Evidence for Topographical Analogy between Methionine-Enkephalin and Morphine Derivatives†

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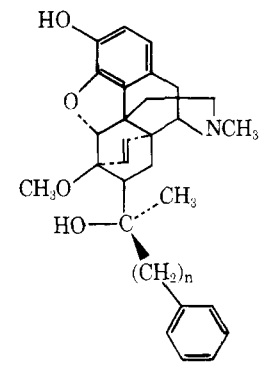
ABSTRACT: Analogues of the endogenous opiate-receptor ligand [5-methionine]enkephalin (H·Tyr-Gly-Gly-Phe-Met·OH) were designed and synthesized for the purpose of testing the proposed similarity in spatial structure between this peptide and morphine derivatives. In the bioassay (inhibition of electrically induced contractions of the mouse vas deferens) [1-*O*-methyltyrosine,5-methionine]enkephalin, [1-*N*-methyltyrosine,5-methionine]enkephalin, [4-tryptophan,5-methionine]enkephalin, and [5-methionine sulfoxide]enkephalin possess, respectively, 0.4, 21, 27, and 67% activity of [5-methionine]enkephalin. These morphinomimetic activities correlate well with the opiate receptor affinities determined by displacement of [^{3}H]naloxone in a guinea pig brain membrane preparation. The effects of O-methylation of the tyrosine residue and N-methylation of the terminal amino group on biological activity and receptor affinity support the hypothesis that the latter two moieties in the peptide correspond to the phenol group and the tertiary nitrogen, respectively, in morphine. Determination of the efficiency of energy transfer from tyrosine in position 1 to tryptophan in position 4 in [4-tryptophan,5-methionine]enkephalin from both tyrosine fluorescence quenching and relative enhancement of tryptophan fluorescence by means of a modified procedure permitted the calculation of an average intramolecular tyrosine–tryptophan separation of 10.0 ± 1.1 Å. Inspection of CPK models showed excellent agreement between this value and both the intrafluorophore distance in the 4 → 1 and 5 → 2 hydrogen bonded β_I-bend models of [4-tryptophan,5-methionine]enkephalin (9–11 Å) and the phenol–phenyl separation in the potent morphine derivative 7α-(1(*R*)-hydroxy-1-methyl-3-phenylpropyl)-6,14-*endo*-ethenotetrahydrooripavine (8–10.5 Å). The ensemble of these findings suggests an analogous topography for [5-methionine]enkephalin and morphine–oripavine derivatives.

The recent discovery (Hughes et al., 1975) of the two pentapeptides H·Tyr-Gly-Gly-Phe-Met·OH ([Met^5]enkephalin)[1] and H·Tyr-Gly-Gly-Phe-Leu·OH ([Leu^5]enkephalin)[1] with potent morphinomimetic activity, their inhibition by the morphine-derived inhibitor naloxone in the bioassay, and their ability to displace morphine derivatives from receptor binding sites (Simantov and Snyder, 1976; Büscher et al., 1976; Cox et al., 1976; Lazarus et al., 1976) led to the hypothesis of an analogous spatial disposition of critical and identical chemical functions in the peptides and in the molecular framework of morphine. Structure–activity relationships have been thoroughly investigated in the morphine field through synthesis of numerous morphine derivatives, including the highly potent oripavines (Lewis et al., 1971). In the series of 7α-(1(*R*)-hydroxy-1-methylphenylalkyl)-6,14-*endo*-ethenotetrahydrooripavines (I), maximal analgesic activity (2200× morphine) is observed with 7α-(1(*R*)-hydroxy-1-methyl-3-phenylpropyl)-6,14-*endo*-ethenotetrahydrooripavine (Ia)[1] (*n* = 2). The fact that both shortening and lengthening of the phenylalkyl chain in I (*n* = 1,3) induces a dramatic loss of activity has led to the proposal of a topographically well defined, second lipophilic binding site on the opiate receptor (Bentley and Lewis, 1972). The recently proposed, hypothetical β-bend model of [Met^5]enkephalin with a hydrogen bond between the amino group of the phenylalanine residue and the carbonyl group of tyrosine would bring the phenol ring of tyrosine, the terminal amino group, the methyl group of the methionine side chain, and the phenylalanine residue into a topographical disposition analogous to that observed for the corresponding phenol group, tertiary amino group, methoxy substituent on C-6, and phenylethyl substituent on C-19 in Ia (Bradbury et al., 1976). Recent NMR studies (Garbay-Jaureguiberry et al., 1976; Roques et al., 1976; Jones et al., 1976) exclude a random-coil structure for methionine-enkephalin and support the existence of a well-defined, preferential conformation of the pentapeptide. Furthermore, these studies suggest an intramolecular hydrogen bond between the amino group of methionine in position 5 and the carbonyl group of glycine in position 2 (5 → 2 hydrogen bonded β_I turn), rather



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[1] Abbreviations used are: [Met^5]enkephalin, [5-methionine]enkephalin; [Leu^5]enkephalin, [5-leucine]enkephalin; [Tyr(OMe)^1,Met^5]enkephalin, [1-*O*-methyltyrosine,5-methionine]enkephalin; [Tyr(NMe)^1,Met^5]-enkephalin, [1-*N*-methyltyrosine,5-methionine]enkephalin; [Trp^4,-Met^5]enkephalin, [4-tryptophan,5-methionine]enkephalin; [Met-(O)^5]enkephalin, [5-methionine sulfoxide]enkephalin; Ia, 7-α-(1-(*R*)-hydroxy-1-methyl-3-phenylpropyl)-6,14-*endo*-ethenotetrahydrooripavine; Dns (= dansyl), 5-dimethylamino-1-naphthalenesulfonyl; ACTH, adrenocorticotropic hormone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; DMF, dimethylformamide, Boc, *tert*-butoxycarbonyl; NMR, nuclear magnetic resonance, UV, ultraviolet.

than the 4 → 1 hydrogen bonded β_I turn proposed on theoretical grounds by Bradbury et al. (1976). The 5 → 2 hydrogen bonded β_I-turn model also accommodates all the critical chemical functions in a spatial arrangement analogous to that present in compound Ia.

In the present investigation, experimental evidence for topographical analogy between [Met^5]enkephalin and morphine–oripavine derivatives is presented which was obtained both through modification of critical chemical functions and by means of a conformational study. In particular, the effect of O- and N-methylation of tyrosine in [Met^5]enkephalin on morphinomimetric activity and receptor affinity is investigated and compared with the analgesic activity of correspondingly modified morphine derivatives. The conformational study is performed with the biologically active [Trp^4,Met^5]enkephalin, which permits the measurement of the intramolecular distance between tyrosine in position 1 and tryptophan in position 4 by evaluation of singlet–singlet resonance energy transfer between the two fluorophores. This experimentally determined intramolecular distance offers an interesting comparison with the corresponding phenol–phenyl separation in the β-bend models of [Met^5]enkephalin and in the rigid oripavine derivative Ia.

Materials and Methods

Synthesis of Amino Acid Derivatives and Peptides. Precoated plates (Silicagel G, 250 μm Analtech, Inc.) were used for ascending thin-layer chromatography in the following systems: (a) chloroform–methanol–acetic acid (CMA) (85:10:5); (b) chloroform–acetic acid (CA) (95:5); (c) 1-butanol–acetic acid–H_2O (BAW) (4:1:5); (d) 1-butanol–pyridine–acetic acid–H_2O (BPAW) (15:10:3:12); (e) *sec*-butyl alcohol–3% ammonium hydroxide (SH) (100:44). Compounds on the chromatograms were detected with the chlorine peptide spray and/or with ninhydrin. Melting points are uncorrected. Proton magnetic resonance spectra were obtained on a Varian EM-390 NMR spectrometer with tetramethylsilane as internal standard. A Beckman Model 25 spectrophotometer was employed for the recording of UV spectra. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter. The deprotected peptides were hydrolyzed for 48 h at 110 °C in 6 N HCl in the presence of phenol, and amino acid analyses were performed on a Beckman Model 121 C amino acid analyzer equipped with a system AA computing integrator.

*tert-Butoxycarbonyl-O-methyl-*L*-tyrosine and tert-Butoxycarbonyl-N-methyl-O-benzyl-*L*-tyrosine.* *O*-Methyl-L-tyrosine was synthesized by methylation of *N*-formyl-L-tyrosine and subsequent hydrolysis as described by Izumiya and Nagamatsu (1952). The measured optical rotation $[\alpha]^{22.5}_D$ −6.9 °C (*c* 2.02, 3 N HCl) is in good agreement with the value published by Jošt and Rudinger (1961) ($[\alpha]^{22.5}_D$ −7.0 °C (*c* 2.02, 3 N HCl)). *tert*-Butoxycarbonyl-*O*-methyl-L-tyrosine was prepared according to the method of Schwyzer et al. (1959). The product was obtained in colorless needles after crystallization from benzene–petroleum ether in 42% yield; mp 96–98 °C; $[\alpha]^{22.5}_D$ +29.66 (*c* 2.124, EtOH); TLC (silica gel) single spot R_f = 0.76 (CMA), R_f = 0.38 (CA); NMR ($CDCl_3$): 1.35 s ($C(CH_3)_3$), 3.0 m (CH_2), 3.65 s (OCH_3), 4.4 m (CH), 6.63 d (aromatic protons), 6.92 d (aromatic protons), 10.05 s (COOH).

tert-Butoxycarbonyl-*N*-methyl-*O*-benzyl-L-tyrosine was prepared essentially according to the procedure published by Olsen (1970). After crystallization from diethyl ether, the product was obtained in 71% yield, mp 128–130 °C; $[\alpha]^{22.5}_D$ −24.9 (*c* 1.004, 90% AcOH); TLC (silica gel) single spot R_f = 0.75 (CMA), R_f = 0.43 (CA); NMR ($CDCl_3$): 1.37 s ($C(CH_3)_3$), 2.72 s (NCH_3), 3.15 m (CH_2-β), 4.4 m (CH-α), 5.01 s (CH_2), 6.88 d (aromatic protons (Tyr)), 7.12 d (aromatic protons (Tyr)), 7.36 s (aromatic protons (Phe)).

Analogues of [Met^5]Enkephalin. The solid-phase method (Merrifield, 1963) was used for the synthesis of peptides with the aid of a Beckman Model 990 peptide synthesizer. Boc amino acids were either purchased from Bachem Inc. or synthesized according to the method of Schwyzer et al. (1959). In the case of tyrosine, the 2,6-dichlorobenzyl group was used for protection of the hydroxyl function. Chloromethylated resin (Bio-Beads S-X1, 200–400 mesh, 1.25 mequiv/g, Bio-Rad Laboratories) was converted to hydroxymethylated resin (1.06 mequiv of OH^-/g) following the method of Bodanszky and Sheehan (1966) and subsequently reacted with *tert*-butoxycarbonylmethionine, whereby a yield of 0.152 mM *tert*-butoxycarbonylmethionine per g of resin was obtained. After acetylation of the resin with acetic anhydride, the coupling of each subsequent Boc amino acid was performed according to the following protocol. The esterified resin was (1) washed with $CHCl_3$; (2) prewashed with 25% (v/v) CF_3COOH in $CHCl_3$ for 5 min; (3) the Boc group removed by treatment with 25% (v/v) CF_3COOH in $CHCl_3$ for 30 min; (4) washed with $CHCl_3$; (5) washed with CH_2Cl_2; (6) neutralized with 10% Et_3N (v/v) in CH_2Cl_2 four times for 1.5 min; (7) washed with CH_2Cl_2 six times for 1.5 min; (8) addition of Boc amino acid in CH_2Cl_2 (2.5-fold excess); (9) addition of dicyclohexylcarbodiimide (2.5-fold excess in CH_2Cl_2) and mixing for 3 h; (10) washed with CH_2Cl_2; (11) washed with DMF; (12) washed with a mixture of DMF–CH_2Cl_2 (1:1); (13) addition of Boc amino acid in a mixture of DMF–CH_2Cl_2 (1:1) (Westall and Robinson, 1970); (14) repetition of step 9; (15) washed with a mixture of DMF–CH_2Cl_2) (1:1); (16) washed with a mixture of MeOH–$CHCl_3$ (1:2) (for removal of precipitated dicyclohexylurea) (Flouret, 1970); (17) washed with DMF. Unless otherwise mentioned, all washings were carried out three times for 1.5 min. After each cycle, completeness of the reaction was checked with the color test (Kaiser et al., 1970). After completion of the last cycle, the peptide was cleaved from the resin and completely deprotected by treatment with HF (Sakakibara, 1971). The reaction was carried out for 1 h at 0 °C with 20 mL of HF and in the presence of 1 mL of anisole and 1 mL of ethyl methyl sulfide as scavengers per g of resin. In the case of [Trp^4,Met^5]enkephalin and [Phe^1,Trp^4,Met^5]enkephalin, 200 mg of skatole per g of resin was added as an additional scavenger. After removal of the HF, the resin was extracted several times with diethyl ether and subsequently with 90% acetic acid. Lyophilization of the latter extract yielded the crude peptide in solid form.

Reduction with aqueous thioglycolic acid (1%) (Hofmann et al., 1966) revealed the presence of methionine sulfoxide peptide in all crude products. Due to the high polarity of the sulfoxide moiety [Met(O)^5]enkephalin could easily be purified in a single step by partition chromatography on Sephadex G-25 with the system 1-butanol–acetic acid–H_2O (4:1:5). For the purification of the nonoxidized [Met^5]enkephalin and its analogues, the following procedure was developed: 400 mg of the crude product was dissolved in 20 mL of aqueous thioglycolic acid (5%) and reduced under nitrogen at 45 °C for 48 h. After subsequent lyophilization, the product plus thioglycolic acid were dissolved in 6 mL of 0.1 N ammonium acetate in 1 N acetic acid and the resulting solution was applied to a SP-Sephadex C-25 column (1.5 × 100 cm) after filtration. Elution was performed by means of a linear gradient with the final buffer reaching a concentration of 0.4 N ammonium acetate

in 1 N acetic acid, and fractions of 14 mL at a flow rate of 7 mL/h were collected. The fractions containing the major product were pooled and lyophilized, and the resulting product was purified by partition chromatography on a Sephadex G-25 column (2.5 × 100 cm) with the system 1-butanol–acetic acid–H_2O (4:1:5), whereby fractions of 7 mL at a flow rate of 70 mL/h were collected. In view of the high purity requirements for spectroscopic studies, the cuts made in the purifications on the basis of thin-layer chromatography of the individual fractions emphasized purity more than yields. After the second purification step, all peptides showed a single major spot on thin-layer chromatograms and a second faint spot with smaller R_f corresponding to the methionine sulfoxide peptide which was estimated to represent less than 5% of the total product. All peptides were obtained as lyophilisates.

[*Met*^{5}]*Enkephalin* has mp 140–143 °C (dec); TLC (silica gel) R_f = 0.54 (BAW), R_f = 0.65 (BPAW), R_f = 0.29 (SH). Amino acid analysis: Gly, 2.05; Met, 1.04; Tyr, 1.00; Phe, 0.99. The overall yield based on initial methionine attached to the polymer was 47%.

[*Met*(*O*)^5]*Enkephalin* has mp 157–159 °C (dec); TLC (silica gel) R_f = 0.27 (BAW), R_f = 0.53 (BPAW), R_f = 0.26 (SH). Amino acid analysis: Gly, 2.03; Tyr, 1.00; Phe, 0.96.

[*Tyr* (*OMe*)^1,*Met*^{5}]*Enkephalin* has mp 157–160 °C (dec); TLC (silica gel) R_f = 0.52 (BAW), R_f = 0.66 (BPAW), R_f = 0.30 (SH). Amino acid analysis: Gly, 2.19; Met, 0.80; Tyr, 0.95; Phe, 1.00. Yield: 77%.

[*Tyr* (*NMe*)^1,*Met*^{5}]*Enkephalin* has mp 208–211 °C (dec); TLC (silica gel) R_f = 0.45 (BAW), R_f = 0.62 (BPAW), R_f = 0.27 (SH). Amino acid analysis: Gly, 2.13; Met, 0.81; Phe, 1.00. Yield: 42%.

[*Trp*^{4},*Met*^{5}]*Enkephalin* has mp 143–146 °C (dec); TLC (silica gel) R_f = 0.52 (BAW), R_f = 0.65 (BPAW), R_f = 0.25 (SH). Amino acid analysis: Gly, 2.08; Met, 0.93; Tyr, 1.00. Yield: 54%. Measurement of the optical densities at 280 and 288 nm of the peptide dissolved in 6 M guanidine hydrochloride and the use of the tyrosine and tryptophan extinction coefficients determined in this solvent (Edelhoch, 1967) permitted the calculation of a Trp/Tyr ratio of 1.09.

[*Phe*^{1},*Trp*^{4},*Met*^{5}]*Enkephalin* has mp 176–179 °C (dec); TLC (silica gel) R_f = 0.51 (BAW), R_f = 0.68 (BPAW), R_f = 0.27 (SH). Amino acid analysis: Gly, 2.00; Met, 1.03; Phe 0.94. Yield: 36%.

Bioassay, Receptor Binding Assay. As bioassay, the inhibition of the electrically excited mouse vas deferens was used as described by Henderson et al. (1972).

For the opiate-receptor binding assay, a membrane fraction from guinea pig homogenate was used. Starting with 8 g of brain tissue, the membrane suspension was prepared by the procedure of Pasternak et al. (1975) with the following modifications: Hepes buffer (25 mM, pH 7.6) was used as standard buffer in place of Tris-HCl, and Trasylol (Boehringer-Mannheim) in a concentration of 3000 kallikrein inactivating units per mL was added to the final suspension (50 mL total volume) in order to reduce proteolytic peptide degradation. The binding assay was based on the displacement of [^{3}H]naloxone by the enkephalin analogues. The total assay volume of 0.4 mL contained 0.2 mL of membrane suspension and 0.2 mL of standard buffer containing the appropriate amounts of [^{3}H]naloxone (New England Nuclear) and enkephalin analogue. Assays were performed at 25 °C for 30 min and the incubation was terminated by filtration over Whatman glass fiber disks (GF/C). After two washings with 3 mL-portions of ice-cold standard buffer and subsequent drying of the disks, the radioactivity was determined with a standard toluene based scintillation fluid. In order to assess the extent of nonspecific binding, the displacement of [^{3}H]naloxone by levorphanol and its (+)-enantiomer dextrorphan at concentrations corresponding to those of the peptides was also determined.

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Hitachi Perkin-Elmer fluorescence spectrophotometer MPF-3L. Temperature was maintained at 25 °C through thermostatting of the cell block with a Haake FK2 circulating water bath. True fluorescence excitation spectra were obtained with the Hitachi Perkin-Elmer corrected spectra accessory; fluorescence emission spectra were not corrected. Approximately 2×10^{-5} M aqueous solutions of peptide were used in all fluorescence experiments.

Quantum yields of tyrosine and tryptophan fluorescence were determined through comparison with emission spectra of tyrosine and tryptophan in aqueous solution, whose quantum yields, ϕ_{AA}, were taken as 0.14 and 0.13, respectively (Chen, 1967). The excitation wavelengths for the tyrosine and tryptophan were 280 and 293 nm, respectively. Relative fluorescence intensities were determined through integration of the spectral area and the quantum yield of the peptide fluorophore, ϕ_{PP}, was calculated with eq 1:

$$\phi_{PP} = \phi_{AA}\left(\frac{I_{PP}A_{AA}}{I_{AA}A_{PP}}\right) \quad (1)$$

where I_{PP} and I_{AA} are the fluorescence intensities and A_{PP} and A_{AA} the absorbances of the peptide fluorophore and the reference fluorescent amino acid, respectively.

Energy Transfer Experiments. The measurements of the intramolecular distance between the donor Tyr^1 and the acceptor Trp^4 in [Trp^4,Met^5]enkephalin followed the procedure recently described in a review article (Schiller, 1975). The intramolecular donor–acceptor separation, r, is related to the transfer efficiency, E, by eq 2:

$$r = (E^{-1} - 1)^{1/6}R_0 \quad (2)$$

where the so-called Förster critical distance, R_0, is defined as that donor–acceptor separation where 50% of the donor excitation energy is transferred to the acceptor.

The transfer efficiency, E, was obtained both from donor fluorescence quantum yield measurements and determination of the relative increase of acceptor fluorescence. In the former case, E is related to ϕ_D and $\phi_D{}^0$, the respective donor quantum yields in presence and absence of energy transfer by eq 3:

$$E = 1 - (\phi_D/\phi_D{}^0) \quad (3)$$

$\phi_D{}^0$, the tyrosine quantum yield in absence of the tryptophan acceptor, is readily determined in [Met^5]enkephalin, while ϕ_D, the tyrosine quantum yield in the presence of transfer, is determined in [Trp^4,Met^5]enkephalin itself. The tyrosine emission in the latter compound can be quantified through normalization at 355 nm of its emission spectra obtained with excitation at 280 and 293 nm, respectively, and subsequent subtraction of the normalized spectra.

On the basis of the relative increase of acceptor fluorescence, the transfer efficiency is obtained through comparison of the fluorescence excitation spectra of the acceptor in the presence and absence of the donor with the absorption spectrum of the donor–acceptor pair after normalization of the three spectra at a wavelength where the donor does not absorb. The transfer efficiency is then calculated with eq 4 (Schiller, 1977):

$$E = \frac{I(\lambda) - I_A(\lambda)}{A_D(\lambda)} \quad (4)$$

where $I(\lambda)$, $I_A(\lambda)$ = intensities of acceptor emission in presence

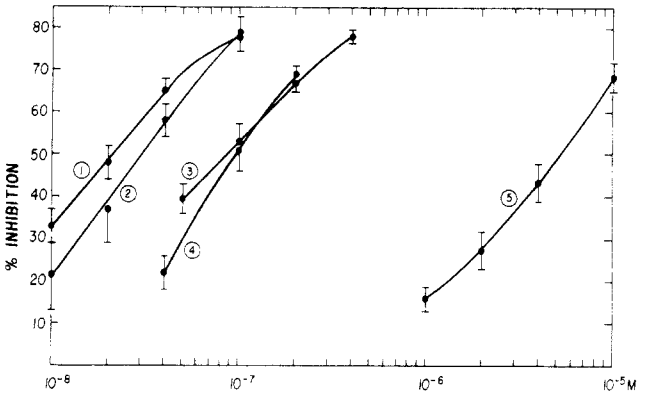

FIGURE 1: Inhibition of electrically evoked contractions of the mouse vas deferns: dose-response curves for [Met^5]enkephalin (1), [Met(O)^5]enkephalin (2), [Trp^4,Met^5]enkephalin (3), [Tyr(NMe)^1,Met^5]enkephalin (4), and [Tyr(OMe)^1,Met^5]enkephalin (5).

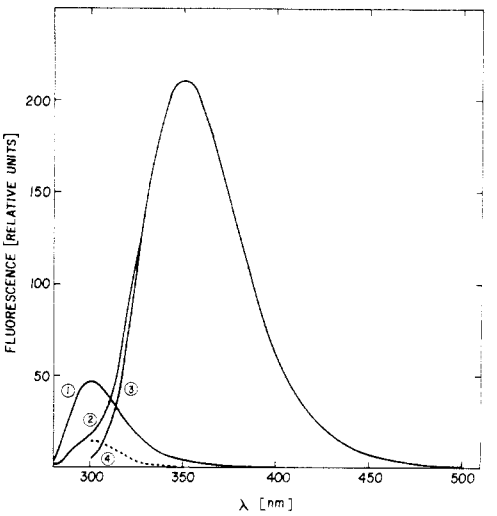

FIGURE 2: Fluorescence emission spectra of [Met^5]enkephalin with excitation at 280 nm (curve 1) and [Trp^4,Met^5]enkephalin with excitation at 280 nm (curve 2) and 293 nm (curve 3). Curve 4 represents the tyrosine emission spectrum of [Trp^4,Met^5]enkephalin obtained by subtraction of spectra 2 and 3 after normalization at 350 nm. Spectrum 1 was corrected for the difference in concentration between the solutions of [Met^5]enkephalin and [Trp^4,Met^5]enkephalin.

TABLE I: IC_{50} Values and Potency Ratios of [Met^5]Enkephalin Analogues.

Analogue	IC_{50} (nm)[a]	Potency ratio: Analogue / [Met^5]Enkephalin
[Met^5]Enkephalin	14.5 ± 2.5	1.00
[Met(O)^5]Enkephalin	21.0 ± 6.0	0.67
[Trp^4,Met^5]Enkephalin	52.0 ± 8.0	0.27
[Tyr(NMe)^1,Met^5]Enkephalin	68.0 ± 8.0	0.21
[Tyr(OMe)^1,Met^5]Enkephalin	3500 ± 600	0.004
[Phe^1,Trp^4,Met^5]Enkephalin	>10 000	

[a] $n = 3$.

and absence of donor (normalized excitation spectra) and $A_D(\lambda)$ = normalized donor absorbance at wavelength λ. The fluorescence excitation spectrum of the acceptor in the absence of transfer is best obtained with an analogue of the peptide under investigation which has the donor substituted by a similar moiety which does not participate in energy transfer in the wavelength region of interest in the energy-transfer experiment. Thus, for the determination of E in [Trp^4,Met^5]-enkephalin the fluorescence excitation spectrum of [Phe^1,Trp^4,Met^5]enkephalin was normalized with that of the former peptide at 293 nm, where tyrosine no longer absorbs. Since tryptophan absorption and fluorescence excitation change rapidly with wavelength in the region around 293 nm, possible small differences in slitwidth and wavelength calibration between absorption and fluorescence spectrophotometer discourage normalization of the absorption spectrum with the excitation spectrum at this wavelength. The absorption spectrum of [Trp^4,Met^5]enkephalin was, therefore, normalized with the fluorescence excitation spectrum of [Phe^1,Trp^4,Met^5]enkephalin at 280 nm on the basis of the known extinction coefficients of tyrosine and tryptophan at this wavelength.

This method was preferred to that proposed by Eisinger (1969) for the reason that no instrument permitting the recording of both fluorescence and absorption spectra was available. Furthermore, the spectrofluorometer used in the latter study required sample concentrations of the order of 10^{-2} M. Such extremely high peptide concentrations represent a serious disadvantage in view of the possibilities of *inter*molecular energy transfer and peptide self-association.

In the computation of R_0, the refractive index of water was used and ϕ_D^0 was determined with [Met^5]enkephalin. A value of $\frac{2}{3}$ was used for the orientation factor under the assumption of complete randomization in donor-acceptor orientation.

Results and Discussion

The dose-response curves of the [Met^5]enkephalin analogues in the mouse vas deferens preparation are presented in Figure 1. With all analogues, a maximal inhibition of 80 ± 10% was reached which could not be exceeded by application of excessive doses. The IC_{50} values were therefore defined as the peptide concentrations which cause 40% inhibition of the electrically evoked contractions in the mouse vas deferens. In Table I, the IC_{50} values obtained from the dose-response curves are tabulated together with the calculated potency ratios. The IC_{50} value for [Met^5]enkephalin ($1.40 \pm 0.25 \times 10^{-8}$ M) is in good agreement with that obtained from Figure 2 in the recent publication by Hughes et al. (1975) (1.0×10^{-8} M in the mouse vas deferens preparation). The potency ratio of 0.67 observed for [Met(O)^5]enkephalin is close to the value (0.5) determined by Lazarus et al. (1976) with a preparation of the myenteric plexus longitudinal muscle of guinea pig ileum. These findings are in contrast to the complete loss of biological activity in ACTH observed after methionine oxidation and suggest that this residue is not directly involved in the interaction with the receptor. However, the observed low potency ratio of [des-Met^5]enkephalin (0.01) (Lazarus et al., 1976) seems to indicate that this residue may be necessary for stabilizing the biologically active conformation or positioning the CH_3S group in a topographical disposition analogous to that of the CH_3O function in morphine derivatives (Bradbury et al., 1976). On the basis of the proposed analogy between [Met^5]enkephalin and the oripavine derivative Ia, the phenylalanine residue in position 4 has been implicated with the proposed second lipophilic binding site on the opiate receptor (Bentley and Lewis, 1972). With regard to this model, the potency ratio of 0.27 determined for [Trp^4,Met^5]enkephalin

would indicate that substitution of tryptophan for phenylalanine does not greatly affect binding to the receptor. The 3.5-fold decrease in opioid activity may be due to increased nonspecific binding induced by the presence of the relatively large hydrophobic indole moiety. The considerable biological activity of this analogue constitutes an important prerequisite for the conformational study described below.

Since [desamino-Tyr[1],Met[5]]enkephalin is devoid of any analgesic activity (Büscher et al., 1976), the terminal amino group seems to be essential for interaction with the receptor. Acetylation of the terminal amino group in [Met[5]]enkephalin (Ling and Guillemin, 1976) or chain extension at the N terminus of the peptide with sarcosine (Chang et al., 1976) led to drastic reductions in biological activity. These findings are in tune with the loss of activity observed by attachment of an acetylseryl residue to the amino-terminal tyrosine of a synthetic opioid peptide (Goldstein et al., 1975) and thus seem to indicate that an intact amino group is essential for biological activity. On the other hand, addition of an arginine residue to the N terminal of [Met[5]]enkephalin (H·Arg-Tyr-Gly-Gly-Phe-Met·OH) reduces activity only by half (Day et al., 1976; Chang et al., 1976). Results obtained by Goldstein et al. (1975) seem to indicate that the positive charge on the tertiary nitrogen of opiates, rather than the electron lone pair, is required for biological activity. Presumably, the positive charge interacts with an anionic moiety on the receptor. On the basis of this observation, it can be argued that the positively charged side chain of arginine in a bent conformation might substitute for the positive, terminal amino group in the interaction with the negatively charged receptor site, which would explain the considerable activity of the arginine hexapeptide analogue. Terenius et al. (1976) declared the amino group of tyrosine as nonessential on the grounds that extension of the N terminal in [Leu[5]]enkephalin with a second tyrosine (H·Tyr-Tyr-Gly-Gly-Phe-Leu·OH) did not alter the relative receptor affinity. However, it may well be that the second amino-terminal tyrosine in the hexapeptide plays the role of the tyrosine in [Leu[5]]enkephalin and we do not consider this argument as conclusive. Obviously, further analogue studies are required for a definite clarification of this point. The potency ratio of 0.21 determined for [Tyr(NMe)[1],Met[5]]enkephalin (Table I) demonstrates that introduction of a secondary amino group, as present in normorphine, does not greatly affect opioid activity. This finding lends further support to the hypothesis that the amino group of tyrosine in enkephalin may correspond to the nitrogen contained in the piperidine ring of normorphine and morphine derivatives.

The low potency ratio of 0.004 observed with [Tyr(OMe)[1],Met[5]]enkephalin shows that O-methylation of tyrosine causes a drastic loss of activity. While this paper was being reviewed, Day et al. (1976) reported a very similar potency ratio (0.002) for the same analogue with the guinea pig ileum bioassay. These values are significantly lower than the potency ratio (0.17) observed in the myenteric plexus bioassay, which was published somewhat later by Ling and Guillemin (1976). In good correlation with the potency ratio observed by us with [Tyr(OMe)[1],Met[5]]enkephalin, a low ratio of 0.011 between the potencies of the O-methylated morphine derivative codeine and morphine had previously been determined by Cox and Weinstock (1966) with a transmurally stimulated guinea pig preparation. In the latter report, a 16-fold loss in in vitro activity with O-methylation of an oripavine derivative to the corresponding thebaine derivative was also demonstrated. This observation is in agreement with the finding that the in vivo analgesic potencies of oripavine derivatives are approximately 10–50 times higher than those of the corresponding thebaine derivatives (Lewis et al., 1971). The observed parallelism between morphine derivatives and [Met[5]]enkephalin with regard to the effect of O-methylation on morphinomimetic potency strongly suggests that the tyrosyl residue in the peptide and the phenol ring in the phenanthrene nucleus of morphine derivatives correspond to each other. It is at the moment unclear whether the hydrogen bonding capacity, acidity, or chemical reactivity of the phenolic hydroxyl group constitutes the essential property for activity. Furthermore, the question whether enzymatic O-demethylation of codeine and thebaine derivatives and of [Tyr(OMe)[1],Met[5]]enkephalin plays a role in the opiate agonist action of these compounds still remains in debate.

TABLE II: Displacement of [^{3}H]Naloxone in the Presence of [Met[5]]Enkephalin Analogues.[a]

Substance	Final Concn (M)	Inhibition of [^{3}H]Naloxone Binding (%)
Levorphanol	1×10^{-6}	91.6 ± 0.26
	1×10^{-5}	93.4 ± 0.27
Dextrorphan	1×10^{-6}	19.7 ± 1.38
	1×10^{-5}	29.3 ± 4.20
[Met[5]]Enkephalin	1×10^{-6}	36.7 ± 3.03
	1×10^{-5}	69.7 ± 0.73
[Met(O)[5]]Enkephalin	1×10^{-6}	22.4 ± 4.19
	1×10^{-5}	54.3 ± 1.48
[Trp[4],Met[5]]Enkephalin	1×10^{-6}	17.8 ± 1.20
	1×10^{-5}	55.3 ± 0.07
[Tyr(NMe)[1],Met[5]]Enkephalin	1×10^{-6}	55.1 ± 1.35
	1×10^{-5}	71.7 ± 0.94
[Try(OMe)[1],Met[5]]Enkephalin	1×10^{-6}	0
	1×10^{-5}	25.0 ± 1.81
[Phe[1],Trp[4],Met[5]]Enkephalin	1×10^{-6}	0
	1×10^{-5}	0

[a] The binding assay values are means of three measurements ± SEM. The concentration of [^{3}H]naloxone was 5×10^{-10} M.

The fact that the phenolic hydroxyl group is essential for activity also becomes apparent from the complete lack of activity observed with [Phe[1],Trp[4],Met[5]]enkephalin (Table I). The latter finding is in agreement with the reported low receptor affinities of [Phe[1],Leu[5]]enkephalin (Terenius et al., 1976) and [Phe[1],Met[5]]enkephalin (Chang et al., 1976) and the low activities of the latter analogue observed in bioassays (Day et al., 1976; Ling and Guillemin, 1976).

The results of the opiate-receptor binding assay are presented in Table II. Evaluation of the data obtained for levorphanol and dextrorphan on the basis of the definition of stereospecificity by Goldstein et al. (1971) yields 60–75% stereospecific binding of [^{3}H]naloxone. In view of the obvious methodologic difficulties, we do not intend to evaluate the data in terms of exact IC_{50} values. However, inspection of the data reveals that the relative receptor affinities of the analogues correlate well with their relative potencies in the bioassay. The one exception is [Tyr(NMe),Met[5]]enkephalin, whose receptor affinity is about five times higher than that of [Met[5]]enkephalin, while exactly the reverse ratio was observed for the potencies of these two peptides in the bioassay (Table I). One possible explanation for this reversal in potencies could be more predominant enzymatic degradation of the peptides in the binding assay, which would explain the relatively higher potency of [Tyr(NMe)[1]]enkephalin as a consequence of reduced degradation by aminopeptidases.

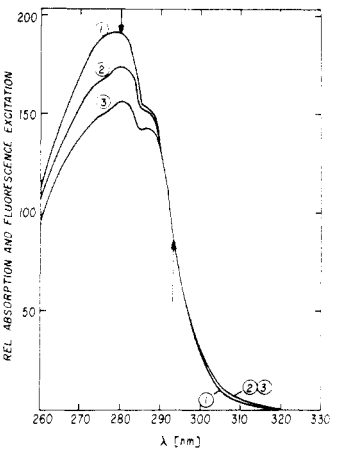

FIGURE 3: Absorption spectrum of [Trp⁴,Met⁵]enkephalin normalized at 280 nm (curve 1) and fluorescence excitation spectra of [Trp⁴,-Met⁵]enkephalin (curve 2) and [Phe¹,Trp⁴,Met⁵]enkephalin (curve 3) normalized at 293 nm. Arrows indicate wavelengths of normalization as described in the text.

The absorption spectrum of [Trp⁴,Met⁵]enkephalin above 260 nm (Figure 3) nearly coincides with the sum of tyrosine and tryptophan absorption and is exactly superimposable on the addition spectrum of [Met⁵]enkephalin and [Phe¹,-Trp⁴,Met⁵]enkephalin. This indicates that the absorption properties of the two fluorophores are not changed as a consequence of incorporation into the peptide chain. The fluorescence emission spectra of both [Trp⁴,Met⁵]enkephalin and [Phe¹,Trp⁴,Met⁵]enkephalin show a maximum at 350 nm (Figure 2) which coincides with that observed for tryptophan in aqueous solution. This finding indicates that the fluorophore is free to interact with the polar water molecules as it is observed with the tryptophanyl residues in most polypeptide hormones of low molecular weight (Edelhoch, 1976). The emission spectrum of [Met⁵]enkephalin shows the typical tyrosine fluorescence maximum at 305 nm. Within the accuracy of the measurement, the same tryptophan fluorescence quantum yields are determined for [Trp⁴,Met⁵]enkephalin and [Phe¹,Trp⁴,Met⁵]enkephalin (Table III). This is a good indication that the tryptophanyl residues in the two peptides are located in structurally identical environments, which justifies the use of [Phe¹,Trp⁴,Met⁵]enkephalin as reference compound in the determination of the transfer efficiency (cf. Materials and Methods). The low tyrosine quantum yield observed in [Met⁵]enkephalin is due to the N-terminal position of this amino acid. A similarly low quantum yield ($\phi_{Tyr} = 0.031$) was calculated for the tyrosine fluorescence of the tripeptide H·Tyr-Gly-Gly·OH on the basis of the reported ratio of fluorescence output of the latter compound with that of free tyrosine (Cowgill, 1976) and the tyrosine quantum yield of 0.14 employed in our study. Obviously, this low quantum yield also lowers the R_0 value for energy transfer between tyrosine and tryptophan, which is an advantage in view of the relatively short distance to be measured.

The use of the values $\phi_D = 0.009 \pm 0.0015$ and $\phi_D^0 = 0.030 \pm 0.001$ (Table III) in eq 3 results in an efficiency of 0.70 ± 0.05 for energy transfer between tyrosine in position 1 and tryptophan in position 4. On the basis of the relative enhancement of tryptophan fluorescence, a transfer efficiency of 0.54 ± 0.10 is obtained by means of eq 4 (cf. Figure 3). The good agreement between these two values indicates that aside from energy transfer no additional quenching of tyrosine fluorescence is engendered by the substitution of phenylalanine with tryptophan in position 4 (cf. Schiller, 1975).

TABLE III: Tyrosine and Tryptophan Quantum Yields of [Met⁵]-Enkephalin Analogues.[a]

Compound	λ_{exc} (nm)	ϕ_{Trp}	ϕ_{Tyr}
[Met⁵]Enkephalin	280		0.030 ± 0.001
[Trp⁴,Met⁵]-Enkephalin	280		0.009 ± 0.0015
	293	0.058 ± 0.0002	
[Phe¹,Trp⁴,Met⁵]-Enkephalin	293	0.060 ± 0.002	

[a] The quantum yields are the means of three measurements ± SEM.

Using the value of 4.8×10^{-16} M^{-1} cm^6 for the overlap integral (Eisinger et al., 1969), an R_0 of 10.7 Å was calculated. The lack of knowledge with regard to the relative orientation of donor and acceptor has often been pointed out as a source of error in the computation of this parameter. However, the assumption of extensive randomization in donor–acceptor orientation is fairly justified in the case of polypeptide hormones with low molecular weight where orientational freedom of side-chain residues relative to the peptide backbone has been demonstrated in several cases either by fluorescence polarization spectroscopy (Schiller, 1972) or by carbon-13 spin–lattice relaxation experiments (Smith and Deslauriers, 1975). The possibility of obtaining upper and lower limits for the orientation factor on the basis of polarized emission spectroscopy has been extensively discussed by Dale and Eisinger (1975). For the dansyl group attached to the lysine residue in position 21 of the tetradecapeptide [Lys(Dns)²¹]$ACTH_{11-24}$, a limiting value of 0.140 for the emission anisotropy was determined by nanosecond fluorescence polarization spectroscopy (Schiller, P. W., and Brand, L., unpublished results). This value permitted the calculation of an angle of 47° through which the transition moment vector of the dansyl group moves with respect to the peptide backbone during the excited-state lifetime. On the basis of the model introduced by Eisinger and Dale (1974), orientational freedom of this magnitude for donor and acceptor gives rise to an uncertainty of about 20% for the value of R_0. In the case of [Trp⁴,Met⁵]enkephalin, such analysis is complicated by the very short lifetimes of tryptophan and tyrosine fluorescence. However, information about internal orientational freedom of side chains can also be obtained by NMR spectroscopy. Recent ^1H NMR studies with [Met⁵]enkephalin demonstrated the existence of a preferred, rigid backbone conformation (Garbay-Jaureguiberry et al., 1976; Roques et al., 1976; Jones et al., 1976). Furthermore, the results obtained by Garbay-Jaureguiberry (1976) indicate that both the tyrosine residue in position 1 and the phenylalanine residue in position 4 have orientational freedom relative to the peptide backbone. For the side chains of both tyrosine and phenylalanine, the existence of three populations of rapidly interconverting rotamers was demonstrated by ^1H NMR analysis (Jones et al., 1976). Recently performed ^{13}C relaxation time measurements indicated orientational freedom for the phenylalanine residue and somewhat more restricted motion for the tyrosine side chain with respect to the peptide backbone (Bleich et al., 1976). The ensemble of these NMR studies provides good evidence for considerable randomization in donor–acceptor orientation and we believe that the value of R_0 calculated with the orientation factor applying to the random situation ($\chi^2 = \frac{2}{3}$) cannot be seriously in error. The NMR data also rule out molecular self-association (Bleich et al.,

1976) and thus eliminate the possibility of *inter*molecular energy transfer.

With R_0 = 10.7 Å and the measured transfer efficiencies of 0.54 ± 0.10 and 0.70 ± 0.05, average donor–acceptor separations of 10.4 ± 0.7 and 9.3 ± 0.4 Å, respectively, are calculated by means of eq 2. These two values are averaged to yield a mean intramolecular distance of 10.0 ± 1.1 Å between tyrosine in position 1 and tryptophan in position 4.

It is interesting to compare this value with the end-to-end distance of a tetrapeptide in fully extended conformation (15 Å) and with experimentally determined end-to-end distances of other tetrapeptides reported in the literature. Edelhoch et al. (1967) determined an intramolecular tryptophan–tyrosine separation of 13.3 Å in H·Trp-Gly-Gly-Tyr·OH at alkaline pH by evaluation of energy transfer between tryptophan (donor) and tyrosinate (acceptor). A similar value (13.5 Å) was determined for the intertyrosine distance of H·Tyr-Gly-Gly-Tyr·OH in glycerol solution by means of fluorescence polarization measurements (Edelhoch et al., 1968). However, the latter value may constitute a slight overestimate due to the large R_0 value employed by these authors, as pointed out by Guillard et al. (1975). In any case, the comparison with our value is complicated by the differences in solvent conditions. Guillard et al. (1975) determined intramolecular tyrosine–tryptophan distances in the tetrapeptides R′·Trp-Ala-Ala-Tyr·R″ (14.5–15.7 Å) and R′·Trp-Gly-Ala-Tyr·R″ (<12.4 Å) in ethanol solution, which demonstrated a significant reduction in intrafluorophore distance by substitution of glycine for alanine in position 2. Theoretical calculations carried out by these authors resulted in two energetically preferred conformations both containing two hydrogen-bonded 3 → 1 and 4 → 2 seven-membered rings. However, it was not ruled out that stacking interactions between the phenol and indole rings which only become feasible in the 4 → 1 hydrogen-bonded β_I-bend-model may render the latter conformation energetically more favorable.

On the basis of theoretical considerations, Bradbury et al. (1976) proposed a β_I-bend conformation with a hydrogen bond between the amino group of phenylalanine and the carbonyl group of tyrosine for [Met^5]enkephalin. As pointed out by these authors, this conformation would position the critical chemical functions of the peptide in a topographical disposition analogous to that existing in the rigid framework of the potent oripavine derivative Ia. The same holds true for the β_I-bend model characterized by a hydrogen bond between the amino group of methionine and the carbonyl group of glycine in position 2. Recent NMR studies seem to favor the latter conformation over the 4 → 1 hydrogen-bonded model (Garbay-Jaureguiberry et al., 1976; Roques et al., 1976; Jones et al., 1976). From inspection of the CPK models of [Met^5]enkephalin in either one of the proposed β_I conformations, a phenol–phenyl distance of 9–11 Å is obtained, which is in good agreement with the experimentally determined average tyrosine–tryptophan separation of 10.0 ± 1.1 Å in [Trp^4,-Met^5]enkephalin. However, this result does by no means provide a definitive proof for the correctness of the β_I-bend model, since the observed intrafluorophore distance could also be brought about by another type of folded conformation which might be devoid of a hydrogen bond (Bleich et al., 1976). The important finding of this study is the quantitative agreement between the measured tyrosine–tryptophan separation in [Trp^4,Met^5]enkephalin and the phenol–phenyl distance (8–10.5 Å) in the oripavine derivative Ia.

In view of the recently reported higher receptor affinity and activity of β-endorphin relative to [Met^5]enkephalin (Birdsall and Hulme, 1976; Ling and Guillemin, 1976), it would be interesting to know whether the two peptides differ in the spatial arrangement of the aromatic residues in positions 1 and 4. However, the enhanced activity of β-endorphin may simply be due to an increased metabolic lifetime or to the presence of the two positively charged lysine residues at the C terminal of the peptide, which might increase its affinity for the opiate receptor through an additional ionic interaction with a negatively charged receptor site.

In summary, we conclude that both the performed chemical modifications in relation to the observed morphinomimetic potencies and the determined intramolecular distance provide strong evidence for an analogous spatial disposition (topography) of the critical chemical functions in [Met^5]enkephalin and morphine–oripavine derivatives.

Acknowledgments

We thank J. Prosmanne and L. Prud'homme for expert technical assistance. Thanks are also due to R. Routhier for the amino acid analyses. The supply of levorphanol and dextrorphan by Hoffmann-La Roche, Ltd., is gratefully acknowledged.

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Effects of Neurotoxins (Veratridine, Sea Anemone Toxin, Tetrodotoxin) on Transmitter Accumulation and Release by Nerve Terminals in Vitro†

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ABSTRACT: Two of the three toxic compounds used in this work, veratridine and the sea anemone toxin, provoke neurotransmitter release from synaptosomes; the third one, tetrodotoxin, prevents the action of both veratridine and the sea anemone toxin. The half-maximum effects of veratridine and sea anemone toxin actions on synaptosomes are $K_{0.5}$ = 10 and 0.02 μM, respectively. Although veratridine and the sea anemone toxin similarly provoke neurotransmitter release, they act on different receptor structures in the membrane. Tetrodotoxin antagonizes the effects of both veratridine and the sea anemone toxin. The half-maximum inhibitory concentration of tetrodotoxin is $K_{0.5}$ = 4 nM for veratridine and 7.9 nM for ATX_{II}. It is very similar to the dissociation constant measured from direct binding experiments with the radioactive toxin. The analysis of this antagonistic action offers an easy in vitro assay for tetrodotoxin interaction with its receptor.

Several neurotoxins now appear to be potentially important tools in studying the mechanism of action potential generation in axons. This group of toxins includes (i) the alkaloids veratridine and batrachotoxin which cause firing and depolarization of nerves by activating the action potential Na^+ ionophore (Albuquerque et al., 1971; Narahashi, 1974; Ulbricht, 1969), (ii) scorpion neurotoxin which markedly slows down sodium inactivation (i.e., the closing of the Na^+ channel) and also alters the steady-state potassium current (i.e., the opening of the K^+ channel) (Romey et al., 1975; Narahashi et al., 1972; Koppenhöffer and Schmidt, 1968), (iii) sea anemone neurotoxin which selectively prevents the closing of the Na^+ channel (Romey et al., 1976a,b), and (iv) tetrodotoxin, the most widely used of all these neurotoxins, which is a specific inhibitor of the action potential Na^+ ionophore (Evans, 1972; Narahashi, 1974).

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