Isolation and Physical-Chemical Characterization of a Humoral, Sleep Inducing Substance in Rabbits (Factor 'delta')

In 1910 it was for the first time suggested that transfusion of cerebro-spinal fluid (CSF) from sleep-deprived to normal dogs induced behavioral sleep in the recipients[1]. Sleep-deprivation was thought to increase the production of humoral sleep transmitting factors. In 1965 it became possible to transmit sleep to recipients by i.v. injection of an extracorporal hemodialysate obtained from rabbits submitted to electrical stimulation of the thalamic sleep area during dialysis[2]. More recently, sleep was again successfully transmitted by intraventricular infusion of CSF from sleep-deprived goats to rats[3,4] or of dialysate from sleeping rabbits submitted to thalamic stimulation to recipients of the same species[5,6]. Simultaneously, evidence was presented by these authors that one or more substances of small molecular weight might be responsible for the humoral transmission of sleep[7]. In order to complete the latter experiments and to check whether

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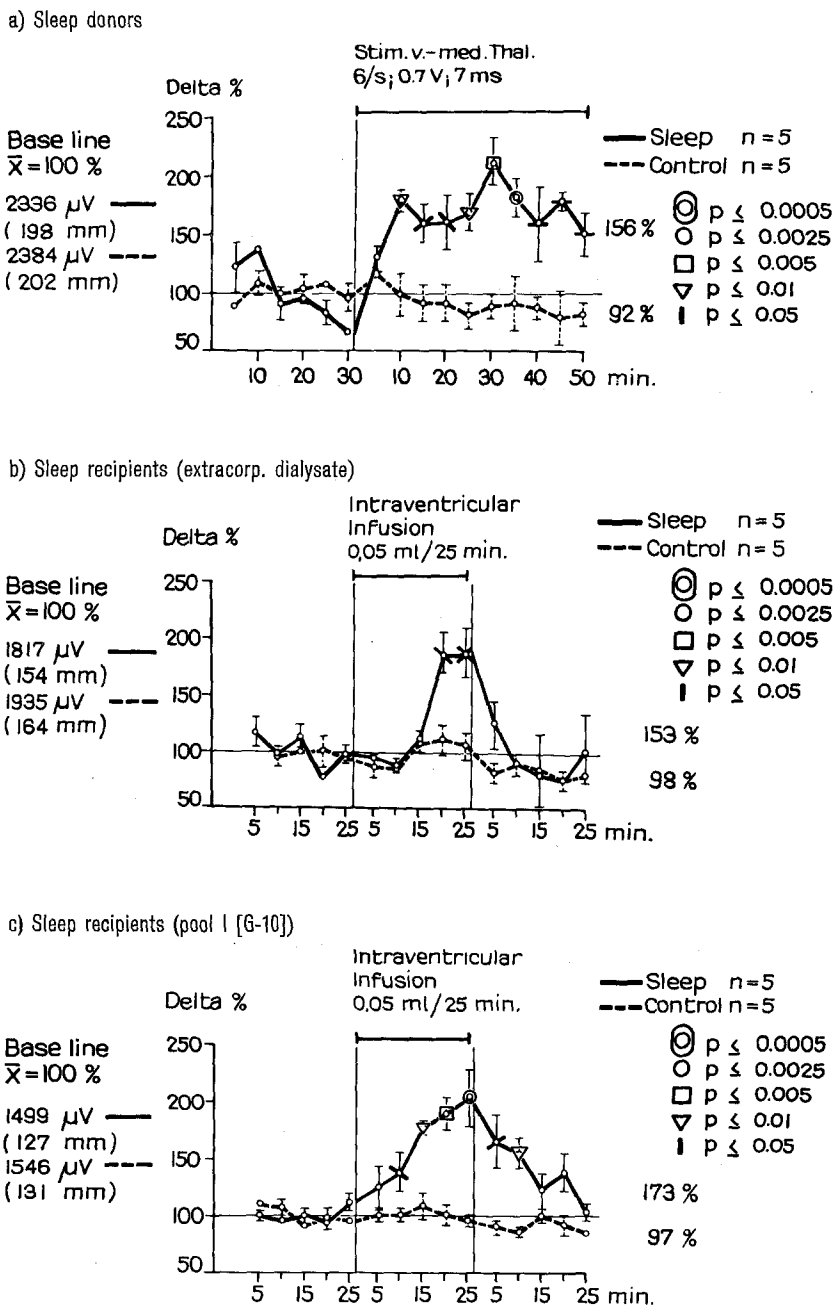

Fig. 1. a) Percentage of the increased delta amount in donor-rabbits during electrical stimulation of the thalamic sleep area (—) compared with non-stimulated control animals (- - -). b) Transmission of sleep ('delta sleep') by intraventricular infusion of an extracorporal dialysate from donors to recipients (—); control = infusion of dialysate from non stimulated donors (- - -). c) Increase of delta amount in recipients (—) after infusion of Pool I (Sephadex G-10). Controls (- - -).

humoral factors are actualyl involved in the phenomenon of sleep, attempts were made to isolate and characterize the active substance, or substances in the extracorporal dialysate.

Methods and results. Rabbits were submitted before and during electrical stimulation of the thalamic sleep area of HESS[8] to an extracorporal hemodialysis according to KUHN[9]. After a prestimulation period of 30 min, they were intermittently stimulated during 50 min: voltage 0.7 V; pulse frequency 6/s; pulse duration 7 ms; stimulation episode: 10 s; total stimulation time 277 s; total stimulation charge (11.6 × 0.25 mA) = 2,9 mC. This mild stimulation induced in the donor an 'orthodox delta sleep' in contrast to the 'paradoxical sleep' described by JOUVET[10].

The dialysate or the fractions obtained therefrom were infused with an automatic pump (0.05 ml/25 min) into the meso-diencephalic ventricular system of mildly restrained or free-moving rabbit recipients. The hypnogenic activity was expressed by the amount of electrical delta activity of the cortex, recorded with an electroencephalograph (EEG) and quantified by an automatic frequency analyzer (μV/5 min)[11,2]. The concomitant decrease in motor activity was recorded with a kinesigraph[12]. The control animals were donor rabbits in which electrodes were implanted in the thalamus, but no stimulation was applied.

The delta-amount was expressed in percent of the average increase (μV/5 min) referred to the average value of the prestimulation period in the donor or the preinfusion period in the recipient (= 100%). The statistical significance of the differences of delta-amounts in sleeping and control animals was calculated[13]. During fractionation, the lyophilized dry material of the corresponding fraction was dissolved in an electrolyte solution, identical to CSF in content, pH and in a concentration proportional (w/w) to the dry substance of the primary sleep-inducing dialysate. Artefacts, eventually introduced by the procedure in the course of fractionation were eliminated by testing the 'blank procedure', i.e. fractionation without substance.

The lyophilized primary sleep dialysate was dissolved in distilled water, centrifuged and the supernatant desalted by gel chromatography on Sephadex G-10 (Pharmacia, Uppsala, Sweden). The column was equilibrated and eluted with distilled water. The higher molecular weight fraction (MW > Cl^-), denominated Ponl I, contained the total hypnogenic activity. In Figure 1a, the increase of delta activity in sleeping donors during stimulation (156%) was compared to the corresponding delta activity of control-donors (92%). The recipients of sleep dialysate and of control dialysate showed a delta difference of 153% to 98%, respectively, (Figure 1b) and recipients of the desalted Pool I (Figure 1c) showed an increase of the delta amount of 173% compared to 97% in the control.

The desalted hypnogenic material (20 mg/5 donors) was separated by preparative thin-layer chromatography (TLC) on commercially available (PSC) Silica-Gel plates (Merck, Darmstadt). Running medium: aceton – water (7 + 3). Six ninhydrin-positive fractions with different Rf-values were thus separated. Only 1 of the 6 fractions, fraction 2, the Rf-value of which differed clearly from that of Serotonin, contained the hypnogenic activity. The analytical high voltage electrophoresis revealed the presence of 6 ninhydrin-positive substances in this fraction. Further fractionation on a Sephadex G-15 column, equilibrated and eluted with water, elicited 3 ninhydrin-positive peaks (Figure 2). Only Peak 1, with the smallest V_e, which was not present in the TLC fractions 1, 3, 4, 5 and 6, contained the whole hypnogenic activity. V_o (Blue Dextran) > $V_{e(1)}$ of the 'hypnogenic peak' suggested a molecular weight below 1500[14]. A more precise evaluation of the molecular weight of the hypnogenic material could be obtained by rechromatography over a longer G-15

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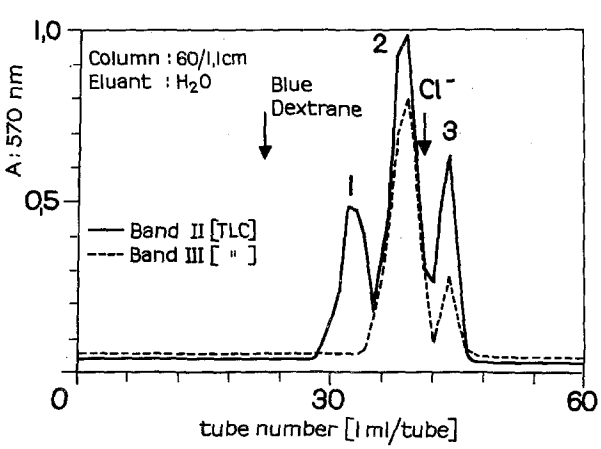

Fig. 2. Gel chromatography on Sephadex G-15 of a hypnogenic fraction 2 (—) separated by thin layer chromatography and a non hypnogenic fraction 3 (---); separation into 3 and 2 peaks, resp., with different molecular weights.

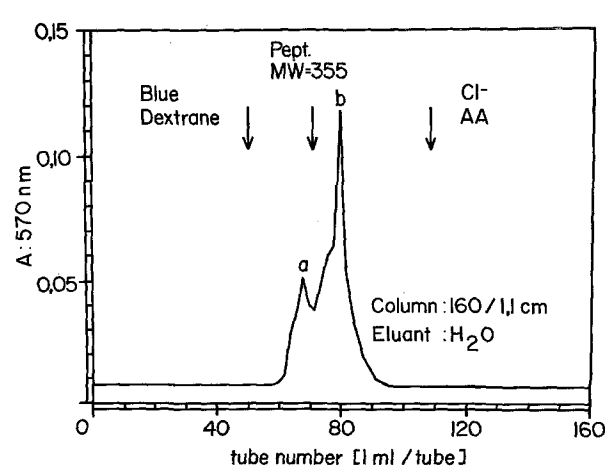

Fig. 3. Estimation of the molecular weight of the hypnogenic fraction (Peak 1a) by analytical Gel chromatography over Sephadex G-15; reference substances: blue dextran (MW 2×10^6), Gly-Leu-Met-NH_2 × *HCl* (MW 355), Alanin (MW 89).

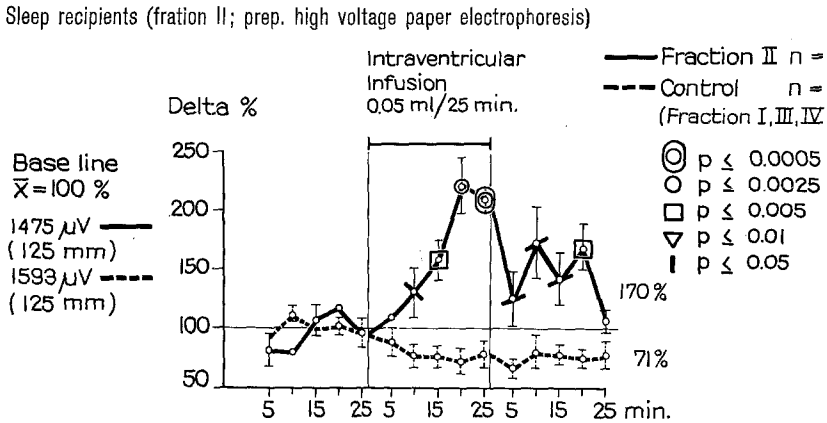

Fig. 4. Percentage of increase of delta amount after infusion of the purified and characterized fraction 2 (—); controls (---) = non hypnogenic fractions with similar physical-chemical properties, separated by high voltage electrophoresis.

column (Figure 3). The column was calibrated with Alanine (MW 89), a tripeptide (MW 355) and blue dextran ($MW > 2 \times 10^6$). A peak with 2 shoulders was eluted. The first apex contained the hypnogenic material and appeared before the peptide marker. The molecular weight must, therefore, be above 355. The separation of the two shoulders was not successful. It was however achieved by preparative high voltage paper electrophoresis on Whatman 2 MM-Paper (20 × 40 cm) at pH 2.0 (2% formic acid; 2500 V/120 mA/75 min). Four electrophoretic different fractions appeared. The hypnogenic acivity resided only in fraction II (from the starting point) with a delta increase of 170% as compared to 71% for the controls (Fractions I, III and IV) (Figure 4).

Both the analysis of the hypnogenic ninhydrin-positive peak 1 (a + b) obtained from Sephadex G-15 (Figure 3), before paper electrophoretic purification and that of the purified fraction 2 after high voltage electrophoresis, on the amino acid analyzer, before and after acid hydrolysis, showed the release of at least 7 different amino acid residues by the hydrolytic procedure[15]. Additionally, fraction 2 showed after dansylation before acid hydrolysis the presence of contaminating amino acids from the electrophoresis paper, but one dansyl-positive substance, which could not be identified, yielded again after elution from the cellulose plate, hydrolysis and redansylation at least 7 free DNS-amino acid residues[16]. A quantitative analysis was not yet achieved, due to the small amount of purified substance available.

Conclusion. Multiple fractionations, at each step of which different physical-chemical properties of the molecules were made use of, showed that the hypnogenic activity was always associated with a ninhydrin-positive fraction. The molecular weight of the hypnogenic active substance could be estimated by Sephadex G-15 and the presence of at least 7 different amino acid residues was revealed after amino acid hydrolysis. On the basis of the latter fact, verified with two different techniques and of other data, we feel entitled to draw the following conclusions: humoral transmission of sleep in our model seems to be due to a *specific transmitter of peptide nature*[7]. The molecular weight of the active substance, which we characterized as 'sleep factor delta', lies between 355 and 1500[17]. The assumption that such a substance might be responsible for humoral transmission of sleep is sustained by the fact that the effective dosis is $10^{-7} M/l$ or 5.8 ng/kg for intraventricular infusion. The ultimate proof will be provided by further production and purification of sufficient quantities of the compound, allowing elucidation of the structure, followed by synthesis of the active sleep substance.

Zusammenfassung. Das Hämodialysat schlafender Kaninchen (unter Einfluss thalamischer Reizung) wurde unter Kontrolle der «Delta-Schlaf»-induzierenden Aktivität fraktioniert. Nach Entsalzung über Sephadex G-10 wurde die hypnogene Fraktion durch anschliessende präparative Dünnschichtchromatographie in 6 verschiedene ninhydrin-positive Fraktionen getrennt ($MG > Cl^-$). Eine davon enthielt die hypnogene Aktivität. Diese Fraktion 2 wurde über Sephadex G-15 in 3 ninhydrin-positive Gipfel aufgetrennt. Nur die Fraktion mit dem geringsten V_e enthielt die hypnogene Aktivität. Durch Vergleich mit dem Elutionsvolumen von Markersubstanzen konnte für die aktive Substanz ein Molekulargewicht zwischen 355 und 1500 geschätzt werden. Präparative Hochspannungs-Papierelektrophorese trennte das aktive Material in vier Unterfraktionen. Nur eine elektrophoretisch und chromatographisch einheitliche Fraktion (2) enthielt die hypnogene Aktivität. Aus dieser Fraktion wurden nach saurer Hydrolyse mindestens 7 verschiedene Aminosäurereste freigesetzt. Dieses Resultat wurde durch Dansylierung vor und nach saurer Hydrolyse verifiziert; gleichzeitig wurden Artefakte durch kontaminierende Aminosäuren aus dem Papier ausgeschlossen. Die Ergebnisse lassen auf die Peptidnatur einer humoralen «Delta-Schlaf»-induzierenden Substanz schliessen (sleep factor 'delta').

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