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Differential Penetration of DSIP Peptides into Rat Brain

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KASTIN, A. J., W. A. BANKS, P. F. CASTELLANOS, C. NISSEN AND D. H. COY. *Differential penetration of DSIP peptides into rat brain.* PHARMAC. BIOCHEM. BEHAV. 17(6) 1187–1191, 1982.—Delta sleep-inducing peptide (DSIP) or five closely related peptides were injected peripherally and the levels of DSIP-like immunoreactivity measured in the brains of 100 g and 500 g rats decapitated a minute later after washout with 0.9% NaCl. Higher concentrations of immunoreactive material were found in brain tissue with injection of desTrp^1-DSIP, D-Ala^4-DSIP, and, after correction for cross-reactivity, with D-Ala^4-DSIP-NH_2 than with the other peptides. Both peptides substituted in the fourth position with D-alanine resulted in higher concentrations in 500 g rats than in 100 g rats, indicating a possible effect of age. The 5–10 fold greater brain to blood ratios of radioactivity after ^{125}I-N-Tyr-DSIP than after ^{125}-I radioiodinated serum albumin (RISA) provided aditional evidence against non-specific leakage or contamination of the brains with substantial amounts of residual blood. The results suggest that penetration of the blood-brain barrier (BBB) by small amounts of peptides, at least DSIP peptides, may be somewhat selective.

Sleep peptide Blood-brain barrier Radioimmunoassay DSIP analogs Age

DESPITE general acceptance that peripherally administered peptides can exert central effects on the brain [7], surprisingly few studies have been published describing how this occurs. In the last few years, our group has focused part of our continuing research in this area [6] on the use of delta sleep-inducing peptide (DSIP) as a model. Although the functional role(s) of DSIP remain unclear [4,11], this nonapeptide has been useful as a tool for exploration of the possibility that at least some peptides can enter the brain in essentially intact form.

Crucial to these studies is the use of an antibody that requires a minimum of eight of the nine amino acids of DSIP for recognition [9]. Measurement by radioimmunoassay (RIA) of DSIP-like immunoreactivity in the brain after peripheral injection, therefore, constitutes strong evidence for passage through the blood-brain barrier (BBB) or the associated blood-CSF barrier. The RIA was used, therefore, to determine whether various DSIP peptides enter the brain to the same extent.

EXPERIMENT 1: 10 mg/kg, 100 g RATS

Method

DSIP, desTrp^1-DSIP, N-Tyr-DSIP, D-Ala^3-DSIP, D-Ala^4-DSIP and D-Ala^4-DSIP-NH_2 were synthesized by solid-phase methods. Each peptide was diluted on ice immediately before use with 0.9% NaCl and injected at a dose of 10 mg/kg into the carotid artery of 5-7 male, albino rats (Blue Spruce Farms, Altamont, NY) weighing 106±1 g at the time of injection. The wash-out method of Experiment 3 of our previous study in this series [8] was used for all rats. Five sec after the rapid intracarotid injection of about 0.1 ml of solution as a bolus, the ipsilateral jugular vein was cut and the flow of 0.9% NaCl into the contralateral jugular vein begun. One minute later (65 sec after injection of the peptide or saline), the rat was decapitated and its brain rapidly removed and frozen.

Extraction of brain tissue involved homogenization in 0.1 M acetic acid and 4% Trasylol for 10 sec with a Brinkman Polytron (setting 4.5), centrifugation for 20 min at 27,500 g, and lyophilization. At the time of assay, the lyophilized tissue was resuspended in Trizma (pH 8.0) buffer with 4% Trasylol and diluted to make an initial concentration of 2 mg/100 μl (0.4 mg/100 μl final dilution). All brain samples were measured in a single RIA and values expressed as pg per mg brain tissue. The results were compared by analysis of variance followed by Duncan's Multiple Range Test.

Results

Figure 1 shows that brain levels of DSIP-like immunoreactivity were highest after injection of D-Ala^4-DSIP followed by those after desTrp^1-DSIP. Analysis of variance revealed the effect of treatment to be significant, $F(6,36)=9.03$, $p<0.001$. The values after D-Ala^4-DSIP were significantly ($p<0.01$) greater than those after any of the other peptides or saline, and the values after desTrp^1-DSIP were significantly ($p<0.05$) greater than those after saline.

EXPERIMENT 2: 1 mg/kg, 100 g vs. 500 g RATS

Method

The only differences from Experiment 1 were the dose and the inclusion of both 100 g and 500 g rats. All peptides were injected at a dose of 1 mg/kg, one tenth that of Experiment 1. Half the rats weighed 111±2 g and half 530±9 g at the

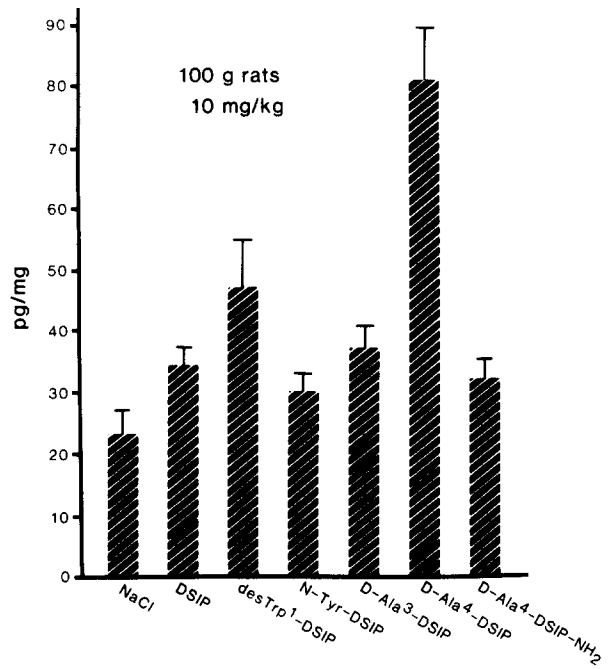

FIG. 1. Mean (±SEM) DSIP-like immunoreactivity in brain after intracarotid injection of 10 mg/kg DSIP peptides to 100 g rats followed by washout.

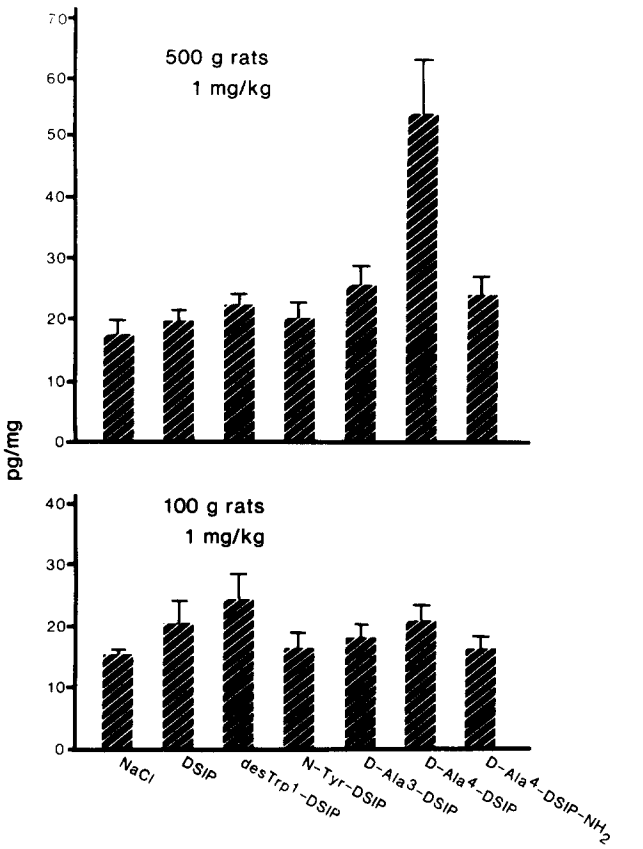

FIG. 2. Mean (±SEM) DSIP-like immunoreactivity in brain after intracarotid injection of 1 mg/kg DSIP peptides to 100 g and 500 g rats followed by washout.

time of injection. Equal numbers of rats at each weight were injected on each experimental day and all samples from both groups measured in a single RIA.

Results

Figure 2 shows that although the D-Ala^4-DSIP and desTrp^1-DSIP groups again ranked highest in brain levels of DSIP-like immunoreactivity in the 100 g rats, the lower dose used in this experiment resulted in very slight, non-significant differences among the treatments. In the 500 g rats, however, injection of D-Ala^4-DSIP produced very high levels of DSIP-like immunoreactivity in the brains. The main effects of treatment, $F(6,70)=11.13$, $p<0.001$, and rat weight, $F(1,70)=20.50$, $p<0.001$, as well as the treatment × weight interaction, $F(6,70)=8.38$, $p<0.001$, can be explained largely by the effects of the D-Ala^4-DSIP injection. Values from this group were significantly ($p<0.0001$) higher than in the brains of any of the other groups of rats from either weight. No other differences were statistically significant.

EXPERIMENT 3: DETERMINATION OF CROSS-REACTIVITIES OF DSIP PEPTIDES AND SUBSEQUENT TRANSFORMATION OF VALUES FROM EXPERIMENTS 1 AND 2

Method

The relative cross-reactivities of DSIP-peptides to DSIP were determined by measuring the immunoreactivity of known quantities of these compounds in the DSIP assay. Comparisons were made at high, low, and midrange concentrations. The relative cross-reactivities were calculated by dividing the mean immunoreactive value by the weight of the peptide added. Corrections for the levels obtained in the brains from Experiments 1 and 2 were then made by dividing the amount of immunoreactivity in each brain that was greater than that of the saline controls by the relative cross-reactivity of the appropriate peptide regardless of its molar concentration or questions of possible alteration of endogenous levels.

Results

Dilution curves for all analogs were parallel to the standard curve but shifted to the right, except for D-Ala^4-DSIP which was shifted to the left, indicating that D-Ala^4-DSIP was more effective at displacing ^{125}I-N-Tyr-DSIP than was DSIP. The mean relative cross-reactivity for each analog was determined to be 0.89 (89%) for N-Tyr-DSIP, 0.85 for D-Ala^3-DSIP, 1.32 for D-Ala^4-DSIP, 0.126 for D-Ala^4-DSIP-NH_2, and 0.45 for desTrp^1-DSIP. Correction of the increase in DSIP-like immunoreactivity in the brain samples after administration of the peptides for their relative cross-reactivity in the RIA resulted in the transformed values shown in Figs. 3 and 4.

After transformation of the values for Experiment 1 (Fig. 3), 10 mg/kg D-Ala^4-DSIP tended to increase DSIP-like immunoreactivity in the brains of 100 g rats, but this did not reach statistical significance ($p<0.07$). The increases after D-Ala^4-DSIP-NH_2 and desTrp^1-DSIP, however, were more pronounced. The main effect of treatment was significant, $F(6,36)=4.90$, $p<0.01$, and the values for D-Ala^4-DSIP- NH_2 and desTrp^1-DSIP were significantly greater than those for

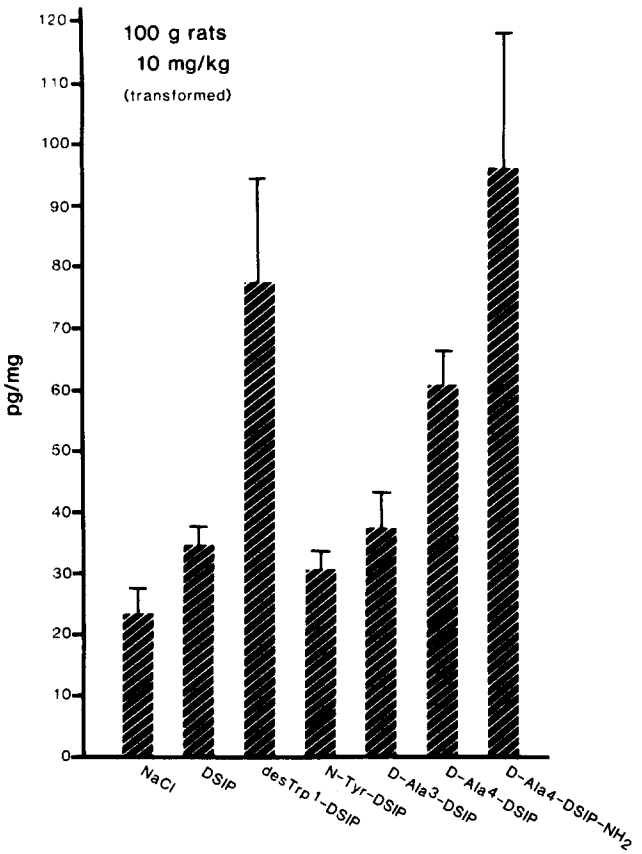

FIG. 3. Values from Figure 1 (Experiment 1) corrected for cross-reactivity with the antibody used in the RIA of DSIP-like immunoreactivity.

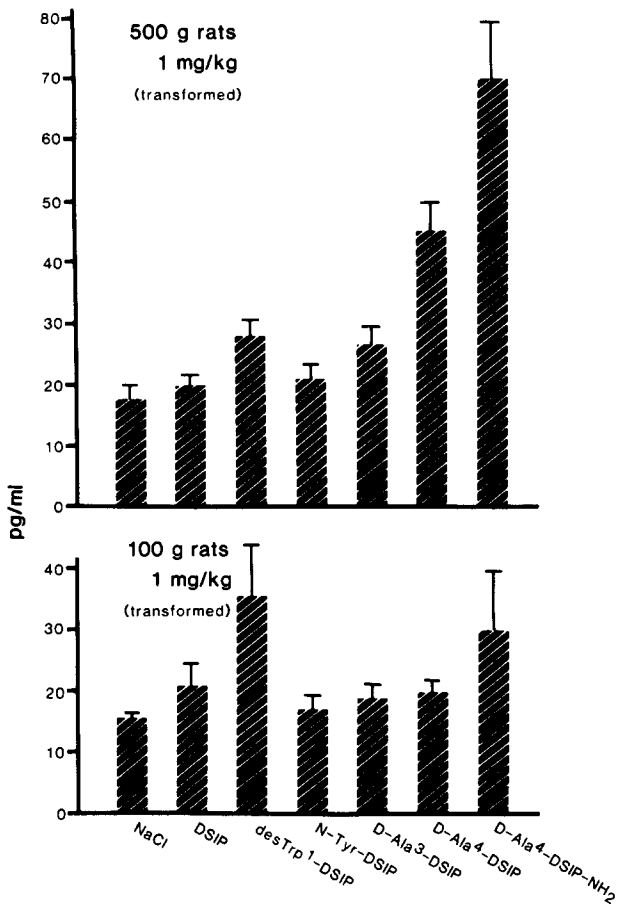

FIG. 4. Values from Figure 2 (Experiment 2) corrected for cross-reactivity with the antibody used in the RIA of DSIP-like immunoreactivity.

DSIP, N-Tyr-DSIP, D-Ala^3-DSIP ($p<0.01$ for D-Ala^4-DSIP-NH_2, $p<0.05$ for desTrp^1-DSIP) as well as those for saline-treated controls ($p<0.01$). An additional correction for molecular weight would have increased the brain values of the octapeptide desTrp^1-DSIP by almost a third, but it would not have substantially changed any of the other values and, therefore, was not performed.

As shown in Fig. 4 (transformed values from Experiment 2), a lower dose (1 mg/kg) of the substances tended to produce a similar pattern in the 100 g rats, but none of the differences reached statistical significance, desTrp^1-DSIP being the closest ($p<0.08$) in the 100 g rats. In the 500 g rats, however, 1 mg/kg of D-Ala^4-DSIP and D-Ala^4-DSIP-NH_2 produced a marked increase in the levels of DSIP-like immunoreactivity as measured by the transformed scores. The analysis revealed a significant effect of treatment, $F(6,70)=6.25$, $p<0.0001$, weight of the rat, $F(1,70)=8.14$, $p<0.01$, and treatment × weight interaction, $F(6,70)=3.25$, $p<0.01$.

The values of the D-Ala^4-DSIP and D-Ala^4-DSIP-NH_2 groups were significantly higher than those of the saline- and the parent DSIP-injected rats ($p<0.0001$ for D-Ala-^{4}-DSIP-NH_2, $p<0.05$ for D-Ala^4-DSIP). The transformed levels after injection of either D-Ala^4-DSIP-NH_2 ($p<0.0001$) or D-Ala^4-DSIP ($p<0.05$) were greater in the 500 g rats than in the 100 g rats.

EXPERIMENT 4: RISA vs. ^{125}I-N-Tyr-DSIP

Method

This experiment was conducted exactly like Experiments 1 and 2 except that ^{125}I-radioiodinated human serum albumin (RISA) or ^{125}I-N-Tyr-DSIP, both containing about 1% free iodine, were injected rather than unlabeled peptides. Before the washout with saline was begun, a few drops of blood were collected from the jugular vein in chilled 12 mm × 75 mm borosilicate tubes containing 25 U of heparin sulfate. The brains were removed, frozen, and processed in the identical manner as the brains that were used to measure immunoreactivity except than an aliquot of the supernatant was counted immediately after centrifugation. The passage into brain of ^{125}I-N-Tyr-DSIP relative to that of RISA was then determined by comparing their brain to blood ratios. Aliquots of brain samples were subsequently chromatographed on a 1 cm × 20 cm column of Sephadex G-25.

Results

The mean (±SEM) blood level in three 500 g rats after injection of 3×10^7 CPM of RISA was 6.55 (±0.838)$\times10^6$ CPM/ml. The mean level in the brain was 7.39 (±1.79)$\times10^3$ CPM/brain. The brain to plasma ratio was 1.14

$(\pm 0.24)\times 10^{-3}$. The CPM in whole brain represented 0.025% of the total CPM injected. With column chromatography, 85% of the brain radioactivity coeluted with RISA.

For the three 100 g rats injected with 1.5×10^{7} CPM of RISA, the mean blood level was $7.78\ (\pm 1.11)\times 10^{6}$ CPM/ml and the mean brain level was $2.20\ (\pm 0.54)\times 10^{3}$ CPM. The brain to plasma ratio was $2.83\ (\pm 0.66)\times 10^{-4}$. The CPM in whole brain represented 0.015% of the total CPM injected. Column chromatography showed that 92% of the brain radioactivity coeluted with RISA.

The mean blood level in four 500 g rats after injection of 6.8×10^{5} CPM of ^{125}I-N-Tyr-DSIP was $2.23\ (\pm 0.38)\times 10^{5}$ CPM/ml. The brain level was $1.24\ (\pm 0.41)\times 10^{3}$ CPM/brain. The brain to plasma ratio was 5.56×10^{-3}. The CPM in brain represented 0.18% of the injected peptide, and 84% coeluted with intact ^{125}I-N-Tyr-DSIP after gel filtration.

Injection of 3.4×10^{5} CPM ^{125}I-N-Tyr-DSIP to four 100 g rats resulted in a mean blood level of $2.73\ (\pm 0.294)\times 10^{5}$ CPM/ml and a mean brain level of $7.45\ (\pm 1.65)\times 10^{2}$ CPM/brain. The brain to plasma ratio was 2.75×10^{-3}. The CPM in brain represented 0.22% of the CPM injected, and 85% coeluted with intact ^{125}I-N-Tyr-DSIP by column chromatography.

DISCUSSION

The results show, in general, that of the six DSIP peptides injected peripherally, brain levels of DSIP-like immunoreactivity were increased most by removal of the first amino acid (desTrp^{1}-DSIP) or substitution of D-Ala in the fourth position. Compared to the parent compound, D-Ala^{4}-DSIP reacted somewhat more strongly with the DSIP antibody but the addition of an amide group to this analog (D-Ala^{4}-DSIP-NH_2) considerably weakened its reactivity. Correction for these cross-reactivities revealed that D-Ala^{4}-DSIP-NH_2 penetrated the brain to a statistically significant extent. Administration of either D-Ala^{4}-DSIP or its amide to 500 g rats caused corrected levels of immunoreactivity to be reliably higher than those of saline controls and those of 100 g rats. The difference between large and small rats raises the possibly of increased permeability of the BBB to peptides with age, although the heavier rats received a greater total amount of peptide.

Increased activity of a peptide is usually explained by increased binding to the target receptor, frequently as a result of changes in conformation, or increased resistance to enzymatic degradation. In addition, because of our interest in the BBB, we have previously considered other factors, such as lipophilicity [2,5] and protein binding [1]. A combination of these factors could be involved in the differential penetration of the BBB, but it is not clear whether any of them fully explains our results.

At the present state of understanding, not much can be said about the relative potencies of DSIP peptides to bind to a DSIP receptor, and protein binding of peptides, particularly in blood, represents a relatively unexplored area [1,3]. Our RIA measures both bound and free DSIP-like immunoreactivity, but not only do their relative amounts apparently differ in different species of animals, but some analogs of DSIP may show less protein binding than the parent DSIP [1]. The amount of free rather than total DSIP-like activity in plasma, therefore, could greatly influence the amount entering the brain.

It appears that the structural conformation of the D-Ala^{4} substituted analogs made them very suitable for crossing the BBB. Although protein binding in blood may be altered, this would seem to favor a saturable or highly specific process for penetration of DSIP peptides into the brain, as opposed to a less specific process such as pinocytosis or sieving, especially since D-Ala^{3}-DSIP showed much less crossing than D-Ala^{4}-DSIP, despite their identical molecular weights. The apparently easier passage across the BBB of desTrp^{1}-DSIP, therefore, may involve considerations other than its smaller size.

Degradation of D-Ala^{4}-DSIP by brain extracts has been tested previously. It was found that the presence of D-Ala in the fourth position of the nonapeptide blocked internal cleavage of amino acids and peptides [10]. This could explain the greater activity of D-Ala^{4}-DSIP than the parent compound but not the much lower activity of D-Ala^{3}-DSIP which is at least equally, if not more, resistant to breakdown than D-Ala^{4}-DSIP [10].

Lipophilicity usually has not been considered for peptides as frequently as binding, conformation, and degradation, probably because of the previous misconception that peripherally administered peptides could not affect the brain [7]. Although D-Ala^{4}-DSIP-NH_2, because of its amide group, may be the most lipophilic peptide tested in these experiments, there does not seem to be any reason to suppose that D-Ala^{4}-DSIP is any more lipophilic than many of the other peptides tested.

Requirements for binding with our antibody seem to differ from the requirements for optimal passage across the BBB. Removal of the tryptophan in the first position in desTrp^{1}-DSIP or addition of an amide group at the last position in D-Ala^{4}-DSIP-NH_2 greatly decreased binding to our antibody but appeared to facilitate crossing of the BBB. Only D-Ala^{4}-DSIP increased both binding and crossing.

Contamination of brain tissue by blood could also explain the increased immunoreactivity after injection of peptides. In addition to being shown previously to be unlikely [8,9], this could not explain the large differences of immunoreactivity in the brains after injection of the different peptides. Nevertheless, to further examine the question of residual blood, rats were injected with either albumin (RISA) or N-Tyr-DSIP, both labeled with ^{125}I. The brain to blood ratios of radioactivity after injection of ^{125}I-N-Tyr-DSIP were about 10 times larger than those after injection of RISA in the 100 g rats and about 5 times larger in the 500 g rats. These results indicate that ^{125}I-N-Tyr-DSIP penetrated the BBB much more readily than RISA even though the smaller unlabeled N-Tyr-DSIP appeared to cross less well than most of the DSIP peptides. The differences among the various DSIP peptides tested does not support nonspecific mechanisms of entry, such as pinocytosis or sieving, and the results with the two radioactive compounds are also consistent with this general suggestion of selectivity.

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REFERENCES

1. Banks, W. A., A. J. Kastin and D. H. Coy. Delta sleep-inducing peptide crosses the blood-brain barrier in dogs: Some correlations with protein binding. *Pharmac. Biochem. Behav.* **17:** 1009–1014, 1982.
2. Coy, D. H., A. J. Kastin, M. J. Walker, R. F. McGivern and C. A. Sandman. Increased analgesic activities of a fluorinated and a dimeric analogue of [D-Ala-2]-methionine enkephalinamide. *Biochem. biophys. Res. Commun.* **83:** 977–983, 1978.
3. Kastin, A. J., P. F. Castellanos, W. A. Banks and D. H. Coy. Radioimmunoassay of DSIP-like material in human blood: possible protein binding. *Pharmac. Biochem. Behav.* **15:** 969–974, 1981.
4. Kastin, A. J., D. H. Coy, A. V. Schally and R. D. Olson. Dissociative actions of peptides on the brain. In: *Functional Regulation at the Cellular and Molecular Levels*, edited by R. A. Corradino. Amsterdam: Elsevier, 1982, pp. 91–103.
5. Kastin, A. J., M. T. Jemison and D. H. Coy. Analgesia after peripheral administration of enkephalin and endorphin analogues. *Pharmac. Biochem. Behav.* **11:** 713–716, 1979.
6. Kastin, A. J., R. D. Olson, E. Fritschka and D. H. Coy. Neuropeptides and the blood brain barrier. In: *Cerebral Microcirculation and Metabolism*, edited by J. Cervos-Navarro and E. Fritschka. New York: Raven Press, 1981, pp. 139–145.
7. Kastin, A. J., R. D. Olson, A. V. Schally and D. H. Coy. CNS effects of peripherally administered brain peptides. *Life Sci.* **25:** 401–414, 1979.
8. Kastin, A. J., C. Nissen and D. H. Coy. Permeability of the blood-brain barrier to DSIP peptides. *Pharmac. Biochem. Behav.* **15:** 955–959, 1981.
9. Kastin, A. J., C. Nissen, A. V. Schally and D. H. Coy. Additional evidence that small amounts of a peptide can cross the blood-brain barrier. *Pharmac. Biochem. Behav.* **11:** 717–719, 1979.
10. Marks, N., F. Stern, A. J. Kastin and D. H. Coy. Degradation of delta sleep inducing peptide (DSIP) and its analogs by brain extracts. *Brain Res. Bull.* **2:** 491–493, 1977.
11. Schneider-Helmert, D., M. Graf and G. A. Schoenenberger. Synthetic delta-sleep-inducing peptide improves sleep in insomniacs. *Lancet* **1:** 1256, 1981.