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[^{3}H]CYCLO(HISTIDYL-PROLINE) IN RAT TISSUES: DISTRIBUTION, CLEARANCE AND BINDING

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SUMMARY: Cyclo(Histidyl-Proline), a metabolite of TRH, has been demonstrated to have a number of biological activities. The clearance, distribution and binding of the peptide in the rat was studied. Cyclo(His-Pro) was cleared from the circulation biphasically (t1/2 = 1.25 and 33 min). Unmetabolized cyclo(His-Pro) appeared rapidly in urine. Accumulation of [^{3}H]cyclo(His-Pro) in adrenal, liver and kidney was demonstrated. Membrane preparations from adrenal and liver, but not from kidney, brain, pituitary, and other tissues were shown to bind cyclo(His-Pro) specifically.

INTRODUCTION: Cyclo(Histidyl-Proline), a metabolite of TRH[1], has been reported to inhibit prolactin release from the pituitary. The peptide also has a variety of central effects including antagonism of central nervous system depression, hypothermia, changes in cyclic nucleotide concentrations, stereotypic behavior and stress-induced eating. Some of these effects have been reviewed recently (1). The possibility that cyclo(His-Pro) has a distinct set of receptors and a mechanism for terminating its action has not previously been explored. The experiments reported here deal with this subject. The peptide is cleared rapidly from the blood and accumulates in the urine of the rat in its original form. It was established that the peptide accumulates in some rat tissues. Binding of [^{3}H]cyclo(His-Pro) to membrane preparations derived from adrenal and liver was demonstrated.

Materials and Methods: Male Sprague-Dawley rats were from Taconic Farms, N.Y. Frozen bovine adrenals were from Rockland Farms, Gilbertsville, Pa. TRH was purchased from Calbiochem. Cyclo(His-Pro) was synthesized as described (2). [^{3}H]Cyclo(His-Pro) (58 Ci/mmole) was prepared by catalyzed halogen-tritium replacement of (2,4-diiodohistidyl)-cyclo(His-Pro) (3). Paper chromatography conditions and the procedure for thin-layer chromatography on

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2. Abbreviations used: histidyl-proline diketopiperazine, cyclo(His-Pro); thyrotropin releasing hormone, TRH; PBS, phosphate-buffered saline (0.1 M potassium phosphate, pH 7.2, and 0.14 M NaCl).

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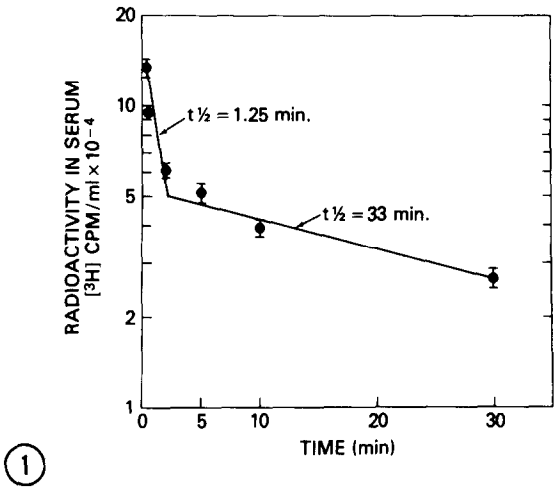

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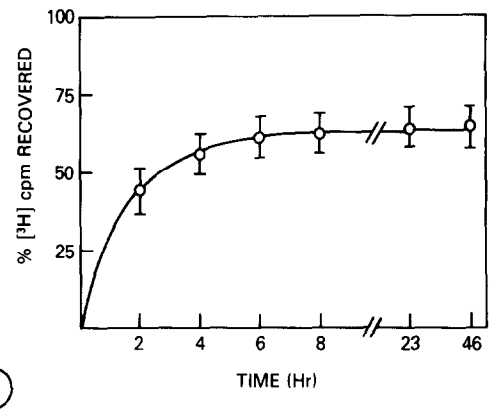

(2)

Fig. 1 Clearance of radioactivity derived from [^{3}H]cyclo(His-Pro) from serum of rats. [^{3}H]Cyclo(His-Pro) (4 x 10^6 cpm in 1 ml of PBS) was injected into the tail vein of Sprague-Dawley male rats (150 gm body weight, n = 8) under light ether anesthesia. At the indicated times, 0.1-0.2 ml of blood was withdrawn by heart puncture. The samples were centrifuged at 12,000 x g in a Microfuge and duplicate aliquots (20 μl) of the serum were counted in a Beckman LS-3150T liquid scintillation counter. Each point represents the mean ± SEM.

Fig. 2 Appearance of radioactivity derived from [^{3}H]cyclo(His-Pro) in urine of rats. [^{3}H]Cyclo(His-Pro) (6 x 10^6 cpm in 750 μl of PBS) was injected into the tail vein of Sprague-Dawley male rats (250 gm body weight, n = 2) while the animals were housed in a restraining cage. The animals were then transferred to metabolic cages. Urine was collected at the indicated times and samples (100 μl), in duplicate, were counted as in Fig. 1. The data are expressed as the cumulative recovery of the radioactivity injected into the animals. Each point represents the mean ± SEM.

reverse-phase plates (Whatman) are described in the legend to Fig. 3. Radioactive cyclo(His-Pro) was detected by scintillation counting while unlabeled cyclo(His-Pro) was detected by spraying with the Pauly reagent (4). Injections of [^{3}H]cyclo(His-Pro) into the lateral ventricle of the brain of rats were performed as described (5). Injection of [^{3}H]cyclo(His-Pro) into the tail vein of rats is described in the legends to Figs. 1 and 2. Preparation of membranes was as described in Table 1.

RESULTS

Radioactive cyclo(His-Pro) was injected into the tail vein of rats and blood was withdrawn at intervals over a period of 30 min (Fig. 1). The kinetics of clearance suggested the involvement of at least two compartments. The first component of the disappearance curve had a t1/2 of 1.25 min, while the second component had a t1/2 of 33 min.

The disappearance of radioactivity from rat serum was paralleled by rapid appearance of radioactivity in the urine. Five minutes after the injection of [^{3}H]cyclo(His-Pro), radioactivity per unit volume in urine was 100 times higher than that in blood. Within 2 hr, 45% of the administered radioactivity was recovered; between 60-70% recovery was observed within 6-8 hr (Fig. 2).

TABLE 1. BINDING OF [^{3}H]Cyclo(HIS-PRO) TO CRUDE MEMBRANE PREPARATIONS OF VARIOUS TISSUES

Tissue	fmol bound/ mg wet weight
Bovine	
Adrenal cortex	26.6
Adrenal medulla	7.7
Rat	
Adrenal	10.5
Liver	10.5
Kidney	0
Small intestine	0
Testis	0
Pituitary	0
Hypothalamus	0
Cerebral cortex	0

Samples (200-300 mg wet weight) of the indicated tissues were minced with scissors and suspended in 20 volumes of 20 mM Tris, pH 7.5. The suspensions were homogenized for 20 sec (Polytron, setting 2.5). The homogenates were filtered through 4 layers of gauze and then centrifuged at 12,000 x g for 20 min. The pellets were resuspended in the same volume of buffer and recentrifuged. The resultant pellets were suspended in buffer by homogenization for 5 sec to a concentration of 20 mg original tissue wet weight/ml. Aliquots (0.3 ml) of homogenate in 10 x 75 mm glass tubes were incubated with [^{3}H]cyclo(His-Pro) (50,000 cpm in 20 μl) and either H_2O (20 μl) or excess unlabelled cyclo(His-Pro) (20 μl) at 0°C for one hour. The final concentration of cyclo(His-Pro) in these incubation mixtures was either 2.5×10^{-9} M or 10^{-4} M. The mixtures were deposited on Whatman GF/B filters (presoaked in gelatin solution; 0.5 mg/ml), the tubes were rinsed with 5 ml of cold Tris buffer and the filters washed with an additional 5 ml of Tris buffer. The filters were dried and counted as described in the legend to Fig. 3. The data are presented as specific binding in fmol bound/mg wet weight of original tissue. Blank tubes containing excess unlabelled cyclo(His-Pro) had binding values never exceeding 10% of the total binding in the absence of excess cyclo(His-Pro). These values were subtracted from the total binding to obtain the tabulated specific binding.

The possibility that cyclo(His-Pro) was metabolized was investigated by chromatographic examination of the radioactive material in rat blood and urine. Figure 3 presents evidence that the radioactive material recovered from both blood and urine behaves like cyclo(His-Pro) by paper chromatography (Fig. 3, panel A) and reverse-phase chromatography (Fig. 3, panels B and C). Similar results were obtained whether [^{3}H]cyclo(His-Pro) was injected intravenously or into the lateral ventricle of the brain. It therefore appears that administered cyclo(His-Pro) is excreted in the urine in an unchanged form.

There was incomplete recovery of administered radioactive cyclo(His-Pro) in the urine of rats (Fig. 2). Furthermore, no more than 3% of the radioactivity was found in feces after 24 hr (data not shown). It was therefore of interest to examine body tissues for possible accumulation of [^{3}H]cyclo(His-Pro).

The distribution of radioactivity in various rat tissues and body fluids one hour after intravenous injection of [^{3}H]cyclo(His-Pro) was determined (Fig. 4). Some tissues contained radioactivity at levels close to that of serum; these include hypothalamus, anterior pituitary and heart as well as

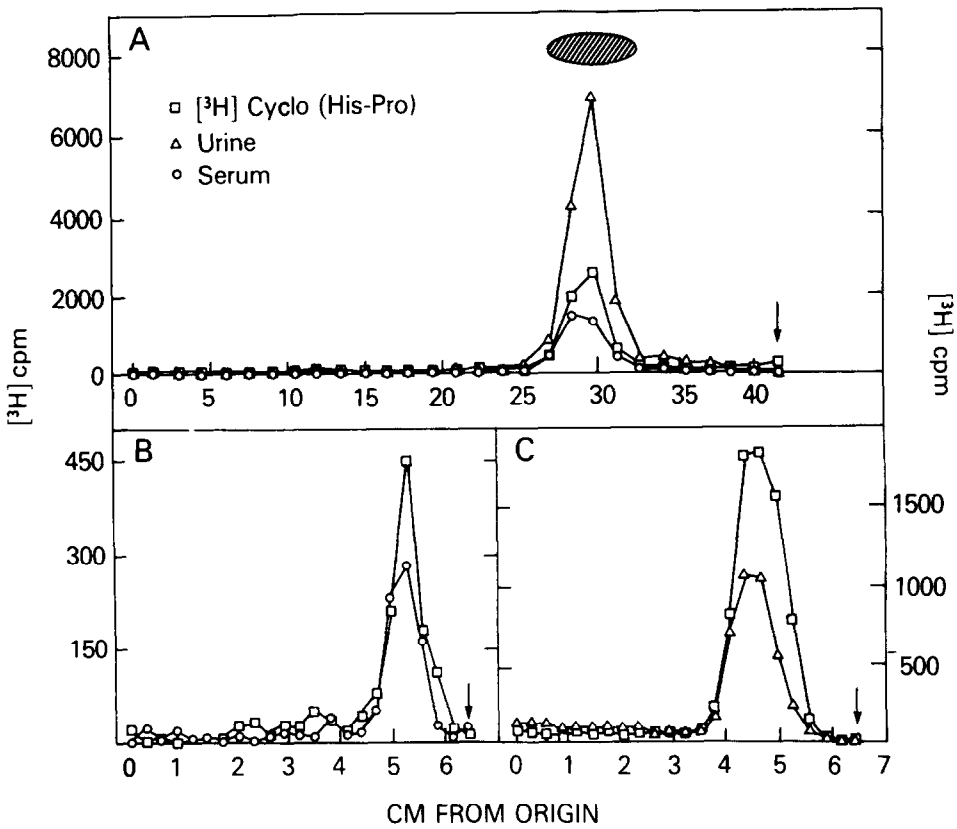

Fig. 3 Chromatography of serum and urine of rats that had received [^{3}H]cyclo(His-Pro). Panel A. [^{3}H]cyclo(His-Pro) was injected into two groups of rats (4 in each group) as described in the legend to Fig. 1. After 5 min, one group of animals was decapitated and the trunk blood was collected and centrifuged. The other group of animals was sacrificed after 60 min and urine was collected by bladder puncture. Urine (one ml) and serum (12 ml) were mixed with 3 volumes of methanol and centrifuged for 15 min at 1000 x g. The precipitates were then washed with one volume of methanol. The combined supernatant solutions were evaporated (Savant Speed-Vac Concentrator, Savant Instruments, Hicksville, N.Y.) to dryness and the residues were extracted with methanol. This procedure was repeated several times and the final residues were dissolved in 0.5 ml of methanol. [^{3}H]cyclo(His-Pro) (1.5 ml in 50 mM Tris, pH 7.5, 6 x 10^6 cpm) was carried through the same procedure. Recovery of radioactivity through the extraction procedure was between 40-55%. Aliquots of the methanol extracts containing 20,000 cpm derived from the original [^{3}H]cyclo(His-Pro), 12,000 cpm derived from serum and 40,000 cpm derived from urine were applied in 2.5 cm stripes to Whatman 3MM paper and ascending chromatography (3) was carried out for 16 hr using a solvent of $CHCl_3$:CH_3OH:H_2O, 5:5:1. The chromatogram was dried and cut into 1.5 cm segments (6 cm wide) spanning the region from the origin to the solvent front. The strips were soaked in 1 ml of 2-methoxyethanol and then counted with 10 ml of triton-toluene based scintillation fluid. Recovery of applied radioactivity in all fractions was approximately 50%. The cross-hatched area represents the position of authentic cyclo(His-Pro) detected by the Pauly reagent (4). The arrow represents the position of the solvent front. Panel B. An aliquot of the methanol extract from serum prepared as described in Panel A containing 1300 cpm was applied to a 1 x 3 in Whatman $MKC_{18}F$ reversed phase thin layer plate. In a second position, a similar aliquot of serum was deposited together with an aliquot of the methanol extract derived from the original [^{3}H]cyclo(His-Pro) containing 1800 cpm. Ascending chromatography was carried out in n-propanol:H_2O (80:20) for approximately 30 min. The plate was dried and segments of 3 mm (12 mm wide) were scraped into scintillation vials, soaked in 1 ml of 2-methoxyethanol and then counted with 10 ml of triton-toluene based scintillation fluid. Approximately 75% of applied radioactivity was recovered

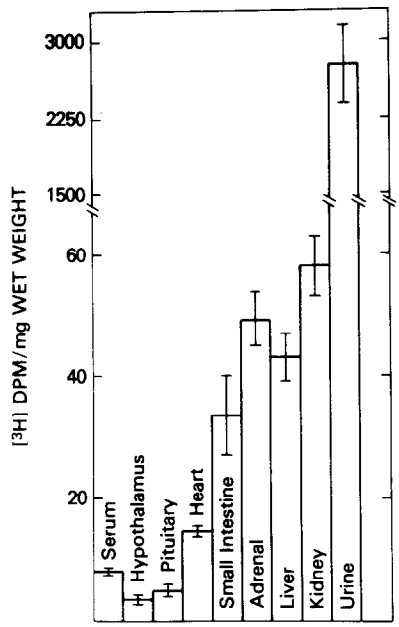

Fig. 4 Distribution of [^{3}H]cyclo(His-Pro) in various rat tissues and body fluids. [^{3}H]Cyclo(His-Pro) (4 x 10^6 cpm in 1 ml) was injected into Sprague-Dawley male rats (200 gm body weight, n = 5) as described in the legend to Fig. 1. One hour later, the animals were decapitated and both trunk blood and urine were collected. Pieces of the indicated organs were removed, weighed, minced and dissolved in 1 ml of tissue solubilizer (NCS, Amersham; 55°C for 24 hr). The samples were then counted in the liquid scintillation counting mixture described by Neame (6). Counts (expressed as dpm/mg wet weight of tissue) were corrected for quenching by use of a quench curve and the external standard channels ratio in a Beckman LS-3150T counter. Each point represents the mean ± SEM.

cerebellum, testis, prostate, muscle, pancreas, lung and duodenum (data not shown). Other tissues contained substantially higher levels of radioactivity than did serum, suggesting a specific accumulation of the peptide in those tissues. These include small intestine, adrenal, liver and kidney. The highest level of radioactivity was found in urine. The nature of the radioactive material associated with liver was examined after extraction of the tissue with methanol; essentially all the radioactivity in the tissue was recovered in the methanol extract. Chromatographic studies (data not shown) indicated that the material was identical with cyclo(His-Pro).

The data in Fig. 4 suggested that some rat tissues have the capacity to accumulate cyclo(His-Pro). These tissues were therefore considered as possible

in the fractions. The values for radioactivity in the [^{3}H]cyclo(His-Pro) were calculated by subtracting the counts found in the corresponding fractions from serum from the counts found in the fraction containing both serum and the [^{3}H]cyclo(His-Pro) standard. Panel C. An aliquot (5 µl) of urine recovered from animals injected with [^{3}H]cyclo(His-Pro) (see Fig. 2) containing 4200 cpm was applied to a reversed-phase thin layer plate as described in Panel B. An aliquot of [^{3}H]cyclo(His-Pro) containing 7000 cpm was dissolved in normal rat urine (5 µl) and applied to a second position on the plate. Ascending chromatography was carried out in ethanol:H_2O (80:20) for approximately 30 min. The plate was processed for counting as in Panel B.

candidates for the demonstration of specific binding sites for cyclo(His-Pro). Crude membrane preparations of a variety of tissues (bovine and rat) were tested for binding of [^{3}H]cyclo(His-Pro). There was no detectable binding to membranes derived from rat kidney, small intestine, testis, pituitary, hypothalamus and cerebral cortex (Table 1), even when the concentration of membranes was increased by a factor of 10 (data not shown). Specific binding of cyclo(His-Pro) was detected in membranes prepared from rat liver or adrenal. The binding activity in adrenal membranes was approximately 10 times higher than in liver membranes. In order to find a more precise localization of the binding activity in adrenal, membranes were prepared from bovine adrenal cortex and bovine adrenal medulla. The data in Table 1 indicate that binding to adrenal cortex membranes is approximately 3.5 times greater than to adrenal medulla membranes. Unlabeled cyclo(His-Pro) displaces [^{3}H]cyclo(His-Pro) from bovine adrenal cortex membranes with a K_D of approximately 1 μM (data not shown).

DISCUSSION

On the basis of the observations that cyclo(His-Pro) regulates a variety of biological activities in the central nervous system, it might be suggested that this peptide functions as a neuromodulator. Such a compound would be expected to bind specifically to membranes in the target tissue and turn over rapidly. The studies reported here provide some insight into these questions.

Cyclo (His-Pro) appears not to be metabolized in rat blood or body tissues (see Fig. 3 and Results section) but accumulates rapidly, in an unchanged form, in the urine (Fig. 2 and Refs. 7 and 8). The kinetics of disappearance of cyclo(His-Pro) from the circulation are biphasic. Similar complex kinetics of disappearance of TRH have been previously reported (9, 10).

Based on the previous studies (1) with cyclo(His-Pro), it might be anticipated that specific receptors for the peptide would be present in hypothalamus and/or pituitary. The studies reported here indicate that there is no observable accumulation or binding of the peptide in rat brain. However, as indicated in Table 1, specific binding of cyclo(His-Pro) is observed in rat liver and adrenal membranes. The apparent accumulation of cyclo(His-Pro) in rat kidney (Fig. 4), unaccompanied by specific binding (Table 1), may be explained on the basis of the rapid accumulation of cyclo(His-Pro) in urine. Burt and Snyder (11) have reported binding of TRH to rat brain, pituitary and liver membrane preparations. It is noteworthy that the binding of cyclo(His-Pro) demonstrated here is displaced by cyclo(His-Pro) but requires about 100-fold higher concentrations of TRH for displacement. Therefore, the binding sites for cyclo(His-Pro) in rat adrenal and liver membranes appear to be different than the previously reported binding sites for TRH. The properties of these binding sites will be reported elsewhere.

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