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Pharmacological Properties of Acetorphan, a Parenterally Active "Enkephalinase" Inhibitor

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ABSTRACT

Acetorphan, *i.e.* N-[(*R,S*)-3-acetylmercapto-2-benzylpropanoyl]-glycine, benzyl ester, is a lipophilic derivative of Thiorphan, a potent inhibitor of "enkephalinase" (EC 3.4.24.11). On purified enkephalinase its inhibitory potency was approximately 1000 fold less than that of Thiorphan but became close to the latter (nanomolar) when it was incubated previously with cerebral membranes. After parenteral administration to mice and rats (1–10 mg/kg) extensive inhibition of cerebral enkephalinase was shown by 1) the depressed enzyme activity in brain membranes from treated animals and 2) the long-lasting potentiation of analgesia elicited by (D-Ala^2,Met^5)enkephalin (i.c.v.). This suggests that acetorphan easily enters the brain where the active Thiorphan is released. Parenteral acetorphan elicited a series of naloxone-reversible, opioid-like effects, most of which were described previously with intracerebral Thiorphan or other enkephalinase inhibitors. Antinociceptive effects were found in some tests (hot plate jump and phenylbenzoquinone-induced writhing) but not in others (hot plate licking and tail withdrawal). "Antidepressant" effect was found in the "mouse despair" test and antidiarrhoeal effect in the rat castor oil test. Acetorphan also elicited significant increases and decreases in turnover indexes of serotonin and noradrenaline, respectively, in mouse cerebral cortex. In mice chronically treated with acetorphan, the antinociceptive activity of the compound was not modified markedly and no overt withdrawal symptom could be observed after either treatment interruption or administration of naloxone.

Receptor-mediated responses evoked by neurotransmitters are controlled by removal of these molecules by various inactivation processes whose inhibition by pharmacological agents provides experimental and therapeutic means to modify neurotransmission, particularly in the monoamine field. Like other neurotransmitters the enkephalins appear to be inactivated rapidly after their release and circumstantial evidence has implicated "enkephalinase" (EC 3.4.24.11; recommended name of the Enzyme Commission: kidney brush border neutral proteinase) in this process (reviewed by Schwartz *et al.*, 1981, 1982, 1984b, 1985; Hersh, 1982; Schwartz 1983; Hui and Lajtha, 1983; McKelvy, 1983). This peptidase, present in cerebral synaptic membranes (De la Baume *et al.*, 1981; Checler *et al.*, 1983; Matsas *et al.*, 1983) efficiently cleaves the Gly^3-Phe^4 amide bond of enkephalins (Malfroy *et al.*, 1978) or the heptapeptide (Met^5)enkephalin-Arg^6-Phe^7 (Patey *et al.*, 1983; Hersh, 1984; Schwartz *et al.*, 1984b) but a variety of other neuropeptides like cholecystokinin (Deschodt-Lanckman and Strosberg, 1983; Zuzel *et al.*, 1985), substance P (Matsas *et al.*, 1983; Skidgel *et al.*, 1984; Horshtemke *et al.*, 1984), neurotensin (Checler *et al.*, 1983) angiotensin (Swerts *et al.*, 1979; Gafford *et al.*, 1983) or oxytocin (Johnson *et al.*, 1984) are also substrates. However a participation of enkephalinase in endogenous neuropeptide inactivation has been as yet only demonstrated in the case of enkephalins; selective inhibitors like Thiorphan, retro-Thiorphan, phosphoramidon or phosphoryl Leu-Phe partially protect the opioid pentapeptides released from brain slices from being extensively hydrolyzed (Patey *et al.*, 1981; De la Baume *et al.*, 1983; Altstein *et al.*, 1983) and elicit naloxone-reversible analgesia (Roques *et al.*, 1980; Chipkin *et al.*, 1983; Greenberg and O'Keefe, 1982; Rupreht *et al.*, 1983; Roques *et al.*, 1983; Kayser and Guilbaud, 1983) and various other opioid-like effects (Algeri *et al.*, 1981; Wood, 1982; Llorens-Cortes and Schwartz, 1984; Chaillet *et al.*, 1983a; Guilbaud *et al.*, 1983; Ukponmwan and Dzoljic, 1984; Hisamitsu and de Groat, 1984; Glimcher *et al.*, 1984). Most of these effects are potentiated by aminopeptidase inhibitors, suggesting that two peptidase activities participate in the inactivation of endogenous enkephalins (Zhang *et al.*, 1982; De la Baume *et al.*, 1983; Chaillet *et al.*, 1983b). In contrast, Thiorphan does not protect endogenous cholecystokinin released from brain slices (Zuzel *et al.*, 1985).

Although inhibitors of enkephalinase have already provided

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ABBREVIATIONS: 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.

important insights on the role of this peptidase in neuropeptide metabolism, their utilization in pharmacological studies has been limited by their poor penetration into brain. For instance, in the case of Thiorphan, its nanomolar potency *in vitro* contrasts with the large parenteral dosages (50–100 mg/kg) required to inhibit the cerebral enzyme (Roques *et al.*, 1980; Chipkin *et al.*, 1983); thus, most *in vivo* studies have been performed using intracerebral injections. In this report, we show that acetorphan, a lipophilic derivative of Thiorphan (in which the thiol and terminal carboxylate groups are esterified by acetyl and benzyl residues, respectively) is able, when administered in low parenteral dosages, to inhibit the cerebral peptidase, reproducing most effects reported previously with intracerebral Thiorphan, as well as eliciting some other opioid-like effects.

Materials and Methods

Animals. Male Swiss albino mice (20–25 g) and male Wistar rats (180–200 g) were purchased from Charles River (St. Aubin-les-Elbeuf, France). They were housed in makrolon cages and had free access to standard semisynthetic diet and tap water. Intracerebroventricular drug administrations were performed under a volume of 10 μl/mouse according to Haley and McCormick (1957). Animals were only used once.

Assay of enkephalinase activity. The activity was evaluated either on a highly purified enzyme preparation or in cerebral membranes from mice treated with acetorphan or vehicle. The enzyme from rat kidney was purified close to homogeneity as described (Malfroy and Schwartz, 1982, 1984). Incubations of about 1 ng of this preparation were performed at 37°C for 60 min in 150 μl of 50 mM Tris-HCl buffer (pH 7.4) in the presence of 40 nM [^{3}H](D-Ala^2,Leu^5)enkephalin and the enzyme product [^{3}H]Tyr-D-Ala-Gly was isolated by chromatography on columns of polystyrene beads (Llorens *et al.*, 1982). This enzyme activity was inhibited completely in the presence of 10^{-7} M Thiorphan.

For assay of cerebral enzyme, animals were decapited at various time intervals after the administration of acetorphan, cerebral regions were dissected out and homogenized in 30 volumes of cold 50 mM Tris HCl buffer (pH 7.4). Half of this homogenate was immediately centrifuged for 3 min at 100,000 × *g* using an airfuge (Beckmann, Paris, France). Supernatants were discarded, pellets superficially rinsed and then sonicated in 30 volumes of the same buffer. Enkephalinase activity was estimated on 50 μl of this particulate fraction (75 μg of protein) in presence of [^{3}H](D-Ala^{2}Leu^5)enkephalin (40 nM), puromycin (0.1 mM) and captopril (1 μM) and in presence or absence of Thiorphan (0.1 μM). After a 30-min incubation at 25°C enkephalinase activity was calculated as the diffence between [^{3}H]Tyr-D-Ala-Gly formation in the absence and the presence of Thiorphan (Llorens *et al.*, 1982).

Radioimmunoassay of (Met^5)enkephalin. Striata were homogenized in 10 volumes of cold 0.4 N $HClO_4$. After centrifugation (20,000 × *g* × min), (Met^5)enkephalin was isolated from the clear supernatant by column chromatography on polystyrene beads (Porapak Q, Waters, Paris, France) as described (Vogel and Altstein, 1977; De la Baume *et al.*, 1983). The radioimmunoassay was performed according to Gros *et al.* (1978).

Assay of monoamines and acidic metabolites. Immediately after decapitation of mice the cerebral cortex was dissected out on ice and sonicated into 0.4 N $HClO_4$ containing 2% ascorbic acid and 2% EDTA. The homogenates were centrifuged and supernatants, adjusted to pH 6.0 with 3 N KOH and 0.2 M potassium-phosphate buffer, were frozen until the next day.

After thawing and centrifugation, the clear extracts were used for assays. Noradrenaline and 5-HT were isolated by ion-exchange chromatography on Amberlite CG-50 microcolumns and assayed spectrofluorometrically (Von Euler and Lishajko, 1961; Curzon and Green, 1970). 5-HIAA, contained in the effluent of the Amberlite column, was isolated on Sephadex G-10 microcolumns and assayed spectrofluorometrically (Curzon and Green, 1970).

Hot plate test. This test was derived from that of Eddy and Leimbach (1953). A plastic cylinder 20 cm high and 14 cm in diameter was used to maintain the mouse to the heated surface of the plate. The plate was heated to a temperature of 55 ± 0.5°C, using a thermoregulated water circulating pump. For each animal the latencies of both the forepaw licking and the jump were determined. To avoid injury, animals not responding within 240 sec were removed from the hot plate.

Tail withdrawal test. This test was that described by Ben Bassat *et al.* (1959). Mice were restrained in individual rigid tubular containers and the tail was immersed into a water bath set at 48°C. The nociceptive endpoint was characterized by a violent jerk of the tail. A 15 sec cutoff time was selected.

Writhing test. This test was derived from that of Sigmund *et al.* (1957). Mice received i.p. 0.2 ml/20 g of a solution of phenylbenzoquinone (0.02% dissolved in 5% ethanol). They were then placed individually in large beakers. The number of body stretches were counted over a 10-min period starting from the 10th min after the injection. A stretch was characterized by elongation of the body, development of tension in the abdominal muscles and extension of the forelimbs.

Mouse "behavioral despair" test. The method "forced swimming" used was that described by Porsolt *et al.* (1977). Briefly, 6 min after drug injection each mouse was introduced into a vertical Plexiglass cylinder (height, 25 cm; diameter, 10 cm) containing at its bottom 6 cm of water at 21–23°C. After a 2-min time lag, the time that the animal spent motionless during the following 4-min period was measured.

Body temperature. Temperature was measured during a thermistor probe (Ellab TE$_3$, probe RM 6, Copenhagen, Denmark) inserted in the colon.

Locomotor activity. Mice were individually introduced in an actimeter (length, 26 cm; width, 20 cm; height, 10 cm) equipped with photoelectric cells (Apelex, Bagneux, France) and locomotor activity was evaluated as described (Boissier and Simon, 1965).

Antidiarrheal activity. Rats were kept overnight without food but with free access to tap water. On the next morning they received acetorphan or vehicle and 15 min later were challenged with 1 ml of castor oil (p.o. administration) and then were placed in individual cages (Niemegeers *et al.*, 1974). The stool weight was determined 45, 60 and 90 min after castor oil administration and total cumulative weight at 90 min was calculated.

Analysis of data. ED_{50} values were calculated by log-probit analysis. IC_{50} values of inhibitors were calculated using a computerized least-square curve fitting procedure derived from Parker and Waud (1971).

Materials. Thiorphan and acetorphan, synthesized as described (Roques *et al.*, 1980, 1981) were provided by Laboratoire Bioprojet (Paris, France). Acetorphan was dissolved in the following vehicle: ethanol (10%)-cremophor EL (10%)-H_2O (80%). Naloxone hydrochloride was provided by Endo Laboratory Inc. (Garden City, New York). [^{3}H]D-Ala^2, Leu^5 enkephalin (30–50 Ci/mmol) was from the Centre d'Etudes Atomiques (Saclay, France). Polystyrene beads (Porapak Q) were from Waters Associates (Milford, MA). (D-Ala^2,Met^5)enkephalin was purchased from U.C.B. (Brussels, Belgium) and castor-oil (pharmaceutical grade) from La Cooper (Melun, France). Reagents (analytical grade) were from Prolabo (Paris, France).

Results

Enkephalinase inhibition. When determined directly on the purified enzyme the IC_{50} value of acetorphan (corresponding to its K_i value because the substrate concentration was well below the K_M value) was 4500 ± 740 nM as compared with 6.1 ± 0.7 nM for Thiorphan (fig. 1). In addition acetorphan (100 μM) was preincubated for 15 min in the presence of membranes from mouse whole brain (0.4 mg of protein per ml of Tris HCl buffer, 50 mM, pH 7.4), the reaction stopped by addition of

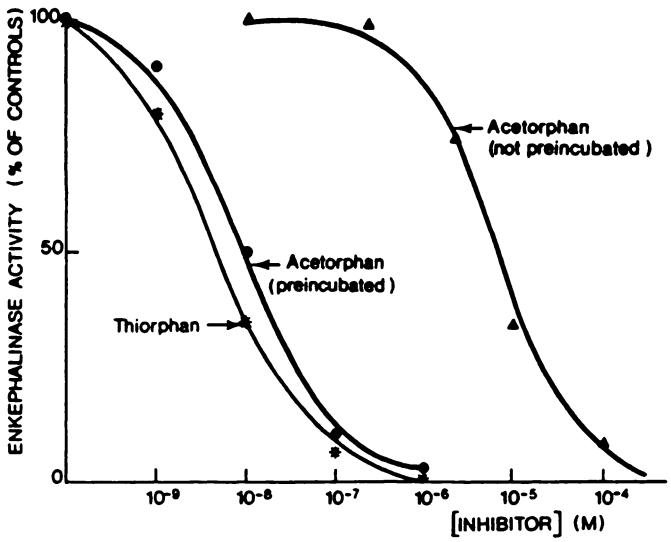

Fig. 1. Inhibition of purified enkephalinase activity from rat kidney by acetorphan and the increase in drug potency after preincubation with mouse brain membranes. Acetorphan was tested against the purified enzyme (1 ng) either directly or after a 15-min preincubation at 37°C with cerebral membranes. Thiorphan was tested directly. Concentrations of the inhibitors refer to those in the final incubation medium.

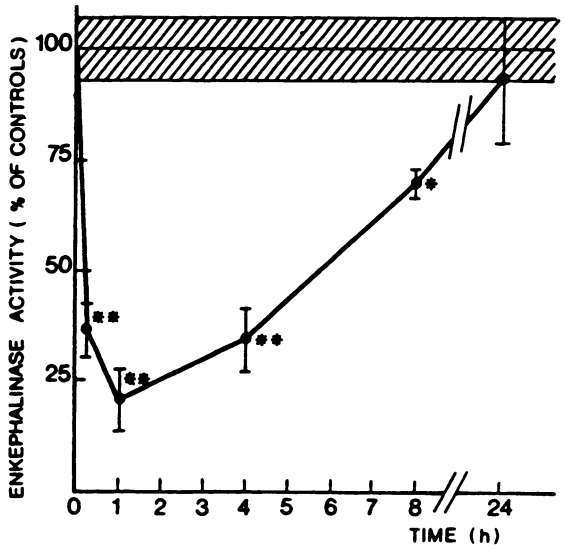

Fig. 2. Enkephalinase activity of striatal membranes from mice treated with acetorphan (1 mg/kg i.v.). Mice were killed at various time intervals after drug administration, the striatum was dissected out and a membrane fraction rapidly prepared for enzyme assays. Enkephalinase activity in controls (hatched area) was 238 ± 18 fmol/mg of protein per min (40 nM [^{3}H](D-Ala^2,Leu^5)enkephalin as substrate, 30 min incubations at 25°C). Means ± S.E.M. of data from three separate experiments. *P < .05; **P < .01 as compared to controls.

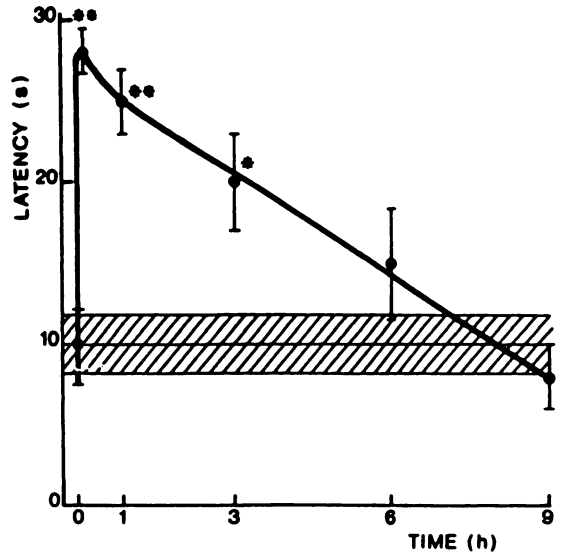

Fig. 3. Potentiation by acetorphan of the antinociceptive activity of (D-Ala^2,Met^5)enkephalin evaluated in the mouse tail withdrawal test. At various time intervals after the administration of acetorphan (10 mg/kg i.v.) or vehicle, mice received (D-Ala^2,Met^5)enkephalin (15 μg i.c.v.) and were tested 20 min later. The hatched area corresponds to control values, *i.e.*, tail withdrawal latencies in vehicle-pretreated, enkephalin-treated mice. Means ± S.E.M. of data from 10 to 20 experiments. *P < .01; **P < .001 as compared to controls. Latencies for mice receiving either the enkephalin or acetorphan alone did not significantly differ from those of controls (not shown).

$HClO_4$ to a final 0.4 N concentration; after centrifugation (20,000 × *g* × min) the resulting supernatant was neutralized, then diluted in Tris buffer and aliquots transferred to incubation media containing the purified enzyme. After such a treatment the concentration-inhibition curve of acetorphan was shifted to the left, its IC_{50} value becoming 8.6 ± 1.8 nM (fig. 1). In contrast when acetorphan was preincubated in the absence of cerebral membranes no significant shift was observed and preincubations of a duration shorter than 15 min led to shifts of intermediate amplitude. In addition the medium in which mouse cerebral membranes had been incubated in the absence of acetorphan, had no inhibitory activity (not shown).

After treatment of mice with 1 mg/kg of acetorphan, the enkephalinase activity of a rapidly prepared striatal membrane fraction was apparently reduced for about 8 hr (fig. 2). In rats, 1 hr after i.p. administration of 10 mg/kg of acetorphan, the apparent enkephalinase activity of hypothalamic membranes prepared in a similar fashion was of 41 ± 7 fmol/mg of protein per min instead of 90 ± 11 fmol/mg of protein per min in vehicle-treated rats (means ± S.E.M. of five experiments, P < .01), corresponding to a 55% reduction; 1 hr after 25 or 50 mg/kg i.p. the apparent reductions were of 68 and 70% respectively.

Antinociceptive activity. Mice and rats received acetorphan in dosages up to 100 mg/kg (i.v. or i.p.) without any overt sign of toxicity, particularly any sign of respiratory depression.

In the **tail withdrawal test** performed in mice treated with either (D-Ala^2,Met^5)enkephalin in subthreshold dosage (15 μg/animal i.c.v., 20 min before test) or acetorphan alone (10 mg/kg i.v., for 10 min to 1 hr before test) no significant change in tail withdrawal latency was observed. However (D-Ala^2,Met^5)enkephalin elicited a significant analgesia in mice treated with acetorphan at the same time or up to 3 to 6 hr before administration (fig. 3).

In the **hot plate test** acetorphan (5 mg/kg i.v.) significantly increased the jump latency, an effect inhibited by naloxone, a compound which decreased the jump latency *per se*. However, no significant effect of any of these compounds was observed in the same animals when the paw licking latency was measured (table 1). Acetorphan (i.v.) increased the jump latencies in a dose-dependent manner, with a significant effect being detected at 1 mg/kg and the maximal effect at 10 to 20 mg/kg; analysis of these data led to an ED_{50} value (calculated by log-probit analysis) of 7 ± 3 mg/kg (results not shown). An antinociceptive effect of the same magnitude as that elicited by 10 μg of

TABLE 1

Effects of acetorphan (i.v.) on the jump and licking latencies (mouse hot plate test)

Groups of 15 mice received acetorphan (or vehicle) 30 min after naloxone (or saline) and 15 min before test. Means ± S.E.M.

Treatments	Latencies	
	Jump	Licking
	sec	
Vehicle	41 ± 4	7.1 ± 0.4
Acetorphan (10 mg/kg i.v.)	83 ± 10***	7.1 ± 0.8 (N.S.)
Naloxone (1 mg/kg s.c.)	29 ± 2*	7.3 ± 0.8 (N.S.)
Acetorphan (10 mg/kg i.v.) + naloxone (1 mg/kg s.c.)	31 ± 3*†	7.1 ± 0.9 (N.S.)

* P < .05; *** P < .001; N.S., nonsignificant as compared to vehicle; † P < .001 as compared to acetorphan alone.

TABLE 2

Effects of thiorphan and acetorphan (i.c.v.) on the jump latencies (mouse hot plate test)

Groups of 10 mice received the drugs or vehicle 10 min before test. Values represent means ± S.E.M.

Treatments	Jump Latencies
	sec
Vehicle	79 ± 7
Thiorphan (10 μg i.c.v.)	105 ± 8*
Acetorphan (10 μg i.c.v.)	107 ± 10*

* P < .05.

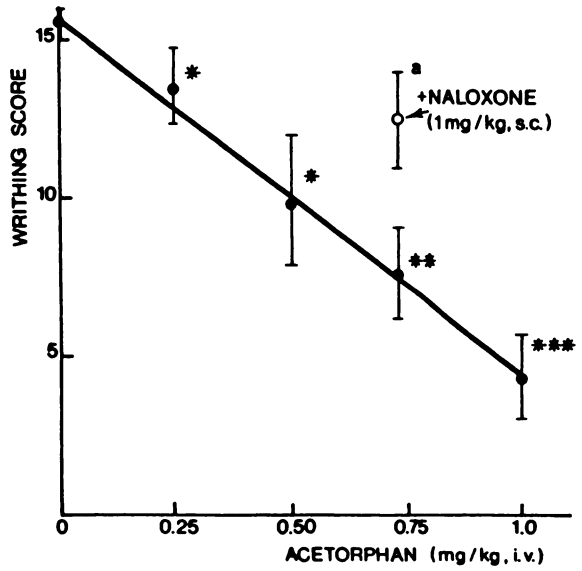

Fig. 4. Antinociceptive activity of acetorphan in the mouse writhing test. Groups of 12 to 30 mice received acetorphan and phenylbenzoquinone (2 mg/kg i.p.) and were scored from 5 to 15 min later. Where indicated naloxone was administered together with acetorphan. *P < .05; **P < .01; ***P < .001 as compared to vehicle. a, P < .05 as compared to acetorphan alone.

Thiorphan (i.c.v.) was observed with 10 μg of acetorphan (i.c.v.) (table 2).

In the **writhing test** a dose-dependent decrease in the frequency of body stretches elicited by phenylbenzoquinone was observed with an ED_{50} value of 0.70 ± 0.15 mg/kg (fig. 4). The analgesic effect of acetorphan (0.70 mg/kg) was reduced significantly by naloxone (1 mg/kg s.c.) which significantly increased the writhing score *per se* (23 ± 2 sec instead of 15 ± 1 sec in controls, means ± S.E.M. of values from 20–30 mice).

Effects in the mouse behavioral despair test. In mice having received acetorphan at either 10 mg/kg (i.v.) (not shown) or 50 mg/kg (i.p.) the immobility time was reduced significantly, a change blocked by naloxone which had no significant effect *per se* (table 3).

Antidiarrheal effect. The castor oil-induced diarrhea was prevented completely in a dose-dependent manner by acetorphan (ID_{50} value about 5 mg/kg i.v.). The effect of 10 mg/kg of acetorphan was antagonized completely by naloxone (2 × 2 mg/kg) (table 4).

Effects on cerebral monoamines and metabolites. Acetorphan (1 or 5 mg/kg i.v.) decreased significantly (−24%) the 5-HT levels in mouse cerebral cortex whereas 5-HIAA levels were increased in a nonsignificant manner; the ratio of 5-HIAA to 5-HT levels in the same animals was increased by about 50%, an effect prevented completely by naloxone (table 5).

The α-methyl-*p*-tyrosine-induced decline of noradrenaline levels in cerebral cortex was reduced by 39% in mice treated with acetorphan (5 mg/kg), an effect antagonized completely by naloxone (table 6).

Effects on striatal (Met^5)enkephalin levels. After the administration of acetorphan (10 mg/kg i.v.) no significant change in striatal (Met^5)enkephalin levels (picomoles per milligram of protein) occurred at either 15 (33.9 ± 0.9) or 60 min (35.1 ± 1.6) as compared to controls (36.0 ± 2.1) (means ± S.E.M. of data from 10 mice in each group).

Effects of chronic treatments with acetorphan. In mice receiving acetorphan (2 × 50 mg/kg i.p. daily) during 10 days no overt sign of toxicity was observed either during treatment or on the day after its interruption. Particularly, body weight and body temperature of these mice did not differ from those of vehicle-treated mice (table 7). Mice were challenged with naloxone (20 mg/kg i.p.) 16 hr after the last acetorphan injec-

TABLE 3

Effects of acetorphan in the mouse behavioral despair test

Mice received acetorphan 60 min before test. Means ± S.E.M. of 24 experiments for each condition.

	Immobility Time
	sec
Vehicle	188 ± 7
Acetorphan (50 mg/kg i.p.)	145 ± 12**
Vehicle + naloxone (1 mg/kg s.c.)	201 ± 5 (N.S.)
Acetorphan (50 mg/kg i.p.) + naloxone (1 mg/kg s.c.)	198 ± 5 (N.S.)***

** P < .01; N.S., nonsignificant as compared to vehicle; *** P < .001 as compared to acetorphan alone.

TABLE 4

Effects of acetorphan on castor oil-induced diarrhea in rats

Rats received 1 ml of castor oil (p.o.) 15 min after acetorphan (i.v.). Stools were collected and weighed 45, 60 and 90 min after castor oil administration and values represent means ± S.E.M. of total cumulative weight at 90 min. Naloxone was administered 10 min before and 25 min after acetorphan (or vehicle). *N*, number of rats.

Treatments	*N*	Stool Wt.
		g
Vehicle	11	2.8 ± 0.7
Acetorphan (5 mg/kg i.v.)	8	1.1 ± 0.6*
Acetorphan (10 mg/kg i.v.)	18	0.6 ± 0.3**
Acetorphan (20 mg/kg i.v.)	8	0**
Vehicle + naloxone (2 × 2 mg/kg s.c.)	8	2.6 ± 0.6 (N.S.)
Acetorphan (10 mg/kg i.v.) + naloxone (2 × 2 mg/kg s.c.)	8	2.9 ± 0.5 (N.S.)

* P < .05; ** P < .01; N.S., nonsignificant as compared to vehicle.

TABLE 5

Effects of acetorphan on 5-HT and 5-HIAA levels in mouse cerebral cortex

Acetorphan and naloxone were administered 20 and 40 min, respectively, before sacrifice. Means ± S.E.M. of number of experiments in parentheses.

Treatments	5-HT Level	5-HIAA Level	$\frac{\text{5-HIAA}}{\text{5-HT}}$
	ng/g	ng/g	
Vehicle (18)	295 ± 8	188 ± 14	0.64 ± 0.05
Acetorphan (1 mg/kg i.v.) (4)	227 ± 22*	233 ± 27 (N.S.)	0.97 ± 0.05*
Acetorphan (5 mg/kg i.v.) (15)	227 ± 13*	216 ± 17 (N.S.)	0.95 ± 0.09*
Naloxone (1 mg/kg s.c.) (7)	309 ± 24 (N.S.)		
Acetorphan (5 mg/kg i.v.) + naloxone (1 mg/kg s.c.) (4)	311 ± 28 (N.S.)	139 ± 20	0.49 ± 0.11 (N.S.)

* $P < .005$.

TABLE 6

Effects of acetorphan on α-methyl-p-tyrosine (α-MPT)-induced decline of noradrenaline in mouse cerebral cortex

α-MPT (200 mg/kg i.p.) was administered 2 hr before sacrifice. Acetorphan (5 mg/kg i.v.) and/or naloxone (1 mg/kg s.c.) were administered 10 and 30 min, respectively, before α-MPT. *N*, number of animals.

Treatments	N	Noradrenaline Levels ng/g	% change
Vehicle	27	233 ± 8	
α-MPT alone	31	131 ± 5	−44
α-MPT + acetorphan	32	156 ± 6**	−33
α-MPT + naloxone	4	117 ± 13 (N.S.)	−50
α-MPT + acetorphan + naloxone	9	135 ± 10 (N.S.)	−42

** $P < .005$; N.S., nonsignificant as compared to α-MPT alone.

tion and the possible occurrence of a series of withdrawal symptoms was assessed. No statistically significant change was observed in acetorphan-treated as compared to vehicle-treated animals regarding body weight, internal temperature, jumping or motor activity and diarrhea did not occur (table 7).

After the same 10-day pretreatment the antinociceptive activity of acetorphan in the hot plate jump test was not greatly modified: there was a tendency to increased responsiveness at low drug dosage (5 mg/kg) but the maximal effect (15 mg/kg) was not modified and log-probit analysis of the data led to an ED_{50} value (7 ± 1 mg/kg) similar to that obtained in vehicle-treated mice (7 ± 2 mg/kg) (table 8). In mice pretreated with 10 mg/kg of acetorphan (i.p.) twice daily for 20 days, the antinociceptive activity of the test-dose (2 mg/kg) was the same as in vehicle-treated mice in the writhing test (table 8); however, in both groups there was a decreased responsiveness to pheynlbenzoquinone (compare to data of fig. 4), presumably attributable to the effects of repeated i.p. injections performed previously.

Discussion

The present study indicates that acetorphan is a parenterally active enkephalinase inhibitor, presumably because it is able to enter the brain and to be hydrolyzed therein into its parent compound, Thiorphan. Indeed acetorphan is approximately 1000-fold less potent than Thiorphan when directly tested on the purified enzyme, whereas it becomes nearly as potent after being incubated with cerebral membranes (fig. 1). This large difference reflects the importance of the free thiol and terminal carboxylate groups of enkephalinase inhibitors by which they presumably interact with the Zn atom and arginine residue, respectively, of the enzyme active site (Schwartz *et al.*, 1981; Malfroy and Schwartz, 1982, 1984; Fournie-Zaluski *et al.*, 1984). On crude membranes from mouse striatum the inhibitory potency of acetorphan increased progressively with the incubation duration, as hydrolysis of both the thioester and ester bonds progressively took place, as indicated by high-performance liquid chromatography analysis (not shown).

That cerebral enkephalinase was inhibited extensively after parenteral administration of acetorphan in relatively low dosage (1–10 mg/kg) is suggested by a variety of observations. Thus, the enzyme activity in striatal membranes prepared rapidly after sacrifice was strongly reduced for 4 to 8 hr after parenteral drug administration (fig. 2). In addition the analgesic effect of (D-Ala^2,Met^5)enkephalin, a good substrate for purified enkephalinase (Malfroy and Schwartz, 1982, 1984), was potentiated in a long-lasting manner (fig. 3), as observed previously after administration of Thiorphan i.c.v. or parenterally at 30 to 100-mg/kg dosages (Roques *et al.*, 1980; Yaksh and Harty, 1982; Chipkin *et al.*, 1982; Baum *et al.*, 1983). Finally, the very similar patterns of behavioral and neurochemical actions of parenteral acetorphan and i.c.v. Thiorphan are consistent with

TABLE 7

Withdrawal symptoms in mice chronically treated with acetorphan

Mice received acetorphan (50 mg/kg i.p.) or vehicle twice a day during 10 days. Body weight and colonic temperature were measured 16 hr after the last injection. Naloxone (20 mg/kg i.p.) was then administered and mice tested at the following postnaloxone intervals: 0 to 10 min (jumping), 10 to 30 min (motor activity), 60 min (body temperature), 0 to 2 hr (diarrhea) and 2 hr (body weight).

	Treatments		
	Chronic vehicle	Chronic acetorphan	Difference
Body weight (g) before naloxone	32.7 ± 0.4	32.6 ± 0.4	N.S.
Body weight (g) after naloxone	29.9 ± 0.4	30.3 ± 0.3	N.S.
Body temperature (°C) before naloxone	37.6 ± 0.1	37.4 ± 0.1	N.S.
Body temperature (°C) after naloxone	37.4 ± 0.2	37.7 ± 0.1	N.S.
Naloxone-induced jumping (n° of animals)	2/40	1/39	N.S.
Motor activity after naloxone (crosses/15 min)	70 ± 7	84 ± 17	N.S.
Naloxone-induced diarrhea	0/40	0/39	N.S.

TABLE 8

Antinociceptive effects of acetorphan in mice chronically pretreated with this agent

Twelve to 18 mice for each condition were pretreated with vehicle or acetorphan (50 mg/kg i.p., twice daily for 10 days in group A or 10 mg/kg i.p., twice daily for 20 days in group B) and the two nociceptive responses were evaluated 20 hr after the last injection. Mice received the test-dose of acetorphan (i.v.) 15 min before placement onto the hot plate or 10 min before receiving phenylbenzoquinone.

Treatments	Chronic Pretreatment	
	Vehicle	Acetorphan
A. Hot plate jump latency (sec)		
Vehicle	59 ± 8	66 ± 9
Acetorphan (5 mg/kg)	81 ± 12 (N.S.)	124 ± 19**
Acetorphan (10 mg/kg)	108 ± 20*	135 ± 22**
Acetorphan (15 mg/kg)	151 ± 21**	159 ± 29**
B. Writhing score		
Vehicle	7.7 ± 2.1	7.0 ± 1.3
Acetorphan (2 mg/kg)	4.2 ± 1.2**	4.3 ± 0.9**

* $P < .05$; ** $P < .001$; N.S., nonsignificant as compared to corresponding vehicle-treated group.

the idea that the two drugs act *via* a common mechanism, *i.e.* enkephalinase inhibition in the central nervous system.

A significant, naloxone-reversible antinociceptive activity of acetorphan, resembling that of Thiorphan, was found in several tests. Like that of Thiorphan it was rather weak and selective in the sense that it could not be observed in all tests in which synthetic opiates are active. For instance a dose-dependent antinociception was elicited in the phenylbenzoquinone-induced writhing (fig. 4) and hot plate jump tests (tables 1 and 8) with ED_{50} values 0.7 and 7 mg/kg, respectively. On the latter test acetorphan and Thiorphan, both given i.c.v., displayed similar potencies (table 2), probably because extensive hydrolysis of acetorphan took place in brain *in vivo*. In contrast there was no significant antinociceptive activity of acetorphan in the tail withdrawal (see "Results") or hot plate licking tests (table 1). Similarly dissociated antinociceptive effects were reported previously with Thiorphan (reviewed by Schwartz *et al.*, 1982) as well as other enkephalinase inhibitors (Algeri *et al.*, 1981; Rupreht *et al.*, 1983) administered either alone or in combination with bestatin, an aminopeptidase inhibitor (Zhang *et al.*, 1982; De la Baume *et al.*, 1983; Chaillet *et al.*, 1983; Schwartz *et al.*, 1983, 1984b; Costentin *et al.*, 1986). Namely, because the dissociated pattern of pronociceptive activity of naloxone on these various tests (reviewed by Sawynok *et al.*, 1979; Hill, 1981; Jacob and Ramabadran, 1981) mirrors that of antinociceptive activity of peptidase inhibitors, it has been assumed that this reflects a selective participation of endogenous opioids in the control of only some nociceptive responses (Schwartz, 1983; Schwartz *et al.*, 1984b; Costentin *et al.*, 1986).

Similar to Thiorphan (i.c.v.) and enkephalins (Ben Natan *et al.*, 1984) parenteral acetorphan elicited a significant naloxone-reversible "antidepressant" effect in the mouse behavioral despair test (table 3) on which opiate receptor antagonists block the effects of some tricyclic antidepressant drugs (Devoize *et al.*, 1982). Also similar to Thiorphan (i.c.v.) (Llorens-Cortes and Schwartz, 1984), parenteral acetorphan elicited significant, naloxone-reversible changes in 5-HT (table 5) and noradrenaline turnover indexes (table 6) which parallel those elicited by synthetic opiates (Yarbrough *et al.*, 1973; Korf *et al.*, 1984; Algeri *et al.*, 1978; Tanaka *et al.*, 1983; Attila and Ahtee, 1983). Finally, acetorphan displayed a pronounced, naloxone-reversible, antidiarrheal activity in the castor oil test (table 4) on which Thiorphan (H. Marcais-Collado, G. Uchida, J.-M. Lecomte and J. Costentin, in preparation) and opiate receptor agonists (Awounters *et al.*, 1983) are active. Interestingly, whereas acetorphan inhibits the cholera toxin-induced intestinal hypersecretion (L. Bueno, personal communication), it does not significantly affect the delay of intestinal transit, as evaluated in the charcoal meal test (H. Marcais-Collado, G. Uchida, J.-M. Lecomte and J. Costentin) on which opiate receptor agonists are active (Niemegeers *et al.*, 1974).

All these data, indicating that acetorphan elicits a variety of naloxone-reversible, opioid-like effects, are consistent with the idea that this compound provides protection of endogenous opioids from hydrolysis by enkephalinase. The absence of any significant change in (Met^5)enkephalin levels in the brain of acetorphan-treated mice (see "Results") does not contradict this hypothesis because metabolism of the endogenous peptide involves not only enkephalinase but also a bestatin-sensitive aminopeptidase activity (De la Baume *et al.*, 1983; Schwartz, 1983), presumably aminopeptidase M (Gros *et al.*, 1985) and possibly another membrane-bound aminopeptidase (Hui *et al.*, 1983; Hersh, 1985). Thus, whereas acetorphan reduces by 70% the endogenous cerebral levels of the tripeptide Tyr-Gly-Gly, a characteristic product of cleavage of the Gly^3-Phe^4 amide bond of enkephalins by enkephalinase, inhibition of both enkephalinase and aminopeptidase activities is required to increase enkephalin levels (Schwartz *et al.*, 1984a; Llorens-Cortes *et al.*, 1985a,b; B. Giros, C. Llorens-Cortes, C. Gros and J.C. Schwartz, in preparation).

One interesting feature of the parenterally active enkephalinase inhibitor is that it allows one to easily assess the effects of chronic treatments. The analgesic activity of acetorphan was not modified greatly after either a 10-day pretreatment (hot plate jump) or a 20-day pretreatment (writhing test) with large doses given i.p. (table 8). Also no overt withdrawal symptoms were observed either at the interruption of a chronic treatment or by administration of a precipitating dose of naloxone (table 7). In the same way interruption of a chronic i.c.v. infusion of Thiorphan in rats failed to elicit the typical pattern of opioid withdrawal symptoms (Bean and Vaught, 1984) and this drug was not recognized in the morphine cue test (Buxton *et al.*, 1982). If one accepts the view that acetorphan acts *via* protection of endogenous opioids, these differences with classical opiates or even exogenous opioid peptides (Martin, 1983) might be due to a rather weak and short-lived stimulation of opiate receptors which might have occurred after the repeated injections of this drug.

Additional studies using other animal species and modes of treatment are currently in progress to establish whether chronic and simultaneous inhibition of the two peptidase activities responsible for endogenous enkephalin inactivation is accompanied by the development of tolerance and dependence.

References

ALGERI, S., ALTSTEIN, M., DESIMONE, M. AND GUARDABASSO, G. M.: *In vivo* potentiation of D Ala^2 Met-enkephalin amide, central effects after administration of an enkephalinase inhibitor. Eur. J. Pharmacol. **74:** 207–209, 1981.

ALGERI, S., BRUNELLO, N., CALDERINI, G. AND CONSOLAZIONE, A.: Effects of enkephalins on catecholamine metabolism in rat CNS. *In* Advances in Biochemical Psychopharmacology, vol. 18: The Endorphins, ed. by E. Costa and M. Trabucchi, pp. 199–210, Raven Press, New York, 1978.

ALTSTEIN, M., BACHAR, E., VOGEL, Z. AND BLUMBERG, S.: Protection of enkephalins from enzymatic degradation utilizing selective metal chelating inhibitors. Eur. J. Pharmacol. **91:** 353–361, 1983.

ATTILA, L. M. J. AND AHTEE, L.: Morphine-induced changes in cerebral noradrenaline turnover depend on the length of chronic treatment. Prog. Neuropsychopharmacol. Biol. Psychiatry **25:** (suppl.) 74, 1983.

AWOUNTERS, F., NIEMEGEERS, E. AND JANSSEN, P. A. J.: Pharmacology of antidiarrheal drugs. Annul. Rev. Pharmacol. **23:** 279–301, 1983.

BAUM, T., BECKER, F. T., SYBERTZ, E. J., SABIN, C. AND DESIDERIO, D. M.: "Enkephalinase A" inhibition by thiorphan: Central and peripheral cardiovascular effects. Eur. J. Pharmacol. **94:** 85–91, 1983.

BEAN, A. J. AND VAUGHT, J. L.: Physical dependence produced by chronic intracerebroventricular infusion of kyotorphin or thiorphan to rats. Eur. J. Pharmacol. **105:** 333–337, 1984.

BEN BASSAT, J., PERETS, E. AND SULMAN, F. G.: Analgesimetry and ranking of analgesic drugs by the reuptake method. Arch. Int. Pharmacodyn. Ther. **182:** 434–440, 1959.

BEN NATAN, L., CHAILLET, P., LECOMTE, J. M., MARCAIS, H., UCHIDA, G. AND COSTENTIN, J.: Involvement of endogenous enkephalins in the mouse "behavioral despair" test. Eur. J. Pharmacol. **97:** 301–304, 1984.

BOISSIER, J. R. AND SIMON, P.: Action de la caféine sur la mobilité spontanée de la souris. Arch. Int. Pharmacodyn. Ther. **158:** 212–221, 1965.

BUXTON, D. A., PRIESTLEY, T., SHAW, J. S. AND TURBULL, M. J.: Failure of enkephalinase inhibitor Thiorphan to produce the narcotic cue. Br. J. Pharmacol. **75:** 105P, 1982.

CHAILLET, P., MARCAIS-COLLADO, H. AND COSTENTIN, J.: Catatonic and hypotonic immobility induced in mice by intracerebroventricular injections of Mu or Kappa opioid receptor agonists as well as enkephalins or inhibitors of their degradation. Life Sci. **33:** 2105–2111, 1983.

CHAILLET, P., MARCAIS-COLLADO, M., COSTENTIN, J., YI, C. C., DE LA BAUME, S. AND SCHWARTZ, J.-C.: Inhibition of enkephalin metabolism by, and nociceptive activity of bestatin, an aminopeptidase inhibitor. Eur. J. Pharmacol. **86:** 329–336, 1983b.

CHECLER, F., VINCENT, J. P. AND KITABGI, P.: Degradation of neurotensin by rat brain synaptic membranes: Involvement of a thermolysin-like metalloendopeptidase (enkephalinase), angiotensin-converting enzyme, and other unidentified peptidases. J. Neurochem. **41:** 375–384, 1983.

CHIPKIN, R. E., BILLARD, W., AHN, H. S., SYBERTZ, E. J. AND IORIO, L. C.: *In vitro* and *in vivo* activities of enkephalinase and angiotension-converting enzyme inhibitors: Relationship of enzyme inhibition to analgesic and cardiovascular effects. *In* Degradation of Endogenous Opioids: Its Relevance in Human Pathology and Therapy, ed. by S. Ehrenpreis and F. Sicuteri, pp. 91–106, Raven Press, New York, 1983.

CHIPKIN, R. E., IORIO, L. C., BARNETT, A., BERGER, J. AND BILLARD, W.: *In vitro* and *in vivo* effects of thiorphan, an inhibitor of enkephalinase A. *In* Regulatory Peptides: From Molecular Biology to Function, ed. by E. Costa and M. Trabucchi, pp. 235–242, Raven Press, New York, 1982.

COSTENTIN, J., VIAICULESCU, A., CHAILLET, P., BEN NATAN, L., AVEAUX, D. AND SCHWARTZ, J.-C.: Dissociated effects of inhibitors of enkephalin-metabolising peptidases or naloxone on various nociceptive responses. Eur. J. Pharmacol., in press, 1986.

CURZON, G. AND GREEN, A. R.: Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain. Br. J. Pharmacol. **39:** 653–660, 1970.

DE LA BAUME, S., PATEY, G. AND SCHWARTZ, J.-C.: Subcellular distribution of enkephalin dipeptidyl carboxypeptidase (enkephalinase) in rat brain. Neuroscience **6:** 315–321, 1981.

DE LA BAUME, S., YI, C. C., SCHWARTZ, J.-C., CHAILLET, P., MARCAIS-COLLADO, H. AND COSTENTIN, J.: Participation of both "enkephalinase" and aminopeptidase activities in the metabolism of endogenous enkephalins. Neuroscience **8:** 143–151, 1983.

DESCHODT-LANCKMANN, M. AND STROSBERG, A. D.: *In vitro* degradation of the C-terminal octapeptide of cholecystokinin by "enkephalinase A." FEBS Lett. **152:** 109–113, 1983.

DEVOIZE, J. L., RIGAL, A., ESCHALIER, A. AND TROLESE, J. F.: Naloxone inhibits clomipramine in mouse forced swimming test. Eur. J. Pharmacol. **78:** 229–232, 1982.

EDDY, N. B. AND LEIMBACH, D.: Synthetic analgesics (II): Dithienylbuteny and dithienylbutylamines. J. Pharmacol. Exp. Ther. **107:** 385–389, 1953.

FOURNIE-ZALUSKI, M. C., LUCAS, E., WAKSMAN, G. AND ROQUES, B. P.: Differences in the structural requirements for selective interaction with neutral metalloendopeptidase (enkephalinase) or angiotensin converting enzyme. Molecular investigation by use of new thiol inhibitors. Eur. J. Biochem. **139:** 267–274, 1984.

GAFFORD, J. T., SKIDGEL, R. A., ERDOS, E. G. AND HERSH, L. B.: Human kidney "enkephalinase," a neutral metalloendopeptidase that cleaves active peptides. Biochemistry **22:** 3265–3271, 1983.

GLIMCHER, P. W., GIOVINO, A. A., MARGOLIN, D. H. AND HOEBEL, B. G.: Endogenous opiate reward induced by an "enkephalinase" inhibitor thiorphan, injected into the ventral midbrain. Behav. Neurosci. **98:** 262–268, 1984.

GREENBERG, R. AND O'KEEFE, E. H.: Thiorphan potentiation of stress-induced analgesia in the mouse. Life Sci. **31:** 1185–1188, 1982.

GROS, C., GIROS, B. AND SCHWARTZ, J.-C.: Identification of aminopeptidase M as an enkephalin-inactivating enzyme in rat cerebral membranes. Biochemistry **24:** 2179–2185, 1985.

GROS, C., PRADELLES, P., ROUGEOT, C., BEPOLDIN, O., DRAY, F., FOURNIE-ZALUSKI, M. C., ROQUES, B. P., POLLARD, H., LLORENS-CORTES, C. AND SCHWARTZ, J.-C.: Radioimmunoassay of methionine- and leucine-enkephalins in regions of rat brain and comparison with endorphins estimated by a radioreceptor assay. J. Neurochem. **31:** 29–39, 1978.

GUILBAUD, G., KAYSER, V., BENOIST, J. M. AND GAUTRON, M.: Depressive effects of morphine and of an enkephalinase inhibitor on responses of ventrobasal thalamic neurones to noxious stimuli. Life Sci. **33:** 545–547, 1983.

HALEY, T. J. AND MCCORMICK, W. G.: Pharmacological effects produced by intracerebroventricular injection of drugs in conscious mouse. Br. J. Pharmacol. **12:** 12–16, 1957.

HERSH, L. B.: Degradation of enkephalins: The search for an enkephalinase. Mol. Cell. Biochem. **47:** 35–43, 1982.

HERSH, L. B.: Reaction of opioid peptides with neutral endopeptidase ("enkephalinase"). J. Neurochem. **43:** 487–493, 1984.

HERSH, L. B.: Characterisation of membrane bound aminopeptidases from rat brain identification of the enkephalin degrading aminopeptidase. J. Neurochem. **44:** 1427–1435, 1985.

HILL, R. G.: The studies of naloxone in the identification of pain control mechanisms operated by endogenous opioids. Neurosci. Lett. **21:** 217–225, 1981.

HISAMITSU, T. AND DE GROAT, W. C.: The inhibitory effect of opioid peptides and morphine applied intrathecally and intracerebroventricularly on the micturition reflex in the cat. Brain Res. **298:** 51–65, 1984.

HORSTHEMKE, B., SCHULZ, M. AND BAUER, K.: Degradation of substance P by neurones and glial cells. Biochem. Biophys. Res. Commun. **125:** 728–733, 1984.

HUI, K. S. AND LAJTHA, A.: Neuropeptidases. *In* Handbook of Neurochemistry, vol. 4, ed. by A. Lajtha, pp. 1–19, Plenum Publishing Corporation, New York, 1983.

HUI, K. S., WANG, Y. J. AND LAJTHA, A.: Purification and characterization of an enkephalin aminopeptidase from rat brain membranes. Biochemistry **22:** 1062–1067, 1983.

JACOB, J. J. C. AND RAMABADRAN, K.: Role of opiate receptors and endogenous ligands in nociception. Pharmacol. Ther. **14:** 177–187, 1981.

JOHNSON, A. R., SKIDGEL, R. A., GAFFORD, J. T. AND ERDOS, E. G.: Enzymes in placental microvilli angiotensin I converting enzyme, angiotensinase A, carboxypeptidase and neutral endopeptidase ("enkephalinase"). Peptides **5:** 789–796, 1984.

KAYSER, V. AND GUILBAUD, G.: The analgesic effects of morphine but not those of the enkephalinase inhibitor Thiorphan are enhanced in arthritic rats. Brain Res. **267:** 131–138, 1983.

KORF, J., BUNNEY, B. S. AND AGHAJANIAN, G. K.: Noradrenergic neurons: Morphine inhibition of spontaneous activity. Eur. J. Pharmacol. **25:** 165–169, 1974.

LLORENS, C., MALFROY, B., SCHWARTZ, J.-C., GACEL, G., ROQUES, B. P., ROY, J., MORGAT, J. L., JAVOY-AGID, F. AND AGID, Y.: Enkephalin dipeptidyl carboxypeptidase (enkephalinase) activity: Selective radioassay, properties and regional distribution in human brain. J. Neurochem. **39:** 1081–1089, 1982.

LLORENS-CORTES, C. GROS, G. AND SCHWARTZ, J.-C.: Study of endogenous Tyr-Gly-Gly, a putative enkephalin metabolite, in mouse brain: Validation of a radioimmunoassay, localisation and effects of peptidase inhibitors. Eur. J. Pharmacol. **119:** 183–191, 1985a.

LLORENS-CORTES, C. AND SCHWARTZ, J.-C.: Changes in turnover of cerebral monoamines following inhibition of enkephalin metabolism by Thiorphan and bestatin. Eur. J. Pharmacol. **104:** 369–374, 1984.

LLORENS-CORTES, C., SCHWARTZ, J.-C. AND GROS, C.: Detection of the tripeptide Tyr-Gly-Gly, a putative enkephalin metabolite in brain, using a sensitive radioimmunoassay. FEBS Lett. **189:** 325–328, 1985b.

MALFROY, B. AND SCHWARTZ, J.-C.: Properties of "enkephalinase" from rat kidney: Comparison of dipeptidyl-carboxypeptidase and endopeptidase activities. Biochem. Biophys. Res. Commun. **106:** 276–285, 1982.

MALFROY, B. AND SCHWARTZ, J.-C.: Enkephalinase from rat kidney. Purification, characterization and study of substrate specificity. J. Biol. Chem. **23:** 14365–14370, 1984.

MALFROY, B., SWERTS, J. P., GUYON, A., ROQUES, B. P. AND SCHWARTZ, J.-C.: High affinity enkephalin-degrading peptidase in brain is increased after morphine. Nature (Lond.) **276:** 523–526, 1978.

MARTIN, W. R.: Pharmacology of opioids. Pharmacol. Rev. **35:** 283–324, 1983.

MATSAS, R., FULCHER, I. S., KENNY, A. J. AND TURNER, A. J.: Substance P and (Leu)enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. Proc. Natl. Acad. Sci. U.S.A. **80:** 3111–3115, 1983.

MCKELVY, J. F.: Enzymatic degradation of brain peptides. *In* Brain Peptides, ed. by D. T. Krieger, M. J. Brownstein and J.-B. Martin, pp. 117–135, John Wiley, New York, 1983.

NIEMEGEERS, C. J. E., LENAERTS, F. M. AND JANSSEN, P. A. J.: Loperamide (R18553), a novel type of antidiarrhoeal agent. Arzneim. Forsch. **24:** 1622–1636, 1974.

PARKER, R. B. AND WAUD, D. R.: Pharmacological estimations of drug-receptor dissociation constants. Statistical evaluations. I. Agonists. J. Pharmacol. Exp. Ther. **177:** 1–12, 1971.

PATEY, G., CUPO, A., CHAMINADE, M., MORGAT, J. L. AND ROSSIER, J.: Release of the heptapeptide Met-enkephalin-Arg^6-Gly^7 and of the octapeptide Met-enkephalin-Arg^6-Gly^7-Leu^8 from rat striatum *in vitro* and their rapid inactivation. Life Sci. **33:** 117–120, 1983.

PATEY, G., DE LA BAUME, S., SCHWARTZ, J.-C., GROS, C., ROQUES, B. P., FOURNIE-ZALUSKI, M. C. AND SOROCA-LUCAS, E.: Selective protection of methionine enkephalin released from brain slices by enkephalinase inhibition. Science (Wash. DC) **212:** 1153–1155, 1981.

PORSOLT, R. D., BERTIN, A. AND JALFRE, M.: Behavioral despair in mice: A

primary screening test for antidepressant. Arch. Int. Pharmacodyn. Ther. **229:** 327–335, 1977.

ROQUES, B. P., FOURNIE-ZALUSKI, M. C., SOROCA, E., LECOMTE, J. M., MALFROY, B., LLORENS, C. AND SCHWARTZ, J.-C.: The enkephalinase inhibitor Thiorphan shows antinociceptive activity in mice. Nature (Lond.) **288:** 286–288, 1980.

ROQUES, B. P., LUCAS-SOROCA, E., CHAILLET, P., COSTENTIN, J. AND FOURNIE-ZALUSKI, M. C.: Complete differentiation between enkephalinase and angiotensin-converting enzyme inhibition by retro-thiorphan. Proc. Natl. Acad. Sci. U.S.A. **80:** 3178–3182, 1983.

ROQUES, B. P., SCHWARTZ, J.-C. AND LECOMTE, J.-M.: Aminoacid Derivatives and their Therapeutic Applications, European Patent 038758-U.S. Patent 4000513009, 1981.

RUPREHT, J., UKPONMWAN, O. E., ADMIRAAL, P. V. AND DZOLJIC, M. R.: Effect of phosphoramidon, a selective enkephalinase inhibitor on nociception and behaviour. Neurosci. Lett. **41:** 331–335, 1983.

SAWYNOK, T., PINSKY, C. AND LABELLA, F. S.: Minireview on the specificity of naloxone as an opiate antagonist. Life Sci. **25:** 1621–1632, 1979.

SCHWARTZ, J.-C.: Metabolism of enkephalins and the inactivating neuropeptidase concept. Trends Neurosci. **8:** 143–151, 1983.

SCHWARTZ, J.-C., COSTENTIN, J. AND LECOMTE, J.-M.: Pharmacology of "enkephalinase" inhibitors. Trends Pharmacol. Sci., **6:** 472–476, 1985.

SCHWARTZ, J.-C., DE LA BAUME, S., YI, C. C., CHAILLET, P., MARCAIS-COLLADO, H. AND COSTENTIN, J.: Enkephalin metabolism in brain and its inhibition into inactive fragments. Prog. Neuropsychopharmacol. Biol. Psychiatry **6:** 665–671, 1982.

SCHWARTZ, J.-C., DE LA BAUME, S. YI, C. C., MALFROY, B., CHAILLET, P., MARCAIS-COLLADO, H. AND COSTENTIN, J.: Physiological roles of "enkephalinase" (enkephalin dipeptidyl carboxypeptidase) and of a bestatin-sensitive aminopeptidase in the inactivation of enkephalins. *In* Degradation of Endogenous Opioids: Its Relevance in Human Pathology, ed. by S. Ehrenpreis and F. Sicuteri, pp. 51–66, Raven Press, New York, 1983.

SCHWARTZ, J.-C., GIROS, B., GROS, C., LEBOYER, M., LLORENS, C., MALFROY, B., PACHOT, I. AND POLLARD, H.: "Enkephalinase" and metabolism of enkephalins. *In* Endocrinology, Proceedings of the 7th International Congress of Endocrinology, ed. by F. Labrie and L. Proulx, pp. 641–644, Elsevier Science Publishers B. V., Amsterdam, 1984a.

SCHWARTZ, J.-C., GIROS, B., GROS, C., LLORENS, C. AND MALFROY, B.: Metabolism of enkephalins and its inhibition. *In* Proceedings International Union of Pharmacology 9th Congress of Pharmacology, vol. 3, ed. by J. F. Mitchell, W. Paton and P. Turner, pp. 277–283, Mc Millan Press Ltd., London, 1984b.

SCHWARTZ, J.-C., MALFROY, B. AND DE LA BAUME, S.: Biological inactivation of enkephalins and the role of enkephalin-dipeptidyl carboxypeptidase ("enkephalinase") as neuropeptidase. Life Sci. **29:** 1715–1740, 1981.

SIGMUND, E., CADMUS, R. AND LU, G.: Methods for evaluating both non-narcotic and narcotic analgesic. Proc. Soc. Exp. Biol. Med. **95:** 729–735, 1957.

SKIDGEL, R. A., ENGELBRECHT, S., JOHNSON, A. R. AND ERDOS, E. G.: Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase. Peptides **5:** 769–776, 1984.

SWERTS, J. P., PERDRISOT, R., PATEY, G., DE LA BAUME, S. AND SCHWARTZ, J.-C.: Enkephalinase is distinct from brain angiotensin-converting enzyme. Eur. J. Pharmacol. **57:** 279–281, 1979.

TANAKA, M., KOHNO, Y., SUDA, A., NAGAKAWA, R., IDA, Y., IMDRI, K., HOAKI, Y. AND NAGASAKI, N.: Differential effects of morphine on noradrenaline release in brain regions of stressed and non-stressed rats. Brain Res. **275:** 105–113, 1983.

UKPONMWAN, O. E. AND DZOLJIC, M. R.: Enkephalinase inhibition antagonizes the increased susceptibility to seizure induced by REM sleep deprivation. Psychopharmacology **83:** 229–232, 1984.

VOGEL, Z. AND ALTSTEIN, M.: The adsorption of enkephalin to porous polystyrene beads: A simple assay for enkephalin hydrolysis. FEBS Lett. **80:** 332–336, 1977.

VON EULER, K. S. AND LISHAJKO, F.: Improved technique for the fluorometric estimation of catecholamines. Acta Physiol. Scand. **51:** 348–355, 1961.

WOOD, P. L.: Phasic enkephalinergic modulation of nigrostriatal dopamine metabolism: Potentiation with enkephalinase inhibitors. Eur. J. Pharmacol. **82:** 119–120, 1982.

YAKSH, T. L. AND HARTY, G. M.: Effects of thiorphan on the antinociceptive actions of intrathecal (DAla^2, Met^5)enkephalin. Eur. J. Pharmacol. **79:** 293–300, 1982.

YARBROUGH, C. G., BUXBAUM, D. M. AND SANDER-BUSH, E.: Biogenic amines and narcotic effects. II. Serotonin turnover in the rat after acute and chronic morphine administration. J. Pharmacol. Exp. Ther. **185:** 328–335, 1973.

ZHANG, A. Z., YANG, H. Y. T. AND COSTA, E.: Nociception, enkephalin content and dipeptidyl carboxypeptidase activity in brain of mice treated with exopeptidase inhibitors. Neuropharmacology **21:** 625–630, 1982.

ZUZEL, K. A., ROSE, C. AND SCHWARTZ, J.-C.: Assessment of the role of "enkephalinase" in cholecystokinin inactivation. Neuroscience **15:** 149–158, 1985.

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