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Porcine pituitary dynorphin: Complete amino acid sequence of the biologically active heptadecapeptide

(endorphin/[Leu]enkephalin/neuropeptide/opioid peptide)

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ABSTRACT **The full primary structure of the very potent opioid peptide dynorphin, from porcine pituitary, has been determined. It is (H)Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln(OH). The synthetic peptide with this sequence behaves identically to natural dynorphin in a number of ways, and it has the same potency in the guinea pig ileum myenteric plexus–longitudinal muscle bioassay. The potency is accounted for by the first 13 residues.**

Dynorphin is an extremely potent opioid peptide which contains [Leu]enkephalin as its NH_2-terminal sequence. Originally isolated in small amount from a commercial porcine pituitary preparation, its amino acid sequence could be determined with certainty only through the 13th residue [Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys] (1). Antisera to synthetic dynorphin-(1–13) were then developed for a highly specific and sensitive radioimmunoassay, with which it has been determined that dynorphin is a widely distributed neuropeptide (2–4) and evidently interacts *in vivo* with its own unique type of opiate receptor (5, 6). By using this radioimmunoassay as a guide to purification, and with the aid of an immunoaffinity procedure, we have now isolated biologically active dynorphin from porcine pituitary in sufficient amount to establish the entire amino acid sequence.

MATERIALS AND METHODS

In the radioimmunoassay (7), synthetic dynorphin-(1–13) (Peninsula Laboratories, San Carlos, CA) was used as the standard for measurement of immunoreactive dynorphin (*ir*-dynorphin). Bioassay used the guinea pig myenteric plexus–longitudinal muscle preparation as described (8, 9). Protein was determined by the Lowry procedure (10) with bovine serum albumin as standard. Amino acid analysis at Palo Alto used standard routines and "Hi-Phi" buffer systems with the Dionex (Sunnyvale, CA) amino acid analyzer kit. Amino acid analysis at Pasadena was carried out on the Durrum D-500 analyzer. Sequence determinations were carried out as described (11).

Two sources of dynorphin were used. The first was the same melanotropin concentrate (MC) from which dynorphin was previously obtained (1). This powder is a dried oxycellulose adsorbate of an extract of whole porcine pituitaries, generously furnished by J. D. Fisher (Armour, Kankakee, IL). Several 20-g batches were processed. The second source was 2500 lyophilized porcine posterior pituitaries (i.e., neurointermediate lobes; PP), purchased from Pel-Freez. Dynorphin from both sources was isolated in a pure state in five steps. Table 1 summarizes the amounts processed, yields, and specific activities at each step.

Step 1. Extraction. Dry PP or MC material was weighed, pulverized, and then extracted in boiling acetic acid (0.1 M or 1.0 M, 50 ml/g) for 30 min with stirring. This solvent yields more *ir*-dynorphin than the acidified methanol used previously. After centrifugation (8000 × *g*, 40 min, 2°C) the supernatant solution was removed, and the pellet was resuspended in cold acetic acid and centrifuged again. The yield from PP was the same with either concentration of acetic acid, but 1.0 M was required for maximal yield from MC.

Step 2a. CM-Sephadex batch procedure. This desalting step was not required for PP extracts. Extracts and wash solutions containing MC material were loaded onto CM-Sephadex (Pharmacia, C-25, prewashed with 1.0 M acetic acid, 1 g per g of MC starting material). After washes with 1.0 M acetic acid and then water (300 ml each), the material was eluted with MeOH/0.3 M HCl, 1:1 (vol/vol), at room temperature. The eluate was diluted with 2 vol of 0.1 M acetic acid. Subsequent procedures were identical for PP and MC.

Step 2b. Precipitation. The 0.1 M acetic acid solution was adjusted to pH 8.6 with NH_4OH. The precipitate that was formed represented 10–20% of the total *ir*-dynorphin for PP and 60–70% for MC. After the precipitate was dissolved in MeOH/HCl and reprecipitated, gel permeation analysis on Sephadex G-50 showed that the precipitate contained a 3-kilodalton (kDal) immunoreactive material, whereas the 1.7-kDal *ir*-dynorphin remained in solution. The pH 8.6 precipitates were set aside for future study. To the supernatant solution, now 0.1 M in ammonium acetate at pH 8.6, was added Triton X-100 (Calbiochem) to 0.1%.

Step 3. Cation exchange. This step takes advantage of the strongly basic character of 1.7-kDal dynorphin, evident from the three arginine and two lysine residues of the dynorphin-(1–13) sequence. The solution from step 2b was loaded onto a preequilibrated CM-Sephadex column and eluted by a linear gradient of ammonium carbonate (0.1–1.0 M) at pH 8.6 (Fig. 1). A prominent peak of *ir*-dynorphin was eluted at 0.4 M ammonium carbonate. Results with PP and MC at this step were virtually identical, and 100- to 400-fold purifications were typical.

Step 4a. Immunoextraction. "Lucia" antiserum, with affinity in the low picomolar range (7), has been used to measure *ir*-dynorphin in tissues (2, 4). Two steps were used to obtain a useful immunoaffinity resin. First, synthetic dynorphin-(1–13) was coupled to cyanogen bromide-activated Sepharose 4B in order to obtain affinity-purified antibodies from the antiserum (12). These were coupled to Sepharose 4B, and this resin was

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Abbreviations: *ir*, immunoreactive; MC, melanotropin concentrate; PP, posterior pituitary; kDal, kilodalton; HPLC, high-performance liquid chromatography; IC_{50}, concentration for 50% inhibition.

Table 1. Steps in purification of dynorphin

Step	Source	Amount of dynorphin, nmol	Recovery of dynorphin in original extract, %	Protein, mg	Purity, %
1. Extract	PP	356	100	1,190	0.064
	MC	261	100	10,800	0.0052
2a. Eluate from CM-Sephadex batch procedure	PP*	—	—	—	—
	MC	154	59	2,830	0.012
2b. Supernatant after pH 8.6 precipitation	PP	319	89	1,190	0.058
	MC	58	22	1,560	0.0080
3. Eluate from cation exchange	PP	178	50	5.29	7.4
	MC	49	19	3.31	3.2
4a. Immunoextraction	PP	114	32	—	—
	MC	29	11	—	—
4b. Eluate from CM-Sephadex adsorption	PP	95	27	—	—
	MC	21	8.0	—	—
5. Eluate from HPLC	PP	29	8.0	0.062†	100
	MC	10	3.8	0.021	100

The numbered steps are described under *Materials and Methods*. Amounts of dynorphin were determined by radioimmunoassay using synthetic dynorphin-(1–13) as standard. Protein was determined according to Lowry *et al.* (10) but was below the detection limit by this method beyond step 3. Percentage purity is 100 times the specific activity (nmol/mg of protein) divided by that of pure dynorphin (465 nmol/mg). Data are for a typical work-up.

* This step was not necessary in the PP work-up.

† Calculated from the amount of pure *ir*-dynorphin.

used for purification of the natural peptide. In both cases, glycine was used to block unreacted active groups. Antiserum from a different rabbit had properties apparently identical to those of "Lucia," and an affinity resin prepared with it was used sometimes.

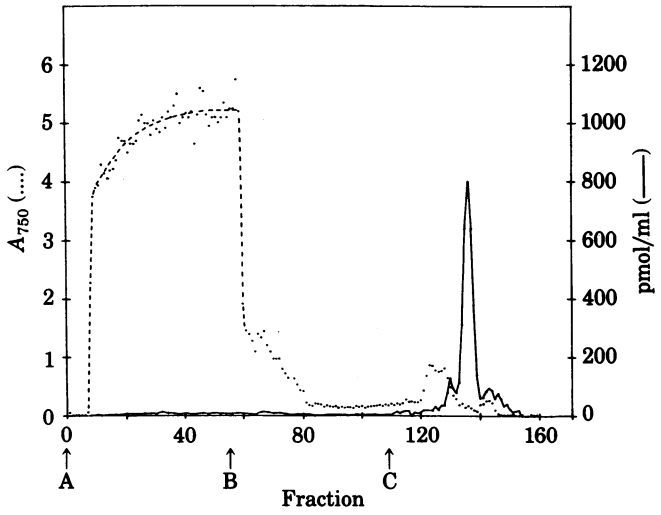

FIG. 1. Purification of dynorphin from PP by cation exchange. Supernatant solution from step 2 (500 ml, pH 8.6) was passed through a CM-Sephadex column (2 × 50 cm, equilibrated at 4°C with 0.1 M ammonium carbonate in 0.1% Triton X-100, 100 ml/hr, 10-ml fractions). A, sample loading; B, 500-ml wash with same buffer; C, start of linear gradient (800 ml) to 1.0 M ammonium carbonate in 0.1% Triton X-100. No more *ir*-dynorphin was eluted by washing column with water and then 1.0 M HCl. Each fraction was assayed for protein (broken line) and *ir*-dynorphin (solid line). Major peak of *ir*-dynorphin, which was eluted at 0.4 M ammonium carbonate, contained 46% (16.7 nmol) of the initial *ir*-dynorphin and 0.25% (1.91 mg) of the initial protein. Overall recoveries: *ir*-dynorphin, 76% (29.1 nmol); protein, 94% (756 mg).

The pooled fractions from step 3 containing *ir*-dynorphin were subdivided for immunoextraction with batches of resin (capacity, 15–30 nmol in 1–2 ml). Incubation was carried out at 4°C overnight, with shaking. Resin was then washed with 200 ml of 0.15 M sodium phosphate (pH 7.4) containing 5% Triton X-100, then with the same volume of phosphate buffer alone, and finally with water. After the overnight incubation, no *ir*-dynorphin remained in solution; 5–10% was detectable in the washes, mostly in the Triton X-100 wash. The peptide was eluted with three 10-ml portions of 0.5 M acetic acid (each for 30 min, 37°C). Overall recovery at step 4a was 60–65% for PP and 55–60% for MC, but the degree of purification is unknown because total protein in the eluate was below the limit of detection by absorbance, and we were unwilling to commit any significant fraction of the material to a destructive assay procedure.

Step 4b. Concentration by CM-Sephadex adsorption. The 0.5 M acetic acid eluate was applied to CM-Sephadex in a small column (0.5 ml packed volume, prewashed with 50 ml each of 1.0 M HCl, 1.0 M NaOH, and MeOH/HCl). The adsorbed peptide was eluted with 2 ml of MeOH/HCl.

Step 5. High-performance liquid chromatography (HPLC). Portions (1 ml) of the MeOH/HCl eluate from step 4b were injected onto a reverse-phase HPLC column. Fig. 2 shows a typical elution diagram for PP material; an identical result was obtained with MC material. A single symmetrical peak of *ir*-dynorphin and absorbance was eluted at exactly the same position as the major peak of *ir*-dynorphin in a crude MeOH/HCl extract of starting material (not shown), indicating that fragmentation or derivatization did not occur during purification.

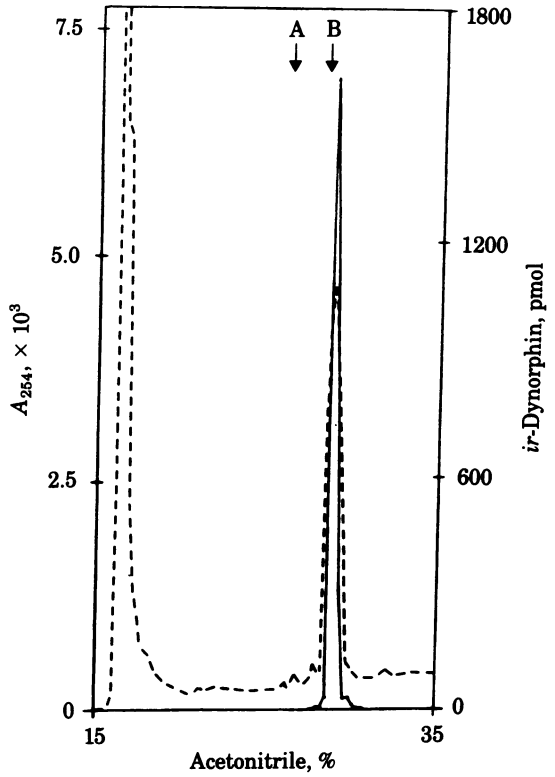

FIG. 2. Preparative reverse-phase HPLC of dynorphin from PP. A Waters Associate system was used with a μBondapak C_{18} column (3.9 × 300 mm). Initial conditions: 15% acetonitrile in 5 mM trifluoroacetic acid. Simultaneous with sample injection, the linear gradient was started, to 35% acetonitrile in 5 mM trifluoroacetic acid in 30 min at 1.5 ml/min; fraction volume, 0.6 ml. Broken line, absorbance at 254 nm; solid line, *ir*-dynorphin. Elution positions of markers: A, dynorphin-(1–13), 26.0% acetonitrile; B, HPLC-purified monoiodinated [^{125}I]dynorphin-(1–13) internal standard, 28.4%. PP dynorphin was eluted at 29.2% acetonitrile.

A second and steeper gradient, from 35% to 90% acetonitrile in 10 min (not shown), yielded no more *ir*-dynorphin. The elution position of natural dynorphin shows that it is more hydrophobic than the synthetic tridecapeptide. Fractions containing *ir*-dynorphin were pooled. Samples from this pool (both PP and MC work-ups) were subjected to bioassay and were sent to Pasadena (frozen on dry ice) for sequence and composition determinations.

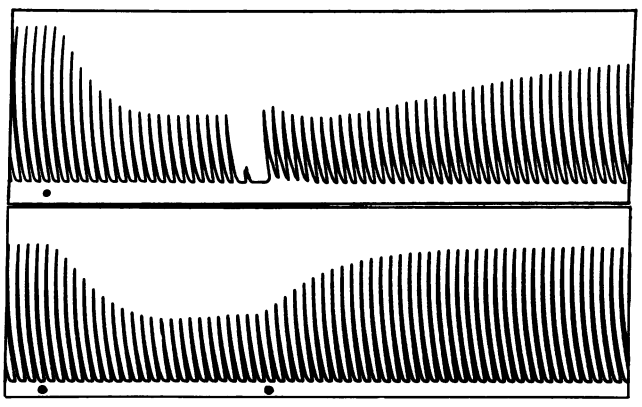

FIG. 3. Bioassay of dynorphin: typical record in the bioassay of pure PP dynorphin (8, 9). Electrically stimulated twitch of guinea pig longitudinal muscle occurs every 10 sec. (*Upper*) Dynorphin from step 5 (see Fig. 2), 2.73 pmol as measured by radioimmunoassay, was added at point marked by dot. Gap in record indicates complete replacement of bath solution by fresh Ringer bicarbonate solution; note slow recovery of twitch amplitude. (*Lower*) Dot at left indicates addition of dynorphin, as above. Second dot shows addition of naloxone hydrochloride to final concentration of 200 nM.

Enzyme Digestion Procedures. Tryptic fragments were produced from pure dynorphin, both PP and MC. Dynorphin (5 nmol) from step 5 was treated with trypsin (Sigma, diphenylcarbamoyl chloride-treated, 10 μg) in 400 μl of 0.15 M sodium phosphate, pH 7.4/0.1% Triton X-100 at 37°C for 2 hr. Dynorphin-(1–13) was treated identically. After acidification with HCl, digests were injected onto an Altex LiChrosorb C_{18} reverse-phase HPLC column (5 μm, 4.6 × 250 mm) and eluted as described in Fig. 3. Absorbance was monitored at 254 and 228 nm. As expected, a single fragment absorbing at 254 nm was obtained from dynorphin-(1–13) (28.0% acetonitrile, A_{228}/A_{254} = 23), which was assumed to be dynorphin-(1–6). Two such fragments were obtained from PP or MC dynorphin. One was identical to that from dynorphin-(1–13). The second fragment in each case was eluted earlier in the gradient (PP, at 23.6% acetonitrile; MC, at 23.8% acetonitrile) and had a different ratio of absorbances at the two wavelengths (PP, 10.6; MC, 10.4), as expected for a tryptophan-containing peptide. These fragments from PP and MC were collected from HPLC, frozen, and sent to Pasadena for sequence determination.

Carboxypeptidase A (Sigma, Type I, diisopropyl fluorophosphate-treated) was used to confirm the identity of the

Table 2. Amino acid sequence determinations

Cycle	Amino acid	PP dynorphin, pmol	MC dynorphin, pmol
		Intact peptides	
1	Tyr	865	965
	Ser	210	205
2	Gly	440	308
3	Gly	450	417
4	Phe	716	765
5	Leu	786	827
6	Arg	460	275
7	Arg	540	351
8	Ile	500	550
9	Arg	400	217
10	Pro	123	135
11	Lys	215	170
12	Leu	330	505
13	Lys	200	198
14	Trp	104	145
15	Asp	55	72
16	Asn	38	66
17	Gln	27	26
18	None		
19	None		
		COOH-Terminal tryptic fragments	
1	Leu	725	590
	Ser	120	77
2	Lys	455	360
3	Trp	122	98
4	Asp	125	84
5	Asn	58	25
6	Gln	47	22
7	None		
8	None		

Method of sequence determination in the spinning cup micro-sequenator is described elsewhere (11). Except for the serine contaminant at cycle 1 and the expected carryover from previous cycles (approximately 2% per cycle), each cycle produced only a single amino acid, in the yields shown. Limit of detection was about 2–5 pmol. Relatively higher yields of hydrophobic than of hydrophilic amino acids are typical of the method.

COOH-terminal amino acid. A suspension of 100 μg of enzyme (5 μl of 20 mg/ml solution) was added to 200 μl of glass-distilled water and centrifuged (2000 × *g*, 10 min, 2°C). The supernatant solution was discarded, and the crystals were dissolved in 200 μl of 2 M ammonium bicarbonate and then diluted to 1.0 ml with water. In a typical experiment, 1 nmol of peptide was dissolved in 30 μl of 0.02 M sodium borate, pH 8.5/0.2 M NaCl/0.1% Triton X-100 and incubated with 1 μg (10 μl) of enzyme at 37°C. Samples (10μl) were withdrawn, diluted with 30 μl of commercial sample diluent ("Na-S," Dionex), and injected directly into the Dionex amino acid analyzer. Blank incubations with enzyme alone provided corrections for free amino acids present at the outset and for those released by autodigestion. Under standard conditions in this system, asparagine and glutamine (which would not be present after acid hydrolysis) are superimposed on the serine peak. By decreasing the temperature to 25°C, diluting the pH 3.25 buffer from 0.2 M to 0.06 M, and slowing the flow rate to 0.1 ml/min, it was possible to obtain complete separation of these three amino acids.

RESULTS AND DISCUSSION

In Fig. 3, showing the bioassay of pure dynorphin from step 5, the upper record depicts the slow reversal on washout (13) that is characteristic of dynorphin; the lower record shows rapid and complete antagonism by 200 nM naloxone.

Quantitative bioassay was carried out on seven such muscle preparations, each estimate of the concentration for 50% inhibition (IC_{50}) being obtained by interpolation into the log(dose)–response curve for synthetic dynorphin-(1–13) obtained on the same preparation. The same solutions of PP dynorphin and dynorphin-(1–13) were diluted serially in MeOH/HCl for parallel determinations in the radioimmunoassay, and aliquots were also taken for amino acid analysis. In the bioassay, IC_{50} of dynorphin-(1–13) standard was 270 ± 40 pM (mean ± SEM), and the solution of PP dynorphin had activity equivalent to 253 ± 34 pmol/ml. Immunoreactivity of the PP dynorphin solution was found to be 273 pmol/ml, referred to dynorphin-(1–13) standard. Thus, the specific biologic activity of dynorphin (i.e., biologic activity/immunoreactivity) was 0.93 ± 0.12, relative to dynorphin-(1–13). Biologic activity entirely comparable to that shown in Fig. 3 was displayed also by MC dynorphin.

An aliquot of the same PP dynorphin was hydrolyzed in 6 M HCl (sealed tube, 120°C, 24 hr) and then subjected to amino acid analysis. The two prominent peaks of aspartic acid (two residues, see below) and phenylalanine (one residue) yielded estimates of 0.93 and 0.82 nmol of peptide, respectively. An identical aliquot subjected to radioimmunoassay contained 0.87 nmol with reference to dynorphin-(1–13) standard. Thus, there is complete equivalence of PP dynorphin and dynorphin-(1–13) with respect to both immunoreactivity and bioactivity, and radioimmunoassay measures the absolute amount of natural dynorphin.

Sequence determinations gave unambiguous results (Table 2). The COOH-terminal tryptic fragment unexpectedly contained leucine-12 as its NH_2-terminal residue. Thus, the Lys^{13}-Trp^{14} bond was resistant to digestion, although the identification of residue 13 as lysine was unequivocal. Although trypsin-resistant lysyl bonds are rare, they have been reported. Review of all relevant sequences in ref. 14 revealed two instances in which a Lys-Trp bond was not attacked by trypsin (15, 16) and two other instances (17, 18) in which the expected tryptic dipeptide Leu-Lys was not produced when the leucine residue was in the NH_2-terminal position, as it would be here if tryptic cleavage occurred first at lysine-11. Trypsin resistance of Lys^{13}-Trp^{14} in dynorphin could reflect some peculiarity of secondary structure.

Amino acid composition analysis (Table 3) was consistent with the sequence determination, accounting for the 16 residues other than tryptophan. It only remained to determine if the COOH-terminal residue is free or amidated and, at the same time, to confirm that this residue is glutamine. Accordingly, digestion with carboxypeptidase A was carried out. To detect any amidase activity in the enzyme preparation, two synthetic dynorphin analogues—one amidated at the terminus and the other not—were treated under the same conditions as natural PP dynorphin. After 22-hr incubation, data for release of amino acid in the Ser/Asn/Gln peak (as pmol/nmol peptide) were: dynorphin-(1–13)-Ser-Asp-Asn-Gln(NH_2), 4; dynorphin-(1–13)-Ser-Asp-Asn-Gln(OH), 827; PP dynorphin, 1257. These data are expressed as glutamine equivalents, and are corrected for

Table 3. Amino acid composition analysis

	PP dynorphin					MC dynorphin				
Amino acid	Amount, nmol	Blank, nmol	Corrected amount, nmol	Residues per mol peptide	Nearest integer	Amount, nmol	Blank, nmol	Corrected amount, nmol	Residues per mol peptide	Nearest integer
Asp	1.16	0.08	1.08	2.1	2	1.41	0.23	1.18	2.0	2
Thr	0.05	0.05	0.00	0.0	0	0.07	0.09	−0.02	0.0	0
Ser	0.15	0.07	0.08	0.2	0	0.27	0.30	−0.03	0.0	0
Glu	0.71	0.11	0.60	1.2	1	1.00	0.35	0.65	1.1	1
Pro	0.51	0.00	0.51	*1.0*	1	0.66	0.07	0.59	*1.0*	1
Gly	1.24	0.14	1.10	2.2	2	1.78	0.51	1.27	2.2	2
Ala	0.16	0.06	0.10	0.2	0	0.19	0.15	0.04	0.1	0
Val	0.12	0.09	0.03	0.1	0	0.13	0.12	0.01	0.0	0
Met	0.00	0.00	0.00	0.0	0	0.00	0.00	0.00	0.0	0
Ile	0.48	0.01	0.47	0.9	1	0.57	0.07	0.50	0.8	1
Leu	0.88	0.02	0.86	1.7	2	1.20	0.14	1.06	1.8	2
Tyr	0.34	0.00	0.34	0.7	1	0.38	0.03	0.35	0.6	1
Phe	0.43	0.00	0.43	0.8	1	0.57	0.03	0.54	0.9	1
His	0.03	0.01	0.02	0.0	0	0.06	0.04	0.02	0.0	0
Lys	1.03	0.03	1.00	2.0	2	1.18	0.11	1.07	1.8	2
Arg	1.46	0.03	1.43	2.8	3	1.72	0.09	1.63	2.8	3
Total residues accounted for					16					16

Approximately 0.5 nmol of each peptide was hydrolyzed (110°C, 24 hr) in 6 M HCl with 0.5% phenol and then processed on the Durrum D-500 analyzer. "Blank" represents fractions collected from HPLC in blank run at same elution volume as for the dynorphin peak. Cysteine and tryptophan are not determined. Residues per mol of peptide are computed relative to the single proline residue.

amounts found in preincubation samples and for autodigestion in the absence of substrate. In the same incubations, the following data were obtained for aspartic acid (residue 15): −10, 94, and 139, respectively.

Amino acid release after a 7-hr incubation was determined by a modified procedure that yielded separate peaks for serine, asparagine, and glutamine. The only amino acid that increased during this incubation was glutamine, from 52 to 368 pmol/nmol of peptide.

We conclude that natural biologically active dynorphin terminates in a free carboxyl group, that the complete amino acid sequence is (H)Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln(OH), and that dynorphin from PP is identical to that from MC.

Synthetic dynorphin with the above sequence was obtained from Peninsula Laboratories (>99% pure by thin-layer chromatography, electrophoresis, and HPLC) and compared with natural dynorphin. In the HPLC system described earlier, the elution position of the synthetic peptide was identical to that shown in Fig. 2. Moreover, a mixture of synthetic and natural dynorphins coeluted as a single symmetrical peak of immunoreactivity. Tryptic fragments were also eluted at identical positions in HPLC; and they had the same amino acid compositions (including the anomalous leucine-12 and lysine-13), confirming the trypsin resistance of the Lys^{13}-Trp^{14} bond in this peptide. Immunoreactivities of synthetic and natural dynorphin were the same and were identical to the immunoreactivity of dynorphin-(1–13). The IC_{50} of synthetic dynorphin in the guinea pig ileum bioassay was 330 ± 60 pM (n = 11), indistinguishable from that of PP dynorphin or dynorphin-(1–13). Naloxone K_e, which is a measure of receptor selectivity (6, 19), was 31 ± 2 nM (n = 6) for synthetic dynorphin and 30 ± 3 nM (n = 6) for dynorphin-(1−13).

The main improvements in the present procedure have been: (*a*) avoidance of lyophilization, a step that we find entails large losses; (*b*) use of detergents to minimize adsorptive losses; (*c*) monitoring by radioimmunoassay instead of bioassay; and (*d*) use of an immunoaffinity resin. The 17-residue length of dynorphin is close to that indicated by apparent M_r on Sephadex G-25 when pituitary opioid peptides were first discovered in this laboratory in 1975 (20). We have here confirmed that the full biologic activity of this remarkably potent opioid peptide is determined by its first 13 residues. The functional significance of the remaining four residues is unknown; they might make the peptide more stable *in vivo*.

That dynorphin plays an important physiologic role is indicated by its potency, which is greater than that of any known endogenous opioid peptide, and by the existence of a highly specific dynorphin receptor in guinea pig myenteric plexus and brain (5, 6, 19). This receptor is evidently the same as the opiate receptor called κ, with which certain benzomorphans are known to interact preferentially.

Note Added in Proof. S. Tachibana has presented (at the International Narcotic Research Conference, Kyoto, Japan, July 1981) a sequence for dynorphin from porcine duodenum that is the same as that reported here.

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