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In Vivo Fate of a Behaviorally Active ACTH 4-9 Analog in Rats After Systemic Administration

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VERHOEF, J. AND A. WITTER. *In vivo fate of a behaviorally active ACTH 4-9 analog in rats after systemic administration.* PHARMAC. BIOCHEM. BEHAV. 4(5) 583–590, 1976. – In vivo fate of a threefold substituted ACTH 4-9 analog with a markedly potentiated behavioral activity, 4-Met(O_2), 8-D-Lys, 9-Phe-ACTH 4-9, was investigated. The radioactive labeled [7-^{3}H-Phe] ACTH 4-9 analog was administered IV, SC and orally in a dose of approximately 40 μg. Plasma concentrations of total radioactivity and intact peptide were determined at various periods after administration in urethane anesthetized rats. Oral administration was also performed with conscious animals. Maximal plasma concentrations were found 8 min after SC injection. After oral administration in anesthetized rats maximal plasma levels were reached 8 hr after administration; in conscious animals this took 4 hr. The initial volume of distribution was 5.9% of body weight and the initial half-life (t½) for intact peptide 4 min. Shortly after IV and SC administration relatively high and stable plasma levels of intact peptide were obtained, reflecting metabolic stability. This stability was also apparent from the metabolite patterns, which were determined in trichloroacetic acid extracts of plasma and brain by paperchromatography and paperelectrophoresis. The plasma profiles indicated increased stability of the labile ^{8}Lys-^{9}Phe bond by the introduction of an ^{8}D-Lys residue in the peptide analog. Enzymatic attack of the analog took place predominantly at ^{6}His-^{7}Phe and ^{7}Phe-^{8}D-Lys. Formation of tritiated water occurred in brain and the gastro-intestinal tract and was considerable; proteolysis in these compartments was higher than in plasma. High uptake of radioactivity was found in the kidney, but urinary excretion was low during the first 30 min. Uptake in brain was low and paralleled uptake in cerebrospinal fluid. Intact peptide concentrations/g fresh tissue were in the order of 10^{-5}–10^{-4} times the administered dose for all three routes.

ACTH 4-9 analog In vivo fate Plasma disappearance Metabolism Systemic administration Uptake in brain

N-TERMINAL ACTH fragments, like ACTH 1-10 and ACTH 4-10 affect acquisition and maintenance of active and passive avoidance behavior in the rat. The steroidogenic activity of these neuropeptides is markedly reduced [7]. The divergence between behavioral and endocrine activity becomes even more pronounced when various amino acid substitutions are introduced in these neuropeptide molecules. This is particularly evident in 4-Met(O_2),8-D-Lys,9-Phe-ACTH 4-9 where the introduction of three structural modifications in ACTH 4-9 resulted in an increase in behavioral activity by at least a factor of one thousand, and a decrease in MSH and steroidogenic activity of a similar magnitude [7]. Evidence was put forward that this increase in behavioral activity could be explained by increased resistance against in vitro proteolytic breakdown [18]. The present report describes the in vivo fate of this particular ACTH 4-9 analog. The studies with this peptide analog were undertaken to investigate uptake in the brain, metabolic fate and possible oral applicability of this substituted hexapeptide.

METHOD

Drugs

4-Met(O_2),8-D-Lys,9-Phe-[7-^{3}H-Phe] ACTH 4-9. The [^{3}H] ACTH 4-9 analog was prepared by catalytic iodine-tritium exchange of the corresponding p-iodophenylalanine peptide. The (radio)chemical purity and biological activity of this tritiated peptide were high (Greven and Witter, submitted for publication). The specific activity was approximately 24 mCi/μmol.

Animals and Procedure

Administration of the [^{3}H] peptide. Male rats of an inbred Wistar strain, weighing 180–240 g, were used. Unless stated otherwise, the animals were anesthetized with 112.5 mg urethane in 0.9 ml saline per 100 g body weight, IP, and received 2.5 mg heparine in 0.5 ml saline per animal, IV, prior to administration of the peptide. IV injection was performed through a cannulated jugular vein with a solution of 44 μg [^{3}H] peptide (50 nmol),

[1] The authors wish to acknowledge the technical assistance of Miss J. M. M. Stolwijk and Miss E. G. Vergeer.

corresponding to 1.2 mCi, in 250 μl saline. This was followed immediately by 150 μl saline. For SC administration a solution of 44 μg [^{3}H] peptide in 0.5 ml saline was used. Prior to oral administration the animals were fasted for 18 hr. The [^{3}H] peptide (36 μg) was mixed with bovine serum albumin (20 mg) and the mixture was dissolved in 1 ml 0.15 M $NaHCO_3$. This solution and the subsequently administered rinsing solution of 2% bovine serum albumin in 0.15 m $NaHCO_3$ (2 ml) were gavaged into the stomach.

Plasma distribution of radioactivity. Blood samples (250 μl) were withdrawn from a cannulated jugular vein, contralateral to the jugular vein used for IV injection. Fifteen sec before each withdrawal, 100 μl blood was taken and discarded, to compensate for the dead space of the cannula and the needle (50–75 μl). A clean propylene syringe was used for each withdrawal. The blood samples were cooled immediately to 0° and plasma was obtained by centrifugation. A 5 μl plasma aliquot was dissolved in 10 ml scintillation mixture, toluene-triton × 100 (2:1, v/v) containing 2,5-diphenyloxazole (4 g/liter) and counted in a liquid scintillation counter (Isocap 300, Nuclear Chicago). Quench corrections were carried out by external standard ratios.

Radioactive metabolites in plasma. To 100 μl of plasma 100 μl of a 2.5% acetic acid carrier solution, containing 10 μg non-radioactive ACTH 4-9 analog and 10 μg of each of the main metabolites Met(O_2)-Glu-His-Phe, Glu-His-Phe, Phe-D-Lys-Phe and Phe, was added. Subsequently 200 μl of a 12% (w/v) aqueous trichloroacetic acid (TCA) solution was added and the mixture centrifuged 30 min later. The precipitate was washed twice with a 6% TCA solution. From the combined supernatants an aliquot was counted for radioactivity to determine recovery. The rest of the solution was washed thrice with an equal volume of peroxide free diethylether to remove TCA. The recovery of this step was determined by counting an aliquot and the rest of the water-layer was lyophilized. All operations were carried out at 0–4°. The lyophilizate was dissolved in 100 μl 2.5% acetic acid and an aliquot counted to determine possible losses due to formation of tritiated water. Radioactive metabolites were investigated with 25 μl samples by paperchromatography in BAPW = n-butanol-acetic acid-pyridine-water (15:3:10:12,v/v/v/v). Radioactivity was located by contact autoradiography on Kodak RP X-omat Medical X-ray film, after a short dip of the chromatogram in a 2.5% solution of 2,5-diphenyloxazole in ether. If contact autoradiography was not feasible because of too low radioactivity concentrations (less than 25 nCi/μl), the chromatogram was cut in 1 cm strips, which were placed in counting vials. Radioactive material was extracted by shaking these vials for 1 hr at 40° with 1 ml of 2.5% acetic acid, after which 10 ml of scintillation mixture was added. In separate experiments located radioactive spots were eluted from the chromatogram by elution chromatography with 25% acetic acid for 4 hr at near 100% relative humidity. The eluates were lyophilized and redissolved in 2.5% acetic acid for further investigation by paperchromatography in BAW = n-butanol-acetic acid-water (4:1:5, v/v/v; organic phase) or paperelectrophoresis in acetic acid-pyridine-water (1:50:950, v/v/v) pH 6.5 for 1½ hr at 2000 V. Identification of radioactive compounds was carried out by cochromatography and coelectrophoresis with synthetic reference peptides.

Tissue distribution of radioactivity. This was determined in separate experiments. Plasma was sampled from trunk blood and counted directly for radioactivity. Liver, kidney and brain were dissected immediately after decapitation, weighed and homogenized ultrasonically for 30 sec at 20 kHz with a cold 6% TCA solution (7.5 ml/g fresh tissue). The homogenate was centrifuged at 4° and the precipitate washed twice with a cold 6% TCA solution. From the combined supernatants an aliquot, usually 1 ml, was counted for radioactivity.

Urinary excretion of radioactivity was measured after IV administration. Shortly before injection of the [^{3}H] peptide the urethra was ligated and both ureters were provided with losely fitting ligatures. Immediately before decapitation the ureters were ligated. After decapitation the urine bladder was dissected, urine removed by aspiration, the bladder washed twice with 200 μl saline and the total volume recorded and counted for radioactivity.

The concentration of radioactivity in the cerebrospinal fluid (CSF) was measured after SC administration. Shortly before injection of the [^{3}H] peptide a polypropylene cannula (id 0.4 mm), connected to a dual infusion-withdrawal pump, was inserted into the cisterna magna. During insertion artificial CSF [4] was pumped through the cannula at 0.3 μl/min. The feasibility of the connection was verified by infusion for 10 min and withdrawal for 30 min. Immediately before decapitation 25–50 μl CSF was withdrawn in 15–30 sec, its volume recorded and the concentration of radioactivity determined. The brain was dissected and cut in four parts by a sagittal section through the midline and a transversal section at the level of the bregma. The four brain segments were washed thrice in icecold saline, 5 ml and for 20 sec each, and frozen.

Radioactive metabolites in brain. To the TCA extract from whole brain tissue was added 100 μl of carrier solution and the mixture washed five times with an equal volume of peroxide free ether. An aliquot of the water layer was counted to establish recovery and the remaining part was lyophilized. The residue was dissolved in 250 μl 0.01 M ammoniumacetate pH 6.5 and an aliquot counted to estimate the contribution of tritiated water to total radioactivity in the TCA extract. The remaining solution was transferred to a Dowex 1 × 2 [H^+] column, 0.7 × 6.5 cm, equilibrated with 0.01 M ammoniumacetate buffer pH 6.5 Elution was performed with the same buffer at 0.4 ml/min and radioactive fractions were pooled, the total volume recorded and a sample counted for recovery. The remaining solution was lyophilized and the residue dissolved in 100 μl 2.5% acetic acid. Metabolites were determined as before by paperchromatographic and paperelectrophoretic analysis.

RESULTS

Distribution of Radioactivity and Intact Peptide in Plasma

IV injection of the [^{3}H]-ACTH 4-9 analog led to a rapid disappearance of radioactivity and of intact peptide from plasma (Fig. 1, A and B). The disappearance rates decreased with time and, accordingly, t½ increased with time. The t½, estimated graphically between 1 and 6 min after administration is approximately 4.6 min for total radioactivity and 4 min for intact peptide. The plasma disappearance curves can probably be represented by the sum of some exponential terms, but the limited number of observations does not allow a realistic analysis by the peeling-off technique. An estimate of the initial volume of distribution

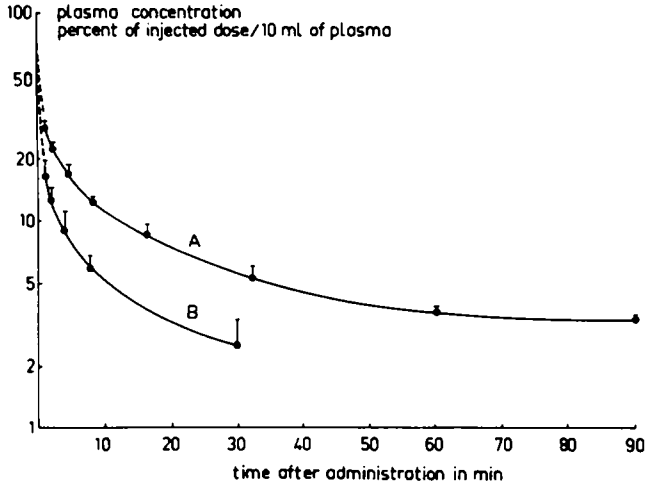

FIG. 1. Plasma concentrationsof radioactivity (A) and intact peptide (B) after IV administration of 44 μg (1.2 mCi) 4-Met (O_2), 8-D-Lys, 9-Phe-[^{3}H-7-Phe] ACTH 4-9 in urethane anesthetized rats. Intact peptide concentrations were determined by paperchromatography of trichloroacetic acid plasma extracts (cf. Fig. 4). Each point represents the mean ± SD of 5 determinations.

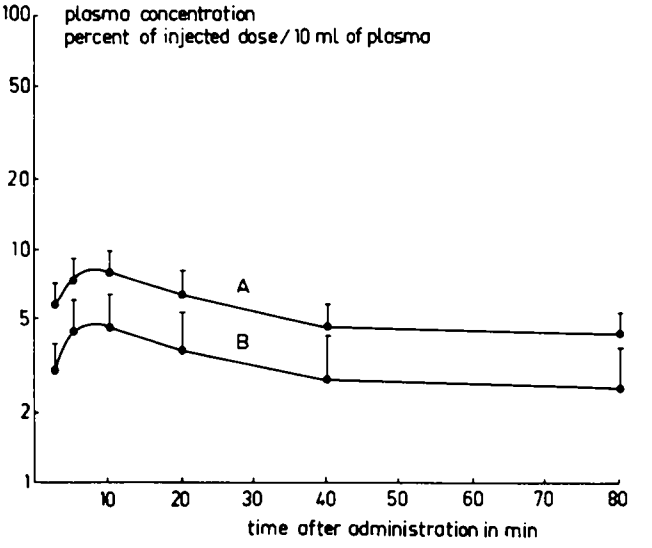

FIG. 2. Plasma concentrations of radioactivity (A) and intact peptide (B) after SC administration of 44 μg 4-Met (O_2), 8-D-Lys, 9-Phe-[^{3}H-7-Phe] ACTH 4-9 in urethane anesthetized rats. For further details see legend Fig. 1.

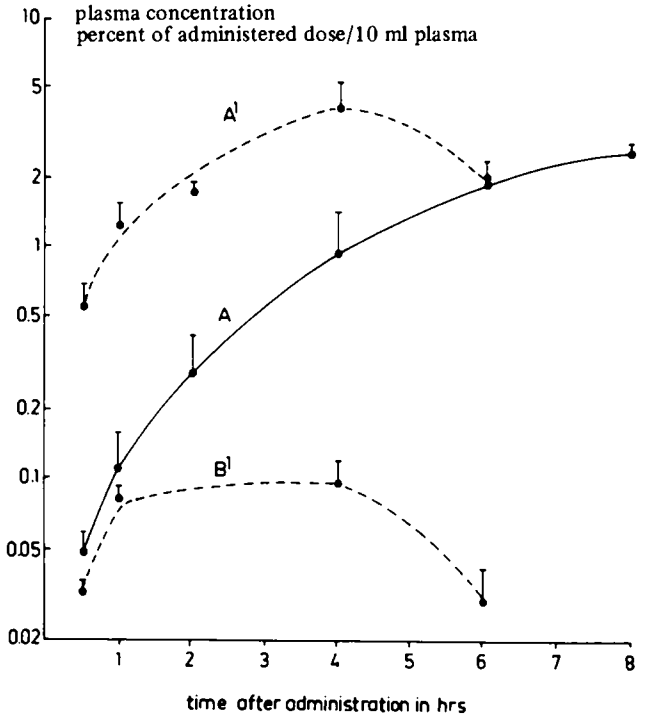

FIG. 3. Plasma concentrations of radioactivity in urethane anesthetized (A) and conscious (A') rats and of intact peptide in conscious animals (B') after oral administration of 36 μg 4-Met (O_2), 8-D-Lys, 9-Phe-[^{3}H-7-Phe] ACTH 4-9. For further details see legend Fig. 1.

was obtained by extrapolation of a third order polynomial approximation of the initial part of the plasma radioactivity disappearance curve to zero time. The coefficients of this approximation were obtained by least square analysis. This volume averaged 5.9% of the mean body weight or 14.2 ml.

The plasma distribution curves for total radioactivity and intact peptide after SC administration are presented in Fig. 2A and B. Both curves run virtually parallel and indicate the appearance of maximal plasma levels 8 min after administration. The plasma disappearance rates are slow and the plasma concentration of intact peptide exceeded the corresponding concentration after IV injection about 15 min after administration and continued to do so (cf. Fig. 2B and 1B).

The initial plasma levels after oral administration (Fig. 3) were much lower than observed after administration by the IV and SC route. However, the plasma concentrations of total radioactivity increased rapidly with time for a considerable period. Accordingly, these concentrations approximated those after IV and SC injection at 3–8 hr after administration (cf. Fig. 3A and A' and Fig. 1A and 2A). A considerable difference in absorption characteristics was observed for anesthetized (Fig. 3A) and conscious (Fig. 3A') rats. The initial absorption in conscious animals was about tenfold the value in anesthetized animals. However, plasma concentrations in conscious animals leveled off after a peak value obtained 4 hr after administration, compared to 8 hr in anesthetized rats. This resulted in similar plasma radioactivity concentrations 6 hr after oral administration for both groups of animals. Such discrepancies in plasma radioactivity levels in anesthetized and conscious animals were not found after IV or SC administration. The intact peptide plasma levels are considerably lower than after IV or SC administration.

Tissue Distribution of Radioactivity

The distribution of total radioactivity over various tissues after IV injection is presented in Table 1. The kidneys show the highest uptake of radioactivity and high levels were reached rapidly after administration. The urinary concentrations were low and showed considerable variations. The contribution of urinary excretion to the elimination of radioactivity in the first 30 min after administration is very small, despite increasing urinary

TABLE 1

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER IV ADMINISTRATION OF [^{3}H] ACTH 4-9 ANALOG

	5 min*	15 min	30 min
Liver	0.66 ± 0.08†	0.41 ± 0.07	0.24 ± 0.04
Kidney	6.33 ± 0.7	6.91 ± 0.9	6.82 ± 1.0
Cerebellum	0.08 ± 0.02	0.06 ± 0.005	0.055 ± 0.006
Brain cortex	0.07 ± 0.01	0.06 ± 0.003	0.055 ± 0.006
Brain stem	0.07 ± 0.01	0.06 ± 0.003	0.05 ± 0.008
Urine	0.001 ± 0.0007	0.01 ± 0.007	0.02 ± 0.01

*Time after i.v. injection of 44 μg [^{3}H] ACTH 4-9 analog in urethane anesthetized rats.

†All values represent the mean ± SD of 4-5 determinations and are expressed as percent of injected dose/g fresh tissue.

concentrations with time. Radioactivity concentrations in the liver were considerably lower than those in the kidneys and they decreased with time. Uptake of radioactivity in the brain was low and evenly distributed over gross brain areas.

The distribution of radioactivity after SC administration is given in Table 2. As before, highest levels were found in the kidneys. At 2 hr after administration the average kidney concentration had increased strikingly, as had the variability in results. The concentration of radioactivity in liver was of a similar magnitude as observed after IV injection, but almost constant in time. Uptake of radioactivity in brain increased with time and ran parallel with the concentration in the CSF.

The distribution after oral administration in conscious animals is tabulated in Table 3. The concentrations in kidney and liver were low and of similar magnitude. They follow resembling time patterns, reaching peak values 4 hr after administration which coincide with a peak in plasma concentration (cf. Fig. 3A[1]). Surprisingly, uptake in brain was of the same order of magnitude as observed after IV and SC administration.

Radioactive Metabolites in Plasma and Brain

The recoveries of the various steps applied in isolating the radioactive metabolites were 91 ± 13% for the extraction with TCA from plasma, 94 ± 8% after washing of the TCA extract with ether, 96 ± 8% after lyophilization of the ether treated extracts, 92 ± 11% for the elution from the Dowex 1 × 2 columns (brain extracts only) and 88 ± 15% for the elution from the chromatograms. These values are given as means ± SD and the number of observations was 25 50. Lower recoveries for the TCA extractions from plasma were found for periods exceeding 1 hr after administration: 76 ± 8%, 80 min after SC injection; 47 ± 8%, 31 ± 2% and 29 ± 2.5% respectively 1, 2 and 4 hr after oral administration. Radioactivity not found in the extract was completely recovered from the TCA precipitate. Therefore, these lower recoveries probably represent losses due to incorporation of liberated [^{3}H]-phenylalanine into TCA insoluble proteins. This was verified by incubating the TCA precipitate, after washing with ether, with a mixture of proteolytic enzymes for 30 h at 38° in 0.1 M phosphate buffer. After incubation the mixture was treated with an equal volume of 12% TCA and less than 0.2% of the radioactivity was found in the precipitate. The supernatant was subjected to paperchromatography and 78% of the recovered radioactivity was identified as [^{3}H] -phenylalanine.

Lyophilization recoveries below 90% were considered to

TABLE 2

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER SC ADMINISTRATION OF [^{3}H] ACTH 4-9 ANALOG

	10 min*	30 min	60 min	120 min
Liver	0.32 ± 0.08†	0.35 ± 0.07	0.31 ± 0.04	0.34 ± 0.09
Kidney	2.15 ± 0.9	1.68 ± 0.5	1.65 ± 0.1	7.39 ± 4.8
Brain	0.03 ± 0.01	0.05 ± 0.01	0.065 ± 0.002	0.09 ± 0.02
CSF	0.03 ± 0.01	0.05 ± 0.02	0.05 ± 0.02	0.10 ± 0.02

*Time after SC injection of 44 μg [^{3}H] ACTH 4-9 analog in urethane anesthetized rats.

†All values represent the mean ± SD of 5-8 determinations and are expressed as percent of injected dose/g fresh tissue.

TABLE 3

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER ORAL ADMINISTRATION OF [^{3}H] ACTH 4-9 ANALOG

	30 min*	1 hr	2 hr	4 hr	6 hr
Liver	0.08 ± 0.01†	0.11 ± 0.03	0.07 ± 0.01	0.17 ± 0.05	0.09 ± 0.01
Kidney	0.06 ± 0.02	0.08 ± 0.01	0.09 ± 0.02	0.20 ± 0.03	0.18 ± 0.02
Brain	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.004	0.07 ± 0.02	0.03 ± 0.01

*Time after oral administration of 36 μg [^{3}H] ACTH 4-9 analog in conscious rats.

†All values represent the mean ± SD of 4 determinations and are expressed as percent of administered dose/g fresh tissue.

indicate losses due to removal of tritiated water. In some control experiments the amount of tritiated water in the ether treated trichloroacetic acid extracts was determined by a different approach, using successive ionexchange chromatography [14]. The values obtained by this procedure were identical to those obtained by lyophilization.

A typical separation profile of radioactive metabolites in plasma or brain extracts is presented in Fig. 4. The distribution of recovered radioactivity over various radioactive metabolites in plasma and brain after IV administration is tabulated in Table 4. The metabolic and distributional events which lead to the metabolite patterns in plasma seem to take place predominantly during the first min after injection. The contribution of intact peptide was high and decreased only slowly with time (cf. Fig. 1B). The main radioactive metabolites were [^{3}H]-Phe-D-Lys-Phe and [^{3}H]-Phe. Initially, the contribution of both metabolites increased with time but at 30 min after administration the concentration of [^{3}H]-Phe-D-Lys-Phe tended to decrease, whereas the concentration of [^{3}H]-Phe continued to increase. the contribution of intact peptide to recovered radioactivity in brain tissue was considerably lower than in plasma and its rate of disappearance much higher. Tritiated water, which could not be detected in plasma, was the main radioactive metabolite. Also [^{3}H]-Phe was a major metabolite in brain, but the contribution of [^{3}H]-Phe-D-Lys-Phe was small.

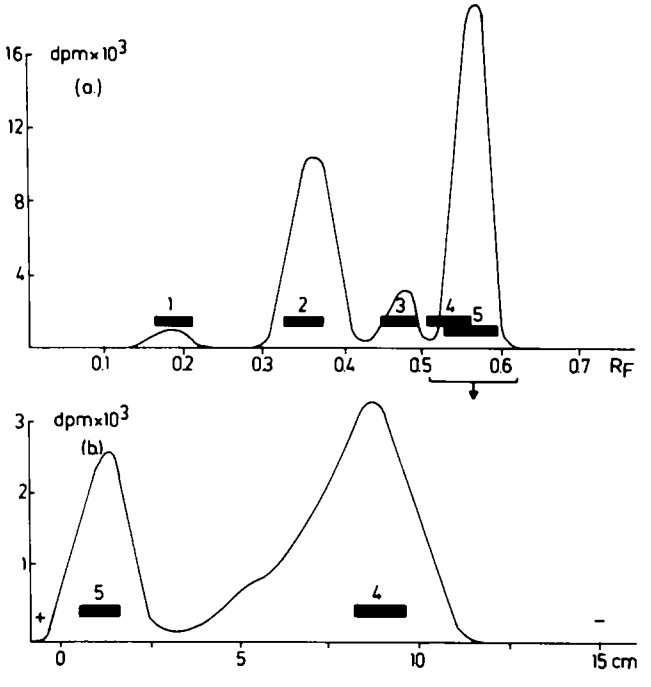

FIG. 4. Separation of radioactive metabolites from trichloroacetic acid extracts from plasma or brain by (a) paperchromatography in n-butanol-acetic acid-pyridine-water (15:3:10:12, v/v/v/v). The area with R_f 0.56 was eluted and investigated further by (b) paperelectrophoresis in acetic acid-pyridine-water (1:50:950, v/v/v) pH 6.5 for 1½ hr at 2000 V. Radioactivity was determined in 1 cm strips of the chromatograms, as described in Method. The positions of reference peptides are indicated by rectangular, numbered bars: 1 = Met (O_2)-Glu-His-[^{3}H]Phe-D-Lys, Glu-His-[^{3}H]Phe-D-Lys; 2 = Met (O_2)-Glu-His-[^{3}H]Phe-D-Lys-Phe; 3 = Met (O_2)-Glu-His-[^{3}H]Phe, Glu-His-[^{3}H]Phe; 4 = [^{3}H]Phe-D-Lys-Phe; 5 = [^{3}H]Phe.

The metabolic profiles after SC administration of the [^{3}H] peptide are given in Table 5. Again, the pattern of radioactive metabolites in plasma is established rapidly after administration. It remains remarkably constant during the 80 min period of investigation. The contribution of intact peptide to recovered radioactivity is well over 50% and the main metabolites in plasma are [^{3}H]-Phe, [^{3}H]-Phe-D-Lys-Phe and (Met (O_2))-Glu-His [^{3}H]-Phe. The contribution of intact peptide in brain was small and rapidly diminishing. Tritiated water was the main metabolite in brain tissue and its relative concentration increased at a similar rate as obtained after the IV route. Again [^{3}H]-Phe was another major metabolite.

The relative concentrations of radioactive metabolites in plasma and brain after oral administration are tabulated in Table 6. The values were obtained from conscious animals. The contributions of intact peptide to total recovered radioactivity were low in plasma and even lower, but still distinctly detectable, in brain. In both brain and plasma tritiated water and to a lesser extent [^{3}H]-Phe were the most abundant radioactive metabolites. The depot character of this route of administration (cf. Fig. 3) is possibly responsible for the presence of (Met (O_2))-Glu-His-[^{3}h]-Phe and (Met(O_2))-Glu-His-[^{3}H]-Phe-D-Lys as radioactive metabolites in both plasma and brain.

An estimate of the average concentration of intact peptide/g fresh brain tissue after IV administration can be obtained by combining the results from Tables 1 and 4. These estimates correspond to 1.1×10^{-4}, 0.7×10^{-4} and 0.3×10^{-4} of the administered dose at 5, 15 and 30 min after administration respectively. Similar figures can be calculated for the SC route from Tables 2 and 5, resulting in average brain concentrations of intact peptide of 0.2×10^{-4}, 0.1×10^{-4} and 0.05×10^{-4} of the administered dose at 10, 20 and 60 min after administration respectively. After oral administration these figures are approximately 0.03×10^{-4} at 30 and 60 min after administration.

DISCUSSION

The present results are generally in accordance with literature data on the in vivo fate of brain oligopeptides [19]. The characteristic feature of the title peptide is its high behavioral activity, introduced by structural modifications. The ACTH 4-9 analog, administered SC in a dose of 1 ng delays extention of a pole-jump avoidance response for several hr after administration [18]. In view of this high potency and the supposed central site of action [17], uptake in the brain is of particular interest. The estimated concentration of intact peptide in the brain is in the order of 10^{-5} times the SC administered dose. The dosage actually used (approximately 40 μg) is much higher than employed in behavioral studies (approximately 1 ng), in order to enable adequate analysis of metabolic patterns in plasma and brain tissue. However, uptake in the brain after SC administration was found to be dose-independent in the range of 100 μg–100 ng; lower dosages were undetectable. If dose-independency is extrapolated to the biological active dose of 1 ng, a brain concentration of approximately 10^{-5} ng of intact peptide/g brain tissue results. This value illustrates the high potency, even though local concentrations might be considerably higher. Compared to regional brain concentrations of thyrotropin-releasing hormone [5,8] and luteinizing hormone-releasing hormone [13] which vary from $1-10^{+5}$ ng/g tissue wet

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN TCA EXTRACTS FROM PLASMA (A) AND BRAIN (B) OVER RADIOACTIVE METABOLITES AFTER IV ADMINISTRATION OF [^{3}H] ACTH 4-9 ANALOG

		Time and Values				
A. Plasma (n=5)		1 min*	2 min	4 min	8 min	30 min
Met (O_2)-Glu-His-	[^{3}H] Phe-D-Lys-Phe	54.9 ± 5.0†	51.1 ± 2.8	48.5 ± 4.2	44.1 ± 3.1	42.0 ± 3.3
(Met (O_2))-Glu-His-	[^{3}H] Phe	5.9 ± 0.7	6.1 ± 1.9	6.9 ± 1.9	7.3 ± 2.9	3.1 ± 0.8
	[^{3}H] Phe-D-Lys-Phe	24.6 ± 2.1	27.8 ± 5.5	28.9 ± 5.6	30.6 ± 0.6	26.5 ± 3.4
	[^{3}H] Phe	15.0 ± 4.3	15.1 ± 4.5	17.1 ± 4.5	21.1 ± 1.6	25.5 ± 1.6
B. Brain (n=2)		5 min*		15 min		30 min
Met (O_2)-Glu-His-	[^{3}H] Phe-D-Lys-Phe	15.7†		11.3		6.2
(Met (O_2))-Glu-His-	[^{3}H] Phe	11.0		7.0		6.3
	[^{3}H] Phe-D-Lys-Phe	5.1		2.3		0.5
	[^{3}H] Phe	26.5		33.9		33.1
	[^{3}H] H_2O	41.6		45.2		54.2

*Time after IV injection of 44 μg [^{3}H] ACTH 4-9 analog in urethane anesthetized rats.
†Values represent the mean ± SD and are expressed as percent of recovered radioactivity.

TABLE 5

DISTRIBUTION OF RADIOACTIVITY IN TCA EXTRACTS FROM PLASMA (A) AND BRAIN (B) OVER RADIOACTIVE METABOLITES AFTER SC ADMINISTRATION OF [^{3}H] ACTH 4-9 ANALOG

		Time and values					
A. Plasma(n=6)		2½ min*	5 min	10 min	20 min	40 min	80 min
Met (O_2)-Glu-His-	[^{3}H] Phe-D-Lys-Phe	54.4 ± 1.7†	60.2 ± 8.7	57.9 ± 8.0	58.1 ± 6.9	59.7 ± 5.7	59.3 ± 9.0
(Met (O_2))-Glu-His-	[^{3}H] Phe	11.4 ± 1.6	10.6 ± 2.1	12.8 ± 2.7	10.4 ± 1.8	10.7 ± 0.9	10.2 ± 1.1
	[^{3}H] Phe-D-Lys-Phe	13.4 ± 5.0	12.7 ± 5.7	13.1 ± 3.7	12.4 ± 4.7	12.4 ± 4.1	11.0 ± 3.3
	[^{3}H] Phe	20.8 ± 5.8	16.7 ± 6.3	16.3 ± 5.9	19.1 ± 4.5	18.2 ± 4.4	19.5 ± 7.2
B. Brain (n=5)			10 min*		30 min		60 min
Met (O_2)-Glu-His-	[^{3}H] Phe-D-Lys-Phe		5.9 ± 2.8†		2.5 ± 1.2		0.8 ± 0.4
(Met (O_2))-Glu-His-	[^{3}H] Phe-D-Lys		2.9 ± 1.0		1.8 ± 0.5		1.7 ± 0.2
(Met(O_2))-Glu-His-	[^{3}H] Phe		9.2 ± 3.4		6.3 ± 1.1		4.3 ± 0.6
	[^{3}H] Phe-D-Lys-Phe		5.6 ± 2.4		1.5 ± 1.4		n.d.‡
	[^{3}H] Phe		31.9 ± 8.1		32.0 ± 3.7		25.7 ± 4.7
	[^{3}H] H_2O		44.6 ± 11.1		55.9 ± 4.8		67.5 ± 5.6

*Time after SC injection of 44 μg [^{3}H] ACTH 4-9 analog in urethane anesthetized rats.
†Values represent the mean ± SD and are expressed as percent of recovered radioactivity.
‡not detectable.

weight, the mean brain concentration of the neuropeptide is strikingly low. The low effective brain concentration of the ACTH 4-9 analog implicates high efficacy and high receptor affinity. The concentration of receptor binding sites is probably high relative to intact peptide concentration in order to obtain sufficient receptor-peptide interaction, but, for the same reason, probably low in absolute terms. This might be translated as high selectivity, indicating receptor locations in small restricted brain areas. The recent interest in a possible function of CSF in the distribution of brain oligopeptides [1, 3, 10] prompted us to investigate the uptake of radioactivity in CSF. After SC administration uptake in CSF parallels uptake in brain. Apparently, equilibrium between the two compartments exists, but conclusions regarding a possible functional role of CSF in distributing the ACTH 4-9 analog through the brain cannot be drawn.

The increased behavioral activity of the ACTH 4-9 analog can be possibly explained by stabilization against proteolytic breakdown. Substantial evidence for this was obtained from the observation that a positive correlation between behavioral activity and in vitro t½ values of 3 different ACTH 4-9 analogs was found [18]. The effect of amino acid substitutions on in vivo stability against proteolysis is not directly evidenct from the initial t½ value of the peptide analog. The value of 4 min is comparable to the approximated values of 3 min reported for bio- and radioimmunoassayable luteinizing hormone-releasing hormone and [2,16] and 5 min for thyrotropin-releasing hormone [11]. It is higher than the estimated t½'s of approximately 1 min for ACTH [9], lysine-vasopressin and oxytocin [15] and thyrotropin-releasing hormone [6]. The rapid uptake of radioactivity in kidney and liver after IV administration demonstrates that distribution contributes considerably to the plasma disappearance of peptide and hence to its relative low initial t½ results. The relatively stable plasma levels of intact peptide then obtained, reflect the high metabolic stability. A similar increase of t½ with

TABLE 6

DISTRIBUTION OF RADIOACTIVITY IN TCA EXTRACTS FROM PLASMA (A) AND BRAIN (B) OVER RADIOACTIVE METABOLITES AFTER ORAL ADMINISTRATION OF [^{3}H] ACTH 4-9 ANALOG

		Time and Values			
A. Plasma (n=3-4)		30 min*	1 hr	2 hr	4 hr
Met(O_2) -Glu-His-	[^{3}H] Phe-D-Lys-Phe	6.0 ± 0.6†	6.7 ± 0.7	4.6 ± 0.4	2.8 ± 0.4
(Met(O_2))-Glu-His-	[^{3}H] Phe-D-Lys	3.9 ± 1.4	4.0 ± 1.2	1.1 ± 0.3	0.5 ± 0.2
(Met(O_2))-Glu-His-	[^{3}H] Phe	15.7 ± 3.2	7.7 ± 0.6	5.0 ± 0.4	2.4 ± 0.5
	[^{3}H] Phe-D-Lys-Phe	2.3 ± 0.3	2.1 ± 0.1	0.2 ± 0.2	n.d.‡
	[^{3}H] Phe	28.5 ± 4.1	33.1 ± 2.4	19.8 ± 6.0	8.8 ± 3.7
	[^{3}H] H_2O	38.7 ± 7.1	44.8 ± 4.1	68.2 ± 5.1	85.3 ± 5.0
B. Brain (n=4)		30 min*	1 hr	2 hr	
Met(O_2)-Glu-His-	[^{3}H] Phe-D-Lys-Phe	0.9 ± 0.3†	0.8 ± 0.1	0.8 ± 0.2	
(Met(O_2))-Glu-His-	[^{3}H] Phe-D-Lys	4.2 ± 0.6	4.7 ± 1.4	2.4 ± 0.4	
(Met(O_2))-Glu-His-	[^{3}H] Phe	6.9 ± 1.7	4.8 ± 0.8	3.0 ± 0.4	
	[^{3}H] Phe-D-Lys-Phe	n.d.‡	n.d.	n.d.	
	[^{3}H] Phe	16.9 ± 3.7	19.7 ± 2.5	15.4 ± 4.7	
	[^{3}H] H_2O	69.3 ± 5.2	68.9 ± 4.6	77.5 ± 4.5	

*Time after oral administration of 36 μg [^{3}H] ACTH 4-9 analog in conscious rats.
†Values represent the mean ± SD and are expressed as percent of recovered radioactivity.
‡not detectable.

time was also observed for other brain oligopeptides. However, the reported plasma concentrations of bio- and radioimmuno-assayable peptide were much lower [11,16] than obtained for the ACTH 4-9 analog in comparable periods after administration. The rather low urinary excretion might further contribute to increased plasma levels of intact peptide.

Another aspect related to the use of a modified peptide analog is its metabolic profile. The position of the label allows detection by radioactivity of 11 out of 20 possible metabolites. Because the applied separation procedures do neither differentiate between Met (O_2)-Glu-His-Phe-D-Lys and Glu-His-Phe-D-Lys nor between Met (O_2)-Glu-His-Phe and Glu-His-Phe, information regarding N-terminal proteolysis is limited. The main degradation sites seem to be the peptide bonds between His-Phe and Phe-D-Lys. Metabolites originating from cleavage of the D-Lys-Phe bond were restricted to brain tissue and were present in low concentrations only. This demonstrates the protective effect of the substituting amino acids, particularly of D-Lys, because the corresponding Arg-Trp bond in natural ACTH is the main site of proteolytic attack [12]. These in vivo data are in accordance with those obtained in vitro [18], both with regard to the metabolic pattern and the finding that proteolysis in brain exceeds proteolysis in plasma. The high transfer of label to tritiated water in brain tissue after peripheral administration is in accordance with observations by others, using ^{3}H-labeled amino acids [14]. The absolute amount of tritiated water in the brain is too small to yield a detectable contribution to plasma metabolites. Apparently, tritiated water is also formed in the gastro-intestinal tract, because high plasma levels of tritiated water occurred after oral administration. The relatively high plasma concentrations of intact peptide after oral administration again demonstrate the stabilizing effect of the amino acid substitutions in the ACTH 4-9 analog against proteolysis and illustrate the possible clinical potential of modified neuropeptides.

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