The Composition of Sleep-promoting Factor Isolated from Human Urine*

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A sleep-promoting substance, Factor S, has been extracted and purified from large volumes of human urine. Urinary Factor S is a small glycopeptide; amino acid-amine analyses of the purified material revealed a substance composed of glutamic acid, alanine, diaminopimelic acid, and muramic acid in molar ratios of 2:2:1:1. The active glycopeptide resembles bacterial peptidoglycans but the composition suggests that it is not simply of adventitious origin. Other reasons for this conclusion are also given. Infusions into the lateral ventricle of the brain of about 5 pmol/kg of body weight induce a 50% increase in slow wave sleep in rabbits. The excess sleep is normal as judged by electrophysiological and behavioral criteria; it resembles the sleep that occurs when animals are allowed to sleep following prolonged sleep deprivation.

The chemical characterization of endogenous substances that promote natural sleep has been a subject of interest for many years (reviewed in Refs. 1–3). Our laboratory has described sleep-promoting factors in extracts of goat cerebrospinal fluid (4–6), brain (6–8), and human urine (8). Intraventricular infusion of picomole quantities of the urinary sleep factor induces excess slow wave sleep for several hours in rats, rabbits, and cats (8, 9). The excess SWS is normal in that it is episodic in nature. The behavior of recipient animals is also normal; they are easily aroused and awaken from time to time for grooming, eating, and drinking (7, 8). In rabbits, the excess SWS is also characterized by slow waves whose amplitude exceeds that of EEG slow waves recorded from the same animals under control conditions (6–8). Such increases in the amplitude of EEG slow waves are also observed during the excess SWS that follows sleep deprivation in rats (10), rabbits (6), and possibly man (11). The chemical and physiological properties of the urinary factor closely resemble the properties of sleep factor found in sterile CSF from sleep-deprived goats (4, 5) or that derived from brains of sleep-deprived animals (6–8). Treatment of the sleep-promoting preparations with proteases eliminated the biological activity (6, 7).

The discovery of sleep factor (Factor S) in urine has made possible the preparation of sufficient biologically active material for systematic chemical studies. We now describe the chromatographic methods used to isolate Factor S from over 3000 liters of human urine. Results of amino acid analyses of the purified product indicate that urinary Factor S is a small glycopeptide. The biological effects in rabbits of administered urinary sleep factor do not change over the course of the purification.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human urine was obtained from healthy male adults. For small scale preparations (i.e., less than 20 liters), the urine was collected in 1-liter bottles, frozen within 1 h, and stored at −20 °C. The frozen material was thawed and filtered through Whatman No. 3 paper just prior to extraction. For large scale preparations (batches up to 400 liters), the urine was collected in large containers placed in lavatories; it was treated with CM-Sephadex resin on the day of collection, without freezing. Between purification steps, samples were stored at −20 °C.

All chemicals were reagent grade. Sephadex resins were purchased from Pharmacia, Beckman buffers were from Beckman Instruments, and fluorescamine (Flurana) was from Roche Diagnostics. Nonpyrogenic saline and water were obtained from Travenol and amino acid standard solutions were from Beckman Instruments and Sigma. Muramyl dipeptide (MDP) was obtained from Vega Biochemicals. [14C] Aspartic acid, [1H] cysteic acid, and [14C] fructose were obtained from New England Nuclear.

**Methods**

**Extraction and Purification of Sleep-promoting Material**

An outline of the procedures developed is shown in Fig. 1, and the following text amplifies that summary:

**A: Extraction of Urine with Carboxymethyl-Sephadex—** Urine was extracted with CM-Sephadex C-25 by a batch operation. The resin was swollen in water, washed with 0.5 N HCl followed by excess NH4OH, and finally equilibrated to pH 7.0 with 50 mM NH4-acetate buffer as previously described (8). The equilibrated resin was mixed with urine (50 ml of swolen resin/liter of urine) at room temperature. Lots sizes ranged from 0.5 liter when pilot studies were conducted to over 300 liters for larger scale extractions. The mixture was gently stirred for 30 min and the resin was then allowed to settle for about 1 h. The liquid phase was decanted and discarded. The resin was poured into a column for further treatment. It was washed sequentially with 3 bed volumes of 50 mM NH4-acetate buffer, pH 7.0, 5 bed volumes of 50 mM NH4-acetate buffer, pH 9.0, and 1 bed volume of water. The sleep-promoting substance was then eluted from the columns using 1 bed volume of pH 1.9 buffer (formic acid/acetic acid/water, 150:100:750, v/v/v). This eluate was reduced in volume by low pressure evaporation at 40 °C.

**B: Gel Filtration—** The concentrated eluates from CM-Sephadex columns were subjected to two successive gel filtrations on G-10 Sephadex columns. The columns were equilibrated and developed in 50 mM acetate acid at 4 °C. Columns of bed volume 0.15, 0.60, 1.0, or 15 liters were used, depending upon the size of the sample to be applied. The volume of the samples did not exceed 7% of the column.
Composition of Sleep-promoting Factor - Urine

A Cation-exchange chromatography
- CM-Sephadex
  - Batch method
  - Elute Factor S with pH 1.9 buffer
  - Volume reduction

B Gel filtration
- G-10 Sephadex

C Anion-exchange chromatography
- DEAE-Sephadex
  - Elute Factor S with 1 M NaCl
  - Volume reduction

D Gel filtration
- G-10 Sephadex

E Cation-exchange chromatography
- SP-Sephadex
  - Elute Factor S with pH 2.83 buffer

F Gel filtration
- G-10 Sephadex

G Ascending paper chromatography
- Elute section: RF 0.1-0.3

H React product with Fluran
- Gel filtration
- G-10 Sephadex

I Amino acid analysis

Abed columns were calibrated with blue dextran 2000 (to determine Vₒ, [14C]fructose, and NaCl (6)).

The fraction saved from the first gel filtration emerged in the eluate from Vₒ to the beginning of the appearance of NaCl. This fraction was lyophilized to dryness, taken up in 50 mM acetic acid, and reapplied to a G-10 Sephadex column. The fraction saved from the second filtration was from Vₒ to the peak of the sucrose marker (Vₐₒ). This fraction was lyophilized in preparation for anion-exchange chromatography. When large scale extractions were performed, the products from several lots were pooled at this stage of purification.

C Anion Exchange Chromatography—The charge properties of Factor S change during the initial preparative steps A and B (7, 8). This enabled us to use an anion-exchange resin equilibrated at relatively low pH values to separate Factor S from other substances that accompanied Factor S in the CM-Sephadex step (above).

DEAE-Sephadex A-25 was swollen overnight in 500 mM NH₄-acetate buffer, pH 5.0. The next day it was washed with at least 10 volumes of 50 mM NH₄-acetate buffer, pH 5.0. The resin was stored at 4°C in this buffer until used.

The lyophilized product from step B was dissolved in an appropriate volume of 50 mM NH₄-acetate buffer, pH 5.0 (1 ml buffer/liter of original urine). This solution was applied to a DEAE-Sephadex column (bed volume of 66 ml/100 liters of original urine). The column was developed by washing with 1 bed volume of buffer, 50 mM NH₄-acetate, followed by 6 bed volumes of 50 mM acetic acid. Factor S remained bound to the resin and the washes were discarded. Sleep-promoting material was eluted from the resin with 3 bed volumes of 1 M NaCl. The volume of this eluate was reduced by low pressure evaporation as above.

G: Paper Chromatography—The lyophilized product from the DEAE-Sephadex column was desalted by gel filtration on G-10 Sephadex, as indicated in B above. In this step, the fraction Vₒ to Vₐₒ was saved; it was concentrated first by low pressure evaporation at 40°C and then by freeze-drying.

E: Gel Filtration—The concentrated eluate from the DEAE-Sephadex column was desalted by gel filtration on G-10 Sephadex, as indicated in B above. In this step, the fraction Vₒ to Vₐₒ was saved; it was concentrated first by low pressure evaporation at 40°C and then by freeze-drying.

Bioassay—Male New Zealand White rabbits were provided with chronically implanted EEG electrodes and a cerebral lateral ventricular guide tube as previously described (6-8). At least 1 week was allowed for recovery from the operation. Rabbits were housed in a room with a 12-h, light-dark cycle. Before each recording period, they were brought to the experimental cages for an overnight acclimation period. Infusions and recordings were performed during the light hours.

Samples were infused intraventricularly at a rate of 3 ml/min for 90 min. EEG, rectified slow wave EEG, and bodily movements were recorded during the infusion period and for the next 6 h as described previously (7, 8). The filtered rectified signal was also electronically integrated and integrals were printed on tape every 2 min.

Recording were analyzed in two ways: 1) Duration of SWS was determined by visual scoring of the EEG and of the filtered rectified EEG recording: 2) Amplitude and slow waves (mean rectified slow wave voltages) averaged over 2 min. SWS periods (Eₛ) and waking periods (Eₐ) were determined for each rabbit using the printed integrals.

Individual control values for duration of SWS, Eₛ, and Eₐ were determined for each rabbit during 1 or more 6-h control recordings.

Monitoring the Progress of the Purification—Bioassays were used to detect sleep-promoting activity in various fractions after each purification step described above. Samples obtained after steps B, D, and F (Fig. 1, “Methods”) were subjected to gel filtration using G-10 Sephadex as described above (in steps D and F) but using sterile nonpyrogenic water. The fraction Vₒ to Vₐₒ was contained biologically active material was desalted by gel filtration on G-10 Sephadex. The concentrate was applied to a 150-ml column equilibrated and developed in 50 mM acetic acid at 4°C. The fraction saved was from Vₒ to Vₐₒ. This eluate was freeze-dried, taken up in 1 ml of water, transferred to a conical test tube, and freeze-dried using a Savant Speed Vac Concentrator.

G: Paper Chromatography—The lyophilized products from step F were taken up in 15 μl of water and each was injected as a single spot (about 0.5 cm in diameter) to Whatman No. 1 paper. Lysine was used as standard and was placed on both sides of the test material. Ascending chromatography was carried out as previously described (7) using a solvent system of acetone/1-propanol/water 5 ml NH₄OH, 262 μl NaNO₃/10H₂O. Three successive additions of 0.25 ml of fluorescamine (0.3 mg/ml acetone) were added with constant stirring. Acetone was then removed using the Savant Speed Vac Concentrator. The aqueous residues (0.5-0.7 ml) were applied to G-10 Sephadex columns (20-ml bed volume) which were equilibrated and developed at room temperature in 50 mM acetic acid. A void volume of 6 ml was discarded and the fraction from 6-12 ml which contained the sleep-promoting activity was saved.

I: Amino Acid Analysis—Aliquots of the product from step H which represented 100 liters of original urine was lyophilized, taken up in 150 μl of Beckman lithium citrate dilution buffer, and applied to a Beckman 121 MB amino acid analyzer. Amino acid analyses were performed on both unhydrolyzed and hydrolyzed samples. The usual hydrolysis conditions were 4 h in HCl, 145°C for 4 h in vacuo sealed tubes. To examine the content of amino sugar in samples, milder hydrolysis conditions were used, i.e., 6 N HCl, 100°C for 6 h in vacuum-sealed tubes. The analyzer was routinely calibrated with standard solutions of amino acids, including diaminopicolinic acid. We also examined N-acetylglycosamine, N-acetylmuramic acid, and muramyl dipeptide after hydrolysis (6 N HCl, 100°C for 6 h) to yield markers for glucosamine and muramic acid.
Parameters were optimized for either single label counting ([\textsuperscript{4}C]sucrose, and fluorescamine steps were performed. Fractions were saved. Blanks for each step of the purification were prepared to control for contaminants that may have been introduced during the purification process. The blanks differed from the process described above only by omission of biological sample. Previously, we reported that the blanks from the gel filtrations, CM-Sephadex, and fluorescamine steps have no significant effects on sleep (8). Blank samples for the DEAE-Sephadex step and the paper chromatographic step were performed in this study. In the case of the SP-Sephadex step, the fractions on each side of those fractions that contained sleep-promoting activity were examined each time these columns were developed, to serve as blanks. These were all negative in the biological assay. Blanks for the gel filtration, SP-Sephadex, paper chromatographic, and fluorescamine steps were also analyzed for amino acids.

**Liquid Scintillation Counting**

An Ansitron liquid scintillation counter was used. Instrument parameters were optimized for either single label counting ([\textsuperscript{4}C]sucrose, step B) or for double label counting ([\textsuperscript{3}H]cysteic acid and [\textsuperscript{14}C]aspartic acid, step E).

**RESULTS**

**Purification**—The first step of purification involved the handling of large volumes of urine; a batch method was adopted after many trials. We demonstrated that most of the cationic sleep-promoting material was extracted using this batch process (13). To elute Factor S from the resin, acetic acid-formic acid buffer, pH 1.9, proved more satisfactory than 1 M NaCl-1 M acetic acid which was used previously (8). We performed experiments designed to test the stability of Factor S at the temperatures that might be encountered in the evaporation and found that it was stable for up to 8 h at 70 °C in the buffer at pH 1.9.

The second step involved desalting and fractionation by gel filtration. Previously we had shown that Factor S elutes in front of sucrose on G-10 Sephadex (6) and these specific fractions were saved. Previously we provided evidence that the charge on Factor S changes during the first two purification steps (7, 8). The events responsible for these charge changes are not known, but we took advantage of this situation by placing an anion-exchange step in series with the cation-exchange step. The anion-exchange step (steps C and D, Fig. 1) was responsible for a 100-fold increase in specific activity (Table I).

Our use of the cation exchanger SP-Sephadex (step E) was designed to separate acidic components from each other; Fig. 2 shows the elution profile of Factor S in relation to the standard markers, cysteic acid, taurine, aspartic acid, and oxidized glutathione. After desalting the active fractions from SP-Sephadex by gel filtration (step F), samples were sufficiently clean to allow us to dissolve them in small volumes and subject them to paper chromatography. Data on steps G and H have been published (7, 8); we now use these steps to achieve the purification needed to obtain definitive amino acid analyses.

Table I is a summary of the purification of urinary Factor S from 1500 liters of urine. On a weight basis, the final product contained only about 10⁻¹⁰ of the solids present in the original urine. Losses of urinary Factor S during the first two steps of purification cannot be evaluated for two reasons: 1) biological assays cannot be performed for two reasons: 1) biological assays cannot be performed for two reasons; 2) the most active fraction was between the elution volumes of 110-119 ml; this fraction is the same one for which amino acid analyses are shown after further purification in Table IV (sample 1) and Table V. The bed volume of this column was 145 ml. The extracts from 2900 liters of urine purified through Step D were pooled, then applied to this column.

![Chemical diagram showing elution profile of Factor S](http://www.jbc.org/)

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**Table I**

<table>
<thead>
<tr>
<th>Purification step (Fig. 1)</th>
<th>Estimated dry weight</th>
<th>Activity recovered</th>
<th>Specific activity [g doses/mg dry weight]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>7.5 × 10⁴</td>
<td>15,000</td>
<td>1</td>
</tr>
<tr>
<td>Steps A + B</td>
<td>1.7 × 10⁴</td>
<td>107</td>
<td>107</td>
</tr>
<tr>
<td>Steps C + D</td>
<td>7 × 10⁻⁵</td>
<td>2,000</td>
<td>5,000</td>
</tr>
<tr>
<td>Steps E + F</td>
<td>4 × 10⁻⁴</td>
<td>833</td>
<td>28,000</td>
</tr>
<tr>
<td>Steps G</td>
<td>3 × 10⁻⁵</td>
<td>500</td>
<td>71,000</td>
</tr>
<tr>
<td>Steps H</td>
<td>7 × 10⁻⁵</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The starting material was 1500 liters of human urine. The total sleep-inducing doses after each purification step are reported (see text).
*Assumes 5% urine solids.
*Dry weight (determined).
*Estimated from total amount of amino acids and amino sugars released by acid hydrolysis.

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**Bioassay of Purified Material**—The effects of extracts of increasing purity on rabbit SWS are shown in Table II. An increase in duration of SWS was observed after intraventricular infusion.
Composition of Sleep-promoting Factor

Effects of urinary extracts of increasing purity on rabbit SWS

<table>
<thead>
<tr>
<th>Step*</th>
<th>Infusate</th>
<th>Dose*</th>
<th>n</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>A + B</td>
<td>Sample</td>
<td>0.1</td>
<td>22</td>
<td>40 ± 1</td>
<td>69 ± 3</td>
</tr>
<tr>
<td></td>
<td>Reagent blank</td>
<td>2</td>
<td></td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>C + D</td>
<td>Sample</td>
<td>0.15-0.2</td>
<td>22</td>
<td>38 ± 13</td>
<td>64 ± 2</td>
</tr>
<tr>
<td></td>
<td>Reagent blank</td>
<td>6</td>
<td></td>
<td>43 ± 2</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>E + F</td>
<td>Sample</td>
<td>0.5-0.75</td>
<td>21</td>
<td>39 ± 1</td>
<td>62 ± 2</td>
</tr>
<tr>
<td></td>
<td>Reagent blank</td>
<td>21</td>
<td></td>
<td>39 ± 1</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>G</td>
<td>Sample</td>
<td>1.8</td>
<td>21</td>
<td>37 ± 1</td>
<td>63 ± 2</td>
</tr>
<tr>
<td></td>
<td>Reagent blank</td>
<td>10</td>
<td></td>
<td>41 ± 1</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>H</td>
<td>Sample</td>
<td>3.0</td>
<td>6</td>
<td>36 ± 1</td>
<td>61 ± 4</td>
</tr>
<tr>
<td></td>
<td>Reagent blank</td>
<td>4</td>
<td></td>
<td>43 ± 3</td>
<td>41 ± 6</td>
</tr>
</tbody>
</table>

See Fig. 1.

Dose is expressed in terms of liters of original urine. The amount shown is the relative amount of extract infused after each stage of purification which induced strong sleep responses, i.e., near-maximal (see Table VI).

*2-6 h postinfusion.

Amino Acid Composition of Active Fractions after Fluorescamine Treatment—After chromatography on SP-Sephadex (steps E and F, Fig. 1), several adjacent eluates were found to be active. Each was taken separately through the remainder of the purification program. Acid hydrolysis of the final purified fractions released glutamic acid, alanine, Dap, and glycine. Three analyses are shown in Table IV. The absolute amounts of amino acid recovered/100 liters of original urine vary because each of these samples represents a separate aliquot from the SP-Sephadex chromatogram. The apparent molar ratios of Glu/Ala/Dap are 2:2:1. The amount of glycine recovered was variable with respect to the amino acids mentioned. Small and variable amounts of aspartic acid, threonine, and serine were also released by acid hydrolysis, but these were always less than 25% of the molar values of Ala, Glu, Gly, and Dap.

Diaminopimelic acid is a constituent of bacterial peptidoglycans (14, 15) and its presence alerted us to the possibility that our purified fractions might contain amino sugars. We, therefore, subjected sample 1 of Table IV to milder hydrolysis conditions followed by amino acid analysis. Mild acid hydrolysis released muramic acid and glucosamine (Table V). The amino acids were also quantitatively released under the milder conditions (cf. Table IV sample 1, and Table V). Muramic acid was equimolar with Dap whereas glucosamine was equimolar with glycine.

Correlation of Biological Effects with Analytic Data—Several different fractions were purified through the fluorescent
TABLE V
Amino acids and amino sugars released by mild acid hydrolysis of purified urinary Factor S

<table>
<thead>
<tr>
<th>Amino acid*</th>
<th>nmol†</th>
<th>Molar ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>1.52</td>
<td>2.0</td>
</tr>
<tr>
<td>Ala</td>
<td>1.53</td>
<td>2.0</td>
</tr>
<tr>
<td>Dap</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.70</td>
<td>0.9</td>
</tr>
<tr>
<td>Gly</td>
<td>1.20</td>
<td>1.6</td>
</tr>
<tr>
<td>glucosamine</td>
<td>1.23</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Hydrolysis conditions were 100 °C, 6 h, 6 x HCl
† Per 100 liters of original urine. Small amounts, less than 100 pmol/100 liters original urine of Asp, Thr, and Ser were also released by hydrolysis.

TABLE VI
Effects of various doses of final purified urinary Factor S on rabbit SWS

<table>
<thead>
<tr>
<th>Rabbit pmol infused*</th>
<th>SWS* Control</th>
<th>SWS* Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>33</td>
<td>43</td>
</tr>
<tr>
<td>59</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>58</td>
<td>33</td>
<td>53</td>
</tr>
<tr>
<td>61</td>
<td>41</td>
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<td>45</td>
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<td>69</td>
</tr>
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<td>54</td>
<td>37</td>
<td>67</td>
</tr>
<tr>
<td>58</td>
<td>33</td>
<td>54</td>
</tr>
<tr>
<td>59</td>
<td>23</td>
<td>36</td>
</tr>
</tbody>
</table>

* Values are picomoles infused based upon the amount of Dap released by acid hydrolysis. Values based on the amount of Gly released by hydrolysis do not correspond with biological activity. From top to bottom, such values were 48, 43, 28, and 39 pmol.
† 2-6 h postinfusion.

The results of infusing different samples of known Dap content are shown in Table VI. The amount of excess SWS observed correlates closely with the Dap-peptide infused. Biological responses were not correlated with the Gly-containing entity. A substantial sleep response was obtained with a 10-pmol dose of the Dap-peptide (presumable muramyl-peptide) and a detectable response was observed with only 1 pmol.

DISCUSSION

The biological and chemical properties of urinary Factor S reported above are in marked contrast to those of other reported sleep-inducing peptides. Monnier and colleagues have isolated a sleep-inducing nonapeptide (DSIP) from blood which causes a transient (20-30 min) increase in slow wave cortical EEG in acute experiments on rabbits (16-18). This nonapeptide differs from Factor S in amino acid composition and the pattern of physiological activity. According to Schoenenberger and Monnier (17), approximately 20 nmol/rabbit are needed to induce a small increase in delta wave activity. In contrast, less than 10 pmol of urinary Factor S are sufficient to cause excess SWS for several hours. In our bioassay, synthetic DSIP had no effect on SWS (8). Nagasaki et al. (19, 20) have reported the partial purification of a sleep-promoting factor from the brain stems of sleep-deprived rats. The molecular weight and charge properties of their material appear similar to those of Factor S and it is possible that group is dealing with the same compound as we are. Pavel (21) has reported that intraventricular infusion of very small amounts of arginine-vasotocin causes a transient increase (10-30 min) of SWS in cats, but it apparently does not induce SWS in rats (22). We have observed a transient effect on SWS in rabbits with Arg-vasotocin (8) but this effect is in marked contrast to the prolonged effects of urinary Factor S.

The purification procedures shown in Fig. 1 were developed over a period of years using CSF, brain, and urine as starting materials (4-8). They represent only a few of the chromatographic systems that we tested. For example, high pressure liquid chromatography and high voltage paper electrophoresis (7) were used in pilot studies on material purified through step D (Fig. 1) but losses in these systems were high. Paper chromatographic systems other than the one reported here and thin layer chromatographic systems were also used but were found to be unsatisfactory. It may be emphasized here that a single biological assay for sleep requires many hours of recording on each of at least two rabbits. The procedures described, together with the procedures which were tested but found wanting, required about 10,000 h of EEG recording associated with about 1,000 assays on more than 200 "chronically" operated rabbits over a period of more than 5 years.

Our final purified fractions probably contained two components, one containing glucosamine and Gly, and the other comprised of Glu, Ala, Dap, and muramic acid. However, it is possible to interpret the amino acid analysis data shown in Table V as showing a single substance which contains Glu, Ala, Dap, muramate, glucosamine, and Gly in molar ratios of 4:4:2:2:3:3. This seems unlikely because: (a) previously we showed that urinary Factor S passes through an Amicon UM05 filter (8) and substances the size of the 2:2:1:glycopeptide (Table V) can pass through UM05 filters (16). A substance the size of the hypothetical 4:4:2:2:3:3 material would presumably be too large. (b) The amount of Gly released by acid hydrolysis of several purified fractions did not correlate with their biological activity (Table VI). (c) In some instances, amino acid-amino sugar analyses were performed on inactive fractions which were adjacent to active fractions. These inactive fractions did not contain the muramyl-Dap substance but did contain the glucosamine-glycine material in higher concentrations than those found in the adjacent active fractions.

Further evidence that the muramyl peptide is indeed responsible for sleep-inducing effects comes from experiments in which synthetic analogs, MDP and MDP linked to L-lysine (MDP-Lys) induce sleep in rabbits and cats. In another paper, we will describe this work in detail. For the purposes of the present paper, it is sufficient to note that intraventricular infusion of 100 pmol of MDP/rabbit induces prolonged excess SWS that is characterized by high amplitude EEG slow waves. If D-alanine is substituted for L-alanine in MDP, sleep-promoting activity is lost. It is clear that MDP is less active than the natural agent (Table VI) and that Dap is not an essential constituent.

Diaminopimelic acid and muramic acid are generally considered to be components of plants and bacteria (23) but not of mammalian cells. However, Dap has recently been identified as a normal component of human urine (24). The components of the purified urinary materials resemble the components of some bacterial peptidoglycans (14). We have had, therefore, to consider the possibility that the glycopeptide(s) we have isolated from urine are derived from bacterial contamination. There are reasons for believing that the material we have isolated may not simply be a contaminant and that the glycopeptide is a normal mammalian natural sleep-promoting substance. The reasons are: 1) the molar ratios of...
constituent units of the glycopeptide we have isolated are different from those of bacterial glycopeptides. The closest natural analogue appears to be the *Escherichia coli* peptidoglycan which is composed of muramic acid, Ala, Glu, and Dap in molar ratios of 1:2:1:1. The urinary sleep-promoting material apparently contains an extra glutamic acid residue. More definitive comparisons await further structural studies on the small amount of urinary material (~20 μg) we have available for that purpose.

2) Approximately the same yield of urinary Factor S has been obtained from at least 20 separate batches of human urine. The negative reagent blanks provide evidence that bacterial growth on columns, in reagent solutions, etc. does not yield sleep-promoting activity. If our material is of bacterial origin, it must have been present in the urine prior to the start of the purification procedures. It is unlikely that a similar amount of contaminant would have been present in each preparation. As indicated under “Materials and Methods,” urine was, in some instances, frozen immediately upon collection from healthy male contributors and fractionated immediately when it was thawed.

3) Urinary Factor S and Factor S obtained from CSF have similar physiological effects, suggesting they are the same. Both induce excess SWS in rats (4, 6, 8), cats (5, 9), and rabbits (6, 8) with a similar time course. Some physical and chemical data are available which also suggest they are the same. Both pass through UM05 ultrafiltration membranes (4, 8), indicating $M_r < 1000$. There is evidence that both are peptides (6), and both elute in a similar position upon gel filtration (4, 6). The original finding of Factor S in CSF was fully controlled to eliminate any possibility of bacterial contamination (4, 5). The direct transfer of sterile CSF from sleep-deprived goats into recipient rats induced excess SWS. The brain and urinary sleep-promoting substances also appear to be identical (8). They both induce prolonged excess SWS in rats (8) and rabbits (8) and have similar effects on EEG slow wave amplitudes in rabbits. Their behavior on every separatory system we have used is identical. These methodologies include ultrafiltration (8), gel filtration (8), paper chromatography (7), cation-exchange chromatography (8), and high voltage paper electrophoresis (8). Neither product is significantly reactive with Fluram (8), and both are peptides (7). It is unlikely that the same contaminant would be obtained using the very different extraction techniques that were used for these two tissues (8). (Fresh or frozen brain was extracted with acetic acid-HCl (6, 7)).

Finally, in this context, muramic acid was readily identified in hydrolysates of our most highly purified sleep-promoting fractions from rabbit brain (6, 7). The identification, on the amino acid analyzer, was based on retention time, peak contour, and the ratio of absorptions at 440 and 570 nm (0.24). There were also indications of diaminopimelate in the hydrolysates of purified active fractions from brain. The quantities of material available were, however, not adequate for establishment of the molar ratios of components (cf. Table V).

4) Bacterial peptidoglycans are reported to inhibit rather than promote SWS and rapid eye movement sleep in rats (25-28). This would be consistent with the possibility that these naturally occurring bacterial peptidoglycans are structural analogs of urinary Factor S and they may share the same receptors but induce opposite effects.

Although all these observations suggest that the material we have isolated is not of adventitious bacterial origin, proof of the source of the urinary sleep-promoting material must necessarily await investigation of possible biosynthesis in mammalian tissue. It is also necessary to consider the possibility that bacterial peptidoglycans, or their components, may be absorbed by the gut and are subsequently used or modified to produce the sleep-promoting substance. Whatever the outcome of such future experiments, it is currently clear that a substance closely resembling bacterial cell-wall glycopeptides is in fact a potent promoter of slow wave sleep.

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