

## ISOLATION FROM BEER AND STRUCTURAL DETERMINATION OF A POTENT STIMULANT OF GASTRIN RELEASE

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**Abstract** — Beer was subjected to five successive chromatographic procedures to isolate the gastrin release-inducing activity, guided by bioassay of the fractions in anaesthetized Donryu rats. The procedures were: (1) hydrophobic interaction chromatography (aqueous effluent with an HP20 column); (2) weak cation-exchange chromatography (1 M acetic acid eluate with a CM Sephadex C-25 column); (3) gel filtration (methanol eluate with a Sephadex LH-20 column); (4) same as (2); (5) high-performance liquid chromatography (YMC-Pack ODS-AM with 7% acetonitrile–0.01 M HCl). The active component finally isolated had a specific activity ~10 000 times higher than that of beer. It was identified by means of mass, <sup>1</sup>H- and <sup>13</sup>C-nuclear magnetic resonance spectral analyses as *N*-methyltyramine (NMT). The dose of NMT giving maximal gastrin-releasing activity was 25 µg/kg, and the 50% effective dose was ~10 µg/kg on oral administration to rats. NMT was isolated and identified as a gastrin release inducer in beer. Its concentration in beer is sufficient to account for most of the activity of beer.

### INTRODUCTION

It has long been known that alcoholic beverages stimulate gastric acid secretion. In the 1940s, it was suggested that ethanol itself was responsible (Beazell and Ivy, 1940), but more recent experiments have failed to confirm any effect of ethanol (Cooke, 1972). Nevertheless, controlled studies on the action of pure ethanol and alcoholic beverages on gastric acid secretion and gastrin release in humans have confirmed that beer and wine are potent stimulants of gastric acid secretion and gastrin release (Lenz *et al.*, 1983; Singer *et al.*, 1983, 1987, 1991; Peterson *et al.*, 1986; Chari *et al.*, 1993), and endogenous gastrin is the mediator of the stimulation of gastric acid secretion

(Singer *et al.*, 1987, 1991; Chari *et al.*, 1993). However, the active component(s) in alcoholic beverages has not yet been identified (Teyssen *et al.*, 1993, 1995, 1997). The aim of this study was therefore to isolate and identify the potent stimulant(s) of gastrin release present in beer.

### MATERIALS AND METHODS

#### Purification

Beer [5% (v/v) ethanol, all malt, bottom-fermented, pale-lager type, pH 4.5] was used as starting material. A flow chart for the purification is shown in Scheme 1. The chromatograms obtained at the third to fifth purification steps are shown in Figs 1–3.

*Step 1.* Two litres of beer (80 g dry weight) were degassed, diluted with 4 l of deionized water and subjected to hydrophobic interaction chromatography (HIC) with an HP20 (Mitsubishi Chemical Co. Ltd, Tokyo, Japan) column (7 × 23.5 cm) equilibrated with deionized water. The column was

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washed with 3 l of deionized water, and then eluted with 3 l of methanol. The aqueous effluent and the methanol eluate were each collected.

*Step 2.* The active fraction (the aqueous effluent) from step 1 was subjected to weak cation-exchange chromatography (WCEC) with a CM Sephadex C-25 (Amersham Pharmacia Biotech UK Ltd, Bucks, UK) column (5 × 30 cm) equilibrated with deionized water. The column was washed with 1.5 l of deionized water and eluted with 2.3 l of 1 M acetic acid. The aqueous effluent and the 1 M acetic acid eluate were each collected.

*Step 3.* The active fraction (the 1 M acetic acid eluate) from step 2 was subjected to gel filtration chromatography (GFC) with a Sephadex LH-20 (Amersham Pharmacia Biotech UK Ltd) column (4 × 91 cm) equilibrated with methanol, and eluted with methanol.

*Step 4.* The active fraction (the eluate fractions from  $K_d$  0.65–0.85) from step 3 was subjected to chromatography using weak cation exchange (WCEC) with a CM Sephadex C-25 column (2 × 26 cm) equilibrated with 1 M acetic acid, and eluted with 1 M acetic acid.

*Step 5.* The active fraction (the eluate fractions from 133 to 140 ml) from step 4 was subjected to high-performance liquid chromatography (HPLC). The HPLC column (YMC-Pack ODS-AM, Yamamura Kagaku Co. Ltd, Kyoto, Japan, 1.0 × 30 cm) was equilibrated with 0.01 M HCl, and eluted with 7% acetonitrile–0.01 M HCl.

### Bioassay

Eight- or 9-week-old male Donryu rats (255–320 g body weight, Charles River Japan Co., Yokohama, Japan) were used ( $n = 6$  or  $n = 8$  in each test group). The rats were maintained under controlled conditions of light (12 h light/12 h dark), relative humidity ( $55 \pm 5\%$ ) and temperature ( $23.5 \pm 2.0^\circ\text{C}$ ) for 1 week. Standard feed [CE-2 (CLEA Japan, Inc., Tokyo, Japan)] and tap water were provided *ad libitum*. The rats were starved for 16 h before each test, but allowed free access to water. Each experiment was started at 10:00. The rats were anaesthetized with 25% (w/v) urethane (Nacalai Tesque Co. Ltd, Kyoto, Japan, 5 ml/kg, intraperitoneally) and, after arterial cannulation, were left to recover for 2 h. During each experiment, test samples (5 ml/kg) were administered orally. Blood was collected in tubes at 0 (just before administration) and 15 min, because the increase of the

plasma gastrin level peaked at 10–20 min after administration (data not shown).

Blood was collected in tubes and left to stand for 30 min. The samples were centrifuged at 10 000 r.p.m. at  $4^\circ\text{C}$  for 10 min, and the plasma was frozen at  $-20^\circ\text{C}$ . Plasma gastrin was measured by the use of GASTRIN-RIAKIT II (Dinabot Ltd, Japan).

### Calculations and statistics

Statistical analyses were performed on incremental gastrin release (Fig. 5), which was calculated as follows.

$$\text{Incremental gastrin release (\%)} = \frac{[\text{PGC at 15 min} / \text{PGC at 0 min}] \times 100}{100} - 100$$

where PGC is plasma gastrin concentration (pg/ml).

The differences between the various samples were evaluated by using Student's *t*-test. A *P* < 0.05 was considered significant. Data are presented as means  $\pm$  SEM.

### Preparation of a large quantity of the active component

A sufficient amount of the active component for structural determination was isolated from 12 l of beer, with the previously isolated active component (Fig. 3) as a standard marker, by using the same five purification steps as described above.

The presence of a peak corresponding to the authentic active component was confirmed by HPLC on an analytical YMC-ODS column (4.6 × 250 mm). Analytical conditions were as follows: equilibration buffer, 0.01 M HCl; eluent, 7% acetonitrile–0.01 M HCl; flow rate, 0.5 ml/min; and detection wavelength: UV 254 nm.

### Structural analysis of the active component

Fast atom bombardment (FAB) mass spectrometry was performed on a JEOL HX-110A sector type spectrometer (Tokyo, Japan) at a resolution of 1000. The accelerating voltage was set at 10 kV. A 1- $\mu\text{l}$  aliquot of sample solution in methanol was mixed with glycerol as a matrix on the tip of the probe.

The  $^1\text{H}$ - and  $^{13}\text{C}$ -nuclear magnetic resonance (NMR) spectra were recorded on a JEOL EX-400 spectrometer (Tokyo, Japan) at room temperature in  $\text{CDCl}_3$ . Chemical shift values were referenced to TMS (tetramethylsilane), used as an internal standard.

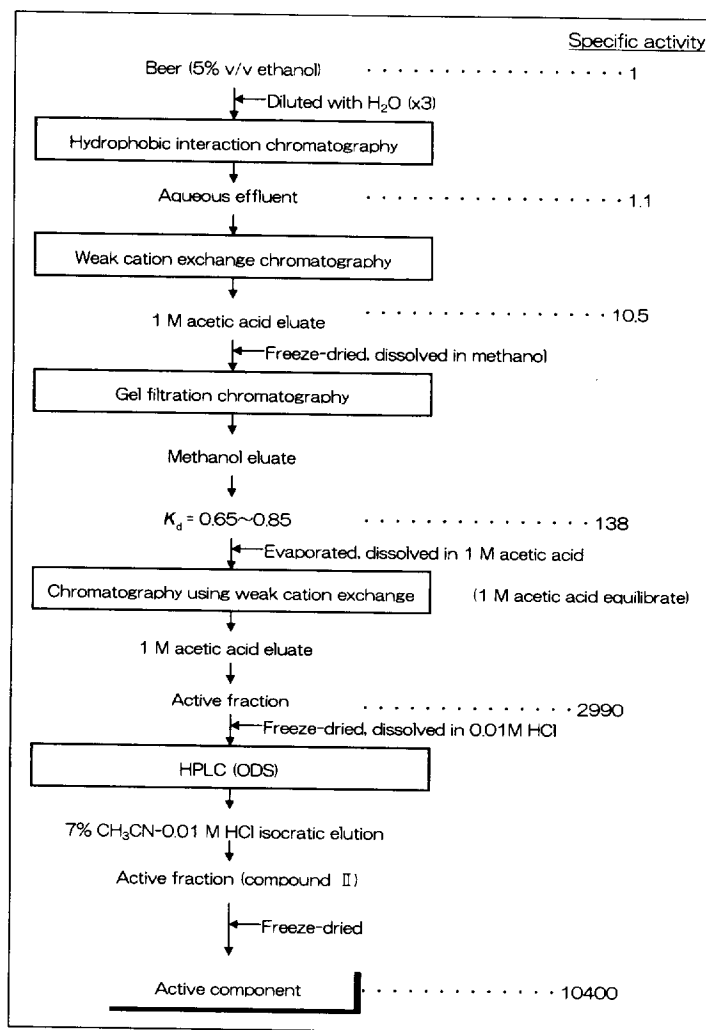
## RESULTS

The basic procedure for purifying the gastrin-releasing activity from beer involved (1) chromatography, followed by freeze-drying of the fractions; (2) activity assay of a part of each freeze-dried fraction dissolved in 5% (v/v) ethanol or distilled water; and (3) selection of fractions showing the most potent activity of gastrin release. The active fractions were pooled and subjected to the next cycle of purification.

## Isolation

The active component was isolated from beer by means of five purification steps shown in the flow chart in Scheme 1.

*Step 1.* The aqueous effluent fraction (64.0 g after freeze-drying) and the methanol eluate fraction (6.0 g after freeze-drying) were obtained from 2 l of beer (80.0 g dry weight). A 1% sample of each fraction was dissolved in 20 ml of 5% (v/v) ethanol to afford a solution of about the same concentration as in beer. The values of the percentage



Scheme 1. Flow chart of purification of the stimulant of gastrin release from beer, and specific activity at each purification step.

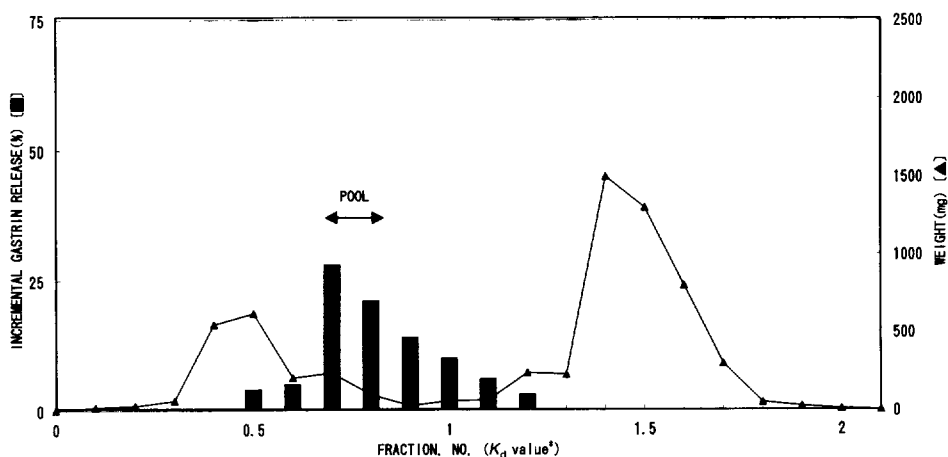


Fig. 1. Result of Sephadex LH-20 gel filtration at step 3 of the purification.

The column was equilibrated and eluted with methanol.  $*K_d = (V_e - V_o)/V_i$ ;  $V_e$  = elution volume of each fraction;  $V_o$  = interstitial volume;  $V_i$  = pore volume. Values of the percentage increment of plasma gastrin release are means of six rats. Values of weight are the dry weight after evaporation of each fraction.

incremental gastrin release (means of six rats) induced by beer, the aqueous effluent fraction and the methanol eluate fraction, were 95, 83 and 5%, respectively. The aqueous effluent fraction was therefore subjected to the next step.

**Step 2.** The aqueous effluent fraction (59.6 g after freeze-drying) and the 1 M acetic acid eluate fraction (2.4 g after freeze-drying) were obtained. A 2% sample of each fraction was dissolved in 20 ml of 5% (v/v) ethanol. The values of percentage incremental gastrin release (means of six rats) of the aqueous effluent fraction and the 1 M acetic acid eluate fraction were 7 and 60%, respectively. Therefore, the 1 M acetic acid eluate fraction was selected for the next step.

**Step 3.** The first and second purification steps were carried out three times to afford 7 g of the 1 M acetic acid eluate fraction mentioned above. This was subjected to gel filtration chromatography on a Sephadex LH-20 column. A 1% sample of each fraction was dissolved in 20 ml of 5% (v/v) ethanol, and the solution was assayed. Activity was observed in the fractions from  $K_d$  0.65 to 0.85 (Fig. 1). The values of the percentage incremental gastrin release (means of six rats) were 28% ( $0.65 < K_d \text{ value} \leq 0.75$ ) and 21% ( $0.75 < K_d \text{ value} \leq 0.85$ ). Therefore, the active fractions of  $K_d$  0.65–0.85 were subjected to the next step.

**Step 4.** A 250 mg aliquot of the above pooled fraction was applied to a CM Sephadex C-25 column. The results are shown in Fig. 2. A 4% sample of each fraction was dissolved in 20 ml of 5% (v/v) ethanol, and activity was detected in the eluates from 126 to 147 ml (from fraction nos. 32–37). The values of the percentage incremental gastrin release (means of six rats) of fraction nos. 34 and 35 were 42 and 58% respectively. Therefore, these fractions were combined for the final purification.

**Step 5.** A 5 mg aliquot of the above pooled fraction was applied to preparative HPLC (Fig. 3). Compounds I–III and the fraction from 0 to 25 min retention time were recovered. A 0.5% sample of each fraction was dissolved in 20 ml of 5% (v/v) ethanol. Compound II showed the same percentage incremental gastrin release as the charged sample. When it was rechromatographed under the same conditions, a single peak was obtained (data not shown). The specific activity of the isolated component for inducing gastrin release was about 10 000 times that of beer (see Scheme 1).

#### *Preparation of a large quantity of the active component*

Using compound II described above as a marker, a larger quantity of compound II was isolated by

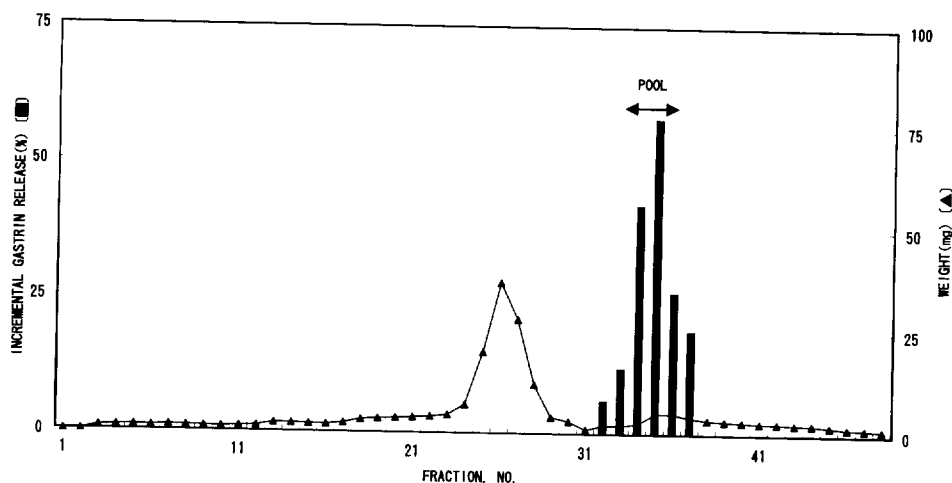


Fig. 2. Result of CM Sephadex C-25 chromatography using weak cation exchange at step 4 of the purification. The column was equilibrated and eluted with 1 M acetic acid. Values of the percentage increment of plasma gastrin release are means of six rats. Values of weight are the dry weight after freeze-drying of each fraction.

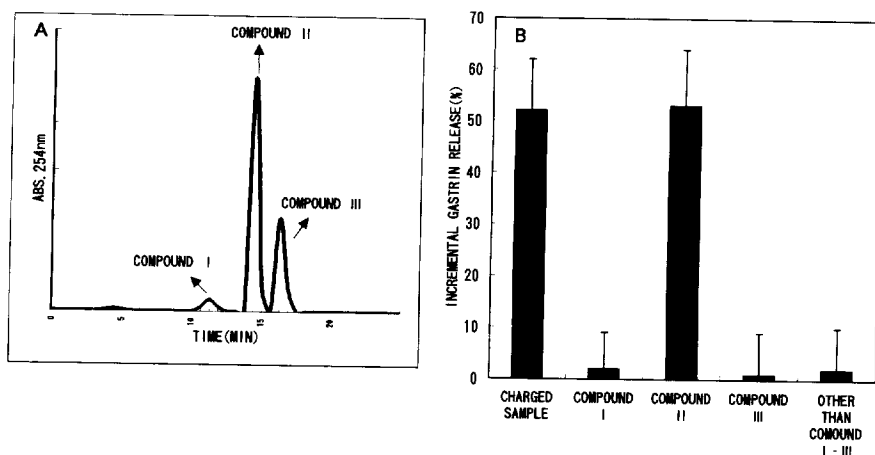


Fig. 3. Result of HPLC at step 5 of the purification.

(A) Conditions: column, YMC-Pack ODS-AM (1 × 30 cm); eluent, 7% acetonitrile–0.01 M HCl; flow rate, 2 ml/min. (B) Percentage increment of plasma gastrin release of each fraction. Values are presented as means ± SEM obtained from six rats. ABS = absorbance.

means of the same procedure from 12 l of beer. Its purity was confirmed by HPLC. The yield of compound II was 5.6 mg.

#### Determination of the structure of compound II

The structural determination of compound II was carried out by FAB mass spectrometry and

NMR spectroscopy. The FAB mass spectrum (glycerol matrix) showed the molecular ion peak (M+H)<sup>+</sup> at m/z 152. The high-resolution FAB mass spectrum indicated a molecular formula of C<sub>9</sub>H<sub>14</sub>ON, m/z 152.1079 [(M+H)<sup>+</sup>, calc. 152.1057].

The <sup>1</sup>H-NMR spectrum was obtained at 400 MHz. In the <sup>1</sup>H-NMR spectrum of compound II, two

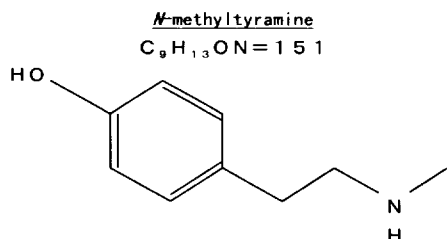


Fig. 4. Chemical structure of compound II.

doublet signals at  $\delta 6.92$  and  $7.23$  with an AB-type coupling pattern were assigned to a *para*-hydroxybenzene proton. The methylene triplet signal at  $\delta 3.30$  was coupled with the methylene triplet signal of the benzyl position at  $\delta 2.97$ . The singlet signal at  $\delta 2.73$  was assigned to an *N*-methyl moiety.

The  $^{13}\text{C}$ -NMR spectrum showed seven carbon signals, which were assigned with the aid of the DEPT (distortionless enhancement by polarization transfer) spectrum. The signals at  $\delta 152.4$  and  $125.9$  were assigned to non-protonated aromatic carbons, and the signals at  $\delta 128.0$  and  $113.6$  to protonated aromatic carbons. The two carbon signals at  $\delta 48.2$  and  $28.7$  were assigned to  $\alpha$ -amino methylene and

benzyl methylene carbons, respectively. The signal at  $\delta 30.7$  was assigned to *N*-methyl carbon.

These results led us to conclude that compound II is *N*-methyltyramine (NMT) (Fig. 4).

#### Chemical synthesis of NMT

NMT was chemically synthesized and crystallized from 2-(4-methoxyphenyl) ethylamine as previously described (Kirkwood and Marion, 1950). Its purity was confirmed by HPLC analysis on a Nucleosil-ODS column ( $4.6 \times 9250$  mm).

#### Confirmation of the activity

The dose-response curve of NMT was sigmoidal, as shown in Fig. 5. Each dosage of NMT was dissolved in 5% (v/v) ethanol. Five per cent (v/v) ethanol, and NMT at concentrations of  $0.25$  and  $2.5$   $\mu\text{g/kg}$ , caused small increases ( $\sim 10$ – $20\%$ ) in gastrin release, as compared with the basal level; none of these changes was statistically significant. NMT at concentrations of  $6.25$ ,  $12.5$  and  $25$   $\mu\text{g/kg}$  caused increases in gastrin release with apparent dose-dependence, and the maximal gastrin-releasing activity of NMT ( $\sim 90\%$  increase) was obtained at  $25$   $\mu\text{g/kg}$ . The increases were statistically significant ( $P < 0.05$ ) at concentrations of  $12.5$ ,  $25$ ,  $250$  and  $2500$   $\mu\text{g/kg}$ . The maximal gastrin-releasing activity of NMT corresponded to that of beer ( $92.0\% \pm 17.0$ ), and addition of NMT

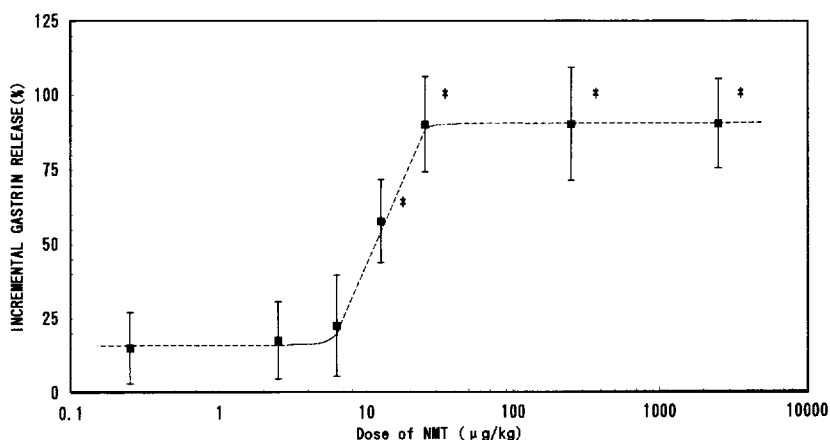


Fig. 5. Dose-response curve of *N*-methyltyramine (NMT).

Chemically synthesized NMT was dissolved at the desired concentration in 5% (v/v) ethanol and the pH was adjusted to pH 4.5 by addition of an appropriate amount of HCl. Each value is the mean  $\pm$  SEM obtained from eight rats. The values of the percentage increment of plasma gastrin release of 5% (v/v) ethanol and beer were  $14.5\% \pm 11.2$  and  $92.0\% \pm 17.0$ , respectively. \* $P < 0.05$  vs 5% (v/v) ethanol.

to beer (2.5 µg of NMT/ml of beer) did not alter the stimulatory effect of beer on gastrin release ( $89\% \pm 10$ ) (data not shown). The 50% effective dose of NMT was calculated to be  $\sim 10$  µg/kg.

## DISCUSSION

The important findings in this study are: (1) the stimulant of gastrin release was isolated from beer and identified as *N*-methyltyramine (NMT); (2) NMT is a potent stimulant, because the maximal gastrin-releasing activity of NMT was obtained at a low dosage (25 µg/kg), and the 50% effective dose of NMT was  $\sim 10$  µg/kg; (3) the amount of NMT in beer could account for most of the gastrin release-inducing activity of beer.

Various attempts have been made to identify the ingredient(s) in beer and wine responsible for the stimulatory effect on gastric acid secretion and gastrin release (Lenz *et al.*, 1983; Singer *et al.*, 1983, 1987, 1991; Peterson *et al.*, 1986; Chari *et al.*, 1993; Teyssen *et al.*, 1993, 1995, 1997). Singer *et al.* (1991) investigated the known ingredients in beer, including putrescine, tyramine and histamine, and found no significant effect of any of them on gastric acid secretion and gastrin release in humans. Teyssen *et al.* (1993) examined stimulants of gastric acid secretion from fermented glucose, and found them to be thermostable, polar, anionic and of low molecular weight ( $<700$ ). The thermostability, polar character and low molecular weight are consistent with the properties of NMT. However, NMT has a weak cationic character at acidic pH. The reason for this apparent discrepancy is not clear. Beer and wine contain varying amounts of a wide range of amines (Maga, 1978), and few studies have been carried out on the stimulatory effect of non-volatile amines on gastric acid secretion and gastrin release (Lichtenberger *et al.*, 1982a,b), though the importance of amines in beer and wine has been discussed by Peterson *et al.* (1986). The present study is the first to show that the non-volatile amine NMT induces gastrin release.

Distilled spirits (e.g. whisky and cognac) have been shown to have no stimulatory effect on gastric acid secretion and gastrin release (Singer *et al.*, 1983, 1987; Teyssen *et al.*, 1995). Because NMT is non-volatile, distilled spirits may not contain it.

Beer is produced from barley extract by alcoholic fermentation using yeast. Singer *et al.* (1991) have

shown that barley extract (both first and finished wort) has some acid secretion-stimulatory action, and that alcoholic fermentation is the most important event for producing the stimulatory substances in beer. NMT has been isolated from some strains of barley (Kirkwood and Marion, 1950), and biogenic amines have been detected in worts and beers (Izquierdo-Pulido *et al.*, 1993, 1994). Further, significant changes in the formation of some amines occur during alcoholic fermentation (Izquierdo-Pulido *et al.*, 1994). We therefore suggest that barley contains certain amounts of NMT or other amines, that these amines are extracted into the worts, and that the subsequent alcoholic fermentation produces more NMT or other amines.

To investigate the structure-activity relationship, we compared the gastrin-releasing activity of NMT, tyramine and *N,N*-dimethyltyramine (the values of percentage increment of plasma gastrin release at the concentration of 83 nmol/kg were  $57.6\% \pm 17.0$ ,  $24.2\% \pm 15.6$  and  $60.0\% \pm 19.3$ , respectively). These results indicated that the methyl group of NMT plays an important role in the activity.

However, the precise mechanism(s) by which NMT induces gastrin release and gastric acid secretion remains to be established.

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