[Chem. Pharm. Bull. 18(2) 255—260 (1970)]

UDC 615.241.015

Influence of Some Drugs Injurious to Gastric Mucosa on Gastric Ammonia Formation

Kazuo Watanabe, Noriko Ishida and Hideomi Fukuda

Faculty of Pharmaceutical Sciences, Nagoya City University[1]

(Received July 17, 1969)

Mechanism of gastric ammonia formation was studied in mice, and the possibility was examined that the injurious effect of some anti-inflammatory drugs on gastric mucosa was due to the gastric urease inhibition. Ammonia formation in the stomach was remarkably augmented by urea irrigation and was inhibited by benzohydroxamic acid, an urease inhibitor. These results indicate the importance of urease in ammonia formation in stomach. Urease inhibiting-activity *in vitro* of three anti-inflammatory drugs, aspirin, phenylbutazone and chinophen, and of caffeine were so weak that their injurious effects to gastric mucosa could not be attributed to urease inhibition.

Gastric ammonia formation *in vivo* was found to be accelerated by irrigation of gastric mucosa with above-mentioned drugs, except aspirin. Gastric ammonia formation was also increased by eugenol, a well-known gastric mucosa irritant, and the effect was completely inhibited by benzohydroxamic acid. From these results it is concluded that injury on gastric mucosa by drugs increases gastric ammonia formation by accelerating the enzymatic reaction between urea and urease.

Gastric urease is the enzyme which is plentiful in gastric mucosa and hydrolyses urea to carbon dioxide and ammonia. The ammonia formed by gastric urease is believed to protect gastric mucosa by neutralizing gastric acid[2] or by transforming into gastric mucus component.[3] On the other hand, there are many opposed discussions on its physiological significance.[4] These discussions, however, seem to lack the conclusive evidence, because it was difficult to control gastric urease activity *in situ*. The authors found benzohydroxamic acid to be a useful inhibitor of gastric urease *in vivo* in mice.[5] In the present paper we utilized this inhibitor for the purpose of showing the functional change of gastric ammonia formation in gastric mucosa damaged by some drugs, and the possibility was examined that the injurious effects of anti-inflammatory drugs on gastric mucosa might be due to gastric urease inhibition.

In the course of the experiment the possibility was found to be ruled out, and on the contrary, it was found that some drugs increased gastric ammonia formation. Thus the relation between ammonia formation and damage of gastric mucosa was studied in detail.

Experimental

Perfusion of Stomach with Drug Solution——Adult male mice weighing 20—30 g were used. Under urethane anesthesia (1.3 g/kg *i.p.*), abdomen of a mouse was opened, and the stomach was ligated at the pylorus. The esophagus was also ligated at the throat. A polyethylene cannula was inserted into the fore stomach, through which 0.5 ml of 0.01N HCl or the test solution was introduced and withdrawn at the interval of 1 hr. The ammonia content of the withdrawn fluid was assayed. Acidic drugs were used as suspension in 0.01N HCl. Eugenol was emulsified in 5% gum arabic solution.

1) Location: *Tanabe-dori, Mizuho-ku, Nagoya.*
2) E.J. Conway, "The Biochemistry of Gastric Acid Secretion," Thomas, Springfield., 1952, p. 166.
3) E.E. Martinson and L.A. Villako, *Biokhimiya*, **27**, 437 (1962).
4) H.L. Kornberg and R.E. Davis, *Physiol. Rev.*, **31**, 169 (1955).
5) K. Watanabe, S. Ohshima and H. Fukuda, *Chem. Pharm. Bull.* (Tokyo), **15**, 1720 (1967).

Assay of Ammonia in the Gastric Perfusates——Ammonia content in the gastric perfusate was assayed by the Conway's microdiffusion method.[6] Five ml of 0.01N H_2SO_4 was taken into the inner chamber of the diffusion appratus. Into the outer chamber 3.0 ml of distilled water, 0.3 ml of the stomach perfusate and 5 ml of saturated K_2CO_3 solution were pipetted. After incubation for 60 min at 38°, 3.0 ml of the acid solution in the inner chamber was transfered to a tube, into which 5.0 ml of 0.01N NaOH and 2.0 ml of water were added. The colour developed by 0.2 ml of Nessler's reagent was measured photometrically.

Assay of Urease-Inhibiting Activity of Some Drugs *in Vitro*——Influences of some drugs injurious to gastric mucosa on jack bean urease (jack bean meal, Difco) were assayed by Van Slyke's method.[7] The jack bean meal was dissolved in saturated H_2S solution in a concentration of 5×10^{-5} g/ml and kept in refrigerator for 2.5 hr at 0° before use. Urea was dissolved in phosphate buffer, pH 7.0 in a concentration of 3%. One liter of the buffer solution contained 400 mM of K_2HPO_4 and 100 mM of NaH_2PO_4. Three ml of the substrate solution and 0.1 ml of the enzyme solution were mixed in a tube, and 1 ml of the drug buffer solution was added. The reaction system was incubated for 15 min at 30°. The enzymatic reaction was stopped by 4 ml of the saturated K_2CO_3 solution. Liberated ammonia was transfered to another tube containing 7.5 ml of H_2SO_4 by air aspiration. Ammonia content in this H_2SO_4 solution was assayed photometrically by using Nessler's reagent.

Assay of Blood Urea Level in Mice——Blood urea level was assayed according to Conway's microdiffusion procedure. The blood specimens were collected from carotid artery into tubes, inner surface of which were covered with thin potassium citrate layer. Urea in 0.3 ml of the blood specimen was hydrolysed with 0.4 ml of 0.05% jack bean meal solution, an excess amount of the enzyme, for 60 min at 38° in Conway's apparatus. Liberated ammonia was assayed in the same way as the case of gastric ammonia assay.

Results

Effects of Urea and Benzohydroxamic Acid on Gastric Ammonia Production

In order to make it clear that gastric ammonia was originated from hydrolysis of urea by urease in gastric mucosa, effects of the substrate and the inhibitor of urease on gastric

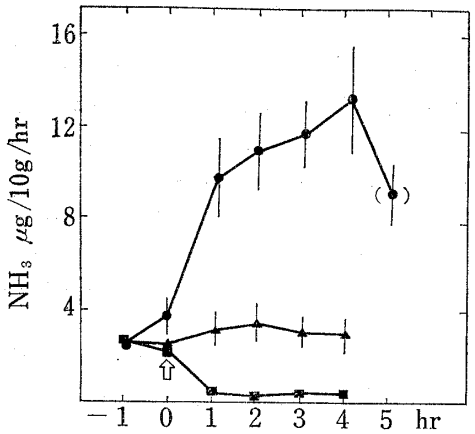

Fig. 1. Effects of Urea and Benzohydroxamic Acid Irrigation on Gastric Ammonia Formation in Mice

The stomachs of urethane anesthesized mice were irrigated with 0.5 ml of the following solutions every 1 hr.
—▲—: 1/100N HCl.
—●—: urea (0.3%) in 1/100N HCl
( ); Urea was eliminated.
—■—: benzohydroxamic acid (0.3%) in 1/100N HCl.
abscissa : time in hr.
At the arrow irrigation of the test solution was started.
ordinate: ammonia content in the perfusate per 10 g of body wieght of mice per hr.
Each point represents the mean value for 10 animals and vertical bars indicate the standard errors.

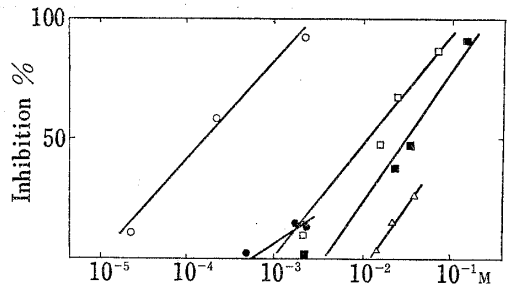

Fig. 2. Inhibitory Effects of Some Drugs Injurious to Gastric Mucosa and Benzohydroxamic Acid on Urease

abscissa : molar concentrations of the test compounds
ordinate: inhibition of urease activity in percent of full activity.
detail of the reaction system is shown in the text.
—○—: benzohydroxamic acid
—●—: phenylbutazone
—□—: chinophen
—■—: aspirin
—△—: caffeine

6) E.J. Conway, "Microdiffusion Analysis and Volumetric Error," 5th ed., Crosby Lockwood & Son Ltd., London, 1962.
7) D.D. Van Slyke and R.M. Archibald, *J. Biol. Chem.*, **154**, 623 (1944).

ammonia production were tested. Basic ammonia production of a mouse stomach was 2.5—3.5 μg/10 g of body weight/hr. During the perfusion of 0.3% urea solution, the ammonia production remarkably increased. An hr after urea application, ammonia in the perfusate increased to 10 μg/10 g/hr and thereafter gradually increased. When urea was eliminated from the perfusate, the ammonia production reduced. After the perfusion of 0.3% benzohydroxamic acid, basic ammonia production was completely inhibited (Fig. 1). In the presence of benzohydroxamic acid, urea perfusion did not cause the increase of ammonia production. Urease activity was not detected in the control perfusates. These results clearly indicate that the ammonia in the perfusate is produced by gastric urease in gastric mucosa.

Effects of Histamine and Atropine on Gastric Ammonia Production

It seemed possible that gastric ammonia might be secreted together with gastric juice. This possibility was examined by stimulating gastric secretion with histamine or by inhibiting it with atropine. Both drugs did not show any remarkable influence on gastric ammonia production (Table I).

Table I. Effects of Histamine and Atropine on Gastric Ammonia Formation

Drugs	Dose (mg/kg)	Gastric ammonia formation (μg/10 g/hr)					
		Before inj.		After inj.			
		1	2	1	2	3	4
Histamine-2HCl	40 *s.c.*	2.7±1.0	3.7±1.2	3.3±1.1	3.3±1.4	4.5±1.0	5.2±1.1
	80 *s.c.*	2.5±0.8	1.9±0.4	2.8±0.5	3.6±1.0	4.6±1.3	4.7±1.2
Atropine-1/2H_2SO_4	10 *s.c.*	2.8±1.0	3.2±0.7	3.0±0.7	3.5±1.1	3.7±1.2	3.9±1.2

No. of animal { histamine, each dose :6; atropine, each dose :5

These results indicate that the gastric ammonia seems to leak out from gastric wall independently of secretory activity of parietal cells.

Effects of Some Drugs Injurious to Gastric Mucosa on Urease *in Vitro* and on Gastric Ammonia Production *in Vivo*

In order to examine the possibility that some drugs are injurious to gastric mucosa because of their urease inhibition, their inhibitory activities on urease were tested *in vitro*. As shown in Fig. 2, all the tested compounds, phenylbutazone, chinophen, aspirin and caffeine, except benzohydroxamic acid, displayed inhibitory activities only in very high concentrations. These results indicate that urease inhibition can not be responsible for gastric mucosa injuring activity of these drugs.

Table II. Effects of Some Gastric Mucosa Damaging Drugs on Gastric Ammonia Formation

Drugs	Dose or concentration	Route of administration	Ammonia formation (μg/10 g/hr)		Change (maximum%)
			Before administration	After administration	
HCl	1/100N	irrigation	2.5±0.5	3.5±0.7	140
Aspirin	0.3%	irrigation	2.3±0.1	3.3±0.6	143
Phenylbutazone	0.3%	irrigation	2.2±0.4	7.8±1.3	355
Caffeine	0.3%	irrigation	5.2±0.8	10.3±2.6	198
Chinophen	0.3%	irrigation	2.9±0.4	6.8±0.9	234
Reserpine	10 mg/kg	*s.c.*	2.3±0.6	3.3±0.7	143

The effects of the drugs on gastric ammonia production *in vivo* were shown in Table II. None of the tested compounds inhibited gastric ammonia production, but, on the contrary, three drugs markedly increased it. Ammonia production was gradually increased after drug perfusion and in 4 or 5 hr it got to the peak response. Phenylbutazone was the most potent and it increased ammonia 3.5 times as much as basic ammonia production. Reserpine and aspirin did not show statistically significant difference to control. Although there were the exceptions of aspirin and reserpine, it was confirmed that some drugs injurious to gastric mucosa increased gastric ammonia production.

The Effect of Eugenol on Gastric Ammonia Production

To elucidate the relation between gastric mucosa injury and gastric ammonia production, the effect of eugenol, a well-known gastric mucosa irritant,[8] on gastric ammonia production was studied.

Eugenol suspension was kept in contact with gastric mucosa for 30 min and washed away thoroughly. After the eugenol treatment, ammonia production was found to increase remarkably (Fig. 3). In the presence of benzohydroxamic acid, however, the ammonia

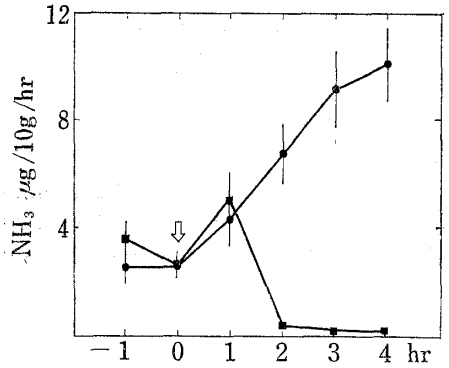

Fig. 3. Effects of Eugenol and Benzohydroxamic Acid on Gastric Ammonia Formation in Mice

Eugenol emulsion (0.3%) was kept in the stomach of a mouse for 30 min. After this treatment, two test solutions were irrigated. Indications of abscissa and ordinate are the same as in Fig. 1. At the arrow, eugenol treatment was started. Each point represents the mean value for 10 animals.
—●—: 1/100N HCl
—■—: benzohydroxamic acid (0.3%) in HCl (1/100N)

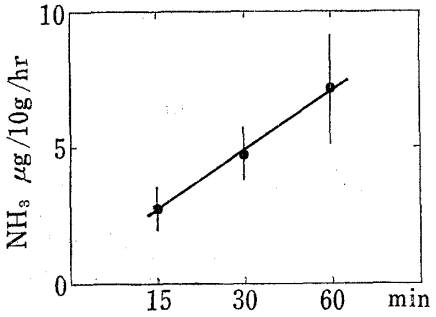

Fig. 4. Relationship between Ammonia Formation and Duration of Eugenol Irrigation

abscissa : duration of eugenol irrigation in min
ordinate: ammonia formation during first an hour after irrigation of eugenol. The unit is the same as that in Fig. 1.

increase after eugenol treatment was not attained. In Fig. 4, the relationship between ammonia formation and the duration of eugenol irrigation was illustrated. In the range of 15 min to 60 min, a linear relationship was shown between them. From these results it was suggested that the injurious effect of eugenol on gastric mucosa resulted in enhancing the gastric ammonia formation by stimulating urea hydrolysis by urease. The increase of ammonia formation by eugenol was not potentiated after additional administration of urea by intravenous injection or by gastric perfusion (Table III).

These results indicate that a considerable amount of urea may exist in gastric mucosa, and that eugenol may promote the enzyme–substrate interaction by destroying some barrier between them. By way of precausion, the relation between blood urea level and ammonia formation was studied under various conditions (Table IV). Blood urea levels did not change so much as ammonia formation, suggesting that they were not the determinant factor of ammonia formation.

8) H.W. Davenport, H.A. Warner and C.F. Code, *Gastroenterology*, 47, 142 (1964).

Table III. Effect of Urea on Gastric Ammonia Formation after Eugenol Treatment

Drugs	No. of animals	Ammonia formation (μg/10 g/hr) Before administration	After administration	Change (maximum%)
Urea (400 mg/kg *i.v.*)	8	2.8±0.8	4.8±0.6	171
Eugenol (2.5%)	12	2.6±0.5	9.0±1.2	346
Eugenol (2.5%)+Urea (0.3% irrigation)	7	2.8±0.6	8.5±1.3	304
Eugenol (2.5%)+Urea (400 mg/kg *i.v.*)	7	4.8±0.9	12.1±2.6	252

Table IV. Relations between Blood Urea Level and Gastric Ammonia Formation

Compounds	No. of animals	Concentration	Route of administration	Blood urea (mg%)	Ammonia formation (μg/10 g/hr)
HCl	6	1/100N	stomach perfusion	91.6± 7.3	2.8
Benzohydroxamic acid	3	0.3%	stomach perfusion	96.6±10.0	0.3
Urea	6	0.3%	stomach perfusion	129.0± 7.5	11.1
Eugenol	6	2.5%	stomach perfusion for 30 min	115.9± 5.8	7.2

Discussion

Therapeutic availability of certain anti-inflammatory drugs is restricted because of their injurious properties to gastric mucosa. Although the untoward effects of many drugs on gastrointestinal mucosa have long been studied, any profitable answer is not obtained. Even the injurious effect of aspirin, which has long history of clinical and foundamental studies, remains undissolved.[9] One of the causes which make it difficult to solve these problems seems to be the complexity of defensive mechanism of gastric mucosa against gastric juice. Among many studies on these problems, ammonia metabolism in gastric mucosa has attracted much interest of researchers in this field.[10] It is believed that gastric ammonia is produced by hydrolysis of urea with urease in gastric mucosa, but physiological significance of gastric ammonia is conflicting. Kornberg[4] denied the role of gastric ammonia in defensive mechanism of gastric mucosa because gastric urease was originated from microorganisms in gastric mucosa. On the other hand, Martinson, *et al.*[3] recently provided the evidences which supported the beneficial action of gastric ammonia.

In the previous paper,[5] we showed that hydroxamic acid derivatives were useful inhibitors of gastric urease in intact mice, and that gastric ammonia formation was closely related to gastric urease activity. It was also shown that repeated administrations of urea stimulated inductive formation of gastric urease in mice. Reserpine-induced gastric ulcers were slightly reduced in the mice, in which urease activity was previously elevated with urea treatment. From these results, gastric ammonia seems to have some significance in protective mechanism of gastric wall, whatever origin the gastric urease has.

In this paper, the relation between gastric ammonia formation and gastric mucosa injury was examined in mice. Gastric ammonia in the perfusate was decreased almost to zero by the irrigation of benzohydroxamic acid and markedly increased by urea (Fig. 1). These

9) D.N. Croft, *J. Pharm. Pharmacol.*, **18**, 354 (1966).
10) C.H. Best and N.B. Taylor, "The physiological Basis of Medical Practice," 6th ed., Williams and Wilkins Co. Baltimore, 1955, p. 522.

facts indicated that gastric ammonia was formed by urease in gastric mucosa. The urease was not derived from the intraluminal microorganisms, because the enzyme reaction between urea and urease might not be able to proceed in the perfusate of 1/100N HCl. Maramaa[11] reported functional change of gastric urease activity which was caused by the stimulation of gastric secretion. In the present experiment, histamine or atropine did not affect on gastric ammonia formation, suggesting that the ammonia was not secreted with gastric juice.

In the early stage of the experiment, we took it for working hypothesis that some drugs gave damage to gastric mucosa by inhibiting gastric urease. This hypothesis, however, proved to be unacceptable because all of the tested compounds showed only very weak urease inhibition (Fig. 2). On the contrary, they were found to increase ammonia formation remarkably (Table II). Aspirin and reserpine were the exceptions of this case. These exceptions might be explained on the smaller solubility of aspirin in 1/100N HCl or on indirect action of reserpine on gastric mucosa.

The increase of gastric ammonia by mucosa irritation with some drugs was clearly confirmed by further experiments with eugenol. Eugenol irrigation remarkably increased gastric ammonia formation when irrigation period of time was elongated. The increase of ammonia production by eugenol was completely inhibited by benzohydroxamic acid perfusion (Fig. 3). These results indicated that the detected ammonia was not discharged from some storing place in gastric mucosa, but formed by accelerated enzyme reaction between urease and urea. The acceleration of the enzyme reaction by eugenol might be effected by damaging some partition wall between the enzyme and the substrate in gastric mucosa. Davenport[8] found that eugenol destructed the gastric mucosal barrier and enabled gastric acid to diffuse back into the tissue. Such an effect of eugenol may take part in increasing gastric ammonia formation. Combined treatment of urea perfusion and eugenol irrigation did not show further increase of ammonia formation. This result indicated that enough amount of endogenous urea became available for urease by eugenol treatment. Blood urea level was not changed by eugenol or urea irrigation so much that they were responsible to the increase of gastric ammonia (Table III).

In conclusion, acceleration of ammonia formation might proceed not for protection of gastric mucosa, but it was effected merely as a result of destruction of the mucosa. The rate of the gastric ammonia formation may be used, from another point of view, as the index of gastric mucosa injury by drugs.

11) S. Maramaa, *Gastroenterology*, **50**, 657 (1966).