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REVIEW

Delta-Sleep-Inducing Peptide (DSIP): An Update

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GRAF, M. V. AND A. J. KASTIN. *Delta-sleep-inducing peptide (DSIP): An update.* PEPTIDES 7(6) 1165–1187, 1986.—The isolation and characterization of delta-sleep-inducing peptide (DSIP) achieved from 1963 to 1977 were reviewed in 1984. The first reports describing sleep as well as extra-sleep effects of DSIP also were included in that work. Only two years later, much additional literature concerning DSIP has accumulated. Besides further sleep-inducing and/or -supporting effects of DSIP in animals, considerable work has been carried out to evaluate the potential use of the peptide for therapeutic purposes such as treatment of insomnia, pain, and withdrawal. Immunohistochemical as well as radioimmunochemical studies provided further insights into the natural occurrence of the nonapeptide and the distribution of DSIP-like material in the body, suggesting possible relations of the peptide to certain diseases. Various physiological functions of DSIP and a possible mechanism of action involving the modulation of adrenergic transmission remain to be established.

Sleep Sleep-factors DSIP Natural occurrence EEG Adrenergic transmission Stress Physiological functions Metabolism Peptides

IN our first review of delta-sleep-inducing peptide (DSIP) two years ago [57], we described the detection, the isolation and some characteristics of DSIP. We also discussed sleep as well as extra-sleep effects of the nonapeptide as far as they were known at that time. Since then, reports about this peptide have doubled. It seems that the concept of DSIP has evolved from a state of a 'strange neuropeptide' into a serious matter of research. Additionally, several clinical studies suggest the usefulness of DSIP as a therapeutic agent. Altogether, the recent results with the peptide provide the basis for further investigations with respect to basic research as well as clinical applications.

This update will review and evaluate the developments

with DSIP that were achieved during the last two years. Most of the results concern the two main questions we raised in our first review [57], i.e., (a) does DSIP exist naturally? and (b) is its main function sleep-induction or something else?

OTHER SLEEP FACTORS

Before discussing recent developments of DSIP research in more detail, some of the advancements made with other sleep factors will be mentioned briefly. This should help in a comparison of the results obtained with DSIP with those of other sleep factors. A more comprehensive review of several putative sleep-inducers has been published recently [87].

Every year, different compounds are proposed as putative sleep factors. This may be due to several reasons: (a) No definition of a sleep factor, though proposed [19,92], has been generally accepted. (b) Sleep consists of many stages and is influenced by many factors, several of them still unknown. (c) Almost any given biologically active compound may have some relation to sleep or a sleep stage, be it through circadian rhythmicity or by interference with transmitter pathways or other biological systems that lead, perhaps only transiently, to influences on sleep. A solution for this problem will necessitate general agreement about a definition of sleep and, in consequence, about basic requirements for 'sleep factors.' Considerations with regard to the problem of endogenous sleep factors have been made by others [18, 19, 34, 86, 228, 229] and some of the more interesting substances have been reviewed previously [57].

The classical neurotransmitters, acetylcholine [50,158], serotonin (5HT) [93,189], norepinephrine (NE) [32,177], and dopamine [237] have been related to vigilance states and gamma-aminobutyric acid (GABA) [192] also seems to affect sleep-wakefulness cycles of different animal species. In addition, different putative neurotransmitters were reported to enhance sleep. For instance, adenosine apparently increased stage S2 as well as paradoxical sleep (PS) in rats [178], and glutamate appears to be correlated with maturation of delta and theta bands [27].

Several peptides have recently been reported to induce sleep. The effect of cholecystokinin on slow-wave sleep (SWS) [184], however, could be mediated by the release of insulin [145,180]. This hormone apparently increased SWS in rats by 25% after chronic, intracerebroventricular (ICV) infusion without changing PS [30], a result not observed by Riou *et al.* [182]. In contrast, a selective increase of PS was found in rats after ICV infusion of somatostatin, whereas administration of cysteamine reduced PS [29]. Whether this indicates that somatostatin is involved in the regulation of PS remains to be confirmed.

A major achievement during the last two years was the characterization and determination of the sequence of urinary sleep promoting factor (FSu) by Krueger, Martin and coworkers [124,144]. From several analogs, the main somnogenic compound was found to be a peptidoglycan of molecular weight 921 with the structure N-acetylglucosaminyl-N-acetyl-anhydromuramyl-alanyl-glutamyl-diaminopimelyl-alanine. Two other analogs were also active as somnogens and further studies revealed that both structures, the hydrated and anhydro forms of muramic acid contained in the structure, are somnogenic whereas amidation of the free carboxyl groups abolished the effects of sleep [124].

Before the sequence of FSu was completely elucidated, its structural similarity to muramyldipeptides (MDPs), usually components of bacterial cell walls, was realized. In consequence, Krueger *et al.* tested several MDP analogs for their somnogenic potency [127]. The authors determined that adjuvant-inactive stereoisomers of MDP also were inactive as somnogens, and periodate oxidation of the muramyl moiety or the replacement of the N-acetylglucosamine moiety of MDP by glucose abolished somnogenic activity [127]. As with FSu, the presence of an unsubstituted amide on the free carboxyl of MDP led to inactivation of the compound suggesting to these authors that amide-synthesizing or -hydrolyzing enzymes exist in the body with the capacity to control somnogenic activity of muramyl peptides. The main features of MDP-induced sleep have been summarized by Krueger in recent reviews [122,123].

Many questions still remain open such as the strong correlation of somnogenic potency with pyrogenic effects, also observed with FSu [124], the suppression of rapid eye movement sleep (REMS) [47,241] and even SWS [121], and the mechanism of action by which MDPs induce SWS. It takes about one hour for excess SWS to appear regardless of the route of administration [123].

According to Inoue *et al.* [89,90], MDP exerts its activity in rats only during the night, which is the active phase of these animals. Such a result, however, seems to be a common feature of several sleep-inducing substances in rats since prostaglandin D_2, sleep-promoting substance (SPS), and uridine also gave the same result [86]. This may also be the reason why Fornal *et al.* [45] did not find any effect of MDPs in rats. In squirrel monkeys, different effects were observed depending upon whether MDPs were administered during night or during daytime [241].

Though sleep induced by intravenous (IV) injections of MDP was claimed to be normal, such treatment produced some toxic effects [128] that also are known to occur with higher doses of MDPs [125]. This led to hypotheses that MDPs exert their activities through endogenous compounds involved in pyrogenicity like interleukin-1 (IL-1) [122,127], the production of which was enhanced by MDPs [134]. IL-1 has been found to induce SWS in a dose-dependent manner [126]. The onset of action was faster than with MDPs but the problem of a concurrent febrile response also arose. Although some evidence was presented that the somnogenic effect was not dependent on the pyrogenicity [126,221], a strong correlation exists between both effects. It is difficult for many scientists to accept compounds as natural sleep-inducers that produce hyperthermia concurrently with sleep. Sleep is usually known to be related with a decrease in temperature [115]. Although individual regulatory systems for body temperature and rest-activity seem to occur [160], both rhythms are intimately correlated [76,241].

It is general knowledge that fever is related to immune function and correlations between sleep and immune function seem to be numerous [128]. This, together with the findings of the sleep-inducing capacity of immunostimulants, led Krueger to develop a theory that normal sleep serves an immune function [128]. Such a hypothesis opens interesting possibilities, but at the present time it remains highly speculative and any proof will require much more work.

Compounds other than IL-1 may be involved in the effects of MDPs. These include the prostaglandins D_2, E_1, E_2, and F_2 that have been found to induce SWS [89, 226, 227] and it was shown that MDPs as well as IL-1 can influence arachidonic acid metabolism, leading to prostaglandin synthesis [122,234].

Another decisive step occurred with the identification of an active component of sleep-promoting substance (SPS) isolated

from the brain of sleep-deprived rats by the group of Inoué *et al.* [86]. SPS-A-1 was finally identified with uridine [117], a nucleoside, and its sleep-inducing capacity was confirmed by ICV infusion [82] as well as IP injection [83] of synthetic uridine into freely moving rats. It is not known whether there is a relation to the sleep effects of adenosine [178], another nucleoside. Other fractions of SPS that exhibit sleep effects remain to be identified. One of them was found to exert sleep-induction at least as strong as that of uridine [89]. SPS fractions seem to be structurally different from DSIP since SPS showed no cross-reactivity in an RIA for DSIP (unpublished data).

Other peptidic factors have been mentioned earlier [57] and are described in different reviews concerning sleep-inducing substances [19, 87, 91, 182]. One of them, vasoactive intestinal peptide (VIP), proposed by Riou *et al.* [182,183] as a sleep-inducing peptide, was also found by Drucker-Colin and coworkers to participate in the regulation of REM sleep [35].

Another substance that has recently obtained some attention was shown to exist in cerebrospinal fluid (CSF) of PS-deprived animals, to induce PS in propranolol-blocked rats [1,229] and in cats treated with parachlorophenylalanine (PCPA) [188], a serotonin synthesis inhibitor. A similar factor was found in extracts of bovine neuro-intermediate lobe. Injected subcutaneously (SC) or ICV, it also was able to induce PS in totally insomniac cats treated with PCPA [190]. In consequence, it was proposed that 5HT is not directly responsible for sleep but, through neurohormonal mechanisms, initiates the biosynthesis and/or release of sleep factor(s) that are stored until they trigger sleep [93]. The PS factor(s) [1, 188, 190] seem to act beyond the noradrenergic step in the regulation of PS [1], a possible mechanism of action proposed for DSIP (see below). Determination of DSIP-like immunoreactivity in the CSF of PS-deprived rats is in progress (G.A. Schoenenberger, personal communication).

NATURAL OCCURRENCE OF DSIP

In our review [57], we concluded that the questions about the natural occurrence of DSIP were not conclusively answered at that time. Though the peptide had been isolated from rabbit blood, it was possible that DSIP represented an isolation-artifact [62,107]. In the meantime, several reports have dealt with this problem and strong evidence for the natural existence of the peptide and/or analogs were obtained by different methods.

IMMUNOHISTOCHEMICAL EVIDENCE

Two independent studies using immunohistochemical techniques have shown DSIP-like immunoreactivity (DSIP-LI) in rat brain. In one study [40], a rather widespread distribution of DSIP-LI was observed. Immunoreactivity was found in neurons of a rostral-caudal band extending from the primary olfactory cortex to the lateral hypothalamus, and also in neurons of the basal ganglia, amygdala, septum, and the thalamus. Labeled neurons were found at even higher concentrations in the brainstem, e.g., the reticular formation and several nuclei like the raphe nuclei, areas known to be active during SWS and PS [141]. In most cases, the immunoreactivity was associated with perikarya and only occasionally were immunoreactive fibers seen [40].

Colchicine pre-treatment increased the number of immunoreactive DSIP neurons without affecting their distribution. In this study [40], the rather diffuse distribution of DSIP-LI suggested an involvement of DSIP with sensory systems, such as general somatic sensation, audition, and vision, and also with visceral brain systems. The results obtained after colchicine-treatment further suggested that DSIP is synthesized in cell bodies and transported in axons, a retrograde transport being unlikely. Direct projections to ventricles and blood vessels, however, may be possible. This point may be related to the influence of DSIP on blood pressure [66]. It is unknown if the location of DSIP-LI in sites with serotonin neurons (raphe), catecholamines (medulla, mesencephalon), or somatostatin (vagal nuclei, basal ganglia, amygdala) may be of functional significance. The possibility of an integrative function of DSIP in sensory information was raised [40]. Most DSIP-LI was found in areas connected with 'awareness,' 'arousal,' and sensory-motor integration so that the peptide might modulate such functions.

DSIP-LI was also found in the hippocampal formation [41]. Immunoreactive neurons were detected in the subicular cortex immediately adjacent to CA1 but not in other regions of the hippocampus or in the fornix. The reaction to three different antisera suggested [41] that most DSIP-LI in these areas exists in a large, bound or aggregated, form. Furthermore, the presence of DSIP-LI in the subiculum, the major outflow tract of the hippocampal formation [21,150], would allow modification of this outflow. Accordingly, it could modulate sensory awareness and brain 'state' [21] and be involved in learning and behavior [140].

The presence of DSIP-LI in hippocampus was reported first by Constantinidis and his colleagues in a different study [25]. These authors found DSIP-LI throughout the hippocampus, with the highest density of DSIP-positive perikarya and neural fibers in the nucleus septilateralis and the precommissural archaic hippocampus. Further DSIP-LI was observed in the indusium griseum, the bandeletta of Broca, and the striae longitudinales of Lancisi. Sleep-selective cells have been described as a subpopulation in the bandeletta of Broca [214]. Little DSIP-positive neuronal bodies and fibers were detected in the nuclei of the brain stem, basal ganglia, hypothalamus (only fibers), whereas no DSIP-LI was found in the medulla and the cerebellum. High specific reactivity, however, was observed in the posterior part of the pituitary.

Constantinidis *et al.* [25] suggested that the indusium griseum (an archaic limbic structure) and also the nucleus septilateralis send DSIP axons through the striae longitudinales of Lancisi and the bandeletta diagonalis of Broca to the postcommissural hippocampus, particularly the gyrus dentatus where, in a different study (unpublished observation), an increased amount of radioactivity was observed after injection of ^{3}H-labeled DSIP. Those authors also postulated another DSIP pathway from the pallidum to the hypothalamus with a possible branch to the hypophysis. It will need further work to determine if the results indicate correlations with other putative neurotransmitter systems such as glutamate, aspartate, cholecystokinin or other peptides, as suggested by Constantinidis *et al.* [25]. They also took into consideration a link between the anatomical distribution of DSIP-LI with regulations of instinctive-affective functions and related disorders like schizophrenia and Alzheimer's disease [26].

The histochemical studies of the two groups disagreed in several aspects: only in the bandeletta of Broca, partly in the hypothalamus, and in small parts of the hippocampus, did both find DSIP-LI. Whereas Feldman and Kastin [40,41] observed DSIP-positive immunoreactivity in midbrain, pons,

medulla and cerebellum, Constantinidis and coworkers [25] did not. A possible explanation was given by Feldman and Kastin when they described the occurrence of DSIP-LI in the hippocampus [41]. It had been noted that some antisera only recognize the 'free' form of DSIP [62]. Such antisera were found by these authors not to be very suitable for immunocytochemistry. Other sera also recognize the 'large forms' of DSIP-LI where the nonapeptide is apparently bound, aggregated, or incorporated in the sequence of a large molecule (see below). The sensitivities as well as the specificities of the antisera used by the two groups were obviously different, leading to different patterns of DSIP-LI in rat brain. Although both groups took care to eliminate nonspecific staining, such artifacts still could be present, as discussed in one study [41]. Nevertheless, the immunohistochemical investigations provide an anatomical basis for the understanding of possible functions of DSIP.

RADIOIMMUNOCHEMICAL EVIDENCE

Further indications for the natural existence of DSIP were obtained by means of radioimmunoassay (RIA). The first RIA for DSIP-LI was developed in 1978 [105] and enabled measurement of DSIP-LI in rat brain (about 10 pg/mg wet tissue). Gel chromatography revealed that the immunoreactive material eluted at the position of the nonapeptide. In the following years, most work involving the measurement and characterization of DSIP-LI was carried out with plasma of different mammals. The early reports have already been discussed [57].

DSIP-LI in Plasma

The first indication of DSIP-LI existing in large molecular form(s) (LF) was found with human plasma [101]. Most of the immunoreactivity eluted with the void volume from a column of Sephadex G-25 and only a minor amount eluted at the position of the nonapeptide.

In 1983, Ekman and colleagues [36] reported that they could not detect any DSIP-LI in plasma that eluted at the position of the synthetic peptide, but that all of their immunoreactive material appeared larger than DSIP. A Japanese group also could not measure DSIP-LI in plasma of rats, monkeys, humans, and dogs or sleep-deprived dogs [110,216]. Later, with an improved enzyme immunoassay, the same group was able to determine DSIP-LI in extracted human and rat plasma [111, 112, 162]. The values of DSIP-LI in human plasma (25–60 pg/ml) measured by Nagaki *et al.* [162] and Kato *et al.* [111] and in rat plasma (about 35 pg/ml) [112] were considerably lower than those published earlier by Kastin *et al.* (2–4 ng/ml) [101]. However, Kato and coworkers found more small-molecular form (SF) of DSIP-LI than LF after chromatography on Sephadex G-25 [111]. How far the specificity of their antibody for the C-terminus of the peptide [110] was responsible for these results is unknown.

High values, similar to those of Kastin *et al.* [101], were reported by Ekman *et al.* (about 1 ng/ml) [36] and by Van Dijk *et al.* (about 4 ng/ml) [232] who used the same antibody as Ekman. Van Dijk *et al.* determined that 70–90% of DSIP-LI consisted of LF [232]. Yet another group, Rozhanets and colleagues from Moscow, found about 200 pg DSIP-LI per ml rat serum [186].

The different reports revealed marked discrepancies regarding the amount as well as the molecular size of DSIP-LI in plasma. We, therefore, studied this problem in more detail. An RIA with a different antibody (No. 607) was developed and used to measure the amounts of DSIP-LI in plasma of 4 different species [60]. The mean values averaged 105 pg/ml in the dog, 155 pg/ml in the rabbit, 260 pg/ml in the human, and 291 pg/ml in the rat. Gel chromatography of all 4 plasmas revealed main peaks at the position of the synthetic nonapeptide in addition to immunoreactive material eluting in the void volume representing LF-DSIP-LI. The peaks containing the SFs were further characterized by high performance liquid chromatography (HPLC) which, in each case, revealed a peak with immunoreactive material at the elution position of DSIP [60].

Thus, the natural occurrence of DSIP (or a structurally closely related compound) in plasma has been demonstrated with high probability. The results suggested that DSIP does exist in plasma in the 'free' form besides other—usually larger—forms. However, several questions still required explanation: How is it possible, that the amount of measured DSIP-LI in plasma varied by a factor of 100 in different laboratories? What was the nature of the large form(s)? Did the LF really contain the sequence of DSIP and, if so, how was the DSIP integrated or attached to the LFs? Do the LFs have a significant influence on the effects of DSIP? Some of these questions have already been discussed in more detail [62].

Large and small forms of DSIP-LI. Different antibodies apparently do not recognize all forms of DSIP, and this can lead to considerable discrepancies concerning the amounts of DSIP-LI in plasma determined with different RIAs. It appears, therefore, that the amount of DSIP-LI determined in plasma is mainly dependent on the antibody used, i.e., if it can recognize both, the 'large' and the 'small' DSIP-LI, or only the SFs. In fact, we found evidence for this [60] when we measured DSIP-LI in plasma with two different antibodies under otherwise similar conditions. Antibody No. 604 yielded high amounts (2–4 ng/ml) with about 90–95% of the immunoreactivity eluting with the void volume (Sephadex G-25), thus indicating larger molecules, whereas antibody No. 607 measured 10 times lower levels with a major peak of DSIP-LI eluting from gel chromatography at the position of the nonapeptide. We had to conclude that the antigenic determinants of LF-DSIP-LI are not easily or completely accessible for all antibodies.

Peculiarities of the two antibodies used in these studies have helped to elucidate the question further [60,62]. Antibody 604 cross-reacted with the sequence consisting of amino acids 2–7 whereas antibody 607 reacted with the amino acids at each terminus. Antibody 607 also cross-reacted well with D-Ala^3-DSIP but not with D-Ala^4-DSIP. The results suggested the existence of a DSIP molecule in an 'open-circle'-shaped folded conformation and the two antibodies apparently reacted with the two opposite sites of the conformation. This assumption based on experimental results coincided with a similar suggestion obtained through theoretical calculations by Popov *et al.* [176]. Furthermore, the fact that antibody 607 recognized the opening of the 'circle' at the same time detecting smaller amounts of DSIP-LI, whereas antibody 604 recognized the opposite site, detecting large amounts including the LFs, led to the conclusion that a major part of the nonapeptide apparently is attached to other molecules at or near the 'cleft' of the folded conformation.

It is, therefore, not surprising that different antibodies measure largely different amounts of DSIP-LI. Based on these considerations, we assume that the 'freely' occurring DSIP in plasma amounts to values of 50 to 100 pg/ml, whereas the total DSIP-like material seems to be in the range of 1–5 ng/ml.

So far, we don't know whether the LFs represent a phys-

iological reservoir of DSIP that can be liberated into 'free' form. Several indications point to such a possibility. For instance, DSIP added to plasma eluted mainly with LF when determined by RIA [101]. More recent investigations with the labeled nonapeptide, however, showed that DSIP itself seems to be rapidly degraded by plasma enzymes in vitro and in vivo (unpublished data with B. Sägesser and G. A. Schoenenberger), whereas analogs of the peptide can yield larger forms, probably aggregates (see below). ^{125}I-Tyr-DSIP mixed with human plasma apparently produced aggregated forms [102] that were reversed by incubation with acetic acid and the chelating agent 1,10-phenanthroline.

Thus, we assume that LF-DSIP-LI represents the sequence of DSIP somehow attached to other molecules at the site of the 'cleft,' which would explain at least in part the large differences of the amounts of DSIP-LI in plasma. However, we still do not know how this attachment is accomplished; there is evidence for aggregation ([102], unpublished data) and a carrier/binding protein) [101], but the existence of (a) precursor(s) must also be considered.

The recent results have added some pieces to the puzzle of DSIP existing naturally, but the picture is still not clear. The problem of the LF-DSIP-LI seems still more complex than anticipated. An indication for the sequence of DSIP existing in LF was the finding that, in peripheral organs, small immunoreactive molecules appeared after tryptic digestion of the LFs without loss of total immunoreactivity [58]. Another indication was obtained when homogenates of peripheral organs of the rat were extracted with charcoal. Increasing percentages of DSIP-LI were removed by this procedure only at more dilute concentrations, suggesting an equilibrium system [58]. Tryptic digestion of the LFs found in milk yielded SFs that eluted at the position of DSIP after HPLC [56]. The question whether DSIP is bound to or incorporated in larger molecules or whether an aggregation takes place remains unclear at the present time.

Circadian rhythm of DSIP-LI. DSIP-LI in human plasma measured in the evening showed higher values compared with other times of day [101,112]. We detected a circadian rhythm of DSIP-LI in rat plasma that peaked at about the transition from light to dark and showed a trough later in the dark phase [43]. This rhythm partly paralleled the circadian rhythm of corticosterone. Under conditions of constant illumination, it became clear that the two compounds were regulated by different factors. A similar rhythm of DSIP-LI in rat plasma was determined by Banks *et al.* [15] who also found a peak before dark and a trough before noon. It is surprising that the rhythm of DSIP-LI in rat and man seems to follow the same pattern with respect to the peak in the evening [43, 101, 111] despite differences in their times of activity. Thus, DSIP possibly fulfills a different function in both species. At least no obvious correlation of DSIP levels in plasma and sleep stages could be observed by Kato in 4 subjects [112].

Whether the circadian fluctuation of DSIP-LI in rat plasma remains the same throughout the year has not been assessed. Variations of the amount of DSIP-LI in the brain of ground squirrels over the annual cycle have been reported by Kramarova *et al.* [120]. We have observed some indications that the effects of the peptide were changing seasonally (unpublished data) and circannual variations were also considered by Sommerfelt [206] as a possible explanation for the reversed activity of DSIP in that study.

DSIP-LI in CSF

Although a major part of the work concerning the measurement of DSIP in mammals has been carried out with plasma, results obtained with other body fluids have also been published. It was found that CSF of rabbits stimulated to sleep could induce delta waves in recipient animals and the effect was hypothetically ascribed to DSIP [51].

Determination of DSIP-LI in CSF has been reported with values between 30 and 560 pg/ml [12, 36, 60, 111, 232]. All studies found 'free' DSIP in CSF and in one study [60], SF-DSIP-LI from chromatography on Sephadex G-100 was subjected to HPLC. Determination of the fractions by RIA revealed a main peak at the position of DSIP, but additional peaks were observed at the position of P-DSIP and N-Tyr-DSIP.

It was suggested that CSF may lack a strong binding protein for DSIP [12]. There were, however, some indications for LF-DSIP-LI in CSF, since LF also appeared with gel chromatography, at least in two studies [36,60]. The reactions of DSIP with other compounds in CSF appear less complex than in plasma. Thus, it may be easier to observe differences in the levels of DSIP-LI in CSF due to physiological or psychological disorders, as discussed below.

DSIP-LI in Urine

DSIP-LI was also determined in human urine and was found to amount from about 100 pg/ml [60] to more than 6 ng/ml [36]. This large difference may be due to phenomena mentioned already (see section III B1), namely preference of antibodies for certain forms of DSIP. One study [36] did not reveal small forms by gel chromatography whereas the other [60] found almost no large DSIP-LI. HPLC of the SF peak showed immunoreactive material at the position of P-DSIP, the phosphorylated analog, with negligible amounts of DSIP [60]. A large peak of unknown origin, however, was observed in the front fractions after HPLC.

Synthetic DSIP can elute immediately with the front from reversed-phase HPLC when a critical concentration of salts is exceeded (unpublished data with B. Sägesser). This phenomenon could account for some of the HPLC patterns observed, since DSIP generally elutes together with salts from Sephadex columns. This represents an additional possibility for false results and emphasizes that extreme care must be applied in the interpretation of HPLC separation of peptides monitored by RIA [44].

DSIP-LI in Milk

The finding that DSIP-LI exists in human milk in large and small forms [56] has been confirmed by a different laboratory [38]. Using HPLC separation and determination by RIA, we found evidence for 'free' DSIP. Tryptic degradation of LF-DSIP-LI followed by gel chromatography, HPLC and RIA revealed peaks at the positions of DSIP as well as P-DSIP, as depicted in Fig. 1.

The existence of DSIP-LI in human milk could be important regarding the possibility of DSIP being absorbed by the intestinal tract [13], at least in the neonate, and crossing the blood-brain barrier [12]. It is remarkable that in plasma of newborn babies, higher amounts of DSIP-LI were found compared with adults [112]. The peptide has been shown to shift circadian rhythms [52, 53, 199, 201, 203, 247], and the development of such rhythms may be partly induced by mothers [39,156], though this question still remains controversial [84].

Besides the fact that DSIP-LI occurs in milk, four other results were reported for the first time in this paper [56]: (a) A circadian rhythm in the level of DSIP-LI with the peak in the

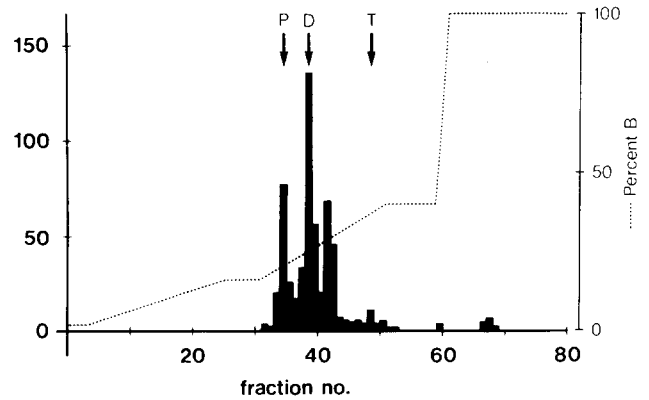

FIG. 1. HPLC of trypsin-digested LF-DSIP-LI from breast milk. The trypsin-digest was first separated on Sephadex G-10. The eluting DSIP-LI was then analyzed by HPLC. Arrows indicate the positions of synthetic P-DSIP (P), DSIP (D), and N-Tyr-DSIP (T). Broken line indicates the gradient of the eluants. The ordinate indicates pg DSIP-LI per fraction as determined by RIA.

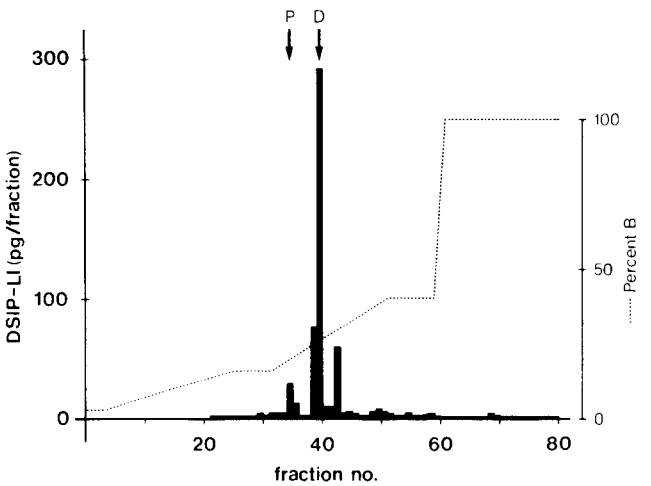

FIG. 2. HPLC of SF-DSIP-LI of rat hypothalamus. The tissue was extracted with 1 N acetic acid. Fractions were analyzed by RIA. P indicates the elution position of P-DSIP and D that of DSIP The HPLC system was the same as in [60].

afternoon was observed. (b) The occurrence of 'free' DSIP was demonstrated by means of HPLC and RIA. (c) Tryptic degradation of LF-DSIP-LI and further separation of this material on gel chromatography produced a peak where the nonapeptide eluted. HPLC of this SF-DSIP-LI monitored by RIA revealed a peak at the position of DSIP as well as P-DSIP. (d) The latter was shown to exist naturally also for the first time. The peak previously eluting with N-Tyr-DSIP [56] was found to elute in a different position with a different HPLC-system (Fig. 1). It is not known whether P-DSIP (-LI) and DSIP (-LI) observed after HPLC of the tryptic digest of LF-DSIP-LI were bound to protein(s), whether they were aggregated through small, trypsin-susceptible molecules, or contained in the sequence of precursor compound(s).

Some of these results have been confirmed. Ernst *et al.* [38] found DSIP-LI as well as P-DSIP-LI in human milk analyzed with different antibodies for each of the analogs. Ultrafiltration with an exclusion limit of about 10,000 MW revealed that more than 60% of DSIP-LI occurred in large forms. The total amount of endogenous DSIP-LI was not changed after 24 hr at 37°C, whereas added, synthetic DSIP was rapidly degraded under these conditions. The half-life for P-DSIP added to human milk appeared to be more than 10 hr, and no decrease in the level of endogenous P-DSIP-LI was observed [38]. In plasma, also, P-DSIP was more resistent to degradation than was DSIP [69,110].

SF-DSIP-LI of human milk was separated on HPLC by Ernst *et al.* [38] and the peak eluting with the nonapeptide was subjected to amino acid analysis which revealed a composition compatible with that of synthetic DSIP (G.A. Schoenenberger, personal communication). The same group also detected DSIP-LI in cow milk, but in this case, exogenous DSIP and P-DSIP did not disappear over time when measured by RIA. Furthermore, the percentage of 'free' DSIP-LI in cow milk seemed to exceed 85% [38].

DSIP-LI in Brain

In brain, DSIP-LI was first observed by RIA [105]. Only slight differences in the amounts of DSIP-LI among the different regions of the rat brain were observed, and gel chromatography of the immunoreactive material suggested that it probably represented the nonapeptide [105]. Similar results were found by other groups. Rozhanets *et al.* [186] determined DSIP-LI in rat brain by RIA and found no major differences among the different regions, hippocampus tending to contain the most (3 pg/mg wet weight tissue) and thalamus the least (1 pg/mg). These authors found, however, marked differences in the subcellular fractions: 1 pg/mg protein in nuclei, 30 pg/mg in myelin, 12 pg/mg in synaptic membranes, 5–8 pg/mg in synaptosomes, and 66 pg/mg protein in mitochondria. So far, this represents the only determination of this kind.

It should be noted that the antiserum used by Rozhanets *et al.* [186] apparently recognized the C-terminal 'cleft-area' of the folded DSIP conformation according to results obtained in cross-reaction experiments with analogs: Tyr^6-DSIP cross-reacted by 100%, Tyr^7-DSIP 5%, Tyr^8-DSIP 0.2% and, most interestingly, substitution of the Gly^3-Gly^4 part by valerianic acid produced a complete loss in cross-reactivity. These results are consistent with our suggestion (see earlier) that such antibodies are likely to produce values markedly lower than those reported by Kastin *et al.* in 1978 [105] or 1984 [103].

Rozhanets *et al.* [186] found clearly different levels of DSIP-LI in the brain of mice and rats and even between two strains of the same species. Column chromatography on Sephadex G-25, LH-20, and DEAE cellulose DE-52 revealed a peak of immunoreactive material that always eluted at the position of synthetic DSIP, which was not the case with liver (see below).

Results comparable to those of the Soviet group were obtained by Kato, Nagaki and coworkers [111, 162, 163]. These authors found a peak of DSIP-LI after chromatography of brain extract on Sephadex G-25 that corresponded to DSIP. The values in the different brain regions were not greatly different and were in the range between 0.7 pg/mg (pons-medulla) and 3.2 pg/mg tissue (nucleus accumbens)

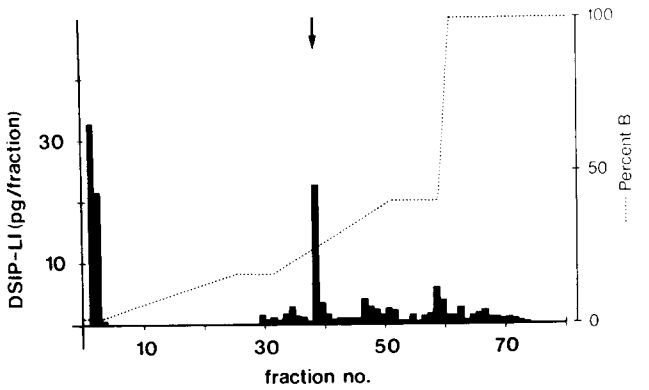

FIG. 3. HPLC of SF-DSIP-LI of rat pituitary. Same legend as with Fig. 2.

[163]. Levels in hippocampus, thalamus, and hypothalamus were between these values. Sleep-deprivation for 24 hr did not change the amount of DSIP-LI in rat brain. According to these authors, the antibody used for the enzyme-immunoassay was sensitive to the C-terminus of the peptide, and they speculated that it recognized only the 'free' form of DSIP [110,111]. Values in the range of 1–2 pg/mg were also found in human brain, but in this case, almost all DSIP-LI eluted as the large form from Sephadex G-50 [112].

In an additional attempt to better characterize DSIP-LI in brain, we extracted brain parts with acetic acid and the supernatants were separated on Sephadex G-100. The fractions representing the peak of small molecular size DSIP-LI were pooled and analyzed by HPLC. Determination by RIA revealed a marked peak of immunoreactive material at the position of DSIP indicating that 'free' nonapeptide is most probably present in thalamus [103], hypothalamus, and pituitary (Figs. 2 and 3)

A positive correlation was detected between the amount of DSIP-LI in thalamus and the time used to run through a 12-choice maze [103]. This is the first report describing a direct correlation between the content of DSIP-LI in a brain area with a distinct behavior. Though the content of an endogenous substance in an organ provides only static information, this result would support asssumptions of DSIP being involved in locomotor [54, 61, 74, 98, 108, 155, 157, 202, 245] and learning behavior [25,41]. Circadian changes in the level of endogenous DSIP-LI in plasma [43] are not necessarily directly related to activity.

Characterization of DSIP-LI in rat pineals was unsuccessful on our first attempt. An immunopositive peak was eluted with the front but no reactivity was found at the position of DSIP (unpublished data with A. J. Fischman). Whether this indicates that no DSIP is present in rat pineal or merely represents a poor preliminary separation remains to be determined. Another group was able to extract and partly characterize DSIP-LI from ovine and bovine pineals (I. Ebels, personal communication). Both forms of DSIP-LI, large and small, were detected in these organs.

DSIP-LI in Peripheral Organs

DSIP-LI was also determined in peripheral organs. In one study [186], the levels were between 0.6 (kidney) and 2.5 pg/mg tissue (muscle). These values were obtained using an antibody specific for 'free' DSIP, but the dilution curve of liver and kidney was not parallel to the DSIP standard dilution curve whereas that of brain was parallel. A further characterization of DSIP-LI in liver extract on Sephadex G-25 revealed a peak of unknown immunoreactive material in addition to a peak at the position of DSIP [186]. Strangely enough, the unknown immunoreactive material eluted later than DSIP from the column, suggesting a smaller form of DSIP-LI. It is not clear whether this represents an immunoreactive fragment of DSIP or an artifact. We have, for instance, observed that one of our antisera was sensitive to high salt concentrations (unpublished data).

In a different study [58], results opposite to those of Rozhanets *et al.* [186] were found when the immunoreactive material of liver eluted with the void volume from a column with Sephadex G-25. Only minor amounts of immunoreactive material were found to elute with DSIP and similar patterns were observed with spleen and jejunum. It is important to know that these results were obtained with an antibody (No. 604) that also recognized LF-DSIP-LI [62]. It could explain why the amounts of DSIP-LI measured in peripheral organs were between 90 pg/mg tissue in muscle to more than 800 pg/mg in stomach, well over 100 times higher than those reported by Rozhanets *et al.* [186]. It is not known whether the ranking of muscle, lowest in 'total' DSIP-LI [58] and highest in 'free' DSIP-LI [186] is of significance to this issue.

The values of 'total' DSIP-LI in peripheral organs seem rather high, especially when compared with the levels in brain measured with the same antibody [105]. They would suggest that, contrary to common belief, perhaps DSIP should be considered more of a peripheral tissue factor than a neuropeptide. This question, however, remains open since extraction of brain tissue with H_2O also produced values of more than 300 pg/mg wet weight tissue comparable to those in the periphery and at least 10 times higher than those reported after acid extraction. Similarly, lower values were found with peripheral organs when acid was used for extraction (unpublished data).

It was possible, however, to digest the LF-DSIP-LI of peripheral organs with trypsin and to produce small fragments without considerable loss in total immunoreactivity [58]. This indicated that the immunoreactive sequence of these fragments (the nonapeptide?) is contained in the LF-DSIP-LI and that the LF do not represent 'nonspecific-interfering' substances unrelated to the sequence of DSIP.

Puzzling was the observation that charcoal treatment of peripheral organ homogenates removed higher percentages of measurable DSIP-LI at more dilute concentrations [58]. Such a result might suggest some sort of equilibrium process. More puzzling was the finding that charcoal treatment of more concentrated homogenates, i.e., more than 2 mg wet weight tissue per ml, produced higher levels of measurable DSIP-LI compared with amounts before treatment [58]. Explanations for this phenomenon such as removal of protease inhibitors, 'hiding' compounds, or nonspecific interfering substances are feasible but, at the present time, only speculative.

DSIP-LI and Disease

The previous sections concerned determinations of endogenous DSIP-LI under normal conditions. Measurement

of levels of DSIP-LI in patients with distinct diseases should help to elucidate possible functions of the peptide. With respect to the effects of DSIP in alcoholic withdrawal [33], it should be mentioned here that chronic alcohol ingestion reduced the level of DSIP-LI in rat brain according to Burov *et al.* [23]. It is of interest that rats which were found to consume ethanol voluntarily had lower values of DSIP-LI in cortex and striatum than those refusing to drink ethanol [24]. It remains to be determined if this occurs in humans. Increased levels of DSIP-LI as well as P-DSIP-LI were detected in CSF of Alzheimer patients as compared with normal controls (G. A. Schoenenberger, personal communication).

Plasma levels of DSIP-LI in 13 primary insomniacs were apparently lowered by repeated injections of the peptide whereas the amount of sleep was increased [199]. Sleep latency in these patients was reported to be positively correlated with higher DSIP-LI values before treatment whereas no such relation was indicated for the post-treatment period. The finding that treatment with a compound lowers the endogenous level of the same substance implies a negative feedback. Such a result could indicate a fine regulatory mechanism controlling the level of endogenous DSIP compounds.

This would suggest the possibility that in chronic insomnia, Alzheimer's disease, and perhaps alcoholism, increased levels of DSIP-LI in plasma or CSF are related with the disorder. The decrease observed in the brain tissue of alcoholic rats [23], might indicate a lack of correlation in levels of brain and plasma [15] but other explanations are possible. Regardless, a correlation in both levels was found when the measurement was made after the injection of exogenous DSIP, indicating a nonspecific permeability through the blood-brain barrier (BBB) [14].

Another piece to the puzzle was added by Lindström *et al.* who reported significantly decreased amounts of the peptide in CSF of schizophrenics (80%) and depressives (85%) compared with controls (100%) [136]. In a different report, they found even lower levels, 58 and 56% of controls [236]. There was an apparent weak correlation between the concentration of DSIP-LI and sleep disturbance in schizophrenics [136], whereas no such correlation was found in depressed patients [236]. The authors concluded that the decrement was nonspecific and supported the view of more general functions of DSIP [136]. The apparent difference in the results concerning schizophrenics and depressives between our unpublished findings of normal values and those of Lindström (decreased levels) may be explained by different RIAs with their inherent problems as discussed in an earlier section.

Still it is possible that altered DSIP levels reported for different diseases might indicate roles of the peptide in the normal functioning of the body. This is consistent with the experience that effects of DSIP appear more marked under disturbed conditions [52, 53, 57, 199, 200, 204, 205].

GENERAL CONSIDERATIONS ABOUT THE NATURAL OCCURRENCE OF DSIP-LI

The last two years have brought about many contributions to the question of the natural occurrence of DSIP. The picture is still complex and puzzling, but it seems safe to conclude that DSIP does occur naturally as the nonapeptide. Analogs like P-DSIP also may exist. It is unknown if there is a fundamental difference in the function of these two peptides. At least their degradation rates appear to be different which may account for the higher potency of P-DSIP when compared with the effects of DSIP (see later).

Besides the small forms of DSIP-LI, which probably represent the nonapeptide in many cases, large molecules apparently bearing the DSIP-sequence were found in plasma, CSF, urine, milk, peripheral organs, and also, in some instances, in brain extracts [36, 38, 56, 58, 60, 101, 103, 111, 112, 186]. Large discrepancies in the levels of DSIP-LI measured in different laboratories are most probably due to peculiarities of the respective antibodies that do not recognize all forms of the peptide.

Whether DSIP or an analog, like P-DSIP, is the naturally active compound still remains open. It is also possible that the LF-DSIP-LI represents neither a precursor nor just a 'reservoir-molecule' but the effective 'DSIP-compound.'

OTHER ASPECTS

STRUCTURAL CONFORMATION OF DSIP

Soviet researchers using fluorescence, Laser-Raman spectra, and nuclear magnetic resonance spectra have actively explored the spatial structure of DSIP and also performed structure-activity studies [170]. In 1982, Akhrem *et al.* [2], based on theoretical calculations, suggested that DSIP (in an aqueous environment) shows two turns in its sequence, namely at Gly^3-Gly^4 and Ser^7-Gly^8, as well as interactions between Ala^2 and Asp^5 and between Trp^1 and Glu^9. Popov and colleagues [176] theoretically calculated the form of DSIP with the lowest energy. The most probable to occur under natural conditions was found to be a folded conformation (pseudocyclic) with the two end amino acids, Trp^1 and Glu^9, close to each other. Asp^5 appears to have an important function for stabilizing the conformation of the peptide, which in a basic or acid environment exists in a more stretched form, but still with β-turns such as between Ala^6 and Glu^9 [161].

As a consequence, Mikhaleva *et al.* synthesized a cyclic analog of DSIP with glycine between Trp^1 and Glu^9 to reduce the high conformational flexibility of the nonapeptide [154]. This cyclo-Gly-DSIP (cGDSIP) proved to be more potent than DSIP itself [153] in inhibiting the spontaneous firing of neuron V 17 of the snail Helix lucorum [152] and also in other paradigms (see later). Whether a particular function of the peptide can be connected with a distinct spatial structure as proposed by Popov [175] remains to be determined. Other structure-activity studies have been performed by a Chinese group, but only with respect to the amino acid sequence of DSIP [138,243].

BLOOD-BRAIN BARRIER

Recent results revealed that the crossing of the BBB by DSIP and analogs [99, 104, 106] is not competitive and probably nonspecific [9,14]. It is, therefore, not surprising that plasma and CSF levels of DSIP were significantly correlated after injection. The contrary appeared to occur for vasoactive intestinal peptide or calcitonin, indicating different ways of penetrating the BBB [9], and specific transport systems for peptides out of the brain have been described by the same investigators.

High doses of aluminum were shown to increase the penetration of ^{125}I-N-Tyr-DSIP through the BBB [8]. Aluminum, as a trivalent metal, could conceivably damage the wall of blood vessels if—for instance by a natural malfunction or lack of transferrin—the metal accumulates in plasma in an

unbound form [224], and then DSIP might leak into the brain. Recent investigations with different substances revealed, however, that aluminum may enhance permeability of membranes to lipophilic material and not just the leakiness of the BBB [10].

The effects of aluminum in decreasing the levels of phosphorus in blood appeared not to be mediated by phosphorus metabolism or to affect the levels of acetylcholinesterase in blood and brain [11]. It remains to be elucidated whether under such harsh conditions peptides still are capable of influencing behavior and also whether a possible circadian change in penetration of the BBB by DSIP [15] is of physiological significance.

METABOLISM OF DSIP

The complex interactions of DSIP with plasma components may be responsible for another unresolved problem: the metabolism of DSIP in blood. After IV injection of ^{3}H-DSIP into rats, radioactivity in blood decreased to about 50% within 15 min and further diminished during the next 45 min. At the same time, radioactivity in different organs increased with two notable exceptions, the pineal gland and the pituitary, where radioactivity also decreased over time [57]. In most organs, including blood, hardly any free ^{3}H-DSIP was recovered after only 5 min (unpublished data with H. P. Lorez and G. A. Schoenenberger). Kato *et al.* found that synthetic DSIP injected IV into dogs, rats, and monkeys disappeared with half-lives of between 2 and 4 minutes, although their antibody also recognized the 2–9 analog which is considered the first metabolite [110].

These results are partly consistent with the earlier observation that DSIP was metabolized in brain homogenates with a half-life of 15 min [85]. Van Dijk *et al.* [233] recently found similar values with preparations of brain membranes that corroborated those results. It seemed reasonable to assume that a crude homogenate would produce the fastest degradation, but according to recent results, plasma or serum appear to be more efficient in degrading the peptide. However, interactions of DSIP with plasma factors, as mentioned before, could also partly explain the fast disappearance of DSIP in blood when measured by RIA.

To further elucidate this problem, another series of experiments was carried out [69]. ^{3}H-DSIP (labeled at Trp^{1}) did not markedly change its elution position on Sephadex G-25 compared with the standard (^{3}H-DSIP in eluant alone) when mixed with serum or plasma for different times in vitro. A rapid degradation of DSIP occurred that was time- and temperature-dependent. Incubation of the peptide with rat plasma at 37°C cleaved ^{3}H-Trp^{1} with a mean half-life of less than 5 min which is comparable to the value reported by Kato *et al.* [110]. In accordance with these authors, we found slower degradation of ^{3}H-DSIP at 37°C in human plasma ($t^{1}/_{2}$=7–8 min) compared with rat plasma ($t^{1}/_{2}$=3–4 min), serum and plasma showing similar values.

When compared with DSIP, ^{125}I-N-Tyr-DSIP was degraded more slowly and ^{125}I-N-Tyr-P-DSIP was found to form aggregates immediately and to be quite resistant to breakdown which apparently required dephosphorylation as the first step. Whether aggregation or degradation takes place may depend on conditions that are still elusive. The considerably longer half-life for P-DSIP compared with DSIP [37] could also explain why P-DSIP seems to be more potent than DSIP in eliciting effects [53, 54, 64, 191, 193].

From these metabolic studies ([69,110], unpublished data), it can be assumed that DSIP is very rapidly degraded by cleavage of the N-terminal Trp, whereas the degradation of P-DSIP is probably slower and apparently requires dephosphorylation as the first step. However, NaF or bromotetramisol, nonspecific phosphatase inhibitors [20], were virtually without effect in rat plasma in preventing dephosphorylation (unpublished data with B. Sägesser) whereas in brain homogenates, dephosphorylation of P-DSIP was considerably slowed down by these compounds (A.M. A. Van Dijk, personal communication). Hence, it can be speculated that the synthesis of DSIP involves phosphorylation of a large precursor molecule. Phosphorylation of neuropeptide-precursor molecules is regarded as a potential control of peptide processing and function [179]. In a second step, P-DSIP would be removed and subsequently dephosphorylated, producing DSIP which in turn would rapidly be degraded to Trp and the 2–9 fragment ([69, 85, 143, 233], unpublished data).

DSIP-'RECEPTOR'

It is now widely accepted that "receptors" (=specific binding sites) are required for a biological function of a given compound. Presumably, this is also true for DSIP. However, reports about binding sites for DSIP are scarce. In a study [80] where ^{3}H-DSIP was incubated with cells cultured from rat brain stem and visualized by autoradiography, it was shown that the label bound to neurons and not to glial cells. The binding appeared to be specific since it was displaced by cold DSIP. Hösli *et al.* [81] reported that a small number of brain stem neurons generally showed binding sites for the peptide and the amount of binding was largely variable. Silver grains were found mainly over the soma and primary dendrites of the neurons. ^{3}H-DSIP was apparently not degraded even after one hour incubation, and addition of puromycin or 1,10-phenanthroline to prevent enzymatic degradation did not change ^{3}H-DSIP binding [81].

Rapid degradation of DSIP in brain homogenates was reported earlier [85,143] and was also found in rat brain membrane preparations (P2-pellet) [232]. The degradation of ^{3}H-DSIP to ^{3}H-Trp and fragments of the peptide emerged as a major obstacle in attempts to characterize specific DSIP binding sites in rat brain [233]. Displacement of ^{3}H-DSIP with an excess of DSIP was strongly correlated with displacement by Trp alone. Inhibition of degradation by physicochemical (pH, temperature) or biochemical means (enzyme inhibitors) also reduced binding of ^{3}H-DSIP in a parallel manner, suggesting that the active site of the enzyme and binding site for DSIP were localized close to each other.

In order to resolve this dilemma, Van Dijk *et al.* [233] used DSIP(2–9) as the displacing compound since it could not produce Trp and thus should not displace ^{3}H-Trp. Any difference in bound radioactivity produced by an excess of des-Trp-DSIP was likely due to displacement of the labeled N-terminus of the peptide. Since degradation of the C-terminal part of DSIP is slow [233], displacement by DSIP(2–9) would reveal 'true' DSIP binding sites. Though this displacement was also inhibited by enzyme inhibitors, the authors could show a specific displacement of ^{3}H-DSIP in rat brain membranes with a Kd of about 15 μM and a Bmax of 260 pmol/mg protein [233]. The affinity seems very weak for a peptide exerting effects at doses of 6 nmoles (ICV) or 30 nmoles (IV) per kg. Displacement with the nonapeptide revealed a binding site with a Kd of approximately 6 μM which still may not be the true value since

degradation strongly interfered. Nevertheless, Van Dijk *et al.* [233] concluded that their results provide convincing evidence for specific binding sites of ^{3}H-DSIP different from ^{3}H-Trp binding sites in rat brain.

However, the strong positive correlation between binding and degradation of ^{3}H-DSIP suggests a functional and anatomical relationship between the two processes. It is even possible that the 'binding site' represents merely the active site of a DSIP-degrading enzyme, in this case an aminopeptidase. Also unusual is the difficulty of reversing the binding when an excess of unlabeled peptide was added at least one hour after the labeled peptide. This could point to an uptake mechanism either for DSIP or for the labeled tryptophan. Such a suggestion is supported by the finding that imipramine strongly inhibited binding of DSIP (Van Dijk, personal communication). The results and different preliminary data render the question of a 'binding site' for DSIP very complex and any characterization of a 'DSIP-receptor' will need more work.

We found a protein fraction in plasma (unpublished results with B. Sägesser and G. A. Schoenenberger) that exhibited marked enzymatic activity with DSIP, cleaving the N-terminal Trp. Preliminary data suggest the existence in plasma of an aminopeptidase that appears to be different from DSIP-degrading enzymes in brain membrane preparations with regard to activity as well as to the spectrum of inhibition [69,233]. No binding of ^{3}H-DSIP or ^{125}I-DSIP in rat brain or in bovine brain membranes was found by Rozhanets *et al.* [186], although these authors apparently used several enzyme inhibitors and different conditions of incubation.

Based on reports that naloxone inhibited DSIP-induced sleep [220,248], a possible interaction of the peptide with some opiate receptors was investigated but the results were negative ([193], J. Zadina, personal communication). The possibility of DSIP modulating adrenergic receptors (see later) could open further dimensions with respect to a 'DSIP-binding site.'

Sleep-induction by benzodiazepines was proposed by Mendelson *et al.* [151] suggesting a receptor-mediated function, and a role in the mammalian circadian system has recently been assumed for these compounds, too [225]. DSIP had no direct effect on ^{3}H-diazepam or ^{3}H-flunitrazepam binding but 10 nM DSIP apparently shifted the affinity constant of chlordiazepoxide, displacing ^{3}H-diazepam towards lower values comparable to GABA [217,218]. Rat brain membranes used in these studies showed more consistent responses when they were pretreated with Triton X-100 (unpublished results with G. A. Schoenenberger). It has not been ascertained whether DSIP also could influence GABA-binding. The peptide was considered a candidate for such a function [173]. A correlation between an anxiolytic action of DSIP and displacement of ^{3}H-diazepam binding was not observed.

EFFECTS OF DSIP

CIRCADIAN RHYTHMS

A circadian rhythm in the level of DSIP-LI in brain and plasma has been found in two different studies [15,43], and influences of DSIP on circadian cycles were described several years ago [52, 54, 55, 68, 73, 199, 203]. Additional support for such influences was obtained when injection of DSIP and melatonin or the combination of both compounds apparently reduced body temperature in a time of day dependent manner, the combination being most effective [247]. In hypophysectomized rats, only melatonin and in pinealectomized animals, only DSIP effects still showed a circadian time dependency. The results suggested that melatonin acted through the pineal whereas DSIP acted through the pituitary [247].

Reduction of amphetamine-induced hyperthermia by DSIP was reported earlier [59,245]. It is not known whether in this experimental set-up a circadian component played a role. One possibility could be circadian changes of the adrenergic receptor [242], since the effects of amphetamine are most probably mediated through such receptors [3,28]. We have recently found direct evidence for an interaction of DSIP with adrenergic receptors that will be discussed in a following section.

ELECTROPHYSIOLOGICAL EFFECTS

In 1982, Normanton and Gent reported direct stimulating and inhibiting effects of DSIP on electrophysiological reactions of neurons in the nucleus gigantocellularis [167]. Recently, Trazcyk and Kosinsky measured hippocampal electrical activity in freely moving chronically implanted rabbits [223]. These authors applied trains of modulated electric pulses to the midbrain reticular formation, thus maintaining a state of EEG threshold continuous arousal pattern (TCAP) at the same time modulating by negative feedback the stimulating pulses when theta rhythms of hippocampal electrical activity appeared. Administration of DSIP, 2.5 and 25 nmol ICV, produced a dose-dependent increase in the amount of energy maintaining the TCAP up to 48 percent after 75 min, but only in the 4–7 Hertz range. The authors concluded that DSIP affected ascending brain systems involved in generating hippocampal theta activity [222].

A specific, significant increase in delta wave electrical activity was found in the brain of rats after IP injection of DSIP [155]. D-Ala^{4}-DSIP-NH_2 (80 μg/kg) produced a similar but more pronounced increase of delta waves at the same time strongly enhancing theta activity. Locomotor activity was clearly reduced by the analog whereas DSIP itself showed no effect on this parameter. Since D-Ala^{4}-DSIP-NH_2 crosses the BBB more readily than DSIP [99], an increased penetrance of the BBB by a DSIP analog may thus indicate a higher efficiency in central effects [155].

In a different study, excitation was found to be mainly induced by DSIP in several brain areas including hippocampus as well as thalamus where only neurons with large receptive fields were affected, half of them excited the other half inhibited [168]. The finding that DSIP can affect hippocampal electrical activity is noteworthy, because Constantinidis *et al.* [26] have shown DSIP-LI in and between neuronal bodies of the hippocampus, in area CA3, pyramidal layer and gyrus dentatus, and Feldman and Kastin found DSIP-LI in the area adjacent to CA1 [41], a major efferent pathway of the hippocampus. This suggested to them possible functions of DSIP in the regulation of behavior and learning. The hippocampus has recently been discussed as an organ with memory functions [219].

It was mentioned earlier in this review that after IV injection of ^{3}H-DSIP into rats, the granular and the pyramidal layer of the hippocampus were preferably labeled (unpublished data with H. P. Lorez and G. A. Schoenenberger). At least in one study, when DSIP-LI was measured by RIA [186], hippocampus exhibited the highest amount of immunoreactivity. It seems, therefore, that this brain area may possess a special relationship with DSIP. Whether this rela-

tionship includes circadian rhythms as noted with hippocampal theta activity [239], is unknown.

DSIP was apparently the only neuroactive substance to mimic inhibitory effects of neurodepressing hormone (NDH) on the electrical activity of crayfish neurons (H. Arechiga, personal communication). Higher concentrations of DSIP than those calculated for NDH were seemingly necessary, but it should be noted that NDH is most probably involved in the regulation of circadian rhythms such as electrical activity of motoneurons and, as a consequence, locomotor activity of crustaceans [5].

SLEEP IN ANIMALS

During the last two years, a few reports were published where no sleep-inducing effect of DSIP was found. Grinjavichus and Milashus [75] could not detect any somnogenic activity of the peptide in rabbits after IV injection and Obal *et al.* did not observe sleep effects in rats after infusion of 7 nmol/kg DSIP ICV [169,171]. Brain temperature in the rats was not affected and an analog, ω-amino-caprilyl-DSIP, also failed to change any of these parameters. Rather, an increase of wakefulness was observed 6–9 hours after the injection of either peptide [171]. An increase in wakefulness with a concomitant decrease in light SWS and REMS was found by Sommerfelt [260] in cats after IP injection of 30 nmol/kg DSIP.

Negative results of DSIP on sleep also were published by Kovalzon and coworkers [118,119]. However, these authors additionally tested other analogs like D-Trp^1-DSIP, D-Tyr^1-DSIP, and D-Ala^2-DSIP which they found to induce a marked increase of mainly SWS at an optimal dose of 7 nmol/kg ICV. Based on their observations, they concluded that prevention of enzymatic degradation of the peptide as well as an intact flow of CSF to reach the fourth ventricle seemed essential for the actions of the peptides. Intravenous administration (3–300 μg/kg) of DSIP analogs were without activity, an effect opposite to that of MDP analogs tested in similar experiments that were effective after IV but not ICV administration [118]. Different effects on sleep and wakefulness of rats were found depending on the time of injection (before lights on or before darkness), suggesting a modulatory role of DSIP on rodent sleep [119].

Results similar to those of Kovalzon *et al.* [118,119] were observed by Obal *et al.* when they injected DSIP and D-analogs ICV at the beginning of the dark period [170]. At the same time, D-Trp^1-DSIP(1–6) immediately produced wakefulness at the expense of NREMS.

In contrast to these partly negative results obtained with DSIP, a majority of reports showed positive effects of the peptide. Ursin [229,230] measured a marked increase in SWS and total sleep in rats, whereas Ye *et al.* [244] found enhanced delta and sigma activity in rabbits. Alyautdin and colleagues observed a strong (230%) increase of delta waves induced by DSIP in unrestrained rabbits for about one hour and comparable effects induced by D-Phe-DSIP [4]. Increase of delta EEG activity in some rabbits was reported by Mikhaleva [152], whereas Susic and Masirevic observed enhanced total sleep (TS) in PS-deprived cats [211]. According to their results, TS was significantly increased due to increased duration of deep SWS at the expense of light sleep and wakefulness [212].

In sleep-deprived rats, Mikhaleva [152] found a sleep-inducing activity of DSIP and a considerable increase of survival as well as stabilization of blood pressure when the animals were subjected to emotional stress. A cyclic derivative, cGDSIP, synthesized as a result of the structural analysis of DSIP, showed the highest activity [2, 154, 176]. It was concluded that the biological actions of the peptide apparently depend on the status of the animal and that they are stronger under disturbed than normal behavioral conditions, as we suggested in our previous review [57].

Electroencephalographic (delta) and behavioral sleep in adult cats and rats was also observed by Karmanova and coworkers [97], but a substantial lagtime (50–90 min) in the onset of PS led these authors to the conclusion that natural sleep and that induced by DSIP were not equivalent. A similar conclusion based on different results was drawn by Demin *et al.* [31] when they evaluated sleep induced by DSIP injected into rat brain. They reported that the induced sleep was not accompanied by the accumulation of proteins and RNA in the supraoptic nuclear glia as it was in natural sleep. Cytoplasmic volume of the neurons in this area was reduced, a phenomenon which, together with the lack of protein synthesis, may indicate a phylogenetically older type of rest such as occurs in lower vertebrates [31].

An intriguing insight into the sleep-inducing effects of DSIP was made by Inoué and his colleagues [89]. They infused DSIP (and other sleep-substances, see section II) into the third ventricle of freely moving rats for 10 hours either from 0700 to 1700 hr or from 1900 to 0500 hr. Recordings included a complete analysis of cortical EEG, neck EMG, and locomotor activity. DSIP, 2.5 nmol/kg, produced a rapid increase in SWS and PS which lasted for almost 8 hr of the dark period but disappeared during the infusion of the peptide [89]. Other sleep substances were tested under the same conditions and produced comparable but distinct effects with regard to the extent of the increase and the time of duration [89]. None of the compounds markedly enhanced sleep in rats during the light period [90].

The disappearance of enhanced sleep 8–9 hours after the start of the infusion [89] was not observed with the other sleep-factors, for which the effect lasted at least until the end of the infusion. This points to a different type of regulation for DSIP. Structures responsible for effects of the peptide seemed to be sensitive only during certain time periods over the 24 hr cycle. Varying efficiencies of DSIP depending on the time of day have also been observed in other studies [52, 64, 197].

An increase in spindle-dominated light NREMS was found in rabbits by Scherschlicht *et al.* [193,194] when DSIP was injected SC at doses of 1 and 3 mg/kg or 25 and 250 μg/kg IV, but not with 0.3 or 10 mg/kg SC. In cats, 100 μg/kg DSIP SC increased total sleep, NREMS and REMS, in a manner comparable to 25 μg/kg IV whereas in both species, administration of 250 μg/kg DSIP IV was ineffective. P-DSIP showed an effect at 5 μg/kg IV which was similar to 25 μg/kg DSIP IV in cats, whereas 25 μg/kg P-DSIP IV was without effect in this species [191].

Thus, both peptides were found again to exhibit an optimal dose-response relation with higher doses not being effective. At the same time, the results made clear that different forms of administration can be used. The optimal dose was dependent on the route of administration, from about 5–10 nmol/kg ICV, 20–40 nmol/kg IV, to about 100–200 nmol/kg SC or IP. Although similar results have been found with different paradigms [57, 59, 63, 64, 71, 72, 74, 98, 108, 159, 174, 191, 193, 195, 199, 202, 220], these doses seem not to work under all conditions. For instance, SC injection of 0.3 mg/kg in rabbit was without effect, whereas 1 mg/kg SC

increased sleep [194]. Since the possibility of several 'windows of active doses' [59] exists, many doses have to be tested before definitive statements can be made.

Recently, Young and Key [248] reported a significant increase in both REMS and NREMS at the expense of waking when DSIP was injected into the lateral ventricle of the brain of rats in 4 sequential doses of 5 μg during the dark period. The effects were blocked by pretreatment with naloxone, corroborating similar findings by Tissot [220]. Increased delta wave electrical activity after IP injection of D-Ala^4-DSIP-NH_2 [155] has already been mentioned in the previous section.

With the various results obtained with DSIP in different laboratories, it seems reasonable to conclude that the peptide can affect sleep in different mammalian species. However, the conditions that limit the consistent demonstration of the effects remain elusive. Some of these factors were mentioned elsewhere [52]. Regardless, DSIP appears to induce delta-wave EEG [4, 89, 95, 97, 152, 155, 197, 211, 244] but at the same time to increase sigma-stage [191,244], light NREMS [191], and also PS [89, 174, 191, 197, 201, 211]. Decreases in wakefulness [130, 212, 230] or locomotor activity [54, 98, 108, 157] have been reported and influences on sweating [33], blood pressure [33,66], and heart rate [33] additionally suggested peripheral effects of the peptide without an anxiolytic action [191]. P-DSIP and other analogs, substituted primarily with D-amino acids to inhibit enzymatic degradation or with an additional Gly to stabilize the presumably preferred folded conformation of DSIP in solution, revealed equivalent or even superior effects compared with the parent peptide [4, 108, 152, 155, 170].

The different DSIP analogs used in these studies were synthesized in various laboratories. It is not known whether discrepancies among different research groups concerning dose or effect of the peptides were due to differing conditions of purity.

The sleep activities of DSIP and its analogs are so broad that no obvious or common feature emerges. With subtle effects or sometimes no action at all for these peptides, it could be speculated that either sleep regulation is not the main task of these peptides or that DSIP does not represent the most active form. Support for the first assumption is obtained by the subtle effects on sleep that are sometimes missed and by the additional activities of the peptide observed with paradigms not directly related to sleep and discussed in the following sections. The second assumption would be supported by the finding of more active analogs like P-DSIP [53, 54, 64, 72, 191], D-Ala^4-DSIP-NH_2 [108,155], and others [152,170]. It is also conceivable that both possibilities exist, whereby a more potent endogenous peptide related to but not identical with DSIP predominantly influences a different parameter than sleep. However, support for this possibility is lacking. Since the regulation of sleep probably requires an extremely fine tuning of different body functions, the effects of DSIP may not be apparent under 'normal' conditions but seem more effective in normalizing a disturbed condition.

EFFECTS OF DSIP IN HUMANS: THERAPEUTIC POTENTIAL

During the last few years, all studies concerning effects of DSIP in humans have examined processes with therapeutic potential. Since the peptide seemed to show greater activity in disturbed conditions, a therapeutic approach to the investigation of the actions of DSIP may be justified.

Sleep

Studies on effects of DSIP in chronic insomia have been conducted during the last two years in at least 4 different clinics. From two of them [48,77] results have been presented at a meeting, but they have not been published so far.

Schneider-Helmert reported effective treatment of chronic insomnia with repeated administration of DSIP [196]. The patients were treated in a double-blind cross-over design with placebo injections before and after the treatment period which consisted of DSIP injections, 30 nmol/kg IV, one hour before bedtime on 7 successive days. Evaluation of the polysomnograms and performance tests of 14 chronic insomniacs revealed an increasing effect of DSIP from night 1 to night 7 with respect to total sleep time (+22.5% final) and sleep efficiency (+26.8%) as compared with baseline. The treatment reduced sleep latency by 53% and awakening after sleep onset by 45%, whereas the performance tests during daytime significantly (Wilcoxon matched-pairs signed-rank test) improved. These effects decreased only slightly the first day after discontinuation of treatment with DSIP [199]. In two different groups of patients, the same beneficial effects were still observed one week after treatment and, in the group with a mean age of 71 years, the substantial increase in TS after treatment was even further enhanced one week later [200].

The results led Schneider-Helmert to the conclusion that DSIP is a somnogenic agent without central nervous system sedation and to the assumption that DSIP might have a modulating function on sleep and waking. A short review of different clinical studies performed by this author using DSIP in the treatment of chronic insomnia was published in 1984 [197] and a more detailed report in 1985 [199]. He concluded that DSIP is capable of normalizing sleep patterns in humans suffering from severe insomnia. The effect was enhanced with repeated injections once a day, whereas two injections per day may have produced adverse reactions. The results further suggested to Schneider-Helmert that DSIP on the one hand may have a pacemaker function for the circadian sleep-activity rhythm and on the other hand that the peptide seems to regulate influences on the rhythmic organization of sleep [197]. Similar conclusions have been drawn already in other studies [68, 73, 203, 205].

These hypotheses led the same author to treat a male narcoleptic with the peptide [198]. Repeated injections of DSIP either in the evening or in the morning improved daytime activity and performance and reduced the frequency of sleep attacks. Night sleep was compressed but improved according to subjective evaluation of the patient. Although the results obtained in this study are very intriguing, they should be judged with extreme caution because it was an open study and only one subject was involved. However, Schneider-Helmert attributed a major part of the improvement to the effects of the peptide [198].

Taking all the results into account, Schneider-Helmert believes that the activating effects of DSIP are not mediated via improvement of sleep but due to neuromodulation of behavioral coordination [199]. Similar assumptions were made by Constantinidis *et al.* [25] and Feldman and Kastin [40,41] based on their immunohistochemical findings. As a working hypothesis, Schneider-Helmert feels that DSIP is needed for adaptive processes serving 'ego functions' [199].

Treatment of severe insomnia by DSIP, 10 injections within 14 days, normalized sleep in 6 out of 7 patients as reported by Kaeser [94] in a different study. In this case, the

treatment remained effective during the follow-up period of 2–7 months. Mood and performance during daytime apparently improved and the best results were obtained after 4 to 5 injections.

In a case of brainstem lesions due to hemorrhage, DSIP was applied successfully in the treatment of concurrent sleep disturbances [130]. Single injections each day increased the combined time of spindle, SWS, and REM sleep to 180% compared with baseline values before DSIP. There was a normalization of sleep stage distribution and improved motor performance in the third week that lasted after discontinuation of treatment, whereas total sleep slowly decreased again. Other substances such as 5-HTP + benserazide, L-DOPA + benserazide (Madopar®), and clonazepam (Rivotril®) were less effective or even provoked adverse effects [130]. It is not clear whether the increasing amounts or the continuous injections of DSIP were responsible for the stepwise improvement observed with each week of treatment. It should also be mentioned that the levels of DSIP-LI in the plasma of the patient were clearly higher before treatment than afterwards, a result also observed with insomniacs in a different study.

It is possible that a comparison of the results of DSIP treatment in disturbed human sleep with the effects of DSIP in animals would reveal that human beings respond better to the peptide. Such a conclusion, however, would be premature, because the number of clinical studies carried out until now is too small, placebo effects may account for positive findings, negative results are not readily published, and failures in treating humans are also known (unpublished data). Although the majority of the available data indicate a positive effect of DSIP on sleep in man, many questions still remain open. For instance, why does the time of day of injection not substantially influence the effects (which apparently include circadian components), whereas two injections per day seem to do so [200]? Why is an elevated level of endogenous DSIP-LI reduced by injection of DSIP which at the same time normalizes the disturbance [130,204]? What explanation is there for the fact that the same substance can enhance sleep and, in a narcoleptic, reduce attacks of sleep [198]? Stabilization and enhancement of the circadian amplitude may contribute to the results [199] but do not seem to provide the full explanation.

Pain

Sleep disorders like chronic insomnia or narcolepsy were not the only disorders to be successfully treated with DSIP. Larbig *et al.* [133] reported that 6 of 7 patients suffering from chronic, pronounced pain episodes responded well to therapy with DSIP. The different types of discomfort included migraine headaches, vasomotoric headaches, tinnitus, and foot pain of psychogenic origin. The patients were injected with 30 nmol/kg DSIP IV once a day for 5 days followed by 5 additional injections at intervals of 2–5 days. They described their pain intensity on a visual analog scale and a questionnaire that assessed mood, stress, and other psychological reactions. The statistical evaluation was individually based on the baseline before treatment. With the exception of one patient with migraine, all others reported reduced pain and improved mood that persisted during the catamnestic period of up to 91 days [133].

Thus, in accordance with other studies [201,205], an effect of DSIP on awake behavior, such as better psychomotor performance and ability to cope with stress, was observed. Mental suppression of pain appears to be reflected in an increase of the density and amplitude of the lower frequency theta band on the EEG [132]. It is not known whether this is related to the results observed with DSIP in the hippocampus by Traczyk and Kosinsky [222] mentioned already.

A major weakness in the study of Larbig *et al.* [133], as noted by them, is the lack of a placebo control group. It is especially true for this type of study where psychogenic factors play a most important role. Support for the assumption of an effect of DSIP on pain was provided by Nakamura *et al.* [165] in their description of a potent analgesic activity of DSIP in mice. In a different study, however, no such effect was observed [193].

The effects of DSIP probably should not be ascribed to antidepressant effects. In a model to test antidepressant activity [166], DSIP was injected IP into mice that were kept in a water tank for 6 min. The activity of the mice that tried to escape the water was registered by means of a water wheel. In the second 3 minutes, when imipramine and other antidepressants produced significantly increased rotations of the wheel [166], DSIP reduced that number, reaching significance at a dose of 150 μg/kg IP [98]. The beneficial effects of the peptide on depression that have been observed on several occasions [133, 199, 200, 201, 205] would seem to involve a different mechanism.

Drug Abuse

Another clinical use of DSIP with therapeutic potential was mentioned already in our previous review [57]: the treatment of withdrawal in alcohol and opiate addicts. The clinical trials were stimulated by the finding that sleep-effects of DSIP were inhibited by naloxone, an opiate antagonist [220,248]. A morphine-like action of DSIP was also assumed by Nakamura *et al.* [165]. They found a strong, dose-dependent analgesic effect of the peptide after ICV administration in mice. Analgesia reached a maximum within 5 min, disappeared 60 min after injection, and was abolished by pretreatment with naloxone.

A study with 41 opiate addicts and 47 alcoholics [33] showed that 97% of the opiate and 87% of the alcoholic patients were successfully treated with DSIP injected IV. The treatment with DSIP was considered superior to the usual treatments of withdrawal (J. M. Gaillard, personal communication). Apparently, the clinical symptoms disappeared or improved markedly and rapidly after administration of DSIP, whereas anxiety was slower to decrease [33]. It is unknown why opiate addicts generally needed more injections than alcoholics.

STRESS

When corticotropin-releasing factor (CRF) was injected IV into stress-blocked rats, DSIP in doses of 5–30 μg/kg administered together with CRF reduced the subsequent increase of corticosterone in plasma [63]. Since the peptide was not able to produce a similar attenuation of ACTH-induced stimulation, it was concluded that the interference of DSIP took place at the level of the pituitary. However, it is not known whether DSIP was interacting with a CRF, adrenergic, or other receptor site thought to be involved in the control of stress-sensitive corticosterone levels [6, 49, 213]. Nevertheless, these results added new support for a stress-reducing activity of DSIP that has been observed in different studies [195, 200, 201, 205, 209].

In the same samples [63], we also determined the amount

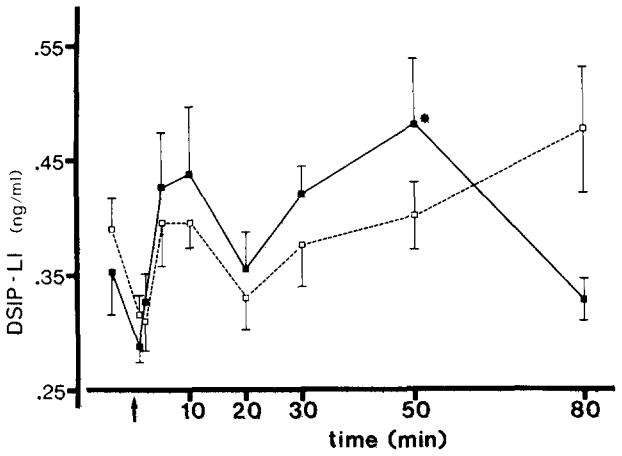

FIG. 4. Effect of ether stress on DSIP-LI in rat plasma. DSIP-LI was determined by RIA [60]. Arrow indicates the time-point when stress was applied. Each point represents the mean±SEM of 8 rats. Broken line and open squares: rats were injected IP with cycloheximide 30 min before stress, whereas saline-injected animals are depicted by the solid line and filled squares.

of growth hormone (GH) in plasma (unpublished data with W. Murphy). A slow but steady increase of GH over time was not significantly different from saline-treated rats compared with CRF-injected animals. However, 40 min after CRF administration, GH levels were significantly reduced by 1–30 μg/kg DSIP and enhanced by 100 μg/kg DSIP, suggesting a modulatory role of the peptide on GH secretion. This would support the observation made by Takahashi *et al.* [215] who found a GH peak 2 hr after intraventricular injection of DSIP.

Recently, Meerson *et al.* [148] found a clear, dose-dependent reduction of the stress reaction in mice after administration of DSIP. DSIP and cGDSIP were injected IP, 1.5 μg per mouse, and natural killer cell activity was determined 24 hr after the stress of immobilization in the dorsal position for 6 hr. The stress-induced decrease of natural killer cell activity was abolished by DSIP and completely reversed by the cyclic analog [147]. The effects of the peptides were compared with those of gamma-oxybutyric acid which was much less effective in the same experiment, although it can suppress the stimulation of the pituitary-adrenal system [146]. It was concluded that DSIP prevented the potentiating effect of stress on the development of tumor cells [210]. A modulation of macrophage-mediated tumoricidal activity by specific stress-related neuropeptides and neurohormones has also been reported by others [116].

In a different study, Meerson *et al.* [149] found a beneficial effect of DSIP and cGDSIP on right-atrial myocardial distensibility and contractility disturbances in left ventricular infarction. DSIP, 20–55 μg/kg, injected into rats one hour before infarction significantly limited infarct-related depression of distensibility and contractility and reduced atrial resistance to hypoxia and excess calcium. Whether the conclusion of these authors is correct that the protective effect of DSIP and cGDSIP consists in limiting the pain-induced stress response in the nonischemic regions remains to be proven. With regard to the blood-pressure (BP) reducing capacity [66] and the probable interaction of DSIP with adrenergic receptors (see below), direct influences of the peptide on heart physiology are also possible without necessarily involving the 'stress-axis.'

The same is true for the results reported by Badikov *et al.* [7]. Although DSIP (and other peptides) administered IV suppressed avoidance and self stimulation responses and inhibited cardiovascular reactions due to electrical stimulation of the hypothalamus, the effects could represent prevention of stress as well as a direct interaction with hypothalamic or peripheral adrenergic receptors.

DSIP has been reported to change body temperature of rats in a cold environment [245] which represents a stress situation. Rats kept for 3 days in the cold exhibit increases of extraerythrocyte hemoglobin and glucose-6-phosphate dehydrogenase activity in serum [17]. These reactions were apparently normalized by DSIP (15 μg/100 g body weight). Bondarenko *et al.* suggested that the peptide stabilized the erythrocyte membrane [17]. In the light of the different effects of DSIP on stress, it is possible that the normalizing action of DSIP was mediated via improved stress tolerance.

The influence of stress on the level of DSIP-LI in rat plasma was investigated in another study (unpublished data with A. J. Fischman). Almost no difference in the stress-induced changes of DSIP levels was found between morning and evening. However, the overall amounts in the evening were clearly higher (325±20 pg/ml) than in the morning (214±45 pg/ml; mean±SEM, n:8), corroborating earlier findings in normal rats [15,43]. Rats injected IP with saline or cycloheximide and exposed to a stressful situation showed slight but significant changes in the level of DSIP-LI in plasma, whereas animals treated similarly but without stress did not reveal any significant alteration.

Exposure to ether for 1 minute elicited a rapid decrease followed by an increase of the mean level of DSIP-LI within 1–15 min, followed by a second increase over the next 50–80 min (Fig. 4). The main influence of the time course after stress on DSIP-LI in plasma was significant, $F(8,136)=3.5$, $p=0.001$, whereas the interaction of the time course by pre-treatment only tended to be significant, $F(8,136)=1.77$, $p<0.09$. The decreased value 1 min after stress was significantly ($p<0.05$) lower than the scores after 5, 10, 30, and 50 min, the last also being significantly higher than the baseline value 4 min before stress. The changes of DSIP-LI after cycloheximide were similar but less pronounced than after saline pre-treatment. Only the value 80 min after stress was significantly ($p<0.05$) increased compared with the scores at 1 and 2 min and also compared with the level in rats 80 min after saline injection.

Prolonged stress such as immobilization in plastic cones for 120 min had a significant influence, $F(6,80)=3.05$, $p<0.01$, on the concentration of measurable DSIP-LI in rat plasma. A decreased level 30 min after the beginning of stress was significantly ($p<0.05$) lower than at 90 min for saline-treated and at 60, 90 and 120 min for cycloheximide-injected rats. In stressed rats, cycloheximide pre-treatment, therefore, did not produce marked differences in plasma DSIP-LI compared with saline controls, but inhibited the stress-provoked increase of corticosterone.

Thus, for almost 60 min, changes in the amounts of DSIP-LI in plasma of stressed rats were probably not dependent on protein synthesis. This assumption is consistent with the view of a DSIP-reservoir in plasma ([62], see earlier). Altered mean levels in some diseases as described in section IIIB7 could also be explained if stress changes endogenous levels of DSIP-LI with the disease being considered stressful.

A MECHANISM OF ACTION FOR DSIP: MODULATION OF ADRENERGIC RECEPTORS?

An indication for a direct relation between the adrenergic neurotransmitter system and DSIP was provided by Benson *et al.* [16]. They reported a markedly reduced norepinephrine turnover in hypothalamic median eminence after pretreatment of rats with DSIP and, 30 min later, α-methylparatyrosine (αMPT). The steady-state level of NE was not altered when measured 60 min after αMPT. The same authors detected a decreased (30% of baseline) level of luteinizing hormone (LH) in serum 30 min after injection of 25 μg/kg DSIP, but the episodic release of LH into plasma might have influenced the results [42].

We found evidence for an interaction of DSIP with adrenergic receptors when investigating the effect of the peptide on the activity of N-acetyltransferase (NAT) in rat pineal [64]. This enzyme exhibits a strong circadian rhythm [187] with a marked increase in activity at the beginning of the dark period. Klein *et al.* have reported that the enzyme is stimulated through postsynaptic β-adrenergic receptors, the effects of which are potentiated by α_1-adrenergic receptors [113]. Vacas *et al.* have further determined that both receptors are interactively involved in the regulation of rat pineal adenosine cyclic 3′,5′-mono-phosphate phosphodiesterase activity [231].

We observed an increase in nocturnal NAT activity that was significantly smaller in rats receiving DSIP (30 nmol/kg IV) than in controls [64]. Similarly, two analogs, P-DSIP and D-Ala^4-DSIP, diminished the natural increase of NAT activity at the same dose, P-DSIP showing a tendency to be effective at a lower dose, too. These results were confirmed in a recent investigation using α-adrenergic (phenylephrine) or β-adrenergic (isoproterenol) agonists, the effects of which were also reduced by the peptide in vivo [71]. The effective doses of DSIP (150 and 300 μg/kg) injected SC were in the same range as those reported by Scherschlicht *et al.* [193].

In vitro, when rat pineals were kept in culture for two days, DSIP inhibited the effect of isoproterenol alone or in combination with phenylephrine, but not the stimulation by phenylephrine alone. At concentrations of 20 to 300 nM, the peptide enhanced the stimulating activity of NE, whereas P-DSIP was effective at 2 nM, but not at 20 nM or more. An increase of the NE-induced stimulation by DSIP was still observed in the presence of propranolol, whereas prazosin apparently abolished that increase [72]. Neither the effects of forskolin nor those of dibutyryl-cyclic AMP were altered by DSIP.

This suggested that the action of DSIP was mediated through the α_1-adrenergic postsynaptic receptor. However, such an interpretation does not fully explain the different results. It is not known by what mechanism α-adrenergic receptors act synergistically with β-adrenergic receptors in this system [113], so that it is possible that DSIP could influence the 'link' between α- and β-adrenergic receptors.

DSIP can interfere with the serotonergic system [246], reduce stress [57, 63, 148, 195, 205, 209], and act as a morphine agonist [165, 220, 248], but an interaction of DSIP with adrenergic mechanisms has never been strongly considered as a mechanism of action. There are now several indications that DSIP can also affect events apparently mediated by adrenergic transmission.

Besides the probably direct action on adrenergic receptors regulating NAT activity in pineal [64,71] and NE turnover in the median eminence [16], it is likely that other effects of the peptide are also mediated through the adrenergic system. These would include the effects on amphetamine-induced hypermotility [74] and hyperthermia [59,245], the attenuation of CRF-stimulated corticosterone release [63], and protection against symptoms of stress [7, 148, 149, 195, 200, 205, 209, 210].

Stress produces an increase in corticosteroids not only through CRF-ACTH, but ACTH is also released by stimulation of α- and β-adrenergic receptors on the pituitary [6]. The increased secretion of CRF under stress appears to involve the release of NE with subsequent activation of α-adrenoceptors [49]. The catecholaminergic system as a whole may be the most important parameter in controlling adrenocorticotropic functions [213]. The reported beneficial effect on opiate and alcoholic withdrawal [33] may also be mediated through adrenergic pathways [129].

As outlined before, influences on circadian rhythms have been reported for DSIP. It is known that levels of epinephrine and NE follow a diurnal pattern [137] and that adrenergic receptors exhibit circadian changes in their maximal binding capacity [242]. Thus, variable effects of a modulating substance on these receptors would be expected over a period of 24 hours.

Even sleep effects of DSIP could be explained by a modulating role on adrenergic receptors. For many years it has been known that noradrenergic transmission can affect sleep stages, especially PS, and also SWS to some extent [32, 158, 177, 204]. The literature about these actions is rather controversial. Sleep experiments with specific adrenergic agonists and antagonists revealed that most probably stimulation of α_1-adrenergic receptors decreases PS while drowsy waking or light sleep is increased [177]. Stimulation of α_2-receptors seems to have a similar effect [114, 135, 207]. By contrast, β_1-stimulation apparently increases PS whereas β_2 stimulation decreases REM sleep [78,207]. Specific inhibition of the different receptor types mainly produced the opposite effect of stimulation [46,207].

However, it must be stressed that sleep is not as mechanistic as presented here; many other factors also are involved in the generation of natural sleep and even stimulation of the same receptor can produce different effects [172,238]. For instance, a strong stimulation of β-receptors increases PS at least in cats, whereas a weak β-adrenergic activity together with a strong α_2 stimulation produces enhanced drowsiness [78]. A well balanced interaction of the different neurotransmitter receptors seems of major importance for the appearance of natural sleep [32,207]. The sensitivity of certain receptors changing over time seems responsible for the appearance of special sleep stages. This could be the point where the effects of DSIP take place.

Considering the influences exerted on sleep through the different subtypes of adrenergic receptors, one could postulate that DSIP possesses an α-agonistic and a β-antagonistic activity. Evidently this assumption is too simple, although indications for both have been obtained. Despite good evidence for an interaction of DSIP (and P-DSIP) with the postsynaptic adrenergic receptor, no definitive conclusion can be drawn as yet regarding the subtype that is mainly involved. It seems that the peptide interferes with both. Furthermore, it cannot be excluded that DSIP interferes with the hypothetical interaction between the two receptors assumed to work in a synergistic way [113,231]. DSIP would then exert a regulatory function on adrenergic transmission.

There also might be an effect of DSIP on presynaptic receptors, although this was presumably eliminated by cul-

turing pineals for 48 hr [71,113]. Rozhanets and Anosov [185] tested the influence of neuropeptides on presynaptic regulation of the release of endogenous NE from synaptosomes during depolarization. The release of NE was significantly reduced by 10^{-5} M Leu-enkephalin but DSIP showed no effect. It remains to be determined whether the peptide only affects postsynaptic mechanisms.

Multiple activities of the peptide have been reported in sleep and extra-sleep events [57, 62, 159, 202] and it seems that a multiplicity of reactions are observed on a molecular level, as we have discussed in our previous review [57]. The pineal NAT stimulating system appears to be an appropriate tool for the elucidation of at least some of the interactions of DSIP with adrenergic regulatory systems.

Appropriate systems, however, may also be found to study other actions of DSIP. We have, for instance, investigated the influence of the peptide on growth of different cells in culture. After 9 days of incubation, the effect of DSIP significantly depended on the concentration of the peptide as well as on the cell-type involved. DSIP increased the amount of DNA/well by 30% compared with the control in T47D cells, and by 68% in ZR-75 cells at a concentration of 150 μg/l medium. With MCF-7 cells, no effect of DSIP was found, whereas with MCF-7 cells cultured in a serum-free medium, 30% increase of DNA was detected at a concentration of 30 μg/l (unpublished data with W. Küng).

Although serotonin still is considered a major sleep factor [158], its role as a sleep neurotransmitter is now being revised [189]. The monoaminergic theory of sleep was unable to withstand the test of time and sleep now appears to result from the concerted action of many different components [172]. Various neurotransmitter systems are known to interact with each other. For instance, NE stimulates serotonin secretion from neurons [235] and, conversely, serotonin axons exert a strong influence on β-adrenergic receptors in rat brain [208].

There is now general agreement that one neurotransmitter cannot be responsible for sleep. Jouvet, in a recent theory about sleep induction and maintenance, considers the existence of a peptide responsible for sleep induction (at least PS) as very probable [93]. It is, thus, conceivable that DSIP exerts distinct functions on certain subsets of sleep, but also serves as 'more than a sleep-peptide' [107], influencing many systems in the organism perhaps through the pineal [181].

BLOOD PRESSURE

If DSIP has an adrenergic modulating activity, the peptide can be assumed to also affect peripheral adrenergic receptors like those involved in vasoconstriction and blood pressure [131,142]. DSIP-positive immunoreactivity has been observed in connection with blood vessels in at least one immunohistochemical study [40]. We observed a patient with a mild secondary hypertension who was treated with DSIP for another disorder. When injections of DSIP began, medication of hypertension was stopped and blood pressure (BP) remained normal without treatment for the next 3–4 weeks, i.e., 2–3 weeks after discontinuation of DSIP (unpublished observation with F. Wicki).

When spontaneously hypertensive rats (SHR) were injected IP with 200 μg/kg DSIP, their BP was slightly but significantly decreased compared with saline controls [66]. Chronic SC infusion of DSIP, 200 μg/kg per day, lowered BP by 10% after one day and these rats showed no further increase over the next 10 days, a significantly different effect from that in the saline controls in which the BP rose steadily. These observations were supported by the finding that blood levels of DSIP-LI in SH rats were significantly higher than those of WKY rats [66] that are the genetic-matched controls of SHR. It represents another example for a disorder with a concurrent increased level of endogenous DSIP-LI.

Elevated BP, tachycardia, tremor, sweating, and lachrymation of opiate and alcohol addicts were also normalized by DSIP [33]. In one histochemical study [40], DSIP-positive neurons with connections to nuclei that control heart function and respiration were detected. Any causal relationship, however, has not been established.

DISCUSSION

The last few years have brought about several distinct advancements in DSIP research. First of all, the natural occurrence of DSIP as the nonapeptide has been demonstrated by different groups with high probability. A better understanding of the discrepancies regarding the amounts of endogenous DSIP-LI has been achieved. The different forms of DSIP-LI seem to be real and not merely artifacts. The accumulated knowledge in this field provides a basis for the final elucidation of the different forms and will help to clarify apparent immunohistochemical differences.

Another basic question, the main effects of DSIP, is still open for speculation. A large variety of different effects have been reported, many of them difficult to repeat. Even such effects that could be repeated often appeared subtle and not as robust as one would like.

For instance, treatment of insomnia in humans seems to yield positive results in almost every case. However, patients who respond well to therapy may have certain characteristics that are still elusive and, as outlined earlier, negative results are not readily published. Environmental conditions, dose, and form of application have also been shown to alter the effects of the peptide and many times no clear influence of DSIP was observed at all. Clearly, the results gathered by different groups until now are not sufficient proof for the effectiveness or usefulness of DSIP as a therapeutic agent. However, they certainly should be sufficient to stimulate further research with this peptide.

It should be emphasized that DSIP did not provide a significant effect in many cases. We like to stress this point, because we favor the view that the effects of DSIP are modulatory. Given recent evidence, a modulating interaction of DSIP with adrenergic receptors is possible. DSIP increased and decreased adrenergic actions at least in the case of induced NAT activity in the pineal [64, 71, 72]. Effects were observed in combination with other, active compounds. The peptide alone showed little if any direct action on the receptors. This is supported by the observation that effects of DSIP usually were more pronounced the further the conditions were from normal.

Another example of a modulatory action of DSIP may be the effect on chronic insomnia. The peptide induced sleep but, depending on the time of day, also more wakefulness [196,199]. The apparent positive influence on narcolepsy [198] would also support our assumption of a modulating interaction of DSIP with bioregulatory systems.

It is quite probable that DSIP has been misnamed, a fate that it shares with other peptides [100]. As such, a false name cannot be responsible for the neglect of the compound by investigators, but it represents a first and sometimes decisive step to a misleading concept [109]. The enhancement of delta

waves by DSIP appears to be just one of multiple effects, probably not even the most prominent one. This could also explain why no clear correlation between sleep-stages, sleep-deprivation, and the amount of DSIP-LI could be detected so far, although central levels were not well studied. More general disturbances may be related to DSIP-levels. It is also possible that other peptides, structurally related to DSIP but unknown as yet, may be biologically more important than DSIP.

In this review, the new contributions to DSIP research published during the last few years are discussed. The numerous reports describing a large variety of results have advanced the understanding of different problems concerning DSIP such as the natural occurrence of the peptide and possible mechanisms of action. Many questions, however, are still open. This, together with the sometimes provocative findings, should stimulate further research about DSIP such that the next few years should resolve many of the issues.

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