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DSIP Occurs in Free Form in Mammalian Plasma, Human CSF and Urine

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GRAF, M. V., A. J. KASTIN AND A. J. FISCHMAN. *DSIP occurs in free form in mammalian plasma, human CSF and urine*. PHARMACOL BIOCHEM BEHAV 21(5) 761–766, 1984.—Although delta-sleep inducing peptide was isolated and characterized several years ago, no definitive evidence has been presented for the natural existence of the free peptide. Several attempts at the partial characterization of DSIP-like immunoreactivity (DSIP-LI) have indicated that a small part of the total immunoreactivity is probably present as the free nonapeptide. Using gel chromatography (Sephadex G-100) and subsequent high performance liquid chromatography on rabbit, human, rat and dog plasma, we now show a distinct peak of DSIP-LI that has the same elution position as synthetic DSIP. Free DSIP was also found in human CSF, whereas in human urine most of the small molecular weight DSIP-LI eluted at a position corresponding to DSIP-P, the phosphorylated analog of DSIP. A newly developed antibody recognizing primarily small molecular weight DSIP-LI was used in a modified, rapid assay to facilitate demonstration of the natural occurrence of free DSIP.

Sleep Peptide Blood HPLC

SINCE the isolation and characterization of delta-sleep inducing peptide in 1977 [15,16], several reports have focused on its occurrence in different locations of the body. We first showed by radioimmunoassay (RIA) the presence of DSIP-like immunoreactivity (DSIP-LI) in rat brain [11] and later in plasma of several mammals including humans [12] and in dog CSF [1]. In plasma, brain, and cerebrospinal fluid (CSF), DSIP-LI was partially characterized with gel filtration chromatography. In plasma [12], CSF [1,12], and peripheral organs of the rat [9], a major peak of apparently high molecular weight DSIP-LI and a minor peak eluting at the position of the free nonapeptide were detected by Sephadex chromatography.

Recently, Ekman *et al.* reported that free DSIP could not be detected in human plasma or urine, whereas the free peptide may occur in CSF [5]. The majority of the immunoreactivity was detected at elution positions corresponding to a higher apparent molecular weight than synthetic DSIP. In the present work, we use high performance liquid chromatography (HPLC) to demonstrate that some DSIP-LI elutes in a position corresponding to free DSIP.

METHOD

The DSIP and N-Tyr-DSIP used in the RIA as well as the fragments and analogs used in the cross-reactivity studies were generously supplied by Dr. D. H. Coy, Tulane University School of Medicine, New Orleans, LA. DSIP-P, a DSIP-analog phosphorylated at the serine in position 7, was obtained by courtesy of Dr. D. Gillessen, F. Hoffmann-La Roche, Basle, Switzerland.

A new antibody (Ab No. 607) to DSIP was generated in rabbits as previously described [11]. Labeled ^{125}I-N-Tyr-DSIP, prepared as in earlier work [12], was used as the tracer. Trasylol® was purchased from Mobay Chemical Co., New York, NY, and bovine serum albumin (BSA) from Sigma Chemical Co., St. Louis, MO. HPLC-grade solvents were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. The water used for HPLC was triple-distilled in glass. Proteins used for cross-reactivity studies were obtained from Sigma Chemical Co., St. Louis, MO. Samples of human plasma and urine from healthy volunteers were collected in the afternoon. CSF was obtained from the clinical chemistry laboratory of the VA Hospital, New Orleans, LA. The samples were originally collected from patients with a variety of clinical disorders. Plasma from rabbits, rats, dogs, and healthy humans was collected in heparinized tubes, centrifuged at 2000×g for 20 min at 4°C, and stored at −20°C until assayed.

DSIP RIA

In the standard RIA procedure, 50 μl aliquots of the different fluids were mixed with 250 μl RIA buffer (0.1 M borate buffer, pH 8.4; 0.1% BSA, 4% Trasylol), antibody (No. 607) in 100 μl buffer (1:20,000 final dilution), and 2 hr later, with 100 μl of ^{125}I-Tyr-DSIP (10,000 cpm) in RIA buffer. After an incubation of 24 hr, 1 ml of charcoal-dextran (0.5%/0.05% in 0.1 M borate buffer) was added and, 30 min later, the tubes centrifuged for 30 min at 2000×g and 4°C. The supernatants were counted in a gamma counter. For studies of parallelism, appropriate amounts of the different

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fluids were diluted with and without added DSIP and assayed as described above.

Gel Chromatography

Samples of plasma (5 ml), urine (5 ml), or CSF (20 ml reconstituted to 5 ml after lyophilization) were adjusted to pH 3.3 with acetic acid, centrifuged, and applied to a column of Sephadex G-100 (2.5×100 cm) that was previously equilibrated with 0.1 M acetic acid containing 0.002% sodium azide. The column was run at a flow rate of 25 ml/hr and 9 ml fractions were collected. One ml of each fraction was lypophilized and assayed for DSIP. The fractions representing the peak of small molecular weight DSIP-LI were pooled, lyophilized, resuspended in 0.1% trifluoroacetic acid (TFA), centrifuged (10 min, 3000×g, 4°C), and analyzed by HPLC.

HPLC

HPLC was performed with a Beckman (Palo Alto, CA) HPLC-system (model 344) equipped with an Ultrasphere™-ODS column (0.46×15 cm). The flow rate was 1.5 ml per min, solvent A was 0.1% aqueous TFA, and solvent B was 0.1% TFA in methanol. Two min after injection, solvent B was increased from 3% to 16% over 11 min, and at 16 min from 16% to 40% over 10 min. After 30 min, the column was flushed with 100% solvent B for 10 min and re-equilibrated with 3% B. Fractions of 0.75 ml were collected, lyophilized, and measured by RIA. Before each analytical run, a blank injection of 250 μl 0.1% TFA was run under identical conditions. The following amounts of the reconstituted samples were injected: CSF, rabbit, and human plasma: 50%; dog and rat plasma, 80%; human urine, 25%.

RESULTS

With the new antibody (No. 607), an incubation time of 24 hr yielded a mean total binding (B_0) of 30%, with a nonspecific binding of 1%. This antibody showed high sensitivity with a detection limit of 2 pg DSIP per tube and an ID 50 (amount of DSIP required to reduce the binding to 50% B_0) of 12 pg per tube. The intra-assay coefficient of variation (CV) was 6.0% for a standard of 80 pg DSIP/tube and 6.1% for 20 pg/tube, and the interassay CV was 9.0% for 80 pg and 11.9% for 20 pg DSIP per tube. Before the use of the 0.1 M borate buffer was adopted, we found that use of phosphate (pH 7.4) or Tris-HCl (pH 7.4) buffer resulted in considerably lower total binding (3–6%) and sensitivity. Exclusion of BSA from the buffer decreased the total binding by about 10-fold.

D-Ala^4-DSIP-NH_2 exhibited about 8% cross-reactivity, whereas D-Ala^4-DSIP, DSIP-P, and N-Tyr-DSIP showed about 100% cross-reactivity with Ab 607. MIF-1 (Pro-Leu-Gly-NH_2), N-Tyr-MIF-1, α-MSH, β-MSH, VIP, CRF (rat), ACTH, neurotensin, CCK, Leu-enkephalin, β-LPH, substance P, motilin, and γ-endorphin did not cross react significantly (<0.1% on a molor basis) with Ab 607 at a concentration of 10 ng/tube; a similar lack of cross-reactivity was also observed with DSIP fragments ($DSIP_{1-3}$, $DSIP_{2-9}$, $DSIP_{4-6}$, $DSIP_{4-7}$, $DSIP_{4-8}$, $DSIP_{6-9}$) and a DSIP analog D-Ala^3-DSIP.

Fourteen different plasma protein fractions (including commercially available albumins, α, β, and γ-globulin fractions of human and bovine plasma, and bovine thyroglobulin) at a concentration of 100 μg/tube did not cross-react significantly (<0.1%) with the antibody. However, a human α-globulin fraction obtained from ICN Pharmaceuticals Inc., Plainview, NJ, showed substantial cross-reactivity with Ab 607. Preliminary experiments suggested that a substance with DSIP-LI may be present in this protein preparation.

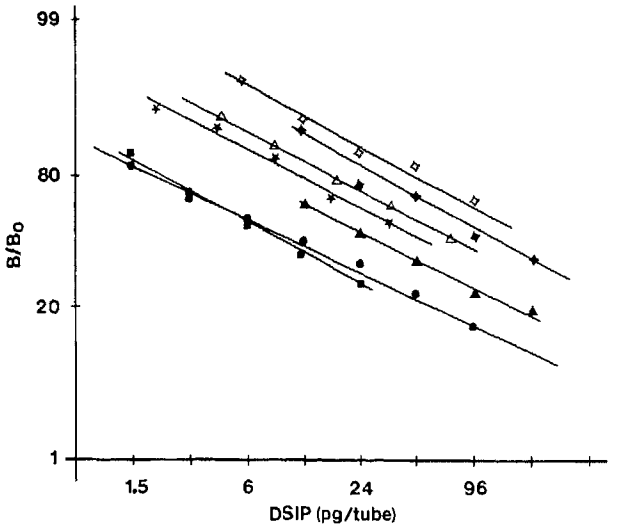

FIG. 1. Log-logit representation of dilution curves of human plasma (■), rabbit plasma (◆), rat plasma (▲), and dog plasma (◇), human CSF (△), and human urine (★). Serial dilutions showed parallelism with the standard (●) curve.

Plasma of human, rat, rabbit, and dog as well as human urine and CSF showed dilution curves that were parallel to standard DSIP (Fig. 1). Addition of DSIP shifted the curves to the left but did not change the slope. Recovery of added DSIP was determined to be 100% in all cases except in rat plasma where it was 75%. Basal levels (mean±SD, pg/ml) of DSIP-LI were as follows: human plasma: 260±58 (n=6); rabbit plasma: 155±34 (n=7); rat plasma: 291±113 (n=13); dog plasma: 105±78 (n=12); human CSF: 33±16 (n=62); and human urine: 108±29 (n=5).

Gel filtration of plasma from all 4 species revealed similar elution profiles of DSIP-LI (Fig. 2) with a first peak at the void volume followed by a peak at the position of synthetic DSIP. With human plasma, a small peak of intermediate molecular size was also observed (Fig. 2A). A different elution pattern of DSIP-LI was observed with human CSF and urine (Fig. 3). In both cases a large peak of DSIP-LI eluted at the position of free DSIP, but little (CSF) or no (urine) immunoreactivity was detected near void volume.

HPLC of all 4 samples of plasma showed a clear peak of DSIP-LI that varied in amount and eluted at the position of synthetic DSIP, indicating that free DSIP can be found in plasma of these species (Fig. 4). What appears to be free DSIP was also detected in human CSF, but in addition 3 other peaks were seen. Two of these peaks eluted at the position of DSIP-P and N-Tyr-DSIP. In urine (Fig. 6), a small fraction of the total detectable DSIP-LI eluted at the position of synthetic DSIP and a larger peak was observed at the position of DSIP-P. In all cases, a large immunoreactive peak eluted at the beginning of the gradient. This is characteristic of highly polar compounds. With human and dog plasma, an additional peak of DSIP-LI appeared late in the gradient (around fractions 67–70) (Fig. 4) where, as indicated by UV monitoring, large proteins elute.

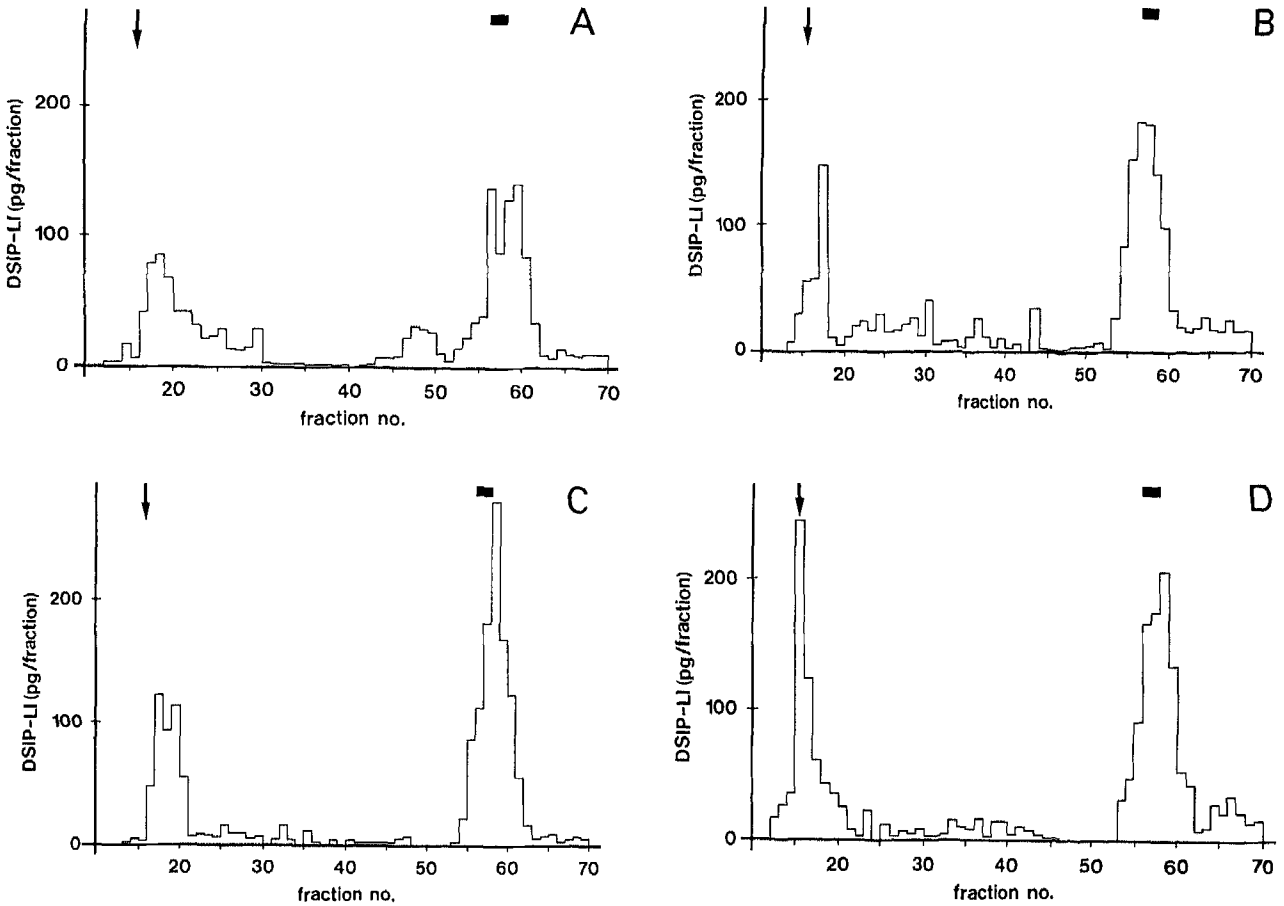

FIG. 2. Gel chromatography of human plasma (A), rabbit plasma (B), rat plasma (C), and dog plasma (D) on Sephadex G-100. The column size was 2.5×100 cm and the fraction volume was 9 ml. The arrow indicates the void volume of the column and the black bar the position where standard DSIP eluted.

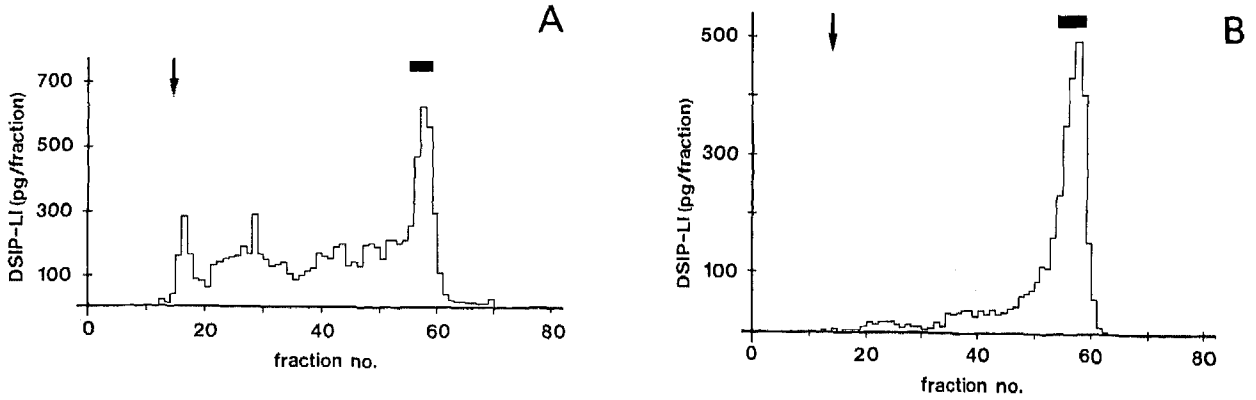

FIG. 3. Gel chromatography of human CSF (A) and human urine (B) on Sephadex G-100. The column size was 2.5×100 cm and the fraction volume was 9 ml. The arrow indicates the void volume of the column and the black bar the position where standard DSIP eluted.

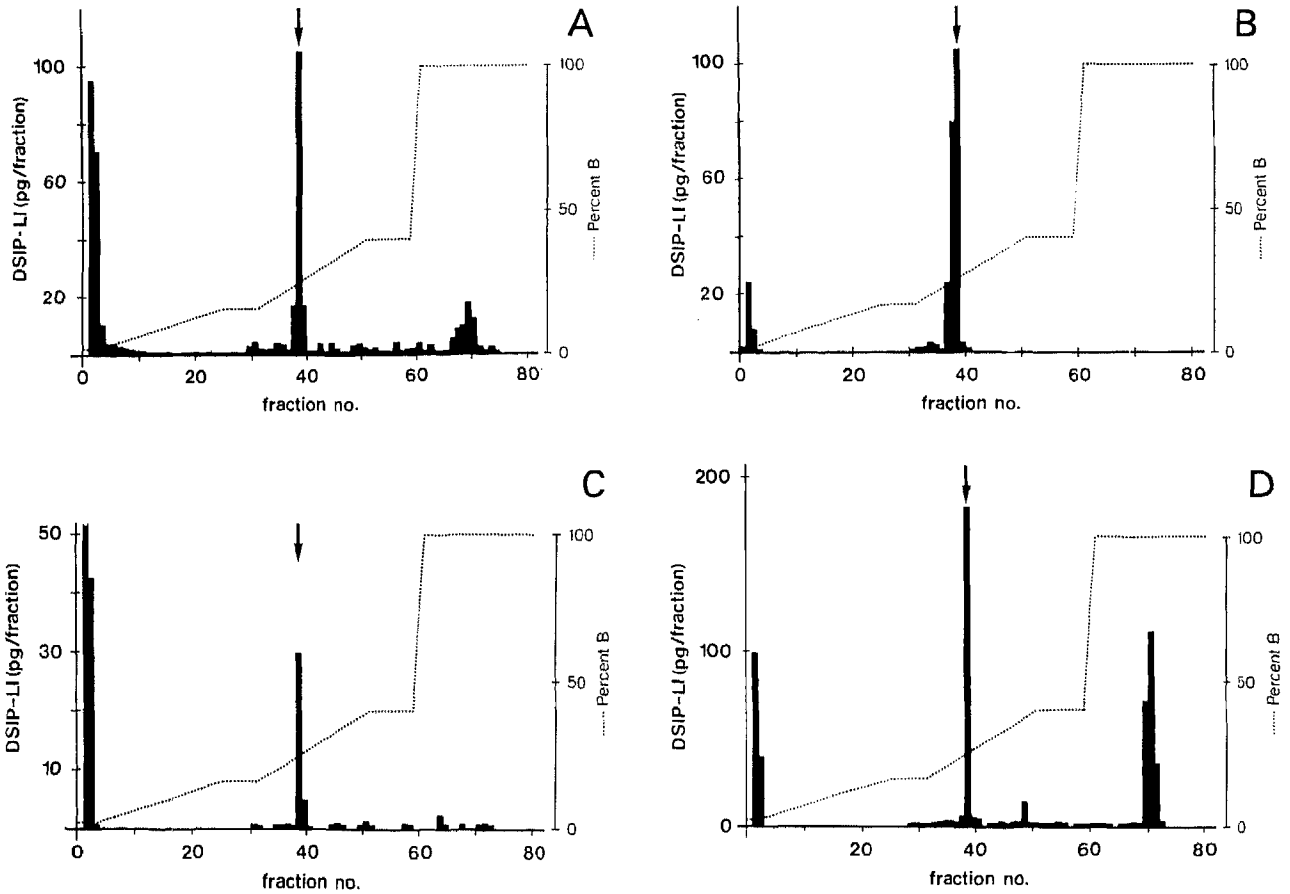

FIG. 4. HPLC of small molecular weight DSIP-LI of human plasma (A), rabbit plasma (B), rat plasma (C), and dog plasma (D). The arrow indicates the position where synthetic DSIP eluted. Fraction volume was 0.75 ml. The gradient is depicted by the broken line.

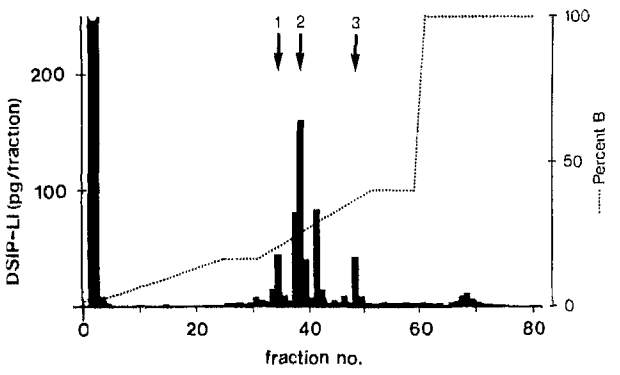

FIG. 5. HPLC of small molecular weight DSIP-LI of human CSF. The arrows indicate the position where standard DSIP-P (1), DSIP (2), and N-Tyr-DSIP (3) eluted.

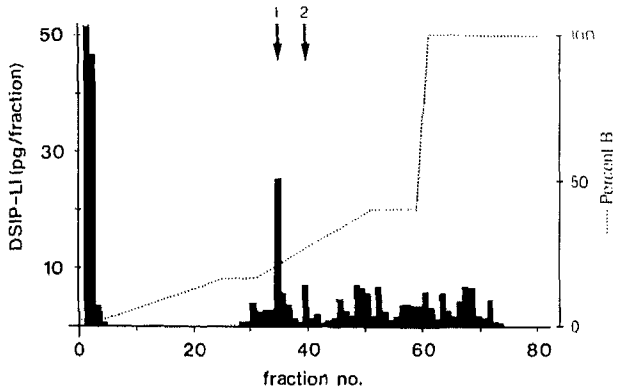

FIG. 6. HPLC of small molecular weight DSIP-LI of human urine. The arrows indicate the position where standard DSIP-P (1) and DSIP (2) eluted.

DISCUSSION

The development of a new antibody (Ab 607) enabled us to readily demonstrate the existence of apparently free DSIP in body fluids. A shorter incubation time (24 hr) as compared to the earlier [12] published procedure (5 days) together with the higher specificity and sensitivity were major advantages of this new antibody.

The cross-reactivities of the DSIP analogs showed an unusual pattern. Antigen recognition by the antibody seems to be sensitive to changes at both the C-and N-terminus (8% cross-reactivity with D-Ala^4-DSIP-NH_2 and <0.5% cross-reactivity with des-Trp-DSIP), but variations in position 4 (D-Ala^4-DSIP) or 7 (phosphorylated serine) do not decrease immunoreactivity. The observation that D-Ala^3-DSIP lacks significant cross-reactivity with Ab 607 may provide insight into the conformational requirements for the antigen-antibody interaction.

Based on detailed studies of protein folding, it has been shown [2, 3, 17] that glycine residues frequently occur at the corner positions of β-turns. When both corner residues are glycine, no particular type of turn is favored. If one of the glycines is replaced by D-alanine, steric considerations indicate that a type II' β turn is favored when the D-residue is placed in the first corner position, as in D-Ala^3-DSIP, while a type II β turn is favored when such a residue is placed in the second corner position, as in D-Ala^4-DSIP. Thus, if a type II β turn conformation for the Ala-Gly-Gly-Asp sequence in DSIP is required for antibody recognition, the observed cross-reactivities of Ab 607 are explained. In addition, this conformation brings the C and N terminal residues into a spatial arrangement suitable for interaction with the antigen-combining site of the antibody. These arguments are compatible with a recent report that DSIP may have a folded conformation in solution [14].

The levels of DSIP-LI measured in the present study are considerably lower than those reported previously [1,12] using a different antibody (Ab 604). This observation as well as the relative specificity of Ab 607 for "free" DSIP are readily explained if the conformational determinants recognized by Ab 607 are not available for binding in other forms of DSIP (bound or aggregated).

Another possible factor that may affect the level of DSIP in plasma is the time of year. Seasonal changes in DSIP-LI levels have been found in hibernating squirrels [13] as well as deer and bear (Morley and Kastin, unpublished observations). We also have found indications for such changes in rat, rabbit, and human plasma. Diurnal variation of the levels of DSIP-LI in plasma [7,12] were taken into account by sampling at the same time of day.

Addition of DSIP to plasma of the rat resulted in recovery of only about 75%, whereas in the other species 100% was found. It was earlier observed [12] that addition of the peptide to mouse plasma also resulted in a recovery of about 70%. It is not known if this decreased recovery is due to a binding phenomenon [1,12], aggregation, or rapid degradation. The last possibility seems unlikely since no rapid changes in endogenous DSIP levels were observed over the time-course of the recovery experiments.

Gel chromatography of the plasma samples on Sephadex G-100 produced elution patterns of DSIP-LI that differed from earlier published results. These reports showed either no small molecular weight material eluting at the position of free DSIP [5] or an amount much smaller than the peak of large molecular weight material eluting at the void volume [12]. In the present study, we found the peak representing small molecular weight DSIP-LI to be much more prominent than observed previously. This peak represented a major component of the total immunoreactivity in plasma, in contrast to the study of Ekman *et al.*[5] in which no DSIP-LI was found to elute with synthetic DSIP. These discrepancies could be due to the different antibodies used. Ab 607 seems to recognize mostly the freely occurring nonapeptide.

In human urine and CSF, only a small amount of large molecular weight DSIP-LI was found by gel chromatography. The larger material may not be freely filtered by the kidney and may not readily pass into the CSF. This finding is consistent with other studies in which smaller amounts of large DSIP-LI were found in CSF than in plasma, the mean levels of DSIP-LI in plasma and CSF also being different by a factor of about ten [1].

HPLC of all samples of the small molecular weight DSIP-LI showed a distinct peak that emerged at the elution position of DSIP. This is consistent with the occurrence of free DSIP, although the possibility exists that an aggregated form of DSIP was dissociated by the chromatographic conditions. The CSF sample also revealed the presence of material that eluted in the position of DSIP-P and N-Tyr-DSIP; a fourth unknown peak also appeared close to the DSIP analogs. In urine, most of the DSIP-LI seemed to occur as DSIP-P; it is not known if this peptide is formed during excretion or if it is more readily excreted by the kidney. Large amounts of DSIP-LI have been found in that organ [9].

In all HPLC runs, the peak eluting first may be due to nonspecific interference from substances like salt, but a DSIP-like compound cannot be completely excluded. An additional peak eluting between fractions 67 and 70 was found in human and dog plasma. This compound could be a protein containing the DSIP sequence or it could be DSIP noncovalently associated with another molecule.

Since the isolation of DSIP several years ago [15,16], questions have been raised not only about its physiologic function but also about its actual existence [5]. It was possible that the nonapeptide represented only a fragment of a larger molecule broken down during the initial isolation procedure. The early attempts at characterization of DSIP-LI suggested that this peptide-sequence existed in brain [11] and plasma [12]. Similar results were also found with human breast milk [10]. Tryptic degradation of the large molecular weight DSIP-LI produced large amounts of DSIP and, to a lesser extent, DSIP-P [10]. Immunohistochemical studies have also detected DSIP-LI in the brain of rats [4,5]. Although the present work demonstrates more conclusively that DSIP occurs in mammalian plasma and CSF, it has not been established whether this nonapeptide represents the original, full sequence of the naturally active compound.

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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. *Pharmacol Biochem Behav* **17**: 1009–1014, 1982.
2. Chandrasekaran, R., A. V. Lakshminarayanan, U. V. Pandya and G. N. Ramachandran. Conformation of the LL and LD hairpin bends with internal hydrogen bonds in proteins and peptides. *Biochem Biophys Acta* **303**: 14–27, 1973.
3. Chou, P. Y. and G. D. Fasman. β-Turns in Proteins. *J Mol Biol* **115**: 135–175, 1977.
4. Constantinidis, J., C. Bouras, R. Guntern, C. H. Taban and R. Tissot. Delta-sleep-inducing peptide in the rat brain: an immunohistochemical microscopic study. *Neuropsychobiology* **10**: 94–100, 1983.
5. Ekman, R., I. Larson, M. Malmquist and J. I. Thorell. Radioimmunoassay of delta sleep-inducing peptide using an iodinated p-hydroxphenylpropionic acid derivative as tracer. *Regul Pept* **6**: 371–378, 1983.
6. Feldman, S. C. and A. J. Kastin. Localization of neurons containing immunoreactive delta-sleep-inducing peptide in the rat brain: an immunocytochemical study. *Neuroscience* **11**: 303–317, 1984.
7. Fischman, A. J., A. J. Kastin and M. V. Graf. Circadian variation of DSIP-like material in rat plasma. *Life Sci*, in press.
8. Graf, M. V. and A. J. Kastin. Delta-sleep-inducing peptide (DSIP): A review. *Neurosci Biobehav Rev* **8**: 83–93, 1984.
9. Graf, M. V. and A. J. Kastin. DSIP (a nonapeptide) exists in peripheral organs of rats in large dissociable forms. *Proc Soc Exp Biol Med*, in press.
10. Graf, M. V., C. A. Hunter and A. J. Kastin. Presence of DSIP-like material in human milk. *J Clin Endocrinol Metab* **59**: 127–132, 1984.
11. Kastin, A. J., C. Nissen, A. V. Schally and D. H. Coy. Radioimmunoassay of DSIP-like material in rat brain. *Brain Res Bull* **3**: 691–695, 1978.
12. Kastin, A. J., P. F. Castellanos, W. A. Banks and D. H. Coy. Radioimmunoassay of DSIP-like material in human blood: Possible protein binding. *Pharmacol Biochem Behav* **15**: 969–974, 1981.
13. Kramarova, L. I., S. H. Kolaeva, R. Y. Yukhananov and V. V. Rozhanets. Content of DSIP, enkephalins and ACTH in some tissues of active and hibernating ground squirrels (Citellus suslicus). *Comp Biochem Physiol* **74C**: 31–33, 1983.
14. Mikhaleva, I., A. Sargsyan, T. Balashova and V. Ivanov. Synthesis, spectral and biological properties of DSIP and its analogs. In: *Chemistry of Peptides and Proteins*, vol 1, edited by W. Voelter, E. Wuensch, J. Ovchinnikov and V. Ivanov. Berlin: de Gruyter and Co., 1982, pp. 289–297.
15. Schoenenberger, G. A., P. F. Maier, H. J. Tobler and M. Monnier. A naturally occurring delta-EEG enhancing nonapeptide in rabbits. X. Final isolation, characterization and activity test. *Pflugers Arch* **369**: 99–109, 1977.
16. Schoenenberger, G. A., P. F. Maier, H. J. Tobler, K. Wilson and M. Monnier. The delta-EEG (sleep)-inducing peptide (DSIP). XI. Amino acid analysis, sequence, synthesis and activity of the nonapeptide. *Pflugers Arch* **376**: 119–129, 1978.
17. Venkatachalam, C. M. Stereochemical criteria for polypeptides and proteins. V. Conformation of a system of three linked peptide units. *Biopolymers* **6**: 1425–1436, 1968.