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Pharmacological Studies on the Mechanism of Gastric
Ammonia Production in Relation to Peptic
Ulcer Remedies.

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1. Relationship between urease activity in gastric mucosa and ammonia content of gastric juice was examined in 3 species of laboratory animals. The close relation was confirmed between them.
2. Urease inhibitory effects of some agents were compared *in vivo* and their influence on gastric ammonia production was examined in mice. Benzohydroxamic acid, a specific inhibitor of urease, distinctively reduced gastric ammonia production.
3. By intravenous injection of urea, ammonia content of gastric juice was markedly increased.
4. Repeated administrations of urea activated gastric urease activity and gastric ammonia production.
5. Repeated administrations of urea reduced the incidence of gastric erosion which was induced by reserpine in mice.
6. All these results may suggest the important role of gastric urease in defensive mechanism of gastric mucosa.

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It has been very difficult to answer the question how the stomach protects itself from its own digestive juice. Recently, some authors tried to approach to this problem from the view of biochemistry of gastric mucosa in relation to the cause of peptic ulcer. Especially "Urea-Urease-Ammonia mechanism" was thought to be one of the probable explanations.¹⁾ The presence of ammonia in the gastric contents was observed by Bidder and Schmidt¹⁾ (1852), and Luck and Seth²⁾ (1925) demonstrated its production by the action of urease upon urea in gastric tissue. They pointed out the possible role of this mechanism in neutralizing gastric acid at the stomach wall. Martinson and Villako³⁾ (1962) postulated the view that the ammonia might be used in hexosamine synthesis in gastric mucosa. FitzGerald⁴⁾ (1950) assayed the urease activity of various kinds of organs in animals of different species and found that the stomach had the highest activity. Recently, Maramaa⁵⁾ (1966) studied on the distribution and functional changes of gastric urease activity.

In this paper, the authors aimed to make some contribution to these developments of this theory, researching for a dynamic phase of ammonia metabolism in gastric mucosa. First, the authors compared the urease activity of gastric mucosa of some laboratory animals in relation to ammonia content of gastric juice. Then, the effect of urease inhibitors on the urease-ammonia system was examined *in vitro* and *in vivo*. Finally, the role of the gastric urease in protecting gastric mucosa from gastric juice was examined using experimental peptic ulcer.

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2) J.M. Luck, T.N. Seth: Biochem. J., **19**, 357 (1925).

3) E.E. Martinson, L.A. Villako: Biokhimiya, **27**, 437 (1962).

4) O. FitzGerald: Biochem. J., **47**, ix (1950).

5) S. Maramaa: Gastroenterology, **50**, 657 (1966).

Methods

1. **Assay of Urease Activity of Gastric Mucosa**—The isolated and washed stomach was homogenized in the mill bowl with a small amount of sea sand and H₂S saturated water (2 ml.) under ice-cooled temperature. This homogenate was served as the test enzyme. According to the Van Slyke's method⁶⁾ (1944), the enzyme and substrate in phosphate buffer solution (3% urea, KH₂PO₄ 1/15M, K₂HPO₄ 1/15M, pH 7.0) were incubated for 40 min. at 30°. The enzyme activity was stopped by the addition of saturated K₂CO₃ solution (5 ml.), and liberated ammonia was transmitted to 4% H₃PO₄ solution (10 ml.) through aspirated air. Air aspiration was continued for 90 min. Ammonia in H₃PO₄ solution was titrated with 0.01N HCl, using 0.1% B.C.G. solution as indicator.

2. **Assay of Ammonia Content in Gastric Juice of Mice**—a) Collection of specimens: Under urethane anesthesia (1.3 g./kg., *i.p.*) abdomen of a mouse was opened, and the stomach was ligated at the pylorus, the oesophagus was also ligated at the throat. Into the forestomach a polyethylene cannula was inserted, through which 0.5 ml. of 0.01N HCl was introduced and withdrawn at interval of 1 hr. This perfusate was served as the specimen.

b) Assay method: Ammonia content in this perfusate was assayed by the Conway's micro-diffusion analysis method.⁷⁾ Five ml. of 0.01N H₂SO₄ was taken into the inner room of the diffusion apparatus. Into the outer room 3.0 ml. of distilled water, 0.3 ml. of the stomach perfusate and 5 ml. of saturated K₂CO₃ solution were pipetted. After 60 min. 3.0 ml. of the acid solution in the inner room was measured into a tube, into which 5.0 ml. of 0.01N NaOH solution and 2.0 ml. water was added. This mixed solution was colored with 0.2 ml. of Nessler's reagent. Then the ammonia content of the perfusate was determined photometrically.

Results

1. Species Difference of Urease Activity of Gastric Mucosa and Ammonia Content of Gastric Juice

Relationship between urease activity of gastric mucosa and ammonia content of gastric juice was searched in three different species of laboratory animals; mice, rats and guinea-pigs.

Ammonia content in gastric juice of rats and guinea-pigs was assayed according to the method in case of mice. The results are shown in Table I. The highest urease activity was found in mice whose gastric juice contained also the highest concentration of ammonia. Then mice seemed to be the most convenient animal for our experiment. The figures in the Table I indicate the close relation between urease activity and ammonia content of gastric juice.

TABLE I. Species Difference in Gastric Urease Activity and Ammonia Content in Gastric Juice

Species	NH ₃ in juice NH ₃ µg./100 g. bw./hr.	Urease activity in mucosa NH ₃ µg./g./hr.
Mouse	39.4 ± 2.0 (34)	575 ± 33.8 (13)
Rat	15.1 ± 1.9 (5)	138 ± 4.6 (4)
Guinea-pig	1.94 ± 0.26 (5)	59.6 ± 2.1 (2)

(): Number of animals

2. Effect of Urease Inhibitors on Ammonia Content in Gastric Juice

1) *In vitro* effect of inhibitors on mice gastric urease: To compare the inhibitory effects of urease inhibitors on mice gastric urease the activities of benzohydroxamic acid,

6) D.D. Van Slyke, R.M. Archibald: J. Biol. Chem., **154**, 623 (1944).

7) E.J. Conway: "Microdiffusion Analysis and Volumetric Error," 5th ed. (1962). Crosby Lockwood & Son, Ltd.

hydroquinone and sodium *p*-chloromercuribenzoate (PCMB) were assayed. Of these inhibitors benzohydroxamic acid was proved to be the most potent inhibitor of mice gastric urease. (Fig. 1) The other two inhibitors needed much higher concentrations to show the distinctive effect.

2) *In vivo* effect of the intravenously injected urease inhibitors on ammonia content of gastric juice: The results are shown in Fig. 2 and Table II. Benzohydroxamic acid (50 mg./kg.) reduced the ammonia concentration to about a half of the control. The time course of ammonia production is shown in Fig. 2. The effect reached the maximum 2 hours after the intravenous administration of the hydroxamic acid. However, the increase in the dose did not elevate inhibitory effect so much. Hydroquinone also reduced the ammonia production distinctively in a dose of 80 mg./kg., but it led some test animals to death by its general toxicity. Sodium *p*-chloromercuribenzoate was administered intraperitoneally in a sublethal dose of 25 mg./kg., but exerted no inhibitory effect.

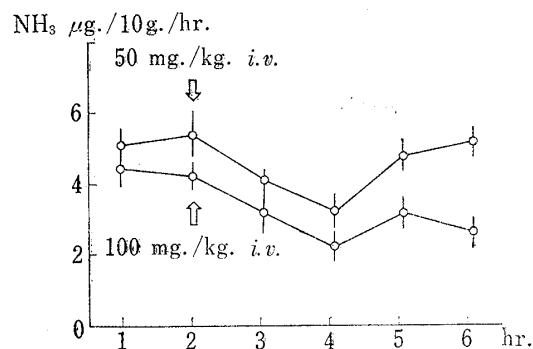
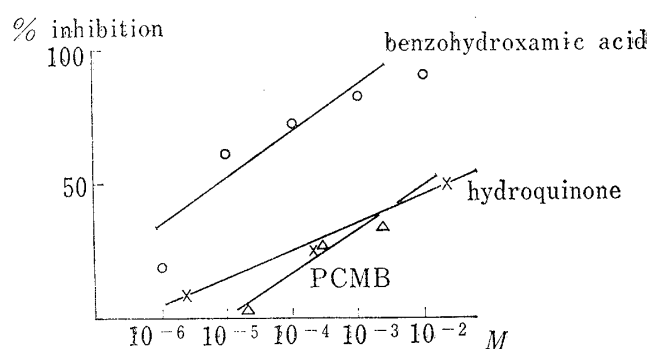


Fig. 1. Comparison of Inhibitory Effect of Some Agents on Gastric Urease in Mice

Fig. 2. The Effect of Benzohydroxamic Acid on Gastric Ammonia Production in Mice

Assay system consists of;

200 mg. of whole stomach homogenate from 10 mice,
5 ml. of phosphate buffer solution (pH 7.0, 1/15M KH₂PO₄, 1/15M K₂HPO₄) which contains 3% of urea.

TABLE II. The Effect of Some Urease Inhibitors on Gastric Ammonia Production in Mice

Compounds	No. of assays	Dose (mg./kg.)	Inhibition (%)	Toxicity
Benzohydroxamic acid	11	50 <i>i.v.</i>	52.1 ± 9.8	—
Benzohydroxamic acid	5	100 <i>i.v.</i>	53.9 ± 7.3	—
Hydroquinone	7	80 <i>i.v.</i>	32.3 ± 7.9	+
PCMB	4	25 <i>i.p.</i>	0	+

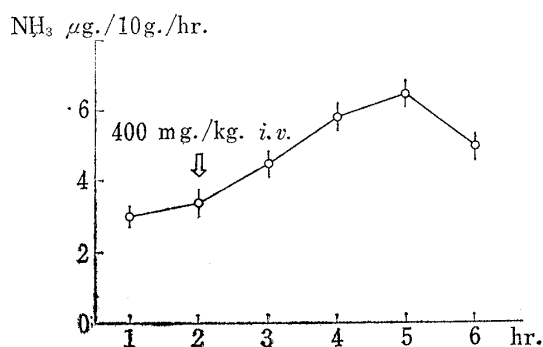


Fig. 3. Increase Gastric Ammonia Production after Intravenous Injection of Urea

3. Effect of the Urease Substrate on Gastric Ammonia Secretion

Ammonia content of gastric perfusate after the intravenous administration of urea was assayed in the same manner as in the case of urease inhibitor. Intravenous administration of urea in a dose of 400 mg./kg. showed a significant increase in ammonia secretion in gastric juice. The time course of ammonia production after urea administration is illustrated in Fig. 3.

Intravenous injection of urea, 400 mg./kg., caused a marked increase in ammonia concentration in the gastric perfusate. Two or three hours after injection the effect of urea got to its peak and thereafter the ammonia secretion gradually decreased. A dose of less than 400 mg./kg., *i.e.* 200 mg./kg. of urea was less effective and the effect at 800 mg./kg. did not show a significant difference from the effect at 400 mg./kg. of urea.

4. Influence of Repeated Administrations of Substrate and Urease Inhibitor on the Activity of Gastric Urease

Under the expectation if the enzyme induction of gastric urease can be attained, the substrate urea or the inhibitor benzohydroxamic acid was administered to mice repeatedly within a week and the change of activity of gastric urease was chased. Benzohydroxamic acid, 50 mg./kg. *s.c.* or urea, 400 mg./kg. *p.o.* was administered to mice once a day. Change in gastric urease activity is shown in Fig. 4. The enzyme

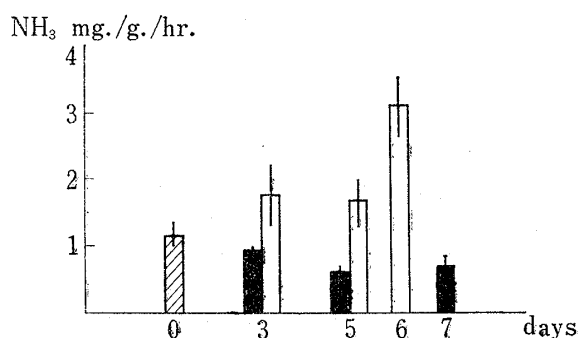


Fig. 4. Changes in Gastric Urease Activity after Successive Administration of Benzohydroxamic Acid and Urea in Mice

■ benzohydroxamic acid
□ urea
▨ control

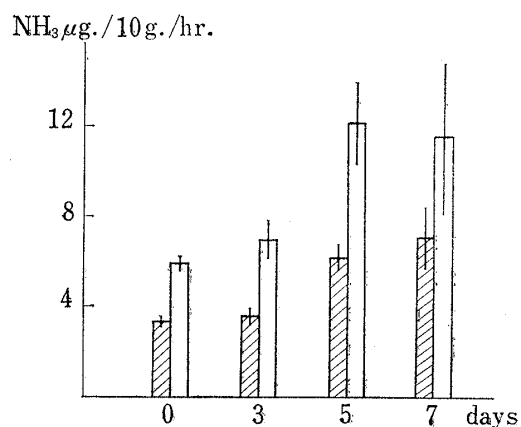


Fig. 5. Changes in Gastric Ammonia Production after Successive Administrations of Urea in Mice

▨ basic NH₃ □ urea 400 mg./kg. *i.v.*
Each column indicates the mean value of 10 animals with standard error.

activity was not changed by benzohydroxamic acid. However, repeated administrations of urea seemed to increase the activity of gastric urease. The mice group which received urea 3 or 5 times showed a tendency of increasing the activity and the last group which received urea for 6 days had the gastric urease activity twice as much as control group.

5. Influence of Repeated Administrations of Urea on Ammonia Content of Gastric Juice

With the mice which were administered urea, 400 mg./kg., everyday, the change in gastric ammonia secretion was assayed. Ammonia secretion during 2 hours before urea injection we called "basic" secretion. After the injection of urea, gastric ammonia content may reflect the gastric urease activity *in vivo*. In Fig. 5, the highest values of ammonia content after urea injection (400 mg./kg.) were plotted. Basic ammonia secretion was increased gradually in course of repeated administrations of urea, and effect of urea injection was also increased more distinctively. On the 5th day ammonia secretion after urea injection reached the value twice as high as the control. These results indicate that gastric urease was activated or induced by the repeated administrations of the substrate.

6. Effect of Urea on the Gastric Haemorrhage induced by Reserpine in Mice

Reserpine, 5 mg./kg., was injected to each mouse which was pretreated with urea, and the gastric mucosa was tested for the haemorrhage after 24 hours. Single dose of urea,

simultaneously injected with reserpine, had little or no effect to prevent gastric haemorrhage in doses of 400 mg./kg. and 800 mg./kg. However, in mice which received 400 mg./kg. of urea once a day for 3 days or for 7 days successively, severity of haemorrhage was apparently reduced. These results may suggest the important role of gastric urease in defensive mechanism of gastric mucosa.

TABLE III. Effect of Urea on Gastric Errosion induced by Reserpine in Mice

Treatment	No. of animals	Severity of haemorrhage			
		+++	++	+	-
Saline	10	6	3	1	0
Urea 400 mg./kg. <i>i.p.</i>	10	5	4	1	0
Urea 800 mg./kg. <i>i.p.</i>	10	3	5	2	0
Urea 400 mg./kg. <i>s.c.</i> for 3 days	6	1	2	1	2
Urea 400 mg./kg. <i>s.c.</i> for 7 days	6	1	1	2	2

Discussion

There are many papers⁴⁾ (1950) which provide the evidence for the theory suggesting that gastric ammonia was produced by the action of gastric urease. They are, however, not enough to explain how much the contribution of gastric ammonia is to the defensive mechanism of gastric mucosa against digestive fluid. The authors aimed to make sure the relationship between gastric urease activity and ammonia content of gastric juice under the influence of enzyme inhibitors or substrates. The authors expected that these relationships will give some clues for declaring the physiological significance of gastric urease and ammonia.

As to the species difference of gastric urease activity, there is the early study⁴⁾ (1950), but it did not deal with gastric ammonia. In this paper the authors have shown the close relation between gastric urease and ammonia content of gastric juice in three species of laboratory animals. Utilization of enzyme inhibitor is surely essential for the study of physiological role of an enzyme. Three well known inhibitors were examined for the possibility if they could be used for *in vivo* experiments. Of these inhibitors benzohydroxamic acid revealed to be the most potent *in vitro* and less toxic when injected intravenously into mice. Hydroxamic acid derivatives were described as specific urease inhibitors by Hase, *et al.*⁸⁾ (1962). Hydroquinone and sodium *p*-chloromercuribenzoate were too toxic for *in vivo* experiment. Decrease of ammonia content in gastric juice was clearly shown when benzohydroxamic acid was injected intravenously. This inhibition, however, was limited within about 50% of normal ammonia content. This result may suggest that there are other ammonia production systems in gastric mucosa. Ammonia production of gastric mucosa was markedly increased after the successive administrations of urea. This increase may owe to the urease activation or induction of the enzyme. In fact, the activity of gastric urease was also increased after repeated doses of urea. In the early stage of experiments, the authors thought that the successive administrations of urease inhibitor might cause the enzyme induction. But it proved to be failure by a series of experiments.

The experimental gastric erosion produced by reserpine in mice was employed for the research on the significance of the gastric ammonia in the defensive mechanism against gastric juice. The reasons why this lesion was employed are as follows :

8) J. Hase, K. Kobashi, K. Uehara : Biochem. Biophys. Acta, **65**, 380 (1962).

gastric urease activity is very high in mice; the gastric erosion is easily induced by reserpine⁹⁾ (1959); this erosion can be prevented by antacid⁹⁾ (1959). In this experiment the distinctive effect of urea was shown in preventing gastric erosion only after successive administrations of the drug. This result supports the propriety of clinical trial for treatment of peptic ulcer by urea administration (1962)¹⁰⁾, (1960)¹¹⁾.

9) J.G. Blackman, D.S. Campion, F.N. Fastier : *Brit. J. Pharmacol.*, **14**, 112 (1959).

10) H. T. Kelly : *The Canadian Med. Assoc. J.*, **86**, 17 (1962).

11) D.J. Goodfriend, C.E. Vanderkleed, T.L. Goodfriend : *Am. J. Gastroenterology*, **33**, 80 (1960).