STUDIORUM PROGRESSUS

# The delta sleep inducing peptide (DSIP). Comparative properties of the original and synthetic nonapeptide[1]

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*Summary.* Both the original and the synthetic nonapeptide Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu enhance, in recipient rabbits, spindle and delta EEG activity as in orthodox slow wave sleep.

In 1963, humoral transmission of sleep was suggested by experiments with cross-circulation[4]. In a next step, rabbit donors, kept 'asleep' by electric stimulation of the ventrocentro-median intralaminar thalamus (somnogenic area of Hess), were submitted during 60 min to extracorporal dialysis of the occipital venous sinus blood under EEG control[5,6]. The hemodialysate thus obtained was first injected i.v. to rabbit recipients; later, the dialysate or its desalted fraction (factor delta) was infused into the meso-diencephalic ventricle of restrained or free moving rabbits[7-9]. This infusion induced electroencephalographic (EEG) and behavioral changes (reduced motor activity), suggesting orthodox slow wave sleep by contrast to paradoxical sleep. The peptide nature of factor delta, ascertained by subsequent purifications, its mol.wt (800–900), amino acid composition with 9 amino acids and presumably tryptophan as amino terminal, were reported in 1974[10,11] as well as its delta EEG sleep effect[12]. This report will deal first with the original DSIP from the last isolated fraction, its quantitative amino acid analyses, molecular weight, amino acid sequence, biological activity, effective dose and specificity. Its properties will then be compared to those of the DSIP synthetized in 1975.

*Methods and material.* I. Orig. DSIP. A. Biochemical methods. The amino acid analyses of the last fraction obtained from the occipital venous sinus blood of stimulated rabbits after 17 fractionation steps, were performed on a Durrum D-500 analyzer with buffers from Pierce. The quantification of the contaminating free amino acids was obtained by analyzing both unhydrolyzed and hydrolyzed (6 N HCl at 110 °C for 22 h under vacuum) portions of the original samples. Norleucine was added as internal standard prior to removing aliquots for either of the sample manipulations (detection limit 0.1 nmole). Trp as amino terminal residue was confirmed after tlc of the end product, by spectrofluorometry, UV-absorption at 280 nm, and disappearance of the DNS-compound after dansylation and hydrolysis[13].

Sequence. 3 different sequences were carried out with 50, 75 and 80 nmoles of peptide, using the subtractive Edman procedure. Dansyl derivatives at each step were identified by thin layer chromatography on polyamide sheets. Tryptophan as amino terminal was confirmed by eluting the purified dansyl peptide from the tlc plate and by the absence of any dansyl amino acid after acid hydrolysis[14].

B. Biological methods. The hemodialysates obtained from thalamic stimulated rabbits, prepared the day before[5,6,15], and their fractions were submitted to 82 EEG and behavioral bioassays on 'chronic' recipient rabbits[7,9,16]. As controls, hemodialysates from non-stimulated or sham-stimulated rabbits were used. The delta EEG activity was quantified with a calibrated automatic frequenzy analyzer in mm deflexion for the 2 and 3 Hz frequencies per time unit (10, 50, 300 sec). The mean integrated voltage of the calibration signal was calculated as the root mean square (RMS) of one-half its peak value. The cortical delta activities were thus expressed in RMS μV. The test consisted of a 20 min pre-infusion period (PrIP), followed by a 20 min infusion (I) (3.5 min) + post-infusion period (PoIP) (16.5 min), taken as reference for the subsequent 50 min period of specific activity (SpAP) (figure 2). The delta EEG values were summed over 5 min, calculated by a Univac computer system in RMS μV and plotted in μV on the ordinate, against the whole experimental time (90 min) on the abscissa. Furthermore, the time integrals, i.e. the activity summed over the time ± SE, served for calculation of the difference between the delta EEG activity accumulated over 90 min in the peptide group and the control group.

II. Synthetic DSIP. A. Biochemical methods. The syntheses of DSIP were performed by Bachem Chemicals Ltd (Liestal, Switzerland) according to our amino acid sequence, as well as those of long and short oligopeptides. Besides, 2 nonapeptide analogues, 1 with Tyr instead of Asp, and 1 with substitution of Arg to Gly and Gly to Asp,

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were synthetized in the Research Laboratories of Merck Sharp and Dohme, USA (Dr C. D. Bennett). The syntheses were carried out by conventional methods, using benzyl esters for protecting the side chains. All the intermediates were purified by crystallization and tlc electrophoresis, and identified by tlc on silicagel. (Reagents: Ninhydrin, Fluorescamine Roche, Ehrlich and Greif-Leadback.) A tripeptide Trp-Ser-Glu was synthetized elsewhere[17].

B. Biological methods. 11 rabbits were infused with DSIP and compared with 14 control rabbits. 24 rabbits received long oligopeptides and 12 short oligopeptides. The activity of all synthetic peptides was tested by intraventricular infusion of 6 nmoles/kg in 0.05 ml csf like solution to recipient rabbits over 3.5 min. Altogether 61 tests were performed under double blind condition. The EEGs from the frontal neocortex and limbic archicortex were recorded both on paper and magnetic tape for further processing. They were analyzed by a digital computer system equipped with A/D converter and submitted to a fast Fourier Transform. The power spectra between 0 and 125 Hz were averaged over 2 min per 1 Hz and further processed on a Univac 1108 computer system. The average spectrum per 2 min finally consisted of 56 values. In a first operation, 3 frequency bands (delta, spindles, beta) were submitted to mathematical analysis. The mean values in RMS μV and time integral were calculated for the 3 frequency bands, as well as the standard error of the mean (SE). The next operations were devoted to factor analysis of the power spectra, considered as multidimensional random variables. A factor analysis model for spectral energy variations in 12 frequency bands of 2 Hz made it possible to describe with only 3 factors more than 90% of the variance from the power spectra of the neocortex and limbic cortex EEG. The values of the factors were submitted to a Wilcoxon-Whitney-Mann test for intergroup comparisons[18].

*Results.* I. The original DSIP from the last isolated fraction. A. Biochemical findings. 3 different sequence analyses carried out on the last fraction of rabbit's hemodialysate revealed the following sequence of the nonapeptide DSIP: Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu. mol. wt: 848.98. Tryptophan was confirmed as amino terminal residue[13,14,19].

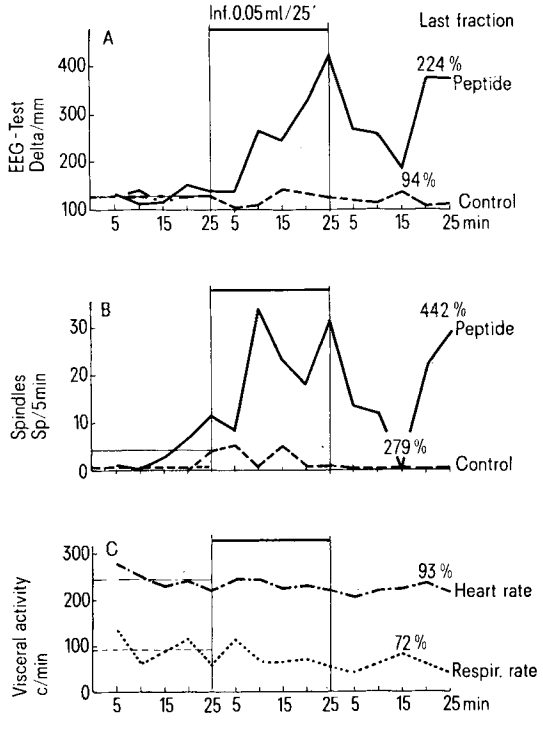


Fig. 1. Example of biological effects of original DSIP from last isolated fraction. *A* In the EEG test, total delta activity in one rabbit infused with DSIP reaches 224% during infusion and post-infusion period. *B* Concurrent increase in spindle activity (442%). *C* Moderate decrease in heart (93%) and respiration rate (72%).

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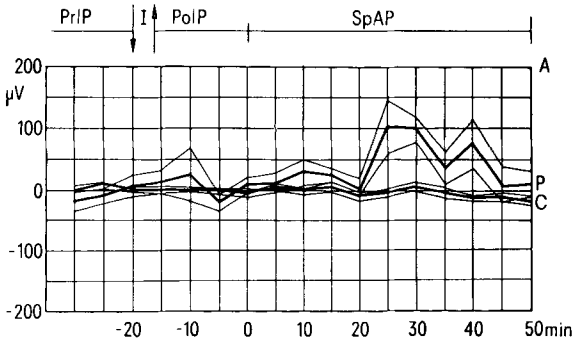
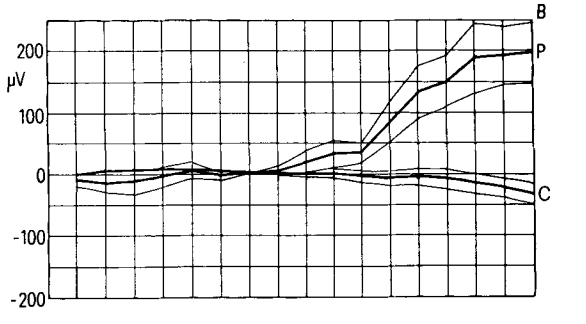
11 Control + 9 tyrosine – peptide VS 4 natural peptide




Microvolts μV ref. to preinfusion period (PrIP)

Bias: Control = 106.27 RMSμV±SE = 0
Peptide = 106.54




Time integral RMS μV ± SE

Control – 6.2 ± 3.4 = – 5.8 ± 3.2%
Peptide + 39.6 ± 9.9 = + 37.3 ± 9.3% + 43.1 ± 6.25%

Fig. 2. Activity of the original DSIP (infused in 4 rabbits compared to 20 control rabbits: 11 infused with csf like solution and 9 with a tyrosine-peptide analogue). *A* Delta EEG activity measured in RMS μV ± SE, plotted on ordinate in μV and on abscissa every 5 min. The values of the specific activity period (SpAP) lasting 50 min are compared to those of the 15 min preinfusion period (PrIP) and 20 min infusion I + post-infusion (PoIP) period for the DSIP group (P) and the control group (C). *B* The time integrals of the values exhibit an increased delta EEG activity in group P; this starts 5 min after onset of the SpAP, becomes significant after 15 min, reaches + 37.3 ± 9.3% after 50 min in group P, against – 5.8 ± 3.2% in group C. Total delta increase in %: 43.1 ± 6.2%.

Yield. The yield of this nonapeptide was 160 nmoles/g. dry dialysate, i.e. 122.2 nmoles/rabbit. This corresponds, for a mol.wt of 848.98, to 0.1037 mg/rabbit. Considering that, after the isolation procedure (7 main fractions), only 30% of the material extracted represents the final yield, and that the extracting capacity of the dialyzer is 78%, it follows that 0.103 mg = 22% (100–78) of the plasma concentration. Therefore, the total plasma concentration of peptide in electrically stimulated rabbits (100%) is 0.471 mg. By contrast, the peptide plasma level of the non-stimulated rabbit (yield = 19 nmoles/g dialysate = 14.55 nmoles/rabbit = 0.01235 mg/rabbit) corresponds to to 0.05 mg (= 100%). This comparison shows that, even in the non-stimulated awakened rabbit, there is a definite peptide level (0.05 mg), which increases by about 9 times (0.47) under the influence of the delta EEG sleep inducing thalamic stimulation.

B. Biological effects of the original DSIP. An example of the biological effects of the original DSIP, infused into the mesencephalic ventricle of one rabbit recipient (6 nmoles/kg in 0.05 csf like solution within 25 min) shows a marked increased of delta activity (224%) during the infusion and post-infusion period (50 min) referred to a pre-infusion period of 25 min (figure 1A). Concurrently with this increase, there is a parallel rise of spindle activity (442%) (figure 1B), with moderate bradycardia (93%) and bradypnea (72%) (figure 1C). A further quantification in absolute RMS μV and integral values was carried out in a group of 4 rabbits treated with original DSIP and a group of 20 control rabbits. This group of control rabbits consisted of 11 animals receiving csf like solution alone, and 9 animals infused with a csf like solution + a synthetic nonapeptide analogue (containing 1 Tyr instead of 1 Asp). Comparison of the delta activity of both groups was necessary to exclude a possible enhancing action of nonapeptide analogues; this comparison showed no significant difference between the RMS μV curves or time integral curves of both control groups. Therefore they were combined as reference level for comparison with the DSIP group (figure 2A). The delta EEG activity of the control group (C) is compared to that of the peptide group (P). In the latter, the delta activity, plotted in μV ± SE, starts to increase 5 min after the onset of the specific activity period (SpAP) and discriminates significantly from the control after 25 min (100–150 μV, referred to the pre-infusion period. Base line 0 = bias 106.54 μV.) In figure 2B, the time integrals of the values shown in figure 2A for the peptide group exhibit a steady increase in delta EEG activity, starting 5 min after onset of the SpAP. The discrimination from the control group becomes significant 15 min after onset of the SpAP. The total absolute delta increase over 50 min in the peptide group is: + 39.6 ± 9.9 RMS μV, against control: −6.2 ± 3.4 RMS μV. This gives a difference of 45.8 ± 6.5 RMS μV between peptide and control groups for the frontal neocortex. The increase in integral percent is, for the peptide group: +

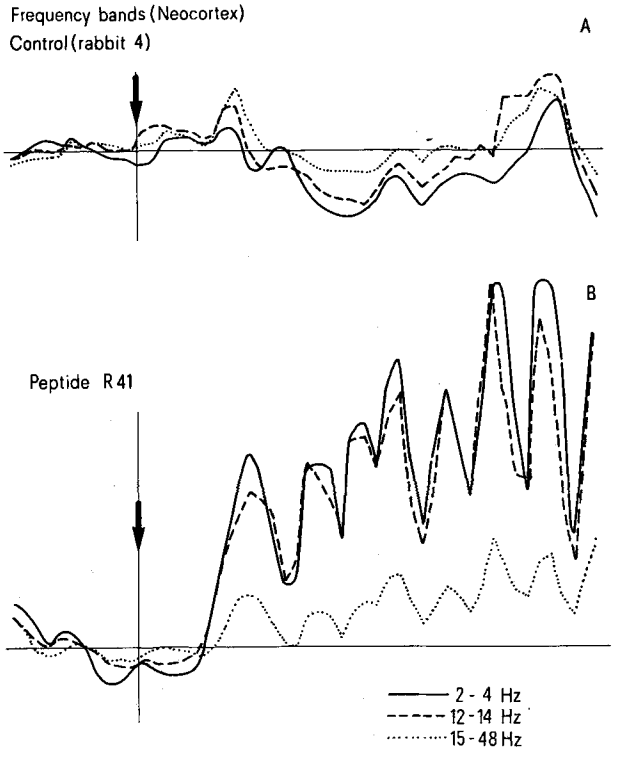



Fig. 3. Activity of synthetic DSIP. Linear power spectra from neocortex EEG for frequency bands delta (———), spindles (–.–.–.–), beta (.....) *A* Control rabbit No. 4. Low level of all frequency bands. *B* Rabbit No. 41, infused with synthetic DSIP: progressive delta + spindles increase with 6 cph biorhythm.

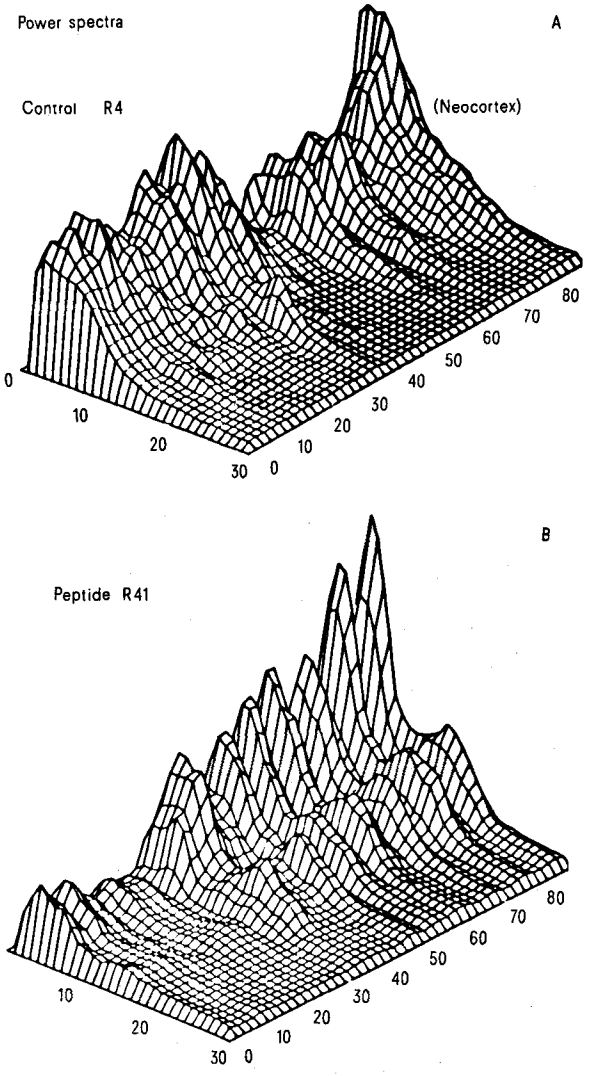



Fig. 4. 3-dimensional power spectra of the corresponding linear power spectra of figure 3. *A* Control rabbit No. 4. *B* DSIP rabbit No. 41: Progressive delta and spindle crests.

37.3 ± 9.3% against control: −5.8 ± 3.2%. This gives a difference of 43.1 ± 6.25% between peptide and control groups.

II. The synthetic DSIP. A. Biochemical findings. Synthetic DSIP showed only one band on Polyacrylamide electrophoresis, and one spot on 2-dimensional tlc and tl-electrophoresis. Analysis gave the following molar content of amino acids referred to Glu = 1 = Asp 0.79; Ser 0.80; Glu 1.00; Gly 2.88; Ala 1.85; ($NH_3$ 1.48). (Ser was corrected for 7% loss during hydrolysis.) A last amino acid analysis by Merck Sharp and Dohme Research Laboratories confirmed the composition, and electrophoresis at pH 1.9 and 6.5 showed only a single spot. However, electrophoresis at pH 3.8 and sequencing both revealed the presence of 2 components in the ratio 20:80, suggesting that the sample contained only 20% of the natural sequence and 80% of an isomer with Asp linked through the beta carboxyl.

B. Biological data. Power spectra and frequency bands. The power spectra for the 3 EEG frequency bands (delta, spindles, beta) over the experimental time are shown bidimensionally for the neocortex of rabbit No. 4 infused with csf like solution (figure 3A). Figure 3B reproduces the power spectra from rabbit No. 41 infused with synthetic DSIP. In contrast to the control, there was a drastic progressive increase in delta and spindle activities with a clear ultradian biorhythmic activity (6 cph), while the high frequency beta activity remained low. In figure 4B, the corresponding tridimensional power spectra in DSIP rabbit 41 confirm the abrupt increase starting 10 min after infusion onset and progressing in form of a high delta crest, with a marked biorhythm (5–6 cph) and a parallel smaller spindle crest. These systematic features are not detectable in the power spectra of the control rabbit (figure 4A).

A comparison of the difference in time integrals (mean values) between the delta frequency bands (2–4 Hz) of the DSIP and control groups showed an increase of 53.9 ± 10.7% for the neocortex (DSIP + 32.7 ± 15.4% against control −21.2 ± 6.1%) (figure 5A). This delta integral increase (+32.7%) induced by synthetic DSIP has the same magnitude as the increase induced by original DSIP (+37.3%) in figure 2B. The significance of the delta increase was above 90% with biorhythmic maxima up to 99.9% ($p<0.01$), as shown by the Wilcoxon-Whitney-Mann test. A similar, but stronger increase was detected for the spindle frequency band of the neocortex (61.8 ± 16.3%) (figure 5B). 3 factors were detected by factor analysis among the variables of the multidimensional power spectra involving numerous frequency bands, reduced to 12 bands of 2 Hz each. Among these 3 factors, understood as coordinates of a tridimensional space, representative of the vigilance state, only factor 1, with its delta and spindle components, was found meaningful of changes in vigilance, with a strong drift towards 'orthodox' spindle-slow wave sleep[18].

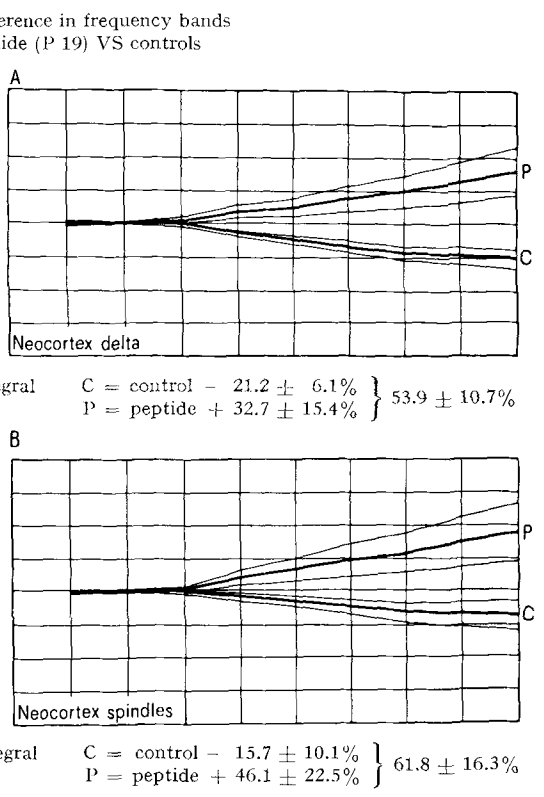



Fig. 5. Difference in time integral of frequency bands delta and spindles from neocortex between DSIP and control groups. *A* In group P, the increasing delta integral reaches + 32.7 ± 15.4%. (This value has the same magnitude as that of the original peptide in figure 2B (+ 37.3 ± 9.3%). In group C, the delta integral decreases: − 21.2 ± 6.1%. Difference = 53.9 ± 10.7%. *B* In group P, the spindle integral reaches + 46.1 ± 22.5% against − 15.7 ± 10.1% in group C. The difference 61.8 ± 16.3% is greater for spindles than for delta.

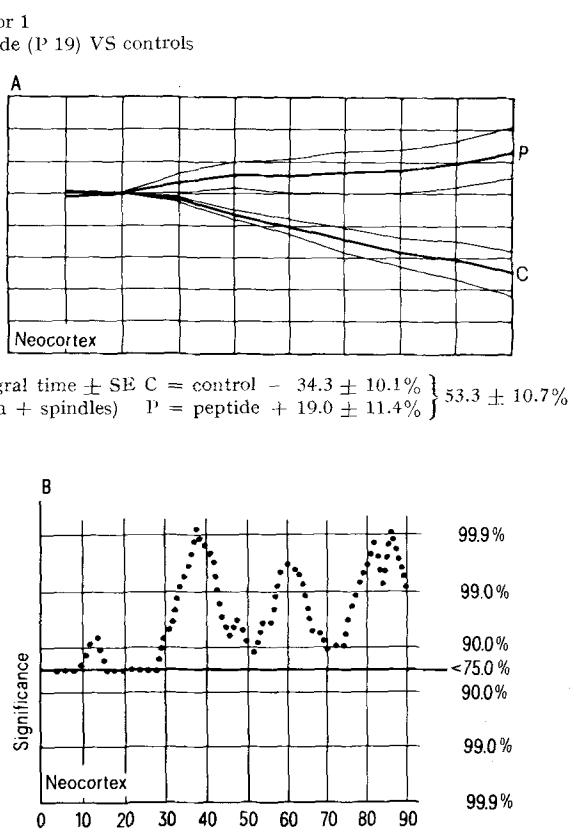



Fig. 6. Difference in time integral of factor 1 from neocortex and significance for synthetic DSIP and control groups. *A* The difference between the maximal DSIP group integral (P) and the minimal control group integral (C) reaches 53.3% ± 10, a value symptomatic of spindle-delta EEG sleep. *B* Significance of the difference in factor 1 values between DSIP group and control group is above 90%, with biorhythmic 3 cph maxima up to 99.9%.

As for the frequency bands, the mean values and SEM were quantified for factor 1 in RMS μV and integrated over the experimental time (figure 6A). The time integral in rabbits receiving DSIP was compared to that in control rabbits. For factor 1 in the neocortex, the difference between integral increase in DSIP rabbits and decrease in controls reached 53.3 ± 10.7% (figure 6A). The statistical significance of factor 1 was calculated with the same nonparametric test as for the frequency bands. The significance of factor 1 increase in neocortex was above 90% ($p<0.01$), with biorhythmic maxima up to 99.9% (figure 6B). In the limbic cortex, for factor 1, the difference was higher than in the neocortex: 78.7 ± 15%. The significance reached a maximum value of 99.9%, without marked biorhythmic oscillations.

*Discussion and conclusions.* The original nonapeptide (mol.wt 848.98), identified in the last fraction of cerebral blood dialysate from thalamus stimulated rabbit donors, induces, besides bradypnea and bradycardia, a delta + spindle EEG sleep, when infused into the mesencephalic ventricle of rabbit recipients. Because of its significant delta EEG enhancing activity (quantified in RMS μV and time integral), this compound was called original 'delta sleep inducing peptide', DSIP. The peptide level in plasma of awakened rabbits (0.05 mg) is 9 times lower than that of rabbits submitted to stimulation of the somnogenic thalamic area (0.47 mg).

Synthetic DSIP induces a similar activity in the delta and spindle frequency bands of the rabbit's neocortex, with ultradian biorhythms of 3–6 cph. The delta + spindle activity is even higher in the limbic cortex. The specificity of the original and synthetic DSIP is established by comparing their delta activity with that of peptide analogues; the latter were found to be inactive.

The original and synthetic DSIP have the same effective dose (~ 6 nmoles/kg intraventricularly infused in rabbit). Definite features suggest that DSIP might act as a programming modulator at supra-operational level rather than as a transmitter at operational level (long latency, activation of latent biorhythmic oscillators, reversibility of the EEG sleep effect under influence of waking stimuli). DSIP might pass the blood-brain barrier, since ultrafiltration through UM-05 filters is possible for peptides with mol.wt above 1000 or bacitracin with mol.wt = 1400. This is also supported by the fact that i.v. injection of synthetic DSIP in free moving rabbits induces an EEG delta activity up to 144% during 5 h following a reference period of 90 min against 126% in control rabbits (unpublished data). Concurrently the motor activity decreases. DSIP differs by its higher mol.wt from factor S of Pappenheimer et al.[20], extracted from the goat's csf or sheep's brain, and from the sleep-promoting material of Uchizono and coworkers[21] extracted from the rat's brain. It furthermore differs from factor S by the fact that, in the intraventricular tests in rabbits, the EEG delta effects are detectable already during the infusion period and without concomitant 'epileptiform episodes'.

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## PRO EXPERIMENTIS

# A method for demonstrating zinc content of the brain using 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene perfusion-staining

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*Summary.* A rapid accurate method for histochemical localization of zinc in the rat brain, utilizing 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene perfusion-staining, is described.

Zinc is an essential element in animal nutrition; deficiency or intoxication produce characteristic symptoms. This element forms an integral part of a number of metalloenzymes such as carbonic anhydrase, alkaline phosphatase, lactic dehydrogenase and alcohol dehydrogenase[2]. The dithizone method is commonly used for histochemical demonstration of zinc[3], but dithizone (diphenylthiocarbazone) forms an insoluble, coloured inner complex salt with a number of heavy metals (Zn, Pb, Ag, Cu, Hg, Au, Cd). Zincon (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene, figure 1) has recently been used for serum zinc determinations[4]. Under carefully controlled conditions, according to Searcy[4], this procedure yields results with an acceptable degree of precision and accuracy. Furthermore, Zincon has been successfully used as an indicator for the spectrophotometric determination of the zinc content of water[5].

*Materials and methods.* Preparation of experimental animals. A total of 35 male albino rats weighing 100–150 g were used for this study. I. p. injections of 5 mg elemental zinc per kg b.wt in the form of zinc chloride were given daily for 5–7 days. Atomic absorption spectrophotometric estimation of the level of zinc in different regions of the brain of 20 rats (10 zinc-treated and 10 control rats receiving equal volumes of normal saline) was carried out using a Perkin-Elmer model 303 atomic absorption spectrophotometer. Details of this experiment form part of a separate communication[6].

1 Acknowledgment. The author is grateful to Dr S. H. Zaidi, Director, I.T.R.C., Lucknow, for permission to use the atomic absorption spectrophotometer and to Mr M. I. Siddiqi, Mr M. Z. Hasan, Mr Mujir and Mr B. Z. K. Sherwani for the technical assistance.
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