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# Hyaluronidase-Inhibitory and Anti-allergic Activities of the Photo-Irradiated Products of Tranilast[1)]

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We found that *N*-(*trans*-3,4-dimethoxycinnamoyl)anthranilic acid (tranilast) is readily transformed by photo-irradiation to *N*-(*cis*-3,4-dimethoxycinnamoyl)anthranilic acid (*cis*-isomer) and a cyclobutane derivative (dimer). Of these photo-irradiated products of tranilast, the *cis*-isomer was found to inhibit hyaluronidase more effectively than tranilast.

From these results, it was expected that the *cis*-isomer might have more effective anti-allergic activity than tranilast; indeed, the *cis*-isomer was found to show more effective prevention than tranilast of (1) histamine release from sensitized rat peritoneal mast cells induced by antigen (egg white albumin), (2) histamine release from rat peritoneal mast cells induced by concanavalin A and calcium ionophore A-23187, and (3) the 48 h homologous passive cutaneous anaphylaxis (PCA) in rats.

**Keywords**——allergy; hyaluronidase; anti-allergic activity; anti-hyaluronidase activity; tranilast; *N*-(*cis*-3,4-dimethoxycinnamoyl)anthranilic acid; dimer of tranilast

The pathological mechanism of type I allergy has been explained in term of antigen-IgE antibody reaction on the membrane of the mast cells and subsequent histamine release.[2,3)] It is considered that the histamine release is induced by the influx of calcium ions into the cells through the calcium channels, and these ions activate certain enzyme reactions. However, the precise mechanism of the intracellular series of enzyme reactions has been clarified only a very limited extent.[4-6)]

We presumed that hyaluronidase might be one of the target enzymes of the influxed calcium ions and have investigated the possibility of modulating the hyaluronidase activity with drugs associated with histamine release. We have reported[7)] that compound 48/80, a histamine-releasing agent, activates hyaluronidase, whereas acidic anti-allergic agents, such as disodium cromoglycate (DSCG),[8)] *N*-(*trans*-3,4-dimethoxycinnamoyl)anthranilic acid (tranilast),[9)] baicalein phosphate[10)] and traxanox,[11)] which are known inhibit the histamine release from mast cells induced by antigen-immunoglobulin E (IgE) antibody reaction, strongly inhibited hyaluronidase.

These results suggested that the anti-hyaluronidase activity of these acidic anti-allergic agents may be a general mechanism of inhibitory action on histamine release from mast cells. Furthermore, if strongly hyaluronidase-inhibitory substances could be discovered, they might have potent anti-allergic activity and would represent leading compounds for developing new anti-allergic agents.

Tranilast is an orally active anti-allergic agent which was developed initially in Japan. This drug was obtained by the chemical modification of cinnamic acid derivatives which were isolated from folk medicine.[12)] We found that tranilast is readily transformed by photo-irradiation to *N*-(*cis*-3,4-dimethoxycinnamoyl)anthranilic acid (*cis*-isomer) and a cyclobutane

derivative (dimer), as was expected from the fact[13] that cinnamic acid derivatives generally photo-react to give their *cis*-isomer and dimer.

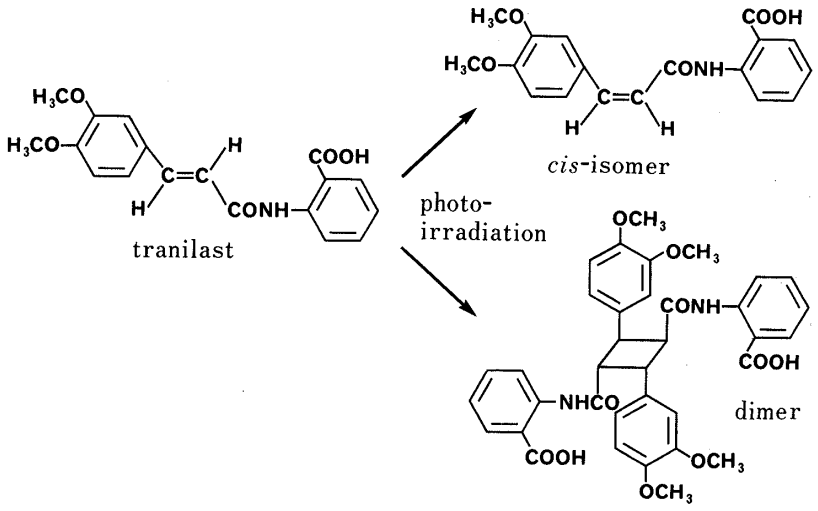


Chart 1

Of the photo-irradiated products of tranilast, the *cis*-isomer was found to inhibit hyaluronidase more strongly than tranilast. Thus, it was expected that the *cis*-isomer might have more effective anti-allergic activity than tranilast, and this was in fact confirmed.

## Results and Discussion

We have reported[14] that it is preferable to use calcium chloride and compound 48/80 rather than sodium chloride, which had been used by most investigators previously, as the activator of hyaluronidase when testing for inhibition of hyaluronidase activity during the development of new anti-allergic agents. Therefore 2.5 mM $CaCl_2$ and 0.1 mg/ml compound 48/80 were used to activate hyaluronidase in the present work.

Figure 1 shows the inhibitory effects of the *cis*-isomer, the dimer and tranilast on hyaluronidase activated by 2.5 mM $CaCl_2$. Figure 2 shows the inhibitory effects of these compounds on the activation of inactive hyaluronidase by 2.5 mM $CaCl_2$. These compounds inhibited hyaluronidase concentration-dependently in both tests. A comparison of the inhibitory effects of these compounds on activated hyaluronidase and on the activation of inactive hyaluronidase demonstrated that all these compounds inhibited the activation of inactive hyaluronidase more effectively than they inhibited the activated enzyme. In both cases, the *cis*-isomer inhibited hyaluronidase most effectively, while the dimer inhibited hyaluronidase as effectively as tranilast.

Figure 3 shows the inhibitory effects of the *cis*-isomer, the dimer and tranilast on hyaluronidase activated by 0.1 mg/ml compound 48/80. Figure 4 shows the inhibitory effects of these compounds on the activation of inactive hyaluronidase by 0.1 mg/ml compound 48/80. In both cases, these compounds inhibited hyaluronidase concentration-dependently in both tests. The *cis*-isomer inhibited the activation of inactive hyaluronidase more effectively than it inhibited the activated enzyme, while the dimer and tranilast were equally effective in both tests. The *cis*-isomer showed the most potent effect on the activation of inactive hyaluronidase. Based on these results, we presumed that the *cis*-isomer might have more potent anti-allergic activity than tranilast and the dimer.

Thus the effects of the *cis*-isomer and tranilast on the histamine release from sensitized rat

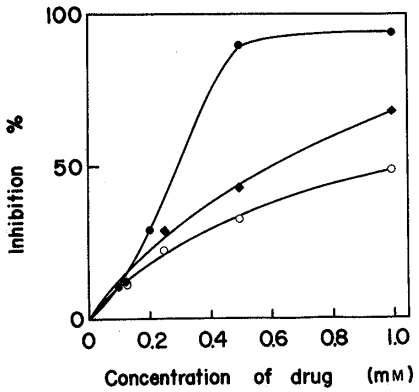


Fig. 1. Inhibitory Effects of *cis*-Isomer, Dimer and Tranilast on Activated Hyaluronidase

$CaCl_2$ (2.5 mM) was used as an activator of hyaluronidase. Each point is the mean of 3 observations.
●, *cis*-isomer; ◆, dimer; ○, tranilast.

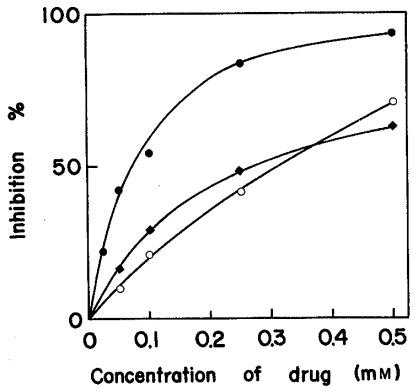


Fig. 2. Inhibitory Effects of *cis*-Isomer, Dimer and Tranilast on the Activation Stage of Hyaluronidase

$CaCl_2$ (2.5 mM) was used as an activator of hyaluronidase. Each point is the mean of 3 observations.
●, *cis*-isomer; ◆, dimer; ○, tranilast.

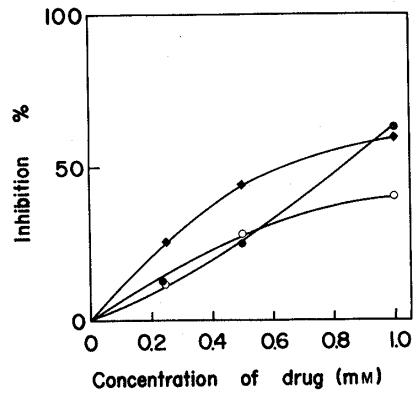


Fig. 3. Inhibitory Effects of *cis*-Isomer, Dimer and Tranilast on Activated Hyaluronidase

Compound 48/80 (0.1 mg/ml) was used as an activator of hyaluronidase. Each point is the mean of 3 observations.
●, *cis*-isomer; ◆, dimer; ○, tranilast.

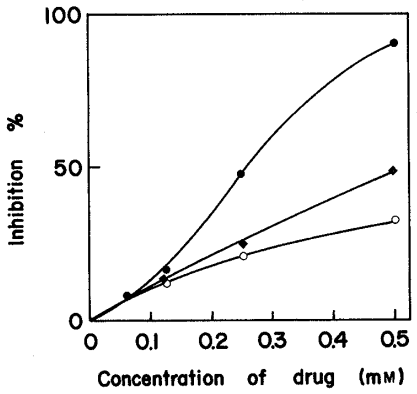


Fig. 4. Inhibitory Effects of *cis*-Isomer, Dimer and Tranilast on the Activation Stage of Hyaluronidase

Compound 48/80 (0.1 mg/ml) was used as an activator of hyaluronidase. Each point is the mean of 3 observations.
●, *cis*-isomer; ◆, dimer; ○, tranilast.

peritoneal mast cells induced by antigen (egg white albumin) were examined, and the results are shown in Table I. The *cis*-isomer and tranilast both inhibited the histamine release from sensitized rat peritoneal mast cells induced by antigen concentration-dependently. The *cis*-isomer showed more effective inhibition than tranilast. The effects of the *cis*-isomer and tranilast on the histamine release from rat mast cells induced by concanavalin A were also examined, and the results are shown in Table II. Further, the effects of the *cis*-isomer and tranilast on the histamine release from rat peritoneal mast cells induced by calcium ionophore A-23187 are shown in Table III. The *cis*-isomer and tranilast inhibited histamine release concentration-dependently in both systems, and the *cis*-isomer was more effective than tranilast. In both cases, the *cis*-isomer showed a significant inhibition of histamine release at more than $1 \times 10^{-4}$ M, while tranilast showed a significant inhibition at more than $3 \times 10^{-4}$ M.

We also examined the effects of the *cis*-isomer and tranilast on the 48 h homologous passive cutaneous anaphylaxis (PCA) in rats, and the results are shown in Table IV. Both compounds inhibited the PCA concentration-dependently. The *cis*-isomer showed a signifi-

TABLE I. Inhibitory Effects of the *cis*-Isomer and Tranilast on Histamine Release from Sensitized Rat Peritoneal Mast Cells Induced by Antigen

| Drug | Concentration (M) | Released histamine (%) |
|---|---|---|
| *cis*-Isomer | $3\times10^{-5}$ | 93.6±2.8 |
| | $1\times10^{-4}$ | 88.5±7.8 |
| | $3\times10^{-4}$ | 81.6±8.1 |
| | $1\times10^{-3}$ | 60.3±3.6[b] |
| | $3\times10^{-3}$ | 48.7±1.3[b] |
| Tranilast | $3\times10^{-5}$ | 96.9±3.8 |
| | $1\times10^{-4}$ | 90.4±5.7 |
| | $3\times10^{-4}$ | 88.7±7.9 |
| | $1\times10^{-3}$ | 74.2±4.8[a] |
| | $3\times10^{-3}$ | 56.6±5.3[b] |

The amount of histamine released in the control experiment was taken as 100%. Histamine release induced by antigen (egg white albumin $1\times10^{-4}$ g/ml) was 35.7±0.6% of the original tissue content. Spontaneous histamine release was 5.0±1.1% of the original tissue content. The reaction mixture in a total volume of 1.0 ml contained $1\times10^{5}$ mast cells. Each value is the mean (±S.E.) of 4—5 observations. *a*) and *b*) Significantly different from the control at $p<0.05$ and $p<0.01$ (*t*-test), respectively.

TABLE II. Inhibitory Effects of the *cis*-Isomer and Tranilast on Histamine Release from Rat Peritoneal Mast Cells Induced by Concanavalin A

| Drug | Concentration (M) | Released histamine (%) |
|---|---|---|
| *cis*-Isomer | $3\times10^{-6}$ | 94.2±3.3 |
| | $1\times10^{-5}$ | 90.7±3.7 |
| | $3\times10^{-5}$ | 89.5±3.5 |
| | $1\times10^{-4}$ | 77.9±4.7[a] |
| | $3\times10^{-4}$ | 64.0±3.6[b] |
| Tranilast | $3\times10^{-6}$ | 97.2±3.6 |
| | $1\times10^{-5}$ | 101.4±4.9 |
| | $3\times10^{-5}$ | 96.8±4.2 |
| | $1\times10^{-4}$ | 93.1±8.3 |
| | $3\times10^{-4}$ | 79.2±7.7[a] |

The amount of histamine released in the control experiment was taken as 100%. Histamine release induced by concanavalin A (20 μg/ml) was 67.3±2.6% of the original tissue content. Spontaneous histamine release was 9.8±2.0% of the original tissue content. The reaction mixture in a total volume of 1.0 ml contained $1\times10^{5}$ mast cells. Each value is the mean (±S.E.) of 4—5 observations. *a*) and *b*) Significantly different from the control at $p<0.05$ and $p<0.01$ (*t*-test), respectively.

cant inhibition of PCA in rats at a dose of 100—300 mg/kg, while tranilast was less effective.

These results suggest that the *cis*-isomer has more effective anti-allergic activity than tranilast. Furthermore, a correlation was found between the anti-hyaluronidase activities of the *cis*-isomer and tranilast and the inhibitory effects of these compounds on the histamine release from rate peritoneal mast cells and on the 48 h homologous PCA in rats. Kojima *et al.* reported[15] that tranilast showed more effective inhibition of histamine release from sensitized rat peritoneal exudate cells induced by antigen (DNP-As) than the *cis*-isomer, which is contrary to the results of our experiments. One possible reason for the difference might be that a different antigen was used in their experiments. However, the results in our experimental systems were well related to the anti-hyaluronidase activities. Thus, we confirmed that potent hyaluronidase-inhibitory substances may have anti-allergic activities and could be excellent

TABLE III. Inhibitory Effects of the *cis*-Isomer and Tranilast on Histamine Release from Rat Peritoneal Mast Cells Induced by Calcium Ionophore A-23187

| Drug | Concentration (M) | Released histamine (%) |
|---|---|---|
| *cis*-Isomer | $3\times10^{-6}$ | 92.9±7.1 |
| | $1\times10^{-5}$ | 95.3±2.4 |
| | $3\times10^{-5}$ | 94.1±2.5 |
| | $1\times10^{-4}$ | 77.7±7.0[a] |
| | $3\times10^{-4}$ | 50.6±7.7[b] |
| Tranilast | $3\times10^{-6}$ | 97.4±1.3 |
| | $1\times10^{-5}$ | 98.7±3.8 |
| | $3\times10^{-5}$ | 100.0±1.5 |
| | $1\times10^{-4}$ | 93.6±1.7 |
| | $3\times10^{-4}$ | 53.9±1.9[b] |

The amount of histamine released in the control experiment was taken as 100%. Histamine release induced by calcium ionophore A-23187 ($1\times10^{-6}$ M) was 51.5±0.7% of the original tissue content. Spontaneous histamine release was 9.8±1.6% of the original tissue content. The reaction mixture in a total volume of 0.1 ml contained $1\times10^5$ mast cells. Each value is the mean (±S.E.) of 4—5 observations. *a*) and *b*) Significantly different from the control at $p<0.05$ and $p<0.01$ (*t*-test), respectively.

TABLE IV. Inhibitory Effects of the *cis*-Isomer and Tranilast on 48 h Homologous PCA in Rats

| Drug | Dose (mg/kg) | Route | Amount of dye (μg/site) | Inhibition (%) |
|---|---|---|---|---|
| Control | | | 18.0±2.33 | |
| *cis*-Isomer | 30 | *p.o.* | 13.2±3.51 | 26.7 |
| | 100 | *p.o.* | 9.8±2.52 | 45.7[a] |
| | 300 | *p.o.* | 9.3±2.44 | 48.4[a] |
| Tranilast | 30 | *p.o.* | 16.3±1.73 | 9.4 |
| | 100 | *p.o.* | 14.5±1.18 | 19.4 |
| | 300 | *p.o.* | 13.5±2.89 | 25.0 |

The *cis*-isomer and tranilast were administered *p.o.* 60 min prior to challenge with the antigen. Each experiment consisted of 5 observations. *a*) Significantly different from the control at $p<0.05$ (*t*-test).

lead compounds for developing new anti-allergic agents.

## Experimental

**Materials**——Hyaluronidase (from bovine testis) was purchased from Sigma Chemical Co., St. Louis; its specific activity was 500 NF unit/mg protein. Hyaluronic acid potassium salt, concanavalin A (lyophilized) and calcium ionophore A-23187 were purchased from Wako Pure Chemical Co., Osaka. Compound 48/80, phosphatidylserine (from bovine brain) and egg white albumin (from chicken eggs) were purchased from Sigma Chemical Co. *Bordetella pertussis* vaccine was from Takeda Chemical Industries Ltd., Osaka. Tranilast was synthesized in this laboratory according to the patent literature,[12] mp 211—213 °C (Buchi 510, Buchi). IR (KBr): 3530, 1690, 1655, 1590 $cm^{-1}$ (IR-27G, Shimadzu). $^1$H-NMR (DMSO-$d_6$) $\delta$: 3.80 (3H, s, $OCH_3$), 3.84 (3H, s, $OCH_3$), 6.74 (1H, d, $J$=15 Hz, olefinic proton), 7.56 (1H, d, $J$=15 Hz, olefinic proton), 6.92—8.66 (7H, m, aromatic protons) (R-42FT 90 MHz, Hitachi). MS *m/e* (DI): 327 ($M^+$), 309, 191, 163 (GC-MS 9000B, Shimadzu). *Anal.* Calcd for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.24; N, 4.28. Found: C, 66.32; H, 5.21; N, 4.22.

**Isolation of the *cis*-Isomer of Tranilast**——Tranilast (100 mg) in MeOH (100 ml) was irradiated by the use of a high-pressure mercury lamp (Riko UVL-100) at room temperature for 20 min with stirring. The solution was evaporated after the irradiation. The residue was dissolved in EtOH (20 ml), and water (40 ml) was then added. The precipitate, which was tranilast, was collected by filtration. The filtrate was evaporated and the residue was purified

by chromatography on silica gel with benzene–AcOH (20:1) to afford a crystalline powder. The product was recrystallized from diisopropyl ether to give the *cis*-isomer (20 mg) as pale yellow needles, mp 138—139 °C (Buchi 510, Buchi). IR (KBr): 3310, 3270, 1690, 1675, 1590 $cm^{-1}$ (IR-27G, Shimadzu). $^1$H-NMR (DMSO-$d_6$) $\delta$: 3.63 (3H, s, $OCH_3$), 3.75 (3H, s, $OCH_3$), 6.03 (1H, d, $J$=12 Hz, olefinic proton), 6.80 (1H, d, $J$=12 Hz, olefinic proton), 6.88—8.62 (7H, m, aromatic protons) (R-42FT 90 MHz, Hitachi). MS *m/e* (DI): 327 ($M^+$), 309, 191, 163 (GC-MS 9000B, Shimadzu). *Anal.* Calcd for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.24; N, 4.28. Found: C, 66.32; H, 5.33; N, 4.16.

**Isolation of the Dimer of Tranilast**——Tranilast (400 mg) in MeOH (100 ml) was irradiated by the use of a high-pressure mercury lamp (Riko UVL-100) at room temperature for 60 min with stirring. The precipitated crystals were filtered off and recrystallized from EtOH to give white crystals (14 mg). mp 273—274 °C (Buchi 510, Buchi). IR (KBr): 3330, 1700, 1670, 1610, 1590 $cm^{-1}$ (IR-27G, Shimadzu). $^1$H-NMR (DMSO-$d_6$) $\delta$: 3.70 (12H, s, $OCH_3$), 4.15 (2H, dd, $J_1$=10 Hz, $J_2$=7 Hz, cyclobutane), 4.50 (2H, dd, $J_1$=10 Hz, $J_2$=7 Hz, cyclobutane), 7.05—8.30 (14H, m, aromatic protons) (R-42FT 90 MHz, Hitachi). MS *m/e* (DI): 636 ($M^+ - H_2O$), 617, 592, 517, 500, 327, 309, 191.

These data indicate that this product is the dimer of tranilast. In particular, the NMR coupling pattern of the cyclobutane ring protons indicates that this dimer is a head-to-tail type and has *trans-cis* configuration (Chart 1). The general photochemical properties of cinnamic acid derivatives and the nuclear magnetic resonance (NMR) data for an analogous dimer[13] are consistent with our conclusion.

**Assay Methods for Hyaluronidase Activity**——The hyaluronidase activity was determined by the Morgan–Elson method[16] as modified by Davidson *et al.*[17] after the incubation of 340 NF unit/ml of hyaluronidase with 0.6 mg/ml of hyaluronic acid potassium salt at 37 °C for 40 min in 0.1 M acetate buffer at pH 3.5. Calcium chloride (2.5 mM) and compound 48/80 (0.1 mg/ml) were used as activators of hyaluronidase.

**Inhibition of Activated Hyaluronidase by the *cis*-Isomer, the Dimer and Tranilast**——The inhibitory effects of the *cis*-isomer, the dimer and tranilast on activated hyaluronidase were determined by the above method after incubation of these compounds at 37 °C for 20 min in 0.1 M acetate buffer of pH 3.5 in the presence of hyaluronidase which had been activated by preincubation with the activator at 37 °C for 20 min in the same buffer.

**Inhibition of Hyaluronidase by the *cis*-Isomer, the Dimer and Tranilast at the Activation Stage**——The inhibitory effects of the *cis*-isomer, the dimer and tranilast on the activation of inactive hyaluronidase were determined by the above method after incubation at 37 °C for 20 min in acetate buffer of pH 3.5 with the activator, following preincubation of hyaluronidase with the compounds at 37 °C for 20 min in the same buffer. Buffer was added in place of the solution of the compounds as a control. The percentage of inhibition was calculated as follows:

$$\text{inhibition (\%)} = \frac{\text{control OD}_{585} - \text{sample OD}_{585}}{\text{control OD}_{585}} \times 100$$

**Isolation of Rat Peritoneal Mast Cells**——Male Wistar rats weighing 200—250 g were decapitated, exsanguinated and injected intraperitoneally with 20 ml of physiological solution consisting of 137 mM NaCl, 2.7 mM $CaCl_2$, 1.0 mM $MgCl_2$ $6H_2O$, 5.6 mM glucose, 1 unit of heparin/ml and 5 mM phosphate buffer, pH 7.2. The abdominal region was gently massaged for about 2 min and then the peritoneal exudate was collected in a siliconized glass vessel. The cells were washed several times with the same physiological solution by centrifugation (300 × *g* for 3 min at 4 °C), and cells obtained from 15 animals were pooled ($5 \times 10^4$ cells/ml).

**Preparation of Sensitized Rat Peritoneal Mast Cells**——A volume of 0.1 ml of 1% egg white albumin solution of saline was injected intramuscularly into male Wistar rats weighing 200—250 g, and then 1 ml of *Bordetella pertussis* vaccine (containing $2 \times 10^{10}$ heat-killed *Bordetella* cells/ml) was injected intraperitoneally according to Mota.[18] Two or three weeks later, the sensitized rat mast cells were prepared and treated as described above.

**Assay of Histamine Release from Sensitized Rat Peritoneal Mast Cells Induced by Antigen**——A volume of 2.5 ml of mast cell suspension prepared as described above was mixed with 0.3 ml of the physiological solution containing various concentrations of test sample and 0.35 ml of the physiological solution containing phosphatidylserine (30 μg/ml), and the mixture was preincubated for 5 min at 37 °C. Then 3.15 ml of the mast cell suspension was incubated for 20 min at 37 °C with 0.35 ml of egg white albumin (antigen) solution ($1 \times 10^{-3}$ g/ml). The mixture was cooled to 4 °C and centrifuged at 2500 × *g* for 10 min at 4 °C. The supernatant was assayed for released histamine according to Shore *et al.*[19]

**Assay of Histamine Release from Rat Peritoneal Mast Cells Induced by Concanavalin A**——A volume of 2.5 ml of mast cell suspension prepared as described above was mixed with 0.3 ml of the physiological solution containing various concentrations of test sample and the mixture was preincubated for 5 min at 37 °C. Then 2.8 ml of the mast cell suspension was incubated for 20 min at 37 °C with 0.7 ml of concanavalin A solution (100 μg/ml). The mixture was cooled to 4 °C and centrifuged at 2500 × *g* for 10 min at 4 °C, and the supernatant was assayed for released histamine according to Shore *et al.*[19]

**Assay of Histamine Release from Rat Peritoneal Mast Cells Induced by Calcium Ionophore A-23187**——A volume of 2.5 ml of mast cell suspension prepared as described above was mixed with 1.0 ml of the physiological solution containing various concentrations of test sample and the mixture was preincubated for 5 min at 37 °C. Then 3.5 ml of mast cell suspension was incubated for 20 min at 37 °C with 10 μl of calcium ionophore A-23187 EtOH solution ($1 \times 10^{-4}$ M). The mixture was cooled to 4 °C and centrifuged at 2500 × *g* for 10 min at 4 °C. The supernatant

was assayed for released histamine according to Shore *et al.*[19]

**IgE-Mediated Passive Cutaneous Anaphylaxis (PCA) in Rats**——Antiserum containing homocytotropic antibody was obtained from rats that had been sensitized with egg white albumine mixed with heat-killed *Bordetella pertussis* vaccine according to Mota.[18] The antibody titer of the serum was 1 : 32 as estimated by the 48 h PCA. The antiserum diluted 10-fold with 0.9% saline was injected intradermally in 0.1 ml doses into 3 sites on the shaved backs of normal rats (male Sprague-Dawley rats weighing 200—250 g). The same dose of 0.9% saline was similarly injected into the other side. After 48 h, the animals were intravenously given 1.0 ml of 0.25% Evans blue solution containing 2.0 mg of egg white albumin. Thirty minutes later, the animals were sacrified by exsanguination and the skins were removed to measure the PCA blueing lesion. The amount of the dye was estimated colorimetrically after extraction by the method of Katayama *et al.*[20] The test sample suspended in 2% gum arabic and had been given orally 60 min prior to challenge with the antigen. In the control group, 2% gum arabic was administered orally in place of the 2% gum arabic suspension of the test sample.

## References and Notes


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