Isolation, Characterization and Opiate Activity of β -Endorphin from the Pituitary Gland of the Ostrich, *Struthio camelus*

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1. Avian β -endorphin was purified from adenohypophyseal glands of the ostrich *Struthio camelus* by a procedure involving acid/acetone extraction, NaCl fractionation, CM-cellulose chromatography, Sephadex G-50 chromatography and paper electrophoresis (pH 6.7). 2. The 31-amino acid peptide behaved as a single substance during polyacrylamide-gel electrophoresis and isoelectric focusing, the isoelectric point being 8.84. 3. Ostrich β -endorphin exhibited significant opiate activity in the guinea-pig ileum preparation.

β -Endorphin is a potent analgesic agent when injected either intracerebroventricularly (Loh *et al.*, 1976) or intravenously (Tseng *et al.*, 1976). Several structurally related peptides with opioid activity have been isolated from a variety of species including camel (Li & Chung, 1976), pig (Bradbury *et al.*, 1976; Gráf *et al.*, 1976), sheep (Seidah *et al.*, 1977), man (Chrétien *et al.*, 1976; Li *et al.*, 1976) and rat (Rubinstein *et al.*, 1977).

The present work describes the purification to homogeneity and the chemical and biological characterization of ostrich β -endorphin.

Experimental

The biological starting material, fractionation and rechromatography on CM-cellulose and chromatography on Sephadex G-50

These were the same as previously described by Naudé & Oelofsen (1977) for the isolation and characterization of corticotropin from the pituitary gland of the ostrich, *Struthio camelus*. A minor fraction obtained during the corticotropin isolation (peak 8a', as defined by Naudé & Oelofsen, 1977) possessed the opiate activity as well as a low but significant adrenal-stimulating potency. Peak 8a' material on polyacrylamide-gel electrophoresis appeared to largely consist of a single electrophoretic component being contaminated with approx. 10% of more basic material.

Preparative paper electrophoresis

Peak 8a' material (2.0 mg from fresh and aged glands) was applied as a band to a sheet of Whatman 3MM paper (305 mm $\times$ 285 mm) for subsequent electrophoresis in a Durrum type Beckman paper-electrophoresis cell with a pH 6.7 buffer (γ -collidine/acetic acid/water, 8.9:3.1:988, by vol.) at 400 V for 2 h. The more acidic band (termed 8a'-P) was detected by spraying suitable guide strips for 0.1% (w/v) solution of ninhydrin in ethanol, and was then eluted from the paper with 0.1 M-acetic acid. The eluate was freeze-dried and assayed for opiate activity.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was performed at pH 4.5 (0.35 M- β -alanine/acetate buffer) for 1.75 h at 6 mA/tube in a Buchler analytical apparatus. The gels (75 mm $\times$ 5 mm) contained 10% (w/v) acrylamide and were maintained at 25°C during electrophoresis. Protein bands were made visible by staining with a 0.5% (w/v) solution of Amido Schwarz 10B in 7% (w/v) acetic acid, followed by electrophoretic destaining.

Isoelectric focusing

Analytic isoelectric focusing of 120 μ g of fraction 8a'-P material was executed in a gel slab apparatus (gel dimensions, 250 mm $\times$ 20 mm $\times$ 2 mm) with a pH gradient ranging between pH 7 and 10 in the presence of 6 M-urea (Righetti & Chillemi, 1978).

Focusing was complete after about 3 h at 1000 V and 10°C. The gel slab was halved lengthwise, the one half being stained in Coomassie Brilliant Blue G-250 (dissolved in 1 M-H₂SO₄/12% trichloroacetic acid solution). The other half was sliced into 10 mm segments, which were then eluted separately overnight in 1 ml portions of cold water for pH determinations.

Amino acid analysis

The amino acid composition of fraction 8a'-P material was determined by the method of Spackman *et al.* (1958) with a Beckman 120B automatic analyser. Sample preparation was done as before (Naudé & Oelofsen, 1977). Tyrosine/tryptophan content was determined spectrophotometrically by the method of Bencze & Schmid (1957).

Biological activity

The bioassay preparation used was the electrically stimulated longitudinal muscle-myenteric plexus strip from guinea-pig ileum. The methods described by Paton & Zar (1968) and Paton & Vizi (1969) were used for the preparation of the strips and for the assay of acetylcholine output. Each strip was suspended in a 4 ml organ bath and was stimulated by means of two platinum ring electrodes (field stimulation) with supramaximal rectangular pulses of 1 ms duration. The contractile responses were recorded isometrically with a force-displacement transducer coupled to a single pen recorder. Opiate activity was measured as described by Kosterlitz & Watt (1968) by the single-dose method, with morphine as a reference standard and naloxone as the specific opiate antagonist. Results were expressed as percentage twitch inhibitions and ID₅₀ values (concentrations producing 50% inhibition of the twitch responses) were determined by interpolation from linear dose-response plots. Morphine sulphate was obtained from Petersen and naloxone by hydrochloride was from Endo Laboratories (S.A.) (Pty.).

Results and Discussion

The main component from the electrophoretogram yielded 1.4 mg of material (fraction 8a'-P material), which on electrofocusing in the pH range 7-10 revealed a single zone with a pI of 8.84 and which also appeared homogeneous during polyacrylamide-gel electrophoresis.

The result of the amino acid analysis of the 20 h hydrolysate of fraction 8a'-P material is shown in Table 1 and the composition of camel, ovine, rat, human and porcine β -endorphins is listed for comparison as well. All the species so far investigated, including the ostrich, seem to produce a 31-amino acid β -endorphin molecule. Ostrich β -endorphin,

Table 1. Amino acid composition (molar proportions) of camel, ovine, rat, human, porcine and ostrich β -endorphins

Serine and threonine values are corrected for losses on hydrolysis. Values in parentheses are the nearest integers.

Species	Amino acid composition (molar proportions)	
	Camel, bird and rat	Ostrich
Asx	2	1.03 (1)
Thr	3	0.95 (1)
Ser	2	2.95 (3)
Glx	3	1.99 (2)
Pro	1	0.88 (1)
Gly	3	3.99 (4)
Ala	2	3.30 (3)
Val†	1	1.61 (2)
Met	1	1.01 (1)
Ile†	2	0.61 (1)
Leu	2	2.02 (2)
Tyr*	1	1.84 (2)
Phe	2	1.94 (2)
His*	1	0
Lys	5	4.18 (4)
Arg	0	2.09 (2)
Trp	0	0
Total	31	31

* In human β -endorphin: Tyr, 2; His, 0.

† In porcine β -endorphin: Val, 2; Ile, 1.

however, seems to differ considerably from the other endorphins studied, in particular when the relative abundance of acidic and basic amino acids is considered. In the ostrich a relative abundance of the basic amino acids (Arg and Lys) is evident, which may explain its relatively high basicity (pI 8.84).

The purified fraction (fraction 8a'-P) produced a typical sigmoid dose-dependent inhibition of the responses of the longitudinal muscle-myenteric plexus strip to electrical field stimulation at 0.1 Hz (Fig. 1). On the basis of the amino acid composition data, a molecular mass of 3389 was used to calculate the endorphin sample concentrations. The ID₅₀ value for inhibition, as derived from linear dose-response plots, was 68.6 ± 4.5 mM for the ostrich endorphin sample compared with 70.1 ± 12.3 nM for morphine in the same system (mean $\pm$ S.E.M. for five experiments in each case). These results are similar to those reported by Waterfield *et al.* (1977) for morphine and porcine β -endorphin. Inhibition was most marked at low frequencies of stimulation (<1 Hz); the contractions induced by high-frequency stimuli (10-30 Hz) were relatively insensitive. This is a feature of opioid inhibition in guinea-pig ileum (Waterfield *et al.*, 1977).

Fig. 1. Graph from a typical experiment showing the dose-related inhibition, produced by the endorphin sample, of the 0.1 Hz twitch response of the guinea-pig ileum longitudinal muscle-myenteric plexus strip

Table 2. Effect of endorphin sample and morphine on evoked acetylcholine release from longitudinal muscle-myenteric plexus strips

Stimulation was at 0.2 Hz for 10 min. Values are means $\pm$ S.E.M. for the numbers of experiments in parentheses.

	Acetylcholine release (ng/g per min)
Control	72.2 $\pm$ 4.1 (8)
Ostrich endorphin (100 nM)	51.0 $\pm$ 7.3 (3)
(1 μ M)	48.2 $\pm$ 5.4 (3)
(3 μ M)	36.5 (2)
Morphine (1 μ M)	44.1 $\pm$ 6.2 (3)

Ostrich β -endorphin, like morphine (Paton, 1957), did not inhibit the contractions elicited by exogenously applied acetylcholine. This indicates that inhibition was not produced by a direct action on the longitudinal muscle, but suggests that the endorphin sample acted on the cholinergic nerves in the myenteric plexus. This finding was confirmed by measuring evoked acetylcholine release in the presence of the sample. Table 2 shows that both the endorphin sample and morphine decreased the acetylcholine release evoked by field stimulation at 0.2 Hz.

The inhibition of the twitch response produced by ostrich β -endorphin was specifically antagonized by naloxone (Fig. 2). The antagonism was competitive in nature and a similar result was obtained with morphine as agonist and naloxone as antagonist.

Fig. 2. Naloxone antagonism of the inhibitory effect of the endorphin sample on the response of longitudinal muscle-myenteric plexus of the guinea-pig ileum to 0.1 Hz electrical field stimulation

In the first tracing, naloxone 10 nM (Nal.) was added at the time indicated after the inhibition produced by 60 nM-endorphin (End.) had reached a maximum. In the second and third tracings, the preparation was pretreated with naloxone (Nal.) at a concentration of 10 and 30 nM respectively for 5 min before addition of 60 nM-endorphin (End.). The preparation was washed between additions.

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