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Delta Sleep-Inducing Peptide (DSIP)-like Material Exists in Peripheral Organs of Rats in Large Dissociable Forms (41932)

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Abstract. The presence of delta sleep-inducing peptide (DSIP) in brain has been shown by radioimmunoassay (RIA) and by immunocytochemistry. We now describe the occurrence of DSIP-like material in the peripheral organs of the rat as measured by RIA. Tissue from 12 areas was extracted with water, and the amounts of immunoreactive material found to be between 86 pg/mg tissue (muscle) and 849 pg/mg (stomach). Recoveries of about 80% of added DSIP were achieved at tissue concentrations of 1 mg/ml or less. This percentage was reduced in liver at higher concentrations. The percentage of small peptide adsorbed by charcoal was greatly increased at lower tissue concentrations in all organs. This effect was significant and linear. Chromatography on columns of Sephadex G-15 and G-25 showed immunoreactive material mostly larger than DSIP. Digestion with trypsin, however, produced small immunoreactive peptides with only a minimal reduction in total immunoreactivity. Thus, DSIP-like material is widespread in peripheral tissues and appears to exist mainly in a large form, probably bound to protein, that can be reduced in size by tryptic digestion and can be dissociated at lower concentrations of tissue to yield small immunoreactive peptides.

Since the isolation and characterization of the delta sleep-inducing peptide (DSIP) (1), several papers have dealt with its potential activities, especially on sleep (2–8). Nevertheless, no clear answer yet has been obtained whether any of these actions constitutes the main effect of the peptide. Additional difficulties involve determination of a dose of DSIP that is active in all situations and of an optimal time for administration of DSIP to induce sleep in human beings (9).

In 1978, DSIP-like material was detected by radioimmunoassay (RIA) in rat brain (10). Later, similar material was found by an RIA in plasma of several species (11), but most of the material seemed to be contained in a larger molecular form than DSIP alone. Recently DSIP-like material was observed in different regions of the rat brain by immunocytochemistry (12, 13). By radioimmunoassay, DSIP-like immunoreactivity was found in peripheral organs of the rat (10), but no further characterization was performed.

In this paper, we describe the distribution in the rat of DSIP-like material in 12 peripheral organs extracted with water instead of the acetic acid used previously (10). As in plasma, most of the immunoreactive DSIP-like material seemed to occur as larger molecules. Therefore, further attempts were undertaken to determine whether the sequence of the nonapeptide is an intrinsic part of a large protein or is bound to larger molecules. By preadsorbing the free peptide with charcoal, we have shown that a binding process may be involved.

Materials and Methods. The antibody used was the same as in previous work (10). DSIP was prepared by solid-phase synthesis and generously provided by David H. Coy. Trypsin (16,950 U/mg) was purchased from Sigma Chemical Company, St. Louis, Missouri.

Male rats weighing about 150 g were obtained from Blue Spruce (Altamont, N.Y.) and kept on a regular light–dark schedule (lights on 0600–1800 hr) for a week before they were decapitated between 1000 and 1200 hr. The organs were quickly removed and placed on ice, rinsed with chilled saline, blotted, and frozen on dry ice. They were stored at −80°C and assayed within a week (unless otherwise indicated). The RIA followed in principle the same schedule published earlier (10). Instead of extraction with acid, the organs were extracted by homoge-

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nization (Brinkman Polytron, setting 6, 10 sec) in water containing 4% Trasylol. The homogenates were centrifuged (4000*g*, 40 min) and the supernatants adjusted to the desired concentrations. For comparison, the acid extraction (0.1 *M* acetic acid) was performed in the same way, but the supernatants were lyophilized before resuspension in water–Trasylol and adjustment to the concentrations used in the assay. Unless otherwise indicated, concentrations of DSIP-like material in tissues are given per milligram wet weight and concentrations of tissue homogenates as milligrams per milliliter. The amount of protein in the tissues was determined according to Lowry *et al.* (14).

To determine the influence of storage on the amount of DSIP-like material in frozen tissues, five organs of five rats were cut into several pieces, frozen on dry ice, and stored either at −20 or −80°C. The same organs were used as for the determination of DSIP-like material described above. The levels of the organs stored at −80°C and analyzed within a week were taken as controls (100%) for the subsequent determinations; after 1, 2, 3, and 5 months of storage at −20 or −80°C, the levels of DSIP-like material were measured in the same organs and the values expressed as percentages of controls.

In the recovery experiments, 50 pg DSIP per final assay volume were added to the homogenates. After standing 20 min on ice, the tubes were centrifuged and the supernatants used for the RIA. Samples containing the same tissues and volumes but lacking the synthetic peptide were assayed in parallel. For calculations of recovery, the amount of added DSIP was determined at the same time and taken as 100%.

A possible enzymatic degradation of the radioactive tracer was determined as follows. Tissue samples and standard peptide at the desired concentrations were prepared and incubated for RIA as described above. One, three, and six days after addition of the radioactive tracer, one set of each sample was extracted with charcoal–dextran, centrifuged, and the supernatant counted for radioactivity. Similarly, all samples were incubated without antibody to determine the nonspecific binding. This procedure shows the displacement of the radioactive tracer by synthetic peptide or endogenous DSIP-like material until the point of equilibrium and also any change in the nonspecific binding over time.

In the preadsorption experiments, samples of homogenate were incubated with charcoal–dextran (CD; 1% charcoal–0.1% dextran) for 30 min, centrifuged at 1000*g* for 30 min, and the clear supernatants were used for the assay. Samples of tissue without charcoal were run at the same time as controls. The amount of DSIP-like material found in the controls was taken as 100% and the decrease or increase due to the charcoal adsorption was calculated accordingly.

For gel chromatography with Sephadex, the supernatant of freshly extracted tissues was put on the columns (1 × 60 cm) and eluted with 0.02 *M* acetic acid. Fractions of 1 ml were collected, lyophilized, resuspended in 0.5 ml of H_2O–Trasylol, and assayed for DSIP by RIA.

For the tryptic degradation, 100 mg of an organ were homogenized in 4 ml Tris buffer (0.02 *M*; pH 8.0) and centrifuged for 20 min at 5000*g* at 4°C. Part of the supernatant (0.5 ml) was chromatographed on Sephadex G-25 as described earlier. Another aliquot of the homogenate was incubated with trypsin, 5 mg/0.5 ml, for 40 min at 37°C. The reaction was stopped by placing the mixture on ice and the homogenate centrifuged (15 min, 2500*g*). From the supernatant, 0.5 ml was chromatographed on G-25 and the fractions determined by RIA at the same time as those of the sample not digested with trypsin.

For statistical calculations, analysis of variance was used followed by Duncan's multiple-range test. For the preadsorption experiments, a regression analysis was applied.

Results. Several media were tested for their efficacy in extracting immunoreactive material. These included different concentrations of acetic acid, water, 1% Triton X-100, 2 *M* HCl, 0.1 *M* NaOH, 1 *M* NH_4OH, 30% methanol, and 30% acetone. Some were tested after boiling, but water at 4°C was found to yield the highest levels. At tissue concentrations of 1 mg/ml or more, none of these solvents liberated considerable amounts of small immunoreactive DSIP structures. Rat organs extracted with acetic acid, as pre-

viously described (10), yielded different amounts of DSIP-like material compared to those extracted with water. In general, the values found were 5–10 times higher after water extraction than after acid extraction. Chromatography on Sephadex G-15 revealed that the fraction eluting just after the void volume and, therefore, of higher molecular size than DSIP was increased most after water extraction. The subsequent fractions were increased proportionally.

All the organs with the exception of muscle and stomach yielded similar and parallel dilution curves to the DSIP standard in the assay shown in Fig. 1. In this assay, muscle exhibited a steeper curve than DSIP and stomach had a flatter slope. In other assays, however, stomach (and on rare occasions muscle) produced parallel dilution curves. In the assay shown in Fig. 1, duodenum was not determined, but parallelism for this organ was established in other assays.

The different concentrations of DSIP-like material in 12 rat organs are indicated in Fig. 2. In Fig. 2a, the amounts are given as picograms of DSIP-like material per milligram wet weight tissue and in Fig. 2b as picograms of DSIP-like material per milligram protein. Adrenal and muscle were analyzed at a concentration of 2 mg/ml, whereas all the other organs were assayed at 1 mg/ml, since, in preliminary experiments, the values for adrenal and muscle measured at a concentration of 1 mg/ml were below the required range. In the later assays, a doubling of the concentration did not change the amount per milligram tissue in the adrenal and reduced it approximately 10% in muscle. Additionally, heart, testis, and lung were assayed in several samples and found to contain DSIP-like material as well. The concentrations in these tissues were less than in any of the organs listed in Fig. 2 except muscle. Even in fat, small amounts of DSIP-like material were found. A similar small value was detected in placenta, being slightly higher than in blood (15). The nonspecific binding at these concentrations was less than 1% of the total radioactivity. The coefficient of variation was 2.6% within assays and 8.9% between assays.

The difference in levels of DSIP-like immunoreactivity among the various organs was highly significant when expressed per milligram tissue ($F(11,99) = 36.32$, $P < 0.0001$) or per milligram protein ($F(11,99)$

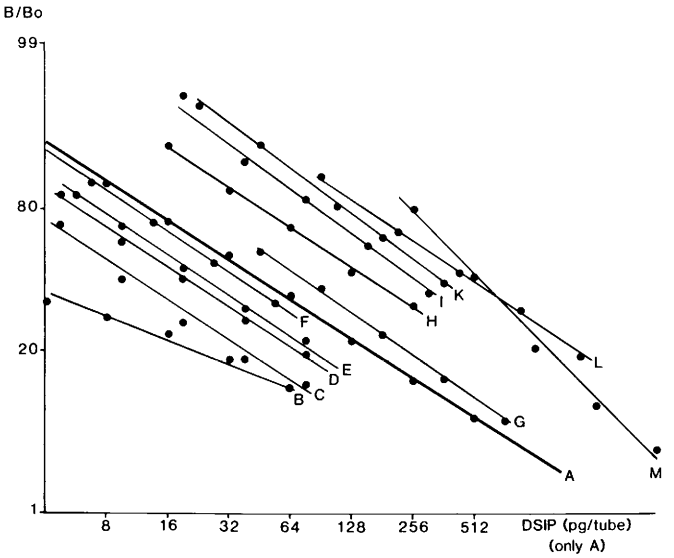

FIG. 1. Log/logit presentation of percent total binding as determined by RIA with dilutions of DSIP-standard (A), stomach (B), jejunum (C), kidney (D), pancreas (E), spleen (F), thymus (G), ileum (H), colon (I), adrenal (K), liver (L), and muscle (M). The organs were extracted with water–Trasylol. For clarity, most curves are shifted from their original position.

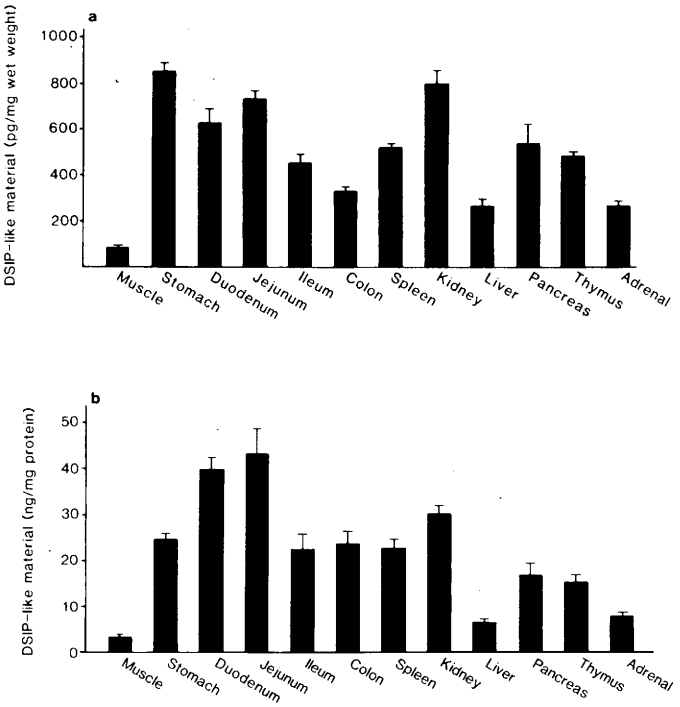

FIG. 2. (a) Mean levels (±SEM) of DSIP-like material in 12 peripheral organs of the rat. The values are expressed per mg wet weight of the tissue. (b) Mean levels (±SEM) of DSIP-like material in 12 peripheral organs of the rat. The values are expressed per mg of tissue protein.

= 31.52, $P < 0.0001$). The ranking of organs was about the same with two noticeable exceptions: on a protein basis, colon possessed significantly more DSIP-like material than thymus and pancreas (and the lower-ranking organs), but this was not the case on the basis of wet weight, where colon was clearly lower than ileum ($P < 0.05$), thymus, pancreas, and all the other tissues ($P < 0.01$), but was not significantly different from liver and adrenal. Stomach was significantly highest ($P < 0.05$) compared to kidney and all other organs ($P < 0.01$) when calculated per milligram wet weight but was significantly lower ($P < 0.01$) than jejunum or duodenum but not kidney, colon, spleen, or ileum when calculated per milligram protein. Although muscle ranked last in both cases, it was not statistically different from adrenal or liver when based on protein but was different from all organs ($P < 0.01$) when based on wet weight. When the organs of a rat were put into a rank order according to the concentrations found either per milligram wet weight or per milligram protein, the correlation among animals was highly singificant ($P < 0.001$).

As seen in Fig. 3, a marked decrease in the level of DSIP-like material occurred during prolonged storage with time ($F(4,189) = 86.9$; $P < 0.0001$). The main effect of the different organs was significant ($F(4,189)$

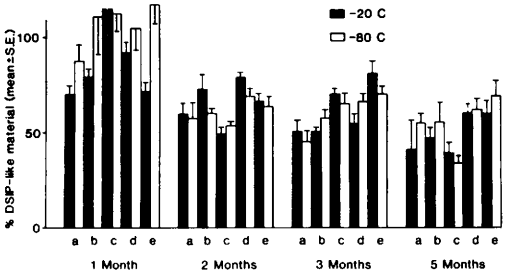

FIG. 3. Decrease of DSIP-like immunoreactivity over time in five organs stored frozen at −20 or −80°C respectively: (a) stomach, (b) jejunum, (c) liver, (d) spleen, (e) thymus. The organs shown in Fig. 2 served as the controls (100%).

= 4.8; $P < 0.001$) as was the interaction of time by organ ($F(16,189) = 2.97$; $P < 0.001$). The influence of the temperature of storage was at the limit of significance ($F(1,189) = 3.9$; $P = 0.049$). This effect of temperature was seen only after the first month when two of the organs stored at −80°C yielded higher scores than at −20°C ($P < 0.01$ for thymus (e) and $P < 0.05$ for jejunum (b)). This is reflected in the significant interaction of time by temperature ($F(4,189) = 4.8$; $P < 0.005$) but not of organ by temperature or organ by time by temperature.

The recovery of DSIP-like material present in the tissues was established by adding 50 pg synthetic DSIP to different concentrations of the homogenates of five organs. The results are shown in Fig. 4. The increase resulting from the amount of added peptide determined in buffer was taken as 100%. In general, the recovery from tissue was about 80%. The main effect on recovery by the organ being assayed was highly significant ($F(4,208) = 6.09$, $P < 0.0001$). The concentration of tissue had no significant main effect ($F(5,208) = 1.44$, $P < 0.2$), but the interaction of organ by concentration tended toward statistical significance ($F(20,208) = 1.56$, $P < 0.07$); this is seen in Fig. 4 which shows that recovery of DSIP added to liver increased with diminishing concentrations but in thymus tended to decrease at lower concentrations. At 4 mg/ml, the recovery from liver was significantly lower than at 1 mg/ml ($P < 0.05$) or

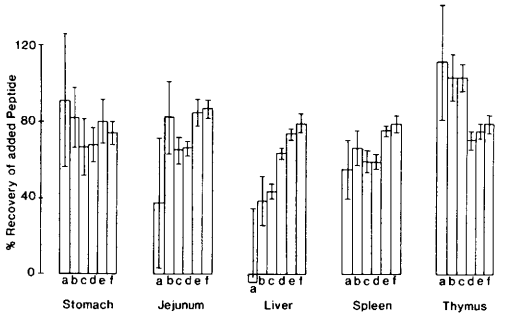

FIG. 4. Percentage recovery (mean ± SEM) of DSIP added to the homogenates of five organs at six different tissue concentrations (a: 4 mg/ml, b: 2 mg/ml, c: 1 mg/ml, d: 0.5 mg/ml, e: 0.25 mg/ml, f: 0.125 mg/ml) from which 100 μl was taken for the RIA. The same amount of DSIP added to the homogenates was determined in buffer and taken as 100%.

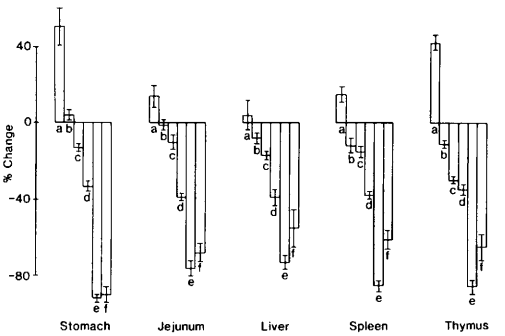

FIG. 5. Percentage change (mean ± SEM) of DSIP-like material found after preadsorption with charcoal-dextran in five organs at six different tissue concentrations (same code as in Fig. 4) as compared to the values without charcoal treatment determined at the same time.

at smaller concentrations ($P < 0.01$), but no enzymatic degradation of the radioactive tracer was observed even at a concentration of 4 mg/ml. The large standard errors for the recovery of added peptide at the higher concentrations of tissue may reflect the fact that the added DSIP represented only a small percentage of the DSIP-like material present in the tissue itself.

Removal of any small molecular forms of DSIP-like material (hereafter called "free peptide") by preadsorption with charcoal–dextran is presented in Fig. 5. The value in the same homogenate before adsorption was taken as 100%. In all of the five examples, there was a marked influence of the concentration of tissue on the recoverable percentage of peptide. The lower the tissue concentration in the homogenate, the larger the percent of free peptide. This correlation was highly significant ($P < 0.001$) for each organ as revealed by regression analysis. At higher tissue concentrations, a "negative" effect of preadsorption was found in which there was an increase of the immunoreactive material. This increase was statistically significant; compared to the smallest change induced by charcoal in an organ (jejunum at the concentration of 2 mg/ml (b)), there was a marked increase in the amount of DSIP-like material at a concentration of 4 mg/ml (a) in stomach ($P < 0.0001$), thymus ($P < 0.0001$), spleen ($P < 0.05$), and jejunum ($P < 0.05$). The linear correlation for the organs revealed that at about 2 mg/ml (b), practically no free peptide was in solution.

Characterization of the DSIP-like material according to size by gel filtration on Sephadex G-15 revealed molecules larger than DSIP with most of the immunoreactive material eluting just after the void volume of the column (molecular mass exclusion limit about 1500 Da). In Fig. 6, the elution pattern of jejunum is shown; other organs produced similar results. On Sephadex G-25, the chromatographic pattern of immunoreactive material generally revealed two major peaks; Fig. 7 illustrates this with spleen. Most of the material eluted at a higher apparent molecular weight than DSIP itself, some of it still being close to the void volume containing molecules of about 5000 Da or more. However, tryptic digestion of the supernatant of the homogenates produced small immunoreactive peptide fragments (Fig. 7, dashed line). After trypsin, the total amount of DSIP-like material was only slightly less than before degradation, and part of the immunoractive material was found at the position where synthetic DSIP eluted. Similar results were obtained with other organs.

Discussion. DSIP-like material is found in peripheral organs of the rat and can be extracted with water (Figs. 1, 2). Previous work with plasma and CSF (11, 16) suggested that the immunoreactive DSIP-like material exists mainly in large forms. It is primarily these large molecular forms of DSIP-like material that were increased by extraction with water as compared to acetic acid, perhaps because some of the larger molecules were precipitated by the acid or coprecipitated

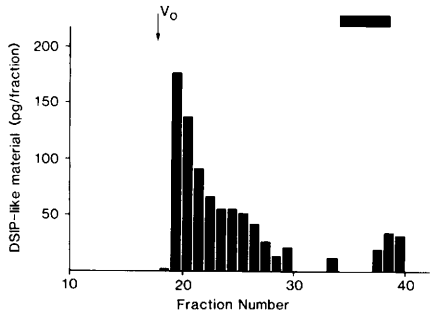

FIG. 6. Gel chromatography on Sephadex G-15 (1 × 60 cm) of DSIP-like material extracted from jejunum with water (containing 4% Trasylol) and determined by RIA. (V_0) indicates the void volume of the column and the black bar the position where synthetic DSIP eluted.

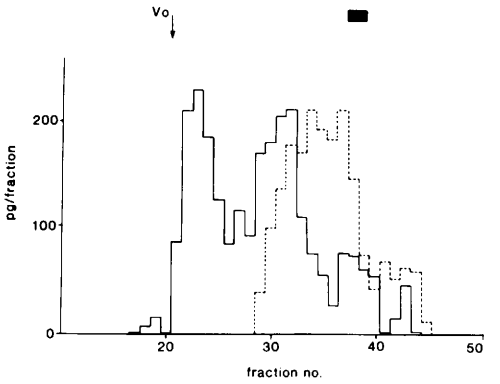

FIG. 7. Gel chromatography on Sephadex G-25 (1 × 60 cm) of DSIP-like material extracted from spleen with Tris buffer (pH 8.0) and determined by RIA. V_0 indicates the void volume of the column and the black bar the position where synthetic DSIP eluted. The dashed line shows the elution pattern of the material after digestion with trypsin.

with other large proteins. The percentage of free peptide did not substantially change under these conditions.

Digestion of the DSIP-like material by trypsin did not destroy much of the immunoreactivity (Fig. 7), whereas a loss of total immunoreactivity would have been expected with nonspecific interfering proteins. Since the DSIP sequence itself should be resistant to tryptic degradation, it is likely that DSIP is either bound to or an intrinsic part of larger protein structures.

DSIP-like immunoreactive material seems to occur ubiquitously in the rat, being found in all organs tested. These were analyzed within a week after storage at −80°C, since longer storage times may lead to deterioration of measurable amounts of immunoreactive DSIP-like material (Fig. 3). A possible contamination by blood could not account for the levels measured in the peripheral organs since they were 10–100 times greater than the amount of DSIP-like material in plasma (11, 16). Extraction of brain tissue by water also yielded amounts of DSIP immunoreactivity in the same range as those in the periphery (unpublished).

The recovery of added DSIP seemed to increase with decreasing concentrations of tissue, at least in the liver (Fig. 4). The finding of negligible recovery of DSIP in liver at a concentration of 4 mg of tissue/ml is

surprising since in the corresponding control of the same tissue without added peptide a value of 250 pg/mg was found. The higher concentrations of tissue could contain increased amounts of enzymatic activity that could have destroyed the added DSIP and radioactively labeled peptide, thus producing false results (17), but we could not detect any enzymatic degradation of the tracer under these conditions, even after 6 days. Another possible artifact could be interfering substances like globulins (18), but such a substance would not explain why the percentage of small DSIP-like material determined by preadsorption with charcoal increased with dilution (Fig. 5). Moreover, the dilution curves of the organs were parallel to the standard curve in buffer (Fig. 1) and tryptic digestion did not destroy the immunoreactivity.

One possible explanation of these unusual results could be provided by a hiding (binding/aggregating) process for "overflow peptide" involving factors present in tissue. This might also help explain the increase of immunoreactive DSIP-like material observed after preadsorption at the highest concentrations of tissue (Fig. 5). Charcoal appeared to adsorb small factors present in the tissues which may help to hide the antigenic determinants of DSIP, perhaps by binding or aggregation. It was previously shown that DSIP added to plasma was later found in larger molecular forms (11,16). An increasing percentage of small DSIP-like structures was apparently freed by dilution, perhaps by an equilibrium process. Since a very small level of immunoreactive DSIP-like material seemed to remain as a large molecule, the lowest concentration of tissue exhibited a slightly reduced percentage of adsorbed material (Fig. 5).

The extent to which these findings (and hypotheses) are applicable to other peptides is unknown, but it has been suggested that some other peptides also exist in different molecular forms (19, 20) and dynamic processes such as binding are also postulated for somatostatin and somatomedin (21, 22). Furthermore, spontaneous aggregation in saline solution of several different peptides has been visualized recently by electron microscopy (23).

DSIP-like immunoreactivity seems to occur in considerable amounts in all rat organs tested. It exists mostly in larger molecular forms, but small DSIP-like material can be freed by diluting the tissue extracts to concentrations less than 1 mg/ml or by tryptic digestion. Compared to the large amount of endogenous material, relatively small doses of injected DSIP (30 nmole/kg) have been reported to induce substantial effects (3, 5, 8, 24–26). It is not known whether a binding phenomenon discussed here may explain this apparent contrast.

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