Pharmacology Biochemistry & Behavior, Vol. 15, pp. 969–974, 1981. Printed in the U.S.A.

Radioimmunoassay of DSIP-Like Material in Human Blood: Possible Protein Binding

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Received 30 September 1981

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(6) 969–974, 1981.—A radioimmunoassay (RIA) for DSIP-like material was established in unextracted human plasma. Most of the immunoreactivity was found in a "large" fraction while a much smaller amount co-eluted with DSIP from Sephadex as a "free" fraction. Both peaks progressively increased with increasing amounts of added DSIP. Acidification, but not treatment with charcoal-dextran, resulted in a relative decrease in the "large" peak and an increase in the "free" peak. This RIA, therefore, appears to measure both bound and free forms of DSIP-like immunoreactivity, the levels of which were higher at 4 p.m. than at 8 a.m.

Humans Dog Radioimmunoassay Peptide Sleep Protein Binding Circadian

When an antibody to delta sleep-inducing peptide (DSIP) that required essentially the entire nonapeptide for recognition was generated [1,2], we initially focused our efforts on the use of the resulting radioimmunoassay (RIA) as a tool for studying passage of peptides across the blood-brain barrier (BBB). In the meantime, the role of DSIP [6] in sleep or other functions still has not been fully established [3,5].

The present report describes the presence in blood of DSIP-like material, most of which appears to be associated with a large protein. The RIA used for detection of DSIP-like-immunoreactivity there should facilitate investigations of the functions of DSIP.

I. CHARACTERIZATION OF RIA

Method

The RIA used to measure DSIP-like immunoreactivity in blood was essentially the same as that previously described for brain tissue [2]. The cross-reactivities of the antibody have been published elsewhere [1]; besides DSIP, it recognizes only [des-Trp^1]-DSIP (=DSIP 2–9) among the peptide fragments of DSIP. Briefly, [N-Tyr]-DSIP is iodinated with chloramine-T and purified by partition chromatography on a column of Sephadex G-25 (fine) with butanol-acetic acid-water 4:1:5 (upper phase). Purity is checked by chromatoelectrophoresis. About 9,500 cpm of the purified labeled peptide is added to test material or DSIP standard (doubling dilutions from 8–512 pg, Fig. 1), buffer (Tris HCl with 0.1% human serum albumin and 4% Trasylol), and antibody (1:1000 initial dilution, 1:5,000 final dilution) and incubated at 4°C. On the fifth day, 0.5 ml of 1% charcoal:0.1% dextran is added to each tube (except that for total count). After incubation for 30 min at 4°C and centrifugation, the supernatant is counted in a gamma counter.

DSIP and its analogs were synthesized by solid-phase methods. Recovery of DSIP added to plasma (about 0.6 ng/ml) was determined by RIA as follows. The level of immunoassayable DSIP in untreated plasma from the dog, rat, mouse, and man was subtracted from the level of this material in plasma to which the DSIP had been added. The difference between the treated and untreated samples was expressed as a percentage of the same amount of peptide added to diluent. The coefficient of variation represents the standard deviation of the variates as a percentage of their mean.

Results

The percent recovery of DSIP added to unextracted plasma was 69% in the mouse and 100% in the dog, rat, and human being. The basal levels were 0.93 ± 0.02 ng/ml in the dog, 2.30 ± 0.15 ng/ml in the mouse, 5.01 ± 0.20 ng/ml in the rat, and 2.61 ± 0.09 ng/ml in the human being. The contribution of varying times of storage of these plasma samples to the relative differences in immunoreactivity was not ascertained. Collection of samples of human blood in Trasylol and EDTA or the later addition of Trasylol or Bestatin did not appear to increase basal values.

The coefficient of variation within assays was 8% and between assays was 13%. No breakdown of labeled peptide occurred during the assay. The maximal non-specific binding (NSB) of the usual amount of plasma (12.5 μl) in the RIA was 2% of the total counts as compared to a range of 1.0–1.5% in buffer. This reduces the likelihood of any substantial interference at this concentration of plasma by an endogenous binding system with the RIA.

The dilution curve for unextracted human plasma was parallel to that of the standard curve of DSIP (Fig. 1). Addition of the same amounts of plasma to the same doses of DSIP shifted the curve to the left, as expected from the displacement of labeled standard by endogenous DSIP. Plasma from the dog was consistently parallel to the standard curve, but that from the mouse or rat tended to have steeper slopes.

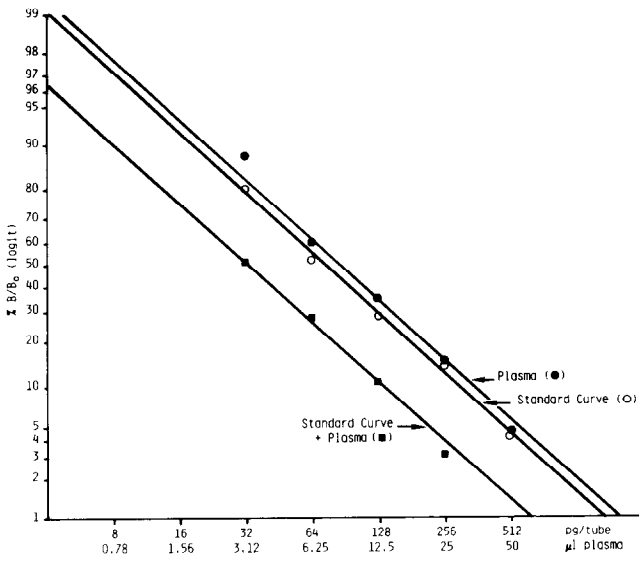

FIG. 1. Parallel dose-response curves of unextracted human plasma, DSIP standard curve, and standard curve+plasma.

II. THE EFFECTS OF CHARCOAL-DEXTRAN AND HEATING ON IMMUNOASSAYABLE DSIP IN HIMAN PLASMA

Method

Plasma was obtained from human volunteers (never treated with DSIP) by collecting venous blood in chilled heparinized tubes. After centrifugation, the plasma was divided into 3 equal parts by volume. The first part was heated at 60°C for 16 hr, the second part received a large excess of the charcoal-dextran mixture (8%:0.8%), and the third part was untreated and kept at 4°C as a control. Samples were then lyophilized and separated into halves by weight. One half of each of the 3 plasma samples was resuspended in buffer alone and half in buffer containing equal amounts of DSIP.

Results

A large excess (8%:0.8%) of charcoal-dextran slightly reduced the basal levels of DSIP-like immunoreactivity in samples of lyophilized plasma equalized by weight from 3.88±0.22 ng/100 mg to 3.27±0.15 ng/100 mg. In preliminary experiments, however, amounts of charcoal-dextran between 1%:0.1% and 8%:0.8% caused essentially no reduction. Heating at 60°C for 16 hr greatly increased basal levels from 3.88±0.22 ng/100 mg to 6.44±0.29 ng/100 mg as was also found in 2 other preliminary experiments. Extraction of the heated plasma sample with charcoal-dextran (1%:0.1%) for 30 min only reduced the immunoreactivity to 5.49±0.07 ng/100 mg. Addition of DSIP increased immunoassayable levels in each of the 3 plasma samples: untreated plasma, plasma extracted with excess charcoal-dextran (8%:0.8%), and plasma heated for 16 hr at 60°C. Added DSIP was completely removed from diluent by this concentration of charcoal-dextran as well as by the usual (1%:0.1%) concentration.

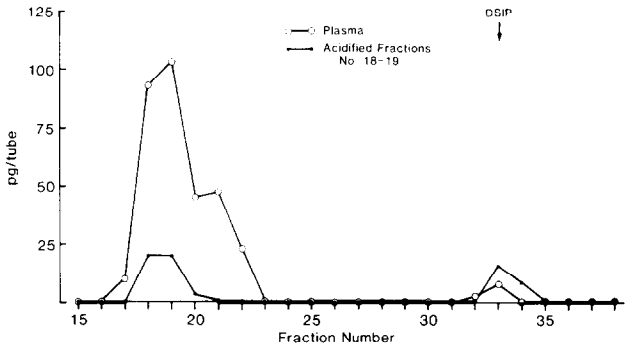

FIG. 2. Elution pattern of immunoreactivity in untreated human plasma (open circles) and acidified fraction numbers 18–19 from the "large" peak (closed circles).

III. EFFECTS OF ACIDIFICATION ON BINDING

Method

Heparinized human blood was obtained by venipuncture for these 2 experiments. Unlabeled DSIP was added to 2 ml of plasma (first experiment, Fig. 2) or [^{125}I-N-Tyr]-DSIP was added to 0.5 ml of plasma (second experiment, Fig. 3). These were incubated at room temperature for 10 min and chromatographed in 0.02 M acetic acid on a column (1×120 cm) of Sephadex G-15 (first experiment, Fig. 2) or Sephadex G-25 (1×20 cm) (second experiment, Fig. 3) and 2 ml fractions collected. Peak activity was determined by RIA (first experiment, Fig. 2) or by counts in a gamma counter (second experiment, Fig. 3). The peaks nearest the void volume (fraction number 5 for cpm, fraction numbers 18–19 for RIA) were acidified with HCl to pH 3.0 and re-chromatographed on the original columns of Sephadex G-15 (Fig. 2) or G-25 (Fig. 3).

Results

Figure 2 shows the elution pattern on a column (1×120 cm) of Sephadex G-15 of plasma as measured by RIA before acidification. The immunoreactivity eluted as a high molecular weight ("large") peak (fractions number 18 and 19) and a smaller molecular weight peak co-eluting with DSIP (fractions number 32 and 33). Acidification of the "large" peak resulted in its reduction, with the reappearance of immunoreactivity co-eluting with DSIP.

Separation of plasma incubated with [^{125}I-N-Tyr]-DSIP by chromatography on a column of Sephadex G-25 resulted in 3 peaks (Fig. 3). One of these peaks co-eluted with the labeled peptide (Peak B) while an apparently "large" fraction (Peak A) appeared soon after the void volume in fraction number 5. An unidentified peak C eluted after peaks A and B and probably represents a degradation product. Plasma to which Trasylol was added before the labeled DSIP showed the same pattern after gel filtration; this tends to reduce the likelihood of a substantial influence of enzymatic damage in binding. Each of the 3 peaks was then acidified and re-chromatographed on Sephadex G-25. Peaks B and C again appeared as single peaks, although slightly shifted to the right (not shown). Gel filtration of the acidified "large" peak

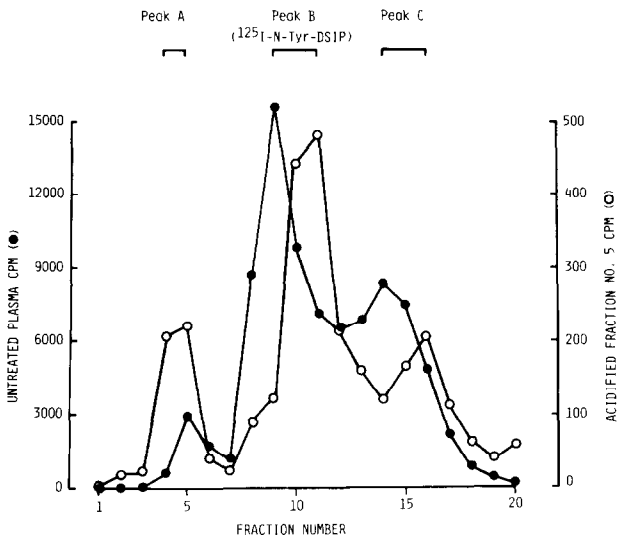

FIG. 3. Elution pattern of radioactivity after addition of [125I-N-Tyr]-DSIP to human plasma and acidified fraction number 5 from the "large" peak. The elution pattern after acidification is represented by a scale (right ordinate) 30 times smaller than that before acidification (left ordinate).

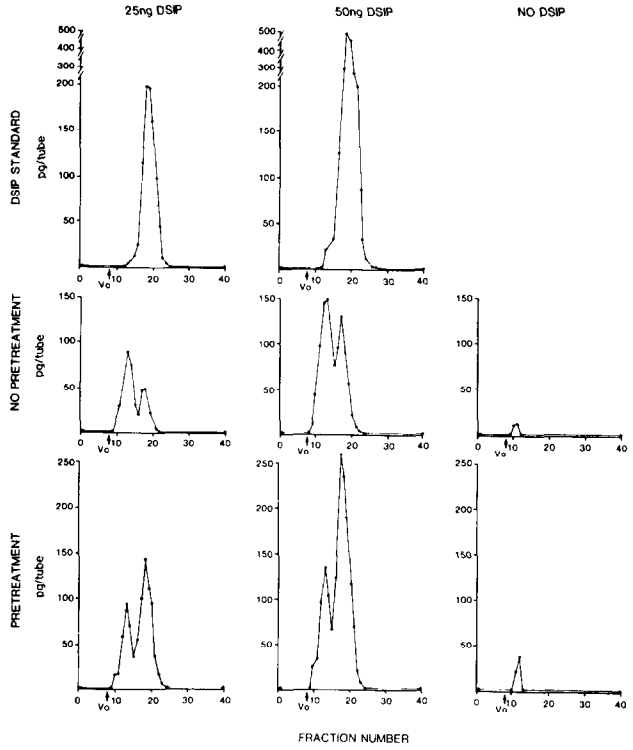

FIG. 4. Elution pattern of 2 doses of DSIP standard without plasma (top panels) and after addition to the "large" peak from untreated human plasma (middle panels) and human plasma pretreated (bottom panels) by incubation with DSIP peptides.

(A), however, resulted in the reappearance of peaks B and C as well as peak A (Fig. 3).

IV. EFFECTS OF ADDITION OF DSIP TO "LARGE" PEAK OF IMMUNOASSAYABLE DSIP

Method

In this experiment (Fig. 4), binding was examined with the unlabeled peptide. Human plasma was divided into 2 parts each of 4 ml. One part was rotated ("pretreated") for 12 hr at 4°C with 100 ng of each of 3 peptides known to cross-react in the RIA (DSIP, [des-Trp1]-DSIP, and [N-Tyr]-DSIP). The other part was mixed with an equal volume of the diluent (0.02 M acetic acid). Each part was added in 0.02 M acetic acid to a column (2.5×60 cm) of Sephadex G-25 (fine), and 4 ml fractions were collected. The first peak of DSIP-like immunoreactivity from each separation was lyophilized, resuspended in 0.02 M acetic acid, and then divided into halves to which was added 25 or 50 ng of DSIP standard. The resulting 4 samples from the first peaks were rotated for 12 hr at 4°C and then chromatographed on a column (1×60 cm) of Sephadex G-15 (fine). The resulting 2 ml fractions were measured by RIA.

Results

The addition of DSIP to plasma from which all but the "large" fraction had been removed by gel filtration on Sephadex G-25 produced more than one peak of immunoreactivity. This is shown in Fig. 4 and similar changes (not shown) were found after addition of DSIP to plasma not previously subjected to gel filtration (50% increase in both the "large" and free fractions after 5 ng DSIP). The panels on the right show the DSIP-like immunoreactivity in the "large" peak (fractions 9–12) obtained from a relatively small amount of plasma after chromatography on Sephadex G-25. Pretreatment with the immunoreactive DSIP peptides substantially increased this peak. The separation patterns of 25 and 50 ng of the DSIP standard without plasma are shown in the top panels. Addition of these amounts of DSIP eluting between fractions 15–20 to the "large" material eluting between fractions 9–12 resulted in the pattern of double peaks seen in the remaining 4 panels. Increases in immunoreactivity were seen in the position of the "large" material as well as in the position of the DSIP standard ("free" DSIP). Addition of 50 ng of DSIP increased each of the peaks substantially more than did addition of 25 ng DSIP. In the samples of plasma incubated with DSIP peptides before gel filtration, however, the relative proportion of the "large" material to the free DSIP was less.

V. EFFECTS OF SEVERAL VARIABLES ON BINDING OF LABELED PEPTIDE

Method

A sample of plasma and [125I-N-Tyr]-DSIP was incubated with charcoal-dextran (1%:0.1%), centrifuged, and the supernatant chromatographed on a column of Sephadex G-25 as in Fig. 3. The percentage of counts in each of the three main peaks was compared before and after treatment

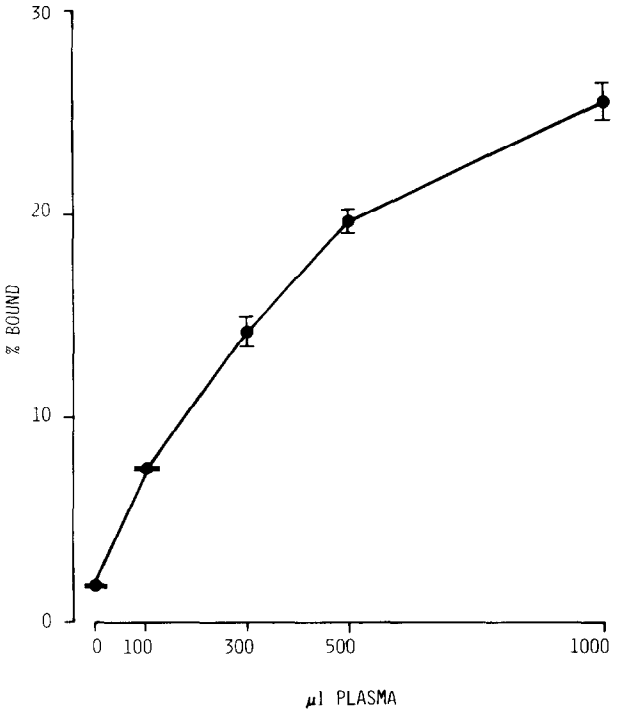

FIG. 5. Effect of increasing amounts of plasma on the percent binding of [^{125}I-N-Tyr]-DSIP as determined by extraction with charcoal-dextran.

with charcoal-dextran. The effects of increasing concentrations of plasma were determined by adding up to 1000 μl of plasma to the incubation mixture containing [^{125}I-N-Tyr]-DSIP and Tris-HCl buffer (Fig. 5). Binding was also examined at 4°C, 25°C, and 37°C and at different pH's between 5.8 and 9.0. At room temperature, the percent binding was determined at 10 min, 1 hr, 16 hr, and 24 hr. Binding was expressed by the following ratio: cpm in the supernatant/(cpm in the supernatant+cpm in the charcoal-dextran residue).

Results

Addition of charcoal-dextran (1%:0.1%) to untreated plasma containing radioactively labeled peptide followed by gel filtration of the supernatant resulted in a 96% decrease in peak C and a 73% decrease in peak B. Only a 19% decrease was found in the "large" peak A. This technique for separating free from bound peptide with charcoal-dextran was used to investigate several variables. Binding of labeled peptide increased with increasing amounts of plasma (Fig. 5). In 0.5 ml of the fresh plasma, the percent labeled peptide binding at pH 7.8 (8.5%) was greater than at the other pH's examined (3.1% at pH 5.8, 4.2% at 6.6, 5.0% at 7.0, 6.8% at 7.5, 7.8% at 8.0, 6.4% at 8.5, and 5.7% at 9.0). More binding occurred at 25°C (11.1%) than at 37°C (7.6%) or 4°C (6.8%). At room temperature, the percent of labeled peptide bound was more at 1 hr (13.6%) than at 10 min (4.4%), 16 hr (10.0%), or 24 hr (8.2%). In general, the percent binding of the [^{125}I-N-Tyr]-DSIP measured by cpm was much lower than that of DSIP measured by RIA.

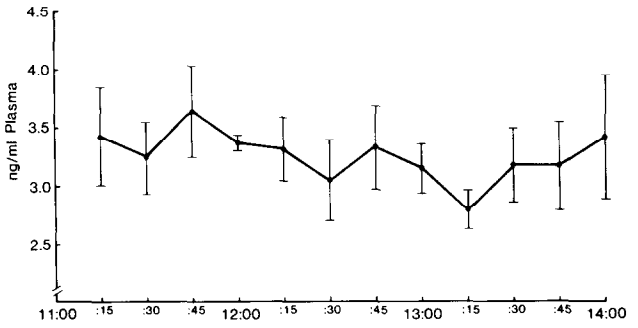

FIG. 6. Levels of DSIP-like immunoreactivity in plasma obtained from healthy human beings every 15 min for a few hr. Lunch was eaten immediately after the sample obtained at noon.

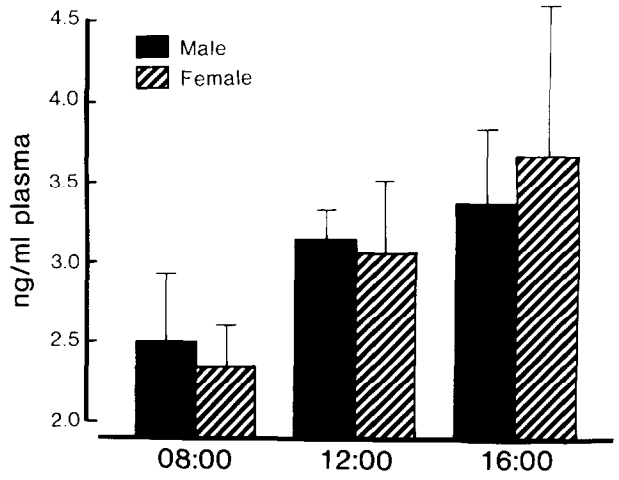

FIG. 7. Levels of DSIP-like immunoreactivity in plasma obtained from healthy human beings at 8 AM, noon, and 4 PM.

VI. LEVELS OF DSIP-LIKE IMMUNOREACTIVITY IN HUMAN PLASMA UNDER SEVERAL CONDITIONS

Method

Human plasma was obtained from healthy volunteers and hospitalized patients under a variety of conditions. To check for pulsatile release of DSIP, blood was taken every 15 min for several hours from 3 normal subjects, in 1 of whom the procedure was repeated on 2 more occasions, each separated by at least a week. The mean of all 5 samples was used for Fig. 6; lunch was eaten between 1200 and 1215. Blood was also taken from 10 normal subjects (5 male, 5 female) with a mean age of 33.1±2.5 years at 0800, 1200, and 1600. The effects of insulin hypoglycemia together with thyrotropin releasing hormone (TRH) were examined in 1 patient and TRH alone in another patient.

Results

Plasma taken every 15 min revealed little evidence for a

pulsatile pattern of DSIP release (Fig. 6). The mean (±SEM) plasma DSIP-like immunoreactivity for these samples in the subject tested on 3 occasions (2.98±0.17, 3.23±0.26, and 4.08±0.24 ng/ml) varied more than that of the other two subjects (2.58±0.15 and 3.36±0.22 ng/ml). The levels of DSIP like immunoreactivity obtained in 10 subjects at 0800, 1200, and 1600 are shown in Fig. 7. Duncan's Multiple Range Test showed that the combined samples obtained at 1600 contained significantly more DSIP-like immunoreactivity than the samples taken at 0800 ($p<0.05$); the levels in the sample obtained at 1200 were not significantly different from those at 0800 or 1600. Heating half of each sample at 60°C for 4 hr increased all values but removed the differences between the samples from 0800 (4.27±0.27 ng/ml) and 1600 (4.33±0.22 ng/ml). There were no differences in samples obtained from 21 black male patients (2.70±0.30 ng/ml) and 17 white male patients (2.73±0.42 ng/ml) and no obvious correlation with age. A dose of insulin (0.15 U/kg) sufficient to lower blood sugar to 50 mg/100 ml only increased the levels of DSIP by 10% in one patient, about the same relative increase seen after TRH in this and another patient.

Several chains of human hemoglobin contain 4–5 amino acids in common with DSIP, although only 3 of the shared amino acids are sequential (National Biomedical Research Foundation, Washington, D.C.). Even though our antibody does not recognize a sequence this small, an attempt was made to correlate hemoglobin levels obtained from 17 patients on admission with plasma levels of DSIP-like immunoreactivity. Instead of a positive correlation, a negative correlation ($r=-0.56$) was found, indicating higher peptide levels with lower hemoglobin levels. No protein sequences were found in the computer search that contained 7, 8, or all 9 amino acids of DSIP. Two substances containing 6 of the 9 amino acids are known, but one of these exists in a plant and the other in a bacterium and in neither is the hexapeptide sequential.

DISCUSSION

Human plasma contains DSIP-like immunoreactivity that dilutes parallel to DSIP (Fig. 1). Essentially all the DSIP added to the plasma can be measured by the RIA. The immunoreactivity in plasma with (Fig. 4) or without (Fig. 2) added DSIP appears mainly in two peaks, the smaller of which co-elutes with DSIP and probably represents free (unbound) DSIP. Evidence that the other peak may represent DSIP bound to a large protein is discussed in the remainder of the paper.

Since charcoal-dextran removes free DSIP, the large amount of DSIP-like immunoreactivity found to remain in human plasma extracted with charcoal-dextran indicates the presence of DSIP in another form. Conversely, the relatively small amount of DSIP-like immunoreactivity removed from plasma by charcoal-dextran indicates that relatively little DSIP circulates in the free form.

Although the endogenous DSIP-like activity in the "large" peak could represent a precursor or pro-hormone containing the DSIP sequence, addition of DSIP to plasma caused an increase in this peak (Fig. 4). Doubling the dose of added DSIP increased the apparent binding of DSIP by the "large" fraction even more. After pre-incubation of the "large" fraction with DSIP peptides, however, the added DSIP resulted in a relatively smaller increase in immunoreactivity in the "large" fraction and a greater increase in the free fraction (Fig. 4). This might be expected if the binding sites on the "large" fraction, or even some proteolytic enzymes, had been partially saturated during pretreatment by immunoreactive peptides or their non-immunoreactive fragments. Consequently, a relatively large proportion of the added peptide would appear in the free fraction that co-eluted with DSIP. These results are consistent with protein binding of much of the added DSIP.

The appearance of radioactivity in the "large" fraction after incubation with [^{125}I-N-Tyr]-DSIP in plasma (Fig. 3) also suggests binding to a large protein. Acidification not only reduced this radioactive peak, as expected, but also produced a peak co-eluting with the labeled peptide, a finding compatible with binding. A peptide cleaved from its precursor protein would not be radioactive. Charcoal-dextran removed most of the radioactivity from the later peaks, but only removed a small amount of peptide from the "large" peak. Thus, this seems to be a useful technique for separating bound from free labeled DSIP. The binding appeared to be dependent upon pH, time, temperature, and concentration of plasma (Fig. 5).

The percent binding measured by cpm in experiments involving [^{125}I-N-Tyr]-DSIP was much less than the percent binding measured by RIA in experiments involving DSIP. Although DSIP may simply bind more strongly than the larger decapeptide, it is also likely that this difference reflects interference by the iodine with binding similar to the decreased biological activity frequently seen with other iodinated peptides. Other explanations include the possibility that some DSIP is degraded to fragments that also may bind to the protein. This could account for the decreased binding of the iodinated peptide since many of the fragments that might be formed would not contain the radioactive label.

All of the above results are best explained by binding of DSIP to a carrier protein. Other processes, however, may also be involved. This is suggested by the finding that plasma heated for 16 hr at 60°C contained much higher levels of DSIP-like immunoreactivity than the controls. Free DSIP liberated by heat from its carrier or precursor protein should have been removed by charcoal-dextran. Since the RIA measures both bound and free peptides, alteration of the relative proportion of bound and free DSIP should not have changed the total amount of immunoreactivity unless some of it had been bound in sites inaccessible to the antibody before heating. It is also possible that a precursor or pro-hormone for DSIP could contain material that was not recognized by the antibody until it was split by the heat into shorter segments, some of which may have been too large to be removed by the charcoal-dextran mixture. The precursor could elute in the same "large" fraction on Sephadex as the protein that apparently binds DSIP.

The heat also could have inactivated an enzyme involved in the metabolism of DSIP, destroyed a large molecule that was interfering in a nonspecific way with the RIA, or increased the immunogenicity of the nonapeptide. Although the excellent recovery of added DSIP tends to make these possibilities less likely, it is known that the DSIP nonapeptide is susceptible to the action of proteolytic enzymes [4]. Regardless, it seems clear that DSIP can circulate in more than one form in blood.

The results suggest that in human plasma most DSIP, like the thyroid hormones, is probably bound to a large protein. Our RIA appears to be able to measure both free and bound forms of DSIP without extraction. This total DSIP-like immunoreactivity increased between 0800 and 1600 (Fig. 7) in a

pattern that cannot be readily explained by differences in pulsatile release (Fig. 6). The implications of the possible protein binding for penetration of the BBB and physiological function(s) of DSIP are not known.

ACKNOWLEDGEMENTS

The authors thank Drs. J. Zadina and R. Moldow for review of the manuscript, M. Reardon for preparation of the manuscript, and N. Kunen and A. Arnold for the art work. The study was supported by the Veterans Administration and NIH.

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