Isolation of a Peptide Material Showing Strong Rectal Muscle-Contracting Activity from Chicken Rectum and Its Identification as Chicken Neurotensin

Seiichi KOMORI, Takashi FUKUTOME and Hidenori OHASHI

Laboratory of Pharmacology, Department of Veterinary Medicine, Faculty of Agriculture, Gifu University, Gifu 501-11, Japan

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Abstract—A peptide with strong activity to contract isolated whole chick rectum, RC-substance, in an acid-acetone extract prepared from 2.0 kg wet weight of chicken rectums was isolated by gel filtration on Sephadex G-25, ion exchange chromatography on a SP-Sephadex C-25 column, and high voltage paper chromatography. The close purity of this substance was established by high performance liquid chromatography (HPLC). The RC-substance displayed the same pharmacological and enzymatic properties as bovine neurotensin (NT). However, it differed from bovine NT in its behavior during the ion exchange chromatography, in its mobility during the electrophoresis, and in its retention time on HPLC. The amino acid analysis by a conventional OPA method revealed that all ten amino acid residues of which molar ratios were integral were common with those of chicken NT. Its biological activities were equipotent to those of bovine NT, suggesting the presence of proline in the COOH-terminal hexapeptide. From these results, it can be concluded that the RC-substance is identical to chicken NT. Exposure to high concentrations of bovine NT rendered the rectal smooth muscle of the chicken insensitive to subsequent applications of the RC-substance as well as bovine NT. This desensitization also reduced specifically the contractile responses of the rectal muscle elicited by non-adrenergic and non-cholinergic (NANC) nerve stimulation, suggesting a possible physiological role of the RC-substance as the neurotransmitter of NANC neurones.

Avian rectal smooth muscle receives a powerful excitatory nervous input arising from the sacral outflow. The excitatory nerve reaches the rectum via the Remak nerve, a unique ganglionated nerve trunk of fowls, and in these ganglia, it has synaptic transmission. The post ganglionic neurone is non-adrenergic and non-cholinergic (NANC) in nature (1–5). The transmitter released from axon terminals of NANC neurones has not been identified yet.

To explore the NANC neurotransmitter, substances which exert a contractile effect on isolated whole chick rectum (WCR) have been extracted from the rectum and Remak nerve of the chicken. The rectum and Remak nerve contain axon terminals and cell bodies of NANC neurones, respectively. Two substances have been already obtained at the stage of partial purification (6–8). On the basis of their chemical and pharmacological properties, one would be a peptide of substance P (SP)-family, which probably share an amino acid sequence required to elicit the biological activity with SP. However, the SP-like substance isolated from chicken rectum has only a low potency in contracting WCR, as does SP. The other substance is also of peptide nature, and it has a potency in contracting WCR 1000 times or so higher than the SP-like peptide. This led us to suspect that the latter peptide is one of the most promising candidates for the neurotransmitter for NANC excitatory neurones.
The substance which has a strong WCR-contracting activity (RC-substance) has been shown to have characteristics that it causes relaxation of the longitudinal muscle of the guinea-pig ileum (GPI), it loses biological activity after incubation with chymotrypsin or carboxypeptidase A, but not with pepsin, and it has a molecular size of roughly 1600 (7, 8). Among naturally-occurring peptides, bovine neurotensin (NT) was found to show the same properties as those of RC-substance.

In the present study, RC-substance prepared from chicken rectum was subjected to further purification, and its highly purified preparation was analyzed to determine whether it is identical with bovine NT and also to determine if it is involved in the responses induced by stimulation of NANC nerves in the chicken rectum.

Materials and Methods

Tissue samples: The rectal region of the intestine was removed from the chicken within 20 min after killing at a slaughter house (a meat product company, Minokashiwa), washed out with ice-cold Tyrode solution to which indomethacin (7.5 μg/ml) was added, collected in a thermos containing the same solution, and brought to the laboratory. The tissues of a total wet weight of 2 kg were prepared for extraction as previously described (8).

Extraction of tissue: The tissue samples were minced to a fine gruel and stirred for 2 hr at room temperature with 10 l of acetone-1 M HCl (100:3, v/v). The extract was filtered under suction and the solid residue was re-extracted for 30 min with 4 l of acetone-0.01 M HCl (80:20, v/v). The filtrates from these procedures were combined (about 20 l), from which acetone and some lipids were removed by extracting 3 times with petroleum ether (3.5 l for each) and then by evaporating down to the aqueous phase (2.7 l) under reduced pressure at 45°C. The aqueous extract was lyophilized.

The lyophilized residue was dissolved in 900 ml of 0.1 M acetic acid and centrifuged at 10,000×g at 4°C for 20 min. The methanol phase which contained all of the active material was evaporated down to dryness under reduced pressure at 60°C. The dried material was dissolved in 1000 ml of distilled water and adjusted to pH 3.0 with 3 M acetic acid. Gel of SP-Sephadex C-25 (10 g), previously swollen in 0.1 M acetic acid, was added to the acidic solution to absorb active substances, stirred for 2 hr at room temperature, and then the gel was filtered under suction. The gel was washed 3 times with a total 200 ml of 0.05 M pyridine/acetate, pH 5.5, and then eluted with 200 ml of 3 M pyridine/acetate, pH 5.5. The eluate was lyophilized and stored at -20°C until used for gel filtration (referred to as the crude extract).

Gel filtration on Sephadex G-25: The crude extract was dissolved in 50 ml of 0.1 M acetic acid and applied to a column of Sephadex G-25 (medium) (4.5×95 cm) equilibrated with 0.1 M acetic acid at room temperature. Elution was made with the same acid solution at a flow rate of 180 ml/hr, and the column eluate was collected in 30 ml-fractions. Every other fraction was tested for activity to contract WCR by injecting a 0.02 ml aliquot of each fraction into the organ bath in which WCR was immersed. All fractions of the eluate region in which the biological activity was detected were pooled (about 300 ml).

To determine molecular sizes of peptides, a smaller column of Sephadex G-25 (1.2×55 cm) was employed, and elution was performed at a flow rate of 21.4 ml/hr. The elution positions for the applied peptides were determined by bioassay using WCR or by measuring UV absorption at 280 nm.

The column eluate was always monitored by measuring UV absorption at 280 nm. The columns were calibrated with blue dextran.

Cation exchange chromatography on SP Sephadex C-25: The pooled active solution after gel filtration was adjusted to pH 3.0 with 3 M acetic acid, and it was applied to a column of SP Sephadex C-25 (0.8×12 cm) equilibrated with 0.1 M acetic acid at room temperature. The column was washed with 40 ml of 0.05 M pyridine/acetate (pH 5.5) and then eluted with a linear gradient molarity...
which was generated using 375 ml of 0.05 M pyridine/acetate (pH 5.5) in the mixing chamber and 375 ml of 2.0 M pyridine/acetate (pH 5.5) in the reservoir. The eluate was collected at a flow rate of 40 ml/hr in 5 ml fractions. A 0.5 ml aliquot of every other fraction was lyophilized to remove the pyridine/acetate, dissolved in 0.5 ml distilled water, and then the WCR-contracting activity was bioassayed.

When synthetic bovine NT was subjected to cation exchange chromatography, the peptide (50 μg) dissolved in 0.1 M acetic acid was applied to the same column as used for the extracted material, and its elution position was surveyed by bioassay using WCR.

**High voltage paper electrophoresis:** The active region of the eluate from the SP Sephadex C-25 column was concentrated by lyophilization and reconstituted with a small volume of the buffer solution and used for high voltage paper electrophoresis of the immersed strip type. The sample solution was applied to a band of Whatman No. 3 MM paper (9 cm in width and 60 cm in length), which had been washed with 20% pyridine overnight and then 20% acetic acid for 2 weeks. Part of the sample solution was spotted on a guide staining paper with a reference amino acid. Electrophoresis was performed at pH 1.9 at 80 volts/cm for 60 min. After electrophoresis, the former paper was cut crosswisely into 1.0 cm strips, and each strip was eluted with 2 ml of 10% acetic acid in a test tube overnight. Aliquots of 0.5 ml of the solution in each test tube were lyophilized, dissolved in 0.1 ml distilled water, and bioassayed for the WCR-contracting activity. The guide staining paper was stained with 0.2% ninhydrin acetone to detect the applied active peptide and reference amino acid.

To compare electrophoretic mobilities of the extracted peptide and bovine NT, two buffers at different pHs, 1.9 and 3.4, were used and electrophoresis was performed under the same conditions as described above. The positions of the substances recovered from the paper were determined by bioassay for the WCR-contracting activity.

**Amino acid analysis:** Amino acid analysis of the RC-substance obtained after electrophoresis was done at the Center for Instrumental Analysis of Hokkaido University. Samples of the RC-substance were hydrolyzed with HCl in evacuated and sealed tubes at 110°C for 24 hr. Spectral analyses were performed by a conventional o-phthalaldehyde method on an amino acid analyzer (Hitachi, 835).

**Measurement of NANC contractile responses:** The whole rectum was removed together with Remak’s nerve and set up in a 10 ml organ bath filled with Tyrode’s solution, kept at 32°C and bubbled with air. The anal end of Remak’s nerve trunk was placed in a bipolar suction electrode and the rectum region into a pair of ring electrodes for field stimulation. For stimulation of the extrinsic and intrinsic nerves, trains for 5 sec of rectangular pulses of 0.5 msec were delivered at various frequencies at supramaximal intensity (Nihon Kohden, MSE-3). Longitudinal changes in tension of the rectum in response to nerve stimulation in the presence...
of atropine (0.4 µg/ml), pyrilamine (1 µg/ml), methysergide (1 µg/ml), phentolamine (1 µg/ml) and propranolol (0.3 µg/ml) were measured isometrically by a force-displacement transducer (Nihon Kohden, SB-1T) and recorded.

Results

Purification: A peptide with WCR-contracting activity, RC-substance, in a crude extract prepared from 2.0 kg wet weight of chicken rectums was isolated by gel filtration, ion exchange chromatography, high voltage paper chromatography and HPLC. The RC-substance at any step in the purification was expressed as an amount of bovine NT exerting a matched contracting activity in WCR.

The crude extract was first subjected to a batch absorption method. It was confirmed by bioassay for the WCR-contracting activity that almost the entire amount of the active substance was recovered. By gel filtration on Sephadex G-25, most of the active substance appeared in the elution volume ranging from 1.3 times to 2.1 times the void volume. The substance at this step in purification, when applied to GPI, elicited a biphasic response that consisted of a small, rapid relaxation followed by a large contraction. The amount of RC-substance in pooled active fractions was estimated to be about 300 µg equivalents of bovine NT.

The solution of pooled active fractions was chromatographed on a SP Sephadex C-25 column, and the profile plotting WCR-contracting activity is shown in Fig. 1. The fractions with the WCR-contracting activity displayed only activity to relax GPI and the elution positions for GPI-relaxing activity and for WCR-contracting activity overlapped each other. Therefore, the ion exchange chromatography effected separation of RC-substance from a substance with GPI-contracting activity, presumably a SP-like peptide (7, 8). At this step in the purification, RC-substance was about 210 µg equivalents of bovine NT.

RC-substance obtained after the ion exchange chromatography was then sub-

![Fig. 1](image-url). Ion exchange chromatography of excitatory material on SP-Sephadex C-25. Active fractions of the eluate after gel filtration of an extract of chicken rectum on Sephadex G-25 were pooled, and this solution was applied to the SP Sephadex C-25 column (0.8×12 cm). Elution was performed with a 750 ml linear gradient concentration of pyridine/acetate (pH 5.5) ranging from 0.05 to 2.0 M. Eluate was collected in 5 ml fractions. Aliquots of each fraction were lyophilized, then each residue was dissolved in distilled water, and the contracting activity was determined using WCR, in the presence of atropine (0.4 µg/ml), pyrilamine (1 µg/ml) and methysergide (1 µg/ml). Ordinate: the contracting activity expressed as an amount of bovine NT that exerts the same effect on WCR as the fraction. The peak elution position for bovine NT, when chromatographed under the same conditions, is indicated by the arrow (↓, NT).
jected to high voltage paper electrophoresis at pH 1.9. Part of the RC-substance corresponding to 15% of the total activity (about 30 μg equivalents of bovine NT) was used for spotting on a guide staining paper. As shown in Fig. 2, RC-substance, which displayed contracting activity for WCR and relaxing activity for GPI, was recovered from only one contiguous region of the paper corresponding to the ninhydrin spot on the guide strip. At this stage, the amount of RC-substance was approximately 110 μg equivalents of bovine NT.

HPLC was employed as the final step in its purification. Figure 3 shows a typical chromatogram of RC-substance of 30 μg equivalents of bovine NT obtained after the high voltage paper electrophoresis. It can be seen that the plot of the biological activity determined using WCR shows a single peak which is associated with the main peak of optical density at 220 nm. The active fractions had again an inhibitory effect on GPI. Fractions corresponding to minor peaks of the optical density caused neither contraction of WCR nor relaxation of GPI (Fig. 3). The recovery rate of RC-substance after HPLC was about 60%.

As HPLC revealed that after the high voltage paper electrophoresis, RC-substance was highly purified, this highly purified preparation was used for the following experiments to compare it with bovine NT, unless otherwise stated.

**Pharmacological properties:** Highly purified RC-substance, like bovine NT, caused contractions of WCR, guinea-pig rectum, rat rectum and rat stomach strip, but elicited relaxation of GPI, in the presence of atropine (0.4 μg/ml), pyrilamine (1 μg/ml) and methysergide (1 μg/ml), as shown in Fig. 4. Furthermore, the responses to both substances observed in each preparation showed close similarities in time course and duration. The contractile responses of rat rectum to the RC-substance and bovine NT were rapid in onset, reached their maximal tension, and then faded even in their continuous presence: The time taken from their onset to termination was only several seconds (Figs. 4 and 6). The excitation and inhibitory effects of these substances on the smooth muscle preparations remained unaltered after application of tetrodotoxin (0.4 μg/ml), indicating their direct actions on the smooth muscle cells. Both substances also exhibited a similar pattern of dose-response curves for the excitatory action on WCR (Fig. 5). The con-

![Fig. 2. High voltage paper electrophoresis of excitatory material. Part of the material (about 180 μg equivalents of bovine NT), obtained after the cation exchange chromatography and originating from the extract in Fig. 1, was applied to the paper (Whatman No. 3 MM, 9×60 cm), and electrophoresis was performed at 80 V/cm for 60 min in formic acid-acetic acid-water (20:80:900, pH 1.9) buffer. After electrophoresis, the paper was cut crosswisely into strips (1 cm in width), then each strip was eluted with 10% acetic acid (2 ml), and the contracting activity was measured using WCR. Ordinate: the contracting activity expressed as in Fig. 1. Abscissa: distance of migration toward the cathode. Also indicated are the positions for the remainder of the material (RC-S) (about 30 μg equivalents of bovine NT) and for serine (Ser), stained with ninhydrin in the guide paper strip.](image-url)
centrations to produce maximal response were 10 arbitrary units for RC-substance (one unit is about equi-potent to 10 ng/ml of bovine NT) and 80 ng/ml for bovine NT, and at concentrations higher than these, a gradual decrease in the magnitude of responses

Fig. 3. High pressure liquid chromatography of highly purified excitatory material. Excitatory material (about 30 μg equivalents of bovine NT), obtained after the high voltage paper electrophoresis in Fig. 2, was chromatographed on a TSK column (ODS-120T type, 0.46×25 cm), and elution was performed with 25% CH₃CN at 1 ml/min. Fractions of 30 sec were collected and lyophilized, then each residue was dissolved in distilled water, and the contracting activity was bioassayed using WCR. Upper trace: the optical density profile at 220 nm, when the sample was injected in 50 μl buffer. Lower trace: the plot showing the WCR-contracting activity expressed as in Fig. 1. Note the main peak of OD₂₂₀ coeluting with the WCR-contracting activity.

Fig. 4. Effects of RC-substance (RC-S) and bovine NT (NT) on five different smooth muscle preparations pretreated with a combination of atropine (0.4 μg/ml), pyrilamine (1 μg/ml) and methysergide (1 μg/ml). RC-S, obtained after the high voltage paper electrophoresis and originating from the extract in Fig. 1, was used. Calibration of tension (vertical bars) indicates 0.25 g. A: WCR, B: GPI, C: isolated rat rectum, D: stomach strip of the rat, E: isolated guinea-pig rectum. Bovine NT: 4 ng/ml in A and B and 8 ng/ml in C, D and E. RC-S: in an amount producing a matched response to the bovine NT-induced one in WCR.
When WCR was exposed to bovine NT in a relatively high concentration (40 ng/ml to 2 μg/ml) continuously or repeatedly at a short interval (less than 3 min), desensitization resulted as manifested by a loss of the obtainable response to subsequent applications of bovine NT (Fig. 6). The desensitization of the preparation to bovine NT resulted in abolition of responses to RC-substance, but left contractile responses to carbamylcholine, histamine, ATP and substance P almost unchanged. Similar results were observed on other smooth muscle preparations (Fig. 6). These findings suggest that the response to RC-substance may be mediated by receptors for bovine NT.

Susceptibility to enzymes: The highly purified RC-substance was used for testing its susceptibility to proteolytic enzymes. The WCR-contracting activity and GPI-relaxing activity of RC-substance were lost after incubation with chymotrypsin or carboxypeptidase A, but survived after incubation with pepsin, confirming the results obtained with crude samples (7, 8). Similar susceptibilities to the enzymes were obtained with bovine NT.

Chromatographic and electrophoretic behaviors: The results are summarized in Table 1. When highly purified RC-substance and bovine NT were chromatographed on Sephadex G-25, similar profiles were obtained, and the ratios of the elution volumes for both substances to the void volume were 1.6, suggesting that RC-substance has a molecular size similar to that of bovine NT (1673).

In the course of purification, RC-substance, when subjected to ion exchange chromatography on SP-Sephadex C-25, eluted at a column buffer molarity of 1.0 M (see Fig. 1). However, bovine NT, when chromatographed on the same column under the same conditions as used for the extract, eluted before RC-substance at a lower molarity of 0.43 M, as indicated by the arrow in Fig. 1.

When RC-substance and bovine NT were applied in parallel to the paper for high voltage paper electrophoresis and electrophoresis was performed at two different pHs (1.9 and 3.4), again these substances did not display equal mobility, as shown in Fig. 7.

Figure 8 shows the optical density profiles for RC-substance and bovine NT on HPLC. In this case, retention times were 8.8 min for RC-substance and 13.3 min for bovine NT. Retention times for the two substances varied slightly from one experiment to another. However, the ratio in retention time between
these two substances was fairly constant with a value of 0.66±0.01 (mean±S.D., n=5).

**Amino acid composition of RC-substance:**

The amino acid composition of RC-substance is presented in Table 2 with those of bovine

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**Fig. 6.** Effects of desensitization of NT receptors on responses to RC-substance. RC-substance, at the same step in purification as used in Fig. 4, was used. Upper panel: contractile responses of WCR to ATP (ATP □, 0.8 μg/ml), bovine NT (NT ○, 8 ng/ml) and RC-substance (RC-S ●, equivalent to 8 ng/ml bovine NT). Middle panel: contractile responses of the isolated rat rectum to bovine NT (NT ○, 80 ng/ml) and RC-substance (RC-S ●, equivalent to bovine NT 80 ng/ml). Bottom panel: relaxations produced by bovine NT (NT ○, 4 ng/ml) and RC-substance (RC-S ●, equivalent to 4 ng/ml bovine NT) in GPI precontracted by substance P (SP □, 4–8 ng/ml). The arrows (NT □) show continuous presence of bovine NT (2 μg/ml in upper and bottom panels, 8 ng/ml in middle panel) used for desensitization. Last pairs in each panel: responses obtained 30 min in the upper panel and 5 min in middle and bottom panels after removal of bovine NT used for desensitization. All the experiments were performed in the presence of atropine (0.4 μg/ml), pyrilamine (1 μg/ml) and methysergide (1 μg/ml).
NT and chicken NT, isolated from chicken small intestines by Carraway and Bhatnagar (9), for comparison. As a conventional o-phthalaldehyde (OPA) method was used because of the scarcity of the purified material, proline was not detected. All ten different amino acid residues of which molar ratios were integral were common with those of chicken NT, and three of them did not share with bovine NT. Other residues such as threonine, serine, glycine, methionine and phenylalanine were present in very low concentrations, being likely to be contaminants.

**Effect of bovine NT on nerve mediated responses of the isolated chicken rectum:** Bovine NT at a concentration of 2 \( \mu \text{g/ml} \) produced a large contraction of the isolated chicken rectum, but its maximal tension was not maintained and subsided to the initial tension or to the level of a slightly increased tension even in the continuous presence of this peptide. Desensitization of this preparation to bovine NT resulted in a marked inhibition of NANC responses elicited by field stimulation of intramural nerves or stimulation of Remak’s nerve trunk (stimulation of NANC nerves at a preganglionic site) (Fig. 9). After removal of the bovine NT, the responses were partially restored.

### Table 1. Chromatographic and electrophoretic behaviors of RC-substance and bovine neurotensin (NT)

| System                          | Property                        | RC-substance | Bovine NT |
|--------------------------------|---------------------------------|--------------|-----------|
| Sephadex G-25                   | \( V_e/V_0 \)                    | 1.60         | 1.61      |
| SP Sephadex C-25                | Elution molarity at pH 5.5       | 1.00         | 0.43      |
| Paper electrophoresis           | Relative mobility               |              |           |
| pH 1.9                         | Mser                            | 1.29         | 1.01      |
| pH 3.4                         | Mlys                            | 0.50         | 0.40      |
| High pressure liquid chromatography (TSK, ODS-1201 type) | Retention time at 25% | 8.8          | 13.3      |

\( V_e/V_0 \): the ratio of elution volumes of the samples to void volume. Mser: relative mobility to serine. Mlys: relative mobility to l-lysine.

Fig. 7. Electrophoretic mobilities of RC-substance and bovine NT. RC-substance, obtained after the high voltage paper electrophoresis, was used. RC-substance and bovine NT were applied to the Whatman No. 3 MM paper (12 \times 60 cm), and electrophoresis was performed at 80 V/cm for 60 min in formic acid-acetic acid-water buffer (20:80:900, pH 1.9) or in pyridine-acetic acid-water buffer (4:40:760, pH 3.4). The plots show the WCR-contracting activities of RC-substance expressed as in Fig. 1. The peak positions for bovine NT are shown by arrows (NT ↓). Abscissa: electrophoretic mobility relative to serine (pH 1.9) and to l-lysine (pH 3.4). The migration distances of serine and l-lysine were 44 cm and 37 cm, respectively.
A peptide, RC-substance, which has strong activity to contract WCR was isolated from an acid-acetone extract of chicken rectums. The substance would belong to a family of NT-like peptides since it displayed biological activities similar to those of bovine NT (10). The COOH-terminal hexapeptide of bovine NT, Arg-Arg-Pro-Tyr-Ile-Leu, has been shown to be essential for eliciting its pharmacological actions in vivo and in vitro (11). Therefore, one would suspect that the RC-substance also has the same COOH-terminal sequence. However, it differed from bovine NT in its behavior during the ion exchange chromatography and in its electrophoretic mobility during the high voltage paper chromatography. A possible reason for the differences may be dissimilarities in the amino acid composition, e.g., presence of amino acid residues which make the RC-substance more basic than bovine NT under the conditions used. The molecular size of about 1600 of the RC-substance estimated by gel filtration (8) suggests that the number of amino acid residues is close to that of bovine NT, a tridecapeptide. To test this, part of the RC-substance was submitted to analysis of amino acid composition.

The amino acid composition of the RC-substance agreed well with chicken NT recently isolated from chicken small intestines.

**Table 2.** Molar ratios of amino acids in RC-substance obtained after high pressure liquid chromatography

| Amino acids | Molar ratios | Nearest integer | Bovine neurotensin | Chicken neurotensin |
|-------------|--------------|-----------------|--------------------|---------------------|
| Asp         | 0.97         | 1               | 1                  | 1                   |
| Glu         | 1.06         | 1               | 2                  | 1                   |
| Ala         | 0.83         | 1               | 0                  | 1                   |
| Val         | 0.97         | 1               | 0                  | 1                   |
| Ile         | 0.98         | 1               | 0                  | 1                   |
| Leu         | 1.99         | 2               | 1                  | 2                   |
| Tyr         | 1.00         | 1               | 2                  | 1                   |
| Lys         | 1.03         | 1               | 1                  | 1                   |
| His         | 0.96         | 1               | 0                  | 1                   |
| Arg         | 2.05         | 2               | 2                  | 2                   |
| Pro         | *            | *               | 2                  | 1                   |

Total 12+* 13 13

The OPA method by which proline is not detected (*) was used for the present amino acid analysis. *: Data cited from the paper by Carraway and Bhatnagar (9).
for which the amino acid sequence was determined to be Glu-Leu-His-Val-Asn-Lys-Ala-Arg-Arg-Pro-Tyr-Ile-Leu-OH (9, 12). In the present experiments, the OPA method by which prolyl residues are not detected was used for the amino acid analysis of the peptide because of the scarcity of the purified preparation. Since the observed strong bovine NT-like pharmacological actions of the peptide could not be elicited unless it shared the COOH-terminal hexapeptide with bovine NT (11), it is highly probable that Pro is present in its amino acid residues. In addition, chicken NT appears to fulfill all the similarities and disimilarities to bovine NT inferred from the observed physicochemical properties of the extracted peptide. Therefore, it can be concluded that the RC-substance is identical to chicken NT.

It has been reported that the corresponding extracts from Remak nerve of the chicken contain a substance which has a high potency to contract WCR (7), and its partially purified preparation displays pharmacological and chemical properties similar to those of the RC-substance. Furthermore, on HPLC, the substance origining from Remak nerve showed the same retention time as the RC-substance (Komori, Fukutome and Ohashi, unpublished observations), indicating that they are identical to each other. Thus, the peptide, chicken NT, is also present in the nerve of Remak.

The ability of bovine NT to specifically inactivate NT receptors on the rectal muscle of the chicken is a useful property of the peptide until specific antagonists of NT become available. This property was used in an attempt to see whether the RC-substance is involved in the NANC responses in the chicken rectum. In the isolated chicken rectum with Remak nerve supplies, nerve stimulation elicited contractions which were usually followed by relaxation in the presence of atropine, pyrilamine, methysergide, phentolamine and propranolol. Desensitization of the preparation to bovine NT resulted in a marked inhibition of the contractile responses to stimulation of NANC excitatory nerves, but left the relaxations probably induced by stimulation of NANC inhibitory nerves (1) almost unchanged. This indicates that the high concentration of bovine NT has no effects on excitability, conduction of impulses, and release of a neurotransmitter in nerve fibers. The COOH-terminal hexapeptide has been reported to function as its binding site to the specific receptor (9, 13, 14) as well as its crucial structure for displaying its biological actions (11, 15). The profound inhibition of nerve-mediated NANC excitatory responses of the chicken rectum is due to desensitization of NT receptors, and it could be taken as evidence for release of a NT-like substance, like chicken NT, following nerve stimulation. This observation together with the potent pharmacological action of the
RC-substance (chicken NT) on the rectal smooth muscle and its distribution in the Remak nerve and rectum in which cell bodies and axon terminals of NANC neurones are located (16) favors the view that it functions as the neurotransmitter of NANC neurones in the chicken rectum. Recently, in the guinea-pig ileum, NT has been proposed to function as a NANC inhibitory neurotransmitter (17). The most important experiment in the efforts to establish NT as a neurotransmitter would be usage of specific antagonists to the peptide. However, they are not available yet.

The distribution and cellular localization of NT-like peptides in the avian gastrointestinal tract have been studied by immunohistochemistry. Many works are concerned with cells with NT-like immunoreactivity. The immunoreactive cells are numerous in the mucosal region throughout the intestine (18–20). However, only few reports are available on the presence of the immunoreactive nerve fibers, and immunohistochemical surveys of such nerve fibers have not necessarily been successful (18, 21). None of the NT immunoreactive nerve fibers could be observed in the gut of the chicken by Sundler et al. (18). On the other hand, Saffrey et al. (21) reported that the nerve fibers occur only in the wall of the upper part of the digestive tract (down to the gizzard) in the chicken. In mammals, the results concerning this subject are also contradictory to each other (in rat stomach, duodenum and caecum; 18, 22) (in guinea-pig ileum; 22, 23). Under these circumstances, lack of immunohistochemical demonstration of the nerve fibers in the chicken rectum could not necessarily contraindicate the possible role of NT as the NANC neurotransmitter in the organ. NT-like peptides located in neurones might not be demonstrable by immunohistochemical techniques, presumably because of their lower concentrations than those in endocrine cells.

Present observations also indicate that bovine NT and chicken NT are powerful stimulants of WCR. Furthermore, it has been already demonstrated that WCR is much less sensitive to many naturally-occurring peptides such as substance P, physalaemin, kassinin, eledoisin, bradykinin, angiotensin II and opioid compounds: their concentrations required to produce a detectable effect on WCR range from $10^{-7}$ to $10^{-5}$ g/ml and are two to four orders higher than that of bovine NT (8). This selectivity of WCR provides a highly sensitive and specific preparation that can be employed for bioassay for NT-like peptides.

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