Biochemical Pharmacology, Vol. 30, No. 12, pp. 1621–1627, 1981.
Printed in Great Britain.

0006-2952/81/121621-07 $02.00/0

A REACTION OF ACETALDEHYDE WITH ENKEPHALINS AND RELATED PEPTIDES

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(*Received* 16 *November* 1980; *accepted* 10 *February* 1981)

Abstract—The naturally-occurring opioid peptides methionine-enkephalin (Tyr-Gly-Gly-Phe-Met-OH) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu-OH), and related peptides with the same N-terminal amino acid sequence react with acetaldehyde in water to form the N-terminal ring-closed imidazolidinone derivative. Experimental evidence for the proposed structure is based on a comparison of the rates of change of the chromatographic, chemical properties and opiate activity of the enkephalins in the presence of acetaldehyde; methionine-enkephalin exhibits a half-time for reaction of about 14 min in aqueous phosphate buffer, pH 7.0 at 25°. Additional studies indicate that the properties of the acetaldehyde-enkephalin reaction are also shared by the larger opioid peptides. In particular, the pituitary opioid, β-endorphin loses opiate activity on exposure to acetaldehyde at a rate comparable to that observed with the enkephalins.

The chemical union of a carbonyl group and an amino group to form a Schiff's base has held the attention of peptide and protein chemists for many years [1, 2]. In this context, the recently described reaction of acetaldehyde with the opioid pentapeptides, methionine-enkephalin ([Met^5] enkephalin) and leucine-enkephalin ([Leu^5] enkephalin), to give stable derivatives with modified opiate activities was of considerable interest [3]. Preliminary structural studies using proton n.m.r. spectroscopy indicated that these acetaldehyde-enkephalin derivatives arise by formation of a 2-methylimidazolidin-4-one as shown in Scheme 1 [4].

In this report we substantiate previous structural work by describing studies which correlate the rates of change of chromatographic and opiate activity of the enkephalins following exposure to acetaldehyde with modification of the α-amino group of the N-terminal tyrosine residue. In addition, the reaction between acetaldehyde and enkephalin is freely reversible; this appears to be a general property shared by several other peptides which react with carbonyl compounds to form the corresponding imidazolidin-4-one derivative [5–9].

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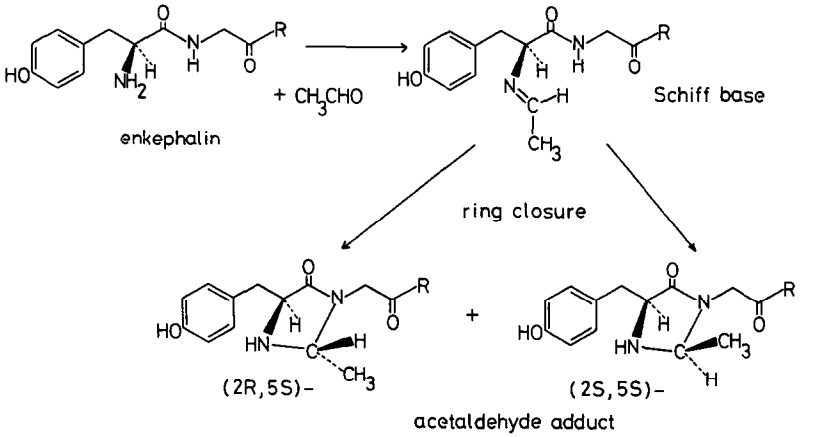
EXPERIMENTAL MATERIALS

[Met^5] Enkephalin and [Leu^5] enkephalin were obtained from Miles Laboratories. [^{3}H]-[Met^5]



methionine-enkephalin, R= -Gly-L-Phe-L-Met-OH

leucine-enkephalin, R= -Gly-L-Phe-L-Leu-OH

Scheme 1

Enkephalin (23.5 Ci/mmole) and [^{3}H]-[D-Ala^2, D-Leu^5]-enkephalin (52 Ci/mmole) were from the Radiochemical Centre, Amersham. [^{3}H]-dihydromorphine (47.5 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, MA). Acetaldehyde was obtained from BDH Chemicals (Poole, U.K.) and purified by standard procedures [10]. Pyridine (BDH Chemicals, Poole, U.K.) was dried over CaH_2 and distilled from α-naphthylisocyanate. TNP-sulphonic acid also from BDH Chemicals was purified as previously described [11]. All other chemicals used were of BDH AnalaR grade or the highest purity available. Phosphate buffers were prepared from potassium dihydrogen phosphate and disodium hydrogen phosphate. Yeast alcohol dehydrogenase (EC 1.1.1.1.), NADH, dithiothreitol, bacitracin and Tyr-Gly-Gly-OH were purchased from Sigma Chemical Co. (St. Louis, MO).

Naloxone hydrochloride was a gift from Endo Laboratories (New York, NY.) β-Endorphin was kindly provided by Dr. C. H. Li of the Hormone Research Laboratory, University of California, San Francisco, CA. Antibody to β-Endorphin was supplied by Dr. D. G. Smyth, National Institute of Medical Research (Mill Hill, London, U.K.).

METHODS

Synthesis of acetaldehyde-peptide adducts. Typically, the peptide (24 μmoles) and acetaldehyde (320 μmoles) were dissolved in 0.05 M phosphate buffer (4.0 ml), pH 7.0 at room temperature. The reaction was monitored by analytical t.l.c. (see below and Table 1). When there was no detectable ninhydrin-positive starting peptide the reaction mixture was diluted with water (0.6 ml) and applied to a short column (3 × 1 cm) of sulphonated polystyrene resin (Dowex-50; 4% cross-linked, H^+ form). The column was washed extensively with water (approximately ten bed volumes), followed by elution with 0.1 M aqueous pyridine. The effluent fractions containing the modified peptide, detected by the Folin-Lowry assay [12] were pooled, freeze-dried and stored anhydrous at −20°. Amino acid analysis (Chromaspek Amino Acid Analyser, Rank-Hilger, Margate, U.K.) of an acid hydrolysate of acetaldehyde-[Met^5] enkephalin (6 M HCl at 105° for 12 hr) gave the following molar ratios: Tyr, 0.94; Gly, 2.03; Phe, 1.02; Met, 1.0.

Thin layer chromatography. Analytical t.l.c. was done on precoated silica gel G aluminium-backed plates (Merck, Darmstadt, Germany). Peptide samples (about 20 ng) were chromatographed in the following solvent systems: (A) chloroform/methanol/acetic acid (9:2:1, by vol.); (B) chloroform/methanol/acetic acid/water (30:20:4:6. by vol.); (C) ethyl acetate/pyridine acetic acid/water (120:20:6:11, by vol.); (D) ethyl acetate/pyridine/acetic acid/water (60:20:6:11, by vol.); (E) butan-1-ol/acetic acid/water (3:1:1. by vol.); (F) butan-1-ol/pyridine/acetic acid/water (60:20:6:24 by vol.). Peptides were detected by spraying the plates with ninhydrin [13] R_f values are shown in Table 1.

Table 1. Chromatographic properties of L-Tyr-Gly-Gly-OH, [Met^5] enkephalin and [Leu^5] enkephalin and their corresponding acetaldehyde adducts

Peptide	Solvent system R_f					
	A	B	C	D	E	F
L-Tyr-Gly-Gly-OH	0	0.21	0	0.16	0.28	0.15
Acetaldehyde adduct	0.15	0.42	0.06	0.43	0.37	0.26
[Met^5] enkephalin	0.10	0.51	0.05	0.61	0.55	0.50
Acetaldehyde adduct	0.35	0.67	0.13	0.75	0.64	0.55
[Leu^5] enkephalin	0.13	0.52	0.06	0.71	0.66	0.57
Acetaldehyde adduct	0.40	0.75	0.21	0.87	0.71	0.61

Peptide assays. [Met^5] Enkephalin was assayed spectrophotometrically by reaction with TNP*-sulphonic acid [14]. The $E_{340}(M^{-1}cm^{-1})$ of TNP-[Met^5] enkephalin was determined to be 13,000 ± 600. Radioiodination and radioimmunoassay of β-endorphin was carried out as described by Snell *et al.* [15].

Acetaldehyde assay. Acetaldehyde was routinely stores at −20° as an aqueous solution at a concentration of 2.0–3.0 M. Fresh solutions were prepared every two weeks and were assayed by determining the yeast alcohol dehydrogenase-catalysed oxidation of NADH at 340 nm. The assay volume was 2.0 ml of 0.1 M phosphate buffer, pH 7.2 containing yeast alcohol dehydrogenase (100 i.u.), NADH (0.05 ml, 10 mg/ml) and dithiothreitol (0.8 μmoles).

Thermal decomposition of acetaldehyde-[Met^5] enkephalin. A solution of acetaldehyde-[Met^5] enkephalin (5.0 mg) in 0.5 M acetic acid (1.0 ml) containing 2,4-dinitrophenylhydrazine (2.0 mg) was heated for 30 min on a boiling water bath. After cooling, the reaction mixture was extracted with ether (2 × 5.0 ml). The ether was dried over anhydrous sodium sulphate and subsequently removed by rotary evaporation. The residue was purified by two consecutive preparative t.l.c. steps on silica gel: developed firstly with chloroform to remove unreacted 2,4-dinitrophenylhydrazine reagent, followed by benzene/ethyl acetate (4:1, by vol.) The major material so obtained co-chromatographed with authentic acetaldehyde-2,5-dinitrophenylhydrazone on silica gel.

Solvent system: chloroform (R_f 0.53); ether (R_f 0.56); petroleum ether (hexane fraction)/ether (1:1, by vol., R_f 0.29); petroleum ether/ethyl acetate (2:1, by vol., R_f 0.26) benzene (R_f 0.25); benzene/ethyl acetate (4:1, by vol., R_f 0.50). The same material also gave a mass spectrum identical to authentic acetaldehyde-2, 4-dinitrophenylhydrazone. Mass spectra were recorded using a Varian CH-5 mass spectrometer linked to a varian 620i computer for peak normalisation. Mass spectrum m-equiv 224[M$^+$, 100%]; 180 (44); 152 (51); 122 (57); 92 (53); 91 (49); 90 (35).

Biological activity. Opiate receptor binding was assayed by inhibition of binding of radiolabelled [^{3}H]-[D-Ala^2,D-Leu^5] enkephalin and [^{3}H]-dihydro-

* Abbreviations used: TNP—sulphonic acid, 2,4,6-trinitrobenzene-1-sulphonic acid.

morphine to opiate receptors in a particulate fraction from rat brain. Male Sprague–Dawley rats weighing about 200 g, were killed by cervical dislocation, each brain (minus cerebellum) was rapidly removed and placed in 15 vol. (w/v) of an ice cold solution of 0.32 M sucrose and 0.005 M Tris HCl buffer, pH 7.4 and homogenised using a motor driven Teflon pestle in Potter-Elvehjem tissue grinder. The crude homogenate was centrifuged for 10 min at 4° and 600 *g* (Beckman Coolspin) and the supernatant retained. The pellet was resuspended in five volumes (original wet weight of brain) and centrifuged again at 4° and 600 g for 15 min. The combined supernatant was centrifuged for 30 min at 4° and 45,000 *g* (Sorvall t-65 rotor) and the pellet finally suspended in 60 vols (original wet weight of brain) of 0.04 M Tris HCl buffer, pH 7.4. In a final volume of 2.0 ml, aliquots (1.7 ml) of this preparation were incubated with either [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin (0.8 nm) or [^{3}H]-dihydromorphine (1.05 nm) and test substance for 2 hr at 0° (ice/water mixture) in the presence of bacitracin (100 μg/ml). The solutions were filtered on Whatman GF/B glass fibre discs, under vacuum, and washed with ice-cold Tris HCl buffer, pH 7.4 (2 × 4.0 ml). The discs were transferred to scintillation phials containing 10 ml of Triton-X-100/toluene-based scintillant (PPO/POPOP), and shaken at room temperature overnight.

Non-specific binding was determined by carrying out parallel incubations in the presence or absence of a large excess (1 μM) of unlabelled [Met^5] enkephalin or naloxone HCl.

Field stimulation of the isolated mouse vas deferens was carried out by the procedure of Hughes *et al.* [16]. Each tissue was suspended in a 2.0 ml organ bath and stimulated by means of two platinum electrodes using a Grass 5–44 stimulator. Force-displacement transduced contractions (Grass FT-03 transducer) were recorded with a Grass model 7C pen recorder.

Bioassay using supramaximal coaxially stimulated longitudinal muscle-myenteric plexus strips from the guinea pig ileum was by the procedure described by Paton [17] and Kosterlitz and Watt [18].

RESULTS AND DISCUSSION

The acetaldehyde-[Met^5] enkephalin reaction

Formation of acetaldehyde-[Met^5] enkephalin may be demonstrated by mixing acetaldehyde and [^{3}H]-[Met^5] enkephalin in aqueous phosphate buffer at room temperature, separating the starting material and product at various times after mixing by t.l.c. and determining the tritium distribution by liquid scintillation counting [Fig. 1(A)]. These reaction conditions also afforded the most convenient method of preparing large quantities of acetaldehyde-enkephalins and related peptides, and the experimental details are described in the Methods Section; under these reaction conditions the conversion of [Met^5] enkephalin to acetaldehyde-[Met^5] enkephalin exhibits a half-time for reaction of about 14 min. The time course for the reaction of [^{3}H]-[Met^5] enkephalin with acetaldehyde was correlated with a progressive loss of N-terminal α-amino group when assayed by derivatisation with TNP-sulphonic acid [Fig. 1(B)]. Means *et al.* [19] have reported that TNP-sulphonic acid reacts specifically with primary amines and the foregoing result therefore provides cogent evidence of a direct role for the α-amino group of [Met^5] enkephalin in the modification reaction.

An interesting consequence of acetaldehyde-enkephalin adduct formation is the observed loss of intrinsic opiate activity of [Met^5] enkephalin and [Leu^5] enkephalin [3]. As shown in Figs 2(A) and

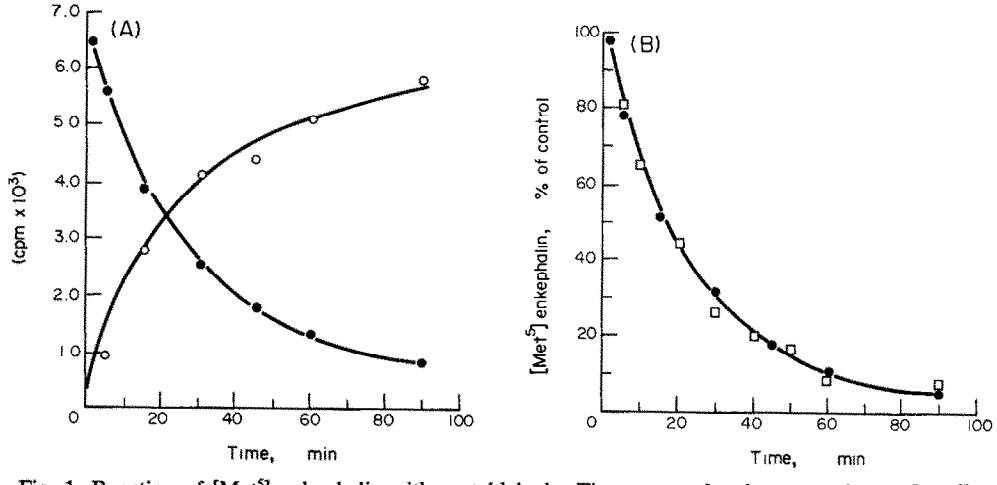


Fig. 1. Reaction of [Met^5] enkephalin with acetaldehyde. Time course for the conversion of [Met^5] enkephalin (●) to acetaldehyde-[Met^5] enkephalin (○). (A) Acetaldehyde (8 μmoles) and [^{3}H]-[Met^5] enkephalin (0.4 μmoles; 2.52 × 10^6 cpm/μmole) were incubated at 25° in 0.11 ml of 0.05 M phosphate buffer, pH 7.0. At various times after mixing a 2 μl aliquot was removed, transferred to a t.l.c. plate, developed (solvent system B) and sprayed with ninhydrin. Ninhydrin-positive spots were scraped off and the radioactivity determined. (B) Residual [^{3}H]-[Met^5] enkephalin (●) was determined as in (A), or by derivitisation of the α-amino group of [Met^5] enkephalin (□): [Met^5]-enkephalin (8 μmoles) and acetaldehyde (16 μmoles) were incubated at 25° in 2.2 ml of 0.05 M phosphate buffer, pH 7.0. At different times, 0.1 ml was removed and assayed using TNP-sulphonic acid [14].

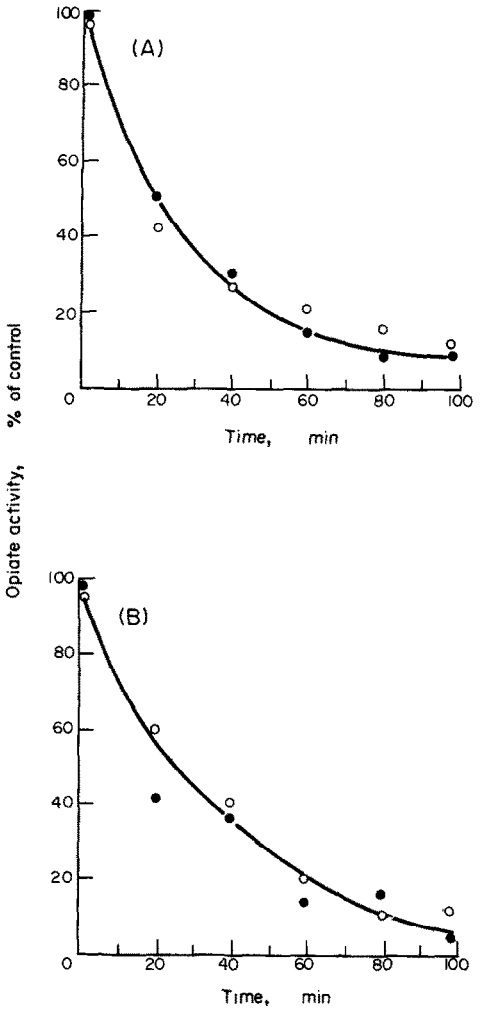


Fig. 2. Changes in the opiate activity of [Met^5] enkephalin (●) and [Leu^5] enkephalin (○) on incubation with acetaldehyde. The enkephalins (4 μmoles each) and acetaldehyde (80 μmoles) were incubated at 25° in 1.1 ml of 0.05 M phosphate, pH 7.0. At the indicated times 0.01 ml was removed, diluted into 30 ml of water and an aliquot (0.01 ml) of the diluted solution was assayed for residual opiate activity by determining the inhibition of stereospecifically bound [^{3}H]-[D-Ala^2-D-Leu^5] enkephalin (2A) and [^{3}H]-dihydromorphine (2B) in a rat brain homogenate as described. The points represent the mean of three separate binding assays.

(B), incubating either [Met^5] enkephalin or [Leu^5] enkephalin with acetaldehyde leads to a loss of opiate activity that is similar in rate to the chromatographic and chemical changes already described. These results are, therefore, fully complementary and further support the view of a specific N-terminal modification of the enkephalins previously derived from structural studies [4]. In the present context, chemical studies have shown that the shortest N-terminal amino acid sequence required for rapid adduct formation with acetaldehyde is Tyr-Gly-Gly and the change in the chromatographic properties of this tripeptide and of [Met^5] enkephalin and [Leu^5] enkephalin are included in Table 1.

Breakdown of acetaldehyde-[Met^5] enkephalin in aqueous solution

Freeze-dried samples of acetaldehyde [Met^5] enkephalin can be stored for short periods under anhydrous conditions at −20° with no apparent decomposition but for biological studies newly-synthesised material must be used [3]. Freshly prepared solutions of acetaldehyde-[Met^5] enkephalin undergo a temperature dependent decomposition to [Met^5] enkephalin; this is very slow at low temperatures but becomes extremely rapid at higher temperatures as shown by an increase of A_{340} on treating a heated solution with TNP-sulphonic acid (Fig. 3). The reversible nature of the reaction and, in particular, the heat-induced decomposition of the acetaldehyde-[Met^5]-enkephalin adduct is shared by several other imidazolidinone derivatives of peptides [5, 9]. This property is perhaps best exemplified by the elegant studies of du Vigneaud and co-workers [6, 7] on the reaction of acetone with oxytocin to give an acetone-oxytocin adduct (see later) and may therefore be used to provide not only additional evidence for the proposed structure of acetaldehyde-[Met^5] enkephalin but also to distinguish alternative structures that could result from the reaction of acetaldehyde with an N-terminal tyrosyl peptide [1, 20]. Consequently, we have undertaken additional experiments to establish the reversible nature of the reaction.

Confirmation that heat-induced decomposition of acetaldehyde-[Met^5] enkephalin also leads to concomitant release of acetaldehyde was obtained by heating an aqueous solution of the former in a sealed tube with 2,4-dinitrophenylhydrazine: the chromatographic behaviour and mass spectrum of the major 2,4-dinitrophenylhydrazone so obtained was identical with an authentic sample of acetaldehyde-2, 4-dinitrophenylhydrazone (see Experimental Section for details). In addition, the recovery of biological activity after heating dilute solutions of the

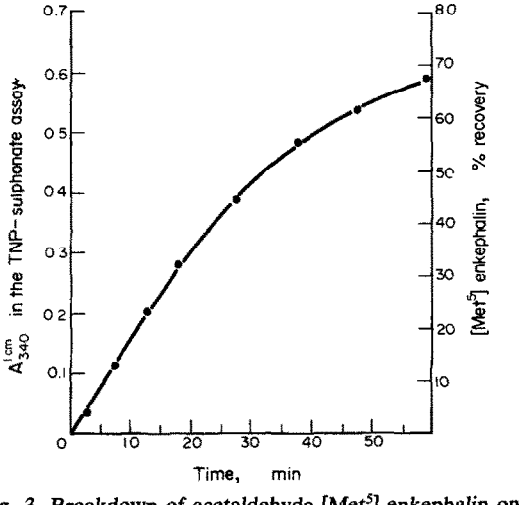


Fig. 3. Breakdown of acetaldehyde-[Met^5] enkephalin on heating. Acetaldehyde-[Met^5] enkephalin in 1.0 ml of 1 per cent acetic acid (v/v) was heated on a boiling water bath. At the times indicated 0.1 ml was removed and assayed using TNP-sulphonic acid [14].

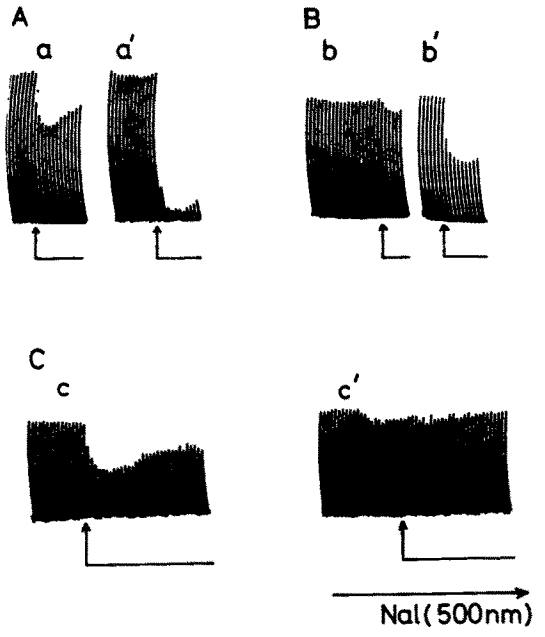


Fig. 4. The inhibiting effect of acetaldehyde-enkephalins on the isolated mouse vas deferens and the guinea pig ileum. (A) Effect of heating an aqueous solution of acetaldehyde-[Met^5] enkephalin at 90° for 30 min on the electrically induced contractions of the guinea pig ileum; (a) before heating (6 μm final concentration); (a') after heating (1 μm, final concentration). (B) Inhibition of electrically induced contractions of the isolated mouse vas deferens by acetaldehyde-[Met^5] enkephalin: before [(b) 300 nm] and after [(b') 30 nm] heating an aqueous solution at 90° for 30 min. (C) Inhibition of contractions of the isolated mouse vas deferens by acetaldehyde-[Leu^5] enkephalin [(c) 8 μm] and antagonism of the inhibiting effects by pretreatment with naloxone (Nal, 500 nm). Tissue preparations were washed between additions. Test substances were added at the points indicated by the arrows.

acetaldehyde adducts of [Met^5] enkephalin and [Leu^5] enkephalin was established by utilising the isolated mouse vas deferens and guinea pig ileum opiate assays. As shown in Fig. 4(A), a heated solution of acetaldehyde-[Met^5] enkephalin causes a significant increase in the inhibition of electrically induced contractions of the guinea pig ileum (a') compared with the same solution before treatment (a). Heating dilute solutions of acetaldehyde-[Met^5] enkephalin facilitated a quantitative evaluation of the recovery of [Met^5] enkephalin; the observed 60 per cent increase in inhibition of contractions of the mouse vas deferens [Fig. 4(B)] compared well with the recovery of [Met^5] enkephalin using the TNP-sulphonic acid assay (Fig. 3). Finally it should be noted that the depressant effect of acetaldehyde-enkephalins on the mouse vas deferens and guinea pig ileum, although very low are presumed to occur by interaction with opiate receptors since these effects are antagonised by naloxone. A typical result is shown in Fig. 4(C) which shows the effect of acetaldehyde-[Leu^5] enkephalin on contractions of the vas deferens (c), and its antagonism by naloxone (c).

The acetaldehyde-β-endorphin reaction

As indicated earlier, the shortest N-terminal amino acid sequence that leads to rapid adduct formation with acetaldehyde is Tyr-Gly-Gly, and this is maintained with the largest opioid peptide, β-endorphin [21], which contains the [Met^5] enkephalin N-terminal sequence [Fig. 5(A)]. This result further supports the view of a specific N-terminal modification reaction that is largely independent of the remaining structural features of the molecule. Additional evidence for the specificity of the acetaldehyde-β-endorphin reaction was obtained by radioimmunoassay of acetaldehyde-treated β-endorphin; during the course of the modification reaction [Fig. 5(B)] there was no observed difference between the control and acetaldehyde-treated samples. Cross-reactivity studies have indicated that the antigenic determinants are at the C-terminal of the β-endorphin molecule [22] (S. L. Lightman personal communication) and this may also be taken as indirect evidence of the specificity of the modification reaction because any non-specific acetaldehyde-promoted decomposition of β-endorphin would most probably involve the amino group of lysine residues

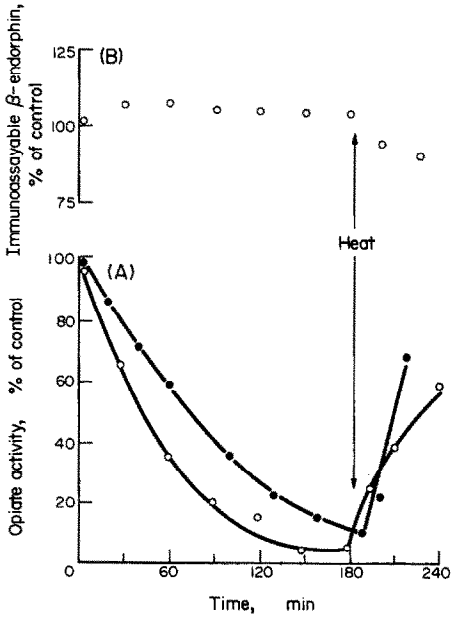


Fig. 5. Loss of the ability of acetaldehyde-treated [Met^5] enkephalin (●) and β-endorphin (○) to inhibit electrically induced contractions of the isolated mouse vas deferens. (A) [Met^5] Enkephalin (2 nmoles) and acetaldehyde (20 μmoles) were incubated at 25° in 0.5 ml of 0.05 M phosphate buffer, pH 7.0. β-Endorphin (200 ng) and acetaldehyde (10 μmoles) were incubated at 25° in 0.2 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.02 per cent bovine serum albumin (w/v). At the indicated times, 0.02 ml aliquots were removed and assayed for residual opiate activity as outlined in the Experimental Section. Experimental points represent the mean of two separate incubations and assayed using two different tissue preparations. (B) Radioimmunoassay of β-endorphin following incubation with acetaldehyde as described above. The points represent the mean of triplicate determinations. The standard curves for β-endorphin in the presence and absence of acetaldehyde were identical (data not shown).

[2], and these are primarily located towards the C-terminal [21]. Another important point which is made clear from this study is the danger of using immunoassay data as an indication of the presence of biologically active opioids. This problem has recently been emphasised by Li *et al.* [23] who have demonstrated a clear dissociation between immunoactivity and biological activity in their studies with synthetic analogues of β-endorphin. They found that deletion of a single amino acid outside the enkephalin segment reduced or abolished immunoreactivity without marked effect on biological activity, whilst deletion of a single amino acid within the enkephalin segment abolished or reduced opiate activity without effecting immunoreactivity. There is already evidence that naturally-occurring N^{α}-acetyl derivatives of endorphins are biologically inactive, but possess immunological potency indistinguishable from the parent peptides [24, 25]). To this we must now add similar findings for the acetaldehyde-β-endorphin adduct. Radioimmunoassay data should therefore be interpreted in the light of careful chromatographic studies and confirmation of full pharmacological potencies.

Interestingly, Doneen *et al.* [26] have reported circumstantial evidence of an acteone-β-endorphin adduct; this adduct was prepared by treating β-endorphin with aqueous acetone under conditions previously described [5] for the synthesis of acetone-oxytocin. This acetone-β-endorphin adduct has approximately 1 per cent the potency of β-endorphin in the guinea pig ileum and rat brain opiate receptor binding assay. Under the same reaction conditions, [Met^5] enkephalin also form a stable acetone adduct with loss of opiate activity, and the proton n.m.r. spectrum of this adduct is indicative of the 2,2-dimethylimidazolidin-4-one derivative [M.C. Summers, M. J. Gidley and J. K. M. Sanders, personal communication]. Heating acetaldehyde-treated solutions of β-endorphin leads to recovery of opiate activity [Fig. 5(A)], confirming the reversible nature of the reaction with the larger peptide. Thus, the results described herein, including preliminary structural studies [4] are consistent with the formation of a 2-methylimidazolidin-4-one derivative in which the initially formed Schiff base experiences nucleophilic attack by the nitrogen of the Tyr-Gly peptide bond as outlined in Scheme 1.

A similar ring closure mechanism has previously been proposed for the reaction between acetone and oxytocin to give an acetone-oxytocin adduct [6, 7]. In this example, ring closure to give a 2,2-dimethylimidazolidin-4-one derivative involves an N-terminal Cys and the amide nitrogen of an adjacent Tyr residue. Curiously, Mean and Feeney [1] have referred to this as a highly specific reaction of oxytocin and related peptides, but in view of the present investigation the reaction may well be more widespread than first envisaged (see also ref. [9]). The loss of biological activity of the enkephalins on reaction with carbonyl compounds is also observed with acetone-oxytocin which possesses about 1/1000 the biological activity of the parent peptide. The large fall in the intrinsic opiate activity of [Met^5] enkephalin and [Leu^5] enkephalin on reaction with acetaldehyde is of considerable interest. Preliminary evidence suggests that the additional presence of an alkyl substituent on the α-amino group contributes greatly to the reduced receptor affinity, because N^{α}-ethyl[Met^5] enkephalin is also considerably less active than [Met^5] enkephalin in several bioassays [3]. There must, however, be additional subtle differences in structure or solution conformation which are not immediately apparent from studies described thus far and we are currently undertaking a comparative proton n.m.r. study of [Met^5] enkephalin and acetaldehyde-[Met^5] enkephalin in dimethylsulphoxide to try to detect any differences in conformation that might explain the low biological activity of the acetaldehyde adduct of [Met^5] enkephalin.

Acknowledgements—Initial experiments related to the work described in this report were carried out while one of us (M.C.S.) was a Beit Memorial Fellow for Medical Research in the Biochemistry Department, Cambridge University. Special thanks go to Professor Keith Tipton and Hans Kornberg for providing laboratory space and equipment. Financial support was provided by the Beit Foundation and H.E. Durham Fund for Biological Research and, more recently, by the Joint Standing Research Committee of the Kensington, Chelsea and Westminster Area Health Authority.

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