

ISOLATION OF LECANORIC ACID, AN INHIBITOR OF  
HISTIDINE DECARBOXYLASE FROM A FUNGUSHAMAO UMEZAWA, NORIO SHIBAMOTO, HIROSHI NAGANAWA, SABURO AYUKAWA,  
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A method of determining histidine decarboxylase activity was established. In this method,  $^{14}\text{C}$ -histamine was separated from  $^{14}\text{C}$ -histidine by Amberlite CG-resin column in the ammonium form. Lecanoric acid was obtained by screening histidine-decarboxylase inhibitors produced by microorganisms. It is the first isolation of this compound from fungi. The inhibition by lecanoric acid was competitive with histidine and noncompetitive with pyridoxal phosphate. Lecanoric acid did not inhibit aromatic amino acid decarboxylase. Though lecanoric acid was hydrolyzed easily both *in vivo* and *in vitro*, its structure yielded an information useful in developing histidine decarboxylase inhibitors of a new structural type.

It is known that histamine is involved in biological processes such as allergic response, microcirculatory hemostasis, gastric secretion, inflammation and some neural functions<sup>1-4</sup>). The turnover of this amine is much slower in mast cells and probably in basophil leucocytes than in the other cells<sup>2</sup>). Histamine is produced from histidine and this process is catalyzed mainly by histidine decarboxylase *in vivo*, though, *in vitro*, aromatic amino acid decarboxylase also catalyzes this reaction<sup>1</sup>). In this connection, a specific inhibitor of histamine biosynthesis would be expected to be a useful tool in the analysis of the biological roles of this biogenetic amine. Therefore, we initiated search for inhibitors of histidine decarboxylase in culture filtrates of microorganisms.

The known methods for determining the activity of this enzyme involve the spectrometric determination of histamine, or the measurement of  $^{14}\text{C}$ -carbon dioxide produced during the conversion. However we could not apply these methods to the screening of the inhibitors in culture filtrates, because the culture filtrates often contained substances which interfered with the results of spectrometric methods and the  $^{14}\text{C}$ -carbon dioxide method was not convenient for many samples. Therefore, we developed a method for the determination of histidine decarboxylation activity based on the measurement of  $^{14}\text{C}$ -histamine after the separation from  $^{14}\text{C}$ -histidine. This method was found to be useful in finding inhibitors in culture filtrates. An inhibitor thus found was identified as lecanoric acid, which had been previously isolated from lichen<sup>5</sup>). This is the first time lecanoric acid has been isolated from fungi. This compound provided a basis for the development of a new structural type of histidine-decarboxylase inhibitor.

In this paper, we will describe this method of determining histidine decarboxylase activity,

the isolation of lecanoric acid from a strain of *Pyricularia* and the kinetic data of the inhibition.

### Materials and Methods

Chemicals: Histidine and pyridoxal-5'-phosphate were purchased from Tokyo Kasei Co., Ltd.  $^{14}\text{C}$ -L-Histidine (45 mCi/mmol) labeled at the 2-position of the ring and  $^{14}\text{C}$ -histamine (54.3 mCi/mmol) labeled at the 2-position of the ring were purchased from Commissariat à l'Énergie Atomique, France.

Preparation of Histidine Decarboxylase: A crude histidine decarboxylase was prepared from fetal rats by the method of HÄKANSON<sup>8)</sup>. Pregnant rats were decapitated 15~18 days after mating and litters were collected. One hundred g of fetal rats were homogenized in 200 ml of 0.1 M sodium acetate buffer, pH 5.5, by a home type mixer (Sharp EM-180, Hayakawa Electric Co., Ltd.) and the homogenate was centrifuged at 20,000 *g* for 20 minutes at 0°C. The supernatant thus obtained was kept at 55°C for 5 minutes in a water bath with occasional stirring and then centrifuged at 20,000 *g* for 10 minutes. Thereafter, histidine decarboxylase was precipitated with ammonium sulfate at 25~42 % saturation. The precipitate was dialyzed against 0.05 M phosphate buffer, pH 7.0, for 16 hours. The enzyme solution thus prepared was stored at -20°C and was stable for several months.

Determination of Histidine Decarboxylase Activity: The reaction mixture, 1.0 ml consisted of  $6.25 \times 10^{-2}$  M potassium phosphate buffer, pH 6.8,  $3.7 \times 10^{-5}$  M pyridoxal phosphate,  $2.4 \times 10^{-4}$  M  $^{14}\text{C}$ -L-histidine (100,000 dpm), the enzyme preparation and an inhibitor solution. After preincubation at 37°C for 5 minutes the reaction was initiated by adding the enzyme solution. Unless otherwise stated, the reaction was carried out at 37°C for 2 hours and the amount of the enzyme added was adjusted to that which produced 2,000~4,000 cpm of  $^{14}\text{C}$ -histamine. The amount of the enzyme added was 1 mg of protein per ml. When culture filtrates were tested, 0.1 ml of the filtrate was added. The reaction was stopped by heating the tube in a boiling water bath for 5 minutes. The reaction mixture and 1 ml of distilled water which was used to rinse the tube were passed through a column (0.5×2.5 cm) of Amberlite CG-50 ( $\text{NH}_4^+$  form, 0.5 ml). The column was washed twice with 10 ml of distilled water, and  $^{14}\text{C}$ -histamine on the resin was eluted into a counting vial with 3 ml of 1 N ammonia. Eight ml of BRAY scintillation fluorophore solution was added to the vial. The radioactivity was counted by Beckmann liquid scintillation system LS-250.

Preparation of Aromatic L-Amino Acid Decarboxylase and Determination of Its Activity: Aromatic L-amino acid decarboxylase was prepared by the methods of AWAPARA<sup>7)</sup>, and the activity was measured by the methods of LOVENBERG<sup>9)</sup>.

Method of Testing the Effect of Lecanoric Acid and Related Compounds on Carrageenin Inflammation: Carrageenin was dissolved in saline at 1.0 %. Lecanoric acid or a related compound, dissolved in a sterilized water, was injected intraperitoneally into rats and 1 hour thereafter 0.1 ml of the carrageenin solution was injected subcutaneously into the hind paw. The extent of edema was measured 3 and 5 hours after the carrageenin injection by the method described by WINTER *et al.*<sup>9)</sup>

Methods of the Shaking Culture and Extraction of Lecanoric Acid: These methods are described in the following section together with the results obtained.

### Results

#### The Method of Determining Histidine Decarboxylase Inhibition in Microbial Culture Filtrates

Before undertaking the study of histidine decarboxylase inhibitors produced by microorganisms, we had to develop a method which could be applied to the determination of inhibitors

in microbial culture filtrates. It seemed likely that culture filtrates would contain the substances which would interfere with the spectroscopic determination of histamine. The method based on the determination of  $^{14}\text{C}$ -carbon dioxide after the histidine decarboxylase reaction could be applied, but it was not suitable for the investigation of a great many culture filtrates. Therefore, we studied a method based on the determination of  $^{14}\text{C}$ -histamine after the reaction. In this case, it was necessary to establish a method for separation of  $^{14}\text{C}$ -histamine efficiently from  $^{14}\text{C}$ -histidine, because during the reaction only less than 5% of the  $^{14}\text{C}$ -histidine was decarboxylated to  $^{14}\text{C}$ -histamine. There is a report by HANKASON who separated  $^{14}\text{C}$ -amines from the original amino acids by adsorption on a column Dowex 50 in hydrogen form<sup>10)</sup> followed by elution with a relatively large volume of 1N hydrochloric acid. DAVIES *et al.* described that the column of Amberlite CG-50 in hydrogen form trapped 100% of histamine<sup>11)</sup>, though histamine was not enough separated from histidine by this resin process. We found that, if Amberlite CG-50 is used in the ammonium form, histidine can be completely washed out with distilled water and the adsorbed histamine can be eluted with aqueous ammonia into the counting vial directly. This eluate was confirmed by high voltage paper electrophoresis to contain  $^{14}\text{C}$ -histamine but not  $^{14}\text{C}$ -histidine.

We studied the recovery of  $^{14}\text{C}$ -histamine which was added to the following mixture: 0.1 ml of the heat-inactivated enzyme solution, 0.1 ml of  $3.7 \times 10^{-4}\text{ M}$  pyridoxal phosphate, 0.1 ml of  $2.4 \times 10^{-3}\text{ M}$  ( $0.05\ \mu\text{Ci}$ )  $^{14}\text{C}$ -histidine, 0.1 ml of  $^{14}\text{C}$ -histamine solution at various concentrations, 0.1 ml of  $6.25 \times 10^{-1}\text{ M}$  potassium phosphate buffer, pH 6.8 and 0.5 ml of distilled water. The inactivated enzyme solution was prepared by heating in boiling water bath for 5 minutes.  $^{14}\text{C}$ -Histamine was dissolved in distilled water and the concentrations in the mixture were  $0.31 \times 10^{-6}$ ,  $0.63 \times 10^{-6}$ ,  $1.25 \times 10^{-6}$ ,  $2.5 \times 10^{-6}$ ,  $5.0 \times 10^{-6}$ , and  $10.0 \times 10^{-6}\text{ M}$ . The mixture was shaken for 2 hours at  $37^\circ\text{C}$  and  $^{14}\text{C}$ -histamine was separated from  $^{14}\text{C}$ -histidine by Amberlite CG-50 resin in

Table 1. Recovery of  $^{14}\text{C}$ -histamine from the reaction mixture

Histamine (nmoles)	Standard solution (dpm)	Standard solution+ Reaction mixture (dpm)	Recovery* (%)
0	$487 \pm 7$ ( $b_2$ )	$937 \pm 7$ ( $b_1$ )	
0.31	$591 \pm 13$ ( $S_m$ )	$1,016 \pm 12$ ( $S_n$ )	75.9
0.63	$685 \pm 3$	$1,081 \pm 3$	72.7
1.25	$1,095 \pm 68$	$1,410 \pm 25$	76.8
2.5	$2,316 \pm 16$	$2,455 \pm 8$	82.9
5.0	$5,656 \pm 6$	$4,965 \pm 40$	77.9
10.0	$10,657 \pm 17$	$8,674 \pm 17$	76.0
		mean	$77.0 \pm 3.1$

The reaction mixture contained 0.1 ml of the inactivated enzyme, 0.1 ml of  $3.7 \times 10^{-4}\text{ M}$  pyridoxal phosphate, 0.1 ml of  $2.4 \times 10^{-3}\text{ M}$  ( $0.05\ \mu\text{Ci}$ ) histidine, 0.1 ml of  $^{14}\text{C}$ -histamine solution at various concentrations, 0.1 ml of  $6.25 \times 10^{-1}\text{ M}$  potassium phosphate buffer, pH 6.8 and 0.5 ml of dist. water.

The condition of the assay was described in the section "Materials and Methods". Ten nmoles of  $^{14}\text{C}$ -histamine showed 11,000 dpm. This amount of histamine is equivalent to that of histamine, which is produced from 4% of  $^{14}\text{C}$ -histidine in the reaction mixture.

$$* \text{ Recovery} = \frac{S_m - b_1}{S_n - b_2} \times 100$$

ammonium form as described above. As shown in Table 1, the recovery of  $^{14}\text{C}$ -histamine was  $77.0 \pm 3.1\%$ . This recovery was inferior to that of  $^{14}\text{C}$ -carbon dioxide, described by LEINWEBER *et al.*<sup>12</sup> However, the standard deviation was small enough to give data which was sufficiently accurate for screening of histidine decarboxylase inhibitors. The radioactivity of the  $^{14}\text{C}$ -histamine formed from  $^{14}\text{C}$ -histidine in the standard reaction described in the previous section was  $5,076 \pm 50.4$  dpm in five replications using the same enzyme solution.

When the enzyme was added to the reaction mixture at  $0.56 \sim 2.1$  mg of protein/ml, there was a complete linear relation at least up to 2 hours between the amounts of  $^{14}\text{C}$ -histamine formed and the time length of the reaction, and the relation between the amounts of  $^{14}\text{C}$ -histamine produced and the amount of the enzyme which was added at  $0.25 \sim 2.5$  mg of protein/ml was also completely linear.

Semicarbazide reacts with pyridoxal-5'-phosphate and inhibits histidine decarboxylase. We studied the inhibition of the enzyme reactions by this inhibitor at the various concentrations (from  $1.25 \times 10^{-5}$  M to  $1.0 \times 10^{-4}$  M). The percent inhibition found was as follows: 21.9 % at  $1.25 \times 10^{-5}$  M; 42.9 % at  $2.5 \times 10^{-5}$  M; 66.7 % at  $5.0 \times 10^{-5}$  M; 85.9 % at  $1.0 \times 10^{-4}$  M. Thus, there was also a linear relation between the concentration of this inhibitor and the percent inhibition of the histidine decarboxylase and it suggested that this method could be used for detecting and determining the inhibitors in culture filtrates and in the extracts.

#### A Fungus, Strain FI-178, Producing Lecanoric Acid

A fungus, which was found by the present authors to produce lecanoric acid, a histidine decarboxylase inhibitor, had been stored in the culture collection of the Institute of Sanraku-Ocean Co., Fujisawa, Kanagawa Prefecture. It was isolated from leaf spot disease of miscanthus plant *Muhlenbergia japonica* and classified as a member of the genus *Pyricularia*. The following is a description of its conidia at the time of isolation: borne at the apex of conidiophore which has 1~3 short sterigmata; claviform or pyriform and septated; hyaline to pale fuliginous;  $19 \sim 35 \mu$  in length and  $6 \sim 13 \mu$  in width at the base. When we received this culture, however, we could not observe the conidia formation.

The characters of the present strains were as follows: On potato dextrose agar at  $25^\circ\text{C}$ : It grew rapidly and radially; the aerial mycelium which covered the growth was brownish gray-colored; the reverse was brownish black, or sometimes pale reddish brown; the substrate hyphae were pale brown and  $7 \sim 10 \mu$  in width, and the interval of the septa was  $20 \sim 30 \mu$ ; the aerial hyphae were hyaline and  $3 \sim 6 \mu$  in width, and the interval of the septa was  $30 \sim 40 \mu$ . On malt extract agar at  $25^\circ\text{C}$ : It grew fairly rapidly, forming woolly, brownish gray-colored colonies; the reverse was

Fig. 1. Production of lecanoric acid by a shaking culture at  $28^\circ\text{C}$

Mycelium was extracted with a volume of methanol equal to the volume of the broth. The activities of the culture filtrate and in the mycelium extract in inhibiting histidine decarboxylase was determined by adding 0.1 ml of culture filtrate or mycelium extract to the reaction mixture described in the section of "Materials and Methods". From the standard curve of the activity of lecanoric acid which was added at 4.0 mcg/ml to the reaction mixture, the amount of lecanoric acid was obtained.

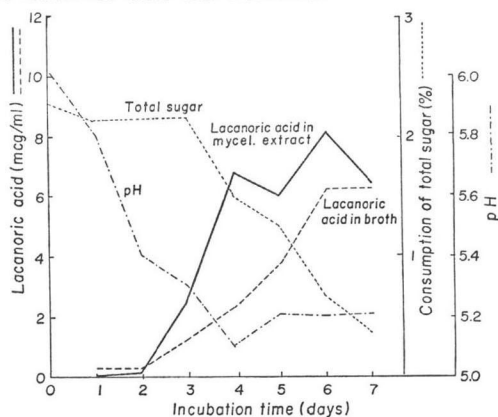
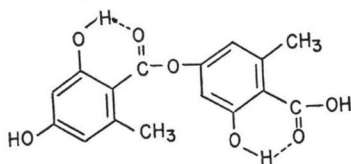


Fig. 2. Lecanoric acid



25  $\mu$  in width.

The optimum growth temperature was 20~30°C, and optimum pH was 5~7.

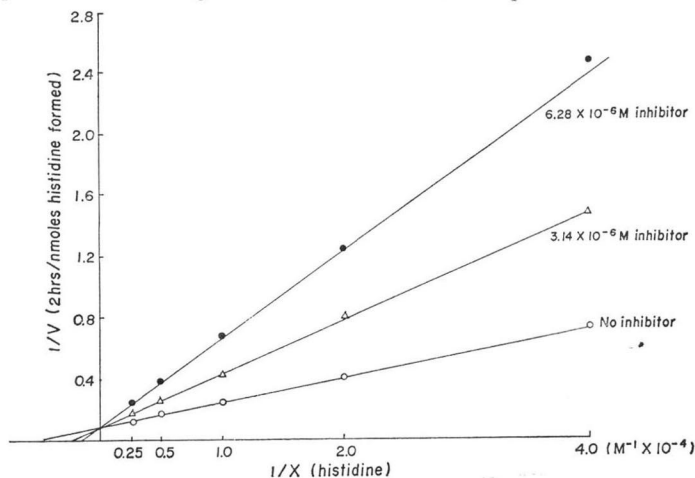
Though we could not observe the conidia formation in the present strain, the other characteristics were essentially the same as those described at the time of isolation. Therefore, it was thought that this strain had lost the ability of conidia formation during transfers on agar media.

#### Production and Isolation of Lecanoric Acid from the Strain FI-178

The strain FI-178 was shake-cultured in a medium containing 2 % potato starch, 1 % glucose, 2 % soybean meal, 0.1 %  $\text{KH}_2\text{PO}_4$  and 0.05 %  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  at 28°C on a reciprocal shaking machine, and the activity of the culture broth in inhibiting histidine-decarboxylase was measured. The time course of the production of the inhibitor, pH, and the consumption of total sugar are indicated in Fig. 1. The production of the inhibitor reached the maximum after 4~6 days of the shaking culture. The active compound in 10 liters of culture filtrate was extracted with 8 liters of butyl acetate at pH 2.0. After washing with acidic water, the active compound was extracted into 4 liters of water at pH 8.0 with 1 N NaOH. The aqueous solution was acidified to pH 2.0 and extracted with 4 liters of butyl acetate. The mycelial cake was extracted with 5 liters of methanol and the solvent was removed *in vacuo*. The tarry substance which remained was dissolved in 5 liters of water and extracted into 5 liters of butyl acetate at pH 2.0.

Fig. 3. LINEWEAVER-BURK plots of histidine concentration against rate of decarboxylation with and without lecanoric acid.

Histidine concentration was varied from  $0.25 \times 10^{-4}$  M to  $4 \times 10^{-4}$  M. The reaction was initiated by addition of the enzyme. The concentration of pyridoxal phosphate was  $3.7 \times 10^{-5}$  M. The reaction was continued for 2 hours at 37°C. The assay was carried out as described in the section "Materials and Methods". The velocities were expressed by nmoles of histamine produced. Each point represents the average of those obtained from duplicate tubes.



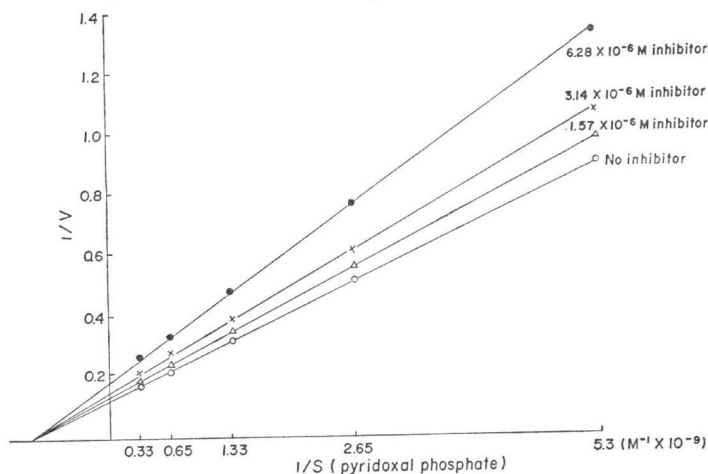
brownish black; the medium is not pigmented; the hyphal size was the same as that observed on potato dextrose agar, except for the formation of the chlamydospores in the substrate hyphae growing on malt extract agar; the chlamydospores were globose or subglobose, brownish-colored, 25~30  $\mu$  in length and 15~

The butyl acetate extracts obtained from the culture filtrate and the mycelial cake were combined and concentrated under reduced pressure. The tarry substance (8.5 g) thus obtained was dissolved in methanol (100 ml) and silica gel (85 g) was added. The solvent was removed from the mixture, and the powder thus obtained was placed on a silica gel column (5×25 cm). The active compound was eluted with chloroform-methanol (100:1). The active eluate was concentrated *in vacuo*, then applied to a Sephadex (LH-20) column chromatography (3×55 cm) which was then developed with methanol. The active fraction thus obtained was concentrated under reduced pressure and it was crystallized as colorless needles (50.2 mg) from chloroform-methanol (20:1).

The active compound decomposed at 174~175°C and had no optical activity. The molecular formula,  $C_{16}H_{14}O_7$  (M.W. 318), was obtained by the elemental analysis (Calcd.: C 60.38, H 4.43, O 35.19. Found: C 60.37, H 4.63, O 35.53), and confirmed by the mass analysis. Its methanol solution showed  $\lambda_{max}$  271 and 306 nm (log  $\epsilon$  4.71 and 4.08). The proton magnetic resonance (pmr) spectrum in hexadeuterodimethylsulfoxide showed two methyl signals at  $\delta$ 2.36 and 2.39, four aromatic protons at  $\delta$ 6.23 and 6.60 and a broad signal at  $\delta$ 10.35. Treatment of the active compound with diazomethane gave a monomethyl ether-monomethyl ester derivative and a dimethyl ether-monomethyl ester derivative as shown by their pmr spectra. The pmr of the monomethyl ether-monomethyl ester derivative in  $CDCl_3$  showed two methyl signals at  $\delta$ 2.57 and 2.62, a methoxy signal and methyl ester signal at  $\delta$ 3.82 and 3.97, four aromatic protons at  $\delta$ 6.37 (2H), 6.60 and 6.72 and two hydrogen-bonded phenol protons at  $\delta$ 11.3 and 11.5. Irradiation of methyl signal at  $\delta$ 2.57 sharpened the signal at  $\delta$ 6.37 and brought about 8 % increase of the integrated area. Irradiation of methoxy signal at  $\delta$ 3.82~3.97 enhanced the signal area of the aromatic protons at  $\delta$ 6.37 by 9 %. The high resolution mass spectroscopy of this derivative gave the molecular peak at  $m/e$  346.1054 (Calcd. 346.1052) for  $C_{18}H_{18}O_7$ . The IR

Fig. 4. LINEWEAVER-BURK plots of pyridoxal phosphate against rate of decarboxylation of histidine with and without lecanoric acid.

Pyridoxal phosphate concentration was varied from  $1.9 \times 10^{-8}$  M to  $3.0 \times 10^{-7}$  M. The reaction was initiated by addition of histidine decarboxylase. The reaction was continued for 2 hours at 37°C. The histidine concentration was  $1 \times 10^{-4}$  M. The assay was carried out as described in the section "Materials and Methods". The velocities were expressed by nmoles of histamine produced. Each point represents the mean of duplicate tubes.



spectrum with KBr showed the hydrogen-bonded phenol ester band at  $1660\text{ cm}^{-1}$  and the hydrogen-bonded phenol band at  $3000\sim 3100\text{ cm}^{-1}$ . The trimethyl derivative (dimethyl ether-monomethyl ester derivative) showed the usual phenyl ester band at  $1750\text{ cm}^{-1}$  in the IR spectrum (KBr). The pmr spectrum of this derivative showed two methoxy signals and a methyl ester signal at  $\delta$  3.83 ( $2\times\text{OCH}_3$ ), 3.92 ( $\text{OCH}_3$ ) and one hydrogen-bonded phenol signal at  $\delta$  11.37. Hydrolysis of the active compound with sulfuric acid gave *o*-orsellinic acid. From these results, the active compound seemed to be lecanoric acid. The identity with authentic lecanoric acid<sup>12)</sup> (Fig. 2) which was supplied by Prof. S. SHIBATA (Univ. of Tokyo) was confirmed by the direct comparison of their IR spectra and by a mixed melting point determination.

#### Kinetic Studies of Histidine Decarboxylase Inhibition by Lecanoric Acid

In the reaction mixture described in the previous section, inhibition of histidine-decarboxylase by lecanoric acid was studied as a function of various concentrations of histidine and pyridoxal phosphate. LINEWEAVER-BURK plots of the data are shown in Figs. 3 and 4. The  $K_m$  value of histidine was  $1.2\times 10^{-4}\text{ M}$  and agreed with the value reported by HÄKANSON<sup>6)</sup>. Inhibition by lecanoric acid was competitive with histidine. The value of  $K_i$  was  $6.9\times 10^{-7}\text{ M}$  and was obtained from the relation of the reciprocal of the  $10^{-9}\text{ M}$  of histamine produced from various concentrations ( $4\times 10^{-4}$ ,  $2\times 10^{-4}$ ,  $1\times 10^{-4}$ ,  $0.5\times 10^{-4}$ ,  $0.25\times 10^{-4}\text{ M}$ ) of histidine to  $3.14\times 10^{-4}\text{ M}$  and  $6.28\times 10^{-4}\text{ M}$  of lecanoric acid. The inhibition by lecanoric acid was noncompetitive with pyridoxal phosphate. The  $K_i$  was  $1.1\times 10^{-5}\text{ M}$ .

#### Inhibition of Carrageenin-Induced Edema in Rat Hind Paw by Lecanoric Acid

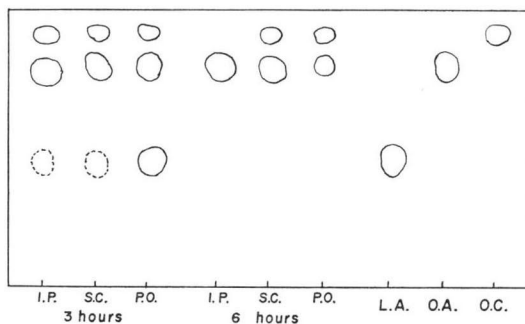
According to the method described in the previous section, 0.1 ml of 1.0 % carrageenin solution was injected into rat hind paw 1 hour after the intraperitoneal injection of various amounts of lecanoric acid, and the volume of the hind paw was determined 3 and 5 hours thereafter. The following percent inhibition was observed at 3 hours: 28.7 % by 100 mg/kg, 24.5 % by 50 mg/kg, 11.2 % by 12.5 mg/kg; at 5 hours: 29.0 % by 100 mg/kg, 20.0 % by 50 mg/kg, 11.2 % by 12.5 mg/kg.

#### Metabolism of Lecanoric Acid in Mice

Lecanoric acid (100 mg) was given to each four mice through intraperitoneal, subcutaneous or oral route and, 3 hours and 6 hours thereafter, the urine was collected. The presence of lecanoric acid, orsellinic acid and orcinol was determined by silica gel thin-layer chromatography developed with ethyl acetate-methanol-2.0 % acetic acid (10 : 1 : 0.15). The spots were detected by spraying Fast blue B salt (purchased from Tokyo Kasei Co., Ltd.) solution. As shown in Fig. 5, lecanoric acid was detected only in urine collected 3 hours after the oral administration. In the other cases, lecanoric acid was not detected, apparently being hydrolyzed to orsellinic acid

Fig. 5. Excretion of lecanoric acid, orsellinic acid and orcinol in the urine after administration of lecanoric acid to mice.

L. A.: lecanoric acid (5 mcg),  
O. A.: orsellinic acid (5 mcg),  
O. C.: orcinol (5 mcg),  
I. P.: intraperitoneal route,  
S. C.: subcutaneous route,  
P. O.: oral administration  
5  $\mu$ l of urine was spotted



which was further decarboxylated to orcinol.

### Discussion

Lecanoric acid has been isolated from lichen<sup>5)</sup>. However, as reported in this paper, we found this compound in a cultured broth of a fungus which had been classified as a member of the genus *Pyricularia*. Its structure suggests the easy hydrolysis of its ester bond in aqueous solution, and we confirmed this hydrolysis at pH 7.0 and 9.0. When lecanoric acid was dissolved at 200 mcg/ml in 1/15 M citrate buffer, pH 3.0, 1/15 M phosphate buffer, pH 7.0 or 1/15 M phosphate buffer, pH 9.0 and kept for 3 or 6 hours at 37°C, silica gel thin-layer chromatography as described above indicated no decomposition at pH 3.0 solution, but the partial decomposition to orsellinic acid at pH 7.0 after 3 hours and complete decomposition to orsellinic acid and orcinol in pH 7.0 after 6 hours. In pH 9.0 solution, lecanoric acid disappeared after 3 hours and the chromatography suggested its hydrolysis to orsellinic acid and further decomposition to orcinol. It may be expected that the instability of lecanoric acid would be one of the reason why this compound had not previously been found in culture filtrates of fungi. Our screening method may be a due to another reason why we found lecanoric acid in culture filtrates of microorganisms. It allowed us to quickly screen 840 strains of fungi and 400 strains of actinomycetes.

Neither the structure nor reactivity of lecanoric acid is related to either histidine or pyridoxal phosphate. The action of lecanoric acid seems to be specific to histidine decarboxylase, because it did not inhibit aromatic amino acid decarboxylase. The 50 % inhibition concentration of lecanoric acid was  $3.7 \times 10^{-6}$  M to histidine decarboxylase, but  $1.1 \times 10^{-4}$  M did not show any significant inhibition of aromatic amino acid decarboxylase in the reaction mixture containing L-dihydroxyphenylalanine as the substrate.

Though there is no structural relationship with histidine, the type of inhibition by lecanoric acid was competitive. As shown in Table 2, 2-hydroxybenzoic acid derivatives showed 100~

Table 2. Inhibition of carrageenin-induced edema in rat hind paw and inhibition of histidine decarboxylase by lecanoric acid and related compounds.

Compound	No. of rats	Inhibition of edema (%)		Inhibition of HDC (%) ( $1 \times 10^{-3}$ M)
		3 hr	5 hr	
2,3-DHBA	5	25.8	26.0	40.4
2,4-DHBA	5	21.3	26.0	32.2
2,5-DHBA	5	42.7	33.3	31.0
2,6-DHBA	5	31.5	34.4	55.3
3,4-DHBA	5	39.3	33.3	14.7
3,5-DHBA	5	35.8	29.2	44.7
Salicylic acid	5	33.7	31.3	19.4
Acetylsalicylsalicylic acid	5	39.3	35.4	65.7
Lecanoric acid	5	24.7	25.0	100*

DHBA: dihydroxy benzoic acid HDC: histidine decarboxylase

Carrageenin (1%, 0.1 ml) was administered in rat hind paw after 1 hour of drugs dosing. Drugs (50 mg/kg) were injected by intraperitoneal route.

Rat: Wistar strain, male. Body weight: mean 136.7 g. ID<sub>50</sub> of orsellinic acid was  $1 \times 10^{-8}$  M.

\* ID<sub>50</sub> of lecanoric acid was  $3.7 \times 10^{-6}$  M.



1,000 times weaker activity than lecanoric acid in inhibiting histidine-decarboxylase. The action of these compounds, though it is weak, suggests that the 2-hydroxybenzoic acid moiety may be involved in the inhibition. *o*-Orsellinic acid at  $5.6 \times 10^{-4}$  M showed 50 % inhibition of histidine decarboxylase. This weak action of orsellinic acid suggests that the whole molecule of lecanoric acid is involved in the inhibition of the enzyme. Inhibition of histidine decarboxylase by NSD-1055<sup>(3)</sup>, indomethacin<sup>(4)</sup> and *p*-chlorobenzylrhodanine<sup>(5)</sup> has been reported to be competitive with histidine, even though there are no obvious structural relationship between these compounds and the substrate. However, lecanoric acid has no structural similarity to these compounds except for the hydroxybenzene moiety in NSD-1055. Moreover, lecanoric acid has no amino group<sup>(6)</sup>.

The orsellinic acid moiety of lecanoric acid somewhat resembles salicylic acid, but 100 mcg/ml of lecanoric acid showed neither antibacterial nor antifungal activity. Lecanoric acid has extremely low toxicity and the intravenous injection of 200 mg/kg to mice did not result in any signs of toxicity.

As shown in Table 2, lecanoric acid inhibited rat hind paw edema caused by carrageenin. However, the magnitude of this action was similar to that of the 2-hydroxybenzoic acid derivatives which had a much weaker level of activity in inhibiting histidine decarboxylase. This weak action of lecanoric acid *in vivo* may be due to its rapid hydrolysis *in vivo*. None of the lecanoric acid injected was excreted intact in mouse urine.

As described above, lecanoric acid is easily hydrolyzed both *in vivo* and *in vitro*, and has only a weak effect in the inhibition of edema caused by carrageenin. However, its structure gave us an important information useful in developing a new type of histidine decarboxylase inhibitor. As will be reported, N-(2,4-dihydroxybenzoyl)-4-aminosalicylic acid showed a little stronger inhibition of histidine decarboxylase and much stronger inhibition of carrageenin edema than lecanoric acid. The amide bond in this compound was more resistant to hydrolysis *in vivo* and *in vitro* than the ester bond in lecanoric acid.

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