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# Sleep-promoting factor S: Purification and properties

(slow-wave sleep/neurohumoral regulation of sleep)

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ABSTRACT **Sleep-promoting factor was purified from acid/acetone extracts of whole brains of rabbits and from brainstems of slaughterhouse cattle. Intraventricular infusion of extracts purified by means of ion exchange and gel filtration induced excess slow-wave sleep in rabbits for 5–10 hr. The procedure is simple and provides material suitable for physiological studies. Further treatment by partition chromatography and electrophoresis yielded an active product that was purified at least 1 million-fold. This product was inactivated by incubation with mixed carboxypeptidases A and B. Amino acid analysis of acid hydrolysates indicated that the effective dose was less than 150 pmol per rabbit and the original concentration in brain tissue was of the order of 30 pmol/g of brain.**

Sleep-promoting factor S is a low molecular weight substance found in brain and cerebrospinal fluid of sleep-deprived animals (1–4). Infusion of factor S into the cerebral ventricles of rats (2) or rabbits (3) induces excess slow-wave sleep (SWS) in the recipients for several hours. Although factor S was not detectable in unconcentrated cerebrospinal fluid from normal animals not subjected to sleep-deprivation (2), it was demonstrable in concentrates of cerebrospinal fluid (3) from such animals. Partial purification of factor S from cerebrospinal fluid and brains of sleep-deprived goats has been described (3), but the quantity and purity of the product were inadequate to permit further chemical studies.

The present paper describes a relatively simple procedure for preparing factor S of sufficient purity for physiological experimentation and a more elaborate procedure for preparing highly purified factor S in amounts suitable for analysis of structure.

## Extraction and purification

The extraction and purification procedures are summarized in Table 1. Because biological assays for factor S are not sufficiently quantitative to establish a unit of biological activity, we express concentrations and doses in terms of gram brain equivalents (GBE) of the original starting material. Recovery of factor S during purification can then be estimated approximately by the number of GBE required to induce substantial sleep responses such as those illustrated in Fig. 1. In order to assess the extent of purification we used the fluorescamine reaction (5) as a measure of the total amount of material (total reacting primary amino groups) present at each stage. The fluorescence developed by 1 GBE in the original acid/acetone extract was taken as 1 relative fluorescamine unit (RFU).

Whole brains from rabbits were obtained from Pel-Freez Biologicals, Inc. (Rogers, AR) in lots of 100–3000 brains. Prior to sacrifice the animals were transported from Texas to the factory in an open-air trailer; it is probable that the animals suffered stress and dehydration in addition to sleep-deprivation during the journey (12 ± 3 hr). After reaching the abattoir in Arkansas the animals were killed within 40 min. Brains were removed rapidly, frozen in liquid nitrogen, and stored in dry ice until the extraction procedure was started. Brainstems from cattle were obtained from a local slaughter house. The previous history of these animals with respect to stress and sleep-deprivation was unknown. The brainstems were frozen about 1 hr after the animals had been slaughtered.

Brains were homogenized and extracted with acid/acetone at 4° as described by Chang and Leeman (6). Usually, the extractions were carried out on less than 500 g of brain tissue; however, one batch of 25 kg was processed by using the large-scale facilities of the New England Enzyme Center. After removal of acetone by extraction with petroleum ether and low-pressure evaporation, the resulting aqueous suspension was centrifuged at 14,000 × *g* in a continuous-flow centrifuge. The supernate was diluted with water until its conductivity was equivalent to that of 0.15 M NaCl; it was then titrated to pH 7.2 with $NH_4OH$. At this stage the concentration of the extract was 1.2 GBE per ml.

The extract was applied to a CM-Sephadex C-25 column having a bed volume of 6.6 ml/100 GBE and equilibrated at room temperature with 50 mM $NH_4OAc$ buffer at pH 7.2. The column was then washed with 1 bed volume of 50 mM $NH_4OAc$ buffer at pH 7; because factor S is retained by the column under these conditions, the wash was discarded. Factor S was eluted with 8 bed volumes of 1 M NaCl followed by 6 bed volumes of 1 M HOAc. The eluates were combined and their volume was decreased to 20% of the original in rotary vacuum evaporators at 38°. The concentrate was desalted and fractionated by four successive gel filtrations on Sephadex G-10 columns calibrated as described (3) with blue dextran, [$^{14}$C]-sucrose, and NaCl. Each retained fraction was lyophilized and taken up in a volume of 50 mM HOAc appropriate for the subsequent filtration. Fractions of increasing selectivity were retained from each successive gel filtration: from column 1, the fraction retained was from the void volume, $V_0$, to the beginning of the ascending limb of the salt peak; from column 2, $V_0$ to 50% of the descending limb of the sucrose peak; column 3, $V_0$ to the sucrose peak; column 4, $V_0$ to 50% of the ascending limb of the sucrose peak. The final fraction was lyophilized and prepared for bioassays and for the next step of purification by paper chromatography.

The ion exchange and gel filtration steps eliminated more than 99.6% of the fluorescamine-reactive groups present in the original acid-acetone extract (Table 1). At this stage the product is free of excitatory amino acids and peptides and is suitable for physiological studies of the sleep factor; intraventricular infusion of 5–10 GBE induced excess SWS in rabbits for several hours (Fig. 1). Ten separate batches of active material have been prepared by this method from whole brains of rabbits and three separate batches from whole brains or brainstems of cattle.

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Abbreviations: SWS, slow-wave sleep; GBE, gram brain equivalents; RFU, relative fluorescamine unit.

Table 1. Summary of procedures for isolation of factor S from whole brains of sleep-deprived rabbits

| Step | Procedure | RFU per GBE |
|---|---|---|
| Extraction | Homogenize and extract with acid/acetone; remove acetone with petroleum ether and low-pressure evaporation; centrifuge residual aqueous extract; dilute supernate to conductivity of 0.15 M NaCl, titrate to pH 7 | 1.0 |
| Ion-exchange | Pass aqueous extract through CM-Sephadex C-25 at pH 7; discard eluate 1 (pH 7 buffer); retain eluate 2 (1 M NaCl) and eluate 3 (1 M HOAc), rotary evaporate eluates 2 and 3 at 38° under low pressure to 1/5th vol | $6 \times 10^{-2}$ |
| Gel filtration | Desalt concentrated eluates from ion exchange on Sephadex G-10 column; freeze-dry; take up in 0.05 M HOAc for three successive fractionations on Sephadex G-10 columns | $4 \times 10^{-3}$ |
| Partition chromatography | Gel filtration product subjected to ascending paper chromatography, acetone/propanol/$NH_4OH$; elute at $R_F$ 0.15–0.35 | $7 \times 10^{-5}$ |
| Electrophoresis | Product subjected to paper electrophoresis pH 1.9, 65 V $cm^{-1}$, 60 min; elute 0.20–0.27 of migration distance of standard marker (Lys) | $5 \times 10^{-7}$ |

Further purification of the material, leading to preliminary chemical characterization of factor S, was achieved by partition chromatography and high-voltage paper electrophoresis. Ascending paper chromatography was carried out on Whatman no. 1 paper in a sealed chamber at room temperature. The solvent system was acetone/*n*-propanol/water/8 M $NH_4OH$, 40:30:20:10 (vol/vol). The product from gel filtration was applied to the origin in amounts of 35 GBE per cm of paper. Development proceeded for approximately 4 hr (20 cm on paper); the chromatograms were dried under a gentle stream of air at room temperature. Sleep-promoting activity was localized to a region of $R_F$ 0.15–0.35. The pooled eluates from multiple runs were subjected to a single gel filtration through a 150-ml Sephadex G-10 column to remove contaminants derived from the paper chromatography. The fraction from $V_0$ to 50% of the ascending limb of the sucrose peak was lyophilized and prepared for bioassay and for the next stage of purification by electrophoresis.

High-voltage electrophoresis was carried out at pH 1.9, 65 V $cm^{-1}$, for 60 min in formic acid/acetic acid/water, 150:100:750 (vol/vol), on Whatman no. 3 paper. The paper was dried overnight at room temperature. Sleep-promoting activity was localized to a strip corresponding to 0.20–0.27 of the migration distance of lysine used as a standard marker. The eluate from this strip was subjected to a final Sephadex G-10 filtration on a 150-ml column. The fraction from $V_0$ to the sucrose peak was lyophilized and aliquots were prepared for bioassay, amino acid analyses, and enzymatic digestion. Hydrolyzed (6 M HCl, 145°, 4 hr) and unhydrolyzed samples were analyzed on a Beckman model 121 MB amino acid analyzer. Enzymatic digestion was carried out with mixed carboxypeptidases A and B (Worthington Biochemicals) at 37° for 4–16 hr in 0.1 M $NH_4HCO_3$ buffer (pH 7.8). Control experiments were done by incubating active factor S with carboxypeptidase that had been boiled for 10 min. The molar ratio of enzyme to substrate, estimated by amino acid analysis, was approximately 1:50. After incubation, the samples were lyophilized, redissolved in water, and again lyophilized, thus removing most of the $NH_4HCO_3$.

Biological assays for sleep-promoting activity were performed on rabbits provided with chronically implanted ventricular guide tubes and epidural screw electrodes for electroencephalography as described (3). Samples for testing were taken up in sterile artificial cerebrospinal fluid and infused intraventricularly at the rate of 3 μl/min for 90 min. After the infusion, the animals were left undisturbed for 6–8 hr while the electroencephalogram and body movements were recorded. SWS was measured in two ways: (*i*) by conventional subjective scoring of the duration of SWS from polygraph records and (*ii*) by digital print-out of integrated mean rectified cortical slow waves (1/2–4 Hz), thus obtaining a measure of the amplitude as well as duration of δ wave activity. Percentage δ activity was computed for hourly intervals by the following equation: % δ activity = $100\ (\bar{E} - E_A) \div (E_S - E_A)$ in which $\bar{E}$ is mean rectified voltage of slow waves obtained from the integrator during the stated hour, $E_A$ is rectified slow wave voltage in the awake animal, and $E_S$ is rectified slow wave voltage during full SWS in the same normal animal without infusion of factor S. $E_A$ is typically about 12 μV and is unaffected by factor S. $E_S$ is typically about 40 μV. Both values are stable within about 10% over a period of weeks in any one animal. However, one effect of factor S is to increase the amplitude of slow waves during sleep as described (3). For this reason, % δ activity (which includes both amplitude and duration) is a more sensitive assay for factor S than is measurement of duration alone, as illustrated in Fig. 1.

Although factor S has been tested successfully in cats (1) and rats (2, 3), we have used rabbits for routine assays. Rabbits have the following advantages for the assay: (*i*) less than 5% of their sleep time is in the form of REM sleep (7), thus simplifying the analysis by restricting it to SWS sleep; (*ii*) intraventricular infusion of sterile artificial cerebrospinal fluid or other control solutions does not affect the subsequent duration or pattern of sleep in rabbits as it does in rats (3), thus permitting valid control measurements to be made frequently on each rabbit; (*iii*) the hourly percentage SWS is relatively independent of the light/dark cycle and normal variations among rabbits are small compared to the changes caused by infusion of factor S (Fig. 1, Table 2).

## Biological activity

More than 800 8-hr recordings on 150 rabbits were made during development of the purification steps but only a small percentage of these involved active fractions. For example, eluates from 12 sections of paper from high-voltage electrophoresis were each tested on two or more rabbits but the eluate from only one section yielded strongly positive results. Adjacent sections yielded slight activity, and none of the remaining sections was significantly different from uninfused controls (Table 3). Positive responses are characterized by gradual increases in percentage SWS and amplitude of δ waves, reaching maximal values about 2 hr after the infusion. For routine assays the recordings were terminated 6 hr after the infusion as illustrated in Fig. 1. However, the sleep-promoting effects often lasted for several more hours. Rabbits sleep in short SWS episodes, and factor S increased the hourly percentage sleep by increasing the number of SWS episodes and, to a lesser extent,

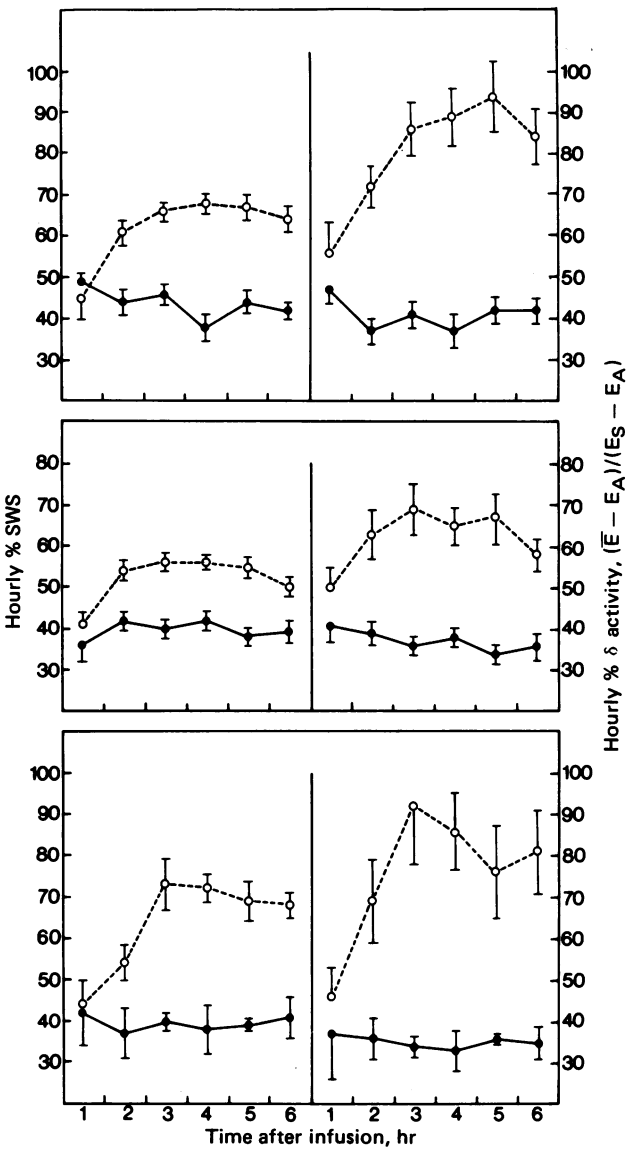


FIG. 1. Effects of factor S on duration of SWS and electroencephalographic δ activity. After infusion of factor S, the hourly percentage of δ-wave activity (*Right*) was greater than the percentage SWS scored from the electroencephalogram record (*Left*). This is because sleep induced by factor S is associated with increased *amplitude* of slow waves as well as increased number and duration of sleep episodes in each hour. ○, Factor S; ●, control. (*Top*) After purification through gel filtration step; 6 GBE. Means ± SE, 10 assays on 8 rabbits. (*Middle*) After purification through paper chromatography step; 15–30 GBE. Means ± SE, 19 assays on 15 rabbits. (*Bottom*) After purification through electrophoresis step; 50–90 GBE. Means ± SE, 6 assays on 6 rabbits.

by increasing the duration of each episode (Table 2).

Substantial losses of factor S were incurred during the purification procedure as shown by the fact that doses of 50–90 GBE after electrophoresis were required to produce approximately the same sleep-promoting effects as 6 GBE after gel filtration. The yield of factor S through these steps is thus about 10%, but the overall yield would be lower if losses in the first two steps could be taken into account. It may readily be calculated from Table 1 and the data of Fig. 1 that purification after the electrophoresis step is several million-fold relative to the original acid/acetone extract and even more if the point of reference is the fresh tissue.

Table 2. Effects of factor S on number and average duration of sleep episodes

| | Mean ± SD | |
|---|---|---|
| | Control | Factor S |
| Duration of SWS, min/hr | 23.5 ± 4.9 | 39 ± 5.9 |
| No. episodes per hr | 14.6 ± 4.2 | 19.6 ± 5.7 |
| Average duration of each episode, min | 1.61 ± 0.36 | 1.99 ± 0.33 |

Data from 5-hr recordings on each of 10 rabbits under control conditions and after intraventricular infusion of purified factor S (6–30 GBE).

## Structural studies

There is evidence that the structure of factor S may be altered by the purification procedure. (*i*) In two preparations the products from the gel filtration step were reapplied to CM-Sephadex columns. In both cases, biological activity was found primarily in the pH 7 eluates, thus indicating that factor S failed to bind to the resin and suggesting that it lost one or more net positive charges during the ion exchange or gel filtration steps. (*ii*) Electrophoresis (pH 1.9 or 3.3) of brain extracts that had been purified only by ultrafiltration through molecular sieves (Amicon UM10 and UM05) and by gel filtration revealed that factor S then migrated close to arginine and lysine (8). In contrast, the electrophoretic mobility of factor S purified as in Table 1 was only 0.20–0.27 that of lysine, further evidence that the charge of factor S had been altered. Nevertheless, the biological effects of the product of the final electrophoresis step were indistinguishable from those obtained using factor S purified only by molecular sieving (3).

The sleep-promoting action of the product of the electrophoresis step was inactivated by incubation with carboxypeptidases (Table 4). This result supports earlier suggestions (3) that factor S may be a small peptide. Several amino acids were released by acid hydrolysis of the active product from the electrophoresis step. Their concentrations ranged from 10 to 150 pmol/100 GBE. "Blank" amino acid values were determined which included the contributions from reagents, solvents, paper, etc. These values were variable, making assignment of specific amino acids to factor S unreliable at this stage. However, if it is assumed that factor S is a peptide, its concentration in the active preparation after electrophoresis cannot exceed that of the most concentrated amino acid in the hydrolysate. On this basis, we can estimate that intraventricular infusion of less than 150 pmol of factor S in a 3-kg rabbit is sufficient to cause a 50% increase in duration of SWS for several hours after the infusion (Fig. 1). From the yield of factor S discussed above and assuming a 50% loss during the first two steps, we can estimate that the original concentration of factor S in whole brain was of the order of 30 pmol/g or 240 pmol per rabbit brain. These estimates, admittedly crude, are similar to values found for

Table 3. Effects of high-voltage electrophoresis eluates on SWS and δ activity*

| | | Mean ± SE | |
|---|---|---|---|
| | *n* | % SWS | % δ activity |
| Controls (no infusion) | 6 | 39 ± 2 | 35 ± 1 |
| Active section ($R_{Lys}$, 0.20–0.27) | 6 | 66 ± 3 | 78 ± 9 |
| Controls (no infusion) | 9 | 35 ± 1 | 33 ± 2 |
| Sections adjacent to active section ($R_{Lys}$, 0.10–0.20 and 0.27–0.35) | 9 | 44 ± 3 | 54 ± 6 |
| Controls (no infusion) | 16 | 37 ± 1 | 34 ± 1 |
| Eluates from 8 other sections | 16 | 42 ± 2 | 38 ± 2 |

* Measured 2–6 hr after infusion.

Table 4. Inactivation of factor S by carboxypeptidases

| Rabbits, no. | Infusion fluid | % SWS* |
|---|---|---|
| 4 | None | 42 ± 2 |
| 6 | 90 GBE factor S | 66 ± 3 |
| 4 | 90 GBE factor S + carboxypeptidases | 46 ± 5 |
| 1 | 90 GBE factor S + denatured carboxypeptidases | 64 |

* Mean ±SE, 2–6 hr after infusion.

other biologically active peptides in brain. For example, the intraventricular infusion of 100 pmol of angiotensin in cats is adequate to elicit a drinking response (9), and the concentrations of substance P(10), neurotensin (11), or thyroliberin (12) are of the order of 10–50 pmol/g of whole brain.

## Comment

It is now clear that the chemical and physiological properties of factor S differ greatly from the properties of δ sleep-inducing nonapeptide (DSIP) isolated from blood by Schoenenberger *et al.* (13). Factor S induces excess behavioral sleep and marked increase of δ wave amplitude in rabbits for several hours after intraventricular infusion of 150 *pico*moles; in contrast, Schoenenberger *et al.* (13) reported that 20 *nano*moles are required to induce a relatively small increase in δ wave activity for periods of less than 1 hr. The quantity of their nanopeptide released to the systemic blood of a rabbit during 20 min of thalamic stimulation was estimated by Schoenenberger *et al.* (13) to be 0.656 mg or 770 nmol. This may be compared with 240 pmol estimated above for the content of factor S in an entire brain of a sleep-deprived rabbit.

Purification of factor S through the gel filtration step involves relatively simple procedures, and the product is suitable for physiological studies. Successive preparations from the same starting material have approximately the same effects in the rabbit assay but at this time neither the purification procedures nor the assay are sufficiently quantitative to allow determination of the relationship between sleep deprivation and accumulation of factor S in various regions of the brain.

The role of factor S in the normal cycle of sleep and wakefulness remains to be investigated in detail. The sleep-promoting activity of cerebrospinal fluid drawn from goats increases gradually during sleep deprivation, reaching a maximum after about 48 hr (2). Cerebrospinal fluid drawn from rats after normal nocturnal wakefulness reduces the locomotor activity of rats entering the active phase at night (14). Intraventricular infusion of purified exogenous factor S into rabbits induces excess sleep that resembles closely the excess sleep observed in the same animals after 16 hr of sleep deprivation (3). These observations support the view that factor S contributes to the normal drive toward sleep after a period of wakefulness. However, details of this mechanism cannot be investigated without adequate supplies of pure material; large quantities of brain tissue are required to obtain small quantities of purified factor S by the method described above, and it may be anticipated that future physiological studies will involve synthetic material.

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