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EFFECTS OF TINORIDINE ON STABILITY OF RAT LIVER AND KIDNEY LYSOSOMES, AND LIVER PARENCHYMAL CELLS*

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Abstract—The stabilizing activity of tinoridine hydrochloride on biomembranes was estimated by means of determining the rate of release of acid phosphatase and aryl sulfatase from lysosomes, or of transaminases from liver parenchymal cells. Tinoridine hydrochloride (10–100 μM), benzydamine hydrochloride (10–100 μM) and phenylbutazone (1–100 μM) stabilized significantly intact lysosomes (700–3500 g fraction), while indomethacin and prednisolone showed only moderate activity. The lysosomes treated with 0.3% hydrogen peroxide were stabilized by tinoridine hydrochloride, phenylbutazone and indomethacin, but not by benzydamine in the concentration range of 1–100 μM. On the fragile liver lysosomes prepared from rats pretreated with carbon tetrachloride and on kidney lysosomes (650–3500 g), only tinoridine hydrochloride had marked stabilizing activity at 1–100 μM among the tested nonsteroidal anti-inflammatory drugs. Prednisolone showed the same activity on these lysosomes as tinoridine hydrochloride. Both tinoridine hydrochloride and prednisolone also stabilized the liver parenchymal cells at 10–100 μM. The elevation of the free activity of lysosomal enzymes in the liver fraction of rats caused by the intraperitoneal injection of carbon tetrachloride (0.5 ml/kg) was really inhibited by the oral treatment with tinoridine hydrochloride (100 mg/kg).

Since the discovery of lysosomes as subcellular organelles by De Duve *et al.* [1], the lysosomal contents such as acid hydrolytic enzymes or cationic proteins are postulated to play an important role as the mediators of inflammation [2–4]. On the other hand, clinically beneficial anti-inflammatory drugs have been reported to stabilize the lysosomes prepared from the liver [5–7] or polymorphonuclear (PMN) leukocytes [8, 9]. However, Brown and Schwartz [10] have reported that anti-inflammatory drugs such as indomethacin labilize rat liver lysosomes.

In our previous paper, it was reported that tinoridine hydrochloride,† an anti-inflammatory drug, stabilizes the rat liver lysosomes [11], rabbit PMN leukocyte lysosomes [9], platelets [12] and erythrocytes [11] *in vitro*. The mode of action of this drug on lysosomes, however, was suggested to be different from that of steroidal anti-inflammatory drugs, for tinoridine hydrochloride and phenylbutazone stabilize only intact lysosomes but steroidal anti-inflammatory drugs stabilize both intact and unstable lysosomes prepared from rabbit PMN leukocytes [9].

The purpose of the present paper is to study, under various experimental conditions, the effect of tinoridine hydrochloride on liver lysosomes, kidney lysosomes and liver parenchymal cells in comparison with that of other anti-inflammatory drugs, and to determine the effect of this drug on biomembranes.

The data presented in this report illustrate that tinoridine hydrochloride stabilizes the liver and kidney lysosomes and the liver parenchymal cells in all experimental conditions employed *in vitro* and *in vivo*, and has a different property from drugs such as benzydamine hydrochloride, indomethacin or prednisolone.

MATERIALS AND METHODS

Drugs. Tinoridine hydrochloride, Y-3923 (debenzyl tinoridine; 2-amino-3-ethoxycarbonyl-4,5,6,7-tetrahydrothieno[2,3]pyridine) hydrochloride, benzydamine hydrochloride, flufenamic acid, indomethacin and phenylbutazone were supplied by our laboratories. Hydrocortisone and prednisolone were purchased from Sigma Chemical Co., Ltd. In the experiments with lysosomes, all drugs were dissolved in ethanol (EtOH). In the experiments with parenchymal cells, the drugs tested were dissolved in dimethylsulfoxide (DMSO), which was used as solvent in the experiment using intact cells such as erythrocytes [11] or platelets [12]. A small amount of EtOH or DMSO employed as vehicle did not influence the stability of lysosomes or parenchymal cells *in vitro*.

Animals. Wistar male rats, weighing between 200 and 250 g, were used in all experiments.

Preparation of liver fraction. Nonfasted rats were decapitated and exsanguinated. The liver was perfused *in situ* with 10 ml of ice-cold 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4) via the portal vein at a rate of approximately 5 ml/min, and then quickly excised and rinsed in the same cold buffer. After weighing, the tissue was cut into small pieces and dispersed in ice-cold 0.25 M sucrose–0.04 M Tris-acetate buffer in a homogenizer. Homogenization was carried out by ten strokes of the Teflon pestle in ice-water. The volume was adjusted to give a 10% (w/v)

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† 2-Amino-3-ethoxycarbonyl-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine hydrochloride.

homogenate, which was centrifuged at 700 *g* for 10 min at 4°. The resultant supernatant (700 *g* supernatant fraction) was centrifuged at 3500 *g* for 15 min at 4°, and the sediment was rinsed once (700–3500 *g* lysosome fraction). This lysosome fraction was suspended to give 0.2 or 0.4 g of the liver equivalent/ml in the above buffer solution. The lysosome-stabilizing activity of certain anti-inflammatory drugs is shown to be more prominent when this fraction is used by Tanaka and Iizuka [6].

In certain experiments, rats were sacrificed 3 hr after intraperitoneal treatment with carbon tetrachloride (0.5 ml/kg), or rats, intraperitoneally injected with carbon tetrachloride (0.5 ml/kg) 1 hr after oral treatment with tinoridine hydrochloride suspension (100 mg/kg) or vehicle (0.5% methylcellulose solution), were sacrificed 3 hr later. The preparation of the liver fractions from these rats was also carried out by the procedures described above.

Preparation of kidney fractions. Since the free activity of lysosomal enzymes in the kidney fraction, obtained by the same method of preparation used for the liver fraction, is higher than that in the liver fraction, it appears that the lysosomes in the kidney fractions become unstable during their preparation. Therefore, the preparation method of the liver fraction was slightly modified for preparing the kidney fractions. Rats were decapitated and exsanguinated. The kidney was quickly excised and rinsed in ice-cold 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4). After weighing, the tissue was cut into small pieces and dispersed in the same ice-cold buffer in a homogenizer. Homogenization was carried out by five strokes of the Teflon pestle in ice-water. The volume was adjusted to give a 10% (w/v) homogenate. All subsequent procedures were carried out by the method described above. The 650–3500 *g* or 650–12,000 *g* fraction was prepared and suspended to give 0.2 *g* of the kidney equivalent/ml in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4). The 650–12,000 *g* fraction in the rat kidney has already been demonstrated to be rich in lysosomal enzymes, such as acid phosphatase, by Shibko and Tappel [13].

Preparation of liver parenchymal cells. The liver parenchymal cells were prepared according to the method of Howard and Pesch [14]. Briefly, the perfused liver with 15 ml of ice-cold enzyme solution (0.05% collagenase and 0.01% hyaluronidase dissolved in Ca^{2+}-free Hanks' solution, pH 7.4) was cut into slices. The slices (2–3 g) were placed in flasks each of which contained 10 ml of the enzyme solution. The flasks were incubated at 37° with shaking in an atmosphere of 95% O_2 and 5% CO_2 for 70 min. At the end of the incubation period, the contents of the flasks were filtered through a single layer of nylon stocking. The filtrate was centrifuged at 50 *g* for 1 min. The supernatant was removed with a pipette, and the cells were washed twice. After washing, the cells were sedimented by centrifugation at 20 *g* for 1 min. After the final centrifugation, the cells were resuspended to give 10 mg of the protein equivalent/ml in the incubation medium.

Assays of lysosome membrane stability. The effects of test drugs on lysosome membranes were studied by determining the release of lysosomal marker enzymes such as acid phosphatase or aryl sulfatase. None of the drugs tested inhibited directly the activity of the marker enzymes. The test solution (20 μl) or EtOH as solvent was added to a 25-ml conical flask containing 2.0 ml of the lysosome suspension (0.2 g of the liver equivalent/ml) in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4). After the incubation mixture was kept for 5 min at 25°, 1.0-ml aliquots were removed to determine the initial activity of free enzymes in the supernatant. The residual mixtures were incubated at 37° for 30 or 60 min with shaking at an agitation cycle of 75/min. After the end of the incubation period, the supernatant obtained by high speed centrifugation at 27,000 *g* for 15 min at 4° was used for the measurement of the marker enzymes released from lysosomes. The total activity of these enzymes was assayed using the supernatant obtained by centrifuging the lysosome suspension incubated at 37° for 30 min in 0.2% (v/v) Triton X-100–0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4).

The effects of hydrogen peroxide and test drugs on lysosomes were ascertained by the following procedures. The test solution (40 μl) was added to a 25-ml conical flask containing 2 ml of the lysosome suspension (0.4 g of the liver equivalent/ml). This mixture was kept at 25° for 5 min. Immediately after the addition of 2.0 ml of 0.6% (v/v) hydrogen peroxide in 0.25 M sucrose–0.04 M Tris-acetate buffer to the above mixture, 2.0-ml aliquots were removed to determine the initial activity of free enzymes and lipid peroxides in the supernatant. The residual mixtures were incubated at 37° for 30 min with shaking, and centrifuged at 27,000 *g* for 15 min at 4°.

The liver fractions obtained from rats treated with tinoridine hydrochloride or vehicle, and with carbon tetrachloride or olive oil as vehicle *in vivo* were incubated alone without addition of the test solution *in vitro* at 37° for 15 or 30 min with shaking. The resulting supernatants were used for the measurement of enzyme activity and lipid peroxides.

Assay of parenchymal cell-membrane stability. The stability of parenchymal cell-membranes was ascertained by determining the release of transaminases. The test solution (10 μl) or DMSO as solvent was added to a 25-ml conical flask containing 2.0 ml of the cell suspension. After the incubation mixtures were kept for 10 min at 25°, 1.0-ml aliquots were removed and then centrifuged at 300 *g* for 10 min at 4°. The residual mixtures were incubated at 37° for 15, 30 or 60 min with shaking at an agitation cycle of 75/min. Incubation was terminated by centrifugation at 300 *g* for 10 min at 4°. The resulting supernatants were used for the measurement of transaminases.

The data showing latency of the marker enzyme of lysosomes and parenchymal cells used in each experiment are in the table or its footnote.

Enzyme assays. Acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase) and aryl sulfatase (EC 3.1.6.1, aryl sulfate sulfohydrolase) activities were assayed using sodium *p*-nitrophenyl phosphate or dipotassium *p*-nitrocatechol sulfate as substrate by the methods described previously [15].

Glutamic-oxaloacetic transaminase (GOT, EC 2.6.1.1, L-aspartate: 2-oxoglutarate aminotransferase) and glutamic-pyruvic transaminase (GPT, EC 2.6.1.2, L-alanine: 2-oxoglutarate aminotransferase) activities

Table 1. Effect of drugs on spontaneous release of acid phosphatase from liver lysosomes *in vitro**

Drug	Per cent inhibition of release of acid phosphatase		
	1 μM	10 μM	100 μM
Tinoridine HCl	11.5 ± 2.2	28.3 ± 1.8†	43.6 ± 0.8†
Y-3923 HCl		5.2 ± 2.8	13.8 ± 1.1†
Benzydamine HCl	11.3 ± 2.2	26.3 ± 4.4†	38.9 ± 3.8†
Phenylbutazone	18.0 ± 1.2†	33.4 ± 1.6†	54.7 ± 1.4†
Indomethacin	6.4 ± 1.3	11.0 ± 2.4	22.6 ± 1.8†
Prednisolone		7.1 ± 1.3	13.8 ± 1.7

* The lysosome suspension (2.0 ml) was incubated with each test solution (20 μl) in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4) at 37° for 30 min. Results are shown as the means ± S. E. of four to six separate experiments. Enzyme activity in controls: before incubation, 2.7; after incubation, 15.2; total activity, 109.9 μg *p*-nitrophenol formed.

† $P < 0.01$ (significant).

were determined according to the method of Reitman and Frankel [16], and expressed as Karmen units.

Assay of lipid peroxide formation. The formation of lipid peroxides was determined by the method of Desai *et al* [17]. Briefly, thiobarbituric acid (TBA) reactants were measured on the supernatant obtained by precipitating the supernatant of the above incubation mixtures with 10% (w/v) trichloroacetic acid and incubating with 0.67% (w/v) TBA in 0.1 N HCl at 100° for 10 min. The red color was read spectrophotometrically at 530 nm and expressed in terms of TBA values.

Calculation of per cent inhibition. The per cent inhibition of the enzyme release or lipid peroxidation by test drugs was calculated by the following formula: $(A - B)/A \times 100$ (A, the free activity of marker enzymes or TBA value increased during the incubation of controls; B, the free activity of marker enzymes or TBA value increased during the incubation of test drugs).

RESULTS

Effects of drugs on stability of intact lysosomes in vitro. The data in Table 1 indicate that tinoridine hydrochloride, benzydamine hydrochloride and phenylbutazone inhibited the spontaneous release of acid phosphatase from the intact lysosomes in the 700–3500 *g* liver fraction. The inhibitory effects of Y-3923 hydrochloride, indomethacin and prednisolone were less potent than those of the above anti-inflammatory agents in this experimental condition.

As shown in the footnote of Table 2, the addition of hydrogen peroxide (0.3% in a final concentration) accelerates the release of enzymes from lysosomes and the formation of lipid peroxides. Under these conditions, some of the above drugs showed different results. Tinoridine hydrochloride, Y-3923 hydrochloride, flufenamic acid, phenylbutazone and indomethacin prevented the release of acid phosphatase or aryl sulfatase, and lipid peroxidation at concentrations of 1–100 μM. Of the drugs tested, only benzydamine hydrochloride showed no effect on either at the above concentrations. At a high concentration of 1000 μM,

Table 2. Effect of drugs on enzyme release and lipid peroxidation from or in liver lysosome fraction induced by hydrogen peroxide *in vitro**

Drug	Marker†	Per cent inhibition of enzyme release and lipid peroxidation			
		1 μM	10 μM	100 μM	1000 μM
Tinoridine HCl	AP	10.5 ± 1.9	19.0 ± 3.4‡	32.2 ± 2.0‡	
	AS	11.0 ± 3.8	25.3 ± 2.2‡	40.9 ± 0.5‡	−68.9 ± 4.2‡
	LP	13.2 ± 1.6‡	33.5 ± 2.8‡	50.0 ± 2.1‡	61.9 ± 2.2‡
Y-3923 HCl	AP	5.3 ± 2.2	14.4 ± 3.3	24.7 ± 3.7‡	
	AS	8.2 ± 3.0	19.3 ± 3.7‡	29.4 ± 3.2‡	
	LP	14.5 ± 3.4	19.3 ± 1.4‡	43.3 ± 2.0‡	
Benzydamine HCl	AP	1.8 ± 1.6	8.2 ± 1.8	10.5 ± 3.5	
	AS	4.8 ± 1.8	7.4 ± 1.9	−8.3 ± 3.7	
	LP	3.1 ± 0.6	−6.9 ± 1.2	−7.7 ± 6.5	
Flufenamic acid	AS	12.2 ± 0.9	34.1 ± 1.2‡	28.1 ± 1.0‡	−19.5 ± 2.5‡
	LP	6.3 ± 4.7	13.9 ± 1.7	26.3 ± 2.2‡	29.5 ± 1.9‡
Indomethacin	AS	10.8 ± 3.4	22.8 ± 1.7‡	40.3 ± 0.8‡	−25.7 ± 1.7‡
	LP	12.9 ± 3.4	23.4 ± 2.3‡	30.8 ± 1.0‡	41.0 ± 1.3‡
Phenylbutazone	AS	12.7 ± 2.3	33.7 ± 0.9‡	53.5 ± 1.6‡	−28.3 ± 9.9
	LP	12.8 ± 1.9	31.2 ± 1.8‡	41.8 ± 0.7‡	47.0 ± 2.6‡
Prednisolone	AS	4.5 ± 1.9	9.5 ± 2.2	12.5 ± 2.1	19.5 ± 2.1‡
	LP	6.1 ± 2.8	8.6 ± 1.5	12.9 ± 3.0	22.6 ± 1.5‡

* The lysosome suspension (1.0 ml) containing each test solution (20 μl) was incubated with 0.3% hydrogen peroxide solution (in final concentration, 1.0 ml) in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4) at 37° for 30 min. Results are shown as the means ± S. E. of four separate experiments. The negative sign preceding certain numbers means the per cent increase of the enzyme release.

† AP, acid phosphatase. Activity in controls: before incubation, 4.1; after incubation, 30.4; total activity, 109.9 μg *p*-nitrophenol formed. AS, aryl sulfatase. Activity in controls: before incubation, 4.1; after incubation, 31.7; total activity, 116.9 μg *p*-nitrocatechol formed. LP, lipid peroxidation. TBA value ($O.D._{530}$) in controls: before incubation, 0.093; after incubation, 0.387.

‡ $P < 0.01$ (significant).

tinoridine hydrochloride, flufenamic acid, indomethacin and phenylbutazone inhibited the latter alone, and they actually increased the former. Prednisolone inhibited significantly both the enzyme release and lipid peroxidation at a high concentration of 1000 μM. The TBA color reaction was not modified significantly with the anti-inflammatory agents tested.

Effects of drugs on stability of unstable liver lysosomes of rats treated with carbon tetrachloride in vitro. The free activity of acid phosphatase or aryl sulfatase before incubation in the intact lysosome suspension shown in Table 2 was 3–4 per cent of the total activity. On the other hand, the free activity of these enzymes before incubation in the liver lysosome suspension prepared 3 hr later from rats treated with 50% (v/v) carbon tetrachloride in olive oil was 13–16 per cent of the total activity (Table 3). The release of acid phosphatase and aryl sulfatase, during the incubation period of 30 min, from the unstable lysosomes thus obtained was inhibited significantly by tinoridine hydrochloride to a degree that was proportional to the drug concentration. In this experiment with the unstable lysosomes, however, phenylbutazone and indomethacin failed to inhibit the release of enzymes.

Anti-inflammatory steroids such as prednisolone or hydrocortisone did inhibit markedly the release of acid phosphatase and aryl sulfatase from the unstable lysosomes at concentrations in the range from 1 to 100 μM for incubation periods up to 60 min.

Effect of tinoridine hydrochloride on the stability of rat liver lysosomes in vivo. The data presented in Table 4 indicate the stability of lysosomes prepared from the liver of rats treated with or without tinoridine hydrochloride 1 hr before the treatment with carbon tetrachloride as a labilizer. The significant increase of free activities of lysosomal enzymes before incubation or after incubation for 15 or 30 min in the 700 g supernatant fraction and the 700–3500 g fraction was observed by the treatment with carbon tetrachloride 3 hr before the preparation of the lysosome fraction. Tinoridine hydrochloride inhibited significantly the increase of free activity of the above enzymes and showed a tendency to prevent the significant decrease of the total acid phosphatase.

Effects of drugs on the stability of kidney lysosomes in vitro. The data in Table 5 indicate that the release of acid phosphatase and aryl sulfatase from two lysosome fractions of the kidney is time-dependent for periods up to 60 min. The free activity of acid phosphatase or aryl sulfatase, before incubation, in the 650–3500 g kidney fraction is 14–15 per cent of the total activity, which is higher than that in the 700–3500 g liver fraction shown in the first footnote of Table 1. Tinoridine hydrochloride showed a marked inhibitory activity on the release of aryl sulfatase from the kidney lysosomes in the 650–3500 g fraction during the 30-min incubation period. However, this drug failed to inhibit the enzyme release from the 650–12,000 g fraction.

Table 6 shows the effects of drugs on the stability of the kidney lysosomes in the 650–3500 g fraction. Tinoridine hydrochloride inhibited the release of aryl sulfatase from the lysosomes to a degree that was

Table 3. Effect of drugs on enzyme release from liver lysosomes of rats treated with carbon tetrachloride *in vitro**

Drug	Concn (μM)	Per cent inhibition of release of: Acid phosphatase 30 min†	Acid phosphatase 60 min†	N‡	Aryl sulfatase 30 min†	Aryl sulfatase 60 min†	N‡
Tinoridine HCl	0.1	6.8 ± 3.9	4.0 ± 1.7	2	10.5 ± 7.9	1.9 ± 2.7	2
	1	21.6 ± 3.8§	4.2 ± 2.4	6	30.8 ± 4.7§	10.3 ± 3.3	5
	10	34.3 ± 2.2§	7.2 ± 2.9	6	44.9 ± 3.2§	17.8 ± 4.9	5
	100	44.9 ± 2.6§	14.2 ± 0.8	4	53.8 ± 3.3§	20.4 ± 7.9	3
Phenylbutazone	1	4.5 ± 3.1	1.0 ± 2.7	2	6.1 ± 4.7	0.3 ± 2.9	2
	10	9.5 ± 2.7	4.2 ± 3.8	2	9.9 ± 3.8	6.3 ± 3.7	2
	100	10.8 ± 4.4	1.2 ± 2.9	4	11.3 ± 5.7	−3.0 ± 2.7	3
Indomethacin	1	1.2 ± 2.0	5.8 ± 3.7	2	4.4 ± 3.9	5.0 ± 4.4	2
	10	4.0 ± 2.1	7.7 ± 3.9	2	5.8 ± 3.9	5.3 ± 7.9	2
	100	5.5 ± 2.7	10.5 ± 4.7	2	10.5 ± 2.1	12.4 ± 1.8	2
Prednisolone	0.1	3.8 ± 1.9	15.0 ± 7.8	2	8.7 ± 4.7	14.7 ± 10.1	2
	1	16.4 ± 4.7	32.1 ± 2.9§	6	27.9 ± 3.7§	28.8 ± 6.0§	5
	10	37.8 ± 1.6§	40.9 ± 3.2§	6	43.2 ± 5.4§	55.7 ± 2.9§	5
	100	46.7 ± 2.0§	47.8 ± 2.1§	4	50.9 ± 7.9§	64.8 ± 3.8§	3
Hydrocortisone	0.1	2.1 ± 0.9	7.8 ± 5.1	2	9.0 ± 3.4	10.4 ± 5.7	2
	1	12.8 ± 2.6	15.4 ± 2.3	6	14.0 ± 3.8	14.3 ± 3.1	5
	10	25.3 ± 3.3§	23.8 ± 4.6§	4	27.2 ± 4.2§	28.9 ± 2.9§	3
	100	33.8 ± 4.7§	39.4 ± 3.9§	4	35.1 ± 7.7§	40.0 ± 2.4§	3

* Rats were sacrificed 3 hr after intraperitoneal treatment with carbon tetrachloride (0.5 ml/kg) and the liver lysosome fraction (700–3500 g) was prepared. The lysosome suspension (2.0 ml) was incubated with each test solution (20 μl) in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4) at 37°. Results are shown as the means ± S. E. of separate experiments. Enzyme activity in controls for acid phosphatase: before incubation, 16.8; after incubation, 30.5 (30 min) and 42.4 (60 min); total activity, 107.5 μg p-nitrophenol formed. Enzyme activity in controls for aryl sulfatase: before incubation, 13.2; after incubation, 33.6 (30 min) and 54.7 (60 min); total activity, 101.6 μg p-nitrocatechol formed.

† Incubation time.

‡ Numbers of separate experiments.

§ $P < 0.01$ (significant).

Table 4. Effect of tinoridine hydrochloride on stability of rat liver lysosomes *in vivo**

Treatment	Marker enzyme†	Per cent of total activity 0 min‡	15 min‡	30 min‡	Total activity§
700 *g* Supernatant fraction					
Vehicle + olive oil	AP	25.9 ± 1.5‖	28.1 ± 1.1‖	32.9 ± 0.7	821.1 ± 13.2‖
Vehicle + CCl_4 in olive oil	AP	37.9 ± 1.5	48.2 ± 2.1	60.4 ± 2.4	757.5 ± 8.2
Tinoridine HCl + CCl_4 in olive oil	AP	30.3 ± 1.0‖	33.2 ± 1.5‖	50.7 ± 1.2	797.9 ± 6.1
700–3500 *g* Lysosome fraction					
Vehicle + olive oil	AP	10.4 ± 0.4	14.2 ± 0.6‖	25.8 ± 1.7‖	114.9 ± 11.0
	AS	6.4 ± 0.4‖	9.6 ± 0.7‖	13.6 ± 1.6‖	81.7 ± 6.3
Vehicle + CCl_4 in olive oil	AP	14.0 ± 1.2	27.6 ± 1.2	42.7 ± 1.4	109.0 ± 6.9
	AS	9.7 ± 0.2	17.0 ± 0.7	32.7 ± 0.8	79.9 ± 3.8
Tinoridine HCl + CCl_4 in olive oil	AP	9.8 ± 1.1‖	18.7 ± 1.5	35.6 ± 1.7	129.6 ± 5.5
	AS	6.8 ± 0.7‖	13.1 ± 1.0‖	22.4 ± 1.1‖	80.5 ± 5.8

* Carbon tetrachloride (0.5 ml/kg) was injected intraperitoneally into ten rats/group 1 hr after oral treatment with vehicle or tinoridine hydrochloride (100 mg/kg), and the rat liver was obtained 3 hr thereafter. The two fractions of liver homogenate were incubated in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4) at 37° for 15 or 30 min. Results are shown as the means ± S. E. of ten rats.

† AP, acid phosphatase; AS, aryl sulfatase.

‡ Incubation time.

§ Amount of *p*-nitrophenol formed for AP or *p*-nitrocatechol formed for AS (in μg).

‖ $P < 0.01$ (significant) vs control.

proportional to the drug concentration. The relative order of the inhibitory activity on the release of the above enzyme was found to be tinoridine hydrochloride > prednisolone > indomethacin = phenylbutazone.

Effects of drugs on stability of liver parenchymal cells in vitro. The data in Table 7 illustrate the rate of release of transaminases from parenchymal cells suspended in each of the incubation medium. Incubation at 37° for 60 min results in the release of 15 per cent of the total GOT activity in Hanks' solution, of 59 per cent of that in Ca^{2+}-free Hanks' solution and 3 per cent of that in Ca^{2+}-free Hanks' solution containing 1% albumin, while 13, 25 and 11 per cent of the total activity are released spontaneously prior to any incubation (0 min) in the respective incubation medium. As long as parenchymal cells were incubated in Ca^{2+}-free Hanks' solution, tinoridine hydrochloride inhibited significantly the release of GOT for incubation periods up to 60 min. On the other hand, incubation at 37° for 15 min results in the release of 20–31 per cent of the total GPT activity in the above three incubation media, while 36–43 per cent of the total GPT activity is released spontaneously prior to

Table 5. Effects of incubation time and tinoridine hydrochloride on stability of two lysosome fractions of rat kidney *in vitro**

Marker enzyme†	Test drug‡	Per cent of total activity 0 min§	30 min§	60 min§	Total activity‖
650–3500 *g* Fraction					
AP	–	14.3 ± 0.7	22.1 ± 1.2	38.0 ± 1.6	43.4 ± 1.9
	+	14.1 ± 0.8	16.4 ± 1.4¶ (33.8)	28.3 ± 1.4¶ (30.1)	
AS	–	14.0 ± 0.8	26.3 ± 0.8	41.3 ± 1.3	60.1 ± 1.4
	+	14.5 ± 0.7	20.3 ± 0.5¶ (47.3)	30.8 ± 0.8¶ (40.2)	
650–12,000 *g* Fraction					
AP	–	14.4 ± 1.3	25.3 ± 2.6	39.6 ± 2.0	60.6 ± 1.7
	+	14.0 ± 0.7	22.6 ± 3.0 (13.8)	35.6 ± 2.1 (9.2)	
AS	–	20.6 ± 0.6	31.8 ± 0.4	49.0 ± 0.7	73.9 ± 1.9
	+	21.1 ± 0.7	29.8 ± 0.9 (18.1)	47.5 ± 0.4 (8.1)	

* Results are shown as the means ± S. E. (N = 4) of two separate experiments; the numbers in parentheses show the per cent inhibition.

† AP, acid phosphatase; AS, aryl sulfatase.

‡ Key: —, control; +, tinoridine hydrochloride (30 μM).

§ Incubation time at 37° in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4).

‖ Amount of *p*-nitrophenol formed for AP or *p*-nitrocatechol formed for AS (in μg).

¶ $P < 0.01$ (significant) vs control.

Table 6. Effect of drugs on enzyme release from kidney lysosomes (650–3500 *g* fraction) *in vitro**

Drug	Per cent inhibition of release of aryl sulfatase		
	1 μM	10 μM	100 μM
Tinoridine HCl	19.3 ± 3.3†	33.8 ± 2.4†	53.6 ± 2.9†
Phenylbutazone	1.1 ± 2.2	5.4 ± 2.8	15.0 ± 2.8†
Indomethacin	2.9 ± 2.8	8.2 ± 2.4	19.0 ± 2.4†
Prednisolone	8.6 ± 0.9	19.5 ± 2.2†	33.3 ± 1.9†

* The lysosome suspension (2.0 ml) was incubated with each test solution (20 μl) in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4) at 37° for 30 min. Results are shown as the means ± S. E. of six to eight separate experiments. Enzyme activity in controls: before incubation, 7.8; after incubation, 14.6; total activity, 57.1 μg *p*-nitrophenol formed.

† $P < 0.01$ (significant).

any incubation. The release of GPT during a 15-min incubation period was inhibited significantly by tinoridine hydrochloride at a concentration of 30 μM in any incubation medium employed.

Table 8 indicates the effects of drugs on the stability of the liver parenchymal cells. The release of both GOT and GPT was inhibited by tinoridine hydrochloride for the incubation period up to 15 min. In this system, hydrocortisone and prednisolone prevented markedly the release of GOT rather than that of GPT. Similar data were obtained on the release of GOT during a 30-min incubation period. The inhibitory activity of prednisolone on the release of transaminases was more potent than that of hydrocortisone.

DISCUSSION

The results in this report, under all experimental conditions employed, illustrate that tinoridine hydrochloride possesses the ability to stabilize lysosomes *in vitro* contained within a crude mitochondrial fraction of both liver and kidney, lysosomes *in vivo* of the rat liver and the liver parenchymal cells *in vitro*. This property of tinoridine hydrochloride on these biomembranes is distinct from that of benzydamine hydrochloride, phenylbutazone, indomethacin or prednisolone, which have only the capacity to stabilize biomembranes *in vitro* under specific experimental conditions.

In our previous paper [11], tinoridine hydrochloride has been reported to prevent markedly the labilization of lysosomes in the 800–20,000 *g* liver fraction caused by the incubation with 2.5% EtOH. The stabilizing activity of the drug in this system was found to be the most potent among nonsteroidal anti-inflammatory drugs tested. On the other hand, Tanaka and Iizuka [6] have reported that the lysosome-stabilizing activity of certain nonsteroidal anti-inflammatory drugs is more prominent when the heavy lysosomal fraction (700–3500 *g*) is used. The same results have been reported by Ignarro [7] that specific experimental conditions are necessary to measure drug-induced stabilization of lysosomes. Specifically, the 600–3500 *g* liver fraction in sucrose–Tris-acetate buffer (pH 7.4) was found to be the most suitable source of the lysosomes. In this system, both steroidal and nonsteroidal anti-inflammatory drugs exhibit the lysosome-stabilizing activity.

The data in Table 1 suggest that tinoridine hydrochloride, benzydamine hydrochloride and phenylbutazone stabilize the lysosomes in the 700–3500 *g* liver fraction in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4), while indomethacin shows only moderate activity, which is similar to the results reported previously by Ignarro [7]. Under these experimental conditions, however, prednisolone failed to stabilize the lysosomes, which is not in accord with the results described by Ignarro [7].

Table 7. Effects of incubation medium, incubation time and tinoridine hydrochloride on enzyme release from rat liver parenchymal cells *in vitro**

Incubation medium	Marker enzyme†	Test drug‡	Per cent of total activity				Total activity‖
			0 min§	15 min§	30 min§	60 min§	
Hanks' solution	GOT	−	12.9 ± 0.4	13.4 ± 1.1	19.4 ± 1.3	28.2 ± 2.4	470 ± 12
		+	12.7 ± 0.5	13.0 ± 0.7	18.3 ± 1.2	25.0 ± 2.0	
	GPT	−	35.7 ± 0.7	59.6 ± 1.5	78.2 ± 2.4		279 ± 18
		+	33.8 ± 0.8	48.4 ± 1.2¶	72.7 ± 1.8		
Ca^{2+}-free Hanks' solution	GOT	−	24.8 ± 0.8	43.7 ± 1.9	62.1 ± 2.8	84.0 ± 3.8	422 ± 16
		+	24.0 ± 0.3	36.6 ± 1.7**	49.1 ± 2.2¶	67.3 ± 2.1¶	
	GPT	−	43.3 ± 0.7	74.2 ± 1.8	88.8 ± 2.9		243 ± 19
		+	41.8 ± 0.6	61.7 ± 1.0¶	81.6 ± 2.2		
Ca^{2+}-free Hanks' solution containing 1% BSA	GOT	−	10.5 ± 0.4	11.0 ± 0.9	13.6 ± 1.0	17.6 ± 1.5	489 ± 16
		+	10.2 ± 0.5	10.7 ± 1.0	13.3 ± 1.1	16.6 ± 1.3	
	GPT	−	36.1 ± 1.2	56.3 ± 2.7	75.6 ± 2.8		238 ± 21
		+	34.7 ± 1.1	46.6 ± 1.4**	70.3 ± 1.0		

* The cell suspension (2.0 ml) was incubated with each test solution (10 μl) at 37°. Results are shown as the means ± S. E. of three separate experiments.

† GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase.

‡ Key: −, control; +, tinoridine hydrochloride (30 μM).

§ Incubation time.

‖ Karmen units/ml/hr for GOT and GPT.

¶ $P < 0.01$ (significant) vs control.

** $P < 0.05$ (significant) vs control.

Table 8. Effect of drugs on enzyme release from rat liver parenchymal cells *in vitro**

Incubation time (min)	Drug	Marker enzyme†	Per cent inhibition of enzyme release 1 μM	10 μM	100 μM
15	Tinoridine HCl	GOT	13.8 ± 4.3	24.2 ± 1.6‡	36.0 ± 1.5‡
		GPT	15.8 ± 2.7	30.4 ± 4.5‡	43.4 ± 6.5‡
15	Hydrocortisone	GOT	1.3 ± 2.4	9.2 ± 1.3	24.6 ± 1.7‡
		GPT		2.1 ± 0.8	8.4 ± 0.9
15	Prednisolone	GOT	16.6 ± 3.2	30.0 ± 1.0‡	42.9 ± 2.8‡
		GPT	4.3 ± 0.9	12.9 ± 2.3	21.7 ± 4.3‡
30	Tinoridine HCl	GOT	14.7 ± 2.9	24.4 ± 1.8‡	33.5 ± 1.0‡
30	Hydrocortisone	GOT	10.8 ± 1.6	14.1 ± 2.0	24.0 ± 1.5‡
30	Prednisolone	GOT	18.8 ± 1.4	28.8 ± 1.8‡	37.7 ± 1.4‡

* The cell suspension (2.0 ml) was incubated with each test solution (10 μl) in Ca^{2+}-free Hanks' solution at 37°. Results are shown as the means ± S. E. of three separate experiments. Enzyme activity (Karmen units) in controls: before incubation, 85.1 (GOT) and 123.9 (GPT); after incubation for 15 min, 186.7 (GOT) and 226.2 (GPT); after incubation for 30 min, 295 (GOT); total activity, 486.2 (GOT) and 226.2 (GPT).

† GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase.

‡ $P < 0.01$ (significant).

Interesting findings have been reported by Desai *et al.* [17] that isolated rat liver lysosomes peroxidize at a comparatively slower rate than either mitochondria or microsomes, but when subjected to induced free radical systems, such as hydrogen peroxide, the lysosomal membranes are extremely labile to such damage as indicated by the release of bound enzymes like aryl sulfatase. Therefore, hydrogen peroxide was used as a labilizer of lysosomes. In the incubation system indicated in Table 2, hydrogen peroxide was found to increase the rate of enzyme release and to cause lipid peroxidation. The data in Table 2 clearly show that nonsteroidal anti-inflammatory drugs, except for benzydamine hydrochloride and Y-3923 hydrochloride, which is one of the metabolites of tinoridine hydrochloride [18], prevented both the labilization of intact lysosomes and lipid peroxidation in the liver fraction. The above findings suggest that benzydamine hydrochloride prevents only mild damage of the intact lysosomes such as that which occurs in incubation alone. The lysosome-stabilizing activity of certain drugs like tinoridine hydrochloride or flufenamic acid is not necessarily proportional to the inhibitory activity on lipid peroxidation. Therefore, it seems likely that the lysosome-stabilizing activity is not due to the prevention of lipid peroxidation, and that lipid peroxides derive mainly from peroxidation of mitochondrial membranes in the 700–3500 *g* fraction employed.

On the mode of action of anti-inflammatory drugs, recent studies of u.v.- and hydrogen peroxide-induced lipid peroxidation and haemolysis in caine erythrocytes have suggested that certain anti-inflammatory drugs may inhibit haemolysis by stabilizing lipoprotein of the membrane, or by absorbing and destroying free radical or peroxides [19, 20]. Tinoridine hydrochloride has been reported by Fugita *et al.* [21, 22] to have the same protective effect as α-tocopherol on changes with fatty acid composition in the liver lysosomal lipids and microsomal lipids obtained from α-tocopherol-deficient rats. It is interesting that both tinoridine hydrochloride and its metabolite (Y-3923) inhibit potently lipid peroxidation rather than the release of lysosomal enzymes (Table 2). Consequently, the mode of action of this agent on biomembranes may be somewhat different from that of the other anti-inflammatory drugs listed in Table 2.

The results of recent studies [9] in our laboratories suggest that both steroidal and nonsteroidal anti-inflammatory drugs have a membrane-stabilizing activity on intact lysosomes, and the former also stabilize the unstable lysosomes prepared from rabbit peritoneal PMN leukocytes *in vitro*. However, the difference between the effects of tinoridine hydrochloride and nonsteroidal anti-inflammatory drugs such as phenylbutazone on these lysosomes remains unclear. In this paper, it is also revealed that only tinoridine hydrochloride among the nonsteroidal anti-inflammatory drugs tested stabilizes the unstable liver lysosomes caused by pretreatment with carbon tetrachloride. The steroidal anti-inflammatory drugs such as prednisolone stabilize also the unstable lysosomes. However, the data in Table 3 suggest that the mode of action of tinoridine hydrochloride is different from that of steroidal anti-inflammatory drugs.

It has already been proven that tinoridine hydrochloride given to rats is distributed highly in the liver and kidney in the form of unchanged tinoridine and debenzylated metabolite (Y-3923) [18]. In preliminary experiments in our laboratories, the increase of both lysosomal enzymes (i.e. aryl sulfatase) and transaminases in sera of rats treated with carbon tetrachloride was found to be inhibited by pretreatment with tinoridine hydrochloride. Therefore, additional experiments were carried out to reveal the effect *in vivo* of this drug on liver lysosomes and the effect *in vitro* on kidney lysosomes and liver parenchymal cells.

The kidney lysosomes in the 650–3500 *g* fraction were found to be more sensitive to tinoridine hydrochloride than those in the 650–12,000 *g* fraction, as is apparent from Table 5. Consequently, this fraction was used as lysosomes of the kidney for determining the effects of drugs on their stability. These lysosomes

were stabilized more potently by tinoridine hydrochloride, moderately by prednisolone and slightly by phenylbutazone and indomethacin (see Table 6). Therefore, it is concluded from the above data that nonsteroidal acidic anti-inflammatory drugs such as phenylbutazone stabilize only intact lysosomes, and steroidal anti-inflammatory drugs such as prednisolone stabilize markedly the unstable lysosomes rather than the intact lysosomes. These conclusions are the same as those reported previously [9].

On the mechanism of action of these drugs on biomembranes, Mizushima *et al.* [23] have proposed that the stabilizing action of nonsteroidal acidic anti-inflammatory drugs on the caine erythrocytes is due to a stabilizing effect of the drugs on certain proteins in the membranes. On the other hand, Lewis *et al.* [24] have reported recently that the stabilizing action of steroidal anti-inflammatory drugs on lysosomes is due to drug–phospholipid interactions rather than drug–protein interactions. All of these findings suggest that nonsteroidal acidic anti-inflammatory drugs stabilize the intact lysosomes by preventing the denaturation of certain proteins in the membranes and that steroidal anti-inflammatory drugs, although being sensitive to unstable lysosomes rather than intact lysosomes, stabilize membranes by preventing the denaturation of certain phospholipids in the membranes.

In contrast, tinoridine hydrochloride was found to stabilize both the intact and unstable lysosomes of the liver or kidney, and inhibit the lipid peroxidation in the liver fraction. Therefore, this drug may prevent injury of the liver or kidney *in vivo*. In fact, tinoridine hydrochloride does prevent labilization of the liver lysosomes *in vivo* (Table 4) and suppresses the increase of both lysosomal enzymes and transaminases as described above. The data in Tables 7 and 8 suggest that this drug actually stabilizes not only lysosomes but also the liver parenchymal cells *in vitro*.

Steroidal anti-inflammatory drugs, such as hydrocortisone, administered to rats have been shown to have a preventive action of leakage of GPT from the liver cells [25]. In the experiments *in vitro*, as shown in Table 8, hydrocortisone and prednisolone prevented the release of transaminases. However, the property of these drugs seems to be different from that of tinoridine hydrochloride.

The conclusion drawn from these experiments was that tinoridine hydrochloride has a membrane-stabilizing activity on the liver and kidney lysosomes *in vitro* or *in vivo*, and on the liver parenchymal cells *in vitro*. In addition, the effect of this drug on biomembranes was suggested to be different from that of benzydamine hydrochloride, phenylbutazone, indomethacin or prednisolone. The mode of membrane-stabilizing action of tinoridine hydrochloride may be due to a stabilizing effect of this drug on both certain proteins and phospholipids in the membranes.

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