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# β-Endorphin: Stability, clearance behavior, and entry into the central nervous system after intravenous injection of the tritiated peptide in rats and rabbits

(brain/hypophysectomy/cerebrospinal fluid/opioid peptide)

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**ABSTRACT** **Rabbits and rats were given intravenous injections of tritiated human β-endorphin. The levels of β-endorphin were followed by the decrease in radioactivity in the plasma of rats or rabbits and by the increase in radioactivity in the cerebrospinal fluid of the rabbit. The results were identical with the tritium label on either tyrosine-1 or -27. The plasma distribution times were 2 and 5 min in the rat and rabbit, respectively, with a later clearance time of approximately 1–8 hr. In the rat, approximately 50% of the radioactivity in the plasma was found to be intact human β-endorphin 45 min after injection. Radioactivity appeared in the cerebrospinal fluid of the rabbit within 30 sec after injection and reached a plateau in approximately 60–90 min after injection. Approximately 75% of the radioactivity in the cerebrospinal fluid of the rabbit was intact human β-endorphin. In the brain hemispheres of the rat and the rabbit, the only significant radiolabeled product was found to be radioactive tyrosine. Moreover, rat plasma levels of β-endorphin decreased dramatically after hypophysectomy, which only slightly lowered the levels in the brain. It appears that β-endorphin, upon entry into the plasma, is either not significantly taken up into the brain or is broken down with extreme rapidity upon entry into the brain, although it apparently does enter the cerebrospinal fluid.**

Biological effects following intravenous (i.v.) injections of β-endorphin (β-EP) (Fig. 1) are well documented (1–9). Immunohistochemical evidence points to the presence of β-EP in the brain (10, 11). It occurs in the pituitary gland (12) and the plasma of human subjects (13–16). Whether β-EP can travel from the pituitary via the plasma to the brain has not been clearly established. The objective of the present investigation was to examine the clearance behavior and fate of β-EP in the plasma of rats and rabbits, its uptake and fate in the cerebrospinal fluid (CSF) of the rabbit, and its uptake and fate in the brains of rats and rabbits by using tritiated β-EP (17).

## MATERIALS AND METHODS

Human β-EPs ($\beta_h$-EP) labeled with tritium on the tyrosine at position 1 or position 27 were prepared as described (17). Specific activities were approximately 50 Ci/mmol (8,000,000 cpm μg; 1 Ci = $3.7 \times 10^{10}$ becquerels). The peptides were homogeneous as evidenced by partition chromatography (18). They were also fully active as assayed by the guinea pig ileum method (19). Nonradioactive $\beta_h$-EP was synthesized as described (2). In the rat, approximately 0.3 μg ($2.5 \times 10^6$ cpm) of tritiated $\beta_h$-EP in 200 μl of 0.1% bovine serum albumin was injected. In the rabbit, approximately 1.5 μg ($12 \times 10^6$ cpm) of tritiated $\beta_h$-EP was injected.

Adult male Long–Evans rats with body weights of 240–260 g were used. At least three animals were used for each experiment. Anesthesia was induced by intraperitoneal injection of sodium pentobarbital (≈12 mg); the tritiated $\beta_h$-EP was introduced by bolus injection into the jugular vein, followed by an equal volume of isotonic saline through an indwelling cannula of PEZO polyethylene. At given times, blood was collected into heparinized tubes at 4°C through a cannula in the carotid artery. After centrifugation at 3000 rpm for 20 min, the plasma radioactivity was measured in PCS scintillator on a TriCarb scintillation counter. To examine the stability of $\beta_h$-EP in the circulation, the procedures above were used except that blood (15 ml) was taken at 45 min. After centrifugation the plasma was made 20% in HOAc and put through a Sephadex G-50 column (3.5 × 85 cm) equilibrated with 20% (vol/vol) HOAc containing 0.01 M $NH_4OAc$. The fraction with the main radioactive peak was lyophilized and then run on a CM-cellulose chromatography column (1.5 × 100 cm) equilibrated with 0.1 M $NH_4OAc$ containing 3% (vol/vol) ethanol. The resulting material with the main radioactive peak was again lyophilized and subjected to partition chromatography on a Sephadex G-50 column (1.5 × 100 cm) in butanol/pyridine/acetic acid/water, 4.0:0.05:1.0:5.0 (vol/vol), as described (17). The estimated percentage of $\beta_h$-EP remaining intact in the circulation after 45 min was based upon the radioactivity in the plasma at 45 min versus the total amount that remained in the position of $\beta_h$-EP after gel filtration on Sephadex G-50, chromatography on CM-cellulose, and partition chromatography on Sephadex G-50.

To examine β-EP uptake into the whole brain minus the cerebellum at a given time, the brain hemispheres, which had been perfused with saline (1–2 min) immediately before removal (removal time, 15–30 sec), were immediately homogenized with 10 strokes of a motor-driven Teflon plunger at 4°C with ≈8.5 ml of 23.5% (vol/vol) HOAc containing 5.9% (vol/vol) HCl (final concentration, 20% HOAc, 5% concentrated HCl). The resulting homogenate was centrifuged at 3000 rpm

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                    5                   10
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-

                   15                   20
Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                   25                       31
Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
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FIG. 1. Amino acid sequence of β-EP.

Abbreviations: $\beta_h$-EP, human β-endorphin; CSF, cerebrospinal fluid; RIA, radioimmunoassay; i.v., intravenous(ly).

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for 30 min, and the supernatant material was put through a Sephadex G-50 column (3.5 × 85 cm) equilibrated with 20% HOAc to determine the amount of intact tritiated $\beta_h$-EP affiliated with the total radioactivity in the brain. This procedure with rat brains was conducted at 5, 15, and 60 min after i.v. injection of tritiated $\beta_h$-EP. Controls (noninjected animals) for the above procedure consisted of: (*i*) homogenization of a brain with 23.5% HOAc containing 5.9% HCl and tritiated $\beta_h$-EP (100,000 cpm); after incubation at 25°C for 15 min, this mixture was centrifuged and chromatographed on Sephadex G-50 as described above; (*ii*) homogenization of a brain in 8.5 ml of 0.1 M phosphate buffer (pH 7.4) containing tritiated $\beta_h$-EP (100,000 cpm); after incubation of this mixture for 15 min at 25°C, the mixture was made 20% in HOAc and 5% in concentrated HCl and was centrifuged and chromatographed on Sephadex G-50 as described above; (*iii*) the procedure in *i* without the concentrated HCl.

Male New Zealand White rabbits (2–3 kg) were anesthetized by intraperitoneal injection of 5–7 g of urethane in 10 ml of isotonic saline; periodic administration of ether ensured anesthesia. Injection routes were the same as those used for the rat except that 1.5 μg (12 × $10^6$ cpm) of tritiated $\beta_h$-EP was injected. The amount of intact $\beta_h$-EP at 45 min in rabbit brain and plasma was examined in a manner identical to that used with the rat. Cerebrospinal fluid (CSF) was periodically taken with a 22-gauge needle inserted into the cisterna magna after surgical exposure of the spinal sheath while the animal was kept immobilized in a stereotaxic apparatus. At given times, 50 μl of CSF was removed and the radioactivity was measured in 4.5 ml of PCS scintillator. To examine for the presence of intact tritiated $\beta_h$-EP in the CSF, it was withdrawn at 60 min until no further clear fluid could be obtained (≈1.3 ml). This was examined in a manner identical to the isolation of tritiated $\beta_h$-EP from rat plasma described above.

To determine the effect of hypophysectomy on plasma and brain levels of β-EP, male Long–Evans rats (150 g) were hypophysectomized and left for 1 month. Control and hypophysectomized animals (20 rats in each group) were anesthetized with sodium pentabarbital (50 mg/kg of body weight) and a cannula was placed in the same vessel leading to the heart so that blood samples could be taken from that organ. About 2.0 ml of blood was taken from each rat and collected into heparinized tubes. For the determination of β-EP content in the rat brain, the head of the rat was then severed from its body and 10 ml of saline was perfused into the head through the carotid artery. The brain was quickly removed (15–30 sec), rinsed twice in saline, and placed in chloroform/methanol (2:1) for defatting. The brain tissue was then extracted in 20% HOAc, the soluble material was lyophilized, and $\beta_h$-EP was determined by radioimmunoassay (RIA) (20). From each of six rats, 1.5 ml of plasma was removed and pooled, and the aggregate was subjected to gel filtration on Sephadex G-50 (3.5 × 80 cm, 0.01 M $NH_4OAc$, pH 4.6). The column fractions were assayed for β-EP by RIA; the fractions showing immunoreactive peaks were pooled, lyophilized, and assayed for total β-EP by RIA.

## RESULTS

The drop in the level of radioactivity in the plasma after i.v. injection of tritiated $\beta_h$-EP into rats is shown in Fig. 2. The distribution half-life was 2.0 min; after this initial period, the clearance half-life was approximately 5–8 hr. The results were the same for $\beta_h$-EP tritiated at either tyrosine 1 or 27. To determine the amount of radioactivity in rat plasma that was still affiliated with intact β-EP, plasma was collected 45 min after injection and chromatographed on successive columns of

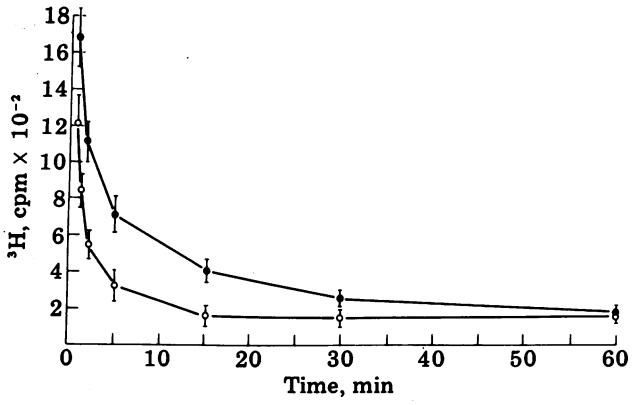


FIG. 2. Clearance rate of [$^3$H]Tyr-1- or [$^3$H]Tyr-27-labeled $\beta_h$-E from rat (○) or rabbit (●) plasma.

Sephadex G-50, CM-cellulose, and Sephadex G-50 partition chromatography (Fig. 3). Approximately 50% of the radioactivity in the plasma at 45 min was recovered in the expected position of $\beta_h$-EP after the above procedures.

The clearance behavior of tritiated $\beta_h$-EP from rabbit plasma is shown in Fig. 2. The initial distribution half-life was 5.0 min with a later clearance half-life of approximately 1 hr. When the

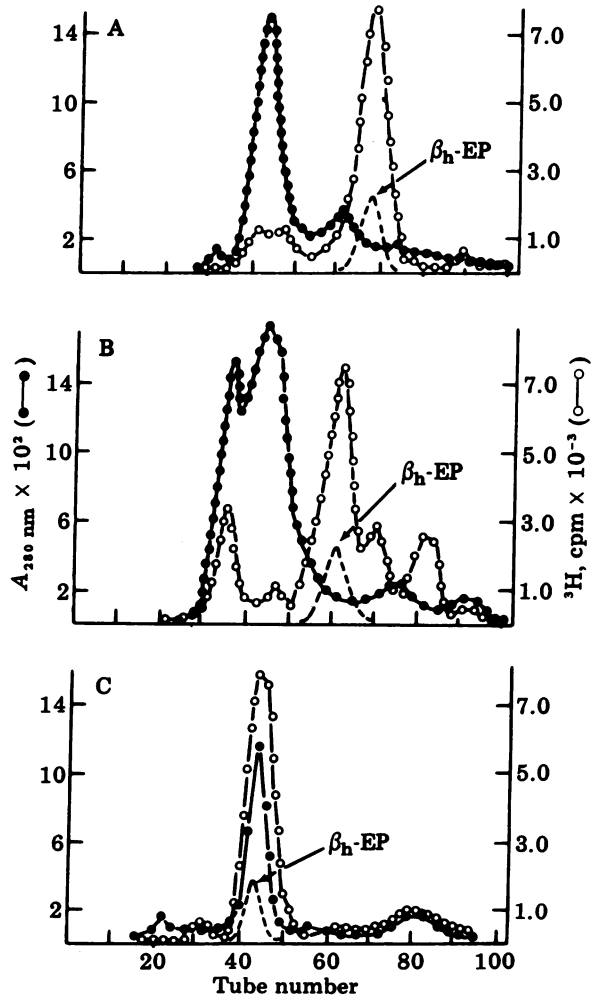


FIG. 3. Chromatographic isolation of tritiated $\beta_h$-EP (---) from rat plasma 45 min after i.v. injection. (*A*) Sephadex G-50 in 20% HOAc; (*B*) CM-cellulose (isocratic elution with 0.1 M $NH_4OAc$) of tubes 62–74 from *A*; (*C*) partition chromatography on Sephadex G-50 (butanol/pyridine/acetic acid/water, 4.00:0.05:1.00:5.00) of tubes 52–65 from *B*.

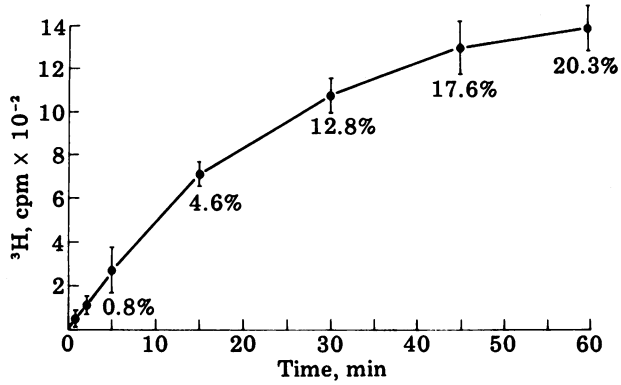


FIG. 4. Uptake of radioactivity into the CSF of rabbit; values indicate the percentage of plasma radioactivity found in the CSF.

recovery of tritiated $\beta_h$-EP in rabbit plasma was examined in a manner similar to that used for rat plasma, 70% of the radioactivity in the plasma at 45 min was intact $\beta_h$-EP. The uptake of radioactivity into the rabbit CSF is shown in Fig. 4. Radioactivity was found in CSF as early as 30 sec after injection. The level of radioactivity in CSF at 60 min after injection was approximately 20–25% of the level found in the plasma. The fate of the tritiated $\beta_h$-EP in the CSF was shown by chromatography on Sephadex G-50 and CM-cellulose. Approximately 75% of the radioactivity in the CSF was intact $\beta_h$-EP (Fig. 5).

The amount of radioactivity in the rat brain was 1%, 5%, and 20% of the level in the plasma at 5, 15, and 60 min, respectively. However, the radioactivity in the brain was found to contain no intact $\beta_h$-EP. The only significant radioactivity was found to be radioactive tyrosine by amino acid analysis and by high-voltage paper electrophoresis. Since our work-up procedure could have resulted in enzymatic breakdown of the $\beta_h$-EP in the brain homogenate, control experiments were performed. [$^3$H]Tyr-27-Labeled $\beta_h$-EP (100,000 cpm) was added to the solution prior to homogenization in 23.5% HOAc 5.9% concentrated HCl. The resulting homogenate was incubated at 25°C for 15 min. After centrifugation and chromatography on a Sephadex G-50 column, no appreciable breakdown of tritiated $\beta_h$-EP occurred. In another control experiment, the tritiated $\beta_h$-EP was added to the solution, but the homogenization was carried out in 0.1 M phosphate buffer (pH 7.4). After the homogenate had been incubated at 25°C for 15 min, it was brought to 20% HOAc and 5% concentrated HCl and subjected to chromatography on a Sephadex G-50 column. There remained at least 50% intact $\beta_h$-EP (Ve/Vo = 1.68). Two other products were also evident: one at Ve/Vo = 2.03 (15%) and the other just after the salt volume Ve/Vo = 2.98 (30%). When the procedure was carried out omitting the concentrated HCl from the homogenization, approximately 35% of the recovered radioactivity was associated with the material with Ve/Vo = 1.99. This material probably corresponds to fragments of $\beta_h$-EP. No material was found in the salt volume area. Similar results were obtained with the rabbit brain.

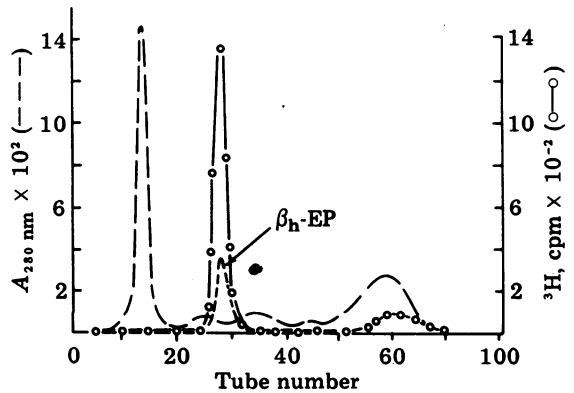


FIG. 5. Exclusion chromatography of CSF of the rabbit on a Sephadex G-50 column in 20% HOAc 60 min after i.v. injection of tritiated $\beta_h$-EP.

In the plasma of normal rats, a level of 260 pg of $\beta$-EP per ml of plasma was found. This value is comparable to that reported by Akil *et al.* (21). In hypophysectomized rats, the level of $\beta$-EP dropped to 12 pg per ml of plasma. In the brains of control animals, $\beta$-EP content was found to be 12.5 ± 1.3 ng per brain. In hypophysectomized rats, the $\beta$-EP content became 10.5 ± 1.5 ng per brain.

The stability of $\beta_h$-EP in rat plasma was examined by incubation at 4, 22, and 37°C for 3 hr. Decreases of approximately 10%, 25%, and 60% of intact $\beta_h$-EP, respectively, were found by RIA.

## DISCUSSION

When injected i.v. in the rat, tritiated $\beta_h$-EP had a distribution half-life of 2.0 min (Fig. 2). This value is lower than that reported previously (9.2 min) by Chang *et al.* (22). This difference may be due to the amount of $\beta$-EP injected. The distribution half-life of injected tritiated $\beta_h$-EP in the rabbit was shown to be 5 min (Fig. 2). In human subjects, the half-life of $\beta_h$-EP was determined by RIA to be 37 min after i.v. administration of 5 or 10 mg (9). In the rat, at least 50% of tritiated $\beta_h$-EP in the plasma after 45 min was intact $\beta_h$-EP. In the same period, about 75% of the $\beta_h$-EP was recovered in the rabbit.

Pezalla *et al.* (23) have reported an increase in the $\beta$-EP level in the CSF of the rabbit after i.v. injection of the peptide, as determined by RIA. We obtained similar results using tritiated $\beta_h$-EP. The uptake of radioactivity into the CSF of the rabbit was found to plateau at approximately 60 min after injection with approximately 20% found in the plasma (Fig. 4). At this time, the radioactivity in the CSF was shown to be at least 75% intact $\beta_h$-EP with other significant breakdown products, including radioactive tyrosine (Fig. 5). When the brain hemispheres of the rat and rabbit were examined, there was no significant amount of $\beta_h$-EP, and radioactive tyrosine was the sole radioactive product. Because these results appeared to support the fact that $\beta_h$-EP does not enter the brain after i.v. injection, we examined the other possible source—namely, the pituitary. Entry of [$^{14}$C]Har-labeled $\beta$-[D-Ala$^2$,Har]EP into the brain regions of the conscious rat has been demonstrated by Rapoport *et al.* (24).

The levels in the plasma decreased dramatically after hypophysectomy, but the levels in the brain hemispheres were only slightly lowered by hypophysectomy. This is in contrast to the recent report that hypophysectomy causes major reduction of $\beta$-EP in the rat brain (25). Control experiments using tritiated $\beta$-EP indicated that no enzymatic breakdown of $\beta_h$-EP occurred during the work-up procedure. In addition, *in vitro* examination of the stability of $\beta_h$-EP in rat plasma indicated that $\beta_h$-EP at 37°C broke down at a slow rate as determined by RIA.

While the site of action for the biological activities of $\beta$-EP after i.v. injection is not clear, it appears from the data reported herein that, although $\beta$-EP enters the CSF from plasma, it does not enter the brain hemispheres from the CSF, or if it does enter the brain, it must be broken down with extreme rapidity.

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