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MEASUREMENT AND CHROMATOGRAPHIC CHARACTERIZATION OF VASOACTIVE INTESTINAL PEPTIDE FROM GUINEA-PIG ENTERIC NERVES

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SUMMARY

The material exhibiting immunoreactivity for vasoactive intestinal peptide in guinea-pig enteric nerves has been characterized by high-performance liquid chromatography in three modes: reversed-phase, cation-exchange and gel permeation. In each case a major portion of the material contained in acetic acid extracts of guinea-pig gut showed the same chromatographic properties as the synthetic porcine peptide of defined amino acid sequence. It is therefore concluded that this immunoreactive material is authentic vasoactive intestinal peptide. The study illustrates a number of the problems encountered in attempting to characterize, and measure reliably, peptides in tissue extracts.

INTRODUCTION

Vasoactive intestinal peptide (VIP) is one of a number of peptides that have been found in, and isolated from, the enteric nervous system [1]. It was first isolated from porcine gut [2] and the amino acid sequence of the peptide from this source has been determined [3]. The amino acid sequence of the VIP-like material in bovine [4], avian [5], human [6] and rat [7] gut has also been determined and has been found to be identical to porcine VIP, although small amounts of apparently variant molecular forms have been reported in a number of species [8, 9].

VIP-like immunoreactivity (VIP-LI) is also present in the guinea-pig small intestine, but it appears to differ from porcine VIP. It has been shown by immunohistochemical techniques, using antisera raised against porcine VIP, to be contained only in nerves [10, 11]. Radioimmunoassay estimation of concentrations in tissue extracts, however, have varied as much as 100-fold [1, 12].

The chromatographic behaviour of guinea-pig VIP-LI has also been reported

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to differ from porcine VIP [12]. Gel filtration chromatography on Sephadex G-50 indicated that the molecular size of guinea-pig VIP-LI was the same as porcine standard, but ion-exchange chromatography on CM-Sephadex indicated that the guinea-pig material was more acidic than the porcine standard.

In the present work, the nature of the VIP-LI material in guinea-pig enteric nerves has been investigated using high-performance liquid chromatography (HPLC). Acid extracts of guinea-pig gut have been analysed in the reversed-phase mode using two different solvent systems, and in the cation-exchange and gel permeation modes.

EXPERIMENTAL

All chromatographic analyses were conducted on a Varian 5020 binary gradient liquid chromatograph equipped with a UV-5 absorbance detector operating at 215 nm. Fractions were collected from the chromatograph using an LKB 2112 Redirac fraction collector. Separations were accomplished on a MicroPak MCH-10 (300 × 4 mm I.D.) column and a MicroPak TSK G2000SW (300 × 7.5 mm I.D.) column, both purchased from Varian Assoc., and on a SynChropak CM300 (250 × 4.1 mm I.D.) column, purchased from SynChrom (U.S.A.). Chromatographic-grade acetonitrile and methanol were purchased from Waters Assoc. Synthetic porcine VIP was purchased from Peninsula Labs. (U.S.A.). All other chemicals were purchased from Ajax Chemicals (Australia).

Tissue processing and extraction

Guinea-pigs (200—300 g) were killed by a blow to the head and bled out. The small intestine, between 20 and 80 cm proximal to the ileo—caecal junction, was taken immediately, opened along the mesenteric border and rinsed in phosphate-buffered saline (PBS) (0.15 *M* sodium chloride, 0.01 *M* sodium phosphate buffer, pH 7.2) to remove the contents. The tissue was then blotted dry on paper towel, wrapped in a piece of pre-weighed aluminium foil and snap-frozen in liquid nitrogen. The sample was weighed frozen and stored at —70°C until it could be extracted.

Frozen tissue was dropped into 5 vols. of boiling 2.0 *M* aqueous acetic acid (with or without 0.01 *M* 2-mercaptoethanol) and was boiled for a further 15 min. The mixture was then cooled in ice and homogenized with 10-sec bursts of a Polytron homogenizer on setting 5 three times. The homogenate was allowed to stand overnight at 4°C and was then centrifuged at 10,000 *g* for 10 min at 4°C. The clear supernatant was decanted, weighed and stored at —70°C until required.

Preparation of tissue extracts for HPLC analysis

Tissue extracts were prepared for chromatographic analysis by the following procedure. Duplicate aliquots (50 μl) were removed and dried down by vacuum centrifugation for estimation of VIP-LI concentration by radioimmunoassay. A portion of the extract (2—3 ml) was washed three times with an equal volume of diethyl ether to remove lipids, and was then loaded onto a pre-wetted Sep-Pak C_{18} cartridge (Waters Assoc.). The cartridge was washed with 20 ml of water to desalt the sample, and the peptides were then eluted with

1—2 ml of methanol. The methanolic eluate was evaporated to dryness and the residue reconstituted in 200 μl of 2.0 *M* aqueous acetic acid for injection into the chromatograph.

Chromatographic analysis of tissue extracts

Reversed-phase analysis was carried out on the MicroPak MCH-10 column, using either of the following solvent gradients: (A) acetonitrile in acid saline (0.15 *M* sodium chloride, pH to 2.1 with hydrochloric acid), 0—2.5 min at 0% acetonitrile, 2.5—5 min 0—10% acetonitrile, 5—45 min 10—40% acetonitrile, 45—50 min 40—60% acetonitrile [13]; or (B) acetonitrile in aqueous trifluoroacetic acid (TFA, 0.5%, v/v, pH 1.9), 0—5 min at 25% acetonitrile, 5—40 min 25—80% acetonitrile, 40—50 min at 80% acetonitrile. All gradient steps were linear and total flow-rate was maintained at 1.0 ml/min for both gradient systems.

Cation-exchange chromatography was carried out on the SynChropak CM300 column, eluting with a gradient of 1.0 *M* potassium chloride in potassium phosphate buffer (0.1 *M*, pH 4.8) containing 10% acetonitrile. The gradient consisted of two linear steps: 0—5 min at phosphate buffer only, 5—30 min 0—50% potassium chloride in phosphate buffer. Flow-rate was maintained at 1.0 ml/min.

Gel permeation chromatography was carried out on the MicroPak TSK G2000SW column, eluting with sodium phosphate buffer (0.2 *M*, pH 2.1) at a flow-rate of 1.0 ml/min.

In all cases 0.5-min fractions were collected from the chromatograph. Glass collection tubes contained 100 μl of bovine serum albumin solution (1%, w/v, in distilled water of fraction V powder from Sigma) to reduce adsorption of peptides to the glass surface.

Fractions collected from gradient analyses (viz. reversed-phase and cation-exchange) were dried down in a vacuum concentrator (Savant Instruments, U.S.A.) and then reconstituted in either distilled water (from acid saline and cation-exchange gradients) or radioimmunoassay buffer (from TFA gradients) before aliquots were taken for measurement of VIP-LI by radioimmunoassay. Fractions collected from the gel permeation column were aliquoted directly into the assay, with an equivalent aliquot of elution buffer added to the standard curve tubes.

The elution position of porcine VIP standard was determined by injection of either 5 μg of standard on-column and monitoring the eluate for absorbance at 215 nm, or by injection of 5 ng on-column and monitoring collected fractions for VIP-LI by radioimmunoassay.

When required, oxidized porcine standard was produced by treating 5 ng of standard in 100 μl of 2.0 *M* acetic acid with 20 μl of hydrogen peroxide (30%, v/v) for 60 min at room temperature prior to injection into the chromatograph.

The recovery of VIP-LI through the pre-column purification and chromatography steps was measured as the proportion of immunoreactive material present in the extract that was recovered in the collected fractions. The recovery from the chromatograph alone was estimated by measuring collected immunoreactive material after injection of standards. After running standards, columns were cleaned by extensive washing with the appropriate solvents (i.e.

blank gradients or elution buffer) until no immunoreactivity could be detected.

Radioimmunoassay

Tissue concentration of VIP-LI was determined from the extracts. Duplicate aliquots were dried down at room temperature in the vacuum concentrator and were reconstituted in assay buffer (0.04 *M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* sodium chloride, 0.01 *M* EDTA and 0.25% bovine serum albumin). VIP-LI was assayed using antiserum 7913 (from Dr. J.H. Walsh) at a final dilution of 1 in 200,000 and using ^{125}I-labelled porcine VIP, iodinated by the iodogen method [14], as tracer. Under these conditions, the assay gave 35% binding of tracer in the absence of VIP standard and measured over the range 10—1000 pg of VIP per tube (100 pg of VIP giving 50% displacement of bound tracer).

The concentration of VIP-LI in fractions collected from the chromatograph was determined as described above.

The cross-reactivity of oxidized porcine VIP in the assay was determined by oxidation of 5 μg of standard with hydrogen peroxide as described above, and then construction of a standard curve using this material (appropriately diluted in assay buffer) together with a standard curve using the normal standard.

RESULTS

Reversed-phase chromatography

Analysis of porcine VIP (pVIP) standard on the reversed-phase column, using a gradient of acetonitrile in acid saline as described above, gave a single sharp peak with retention time of 38.5 min (Fig. 1A). Recovery of peptide from the chromatograph was 95% as measured by radioimmunoassay. Analysis of an extract of guinea-pig small intestine (extracted with 2.0 *M* acetic acid only) under the same conditions gave one major peak of VIP-LI, corresponding in retention time to pVIP and accounting for only 55% of recovered material, together with five other peaks, all eluting earlier (Fig. 2A). Total recovery of immunoreactive material from the chromatograph was 82% of that originally present in the extract. Oxidation of pVIP with hydrogen peroxide resulted in the formation of multiple immunoreactive peaks when chromatographed under these conditions (Fig. 2B), including peaks corresponding to those seen in the extract. Total recovery of immunoreactive material was 110%. The inclusion of 0.01 *M* 2-mercaptoethanol in the extraction medium resulted in only one peak of immunoreactivity being detected, corresponding to 57% of immunoreactive material present in the extract, and this peak corresponded to the elution position of authentic porcine standard (Fig. 3A).

Reversed-phase analysis of pVIP on the same column but with a different gradient of acetonitrile in aqueous TFA also gave a single peak for this material, but with a retention time of 18 min (Fig. 1B). Recovery of immunoreactive peptide was quantitative. Analysis of the extract containing 2-mercaptoethanol with this system gave one peak of VIP-LI, corresponding in retention time to the pVIP standard (Fig. 3B), and with 65% of immunoreactive material originally present in the extract being recovered.

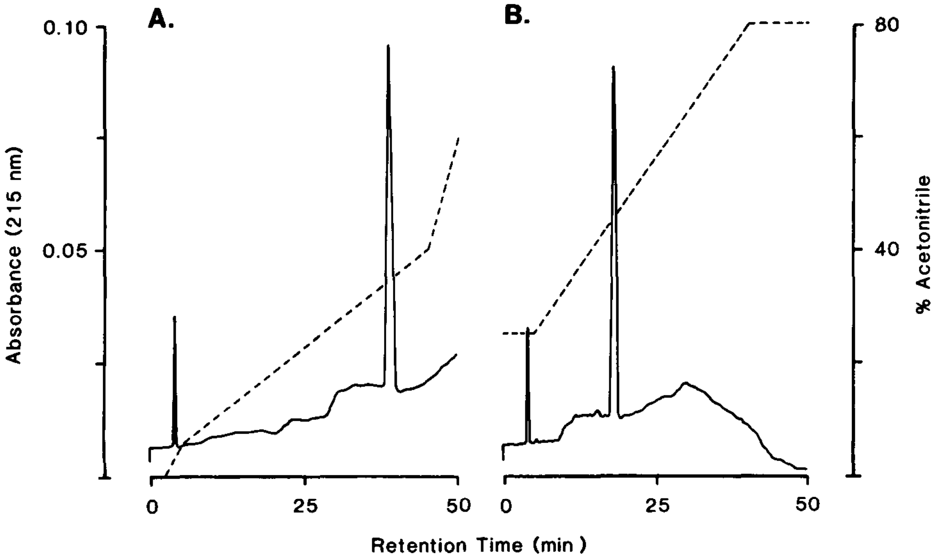

Fig. 1. Reversed-phase HPLC analysis of synthetic porcine VIP. Starting buffers were (A) 0.15 *M* sodium chloride (pH 2.1 with hydrochloric acid), and (B) 0.5%, v/v, TFA (pH 1.9), and the elution gradient (- - -) is expressed in terms of percentage acetonitrile (right hand ordinate). The peptide (5 μg) was injected onto the column and its elution position detected by its absorbance at 215 nm (left hand ordinate) (—).

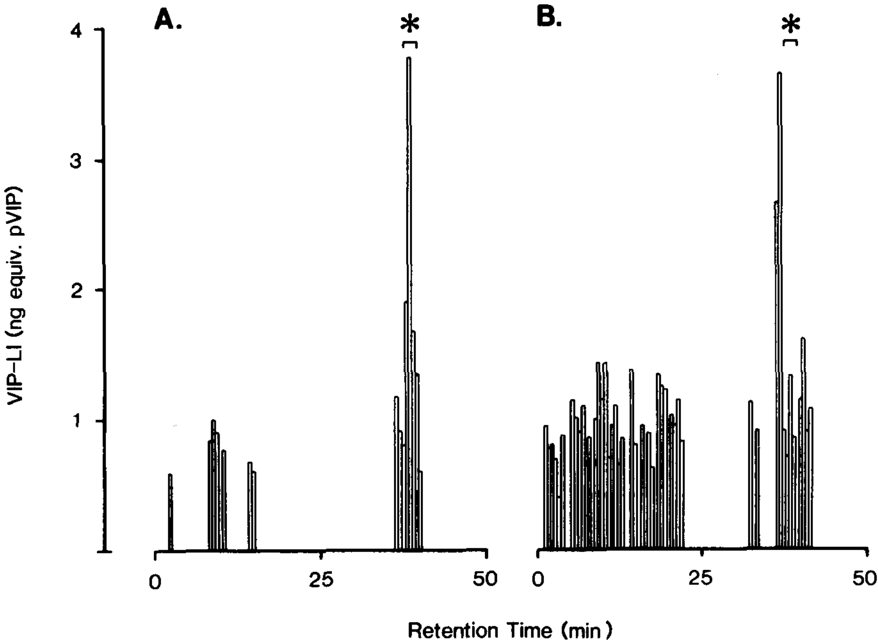

Fig. 2. Reversed-phase analysis of guinea-pig gut extract and oxidized porcine VIP. The histograms show VIP-like immunoreactive material recovered after reversed-phase HPLC analysis (using a gradient of acetonitrile in acid saline) of (A) a 2.0 *M* acetic acid extract of guinea-pig ileum, and (B) oxidized porcine VIP standard. The elution position of authentic pVIP is shown (*). The ordinate gives the concentration of immunoreactive material in ng equiv. of pVIP per 0.5-ml fraction.

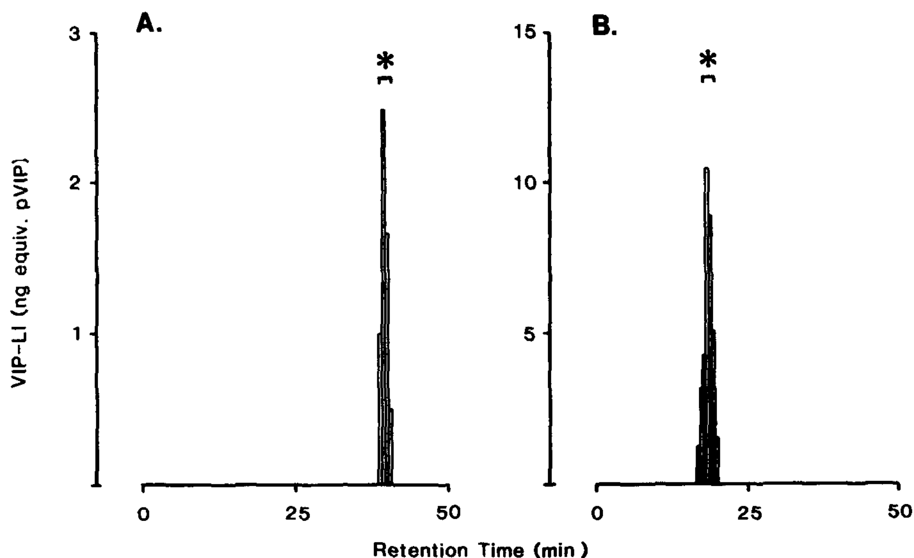

Fig. 3. Reversed-phase analysis of oxidation-protected guinea-pig gut extract. The histograms show VIP-like immunoreactive material recovered after reversed-phase HPLC analysis of a 2.0 *M* acetic acid extract (containing 0.01 *M* 2-mercaptoethanol) of guinea-pig ileum using a gradient (as shown in Fig. 1) of acetonitrile in (A) 0.15 *M* sodium chloride (pH 2.1 with hydrochloric acid), and (B) 0.5%, v/v, TFA (pH 1.9). The elution position of authentic pVIP is shown (*). The ordinates give the concentration of immunoreactive material in ng equiv. of pVIP per 0.5-ml fraction.

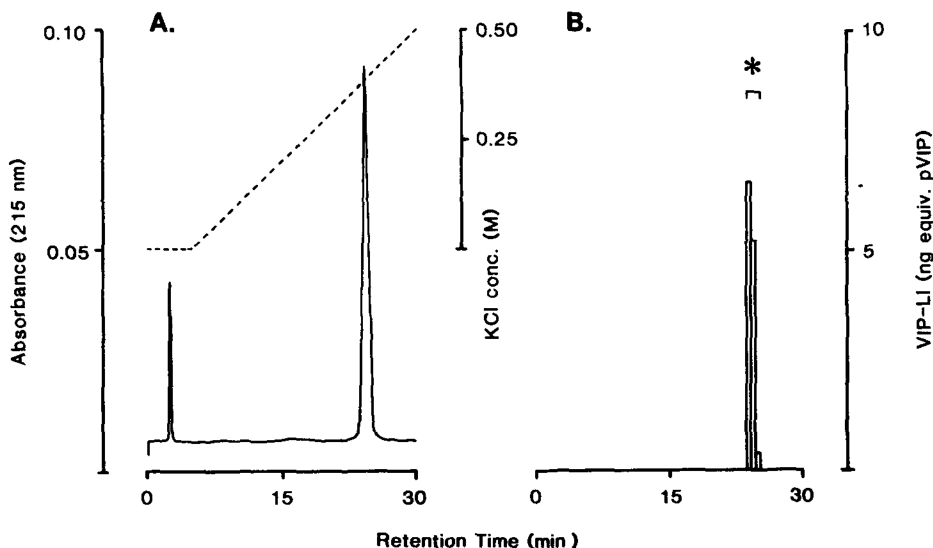

Fig. 4. Cation-exchange analysis of porcine VIP and guinea-pig gut extract. (A) Analysis of pVIP standard using a gradient of increasing potassium chloride concentration in 0.1 *M* phosphate buffer (pH 4.8), containing 10% acetonitrile. (- - -) Gradient profile (inset ordinate); (—) elution profile monitored by absorbance of the eluate at 215 nm (left hand ordinate), baseline-corrected. The amount of peptide injected on-column was 5 μg. (B) Histogram of VIP-like immunoreactivity recovered after cation-exchange analysis, using the above-mentioned gradient, of a 2.0 *M* acetic acid extract (containing 0.01 *M* 2-mercaptoethanol) of guinea-pig ileum. The concentration of material recovered is expressed in terms of ng equiv. of pVIP per 0.5-ml fraction (right hand ordinate). The elution position of authentic pVIP is shown (*).

Cation-exchange chromatography

Analysis of pVIP on the cation-exchange column, using an increasing gradient of potassium chloride in phosphate buffer, gave a single peak for the peptide (which was recovered quantitatively) with a retention time of 24.5 min (Fig. 4A). Analysis of an extract of guinea-pig small intestine, protected from oxidation with 2-mercaptoethanol, showed only one peak of immunoreactive material, eluting with the same retention time as authentic standard (Fig. 4B). Recovery of immunoreactive material was 70%.

Gel permeation chromatography

Analysis of pVIP on the gel permeation column showed only one peak for the peptide, eluting with K_0 = 0.65 (Fig. 5A), which was recovered quantitatively. Analysis of an extract of guinea-pig small intestine (extracted with aqueous acetic acid containing 2-mercaptoethanol) showed only one peak of immunoreactive material, again eluting in the same retention volume as the authentic standard (Fig. 5B). Recovery of immunoreactive material was 64%.

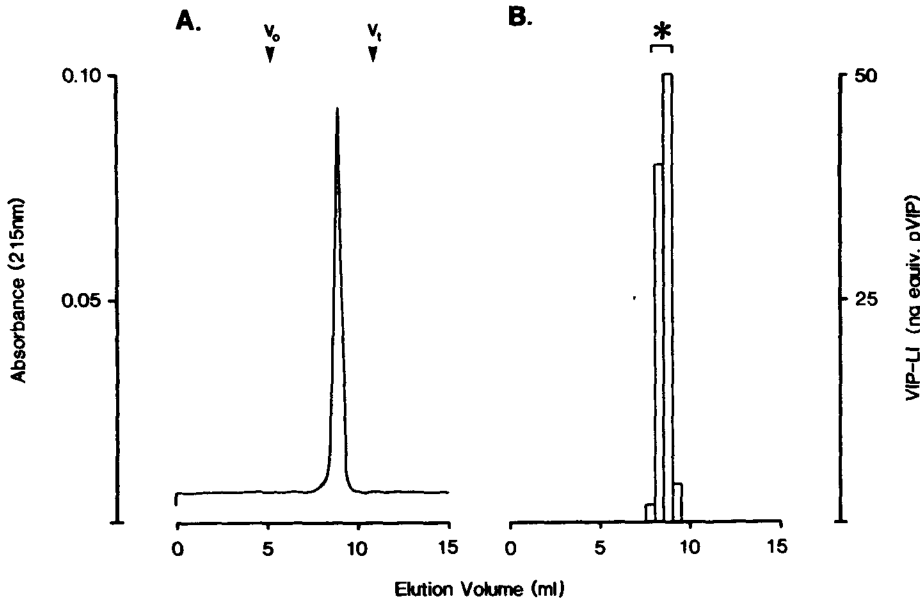

Fig. 5. Gel permeation analysis of porcine VIP and guinea-pig gut extract. (A) Analysis of pVIP standard eluted in 0.2 *M* sodium phosphate buffer (pH 2.1). The peptide (5 μg) was injected onto the column and its elution position detected by its absorbance at 215 nm (left hand ordinate). The void volume (V_0) and total volume (V_t) of the column are shown. (B) Histogram of VIP-like immunoreactivity recovered after gel permeation analysis of a 2.0 *M* acetic acid extract (containing 0.01 *M* 2-mercaptoethanol) of guinea-pig ileum. The elution position of authentic pVIP is shown (*). The right hand ordinate gives the concentration of immunoreactive material in ng equiv. per 0.5-ml fraction.

Radioimmunoassay of extracts

The mean concentration (± standard error) of immunoreactive material in extracts of whole wall of the guinea-pig small intestine was 66 ± 4 pmol/g wet weight of tissue for six animals.

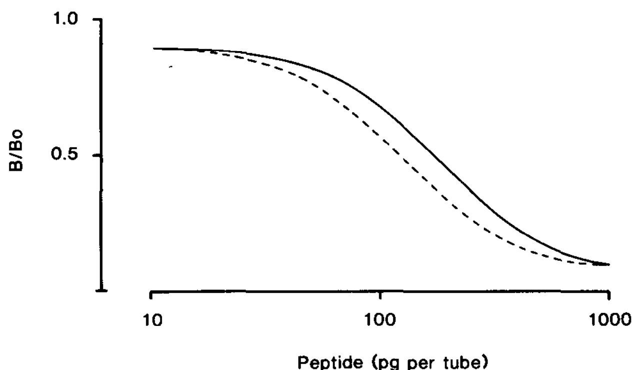

Fig. 6. Standard curves for radioimmunoassay of pVIP (—) and oxidized pVIP (- - -), plotted over the range 10—1000 pg of peptide per assay tube (abscissa). Binding of tracer (B) is plotted on the ordinate as a fraction of the amount bound in the absence of exogenous peptide (B_0). Displacement of 50% of bound tracer occurred with 170 pg of pVIP and 120 pg of oxidized pVIP.

The cross-reactivity of oxidized pVIP standard in the assay was found to be 140%, as shown in Fig. 6.

DISCUSSION

HPLC analysis indicates that the VIP-like immunoreactive material in extracts of the guinea-pig small intestine is identical to porcine VIP. Thus in the guinea-pig, which has been used extensively in studies of the physiological effects of neuropeptides, this clarification of the nature of the VIP-like immunoreactivity in nerves in the gut should lead to a better understanding of its postulated biological role as an enteric neurotransmitter [15].

The presence of an anti-oxidant such as 2-mercaptoethanol in the extraction medium has already been shown to be essential to maintain the chemical integrity of easily oxidized peptides [16], and this also appears to be the case for VIP. In the absence of an anti-oxidant, the apparent recovery of immunoreactive material is higher than when such a compound is included in the extraction medium (82% compared with 57%). However, only slightly more than half of this material chromatographs in the same place as the porcine standard. This apparent increase in recovery implies that the chemically altered forms of VIP (presumably oxidation products) are recognized and measured by this antiserum with greater efficacy than the native compound. That this explanation is correct is also indicated by the relative apparent recoveries of standard (95%) and oxidized standard (110%) from the chromatograph, and it was confirmed by comparison of standard curves constructed using both oxidized and native porcine standard (see Fig. 6). This difference in interaction has been noted for other, easily oxidized peptides, particularly methionine enkephalin [17], and may arise because of unintentional oxidation of a proportion of the peptide molecules during coupling to carrier molecules. Thus the antiserum raised in this case would be a mixture of antibodies directed at oxidation products in addition to the native peptide.

The reliability with which a particular antiserum can be used to measure, in

tissue extracts, the peptide against which it was raised will, then, depend upon two major factors: first, how well the chemical integrity of the peptide is maintained during conjugation to carrier, and secondly, how well the peptide's chemical integrity is maintained during extraction. VIP in particular is not only susceptible to chemical change during extraction, but has also been shown to be altered by some chromatography solvents [18].

A third factor which must also be taken into account is the cross-reactivity of the antiserum with peptides sharing some sequence homology with the antigen. Polyclonal antisera raised against large peptides (such as VIP) will contain components showing an immunochemical response to multiple sites in the amino acid sequence of the peptide, and consequently will increase the opportunities for cross-reactivity with other peptides. Thus precursors and/or metabolites of the peptide in question may contribute at least a portion of the measured tissue levels of immunoreactive material, and other unrelated peptides with limited sequence homology may similarly result in over-estimates of tissue concentrations.

Such differences in immunogenecity and chemical form may explain the large variation in reported tissue concentrations of VIP-LI in guinea-pig gut [1, 12], and suggest caution in the comparison of values obtained using different antisera and differing methods of extraction. Thus careful characterization of antisera, and the use of physicochemical techniques such as HPLC to separate peptides on the basis of their chemistry, are essential to the preliminary characterization of peptides. Isolation, purification and sequencing provide the ultimate chemical characterization of a peptide, and allow a better understanding of its biological role.

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