosome*	Exp.	Drug	Initial free activity†		Free activity†, released during incubation between			
					0 and 30 min		30 and 90 min	
				Ratio (%)		Ratio (%)		Ratio (%)
Heavy lysosome	I	—	5·44		2·80		10·97	
		+	5·31	98	2·86	102	4·17	38
	II	—	7·04		4·88		9·43	
		+	7·07	101	4·46	91	3·00	32
	III	—	8·35		3·78		8·50	
		+	8·16	98	3·36	89	3·82	46
Light lysosome	I	—	7·29		4·30		4·26	
		+	7·65	105	3·17	74	3·80	89
	II	—	4·25		2·33		8·69	
		+	3·88	91	2·43	104	7·09	82
	III	—	5·63		2·79		4·62	
		+	5·96	105	2·38	85	4·04	87

* Heavy and light lysosomes indicate those lysosomes precipitated in the heavy and the light mitochondrial fractions, respectively.

† Expressed by per cent of total activity. Free activity means the activity being detectable in the supernatant. Concentration of acetylsalicylic acid was 10^{-3} M.

Incubation mixture: 0·04 M Tris-acetate buffer (pH 7·4), 0·25 M sucrose, 0·5% ethanol, and lysosomes at 0·25 g and 0·4 g of liver equivalent/ml for the heavy and the light lysosomes, respectively.

contrast, the heavy lysosomes precipitated in the heavy mitochondrial fraction were quite sensitive to these drugs. Namely, the release of acid phosphatase from lysosomes was significantly suppressed by any of the drugs such as acetylsalicylic acid at 10^{-3} M, phenylbutazone at 3×10^{-4} M, or flufenamic acid at 10^{-5} M. Such a kind of suppression as is mentioned above on enzyme release became prominent after the

TABLE 3. EFFECT OF PHENYLBUTAZONE ON RELEASE OF ACID PHOSPHATASE FROM HEAVY AND LIGHT LYSOSOMES

Lysosome*	Exp.	Drug	Initial free activity†	Free activity†, released during incubation between			
				0 and 30 min		30 and 90 min	
				Ratio (%)		Ratio (%)	
Heavy lysosome	I	—	5·44		2·80		10·97	
		+	5·69	105	2·91	104	6·34	58
	II	—	7·04		4·88		9·43	
		+	7·55	107	4·14	85	4·14	44
	III	—	8·35		3·78		8·50	
		+	8·09	97	3·30	87	4·79	56
Light lysosome	I	—	7·29		4·30		4·26	
		+	7·25	99	4·09	95	3·15	74
	II	—	4·25		2·33		8·69	
		+	4·04	95	1·97	85	8·24	95
	III	—	5·63		2·79		4·62	
		+	5·83	104	2·45	88	5·06	109

*, †, and other experimental conditions were the same as in Table 2.
Concentration of phenylbutazone was 3×10^{-4} M.

TABLE 4. EFFECT OF FLUFENAMIC ACID ON RELEASE OF ACID PHOSPHATASE FROM HEAVY AND LIGHT LYSOSOMES

Lysosome*	Exp.	Drug	Initial free activity†	Free activity†, released during incubation between			
				0 and 30 min		30 and 90 min	
				Ratio (%)		Ratio (%)	
Heavy lysosome	III	—	8·35		3·78		8·50	
		+	8·50	102	3·41	90	4·79	56
	IV	—	10·61		5·08		10·31	
		+	10·63	100	5·29	104	7·25	70
	V	—	9·66		4·88		10·77	
		+	9·78	101	5·03	103	7·05	65
Light lysosome	III	—	5·63		2·79		4·62	
		+	5·75	102	3·07	110	5·28	114
	IV	—	8·18		4·45		2·87	
		+	8·56	105	4·45	100	2·65	92
	V	—	8·00		4·41		3·60	
		+	8·17	102	4·17	94	3·16	88

*, †, and other experimental conditions were the same as in Table 2.
Concentration of flufenamic acid was 10^{-5} M.

lapse of 30 min from the beginning of incubation with drug in every experiment about all three drugs.

The lysosomes in the light mitochondrial fraction were suggested by Straus[14] as to be derived mainly from the parenchymal cells, whereas the larger lysosomes from

Kupffer cells sedimented with the heavy mitochondrial or nuclear materials. Taking the general participation of reticuloendothelial cells in the inflammation processes into account, the cellular origin of the drug-sensitive lysosomes will be a matter of the further research.

Dose-response curves of non-steroidal anti-inflammatory drugs as lysosome-stabilizer

As shown in Fig. 5, the enzyme release was suppressed by various concentrations of acetylsalicylic acid. The values expressed in terms of the degree of inhibition (per cent) were found to increase with increasing concentration of acetylsalicylic acid up to 3×10^{-3} M.

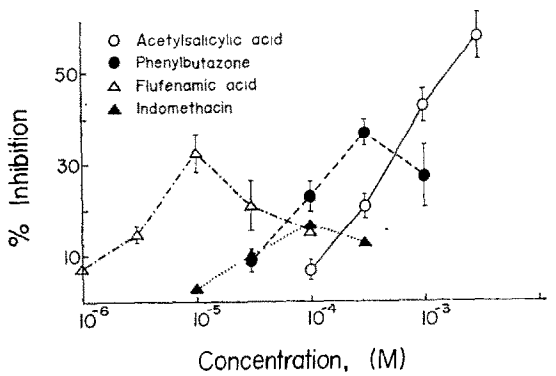

FIG. 5. Dose-response curves of non-steroidal anti-inflammatory drugs as lysosome-stabilizer *in vitro*. Ordinate: suppression of acid phosphatase release from lysosomes during the first 90 min of incubation; expressed as % ± S.E. The heavy lysosomal fraction was used as lysosome preparation. Other experimental conditions were the same as in Table 2. The following S.E. were not illustrated in the figure, in order to avoid a messy confusion on the figure:
± 9·2 for flufenamic acid 10^{-4} M; ± 10·0, ± 5·2,
± 5·2, and ± 3·8 for indomethacin 3×10^{-4} M, 10^{-4} M,
3×10^{-5} M, and 10^{-5} M, respectively.

Phenylbutazone and flufenamic acid, in contrast, exhibited another pattern in their dose-response curves. Namely, there existed an optimum concentration for maximum response. Thus, the curve consists of two parts: an upward part and a downward one. Indomethacin was less active than the other three drugs were.

Taking these data into account, the stabilizing effect of a non-steroidal anti-inflammatory drug on lysosomes seems to be evaluated from the following two aspects: (a) the grade of suppression should be marked, being aside from the concentration of drug, and on the other hand, (b) the drug concentration required for some definite grade of suppression, should be focussed. Namely, an evaluation of the stabilizing effect of drugs can be expressed by using the following two parameters: the attainable maximum grade of suppression (parameter 'A') and the minimum concentration required for some definite grade of suppression (parameter 'B').

In the term of parameter 'A', acetylsalicylic acid was most effective, showing the value of more than 58 per cent of inhibition, whereas phenylbutazone and flufenamic acid showed the maximum suppression as 36·9 and 32·6 per cent of inhibition, respectively.

When ED_{30} of these three drugs were calculated as an adequate index expressing parameter 'B', flufenamic acid was revealed to be the most effective drug of the three. The calculated values of ED_{30} were shown in Table 5, together with those of *in vivo* effect of these three drugs such as on carrageenin edema, mustard edema and cotton pellet granuloma of rat.[15]

TABLE 5. COMPARISON OF THE *in vitro* EFFECTS WITH THOSE *in vivo* OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Drug	ED_{30}			
	*in vitro**	*in vivo*†		
	Enzyme release from lysosome	Carrageenin edema	Mustard edema	Cotton pellet granuloma
	(μM in suspension)	(μmole/kg body wt.)		
Flufenamic acid	8	9	16	12
Phenylbutazone	170	80	160	75
Acetylsalicylic acid	500	260	560	1,100

* Calculated from the data shown in Fig. 5.
† Calculated from the data reported by Winter.[15]

As is obviously seen in Table 5, so far as parameter 'B' is concerned, there is a close correlation between the effect as lysosome-stabilizer *in vitro* and the effect as anti-inflammatory agent *in vivo*.

The experiments were carried out through the seasons between September and next February.

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