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Neurohumoral Correlates of Sleep: Further Biochemical and Physiological Characterization of Sleep Perfusates

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SPANIS, C. W., del CARMEN GUTIÉRREZ, M. and R. R. DRUCKER-COLÍN. *Neurohumoral correlates of sleep: Further biochemical and physiological characterization of sleep perfusates.* PHARMAC. BIOCHEM. BEHAV. 5(2) 165–173, 1976. – Twenty cats were prepared surgically with electrodes for recording the EEG, Eye Movements and EMG and a push-pull cannula system in the midbrain reticular formation (MRF) allowing the extraction of perfusates during wakefulness or REM sleep. Proteins in the perfusates were analyzed by Isoelectric Focusing (IEF) polyacrylamide gels and Sodium Dodesyl Sulphate (SDS) slab gels. In addition analysis of glycoproteins was done by gas chromatography. In some cats the contribution of cerebrospinal fluid (CSF) proteins to perfusate proteins from brain tissue was studied by intraventricular injections of labelled leucine. The effect of sleep alterations on the protein cycle during sleep and wakefulness was also studied. The results of these experiments showed that most of the proteins in the perfusates are acidic, and that REM sleep perfusates contain 2 proteins M.W. 73.000 and 45.000 not present in awake perfusates, CSF or serum. It was also shown that CSF proteins do not appear to contribute to the proteins in perfusates, and that altering the sleep wake cycle, induces changes in the rhythm of protein release in perfusates. It is suggested that some relatively large polypeptides may participate in the regulation of REM sleep.

REM REM-sleep Sleep Push-pull cannula Midbrain reticular formation Reticular formation Protein cycle and sleep Sleep-wake cycle

IMPROVEMENTS in the "Push-pull" cannula technique originally developed by Gaddum [10], have permitted the extraction of a variety of substances from specific brain sites in freely moving animals [15]. Most importantly this technique allows correlations between certain biochemical events and physiological functions, or drug effects. As such acetylcholine has been assayed from perfusates in relation to sleep [11], serotonin in relation to temperature regulation [16], and catecholamines in relation to feeding behavior [16] and to chlorpromazine and oxotremorine administration [14]. Utilizing the push-pull cannula system it has been shown that perfusates obtained from donor sleeping cats can induce sleep in awake recipient cats [4,8]. More recently it has been reported that during 24 hr perfusion-sleep monitoring sessions, protein content in perfusates vary in a cyclic fashion, and that the peaks of proteins correspond to periods of increased REM [5,7]. The increase of protein levels in perfusates associated with REM sleep have been further confirmed [6]. The relationship between proteins and REM sleep has been indirectly substantiated by studies which have shown that inhibition of protein synthesis reduces significantly REM sleep in mice [17], and in rats [9], while growth hormone and anabolic hormone increases REM sleep in rats [9] and cats [18]. In view of the important role that proteins appear to play in the regulation of REM sleep, the aim of the experiments to be reported below was to characterize and isolate REM related proteins and to determine their possible source.

GENERAL MATERIALS AND METHOD

Surgical Procedures

Twenty cats of either sex (8 males and 12 females) weighing between 2.0 and 3.5 kg were used in these studies. All animals were stereotaxically implanted with a push-pull cannula system in the midbrain reticular formation (MRF) (A:O, L:2V: -2) and electrodes for recording EEG, eye movements (EM) and EMG, as previously described [4]. Three cats were additonally implanted with a push-pull cannula in the preoptic area (PO). (A:14, L:2, V: –3) and a simple cannula in the third venticle (A:7, L:0, V:4). Another group of 3 cats were implanted with bipolar electrodes bilaterally in the preoptic area, instead of the PO and ventricular cannulae.

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EXPERIMENTAL SERIES 1

Procedure for Characterization of Protein in the Perfusate

Fourteen cats were perfused from the MRF with the push-pull cannula at a flow rate of 20 μl/min as previously described [6]. In this series of experiments the perfusates were collected under two conditions: either when the animals were awake or when they were in REM sleep. Slow wave sleep (SWS) was disregarded. The detection of these two states was polygraphically determined. Each cat was perfused under these conditions 5 different times. On each occasion 3 4 ml of awake perfusate and 1.5–2.5 ml of REM perfusate was obtained during 12 14 hr of recording time. All individual samples were frozen (−6°) immediately after collection and the samples from 2 or 3 cats were subsequently pooled together. Each pooled sample yielded 40 ml of awake perfusate and 30 ml of REM perfusate. Thus 5 such pooled samples were obtained for analysis. For comparison purposes blood serum and cerebrospinal fluid (CSF) were obtained from 8 cats.

Isoelectric focusing of proteins. Perfusate proteins were separated in pH gradients of carrier ampholytes formed in disc polyacrylamide gels. A 30 ml solution, adequate for 12 tubes, was prepared containing final concentration of 5% (2/v) acrylamide (Bio-Rad. Richmond, Calif.), 0.2% N-N-methyline-bis-acrylamide (Bio-Rad), 6 M Urea and 2% ampholite (Bio-Rad) degassed, then 10 mg of ammonium persulfate and 3 of N, N, N'-tetramentlylethylenediamine (TEMED) added. The solution was poured into 120 × 4 mm (I.D.) glass tubes, precoated with 1% siliclad, to a height 20 mm below the top of the tubes, and the gels polymerized for 1 hr. A few drops of 3 M urea were added to the tops of the tubes prior to polymerization to act as preservative and to produce a flat surface. The tubes were used within a few hr or stored at 10°C overnight. Bio-Rad and LKB ampholine carrier ampholites were used in these studies of pH ranges 3/10, 3/5, 5/7, 5/8, 7/9, 8/10.

Prior to analysis all samples were desalted by ultrafiltration or an "Amicon" UF system, Model 8 MC, employing UM 05 (MW<500) membranes. Following ultrafiltration the proteins were resuspended in 6 M urea and centrifuged at 800 g for 10 min. Two hundred μl samples containing 50 to 150 μg protein (by Lowry) were overlaid to the gels via a capillary tube, after the tubes had been placed in an electrophoresis cell, and submerged in buffer. The upper bath contained 2% (v/v) ethylene diamine, pH 11.9 and the lower bath contained 0.2% (v/v) H_2SO_4, pH 1.5. Gels were subjected to 150 volts (constant voltage) for 13 hr in a cold room (6°C). At the termination of the run the gels were removed from the tubes and the proteins precipitated with 15% Trichloroacetic acid (TCA) and washed for 24 48 hr. Bio-Rad ampholines eluted well during this time. The gels were then stained for 6 hr in a 45% (v/v) methanol, 9% acetic acid, 45% water and 0.5% (w/v) Coomassie Blue (Brilliant Blue "R" Sigma No. 13-0630) solution. The gels were destained in 7.5% acetic acid and 5% Methanol for 4 days. Activated charcoal was added to hasten destaining. All evaluations between bands were made according to pH gradients and not according to numerical distances between bands, since gels tend to stretch and distort on removal from the glass tubes as well as later handling for photography.

Sodium dodesyl sulphate (SDS) slab gels. Molecular weight sizes of perfusate proteins were determined using discontinuous slab gels with SDS according to Reid and Bielesky [18]. Twelve percent were prepared by this method and 2.5 to 20 μg of perfusate, CSF, serum and marker proteins in 60 μl sample buffer were pipetted to the columns. The slabs were run at room temperature at 70 volts for 4 5 hr (until the marker dye reached bottom of slab), then stained for 1 hr in a solution of 0.2% (w/v) Coomassie Brilliant Blue R 250 (Colab 65 72.1), 50% (v/v) Methanol 7% (v/v) acetic acid, and destained in 7% acetic acid 5% Methanol activated charcoal, for 3 days.

Molecular weight of proteins were estimated from a standard curve of the markers.

Gas Chromatography analysis for glycoproteins. Ultrafiltrated samples were resuspended in 2 N Trifluoroacetic acid (TFA) placed in ampules, sealed and autoclaved for 1 hr. The seals were then broken and the samples dried by vacuum dessecation.

The samples were then washed and dried 3 times, to remove the TFA, then Tris buffer pH 7.0 added. The solutions were passed through cation and anion exchange resins to remove amino acids. Test for sugars was then made by standard sugar analysis preparative methods for gas chromatography according to the method of Kim [12]. An integrated gas chromatograph was used (courtesy Dr. Chow, Dept. of Biology, UCSD). 100 μg samples of standard sugar and samples, were resuspended in 200 μl dichloromethane and injected in 2 μl volumes to a 3% ECNSS-M Gas-Chrom Q Colum.

Examinations of low molecular weight component of perfusates. Filtrands of REM and awake perfusates ultrafiltrated on a UM 05 membrane were pooled separately (25 ml volumes) and lypholyzed to dryness. The samples were then resuspended in 2 ml of water and desalted and fractionated by gel filtration on sephadex G-10 (Farmacia Uppsala, Sweden) columns at room temperature. Eluded samples were developed with ninhydrin and absorbance at 570 μm measured with a Spectrophotometer.

EXPERIMENTAL SERIES 2

Procedure for Further Testing the Relation Between Sleep and Proteins

In this second series of experiments the perfusion and recording conditions were slightly modified. Animals were continuously recorded polygraphically and simultaneously perfused without regard to the state within the sleep-wake cycle for 10 12 hr. Then at the end of the perfusion-recording session the percent of wakefulness, SWS and REM was calculated within each hour, and these events compared with perfusate contents sampled within each individual hour.

Tracing source of proteins in perfusates. In this experiment the 3 cats with a simple ventricular cannula and push-pull cannulae in the PO and MRF were used. It should be noted that the push-pull cannulae were equidistant to the ventricular cannula. The PO cannula was 7 mm in front of the ventricular cannula, while the MRF cannula was 7 mm posterior to it. Just prior to perfusion the cats were administered, through the ventricular cannula, a 400 μl solution containing 40 μ Ci L- U- ^{14}C-leucine (299 mc/mmole). New England Nuclear, Boston, Mass., at a rate of 100 μl/min. Immediately following injection the ventricular cannula was sealed and the animals were perfused through the MRF and PO push-pull cannulae, for a period of 10 hr (9:00 a.m.–7:00 p.m.) at a rate of 20 μl/min.

Throughout this time the animals were continuously

monitored with a Model 6 Grass EEG. During the perfusion, samples were removed hourly at the end of each hour from the pull line by means of a two-way valve system designed to prevent change in flow rate. The samples were divided and analyzed for protein content (Lowry) and also prepared for scintillation counting by the Bray method for aqueous samples. Activity was counted on a Packard Tri Carb liquid scintillation spectrometer Model No. 2425. The counting efficiency was 61 percent.

Effect of bilateral preoptic lesion on protein cycle in MRF. Three cats were perfused in the MRF and the sleep-wake cycle continuously monitored for 12–24 hr. At each hour samples were removed as previously described [7] and analyzed for protein content by the Lowry method. At the end of this session a 4 mA current was passed for 15 sec through the electroded located in the preoptic area. Three days later the cats were again perfused in the MRF, and protein content of perfusates as well as the sleep-wake cycle reassessed.

Verification of Stereotaxic Placements

At the end of the experiments the brain of each cat was perfused *in situ* with 10% Formalin, with the animal under deep barbiturate anesthesia. The brain was then removed and fixed in 10% Formalin for at least 2 weeks. Thereafter, frozen sections of 50 μm thickness were cut mounted and photographed unstained in order to verify electrode placements.

RESULTS

EXPERIMENTAL SERIES 1

Characterization of Proteins

Isoelectric Focusing gels (IEF). Comparison of IEF gels containing separated proteins or polypeptides from sleep and awake samples indicate that most of the bands are identical. Figure 1 represents an example of one of the pooled samples.

The similarity between serum, CSF and perfusate proteins would be expected since many soluble proteins in brain are also found in serum and CSF [13].

IEF gels revealed 16 similar bands by pH and stained analysis (Fig. 1). However, the REM gels have 3 additional bands not evident in the awake gels. Such bands are indicated by arrows in Fig. 1. Such differences were seen in all 5 of the pooled samples. One of these bands when solubilized for 2 hr in 2 × dist. H_2O and measured with an Orion ionalyzer Model 801 digital pH meter, was shown to have a pH value of between 4.0–4.4, while the other two between 6.2 and 6.6 Gels with serum samples contained 8 more bands than the REM perfusates samples. The CSF gels contained several bands in similar locations to the other gels but fewer number of bands than expected. In addition, it should be noted that the perfusate proteins are predominantly acid (Fig. 2). Most of the bands in the biolite 3–5 gels are intact, however, as the pH range is increased to basic values only 2 - 3 bands are found on the gels.

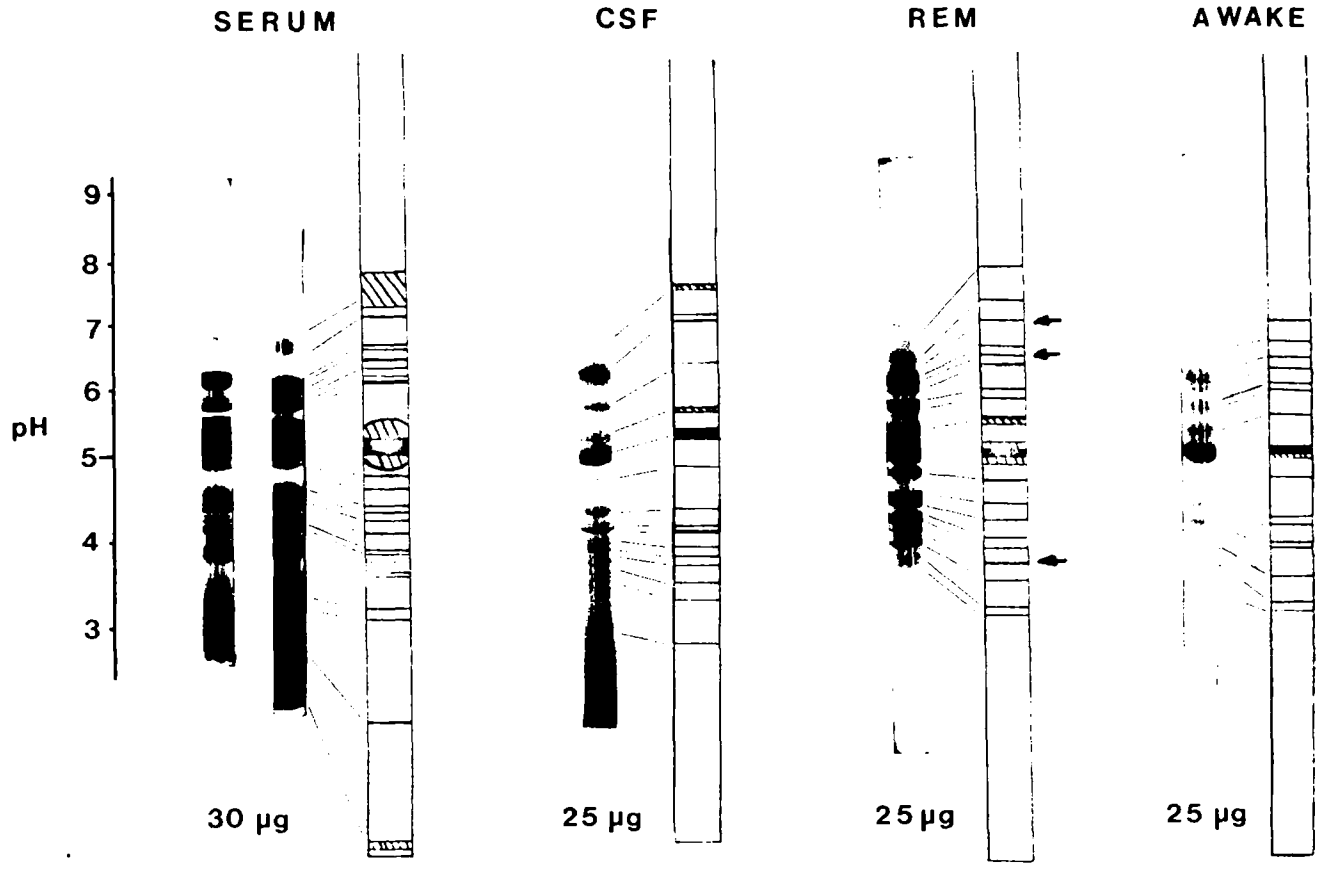

FIG. 1. Comparison of polypeptides (observed as bands) from REM and Awake perfusates, CSF and Serum as separated on IEF polyacrilamide gels. Bands on photographed gels are clarified with line drawings. The arrows indicates differences seen in REM.

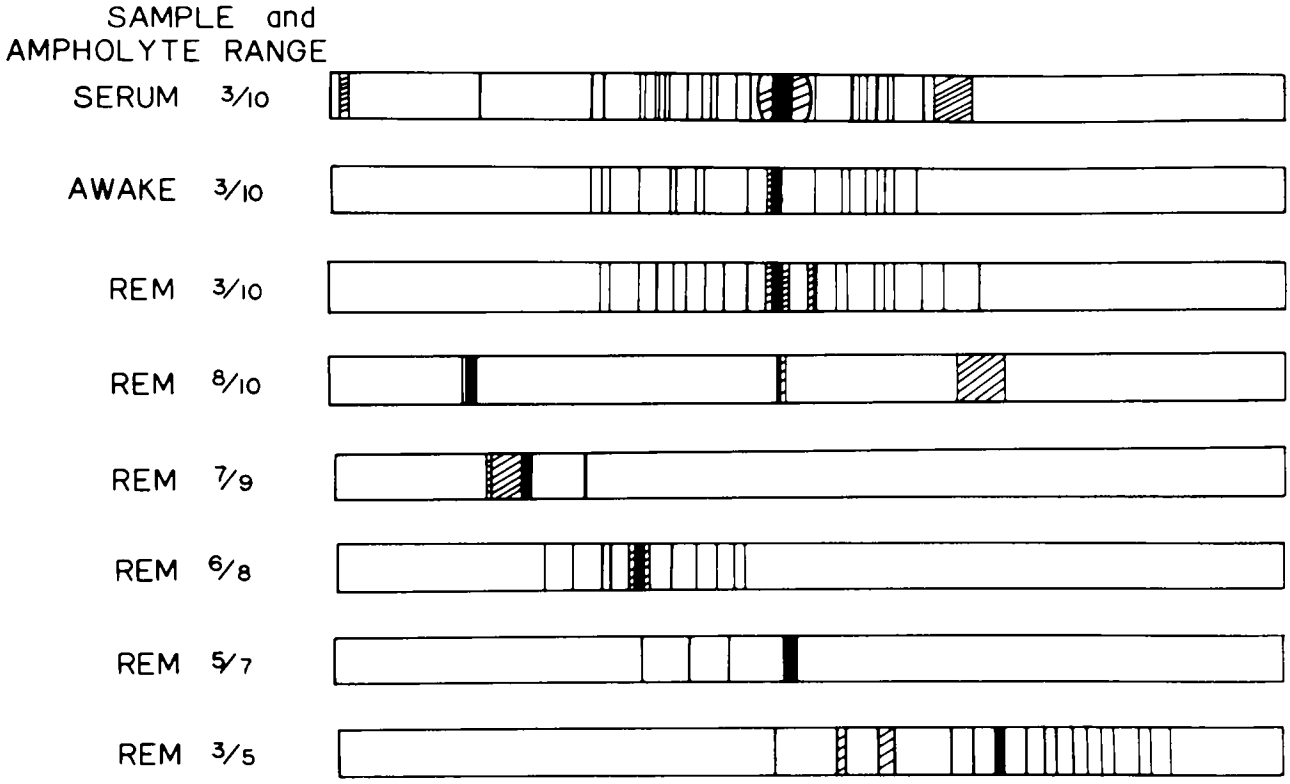

FIG.2. Comparison of polypeptides from Serum Awake and REM proteins as separated on IEF gels using wide and narrow range ampholytes. Relative concentrations of polypeptides are shown by solid bar thickness. Hatched areas indicate lightly diffuse bands. Actual length of gels was 110 mm.

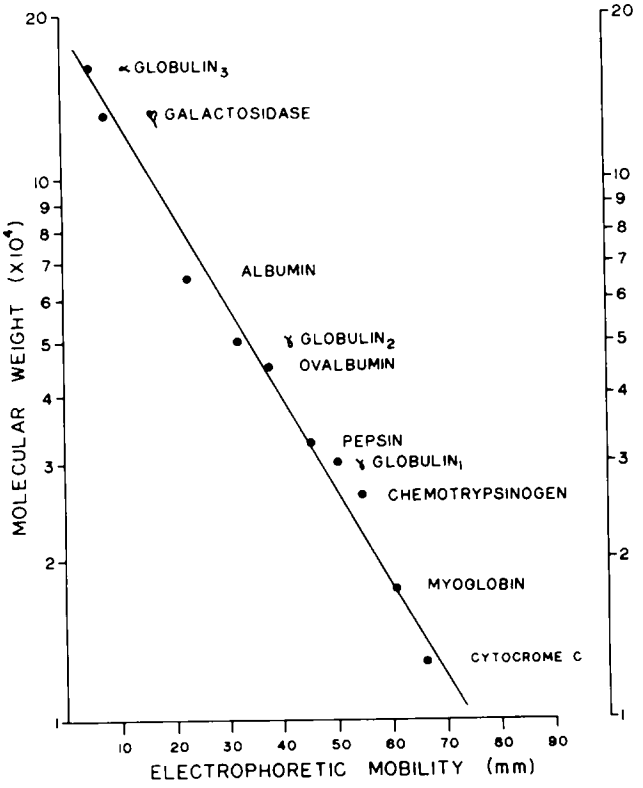

FIG. 3. Electrophoretic mobility in mm of compounds of known molecular weights on SDS slab gels, and used to compare with proteins from perfusates.

SDS slab gels. Molecular weight estimation of sleep and awake perfusates on SDS slabs as evaluated from standard curves of markers is shown in Table 1 and Fig. 3. The separated perfusate proteins were considerably fewer in number when compared to those on IEF or disc acrylamide gels. However, it is important to note that two bands were observed on the sleep perfusate column that were not evident on the awake column in all pooled samples. These were assessed at M.W. 73.000 and 45.000 respectively. The remaining bands were similar and appeared comparable to bands on the serum column. The molecular weight of these are listed in Table 1.

Gas Chromatography analysis for glycoproteins. The presence of glycoproteins in the sleep and awake perfusates was demonstrated by this method. Figure 4 shows that the same sugars were present in both types of perfusates. However, a preponderance of galactose was noted in the REM sample which was 5 times greater than in the awake sample at the same initial protein concentration. Low levels of manose, glucose and ribose, were also observed. Two other low level compounds were unidentified.

Examination of low M.W. components in the perfusate. Spectrophotometric analysis of 25 ml perfusate samples preconcentrated to 2 ml, fractionated and desalted on the G column, reconcentrated by lypholyzation to 1 ml revealed a concentration of less than 1 μg amino acid per 25 ml perfusate.

This is essentially a negligible amount when compared to the standard amino acids cystine, isoleucine and serine which were also eluded through the column at a concentration of 100 μg/ml, with an 80% recovery.

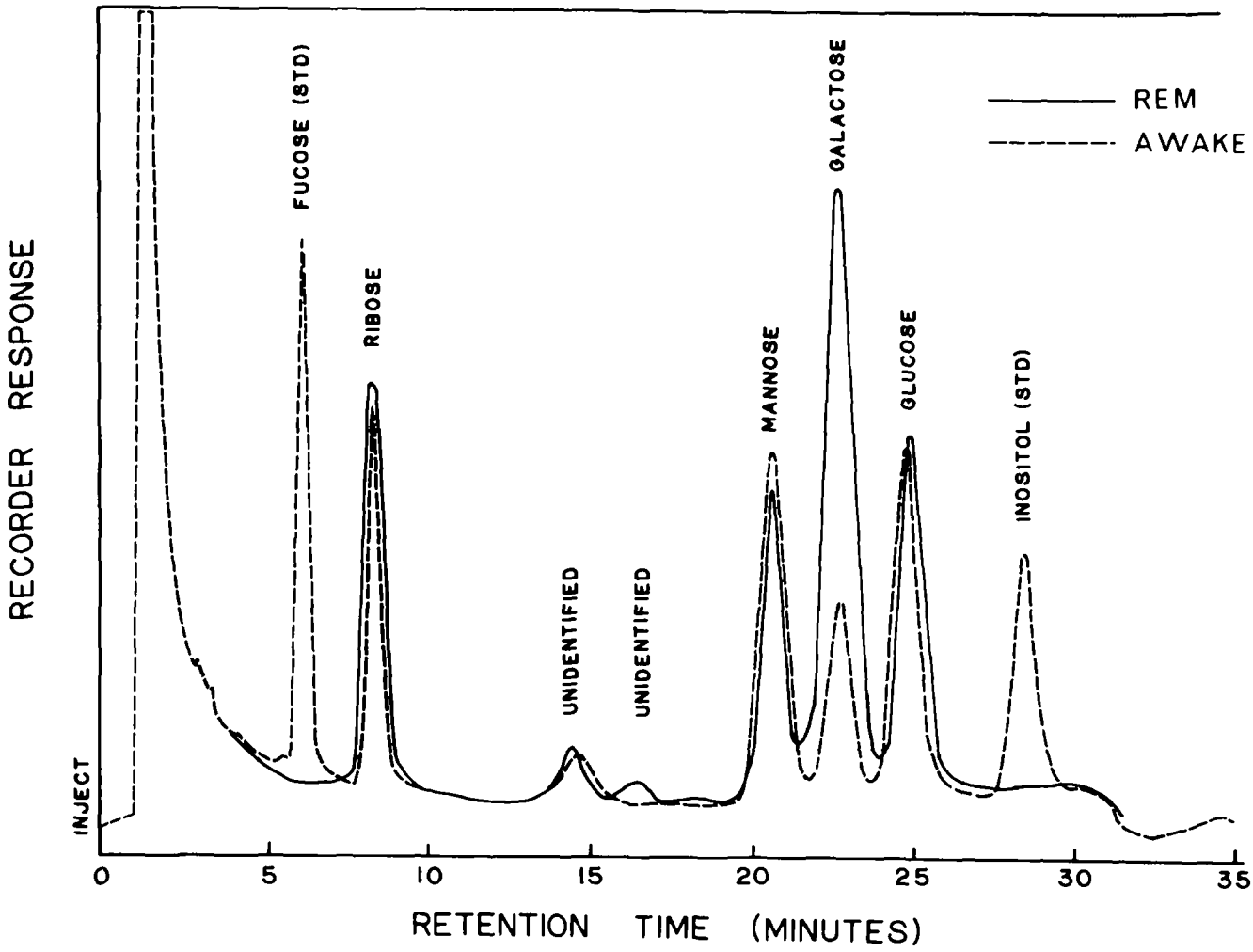

FIG. 4. Gas chromatrographic analysis of the perfusates from the midbrain reticular formation of freely moving cats in REM of Awake states demonstrating the presence of glycoproteins Standards for actual sugars observed are not shown. Comparison of galactose in REM and Awake is shown. Actual concentration of sugars were calculated with integrator of instrument. Gas chromatographic conditions: Column: 3% ECNSS-M Gas – Chrom Q; Range: 10; Attenuation: 4×10^{-4} A.F.S. Carrier Gas: N_2 = 50, H_2 = 28, Air = 16; T_1 = 170C at 8 min.; T_2 = 210 C at 24 min. Injected sample volume is 2 ul.

Samples of M.W. between 500 and 10.000 treated in the same way gave a reading of 12 μg/ml, for REM and 8 μg/ml for awake.

EXPERIMENTAL SERIES 2

Relationship Between Sleep and Proteins

Tracing source of protein in perfusate. During the 10 hr perfusion following injection of C^{14} leucine into the ventricle, the level of radioactivity in the MRF and PO perfusates in the 3 cats tested was negligible. Figure 5 illustrates such an experiment from one cat. As we can also note from this figure, although there was a cyclic appearance of proteins in the perfusates with a peak on the 6th hr, the counts per min remained low throughout the 10 sampled perfusates.

Effect of bilateral preoptic lesion on protein cycle in MRF. In a series of experiments whereby cats were kept awake by manual procedures for a period of 8 hr, a loss of cyclic nature of proteins in the perfusates could be seen. Figure 6A illustrates this effect from one cat. In a cat who sustained bilateral lesions of the preoptic area, which induced long periods of arousal, the cyclic pattern of proteins in the perfusates was absent after the 14th hr, at which time the cat had almost complete insomnia (Fig. 6B). Similar effects were seen in the other two cats during those periods in which no sleep was present.

Verification of Stereotaxic Placements

Representative histological samples of MRF cannula implants and basal forebrain lesions are shown in Figs. 7 and 8 respectively. Examination of serial cuts showed that the perfusion cannulae placements varied from AO to P2; L2 to L4 and V 2 to V-4, while basal forebrain lesions extended from A12 to 15.5, LO.5 to 3 and 3 mm horizontally from the base of the brain.

TABLE 1

MOLECULAR WEIGHT ESTIMATIONS OF THE POLYPEPTIDE FRACTION FROM REM, AWAKE, CSF, AND SERUM PERFUSATES OF CATS BY SDS SLAB GEL ELECTROPHORESIS

Band No.	REM	Awake	CSF	Serum (cat)	Serum (human)
1					160,000
2	160,000	160,000		160,000	160,000
3				150,000	
4				133,000	133,000
5					116,000
6				100,000	
7					82,000
8				80,000	
9				78,000	
10	73,000				
11					70,000
12					66,000
13	65,000	65,000	65,000	65,000	
14					60,000
15				55,000	
16			54,000		
17	53,000	53,000			
18				52,000	52,000
19	47,000	47,000	47,000	47,000	
20					46,000
21	45,000				
22			42,000		
23			35,000	35,000	
24					27,000
25		26,000	26,000		
26	25,000	25,000		25,000	
27					19,000
28					
29					1,000

Estimations were made by measurement of band to origin and compared to the standard curve of markers of known molecular weights (Fig. 3). Samples contained 20 μg total protein (by Lowry).

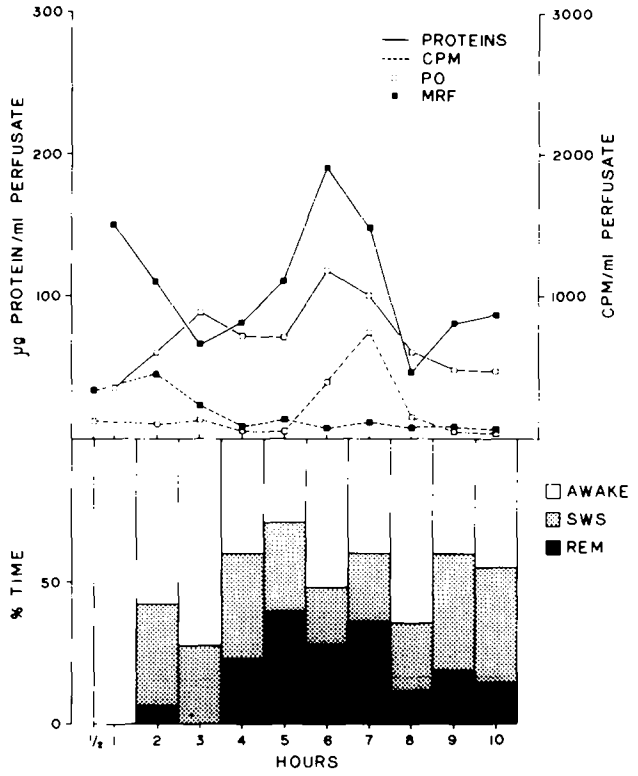

FIG. 5. Graph representing a 10 hr continuous perfusion-EEG monitoring session following the administration of 40 uCi of C^{14} leucine into the 3rd ventricle. Note in the upper half of the graph the cyclic release of proteins in the Midbrain Reticular Formation (MRF) not so apparent in the Preoptic Area (PO) and its relative correlation with increases in time spent in REM as shown in the bottom half of the graph were the percent of time spent in wakefulness, slow, wave sleep (SWS) and REM was calculated for each hour. Also note that despite increase in proteins in perfusates radioactive leucine appears in negligible quantities.

DISCUSSION

The results of our experiments show that the soluble proteins in the perfusates are predominantly acidic. Furthermore differences in types of proteins are present in each of the 5 pooled perfusates samples obtained from cats during REM sleep. These differences are represented by two polypeptides M.W. 45,000 and 73,000 on SDS gels and 3 protein in bands on IEF gels having pH values of 4.0 to 4.4 and 6.2 to 6.6. The bands on the SDS and IEF gels may not necessarily be the same polypeptides. In addition, we further noted that the numbers of free polypeptides under M.W. 10,000, was negligible. We have also shown that the proteins obtained in our perfusates appear to reflect neural processes, since radioactive leucine injected into CSF did not appear in the MRF and PO. Furthermore these neural processes appear to be related to sleep in view of the fact that the cyclic release of proteins usually observed during the normal sleep-wake cycle [7] is disrupted as a result of maintaining the animals awake either by manipulation or by preoptic area lesions.

From our results it appears that many of the proteins from CSF, serum and brain are the same. This agrees with the findings of other authors [13]. In view of this, two possibilities arise to explain the origin of our proteins. Either our perfusates are contaminated by serum or CSF proteins, or else since all components have similar proteins, we would expect to find comparable families of macromolecules. From our labelling experiments the source of proteins as arising from CSF can be discounted, although serum cannot. However, since some bands in our perfusates are not evident in serum this would indicate that these specific proteins, if not all the proteins in our perfusates, arise from neural tissue. Moreover serum gels contain considerably more bands than perfusate gels. Should our perfusates be contaminated by serum, we would expect a much larger number of correlated bands, particularly since our initial concentration of sample proteins placed on the gels is the same.

The presence of glycoproteins in the perfusates and specifically the large quantity of galactose observed during REM sleep would suggest the importance of further investigating these compounds, in view of the prominent role of galactosamine in important biosynthetic pathways.

These results are in agreement with our previous

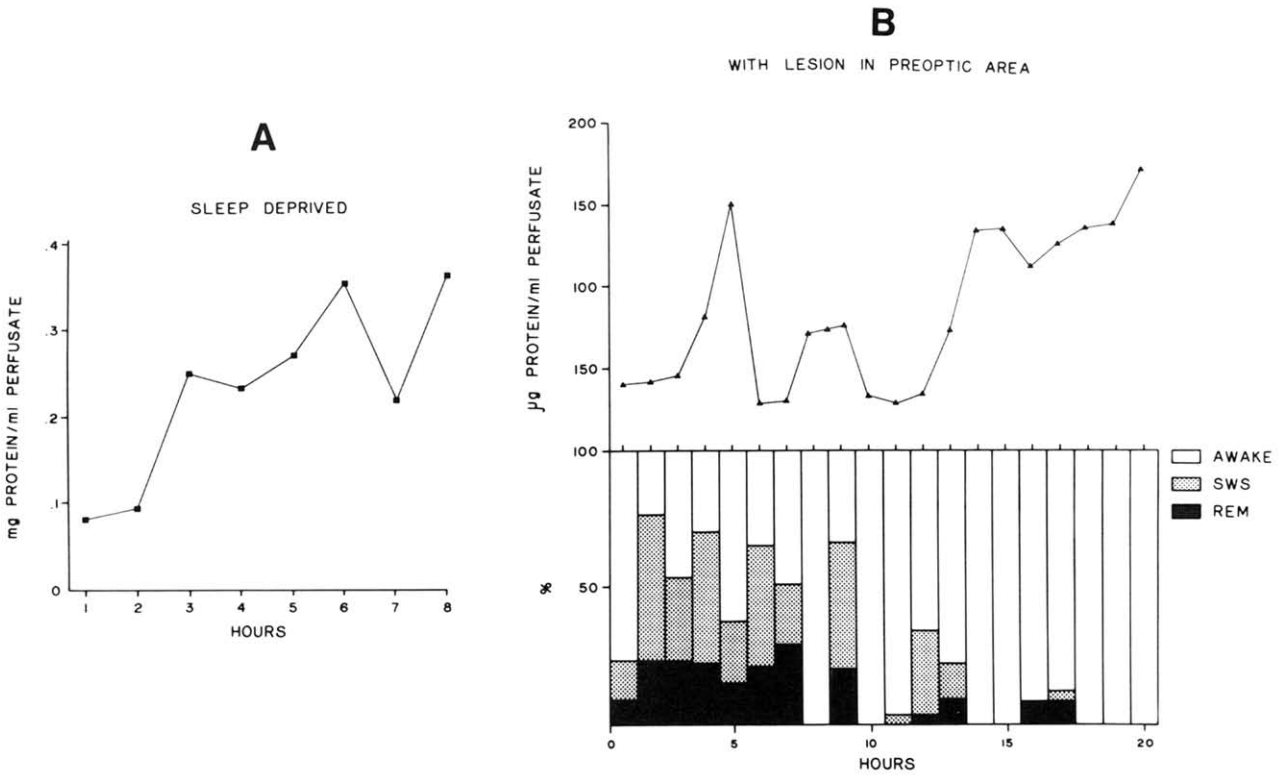

FIG. 6. Graph representing effects of sleep alterations on protein contents in perfusates. A - sleep deprivation was done by manual procedures B - insomnia produced by bilateral preoptic area lesions. Note in both cases disappearance of cyclic pattern.

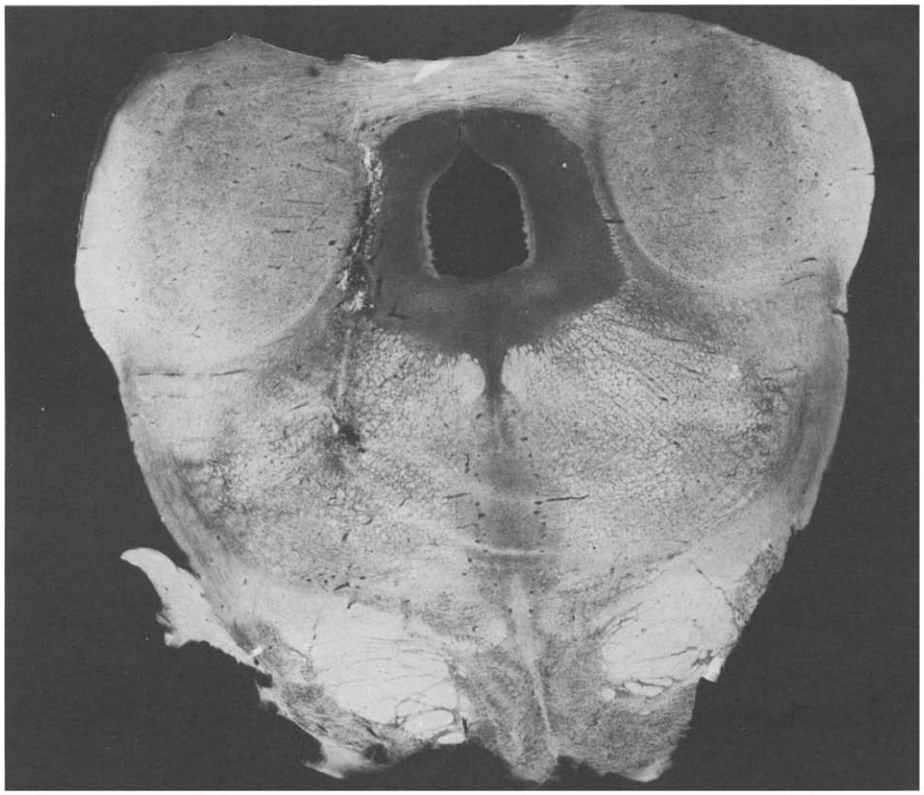

FIG. 7. Photograph of an unstained histological section showing localization of cannula in the MRF.

FIG. 8. Photograph of an instained histological section showing bilateral lesion of basal forebrain.

observations that REM sleep is associated with increases in protein levels [6] thus suggesting that REM sleep reflects a period of increase biosynthesis. Such a concept is further supported by studies which have shown a specific decrease in REM sleep following administration of protein synthesis inhibitors [1, 9, 17]. In addition our results point toward the possibility that some relatively large polypeptides may be involved in the regulation of REM sleep. This would agree with the suggestion made by Stern and Morgane [19] that REM sleep may play a role in the maintenance of only certain types of proteins in the CNS. Finally although it would be interesting to find a relationship between the proteins in this study and Monnier's sleep factor Delta [15], at this point it is very difficult to even suggest a relationship. First of all our proteins are extracted from brain tissue during REM sleep, whereas factor Delta is extracted from blood in relation to cortical slow waves, and secondly factor Delta is a small peptide while our proteins are macromolecules.

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