THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 21, Issue of November 10, pp. 10290–10298, 1980
Printed in U.S.A.

The Subcellular and Organ Distribution and Natural Form of Histidyl-proline Diketopiperazine in Rat Brain Determined by a Specific Radioimmunoassay*

(Received for publication, April 16, 1980, and in revised form, July 20, 1980)

Tadashi Yanagisawa‡, Chandan Prasad§, and Alan Peterkofsky¶

From the Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Histidyl-proline diketopiperazine is produced in brain as a product of the metabolism of thyrotropin-releasing hormone. A number of the previously observed central nervous system and pituitary activities resulting from an exposure to thyrotropin-releasing hormone appear to involve the conversion of the releasing factor to the cyclic dipeptide. In the present study, the development of a rabbit antiserum that is highly specific for histidyl-proline diketopiperazine is described; the antiserum has essentially no capability to bind thyrotropin-releasing hormone or a number of other related peptides. The antibody can also distinguish between the natural form of the cyclic dipeptide and a diastereomer containing D-proline. A procedure for extraction, with high yield, of histidyl-proline diketopiperazine from brain is described. With the aid of the specific antiserum it was found that the preponderance of the cyclic dipeptide in rat brain is bound to high molecular weight material, mainly in the range of M_r = 70,000; histidyl-proline diketopiperazine can be dissociated from this material by boiling in salt/methanol solution. The concentration of the dipeptide in rat brain is in the range of 275 to 565 pmol/brain, approximately 2.5 times the concentrations determined for thyrotropin-releasing hormone (113 to 210 pmol/brain). A study of the subcellular distribution of histidyl-proline diketopiperazine and thyrotropin-releasing hormone suggests that the releasing factor is concentrated in synaptosomal vesicles while the diketopiperazine is not. A determination of the regional distribution of thyrotropin-releasing hormone and histidyl-proline diketopiperazine indicated that both peptides are found in highest concentrations in pituitary and hypothalamus, but are detectable in other areas of brain as well.

Thyrotropin-releasing hormone is a modified tripeptide (pyroglutamyl-histidyl-prolineamide) found in the brain as well as some other organs (1). Its biological roles include regulation of release from the pituitary of thyrotropin, growth hormone, prolactin, adrenocorticotropic hormone, and follicle-stimulating hormone. At the level of the central nervous system, TRH[1] elicits a multitude of effects ranging from antidepressant to behavioral to thermoregulatory. The biochemical basis for most of these complex effects is only superficially understood.

A new direction in investigations concerning TRH was developed with the recent discovery of a biologically active metabolite of TRH, histidyl-proline diketopiperazine (2). This compound has been shown to be more potent than TRH in the antagonism of ethanol narcosis in rats (3). It also elicits hypothermia, which is opposed by TRH (4). Intraperitoneal injection of the dipeptide into rats also produces a transient increase in the level of cGMP in brain (5). Cyclo(His-Pro) also inhibits sodium-dependent catecholamine transport into nerve endings; the mechanism of this effect apparently involves an inhibition of the sodium pump (6). In the pituitary, the diketopiperazine inhibits, while TRH promotes, prolactin release (7, 8).

A number of aspects of the physiology and pharmacology of this interesting peptide have not been amenable to study because of the unavailability of a rapid and specific assay for cyclo(His-Pro). This report describes the development of a specific antiserum to cyclo(His-Pro) and the use of this reagent to measure the concentration of the peptide in brain. These investigations have led to the finding that cyclo(His-Pro) occurs naturally in a bound form.

MATERIALS AND METHODS

A variety of peptides were used in these studies. Cyclo(His-Pro) was synthesized as described (3, 9). [*Pro*-^{3}H]Cyclo(His-Pro) was prepared from [*Pro*-^{3}H]TRH (9). [*His*-^{3}H]Cyclo(His-Pro) was prepared by catalytic dehalogenation of diiodo-cyclo(His-Pro) (9–11) by New England Nuclear. TRH and LHRH were from Calbiochem. TRH, free acid, and histidyl-proline were from Chemical Dynamics Corp., South Plainfield, N. J. Cyclo(His-Gly) was a product of Bachem. His-ProNH_2 was generously provided by Dr. A. O. Geiszler of Abbott Laboratories. Poly-L-lysine (molecular weight range > 70,000) was a product of Miles Laboratories. Bio-Gel P-2 and A-1.5m were from Bio-Rad, Sephadex G-10 and SP-Sephadex C-25 were Pharmacia products, and DEAE-cellulose (DE-23) was from Whatman. ^{125}I-Na was from New England Nuclear. Keyhole limpet hemocyanin was a Calbiochem product. Complete Freund's adjuvant and pertussis vaccine were generous gifts of Dr. Charles Manclark, Bureau of Biologics, National Institutes of Health. Incomplete Freund's adjuvant was from Difco. Rabbits for immunization were of the New Zealand type from the National Institutes of Health animal colony. Rats were Sprague-Dawley from Taconic Farms and varied in body weight from 140 to 500 g. For preparation of brain extracts, the rats

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Utsunomiya University, Utsunomiya, Japan.

§ Present address, Louisiana State University Medical Center, New Orleans, Louisiana.

¶ To whom requests for reprints should be addressed at Building 36, Room 4C-11, National Institutes of Health, Bethesda, Maryland 20205.

[1] The abbreviations used are: TRH, thyrotropin-releasing hormone; cyclo(His-Pro), histidyl-proline diketopiperazine; phenylazo-cyclo(His-Pro), 2- or 4- or 2,4-bis-im(4-carboxyphenylazo)cyclo(His-Pro; LHRH, luteinizing hormone-releasing hormone; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline (0.1 M potassium phosphate, pH 7.2, and 0.14 M NaCl).

(mildly etherized) were decapitated with a guillotine, and the brains were rapidly removed followed by homogenization, usually in PBS, for approximately 5 min in a motor-driven Teflon-glass homogenizer.

Cyclo(His-Pro) Antiserum

Preparation of Haptens and Antigens for Immunization of Rabbits

Im(4-carboxyphenylazo)-Cyclo(His-Pro)—Diazotized *p*-aminobenzoic acid (4-carboxyphenyl diazonium chloride) was prepared by mixing 10 ml of 3.5% $NaNO_2$ with 90 ml of 0.3% *p*-aminobenzoic acid, 0.2 N HCl. This solution was stirred for 60 min in an ice bath. It was then added to a solution (500 ml) of cyclo(His-Pro) (1 mg/ml in 0.16 M sodium borate, pH 9.0). In order to follow the course of the reaction, the solution also contained 5×10^6 cpm of [*His*-^{3}H]cyclo(His-Pro). Stirring was continued for 1 h in an ice bath. The reaction mixture was then applied to a column (4.5 × 36 cm) of DEAE-cellulose, previously equilibrated with water. The column was washed successively with water (600 ml), then 0.1 N HCl (700 ml). A further wash with 200 ml of 0.1 N HCl eluted approximately 17% of the radioactivity. This fraction was concentrated to dryness by evaporation at 45°C followed by lyophilization. This material had an absorption spectrum in 0.1 N HCl with a maximum at 345 nm. Analysis for C, H, and N gave values of 45.22%, 4.17%, and 15.76% (calculated for the dihydrochloride of phenylazo-cyclo(His-Pro): 47.68%, 3.97% and 18.54%). This material (crude phenylazo-cyclo(His-Pro)) was used for coupling to polylysine.

Antigen I: Phenylazo-Cyclo(His-Pro) Coupled to Polylysine—Poly-L-lysine (200 mg dissolved in 60 ml of water and adjusted to pH 7 with NaOH) was mixed with EDC (100 mg). Then a solution of crude carboxyphenylazo-cyclo(His-Pro) (100 mg dissolved in 10 ml of 30% MeOH) was added dropwise, and the pH was maintained at 7 using NaOH (0.1 N). A further addition of 100 mg of EDC was made, maintaining the pH at 7. The reaction mixture was stirred for 18 h and then concentrated to 10 ml by evaporation under vacuum at 45°C. The material was then dialyzed at 4°C against six changes of 4 liters of water over a period of 2 days. The material was then lyophilized to yield 180 mg of conjugate.

Antigen II: Cyclo(His-Pro) Coupled to Keyhole Limpet Hemocyanin using a Bridge of Bis-diazotized Benzidine—Sodium nitrite (5.0 ml of 3.5% solution in water) was mixed with benzidine·2 HCl (45 ml of 0.5% solution in 0.2 N HCl). This mixture was stirred for 30 min at 4°C. Twenty milliliters of the solution of diazotized benzidine was added to 50 ml of sodium borate (0.16 M, pH 9) containing 0.15 M NaCl. This solution was added slowly at 4°C to a solution (50 ml in the sodium borate/NaCl buffer) containing 500 mg of keyhole limpet hemocyanin and cyclo(His-Pro) (100 mg containing 5 μCi of [His-^{3}H]cyclo(His-Pro)). After stirring for 2 h, the sample was dialyzed against seven changes of 4 liters of water over a period of 2 days and then lyophilized.

Immunization

Four New Zealand White rabbits were immunized with 2.3 mg of Antigen I in 0.9% NaCl solution (saline) emulsified with complete Freund's adjuvant (1:1, total volume, 1 ml). Injections were given both sub- and intracutaneously at multiple sites on the backs. The animals were also given injections of 1 ml of pertussis vaccine containing 20 opacity units, injected intramuscularly at two sites on the thighs. After 2 months and at weekly intervals thereafter for 3 months, the animals were given intravenous injections of 1 mg of Antigen I in saline. At this point, one of the animals (Rabbit 96) showed a weak response in tests for binding of radioactive cyclo(His-Pro). After 1 year, the animal was boosted with 1 mg of Antigen II in saline emulsified in incomplete Freund's adjuvant (1:1, total volume 1 ml) injected subcutaneously and intracutaneously at multiple sites on the back. A bleeding after 3 weeks produced a serum that showed enhanced binding of radioactive cyclo(His-Pro). The greatest dilution at which detectable binding occurred was 1:40. On the basis of the low titer of the serum from Rabbit 96, the inactivity of the serum in the other three animals, and an unsuccessful trial with five animals injected with Antigen II, it appears that cyclo(His-Pro) is a poor antigen. This is in marked contrast to the ease with which potent antisera against TRH have been prepared by similar methods (12–15). Unfortunately, rabbit 96 died in July, 1980.

Iodination—Iodination of cyclo(His-Pro) or carboxyphenylazo-cyclo(His-Pro) was carried out in a total volume of 200 μl containing potassium phosphate (pH 7, 50 μmol), substrate (generally 25 nmol), NaI (12.5 nmol), and chloramine T (a total of 25 nmol added in 3 equal portions with periods at room temperature of 5 min between each addition). For the preparation of [^{125}I]iodo-cyclo(His-Pro), the same protocol was followed, but 0.2 mCi of [^{125}I]NaI was included.

Radioimmunoassay—Radioimmunoassay for TRH was as previously described (2). The radioimmunoassay for cyclo(His-Pro) was carried out in a volume of 200 μl containing PBS, [^{125}I]iodo-cyclo(His-Pro) (approximately 10,000 cpm, 0.5 pmol), rabbit antiserum to cyclo(His-Pro) (generally 5 to 10 μl), and, where desired, a sample to be tested for competition of binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum. The incubation mixtures were incubated on ice for 1 h and deposited on Millipore filters (25 mm, 0.45 μ) which were then washed with 10 ml of cold PBS. The filters were then dissolved in 1 ml of methylcellosolve and counted with 10 ml of scintillation fluid. Depending on the titer of the antiserum, binding of [^{125}I]iodo-cyclo(His-Pro) in the absence of competitors was 4 to 65% of the added counts per min.

Standard Procedure for Extraction of Cyclo(His-Pro) from Brain—A rat brain or other tissue was homogenized in PBS:MeOH (1:1) containing approximately 30,000 cpm of [^{3}H]cyclo(His-Pro) (approximately 5 volumes per g wet weight of tissue). The homogenate was incubated in a boiling water bath for 10 min. After addition of 2.5 volumes per g wet weight of tissue of MeOH, the homogenate was held for an additional 10 min in a boiling water bath, then centrifuged at 20,000 × *g* for 20 min. The supernatant solution was diluted to 90 ml with water, and the pH was adjusted below 4.0 with 1 N CH_3COOH. The solution was then applied to a column (1.5 × 20 cm) of SP-Sephadex C-25 adjusted to pH 3.5 and equilibrated with 0.01 M ammonium acetate, pH 3.5. The column was washed with 50 ml of the pH 3.5 buffer, then with 50 ml of 0.01 M ammonium acetate, pH 7.2. Elution with 0.2 M ammonium acetate, pH 7.2, was then initiated, collecting 5-ml fractions. The fractions were checked for radioactivity; generally the fractions from 30 to 60 ml contained most of the radioactive cyclo(His-Pro). Those fractions were pooled and lyophilized. The lyophilized material was then dissolved in PBS (typically 2 ml), and appropriate aliquots were tested for cyclo(His-Pro) content in the radioimmunoassay.

RESULTS

Purification of Phenylazo-Cyclo(His-Pro)—The crude carboxyphenylazo-cyclo(His-Pro) was characterized by high pressure liquid chromatography. Chromatography on a μBondapak C_{18} column (Waters Associates) using an elution program of 5% MeOH:95% NH_4HCO_3 (0.01 M) at 2 ml/min for 22 min followed by a linear gradient to 30% MeOH for the next 20 min led to a resolution of the material into six fractions (labeled Peaks 1 to 6). Absorption spectra through the range of 240 to 450 nm and the proline content of the fractions were determined. Taking advantage of the previous studies of Nagai *et al.* (16) on the absorption characteristics of azo-imidazoles, a tentative assignment of the structures of the compounds was made as follows: Peak 1, 2,4-bis-im(4-carboxyphenylazo)-cyclo(L-His-L-Pro); Peak 2, 2,4-bis-im(4-carboxyphenylazo)-cyclo(L-His-D-Pro); Peak 3, 4-im(4-carboxyphenylazo)-cyclo(L-His-L-Pro); Peak 4, 2-im(4-carboxyphenylazo)-cyclo(L-His-L-Pro); Peak 5, 4-im(4-carboxyphenylazo)-cyclo(L-His-D-Pro); and Peak 6, 2-im(4-carboxyphenylazo)-cyclo(L-His-D-Pro).

Molar concentrations of the fractions were calculated from their proline content determined by amino acid analysis after acid hydrolysis. These fractions were tested for their capacity to compete with [^{3}H]cyclo(His-Pro) for binding to the antiserum (Fig 1). In this test system, 4 pmol of unlabeled cyclo(His-Pro) produced 50% displacement of binding of [^{3}H]cyclo(His-Pro). Peaks 1, 2, 5, and 6 showed essentially no capability to displace cyclo(His-Pro) from the antiserum, while Peak 3 (the 4-azo derivative) produced 50% displacement with 25 pmol. Peak 4 (the 2-azo derivative) produced 50% displacement with approximately 1.3 pmol. Although a conjugate prepared from the crude mixture of the azo compounds had been used for immunization, it appears that the immune response was directed primarily against the Peak 4 compound which is recognized by the antiserum approximately 3 times more effectively than is cyclo(His-Pro).

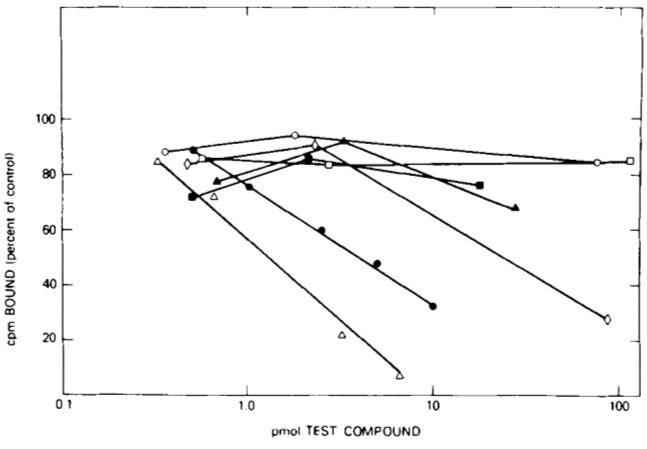

FIG. 1. **Competition of carboxyphenylazo derivatives of cyclo(His-Pro) for binding of cyclo(His-Pro) to antiserum.** [^{3}H]-Cyclo(His-Pro) (approximately 10,000 cpm, 0.5 pmol) was incubated with low titer rabbit antiserum (10 μl) in PBS (total incubation volume equals 200 μl). Where indicated, the incubation mixtures also contained the designated amounts of the indicated test compounds. The incubation mixtures were processed for radioimmunoassay as described under "Materials and Methods." ●——●, cyclo(His-Pro); ■——■, Peak 1, 2,4-bis-im(4-carboxyphenylazo)-cyclo(L-His-L-Pro); ▲——▲, Peak 2, 2,4-bis-im(4-carboxyphenylazo)-cyclo(L-His-D-Pro); ◇——◇, Peak 3, 4-im(4-carboxyphenylazo)-cyclo(L-His-L-Pro; △——△, Peak 4, 2-im(4-carboxyphenylazo)-cyclo(L-His-L-Pro); ○——○, Peak 5, 4-im(4-carboxyphenylazo)-cyclo(L-His-D-Pro); and □——□, Peak 6, 2-im(4-carboxyphenylazo)-cyclo(L-His-D-Pro).

Development of Radioimmunoassay for Cyclo(His-Pro)—For the development of a sensitive radioimmunoassay for cyclo(His-Pro), it would be advantageous to use a radioactive ligand that has a high affinity for the antiserum. The data of Fig. 1 indicated that Peak 4 (the 2-azo derivative of cyclo(L-His-L-Pro) was approximately 3 times more effective in displacing [^{3}H]cyclo(His-Pro) from the antiserum than was unlabeled cyclo(His-Pro). If iodinated Peak 4 material continued to displace bound radioactive cyclo(His-Pro), then a sensitive assay for cyclo(His-Pro) could be developed based on the displacement of [^{125}I]iodo-Peak 4. Samples of iodinated cyclo(His-Pro) and Peak 4 were, therefore, prepared and tested for displacement of binding of [^{3}H]cyclo(His-Pro) to antiserum (Table I). The data show that iodination of cyclo(His-Pro) produces no significant change in its capability to compete for binding to antiserum; however, iodination of the azo derivative results in a substantial loss of the compound's capability to bind to antiserum. Approximately 6 times the concentration of iodinated azocompound as cyclo(His-Pro) is necessary to produce comparable displacement of [^{3}H]cyclo(His-Pro). It was, therefore, concluded that iodinated Peak 4 was not the most desirable ligand, and our attention was directed to radioiodination of cyclo(His-Pro).

Cyclo(His-Pro) was iodinated in the presence of [^{125}I]NaI and then fractionated on a column of Sephadex G-10 (Fig. 2). Four peaks of radioactive material were separated; pools corresponding to the *horizontal bars* in Fig. 2 were made and tested for binding to the antiserum against cyclo(His-Pro). Only the 4th peak showed significant binding to antiserum. The material from this fraction was further tested for its use as a radioimmunoassay reagent.

Competition curves of varying concentrations of cyclo(His-Pro) for displacement of bound [^{3}H]cyclo(His-Pro) or [^{125}I]-iodo-cyclo(His-Pro) were compared (data not shown). The displacement curves were essentially identical. Therefore, a standard protocol for the radioimmunoassay of cyclo(His-Pro) was adopted which involved the use of [^{125}I]iodo-cyclo(His-Pro) as the radioactive ligand (see "Materials and Methods").

TABLE I

Effect of iodination of peptides on reactivity with antiserum to cyclo(His-Pro)

Cyclo(His-Pro) or Peak 4 material (25 nmol) was iodinated as described under "Materials and Methods." The samples were then diluted 100-fold with PBS and tested for displacement of binding of [^{3}H]cyclo(His-Pro) to antiserum (see "Materials and Methods"). Where indicated, the compounds before iodination were also tested. In the absence of competitors, the binding to antiserum was 710 cpm (corresponding to 9.9% of the input counts per min).

Compound	Picomoles	Counts per min bound
		% of control
Cyclo(His-Pro)	5	42
Iodinated cyclo(His-Pro)	5	53
2-im(4-Carboxyphenylazo)cyclo-(L-His-L-Pro) (Peak 4)	6.5	7.2
Iodinated derivative of Peak 4	32.3	63

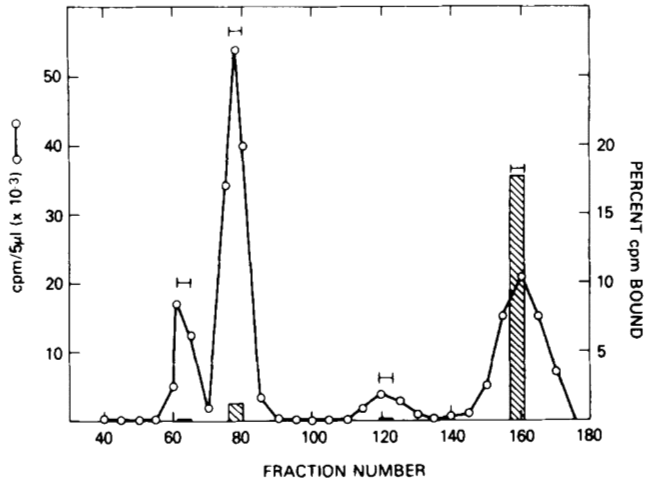

FIG. 2. **Sephadex G-10 chromatography of iodinated cyclo(His-Pro).** After iodination of 25 nmol of cyclo(His-Pro) in a medium containing 0.2 mCi of ^{125}I-NaI, the reaction mixture was deposited on a column (0.9 × 50 cm) of Sephadex G-10 equilibrated with potassium phosphate, pH 7 (0.05 M). The column was washed with the same buffer, and fractions (0.5 ml) were collected; aliquots (5 μl) were counted by scintillation counting. The fractions indicated by the *horizontal bars* for the four isolated peaks were pooled. Aliquots (5 μl) of each of the pools, corresponding to 13,000, 50,000, 4,000, and 20,000 cpm, respectively, were tested for binding to antiserum against cyclo(His-Pro). The *cross-hatched bars* indicate the fraction of the counts in each pool that bound to antibody. Incubation mixtures for binding to antibody (200 μl total volume) contained 70 μl of a 1:10 dilution of the high titer antiserum, 5 μl of the indicated fractions, and 125 μl of PBS. The mixtures were processed as described under "Materials and Methods."

Specificity of the Antiserum—Using the procedure described above (see under "Materials and Methods"), competition curves for a number of peptides related to cyclo(His-Pro) were constructed (Fig. 3). Fifty percent displacement of [^{125}I]iodo-cyclo(His-Pro) was accomplished with 3 pmol of cyclo(His-Pro) but required approximately 27 pmol of His-ProNH$_2$. The observed competition by His-ProNH$_2$ is probably due to contamination with cyclo(His-Pro). Examination of the preparation of His-ProNH$_2$ by HPLC showed that 10% of the absorbance at 210 nm was accounted for as cyclo(His-Pro). A further incubation of the solution of His-ProNH$_2$ in saline overnight in the freezer followed by examination by HPLC indicated that 25% of the compound had now been converted to cyclo(His-Pro). This preparation now also reacted more avidly with the antiserum. It appears, therefore, that His-ProNH$_2$ is readily converted to cyclo(His-Pro), which reacts with the antiserum while the original His-ProNH$_2$ does

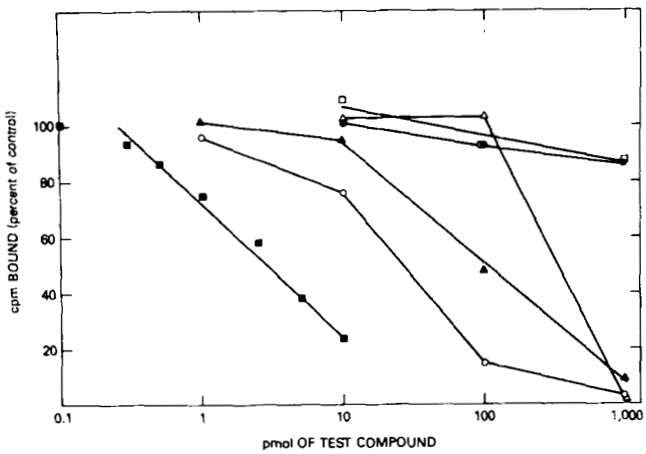

FIG. 3. **Competition of peptides for binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum.** Approximately 10,000 cpm of [^{125}I]iodo-cyclo(His-Pro) were incubated with antiserum (5 μl) as described in Fig. 2. Where indicated, the incubation mixtures also contained the designated amounts of unlabeled test compound. The mixtures were processed for radioimmunoassay of cyclo(His-Pro) as described under "Materials and Methods." The values shown for the different test compounds are derived from a series of experiments; in the absence of competitors, the binding to antibody was from 26% to 67% of the input in different experiments. ■—■, cyclo(His-Pro); ●—●, LHRH; □—□, TRH; △—△, TRH, free acid; ○—○, His-ProNH$_2$; ▲—▲, His-Pro.

not. Fifty percent displacement of [^{125}I]iodo-cyclo(His-Pro) binding to antiserum was observed with 110 pmol of His-Pro, corresponding to a relative effectiveness of His-Pro:cyclo(His-Pro) of 0.027:1. Examination of this compound by HPLC indicated that it contained at most 0.5% cyclo(His-Pro). Therefore, it appears that His-Pro is recognized by the antiserum, but approximately 40 times less effectively than is cyclo(His-Pro).

TRH, free acid, showed a weak cross-reactivity with the antiserum, but was approximately 100 times less potent than cyclo(His-Pro) in displacing [^{125}I]iodo-cyclo(His-Pro) from the antiserum. HPLC analysis of this compound indicated that it contained less than 1% contamination with cyclo(His-Pro). LHRH and TRH showed no sign of displacing cyclo(His-Pro) from the antiserum even at doses of 1000 pmol. Histidine and proline (tested at 500 nmol) and cyclo(His-Gly), lysine, and *p*-aminobenzoic acid (tested at a concentration of 10 nmol) also showed no competition. It was concluded that the antiserum is quite specific for cyclo(His-Pro). Only His-Pro and TRH, free acid, showed any evidence for a possible low level of interference with cyclo(His-Pro) binding to the serum.

Stereoisomers of Cyclo(His-Pro)—Examination of preparations of chemically prepared cyclo(His-Pro) (3, 9) by high pressure liquid chromatography indicated that there were two separable components. The elution profile of a typical preparation is displayed in Fig. 4, *Panel A*. The peak eluting at about 6 min (labeled Peak I) accounts for about 10% of the total material, the remainder eluting at approximately 9 min (Peak II). The material in the two fractions appeared to be structurally related, since heating a preparation of cyclo(His-Pro) in alkaline solution led to an increase in the proportion of the total material accounted for as Peak I. It was, therefore, of interest to determine the nature of these two fractions and to establish whether the antiserum reacted with either of the fractions.

A preparation of cyclo(His-Pro) was heated in alkaline solution. Examination of the heated material on HPLC now indicated that there were approximately equal concentrations of Peaks I and II (data not shown). Repeated application of

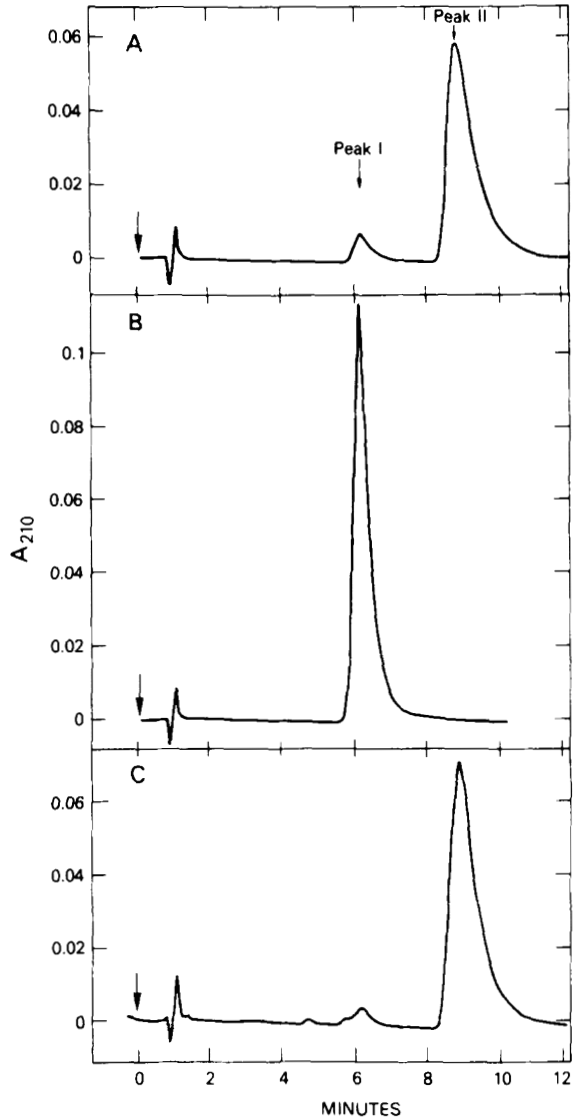

FIG. 4. **Separation of isomers of cyclo(His-Pro) by high pressure liquid chromatography.** High pressure liquid chromatography was run on Waters Associates equipment using a μBondapak C$_{18}$ column (Waters). Isocratic elution with MeOH:NH$_4$HCO$_3$, 0.01 M (5:95) was run at a flow rate of 2.5 ml/min. The *vertical arrow* indicates the point at which 5 μl of a 5 mM solution of the indicated compounds were injected onto the column. *A*, elution profile of chemically synthesized (3, 9) cyclo(His-Pro); 300 mg of cyclo(His-Pro) was heated in alkaline solution, lyophilized, and dissolved in 5 ml of water. Aliquots of 100 μl each were fractionated as shown in *A*, and the fractions corresponding to Peaks I and II were collected. After lyophilization, the peak materials were dissolved in saline. *B*, elution profile of purified Peak I material. *C*, elution profile of purified Peak II material.

this material to the HPLC column with collection of the appropriate fractions resulted in purified preparations of Peak I (Fig. 4, *Panel B*) and Peak II (Fig. 4, *Panel C*).

The possibility that Peaks I and II were related stereoisomers of cyclo(His-Pro) was tested. Samples of the purified peaks (see Fig. 4, *Panels B* and *C*) were hydrolyzed in acid. Circular dichroism spectra of the hydrolysates and control mixtures of D and L isomers of histidine and proline were taken. The results of these studies (Fig. 5) establish that Peaks I and II differ in their stereoisomeric constitution. The spectrum of Peak II (*Panel A*) is similar to that of the mixture of L-histidine and L-proline (*Panel B*), indicating that the major component seen in HPLC chromatograms (Fig. 4, *Panel A*) is probably identical with the naturally occurring form of cy-

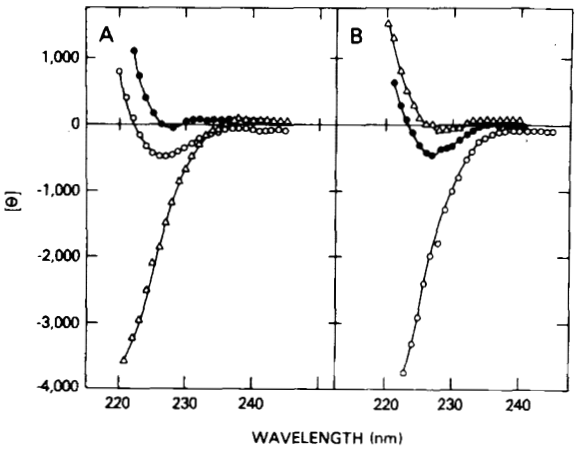

FIG. 5. **Circular dichroism spectra of hydrolysates of isomers of cyclo(His-Pro).** Samples (0.5 mg) of the isolated Peak I and Peak II fractions from the HPLC purification of cyclo(His-Pro) (see Fig. 4) were dissolved in 1 ml of 6 N HCl and hydrolyzed under N_2 at 105°C for 16 h. Control mixtures containing 0.25 mg of the D or L isomers of histidine and proline were treated similarly. After hydrolysis in acid, the samples were evaporated to dryness and dissolved in 3 ml of potassium phosphate (0.1 M, pH 7.0). Circular dichroism spectra were taken in a Cary 60 polarimetric instrument in a cell with a 1-cm path length. *A*, ○——○, L-histidine plus D-proline; △——△, D-histidine plus D-proline; ●——●, Peak II. *B*, ○——○, D-histidine plus L-proline; △——△, L-histidine plus L-proline; ●——●, Peak I.

clo(His-Pro). The spectrum of Peak I (*Panel B*) is similar to that of the mixture of L-histidine and D-proline (*Panel A*), indicating that alkaline treatment of cyclo(L-His-L-Pro) results in racemization of the proline residue. These data are fully in accord with previous observations on the stereoselective equilibration of proline-containing diketopiperazines (17–19). The general finding has been that, in alkaline media, diketopiperazines containing proline undergo isomerization by epimerization of the α-carbon of the proline residue, while the other amino acid retains its original absolute configuration.

The phenylazo derivative of cyclo(His-Pro) used to prepare the antigen for immunization could be separated by HPLC into six fractions (Fig. 1), some of which contain the D isomer of proline. It was, therefore, essential to establish whether the antiserum could distinguish one isomer of cyclo(His-Pro) from the other. The preparations of isolated Peaks I and II from the HPLC column (Fig. 4) were compared with unfractionated cyclo(His-Pro) containing approximately 90% Peak I for their capability to displace [Pro-^{3}H]cyclo(His-Pro) from the antiserum (Table II). It is apparent that Peak II, the form that probably exists naturally, is recognized by the antiserum, while peak I, the unnatural isomer, is not. These data indicate that, if care is taken to completely extract the cyclo(His-Pro) from tissue under conditions that avoid isomerization, the antiserum offers a highly specific tool for the detection of this peptide.

It should be noted that, while the antiserum used here can distinguish between the diastereomers of cyclo(His-Pro), both of the forms appear to be biologically active in at least one test system. Both peptides produce hypothermia in cold-exposed rats (4), although Peak II appears to be somewhat more effective (data not shown).

The Amount and Condition of Cyclo(His-Pro) in Brain Extracts—The amount of cyclo(His-Pro) in an extract of rat brain was in the range of 275 to 565 pmol as determined by immunoassay of supernatant solutions of extracts. Similar values were obtained whether the brain was extracted with water, 0.5 N CH_3COOH, 0.5 N NH_4HCO_3 (pH 7.5), 0.5 N NH_4OH (pH 10.3), or PBS. Similar amounts were recovered

TABLE II

Test of reaction of diastereomers of cyclo(His-Pro) with antiserum

Material from HPLC Peaks I and II (see legend to Fig. 4) was diluted with PBS to a concentration of 0.1 μM. It was then tested together with a preparation of cyclo(His-Pro) not previously purified by HPLC for competition of binding of [*Pro*-^{3}H]cyclo(His-Pro) to antiserum. The conditions for the radioimmunoassay were as described under "Materials and Methods." In the absence of competitors, the binding to antibody was 652 cpm (corresponding to 6.2% of the input counts).

Compound	Picomoles	Counts per min bound
		% of control
Unfractionated cyclo(His-Pro)	1	77
Unfractionated cyclo(His-Pro)	5	50
Cyclo(L-His-D-Pro) (HPLC Peak I)	1	103
Cyclo(L-His-D-Pro) (HPLC Peak I)	5	113
Cyclo(L-His-L-Pro) (HPLC Peak II)	1	77
Cyclo(L-His-L-Pro) (HPLC Peak II)	5	53

TABLE III

Distribution of bound cyclo(His-Pro) across membrane filter

The brain from one rat was homogenized in 9 ml of PBS containing 30,000 cpm of [^{3}H]cyclo(His-Pro). The homogenate was centrifuged at 20,000 × *g* for 20 min, and the supernatant solution was concentrated to 1.3 ml by passage through an Amicon PM-10 membrane. The filtrate (6.5 ml) and retentate were assayed for radioactivity and for material that would compete for binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum (immunoassayable cyclo(His-Pro)). (See "Materials and Methods.")

Fraction	Immunoassayable cyclo(His-Pro)		Carrier [^{3}H]cyclo(His-Pro)	
	Picomoles	Percentage of total	Counts per min	Percentage of total
Retentate	493	87	5,700	19
Filtrate	72	13	24,300	81

whether the brain was extracted with cold or boiling PBS. However, an indication for an unusual property of endogenous cyclo(His-Pro) was obtained under conditions of MeOH extraction. TRH was recovered in good yield, but only 20% of the cyclo(His-Pro) was extracted.

The data of Table III provide evidence that endogenous cyclo(His-Pro) is almost exclusively recovered in a bound form. A PBS extract of rat brain was concentrated approximately 7-fold by pressure ultrafiltration through an Amicon PM-10 membrane (molecular weight exclusion limit approximately 10,000). While the added [^{3}H]cyclo(His-Pro) passed freely through the membrane, only 13% of the cyclo(His-Pro) detected by the immunoassay was found in the filtrate; most of the cyclo(His-Pro) was associated with some material of molecular weight greater than 10,000.

A further demonstration of the association of endogenous cyclo(His-Pro) with a high molecular weight fraction involved gel filtration on Bio-Gel P-2. An aliquot of a PBS extract of rat brain was applied to a Bio-Gel P-2 column, and elution was carried out with PBS. As expected, added carrier [^{3}H]-cyclo(His-Pro) was included in the column and appeared at approximately Fraction 26 (Fig. 6, Panel *A*). In contrast, the material from the brain extract which displaced [^{125}I]iodo-cyclo(His-Pro) from the antiserum was found in the exclusion volume (approximately Fraction 15). This is consistent with the interpretation of the data from Table III that endogenous cyclo(His-Pro) is in a bound form.

Bound cyclo(His-Pro) can be converted to free cyclo(His-Pro) by boiling in PBS:MeOH (1:1). When a PBS extract of rat brain was boiled with MeOH and then fractionated on Bio-Gel P-2, the immunoassayable material was now included

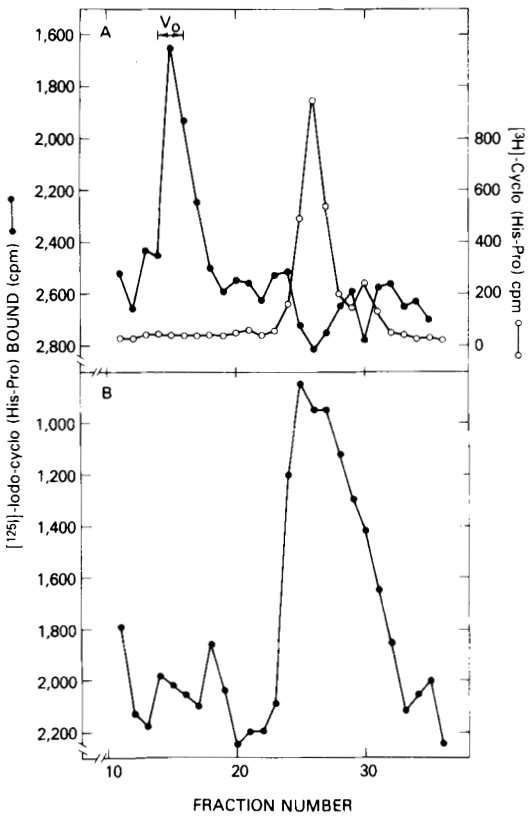

FIG. 6. **Chromatography on Bio-Gel P-2 of a rat brain extract.** *A*, one rat brain was homogenized for approximately 5 min in a motor driven Teflon-glass homogenizer in PBS (9 ml) containing [^{3}H]cyclo(His-Pro) (30,000 cpm), then centrifuged at 20,000 × *g* for 20 min. An aliquot (1 ml) of the supernatant solution was applied to a column (0.9 × 50 cm) of Bio-Gel P-2 previously equilibrated with PBS. Fractions (1.2 ml) were collected, and aliquots (0.1 ml) were assayed for competition of binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum as described under "Materials and Methods" (●——●). ○——○, radioactivity in the fractions corresponding to the elution profile of the added [^{3}H]cyclo(His-Pro) was determined by counting 0.1-ml aliquots of the fractions. V_0 corresponds to the region where blue dextran (Pharmacia) elutes from the column. *B*, an aliquot (4.5 ml) of the PBS extract described in *A* was mixed with MeOH (4.5 ml) and boiled for 10 min. An additional 4.5 ml of MeOH was added followed by another boiling treatment for 10 min. The mixture was then centrifuged at 20,000 × *g* for 20 min, and an aliquot (1.5 ml) of the supernatant solution was applied to a Bio-Gel P-2 column and fractionated as in *A*. Aliquots (200 μl) were tested for competition of binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum as in *A*.

in the column and appeared in the same fractions as [^{3}H]-cyclo(His-Pro) (Fig. 6, Panel *B*).

The size of bound cyclo(His-Pro) was estimated by filtration through Bio-Gel A-1.5m (Fig. 7). An aliquot of the supernatant solution of a PBS extract of rat brain was filtered through a column of Bio-Gel A-1.5m, and fractions were assayed for displacement of binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum. The distribution of immunoassayable material was heterogeneous. Fractions in the void volume (corresponding to molecular weight greater than 1.5 million) were positive, suggesting that endogeneous cyclo(His-Pro) might be associated with some large structures. However, the major portion of the immunoreactive material was eluted from the column in a region similar to that of bovine serum albumin, suggesting that bound cyclo(His-Pro) is primarily in the range of M_r = 70,000.

A set of conditions that resulted in extraction of cyclo(His-Pro) from rat brain in good yield and in a form that behaved on columns as did authentic cyclo(His-Pro) was developed (see "Materials and Methods"). The first part of the procedure involved two cycles of boiling a rat brain homogenate made in PBS:MeOH, followed by centrifugation. The properties of the supernatant solution were examined after chromatography on Bio-Gel P-2 (Fig. 8, *Panel A*). There was only a small amount of bound cyclo(His-Pro) detected by the radioimmunoassay for cyclo(His-Pro). The major portion of the immunoreactive material was in the region that corresponded to cyclo(His-Pro). However, the extract also contained another immunoreactive component that was eluted from the column later than cyclo(His-Pro).

In order to test whether the extraction of cyclo(His-Pro) from rat brain by the hot PBS:MeOH procedure described above was complete, the residue was re-extracted under the same conditions. Examination on Bio-Gel P-2 of the supernatant fraction after the second extraction showed no evidence for a peak of immunoreactive material (Fig. 8, *Panel B*). It was concluded that a single extraction with hot PBS:MeOH results in a quantitative release of cyclo(His-Pro) from the bound form but that another interfering material is also released into the extract.

The identity of the material in the second peak shown in Fig. 8, *Panel A* is not clear. It is not derived from cyclo(His-Pro), since boiling of [^{3}H]cyclo(His-Pro) in PBS:MeOH does not produce a second peak of radioactivity. It is not an artifact of the solvents, since boiling PBS:MeOH in the absence of brain homogenate produces no immunoreactive material. As indicated in Fig. 6, the supernatant solution from cold PBS extraction of brain subjected to boiling with MeOH produces no second peak, only cyclo(His-Pro). Therefore, it was concluded that the second peak is produced by solubilization of some factor derived from brain particulate material. The possibility that the second peak is derived from free histidine or proline has been excluded, since boiling of histidine or proline with PBS:MeOH followed by radioimmunoassay resulted in the formation of no detectable immunoreactive material. Neither does the material in the second peak appear

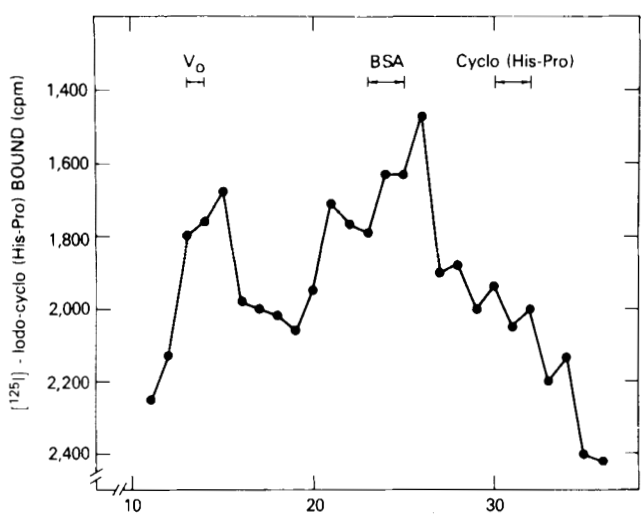

FIG. 7. **Chromatography on Bio-Gel A-1.5m of a cold PBS extract of rat brain.** One rat brain was homogenized with PBS (9 ml). The homogenate was centrifuged at 20,000 × *g* for 20 min. An aliquot (1.5 ml) of the supernatant solution was applied to a column (0.9 × 50 cm) of Bio-Gel A-1.5m (100 to 200 mesh) equilibrated with PBS. Elution was continued with PBS, and fractions (1.2 ml) were collected. Aliquots (0.2 ml) were assayed for competition of binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum (see "Materials and Methods"). The position of elution of standards of blue dextran, bovine serum albumin, and [^{3}H]cyclo(His-Pro) are shown by the *horizontal bars* labeled V_0, *BSA*, and *Cyclo(His-Pro)*, respectively.

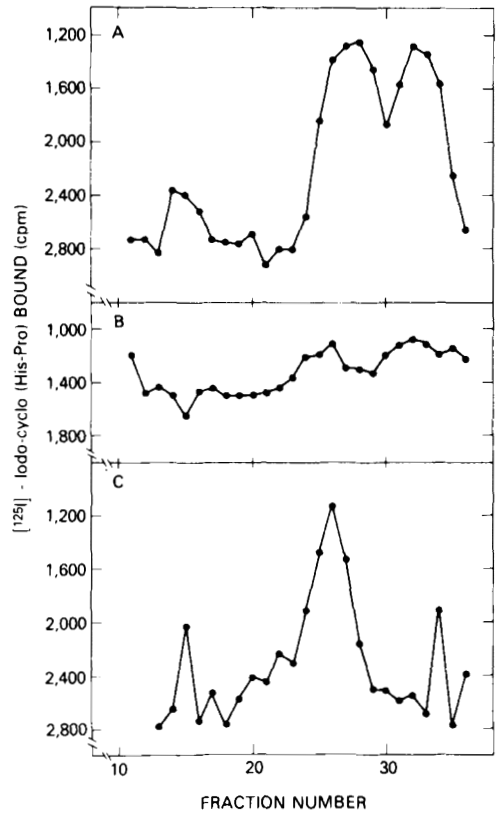

FIG. 8. **Chromatography on Bio-Gel P-2 of a PBS:MeOH extract of rat brain.** *A*, one rat brain was homogenized with 9 ml of PBS:MeOH (1:1), and the suspension was boiled for 10 min. After addition of another aliquot (4.5 ml) of MeOH, the suspension was boiled for another 10 min, then centrifuged at 20,000 × *g* for 20 min. An aliquot (1.5 ml) of the supernatant solution was fractionated on Bio-Gel P-2 as described in Fig. 6. Aliquots (0.2 ml) of the fractions (1.2 ml) were assayed for competition of the binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum (see "Materials and Methods"). *B*, A precipitate from one rat brain that had been subjected to extraction with PBS:MeOH as described in *A* was resuspended in 18 ml of PBS:MeOH and extracted by boiling for 10 min. After adding more MeOH (9 ml), the mixture was boiled for an additional 10 min, then centrifuged at 20,000 × *g* for 20 min. An aliquot (1.5 ml) was fractionated on Bio-Gel P-2 as described in Fig. 6. Aliquots (0.2 ml) were tested for displacement of binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum (see under "Materials and Methods"). *C*, a PBS:MeOH extract from one rat brain was prepared as described in *A*. The supernatant solution was diluted to 90 ml with water and fractionated on SP-Sephadex as described under "Materials and Methods." The fractions containing [^{3}H]cyclo(His-Pro) were pooled and lyophilized. The material was then dissolved in PBS (4.5 ml), and an aliquot (1.5 ml) was fractionated on Bio-Gel P-2 as described in Fig. 6. Aliquots (0.2 ml) of the fractions were tested for competition of the binding of [^{125}I]iodo-cyclo(His-Pro) to antiserum (see "Materials and Methods").

to be related to TRH, since it does not compete with TRH for binding to TRH-specific antiserum. The identity of the second peak, therefore, remains to be established.

Chromatography of the hot PBS:MeOH extract on SP-Sephadex was used to prepare samples of cyclo(His-Pro) from brain extracts that behaved as relatively pure cyclo(His-Pro) (Fig. 8, Panel *C*). The standard procedure that was adopted for preparation of samples of cyclo(His-Pro) for radioimmunoassay, therefore, involved extraction of the tissue with hot PBS:MeOH, fractionation of the supernatant solution on SP-Sephadex, lyophilization of the appropriate fraction, and immunoassay (see "Materials and Methods").

Further documentation that the radioimmunoassayable material prepared by the SP-Sephadex procedure is essentially all cyclo(His-Pro) was obtained by HPLC analysis. A sample of rat brain extract supplemented with carrier [^{3}H]-cyclo(His-Pro) was subjected to the SP-Sephadex purification procedure to produce a sample with a specific activity of 46 cpm/pmol of immunoassayable material. An aliquot of this material containing 24 pmol of immunoassayable cyclo(His-Pro) was fractionated by HPLC, and fractions were assayed for both radioactivity and immunoreactive material. The peak of radioactivity was superimposable on the peak of immunoassayable material; the specific activity was 55 cpm/pmol. These data indicate that the immunoreactive material is actually cyclo(His-Pro) and that the purity of the material prepared by SP-Sephadex is greater than 80%.

Subcellular and Regional Distribution of Cyclo(His-Pro) in Rat Brain—Studies on the subcellular distribution of TRH have supported the notion that TRH is enriched in synaptosomal vesicles (20). Two rat brains were homogenized in 0.32 M sucrose, the homogenate was then centrifuged at low speed, and the sedimented nuclear fraction was discarded. The supernatant solution was then centrifuged at 20,000 × *g*, and the pelleted fraction (crude mitrochondrial fraction) was resuspended in 10 ml of 0.32 M sucrose. Samples of the original homogenate and of the crude mitochondrial fraction were subjected to the standard procedure for extraction and purification of cyclo(His-Pro) from brain tissue (see "Materials and Methods" and Table IV). The purified material was assayed for cyclo(His-Pro) content by radioimmunoassay. In essential agreement with the studies of Winokur *et al.* (20), the data in Table IV show that approximately 60% of the total TRH is localized in the crude mitochondrial fraction (which contains the synaptosomes), resulting in an approximately 50% enrichment of TRH in this fraction. In contrast only about 20% of the total cyclo(His-Pro) is found in the crude mitochrondial fraction such that the concentration of the peptide is only 50% as great as in the original homogenate. It thus appears that, while TRH is substantially concentrated in synaptosomes, cyclo(His-Pro) is not.

The distribution of TRH and cyclo(His-Pro) in various regions of rat brain was determined (Table V). In agreement with a number of other studies, TRH occurs in highest concentration in posterior pituitary and in hypothalamus (21). Cyclo(His-Pro) shows a similar distribution; pituitary and hypothalamus have the highest concentrations. However, the concentration of cyclo(His-Pro) in both anterior and posterior pituitary is higher than in hypothalamus; this is not the case

TABLE IV

Subcellular distribution of TRH and histidyl-proline diketopiperazine

The brains from two rats were homogenized in 40 ml of 0.32 M sucrose, and the homogenate was centrifuged for 10 min at 1,000 × *g*. The precipitate (nuclear fraction and debris) was discarded, and the supernatant solution was centrifuged for 20 min at 15,000 × *g*. The supernatant solution was discarded, and the precipitate (crude mitochondrial fraction) was resuspended in 10 ml of 0.32 M sucrose. After addition of PBS (20 ml), the solution was heated in a boiling water bath for 10 min and then centrifuged at 20,000 × *g* for 20 min. The supernatant solution was fractionated on SP-Sephadex (see "Materials and Methods," "Standard Procedure for Extraction of Cyclo(His-Pro) from Brain" for radioimmunoassay) to purify the cyclo(His-Pro). A similar purification was followed for an aliquot of the homogenate. The concentrations of TRH and cyclo(His-Pro) in the homogenate and crude mitochondrial fractions were determined by radioimmunoassay (see "Materials and Methods.")

Fraction	Total protein	TRH	Cyclo-(His-Pro)	TRH	Cyclo-(His-Pro)
	mg	*pmol/brain*		*pmol/mg protein*	
Homogenate	227	80	547	0.35	2.40
Crude mitochondrial fraction	78	47	105	0.60	1.34

TABLE V

Distribution of TRH and cyclo(His-Pro) in regions of rat brain

The brains from 10 rats (approximately 300 g of body weight) were removed and dissected as indicated. The regions from the 10 animals were pooled, weighed, and homogenized in PBS:MeOH (1:1) containing [^{3}H]cyclo(His-Pro) (60,000 cpm for other brain areas; 30,000 cpm for all other samples). The volumes used for homogenization were: anterior and posterior pituitary, 2 ml; ventral and dorsal hypothalamus, 6 ml; cerebellum and pons medulla, 20 ml; other brain areas, 60 ml. The homogenates were subjected to the standard procedure for extraction of cyclo(His-Pro) (see "Materials and Methods"). The purified samples were then dissolved in PBS and assayed for cyclo(His-Pro) and TRH content by the radioimmunoassay (see "Materials and Methods"). The values reported are corrected for recovery of the added carrier [^{3}H]cyclo(His-Pro). The two sets of figures were obtained from assays from a duplicate set of 10 animals.

Brain region	Wet weight (10 brains)	TRH	Cyclo(His-Pro)
	mg	*pmol/mg*	
Anterior pituitary	110; 88	0.125; 0.107	1.027; 1.033
Posterior pituitary	27; 16	0.382; 0.714	2.011; 2.394
Ventral hypothalamus	516; 572	0.388; 0.570	0.514; 0.353
Dorsal hypothalamus	562; 627	0.237; 0.248	0.376; 0.258
Pons and medulla	2,876; 2,918	0.075; 0.073	0.242; 0.203
Cerebellum	2,927; 2,757	0.01; 0.034	0.187
Other brain areas	12,710; 12,289	0.033; 0.041	0.139; 0.139
Total picomoles per brain		102; 131	365; 372

for TRH. While the cerebellum and other brain areas have very low concentrations of TRH, these regions have significant concentrations of cyclo(His-Pro).

A determination was made of the total amount of TRH and cyclo(His-Pro) in brains of rats (n = 15, body weight range 140 to 450 g) extracted by the standard procedure (see under "Materials and Methods"). The range of values for TRH was 113 to 210 pmol/brain. This is somewhat higher than values reported in the literature (13, 20, 21) for rat brain extracted with MeOH (40 to 120 pmol/brain). In our hands, extraction of rat brain with hot MeOH or water gave a range of 45 to 64 pmol of TRH/brain. This suggests that the standard procedure described under "Materials and Methods" results in a somewhat higher yield of TRH than that obtained by methods generally used. The range of concentration of cyclo(His-Pro) we detected in rat brain was 275 to 565 pmol/brain. These studies indicate that rat brain contains approximately 2.5 times as much cyclo(His-Pro) as TRH.

DISCUSSION

Histidyl-proline diketopiperazine has a variety of effects in the central nervous system; it plays a role in regulation of body temperature (4), in modulation of cGMP levels (5), and as an anti-depressant factor (3). In the pituitary, the peptide operates as a prolactin inhibitory factor, opposing the releasing factor activity of TRH (7, 8).

Many of the experiments that might be visualized to explore further the biology of cyclo(His-Pro) depend on the availability of a specific and sensitive assay for this compound. As a result of development of the specific rabbit antiserum for cyclo(His-Pro) reported here, such an assay is at hand. This antiserum could be used to determine not only normal levels and organ distribution of the peptide in brain as described here, but also to explore changes in the concentration of cyclo(His-Pro) under various physiological conditions. Administration to animals of other factors such as hormones and drugs, followed by assay for cyclo(His-Pro) in brain or body fluids, might reveal some of the regulatory processes that influence the metabolism of TRH. TRH has been detected not only in brain, but in pancreas (22) and gastrointestinal tissue (21). TRH immunoreactivity has been reported to be present in blood and urine (23), but the identity of this material with authentic TRH has been questioned (24). The occurrance of cyclo(His-Pro) in these tissues remains to be explored but is amenable to study with the techniques described here.

A neurotransmitter role has been assigned to TRH based on the localization of a major fraction of the peptide in synaptic vesicles and the demonstration that TRH can be released from synaptosomes by high concentrations of K^+ (25). The studies described here suggest that while cyclo(His-Pro) is present in nerve tissue, the major fraction of the peptide is not packaged in synaptic vesicles. Therefore, while the peptide does produce a variety of central nervous system effects, a classical neurotransmitter role (26) for it is in question.

The major fraction of cyclo(His-Pro) is not found in a free form in brain. Most of the peptide extracted from rat brain under mild conditions is associated with a high molecular weight material. The nature of this material remains to be characterized. All that is known thus far is that the molecular weight is in the range of 70,000 and that the linkage of cyclo(His-Pro) to the factor is stable to high or low pH and to boiling. The material is soluble in aqueous buffer but insoluble in MeOH. The peptide can be dissociated from the factor by boiling in PBS:MeOH (1:1). These data suggest that cyclo(His-Pro) is linked to the factor in association with some lipid rather than in an ionic or covalent form. There is precedence for a number of hormones such as steroids (27) to interact with high molecular weight factors. Some of the factors in tissues are receptors; others may be carriers or reservoirs. Blood serum contains proteins (corticosteroid-binding globulin, testosterone-estradiol-binding globulin) which form stoichiometric complexes with steroid hormones. These proteins vary in molecular weight between 50,000 to 90,000. The binding which is noncovalent and dissociable is accounted for mainly by hydrophobic forces and hydrogen bonds. The complexes of steroids with the binding globulins are biologically inactive; dissociation to the free hormone is associated with biological activity. It has been suggested that the binding to globulins allows a large amount of steroid to be carried in a storage form. The biological activity of the bound form of cyclo(His-Pro) or whether it may act as a storage form of cyclo(His-Pro) remains to be established. It is also conceivable that the bound form of cyclo(His-Pro) might serve as a precursor to free cyclo(His-Pro). If so, it is unlikely that such a precursor is structurally related to TRH, since TRH is stable to boiling in PBS:MeOH (1:1).

TRH is found in highest concentration in pituitary and hypothalamus, consistent with its role as a hypothalamic-releasing factor (21). The highest concentrations of cyclo(His-Pro) are also found in these two areas. The metabolism of TRH to cyclo(His-Pro) in pituitary may play an important role in effectively terminating its action, since the dipeptide has been shown to oppose the action of TRH in promoting the release of prolactin (7, 8).

The total amount of the diketopiperazine in rat brain plus pituitary (275 to 565 pmol/brain) is approximately 2.5 times that of TRH. These findings, together with the observation that cyclo(His-Pro) is more potent than TRH in reversing the central nervous system depressant action of ethanol emphasizes the important physiological role played by this unique dipeptide.

Acknowledgment—Drs. Irwin Chaiken, Louis Cohen, John Folk, Erhard Gross, Werner Klee, and Kenneth Kopple generously pro-

vided their time for valuable discussions. We thank Gladys Deibler of the National Institute of Mental Health for the amino acid analyses.

REFERENCES

1. Morley, J. E. (1979) *Life Sci.* **25,** 1539–1550
2. Prasad, C., and Peterkofsky, A. (1976) *J. Biol. Chem.* **251,** 3229–3234
3. Prasad, C., Matsui, T., and Peterkofsky, A. (1979) *Nature* **268,** 142–144
4. Prasad, C., Matsui, T., Williams, J., and Peterkofsky, A. (1978) *Biochem. Biophys. Res. Commun.* **85,** 1582–1587
5. Yanagisawa, T., Prasad, C., Williams, J., and Peterkofsky, A. (1979) *Biochem. Biophys. Res. Commun.* **86,** 1146–1153
6. Battaini, F., and Peterkofsky, A. (1980) *Fed. Proc.* **39,** 594
7. Bauer, K., Gräf, K. J., Faivre-Bauman, A., Beier, S., Tixier-Vidal, A., and Kleinkauf, H. (1978) *Nature* **274,** 174–175
8. Enjalbert, A., Ruberg, M., Arancibia, S., Priam, M., Bauer, K., and Kordon, C. (1979) *Eur. J. Pharmacol.* **58,** 97–98
9. Matsui, T., Prasad, C., and Peterkofsky, A. (1979) *J. Biol. Chem.* **254,** 2439–2445
10. Ling, N., Leppäluoto, J., and Vale, W. (1976) *Anal. Biochem.* **76,** 125–133
11. Pradelles, P., Morgat, J. L., Frogmageot, P., Oliver, C., Jacquet, P., Gourdji, D., and Tixier-Vidal, A. (1972) *FEBS Lett.* **22,** 19–22
12. Bassiri, R. M., and Utiger, R. D. (1972) *Endocrinology* **90,** 722–727
13. Winokur, A., and Utiger, R. D. (1974) *Science* **185,** 265–267
14. Koch, Y., Baram, T., and Fridkin, M. (1976) *FEBS Lett.* **63,** 295–298
15. Visser, T. J., Klootwijk, W., Docter, R., and Hennemann, G. (1976) *Neuroendocrinology* **21,** 204–213
16. Nagai, W., Kirk, K. L., and Cohen, L. A. (1973) *J. Org. Chem.* **38,** 1971–1974
17. Liberek, B., Bednarek, M., Kitowska, A., Kozlowska, M., and Macikowska, A. (1976) in *Proceedings of the Fourteenth European Pepide Symposium* (Loffet, A., ed), pp. 641–646, University of Brussels
18. Ott, H., Frey, A. J., and Hoffman, A. (1963) *Tetrahedron* **19,** 1675–1684
19. Vicar, J., and Blaha, K. (1973) *Collect. Czech. Chem. Commun.* **38,** 3307–3311
20. Winokur, A., Davis, R., and Utiger, R. D. (1977) *Brain Res.* **120,** 423–434
21. Leppäluoto, J., Koivusalo, F., and Kraama, R. (1978) *Acta Physiol. Scand.* **104,** 175–179
22. Koivusalo, F., and Leppäluoto, J. (1979) *Life Sci.* **24,** 1655–1658
23. Jackson, I. M. D., and Reichlin, S. (1974) *Life Sci.* **14,** 2259–2266
24. Youngblood, W. W., Lipton, M. A., and Kizer, J. S. (1978) *Brain Res.* **151,** 99–116
25. Warberg, J., Eskay, R. L., Barnea, A., Reynolds, R. C., and Porter, J. C. (1977) *Endocrinology* **100,** 814–825
26. Snyder, S. H., and Innis, R. B. (1979) *Annu. Rev. Biochem.* **48,** 755–782
27. Westphal, U. (1978) in *Receptors and Hormone Action* (O'Malley, B. W., and Birnbaumer, L. eds) Vol. II, pp. 443–472, Academic Press, New York