Br. J. Pharmac. (1984), **82**, 701–707

Determination of imidazole acetic acid and its conjugate(s) levels in urine, serum and tissues of rats: studies on changes in their levels under various conditions

Ikuo Imamura, Kazutaka Maeyama, Hiroshi Wada & Takehiko Watanabe

Department of Pharmacology II, Osaka University School of Medicine, 3–57 Nakanoshima 4–chome, Kita-ku, Osaka 530, Japan

1 A convenient and reproducible method for assay of imidazole acetic acid (ImAA) was developed as a modification of that described previously (Watanabe *et al.*, 1983). ImAA conjugate(s) (ImAA-C), mainly consisting of imidazole acetic acid riboside, could be measured by this method after its hydrolysis to ImAA.

2 The ImAA and ImAA-C levels in various tissues of rats were measured and the effects of various agents on these levels were studied.

3 The renal clearance values of ImAA-C in rats and man were similar to the creatinine clearance values, but those of ImAA were 1/40 of those of ImAA-C, suggesting that the latter is readily excreted in the urine.

4 Consistent with this idea, the urinary excretion of ImAA-C was found to increase much more than that of other histamine metabolites during late pregnancy, when the foetus produces much histamine.

Introduction

Imidazole acetic acid (ImAA), a metabolite of histamine, is excreted in the urine as such or as its riboside (Schayer, 1959; Wetterqvist, 1978). ImAA may also be produced from imidazole pyruvate in histidine metabolism, but the enzyme involved in oxidative decarboxylation of imidazole pyruvate has not been well characterized (Greenberg, 1969). As no simple and convenient method is available for assay of ImAA, only its level in urine has been reported (Vugman & Rocha e Silva, 1966; Granerus *et al.*, 1977; Wetterqvist, 1978). We recently developed an enzymatic method for assay of ImAA (Watanabe *et al.*, 1983) based on the strict substrate specificity of ImAA mono-oxygenase [imidazolylacetate, NADH: oxygen oxidoreductase (hydroxylating) E.C. 1.14.13.5], which catalyzes the hydroxylation of ImAA to imidazolone acetic acid with concomitant oxidation of NADH to NAD^+ in the presence of molecular oxygen (Maki *et al.*, 1969). Thus, NADH equivalent to ImAA is decreased in the presence of the enzyme.

In this work, using this method, we determined the levels of ImAA in urine and serum of rats and man and in various tissues of rats. We also determined ImAA conjugate(s) (ImAA-C), mainly composed of ImAA riboside, after its hydrolysis with acid to free ImAA (Karjala, 1955). This allowed us to determine the renal clearance of ImAA and ImAA-C in man and rats. We also measured the levels of N^{π} methylhistamine, another metabolite of histamine (Lindell & Westling, 1966; Axelrod, 1971) at the same time and examined the changes in histamine metabolism after administration of various drugs and during pregnancy.

Methods

Improved method for preparation of tissue samples for enzymatic imidazole acetic acid assay

Tissue extracts for ImAA assay were prepared as described previously (Watanabe *et al.* 1983) with slight modifications as follows. Tissues of rats (Wistar, Shizuoka Laboratory Animal Research, Shizuoka, Japan) or +/+ or W/W^v mice (Jackson

Laboratory, Bar Habor, Maine, U.S.A.) were homogenized in 3 volumes (v/w) of 3% perchloric acid with a Polytron homogenizer (Kinematica, Lucern, Switzerland) for two 10 s periods in an ice bath. The homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant retained. It was applied to a Bio-Rad AG-50 column (H^+ form) (0.5 ml of the bed volume) equilibrated with 0.5 M HCl in place of Bio-Rad AG-1 used previously. The column was washed with 5.0 ml of 0.02 M HCl and the ImAA was eluted with 2.0 ml of 5 M NH_4OH. The eluate was deaerated in a boiling water bath for 30 min and then dried in a dessicator *in vacuo* at 45°C. The residue was taken up in 1.0 ml of water and stored at − 20°C until use. Serum (1.0 ml) was mixed with 0.05 ml of 60% perchloric acid and treated similarly. Urine was collected with one drop of conc. HCl per 10 ml of urine and before use 1.0 ml of urine was mixed with 1.0 ml of 0.4 M HCl and applied directly to a column after brief centrifugation, if necessary.

Conversion of imadazole acetic acid conjugates to free imidazole acetic acid

ImAA-C was hydrolyzed to free ImAA with HCl as described by Karjala (1955). Urine (1.0 ml) was mixed with 0.2 ml of conc. HCl in a thick Pyrex glass tube and the tube was evacuated *in vacuo* and sealed. It was then heated at 145°C for 5 h to hydrolyze ImAA-C to ImAA completely. The hydrolyzate was diluted 4 fold with water and applied to a Bio-Rad AG-50 column as described above. For assay of ImAA-C in serum, 0.5 ml of the concentrated eluate from the column, which was taken up in 1.0 ml as described previously, was mixed with 0.1 ml of conc. HCl and hydrolyzed as described above.

Assays of imidazole acetic acid and its conjugates

ImAA was assayed by measuring decrease in absorbancy at 340 nm of NADH in the presence of ImAA mono-oxygenase as described previously (Watanabe *et al.*, 1983) with the following modifications. The pH of the Tris buffer in the reaction medium was changed from 7.0 to 9.0, because (a) as described above, ImAA was eluted with 5 M NH_4OH and thus catechol derivatives, which caused false positive values for absorbance at 340 nm at pH 9.0, are decomposed under alkaline conditions; (b) ImAA mono-oxygenase was more active at pH 9.0 than at pH 7.0 and thus the enzyme could be saved; (c) the intrinsic NADH oxidase activity of the enzyme was lower at pH 9.0 than at pH 7.0 and thus a lower blank value was obtained. The NADH concentration was reduced from 0.2 mM to 0.01 mM, resulting in a low blank value and a stable base line.

ImAA-C was measured as the difference between the values for total ImAA after acid hydrolysis of ImAA-C and free ImAA before acid hydrolysis.

Assays of histamine and N^τ-methylhistamine

Histamine and N^τ-methylhistamine were assayed by the *o*-phthalaldehyde (Shore *et al.*, 1959; Yamatodani *et al.*, 1982) and dansylation (Yamatodani *et al.*, 1977) methods, respectively. In some cases an h.p.l.c. system was used (K. Maeyama, unpublished).

Table 1 Imidazole acetic acid levels in rats

	Imidazole acetic acid				
	Male	*Pregnant*	*Non-pregnant*	*Foetus*	*New-born*
Brain	18.1 ± 2.2	10.0 ± 2.4	10.7 ± 2.4	3.8 ± 0.1	4.7 ± 1.3
Heart	6.0 ± 1.3	43.0 ± 0.9	13.8 ± 3.6	68.5 ± 1.2	87.2 ± 3.8
Lung	8.0 ± 1.2	17.5 ± 3.2	11.6 ± 1.5	25.7 ± 1.4	22.7 ± 6.4
Stomach	8.5 ± 1.4	13.1 ± 2.7	31.2 ± 4.1	13.4 ± 8.6	13.6 ± 0.2
Ileum	3.8 ± 0.8	18.7 ± 1.0	43.2 ± 8.1	16.6 ± 4.7	10.8 ± 0.9
Rectum	9.3 ± 1.9	31.9 ± 3.3	39.5 ± 3.4		
Spleen	7.4 ± 0.8	23.7 ± 5.1	22.1 ± 4.7	92.3 ± 26.5	62.5 ± 13.1
Liver	–	18.7 ± 3.5	22.1 ± 4.1	16.6 ± 1.1	12.1 ± 1.8
Kidney	3.8 ± 0.7	13.1 ± 1.8	9.9 ± 1.2	48.9 ± 11.4	46.8 ± 9.8
Skin	8.5 ± 1.4	8.7 ± 2.1	6.8 ± 1.0	4.0 ± 0.6	10.0 ± 0.5
Uterus	–	18.1 ± 2.0	40.6 ± 5.4	–	–
Adrenal gland	–	33.2 ± 8.5	134.0 ± 11.0	–	–
Thymus	9.0 ± 1.2	43.0 ± 11.6	13.8 ± 1.0	114.0 ± 37.0	128.0 ± 17.0
Ovary	–	30.1 ± 10.1	127.0 ± 30.0	–	–
Testis	3.0 ± 0.3	–	–	–	–
Serum	2.5	2.1	1.7	5.1	3.1

Figures are means ± s.e. ($n = 5-6$ in nmol g^{-1} wet weight of tissue or ml^{-1})

Determination of creatinine

To normalize changes in volumes of urine excreted, creatinine in the urine was measured as described by Bonsnes & Taussky (1945) and the amounts of ImAA and ImAA-C were expressed per μmol of creatinine in some experiments.

Administration of drugs

Male rats (Wistar, ca. 250 g) were given cimetidine at 2.5 mg ml^{-1} of a drinking water, which was supplied *ad libitum*. The daily water intake was about 10 ml. Rats or mice (body wt, 20 g) were injected with aminoguanidine, SKF Compound 91488 (S-[4-

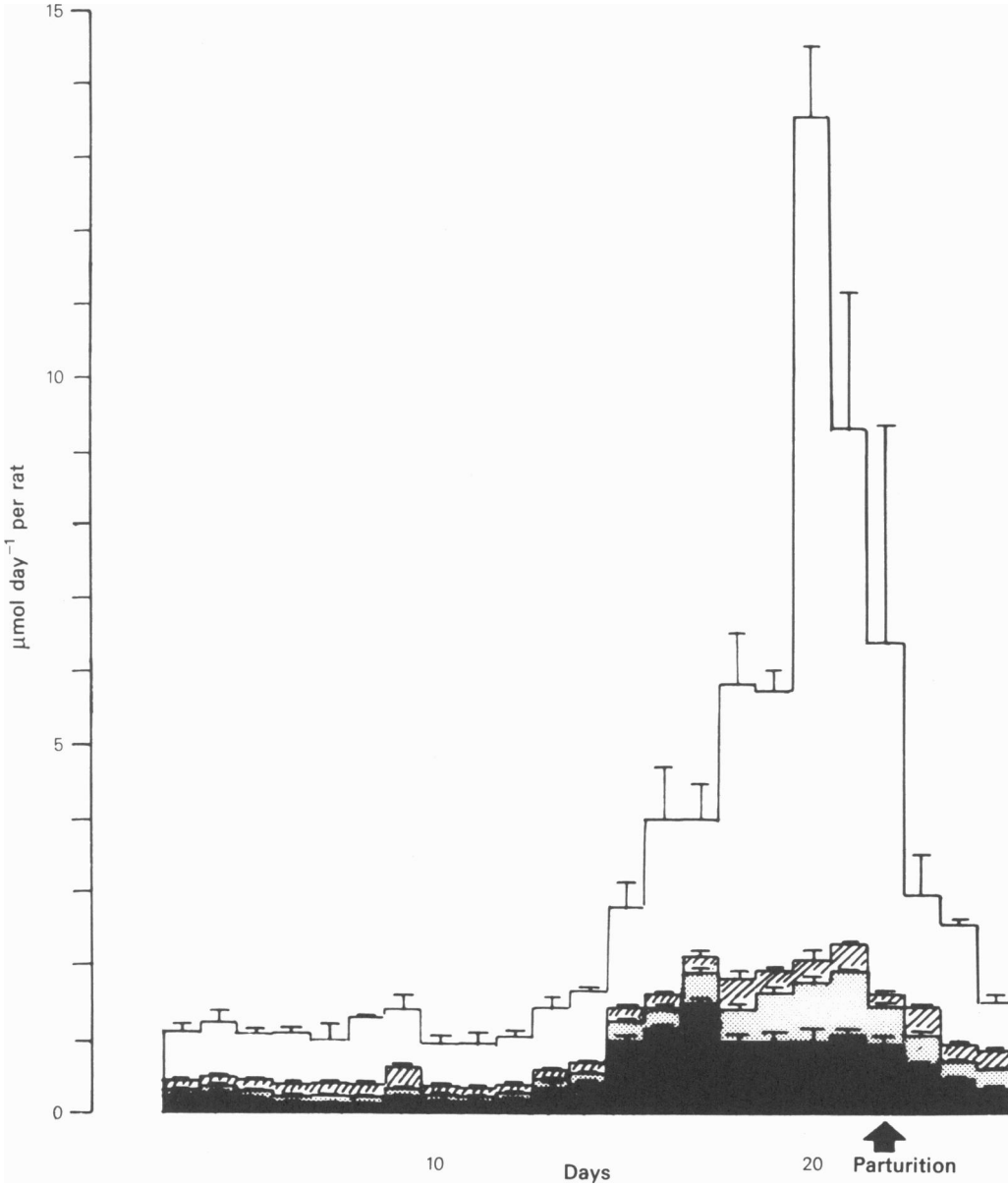

Figure 1 Urinary excretion of histamine (solid columns), N^{τ}-methylhistamine (stippled columns), imidazole acetic acid (hatched columns) and imidazole acetic acid conjugates (open columns) in rats during pregnancy and after parturition. Urine samples of rats were collected during pregnancy and after parturition and histamine and its metabolites were measured as described in Methods.

(N,N-dimethylamino)-butyl] isothiourea) or ImAA at 50, 500 and 10 mg kg^{-1}, respectively, i.p. Twenty-four hour samples of urine of one rat or 5 mice were collected in metabolic cages.

Reagents

ImAA was from Sigma (St. Louis, Mo.). ImAA mono-oxygenase was purified from *Pseudomonas sp.* (ATCC 11299B), as described previously (Watanabe *et al.*, 1983). Cimetidine and SKF Compound 91488 were gifts from Dr A. Inoue (Fujisawa Pharmac. Co., Osaka, Japan) and Dr M. E. Parsons (SKF Research Ltd., Herts, England), respectively. Other chemicals were commercial products.

Results

Analyses of imidazole acetic acid levels in rat tissues

For assay of ImAA in tissues, we modified a previous method (Watanabe *et al.*, 1983) by pretreatment of tissue extracts as described in Methods. The use of a cation exchanger (Bio-Rad AG-50) in place of an anion exchanger (Bio-Rad AG-1) permitted more efficient purification of ImAA from biological samples. 5-Hydroxyindole acetic acid, which was 0.5% as active as ImAA as a substrate of this enzyme and was present in appreciable amount in tissues, was completely removed by this modified method. Use of 5 M NH_4OH as an eluent resulted in decomposition of catechol compounds that caused false increases in absorbance at 340 nm at pH 9.0. Thus, we could measure levels of 2 nmol of ImAA in tissues more precisely and reproducibly than with the previous method. Table 1 summarizes the ImAA levels in various tissues of male, pregnant (day 19 of pregnancy) and non-pregnant female, foetal (day 19 of gestation) and new-born (1 day after parturition) rats. Considerable differences in the ImAA concentrations in various tissues of rats, i.e., heart, spleen, kidney, and thymus, were observed, but generally speaking, female tissues contained more ImAA. It should be noticed that the brain, in which N^{τ}-methylhistamine is a major metabolite, also contained appreciable ImAA. *W/W^v* mice, which were shown by Kitamura *et al.*, (1978) to be devoid of tissue mast cells, had the same amount of ImAA in the brain as congenic *+/+* mice [10.6 ± 1.0 and 16.9 ± 3.9 nmol g^{-1} wet weight, respectively ($n = 5$)].

Analyses of imidazole acetic acid and its conjugates in urine

ImAA-C was determined by measuring increase of ImAA after acid hydrolysis of urine. Figure 1 shows the increases in excretion of ImAA and ImAA-C during late pregnancy in rats. Urine of pregnant rats contained 2–3 and 10–15 times more ImAA and ImAA-C, respectively, than that of non-pregnant rats. The ratio of ImAA-C to ImAA was also increased from 4–5 in the urine of non-pregnant rats to 20–30 in that of pregnant ones. In male rats, the ratio of ImAA-C to ImAA in the urine was 4–5 (also see Table 3). As found previously (Kahlson & Rosengren, 1968; Watanabe *et al.*, 1981), histamine excretion was also increased during pregnancy, but the increase in N^{τ}-methylhistamine excretion was much more than that of histamine and was later than the latter (Figure 1).

As shown in Table 2, the renal clearances of ImAA and ImAA-C in man are 3.0 ± 0.2 and 107 ± 15 ml min^{-1}, respectively. Table 2 also shows the ImAA and ImAA-C clearances in rats normalized for body weight. The value for ImAA-C was of the same order as that in man.

Effects of drugs on excretion of imidazole acetic acid and conjugates in urine

When ImAA 10 mg kg^{-1} was given i.p. to rats every day for 1 week, the urinary excretion of ImAA and ImAA-C increased considerably (about 300 and 65

Table 2 Urinary clearance of imidazole acetic acid (ImAA) and its conjugates (ImAA-C) in healthy men and pregnant and non-pregnant rats

Compound	*Subjects or animals*	*Plasma* (nmol ml^{-1})	*Urine* (nmol min^{-1})	*Clearance* ($ml\ min^{-1}$)	($ml\ min^{-1}$, kg^{-1})
ImAA	Man	2.2 ± 0.1	6.4 ± 0.3	3.0 ± 0.2	0.05 ± 0.01
	Non-pregnant rats	1.7 ± 0.2	0.04 ± 0.01	0.03 ± 0.005	0.18 ± 0.03
	Pregnant rats	2.1 ± 0.4	0.13 ± 0.01	0.06 ± 0.01	0.29 ± 0.06
ImAA-C	Man	0.20 ± 0.02	18.2 ± 1.2	107.00 ± 15.00	1.78 ± 0.25
	Non-pregnant rats	0.97 ± 0.36	0.24 ± 0.03	0.25 ± 0.01	1.69 ± 0.66
	Pregnant rats	5.29 ± 0.57	2.33 ± 0.15	0.44 ± 0.06	2.08 ± 0.26

Figures are means ± s.e. ($n = 10$, 5 and 5 for man and non-pregnant and pregnant rats, respectively).

Table 3 Effects of imidazole acetic acid (ImAA) loading on urinary excretion of ImAA and its conjugates (ImAA-C) in rats

	Without treatment			*ImAA loading*		
	ImAA	*ImAA-C*	*Ratio*	*ImAA*	*ImAA-C*	*Ratio*
Male	0.38 ± 0.11	1.56 ± 0.40	4.11 ± 0.27	95.1 ± 13.9	440 ± 46	4.92 ± 0.57
Female non-pregnant	1.51 ± 0.18	7.94 ± 1.13	4.96 ± 0.36	107.0 ± 16.0	515 ± 56	5.22 ± 0.94
Pregnant	4.96 ± 1.03	126.00 ± 15.00	27.4 ± 3.1	20.1 ± 3.3	604 ± 87	30.1 ± 2.8

Figures for ImAA and ImAA-C are means ± s.e. in nmol μmol^{-1} creatinine ($n = 3-5$). Ratio indicates the ImAA-C/ImAA ratio. Rats were given ImAA 10 mg kg^{-1} and ImAA and ImAA-C in the urine were assayed as described in Methods. Pregnant rats were examined on day 18 of pregnancy.

times in male and female rats, respectively; Table 3), but the ratio of ImAA-C to ImAA was 4–5, which was the same as in control rats. In pregnant female rats, ImAA and ImAA-C excretion increased, even without ImAA loading, as described above, and their ratio became higher than that of non-pregnant females (27.4). ImAA loading in pregant rats caused a further 5 fold increase in excretion of ImAA and ImAA-C but the ratio of the two remained similar to that of control (30.1).

We examined the effect of blockade of the histamine H_2-receptor with cimetidine on the metabolism of histamine (Table 4). When cimetidine 2.5 mg ml^{-1} was included in the drinking water of rats, their urinary excretion of ImAA and ImAA-C was significantly higher (0.61 ± 0.06 and 3.03 ± 0.37 nmol μmol^{-1} creatinine, respectively) than that in untreated rats (0.38 ± 0.11 and 1.56 ± 0.40 nmol μmol^{-1} creatinine, respectively).

The effects of inhibitors of histamine-metabolizing enzymes were tested. When aminoguanidine 50 mg kg^{-1}, an inhibitor of diamine oxidase (histaminase, E.C.1.4.3.6), was given i.p. to rats, the urinary excretion of histamine increased and that of ImAA and ImAA-C decreased very much, but that of N^{τ}-methylhistamine did not change (Table 4). When SKF Compound 91488, an inhibitor of histamine N-methyltransferase, [E.C.2.1.1.8] was given to mice, the most striking change was that the ratio of ImAA-C to ImAA decreased to 0.13 from 0.57 in control mice (data not shown).

Discussion

The enzymatic method for assay of ImAA described previously (Watanabe *et al.*, 1983) was modified so that the tissue levels of ImAA could be measured more conveniently and reproducibly than before. We were also able to measure ImAA-C after its hydrolysis to ImAA (Karjala, 1955). ImAA riboside may be a major compound of ImAA-C, but we have no data showing that ImAA riboside is the only conjugated form of ImAA. Therefore, we have used the term ImAA-C in this paper. Using this method, we showed that the tissue level of ImAA was higher in female than male rats (Table 1), which is consistent with the finding that female rats have higher histaminase activity (Westling & Wetterqvist, 1962). It should also be mentioned that the brain of rats and mice, in which the methylation pathway of histamine degradation is predominant (Schayer, 1959), contained appreciable amounts of ImAA. ImAA may be produced by histidine catabolism, not via histamine. By the present method ImAA-C was detected only in the livers of pregnant rats.

It is known that histamine excretion in the urine increases considerably during the later period of pregnancy in rodents and decreased to the original level after parturition (Kahlson & Rosengren, 1968; Watanabe *et al.*, 1981). We found that N^{τ}-methylhistamine and ImAA increased more than histamine in the urine in this period and that ImAA-C increased over one order of magnitude more than

Table 4 Effects of various compounds on urinary excretion of histamine and its metabolites in rats

	Histamine	*N^{τ}-methylhistamine*	*ImAA*	*ImAA-C*
Control	0.26 ± 0.10	1.14 ± 0.42	0.38 ± 0.11	1.56 ± 0.40
Cimetidine	0.36 ± 0.09	0.35 ± 0.06	0.61 ± 0.06	3.03 ± 0.37
Aminoguanidine	1.44 ± 0.20	0.95 ± 0.07	0.08 ± 0.02	0.14 ± 0.03

Values are means ± s.e. (nmol μmol^{-1} creatinine) ($n = 3-5$).
Rats were treated with drugs, and histamine and its metabolites in the urine were assayed as described in Methods.

either (Figure 1 and Table 3). This is consistent with the finding that the renal clearance value of ImAA-C in man is higher than that of ImAA and is almost equal to that of creatinine; that is, ImAA-C is not absorbed in the renal tubules. Similarly in rats, ImAA-C is excreted in the urine more than ImAA, and its clearance value is almost the same as that in man on the basis of body weight (Table 2).

We examined the effects of administration of several compounds (Tables 3 and 4) on the ImAA and ImAA-C levels in rats and mice. Cimetidine, an H_2-antagonist, increased the urinary excretion of ImAA and ImAA-C, suggesting that the blockade of an H_2-receptor facilitates histamine metabolism. Detailed studies on this effect are necessary. The ratio of ImAA-C to ImAA was 4–5 in male and non-pregnant female rats, but increased to 20–30 in pregnant rats, suggesting that the enzyme that conjugates ImAA, like ImAA phosphoribosyltransferase (E.C.6.3.4.8), is induced by ImAA, which increases as a result of increase of histamine in foetuses. However, long-term administration of ImAA to male and female rats greatly increased the ImAA-C level (over 300 and 65 times, respecitvely), but did not affect the ratio of ImAA-C to ImAA (4–5) (Table 3), thus giving no evidence for enzyme induction by ImAA. Probably, the enzyme for conjugation, i.e. phosphoribosylation, of ImAA is abundant and/or its *Km* value for ImAA (0.37 mM) is higher than the level of ImAA (Crowley, 1970). However, in pregnant rats the ratio of ImAA-C to ImAA increased to 27, but did not increase further on ImAA loading, confirming the idea that ImAA does not induce a conjugating enzyme. Finally, we tested the effects of inhibitors of histamine metabolism. Aminoguanidine, an inhibitor of histaminase, decreased excretion of both ImAA and ImAA-C in the urine to almost zero, indicating that most of the ImAA is derived from histamine and that the contribution of imidazole pyruvate is negligible at least in rats. The enzyme catalyzing the oxidative decarboxylation of imidazole pyruvate in rats and other animals has not been characterized (Greenberg, 1969). In this respect, it is interesting that we found that the urine of histidinemic patients contained abnormally high amounts of histamine and ImAA (Imamura *et al.*, unpublished data). The effect of SKF Compound 91488, an inhibitor of histamine *N*-methyltransferase (Beaven & Shaff, 1979), on histamine metabolism of mice was not clear because the amount of the compound was limited, but the finding that this compound decreased the ratio of ImAA-C to ImAA 4 fold in mice suggests that it inhibits conjugating enzyme(s) of ImAA.

We thank Dr M.E. Parsons, Smith Kline & French Research Ltd. and Dr A. Inoue, Fujisawa Pharmac. Co. for gifts of Compound 91488 and cimetidine, respectively and Mrs K. Tsuji for typing.

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(Received December 14, 1983.
Revised February 1, 1984.)