Elevation of Plasma Neurotensinlike Immunoreactivity after a Meal

CHARACTERIZATION OF THE ELEVATED COMPONENTS

ROBERT A. HAMMER, ROBERT E. CARRAWAY, and SUSAN E. LEEMAN, *Departments of Physiology and Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01605*

ABSTRACT The detection of an elevation in neurotensinlike immunoreactivity in peripheral plasma for several hours after a meal has been confirmed and shown to be primarily due to the presence of amino-terminal fragments of neurotensin (NT) rather than to NT itself. We have developed a procedure to separate and characterize these N-terminal cross-reacting substances, and to estimate the contributions of these constitutents to plasma neurotensinlike immunoreactivity. Gel chromatography of pooled plasma extracts on Sephadex G-25 followed by reverse-phase high pressure liquid chromatography indicated that peptides coeluting with NT and its N-terminal partial sequences NT(1–8) and NT(1–11) were present in plasma. Comparison of plasmas collected before and 1 h after a defined meal, in five experiments, demonstrated no change in C-terminal immunoreactivity and an 8- to 10-fold rise in N-terminal immunoreactivity. Chromatographic analysis of pooled pre- and postmeal plasma in four experiments showed that essentially all of this elevation in neurotensinlike immunoreactivity measured with an N-terminal directed antiserum was due to increases in NT(1–8) and NT(1–11), while NT itself, measured using a C-terminal directed antiserum, did not increase appreciably in peripheral plasma 1 h after the meal. Generation of tritiated substances with the same elution times as NT(1–8) and NT(1–11) did occur after incubation of [^{3}H]NT with whole blood in vitro, providing supporting evidence that these fragments are metabolites of NT. The marked elevation in the circulating levels of these fragments reflects that an increased secretion of NT occurred in response to the test meal. The secreted NT may have acted as a hormone before it was metabolized, or it may only have had a local (paracrine) effect.

INTRODUCTION

The tridecapeptide neurotensin (NT),[1] which has been isolated from bovine hypothalamus (1) and bovine as well as human small intestine (2, 3), has been localized to a population of endocrinelike cells in the distal small intestinal epithelium of many avian and mammalian species, including man (4–7). Plasma neurotensinlike immunoreactivity (NTLI) is composed of several immunoreactive substances, including NT, that cross-react with N-terminal and C-terminal region-specific antisera (8). As demonstrated by gel chromatography of extracted bovine plasma, NT comprises about one-third of the C-terminal (antiserum HC-8) and one-half the N-terminal (antiserum TG-1) immunoreactivity present in the extracts. Using other N-terminal directed antisera, an arteriovenous gradient of NTLI has been demonstrated across the distal small intestine of anesthetized dogs (9) and elevations of the NTLI level in peripheral plasma have been measured for at least 3 h after ingestion of a large meal (10–12), but analysis of the immunoreactive components of this NTLI has not been reported.

This rise in plasma NTLI (measured with N-terminal directed antisera) after a meal has been inter-

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Dr. Hammer is the recipient of Clinical Investigator Award K08 AM00532. His present address (to which all correspondence should be sent) is Gastroenterology Section, 1420 Wesley Pavilion, Northwestern University School of Medicine, Chicago, IL 60611.

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[1] *Abbreviations used in this paper*: buffer A, 0.01 M KH_2PO_4, pH 4.6; HPLC, high pressure liquid chromatography; NT, neurotensin; NTLI, neurotensinlike immunoreactivity; RIA, radioimmunoassay.

preted by some investigators as supporting a hormonal role for NT or one of its metabolites as an enterogastrone (13, 14). Whether this elevated immunoreactivity includes any biologically active material is not known. Thus far, all of the biological activities of NT that have been studied with synthetic partial sequences, including receptor binding (15, 16), contractility of intestinal (17) and vascular (18) smooth muscle, and induction of hyperglycemia (17), depend highly upon functional groups located within the C-terminal half of the molecule. To assess the biological activity of the components of the elevated NTLI, it is necessary to establish their identity. Therefore, we have used both N- and C-terminal directed antisera toward NT in combination with high pressure liquid chromatography (HPLC) to characterize several of the components of NTLI in peripheral plasma, and to compare the concentrations of these components before and after ingestion of a defined meal.

METHODS

Experimental design. Nine healthy volunteers, age 22–50, participated in these studies after giving their informed, written consent. Subjects fasted for 14 h before the meal, which was eaten at 12 noon. Basal blood samples of 15 ml were drawn from an antecubital vein into chilled heparin-containing Vacutainer tubes just before each subject ate, and sometimes 15 min before that. Samples were kept at 4°C while they were spun in a clinical centrifuge for 5 min. Plasma was separated and added to 4 vol of acetone/1N HCl, 100:3. Extracts were centrifuged at 36,000 *g* for 20 min in a refrigerated centrifuge, the supernatants decanted and extracted two times with petroleum ether. After the ether phase was discarded and residual acetone evaporated, the samples were lyophilized. This procedure results in an 80% recovery of added NT (8). Timed postmeal samples of 15 or 30 ml were extracted in the same way. In some experiments, 200 ml of blood was obtained from each subject 1 h after the meal and collected in heparin-treated evacuated 1-liter bottles. Final heparin concentration was 25 U/ml; samples took 5–10 min to be collected, and were kept on ice. The 1-h time was chosen for careful study because our results (Table I) and those of Rosell (11) indicated that plasma NTLI was elevated by that time and remained at a plateau for several hours.

The meal consisted of an omelet with three large eggs and 28 g cheddar cheese, cooked in 28 g butter and served with 180 ml whole milk, 240 ml orange juice, and toast (46 g) with butter (21 g). This was consumed in ~10 min. Coffee was intentionally avoided. The composition of the meal was 37 g protein, 59 g carbohydrate, and 76 g fat, totalling 1,050 calories, and was designed to approximate the composition of meals previously reported to induce elevations of plasma NTLI (10–12).

Radioimmunoassay (RIA) procedure. RIA for NT has been previously described (19). The C-terminal directed antiserum, HC-8, can detect 2–3 fmol NT (10% displacement of trace) and requires 12–15 fmol for 50% displacement. Interassay coefficient of variation was 13% (19). The cross-reactivity of HC-8 with partial sequences was 100% with NT(1–13), the entire molecule; 2% with NT(1–12); 2% with NT(9–13); 50% with NT(8–13); and 100% with NT(6–13) or longer C-terminal partial sequences. The N-terminal directed antiserum, TG-1, can detect 3–4 fmol NT and requires 25–30 fmol for 50% displacement. Interassay coefficient of variation was 9%. The cross-reactivity of TG-1 with partial sequences was as follows: NT(1–13), 100%; NT(1–12), 120%; NT(1–11), 60%; NT(1–10), 50%; NT(1–8), 18%; NT(8–13) or shorter C-terminal partial sequences, 0.01%.

To prepare samples for RIA, aliquots of extracts or column fractions were lyophilized and dissolved in buffer (0.15 M NaCl, 0.05 M NaH_2PO_4 and Na_2HPO_4, pH 7.4, 0.1% gelatin, 0.02% NaN_3). To accurately assay the amounts of peptide present in fractions obtained after HPLC of small volumes of plasma (15–50 ml before extraction), the lyophilized fractions were assayed without transfer from their collection tubes, for a single determination on each fraction and no loss of peptide. Measurements of 5 fmol/fraction were considered reliable.

Chromatographic procedures. Samples were dissolved in the minimum volume of distilled water, adjusted to pH 7, and applied in <2% of bed volume to columns of Sephadex G-25. The columns were equilibrated and run in 0.2 M acetic acid, with flow rates <7 ml/h per cm^2 cross-sectional area.

The lyophilized pooled fractions collected after gel chromatography were dissolved in distilled water and loaded in 0.5–1.5 ml in a Waters U6K injector (Waters Associates, Inc., Milford, MA), then applied to a Waters μBondapak C18 column, 3.9 × 300 mm, run at a flow rate of 2 ml/min. Absorbance at 210 nm was monitored with a model 450 variable wavelength detector (Waters Associates Inc.), and fractions of 0.4 ml (0.2 min) were collected in new 13 × 100-mm glass tubes on a Gilson microfractionator (Gilson Co., Inc., Worthington, OH). Initially, separations were performed using 20% acetonitrile in buffer A (0.01 M KH_2PO_4, pH 4.6).

Development of a satisfactory HPLC gradient. Since the initial composition of the mobile phase did not resolve the components of NTLI with retention times close to that of standard NT(1–11) on the HPLC column, material from this region (Fig. 2B) was pooled, redissolved in distilled water, and injected while the column was being eluted with buffer A. A linear gradient elution program (0–30% acetonitrile in buffer A) was only partially successful; standard NT(1–11) was resolved well, but NT(1–8) and NT(1–10) eluted at the same time as did one of the components of NTLI. Therefore, fractions from the region containing this component were pooled, lyophilized, redissolved, and analyzed using a gradient protocol that was found to be satisfactory to separate all the available immunoreactive N-terminal partial sequences of NT. This protocol consisted of injection of sample while the column was being eluted with buffer A, followed after 2.5 min by gradient number 3 to 15% acetonitrile in 20 min, 2 min at 15% acetonitrile, and gradient number 6 to 30% acetonitrile in 3 min. For these gradient runs, fractions were collected for 0.5 min each. Coinjections of portions of these pooled samples with partial sequences of NT were performed to determine whether any of the endogenous material coeluted with NT(1–8), NT(1–10), or NT(1–11).

Generation of fragments in vitro. Generation of fragments during the usual procedure for the preparation of samples was tested by the addition of ~100 fmol [^{3}H]NT to a sample of whole blood, followed by analysis of the extracted plasma by HPLC. The generation of metabolites of NT in vitro was studied by the addition of ~100 fmol [^{3}H]NT (~10,000 cpm) to 15-ml portions of whole blood that were incubated at 37°C for 10–60 min. The plasma obtained from each blood sample was extracted and applied directly to HPLC using the protocol just described. Half of each fraction

collected after HPLC was used for scintillation counting, and the rest was assayed.

Direct application of plasma extracts to HPLC. A concentrated extract of 102 ml of plasma collected 1 h postmeal was divided into three portions and applied to the HPLC column alone, together with NT(1–8), and together with NT(1–11). Comparison of the amounts of the components of NTLI present in the plasma of fasting and fed (1 h postmeal) subjects was performed by direct application of concentrated pooled extracts of ~30 ml plasma to the HPLC column. Before injection of any samples each day, two blank injections and elutions were performed to check that no UV-absorbent or immunoreactive material was contaminating the injector or eluting from the column. Standards were injected as the last run of each day, and identified by RIA as well as by absorbance at 210 nm. Recovery of peptides in fractions from HPLC was 75% for NT(1–8), 95% for NT(1–10) and NT(1–11), and 99% for NT(1–12) and NT.

Generation of partial sequences of synthetic NT. For the preparation of partial sequences of NT for use as standards on HPLC, 100 nmol of synthetic NT (Beckman Instruments, Inc., Fullerton, CA) were incubated with a 1:100 molar ratio of carboxypeptidase A (Worthington Biochemical Corp., Freehold, NJ):NT at 37°C in 500 μl of 1% NH_4HCO_3, pH 8.3, for 10 min. One drop of glacial acetic acid was added to stop digestion and the sample was injected directly onto HPLC at 10% acetonitrile in buffer A. After 7.5 min, a 20 min gradient was run (curve 6) to 30% acetonitrile. In this system, the NT(1–10) fragment eluted at 12.5 min, NT(1–11) at 15.5 min, NT(1–12) at 19 min, and NT at 21.5 min. Amino acid analysis confirmed the identity and purity of the two fragments obtained, NT(1–10) and NT(1–11). Synthetic NT(1–12) standard was the gift of Dr. P. Kitabgi (Institut National de la Santé et de la Recherche Medicale, Nice, France). NT(1–8) and NT(9–13) were prepared by trypsin digestion of synthetic NT, as previously described (20). Tritiated NT was prepared by Beckman and repurified on HPLC. It had a specific activity of 100 Ci/mmol (measured at 49% efficiency in a Packard Tri-Carb liquid scintillation counter, Packard Instrument Co., Inc., Downers Grove, IL), and was stored at −70°C in 50% methanol.

TABLE I
Plasma NTLI Measured in Five Volunteers after Ingestion of a High-Fat Meal

Time	NTLI* TG-1	NTLI* HC-8
	fmol/ml	
Basal	2.8±1.3‡	24.2±2.3
Basal	3.5±2.5	19.7±3.8
	Meal	
45 min.	22.8±4.9‡	25.8±1.0‡
1.5 h	39.2±6.3	26.3±2.7
3 h	33.8±4.9	24.2±1.8

Blood was collected before and, at the times indicated, after a test meal (Methods). Plasma from each sample was extracted, lyophilized, and dissolved in a volume of assay buffer equal to one-fifth the original volume of plasma. Aliquots of 200 μl were assayed in duplicate with each antiserum. No nonspecific binding of NT trace occurred in aliquots incubated without added antiserum.

* Data represent mean±SEM.

‡ Only four samples.

RESULTS

Measurements of plasma NTLI with a C-terminal directed antiserum (HC-8) did not change from basal levels, while N-terminal immunoreactivity (TG-1) rose 10-fold after ingestion of a test meal (Table I).

Analysis of a pool of all the postmeal samples by gel filtration and HPLC (using 20% acetonitrile in buffer A) demonstrated the presence of NT and another peak of N-terminal immunoreactive material that eluted earlier from the reverse-phase column than NT or the NT(1–12) fragment (data not shown).

Chromatographic characterization of NTLI in postmeal plasma. A large volume of plasma was obtained 1 h after a test meal in order to extract sufficient material for sequential analysis. Mean (±SEM, $n = 8$) plasma NTLI (HC-8) was 17.3±2.3 fmol/ml, and NTLI (TG-1) 35.4±3.0 fmol/ml in these subjects. Several peaks of C-terminal immunoreactivity (HC-8) were detected upon gel chromatography (Fig. 1), one of which eluted in the region of [^{3}H]NT (region I). N-

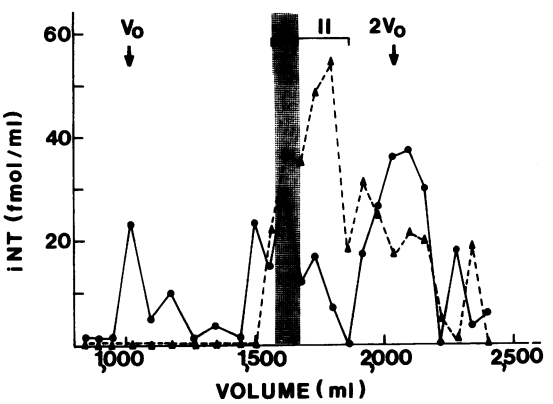

FIGURE 1 Gel permeation chromatography on Sephadex G-25 of an extract of 740 ml pooled plasma collected 1 h after a test meal. Plasma from eight subjects was extracted, and the lyophilized extracts were dissolved in 50 ml distilled water, adjusted to pH 7 with NaOH, and applied to a 2.12-liter (4 × 170 cm) column of gel, which was then eluted with 0.2 M acetic acid at a flow rate of 75 ml/h. After 500 ml were discarded, fractions of 20 ml were collected and aliquots of 250 μl were lyophilized and assayed in duplicate with antisera TG-1 (▲ - - - ▲) and HC-8 (● —— ●). The shaded area indicates the elution region of synthetic NT containing trace amounts of [^{3}H]NT run on the column the previous day. Fractions from regions I and II were pooled separately for subsequent HPLC. V_0 and $2V_0$ indicate multiples of the void volume of the gel bed.

Terminal immunoreactive material (TG-1) had a broad elution pattern including the region of [^{3}H]NT and the region immediately following (region II).

Material that coeluted with [^{3}H]NT after HPLC was recovered from both region I (Fig. 2A) and region II (Fig. 2B). This material was essentially equally reactive with the C-terminal (HC-8) and N-terminal (TG-1) antisera; it comprised all of the C-terminal and half the N-terminal immunoreactivity from region I, and was a minor component of region II.

Other N-terminal immunoreactive material(s), with a retention time between 2 and 4 min, was also recovered after HPLC; about one-third of this material had been in region I (Fig. 2A), and two-thirds in region II (Fig. 2B). Further characterization of this N-terminal immunoreactive material was performed because NT(1–8) and NT(1–10) could not be distinguished from NT(1–11) using the protocol in Fig. 2 (data not shown). Results obtained during the development of an HPLC gradient indicated that the region between 2 and 4 min on Fig. 2B contained two immunoreactive substances, one which coeluted with NT(1–11) and one which coeluted with NT(1–8) (data not shown). Confirmation of this result was obtained by comparison of portions of another postmeal plasma extract applied directly to the HPLC column alone and in combination with partial sequences of synthetic NT (Fig. 3).

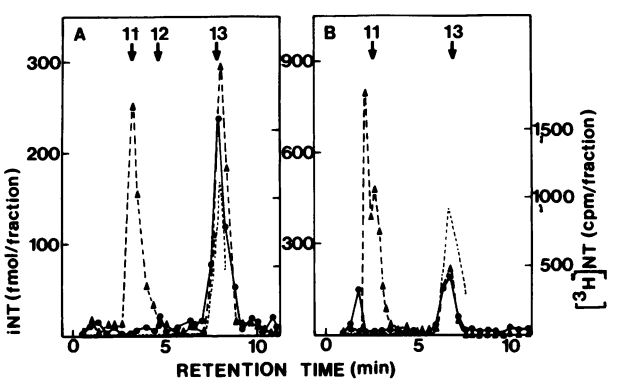

FIGURE 2 HPLC of material pooled after gel chromatography. A. Lyophilized material from region I of Fig. 1 was dissolved in 1 ml of distilled water, a trace amount of [^{3}H]NT was added, 800 μl were injected on the column, and fractions were collected as described in Methods. Column buffer was 20% acetonitrile in buffer A. Aliquots of 50 μl were assayed in duplicate with antisera TG-1 (▲ - - - ▲) and HC-8 (● —— ●); 100 μl of every other fraction was dissolved in Aquasol for determination of ^{3}H activity (• • • • •). B. Lyophilized material from region II of Fig. 1 was dissolved in 1 ml of distilled water, a trace amount of [^{3}H]NT was added, and 450 μl were injected on the column. The same column buffer and assay procedures were used as for A. Note the difference in scale of the ordinates. The peak retention times of standards NT(1–11), NT(1–12), and NT itself (13 residues) are indicated by arrows at the top of each panel.

Generation of NT metabolites in vitro. 80% of the radiolabel added to whole blood as [^{3}H]NT was recovered in extracts of plasma after the usual procedure for the preparation of samples. [^{3}H]NT was the only labeled substance detectable in these extracts after analysis by HPLC (Fig. 4A). Recovery of labeled material in extracts of plasma after incubation of [^{3}H]NT in whole blood for 60 min was only 50–60%. Also, only 50% of the radioactivity in such an extract injected onto HPLC could be recovered in the fractions that were collected. The distribution of labeled material that was recovered in one such experiment, representative of two others, demonstrated that 28% of ^{3}H activity corresponded to NT(1–8), 24% to NT(1–11) or NT(9–13), and 47% to NT (Fig. 4B). Similar analysis of extracts prepared from blood incubated with [^{3}H]NT for 10 min demonstrated that 95% of recovered radioactivity corresponded to NT, and only 5% to labeled fragments of NT.

Evaluation of components of NTLI after a meal. Plasma extracts collected before and 1 h after subjects ate a test meal were assayed (Table II) and analyzed by direct application of extracts to HPLC. There was no change in C-terminal immunoreactivity (antiserum HC-8), but a mean ninefold rise in N-terminal immunoreactivity (antiserum TG-1). In a typical experiment (Fig. 5), immunoreactive material corresponding to NT(1–8) rose from 2 to 5 fmol/ml (11–28 fmol of peptide/ml), that corresponding to NT(1–11) rose from 1 to 5 fmol/ml (2–9 fmol of peptide/ml), and that corresponding to NT rose from 5 to 7 fmol/ml original plasma.

DISCUSSION

The results reported here confirm the observation that NTLI measured with an N-terminal directed antiserum increased markedly in the peripheral plasma for several hours after a large meal. Evidence is presented that this elevation of NTLI represents the NT(1–8) and NT(1–11) metabolites of NT, and reflects the fact that NT was released in response to the meal, although levels of NT in peripheral plasma were not elevated.

The results presented here and previously (8) demonstrate the heterogeneity of plasma NTLI and the inability to measure NT accurately in unfractionated plasma using either an N- or C-terminal antiserum. An accurate measurement of NT in plasma requires either a two-site RIA (specific exclusively for NT) or a chromatographic fractionation such as used in this study. The latter approach allows the measurement of metabolites and variants of NT in the same sample, and was utilized here to establish that N-terminal

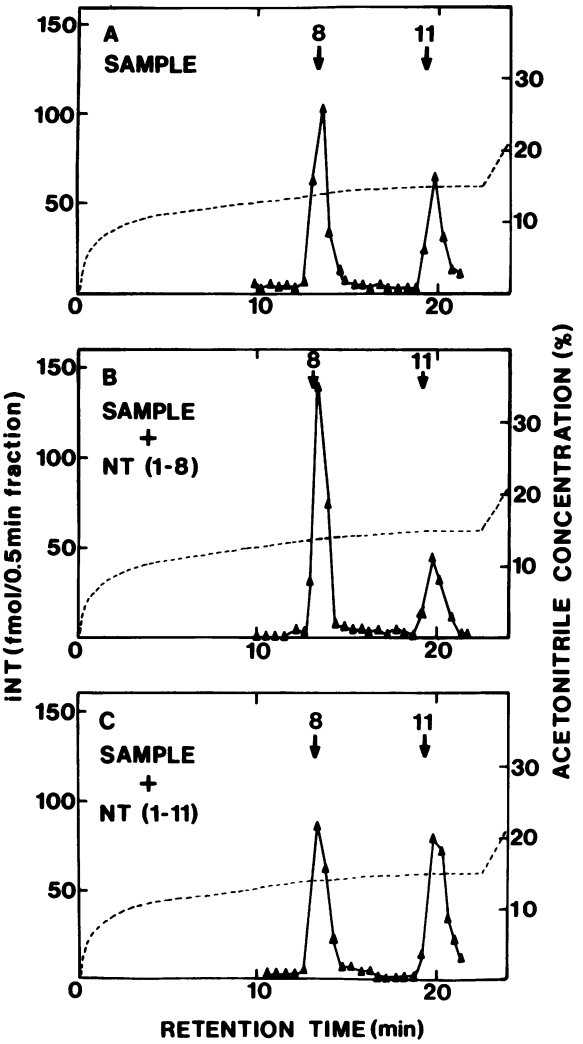

FIGURE 3 Demonstration that the components of NTLI in plasma collected after a test meal coelute on HPLC with partial sequences of NT. A. The lyophilized extract of 102 ml of plasma was reconstituted in 5 ml distilled water. A portion (1,250 μl) of this sample was analyzed with the satisfactory gradient program described in the text. Fractions collected between 10 and 22 min after injection were assayed with antiserum TG-1 (▲ —— ▲). The amount of immunoreactive material corresponding to NT(1–8) was 200 fmol, and the amount corresponding to NT(1–11) was 155 fmol. B. Another portion (900 μl) of the same sample was injected together with 1 pmol of NT(1–8) diluted in 100 μl buffer A. RIA of this dilution confirmed that 200 fmol of immunoreactive standard was injected with the sample. Elution protocol and processing of fractions were identical to those for panel A. The amount of immunoreactive material corresponding to NT(1–8) was 255 fmol (amount expected from sample alone: 135 fmol), and the amount corresponding to NT(1–11) was 105 fmol. C. Another portion (1,120 μl) of the same sample was injected together with 350 fmoles of NT(1–11) diluted in 20 μl buffer A. RIA of this dilution confirmed that 200 fmol of immunoreactive standard were injected with the sample. Elution protocol and processing of fractions were identical to those for panel A. The amount of material corresponding to NT(1–8) was 180 fmol, to NT(1–11), 225 fmol (amount expected from sample alone: 140 fmol). The peak retention times of standards NT(1–8) and NT(1–11) are indicated by arrows. Percent acetonitrile concentration, (– – –).

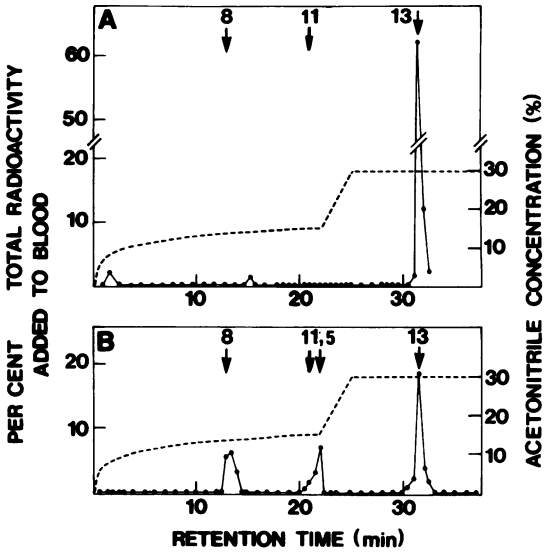

FIGURE 4 Distribution and recovery of radioactivity by HPLC after addition of [^{3}H]NT standard to samples of whole blood collected 1 h after a meal. (A) Blood centrifuged and extracted immediately (Methods) (8,000 cpm added to 2.5 ml blood). 80% of the ^{3}H activity was recovered in the extract. (B) Blood incubated at 37°C for 60 min before being spun and extracted (30,000 cpm added to 5 ml blood). 56% of the ^{3}H activity was recovered in the extract. Both extracts were applied to HPLC and the ^{3}H activity in each fraction was counted and expressed in relationship to the amount of ^{3}H activity added to the original sample. The peak retention times of standard NT(1–8), NT(1–11), NT(9–13) (5, the C-terminal pentapeptide), and NT are indicated by arrows. Percent total radioactivity added to blood, (——); percent acetonitrile concentration, (– – –).

metabolites of NT comprise 72–84% of the identified components of NTLI (TG-1) in peripheral plasma in both the fasting and postprandial states. The variants of NT detected with a C-terminal directed antiserum have previously been shown to comprise two-thirds of the NTLI (HC-8) present in peripheral plasma (8). These substances, which resemble the C-terminal region of NT, have not been characterized chromatographically yet. Although the data presented here did not demonstrate an increase in either NT itself or in C-terminal immunoreactivity (HC-8) in peripheral plasma, such an elevation may have occurred in the portal circulation (21) or in the peripheral circulation at times other than those at which samples were collected.

TABLE II
Comparison of NTLI Levels in Plasma Collected before and 1 h after a Test Meal

No. of subjects	NTLI*			
	TG-1		HC-8	
	Fasting	Postmeal	Fasting	Postmeal
	fmol/ml			
3	2.0±0.9	11.9±1.4	9.5±0.8	10.0±2.1
3	ND	23.1±2.8	8.5±1.1	13.9±0.6
4	1.6±0.6	17.6±2.8	13.8±0.9	14.7±1.4
4	2.0±1.1	18.2±2.9	14.0±1.1	15.1±0.9
3	4.0±1.2	12.2±1.4	8.6±1.2	7.6±1.6
22	2.0±0.4‡	18.1±1.5‡	13.0±1.1‡	14.8±1.2‡

Measurements were made with both N-terminal (TG-1) and C-terminal (HC-8) directed antisera. Treatment of samples was the same as that described in the legend to Table I, except that 300-μl aliquots (the equivalent of 1.5 ml plasma) were assayed. ND, nondetectable.

* Data represent mean±SEM.

‡ Data represent means of all individual values, including data from subjects in Table I (postmeal samples collected at 45 min).

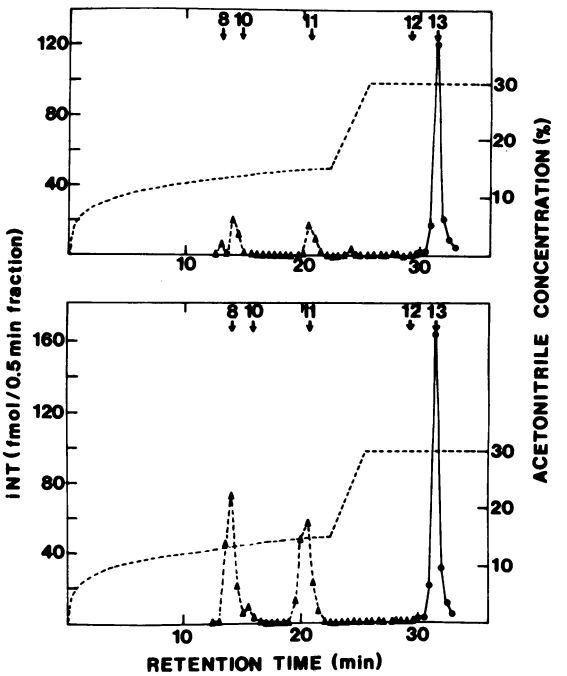

FIGURE 5 Comparison of the components of NTLI present in plasma during a fast and 1 h after a test meal. Top panel: The extracts from 31 ml of plasma obtained from four fasting subjects were dissolved in 1 ml of HPLC buffer and analyzed as described in Methods (by the final HPLC program). Bottom panel: The same volume of plasma was obtained 1 h after the meal, and processed the same way as the sample collected during the fast. Fractions from the region where partial sequences of synthetic NT eluted (10–30 min retention time) were assayed with antiserum TG-1 (▲ - - - ▲), and fractions in the NT region were assayed with antiserum HC-8 (● —— ●). Retention times for the partial sequences of synthetic NT are indicated as described in the legends to Figs. 2 and 3. Estimates of the amount of each immunoreactive component present in the extracts were calculated by dividing the area under each peak by 31 ml, and expressed as femtomoles of immunoreactive peptide per milliliter of plasma. Percent acetonitrile concentration, (- - -).

Since it was possible to demonstrate an increase in N-terminal NTLI (TG-1) after a meal (Tables I and II), chromatographic analysis was designed to separate the other components of this NTLI from NT and to determine which components contributed to the postmeal increase. Blackburn and Bloom (10) also examined the immunoreactive components of plasma before and after a test meal using an N-terminal directed antiserum; they found a major peak of material eluting from Sephadex G-50 in the region where NT eluted. They demonstrated that this material made up the entire elevation of plasma NTLI after the meal. It is likely that this material was actually a combination of the substances that we have resolved using HPLC, since a comparison of Figs. 2A and 2B shows that NT and the other TG-1 immunoreactive material(s) were not resolved in region I or region II after gel chromatography on Sephadex G-25 (Fig. 1).

We demonstrated that the components of plasma NTLI (TG-1) coeluted from a reverse-phase HPLC column with NT(1–8), NT(1–11), and [^{3}H]NT. Demonstration of coelution (rather than simply equal retention time) is important when sample and standard are injected in solvents of different composition or ionic strength. NT from plasma has been previously characterized by HPLC (8). From the chromatographic evidence presented here (Fig. 3), it is reasonable to assume that the other immunoreactive components of plasma NTLI (TG-1) are NT(1–8) and NT(1–11), although a rigorous identification of these components would require at least enough material to determine their amino acid composition.

The origin of these immunoreactive substances was not determined in this study, but several lines of evidence suggest that they are metabolites of NT. We have previously shown that gel chromatography of extracts of plasma collected after infusion of NT into rats resulted in a broadened peak of TG-1 immunoreactive material similar to that found in regions I and II (Fig. 1) (8). Analysis by HPLC of the metabolites generated in vivo from NT injected in rats demonstrated rapid formation of NT(1–8) and NT(1–11).[2]

[2] Aronin, N., R. Carraway, C. F. Ferris, R. A. Hammer, and S. E. Leeman. Unpublished observations.

Formation of NT(1–8) as a metabolite of endogenous NT was suggested by the finding that stimulation of NT release into the mesenteric vein of rats (21) also resulted in increased concentrations of NT(1–8) in plasma from the mesenteric vein compared to the femoral artery.[3] It is also possible, however, that these peptides are synthesized, stored, and released as such by their source tissues, or that they are fragments of other substances related to NT. Arguing against these suggestions is the finding that the levels of NT(1–8) and NT(1–11) in extracts of human (3) and rat intestine are <1% that of NT.

Generation of these fragments of NT can be readily achieved in vitro by incubation with enzyme solutions (3, 20). Incubation of NT with carboxypeptidase A results in rapid formation of NT(1–11), and trypsin cleaves NT between Arg^8 and Arg^9, forming NT(1–8) and NT(9–13). The latter fragment has 1% of the biological activity of NT and 2% cross-reactivity with antiserum HC-8 (19), so it would not have been detectable if it were present in concentrations similar to those of NT(1–8) in plasma. Tritiated NT(1–8) was generated in vitro when [^{3}H]NT was incubated in whole blood for 1 h (Fig. 4B). The second peak of radioactive material that was generated was due either to the (1–11) or (9–13) fragment, which were not fully resolved using the final HPLC gradient protocol.

The amount of metabolism of NT that occurs in vitro after blood samples are drawn is small (5% after 10 min at 37°C) and should not affect the measurement of plasma NTLI or the analysis of immunoreactive components, particularly if the blood is kept on ice and centrifuged as soon as possible, in which case no metabolism was found to occur (Fig. 4A). Our observations apply to plasma that is extracted before analysis; recovery of NT as measured by assays in unextracted plasma was not evaluated here.

NT is the major component of NTLI (TG-1) in plasma after a 14-h fast (Fig. 5, top panel). However, when the known cross-reactivities of the metabolites of NT with antiserum TG-1 are used to calculate the actual concentrations of peptides, NT is recognized as a minor component of the material detected as NTLI, even during fasting. Thus, for example, NT comprised 5 of 18 fmol of peptide/ml during fasting (Fig. 5, top), and 7 of 44 fmol of peptide/ml 1 h after a meal (Fig. 5, bottom).

An N-terminal directed antiserum that has a high degree of cross-reactivity with NT(1–8) and NT(1–11) would measure higher values of NTLI due to the contribution of metabolites of NT in plasma collected both during fasting and after a meal. In addition, the metabolites of NT probably have a longer half-life in the circulation than does NT itself.[4] Thus, estimation of the amount of NT released in response to a physiologic stimulus may be complicated by the slow elimination of these immunoreactive metabolites from the circulation. Moreover, neither of these metabolites has yet been demonstrated to have the biological activities of enterogastrone. Experiments have been performed (13, 14) to evaluate the effect of elevated levels of plasma NTLI on gastric motility and acid secretion by infusing NT in sufficient amounts to reproduce the level of NTLI that was found in peripheral plasma after a meal. In light of the data presented here, the biological role of each of the components of NTLI must be evaluated separately. Until this is done, the function(s) of NT released in response to a meal is still uncertain. Although it may turn out to be a hormone, NT is just as likely to have local (paracrine) effects in the small intestine.

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