to the brain on step-through latencies of untrained mice on day 1. This puncture decreased the initial median step-through latencies in both time intervals used (6 and 1 hours before the trial). The decrease in both cases is significant.

Our study showed that a small brain injury in the region of the hippocampus can interfere with a recently learned simple task. However, this effect depends upon proximity in time to the learning trial; groups treated 6 or 18 hours after the original learning trial showed no impairment. When the temporal gradient obtained in this experiment is compared to that obtained when ECS was used to disturb the memory trace (*6*), it is obvious that the brain injury induced by needle is less effective than that induced by ECS. The whole curve is steeper, and the starting median latency is also higher. Needle puncture was most effective in interfering when performed either immediately after or before learning. Extending the time interval in either direction lessened its influence.

We cannot attribute this effect solely to injury of the hippocampus in view of the fact that adjacent neocortical structure was necessarily damaged too. Neocortical spreading depression has been shown to be an effective method of producing retrograde amnesia (*9*). However, some investigators have shown with different behavioral tests that neocortical influences in most cases are less pronounced than those from the limbic system (*10*). Gardner (*11*) reported that KCl injected through a cannula into the hippocampus immediately after each learning trial of an avoidance response disrupted consolidation of that response. However, KCl injected into the amygdala or septum was without an effect on the same task. Our objective at this point was to demonstrate a temporal gradient resembling that obtained with ECS to reveal a possible common mechanism.

The initial latencies of the group with acute brain injury inflicted 6 hours before their first exposure to the step-through situation were still significantly different from controls (Table 2), whereas the effect of the same injury on their learning ability (measured by their retest latencies) was not pronounced (Fig. 2). The amount of proactive effect of the needle inserted into the hippocampal region 1 hour before the learning trial was approximately the same as that of the corresponding retroactive effect of this treatment.

Apparently, acute brain injury in the region of the hippocampus will produce retrograde amnesia, impair learning, or disinhibit motor activity in a passive avoidance test, depending on the time of the injury.

MILADA BOHDANECKA
ZDENEK BOHDANECKY
MURRAY E. JARVIK

Department of Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

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Sleep: Effects on Incorporation of Inorganic Phosphate into Brain Fractions

Abstract. *During sleep there is a two- to threefold increase in the incorporation of inorganic orthophosphate-^{32}P into a chemical fraction of the brain of the 20-day-old rat. This increase is not in the lipids or nucleic acids, but is associated with an acid-labile phosphate entity of the tissue residue after extraction of these fractions and phosphorus-containing substances of low molecular weight.*

Little is known about the biochemistry of the nervous system during sleep. Chemical analyses of brain and cerebrospinal fluid from sleeping and waking animals have so far failed to differentiate between the two states (*1*). Tracer techniques have not been exploited to any degree to study the effect of sleep upon brain metabolism (*2*). We report a marked (two- to threefold) increase in the incorporation of inorganic orthophospate into a chemical fraction of whole brain of immature rats during sleep.

Littermates, 20 days old, were kept awake for 90 minutes and then injected with orthophosphate-^{32}P in the tail vein (4 μc per gram of body weight). The litters were then divided into two groups of equal number. One group (S) was placed in quiet cages where the animals fell asleep spontaneously in 2 to 3 minutes. The other group (W) was kept awake by gentle manual stimulation. At the end of 30 minutes the rats were decapitated, a blood sample was taken from the neck, and each brain was removed and homogenized in chloroform-methanol (2 : 1) within 2 minutes post mortem.

The brains were separated into three main chemical fractions: (I) the chloroform-methanol extract, (II) the hot trichloroacetic acid extract, and (III) the sodium hydroxide digest of the tissue residue. The chloroform-methanol extraction followed standard methods for lipid extraction (*3*). The residue was washed three times at room temperature with 95 percent ethanol, once with water at 4°C, and three times with 2.5 percent trichloroacetic acid at 4°C; it was then extracted with 5 percent trichloroacetic acid at 90°C for 30 minutes (*4*). The residue was then washed three times at room temperature with 95 percent ethanol, once with water, and then digested in 0.5*N* NaOH at 37°C for 24 hours. The volumes were 5 ml throughout. The specific activity of each fraction was obtained by standard methods of scintillation counting (5) and by assay of total phosphorus (*6*). Optical density at 260 μ was used to measure nucleic acids in fraction II, and total protein was assayed (*7*) in fraction III. For purposes of comparison, the data from each animal were adjusted to a standard blood level of radioactivity of 1×10^5 count/min per milliliter of blood.

Figure 1 presents the results of a series of seven experiments involving a total of 70 animals. Ten additional experiments slightly modified for a different purpose, including 100 animals, have given essentially the same results. Fraction II, the hot trichloroacetic acid extract, showed a two and a half fold increase in the specific activity of phosphorus during sleep as compared to waking ($P < 0.001$). The specific activities of fraction I and fraction III

did not vary significantly between sleep and wakefulness. There was also no significant variation in the blood levels of radioactivity (Table 1). The ethanol washings prior to fraction II contained negligible counts. The aqueous washings contained roughly three times the total activity of fraction II. In the washings with 2.5 percent trichloroacetic acid, diminishing amounts of activity were recovered in the repeated operation until the third washing contained roughly one-twentieth of the total activity of fraction II. We did not observe an increased specific activity with respect to sleeping animals until the third cold 2.5 percent trichloroacetic acid extraction.

We established by paper chromatography that the effect in fraction II was due to increased activity of inorganic phosphate. Two chromatographic systems were used: (i) isopropanol-ammonia-water (5 : 3 : 2) (*8*) and (ii) tertiary amyl alcohol-formic acid-water (3 : 2 : 1) (*9*). Chromatography was conducted both before and after hydrolysis of the hot trichloroacetic acid extract in 0.5*N* potassium hydroxide, a method for the hydrolysis of polynucleotides to mononucleotides (*10*). Extracts from S and W animals did not differ qualitatively in the chromatographic systems cited.

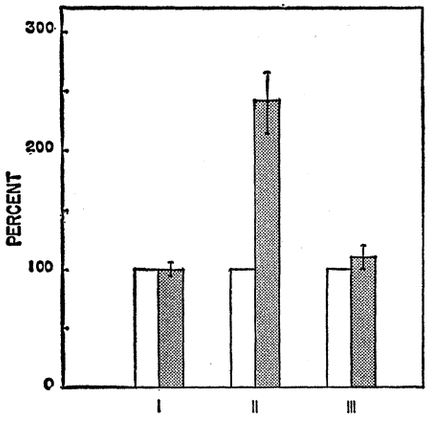

Fig. 1. Effect of sleep upon the specific activities of extracts of rat brains following intravenous injections of $^{32}P_i$. Fraction I is the chloroform-methanol (2:1) extract, fraction II is the hot 5 percent trichloroacetic acid extract, and fraction III is the 0.5*N* NaOH digest of the residue after trichloroacetic acid extraction. The data are from seven experiments involving a total of 70 rats equally divided into sleeping (S) and waking (W) groups. The S and W animals were paired randomly within each experiment, and the data from each S animal were expressed as a percentage of the paired W animal. In this graph the average of the S data is presented by the shaded bar for each fraction. The open bar is the W data set to 100. The 142-percent increase in S in fraction II was significant at a level of $P < 0.001$. The S and W groups did not differ significantly in fractions I and III. The bar indicating variation is the standard error of the mean.

After exploration of several types of experimental animals we found that 20-day-old rats are ideal for the study of normal sleep. These animals sleep spontaneously under laboratory conditions, and such frequently used modifications as sedation, anesthesia, extreme sleep deprivation, stress, or fatigue can be avoided. We carefully observed the daytime sleep-wakefulness cycle of our rats and found that 30 minutes of sleep alternated with 40 to 60 minutes of wakefulness. Our experimental sleep period was designed to coincide with the natural occurrence of sleep; the rats were kept awake through one complete 90-minute cycle and injected at the start of the next sleep period. Exertion and agitation were avoided. When the animals appeared sleepy during this 90-minute preparatory period they were allowed to explore the bench top, were moved to new cages, or were gently handled. They could eat and drink ad libitum. In spite of the mild sleep deprivation in this 90-minute period the rats appeared to be in good condition throughout the experiment and at all times were capable of full arousal. This one departure from our aim to study unmodified sleep was needed to insure the prompt onset of sleep after injection and represented the smallest perturbation of the normal situation that we could devise.

We questioned whether the observed effect was due to variables other than sleep. The use of littermates obviated the possibility that the effect was due to such factors as prior handling, housing, feeding, age, size, or genetic differences. However, the S and W groups varied with respect to muscular activity during the experimental period. We conducted control experiments to determine whether the effect in fraction II might be due to differences in activity and not to sleep per se. After the usual preliminary period of 90 minutes of wakefulness, rats were injected with $^{32}P_i$ as described above and divided into what we termed peaceful and active groups. Both groups remained awake for the 30-minute experimental period. The "peaceful" animals were allowed to rest quietly but were kept awake as described above. The "active" animals were kept in a state of intensified activity by exaggeration of our usual methods for maintaining wakefulness. Differences in the activity of the animals had no influence on the specific activity of the phosphorus of fraction II. There were also no changes in the blood level of radioactivity due to differences in activity.

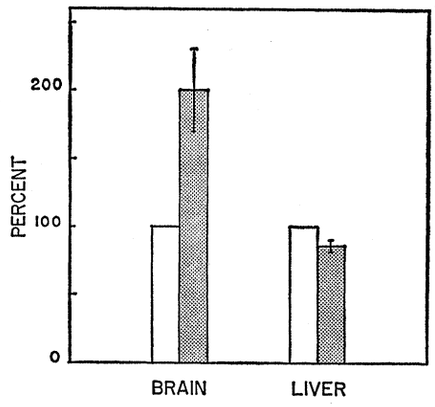

Fig. 2. Comparison of the effect of sleep upon the specific activity of fraction II (hot trichloroacetic acid) of brain and liver. The S and W animals were paired for statistical purposes as described in Fig. 1. The 100-percent increase in brain during sleep was significant at the $P = 0.05$ level. There was no significant difference between S and W in respect to the liver. The shaded bars represent the S and the open bars the W groups. $N = 20$ animals.

In a second series of control experiments, we sought to determine whether the effect in fraction II was confined to the central nervous system or was seen in nonneural tissues as well. Samples of brain, liver, and muscle were removed from S and W animals after injection with orthophosphate-^{32}P and were extracted according to the procedure outlined above. The results for brain and liver are presented in Fig. 2. The specific activity of fraction II of liver showed no difference between the S and W animals, while the brains showed the usual effect noted during sleep. The specific activity of fraction II of muscle was too low to provide conclusive data. However, there was no indication of a sleep effect in the muscle extract.

We questioned whether the increase in the specific activity of phosphorus in fraction II might be secondary to an increase in the availability of phosphorus to the brain during sleep. This possibility seemed unlikely, since blood levels of ^{32}P did not vary with sleep and wakefulness either 10 minutes after the onset of sleep or at the end of the experimental period (30 minutes) (Table 1). Changes in local cerebral

Table 1. Radioactivity in the blood of sleeping and waking animals, in counts (multiplied by 10^{-5}) per minute per milliliter.

State of animals	Time after injection	
	10 min*	30 min†
Sleeping	5.3 ± 0.4	3.8 ± 0.3
Waking	4.9 ± 0.1	3.7 ± 0.3

* $N = 16$. † $N = 70$; these animals are those from which the data of Fig. 1 were obtained.

blood flow or in the permeability of the blood-brain barrier to phosphate during sleep might be expected to affect the specific activity of inorganic phosphate in the brain and, consequently, the specific activity of more than one of the brain fractions we studied. However, only fraction II exhibited changes due to sleep. To check whether the effect might in some way be due to $^{32}P_i$ in blood pooled in the brain post mortem, we conducted the following experiment. We added a known amount of $^{32}P_i$ to nonradioactive rat brain homogenate in chloroform-methanol (2 : 1) and followed our usual procedures to the hot trichloroacetic acid extract. Less than 0.05 percent of the initial radioactivity remained, an amount that would correspond to less than 0.4 percent of that found in fraction II in the experiments on the effect of sleep. The effect thus indeed appears to be the result of an increase in the incorporation of phosphate by a constituent of brain tissue during sleep, and not a reflection of the availability of $^{32}P_i$.

The component of fraction II in which the sleep effect was found was shown above to be inorganic phosphate. In view of the previous treatment of the tissues and the experiment just cited, this inorganic phosphate was split probably from a more complex substance by the hot acid. We are attempting to characterize the constituent implicated by the application of specific extraction procedures.

Extraction of RNA (see *11*) and of phosphoinositides (*12*) from the brains of S and W animals indicates that these compounds are not involved in the sleep effect. Pronase (*13*) treatment of the residue after chloroform-methanol extraction fails to solubilize a labeled entity with increased activity during sleep. The phosphate group involved is not susceptible to the action of alkaline phosphatase (*14*). Alkaline hydrolysis at *p*H 8 and *p*H 12 for 15 minutes at room temperature fails to release the relevant phosphate. We conducted all these procedures on brains split down the midline and ran parallel extractions by our usual methods with trichloroacetic acid in order to insure that the sleep effect in fraction II was present. Phosphoproteins are the most likely possibility for the fraction in which the effect is manifested.

Many questions are raised by this report. Not only is the parent compound from which the inorganic phosphate is split in fraction II still to be defined, but the relationships of the observed effect to the phases of sleep and the age and species of the experimental animal remain to be investigated. We report the effect of sleep on the metabolism of a phosphate-containing fraction of brain at this stage because it is reproducible and because of the paucity of available information on brain metabolism during sleep.

PETER REICH
JUDITH K. DRIVER
MANFRED L. KARNOVSKY
Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

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Toxicity of Antibiotics in Laboratory Rodents

Killby and Silverman [*Science* **156**, 264 (1967)] were apparently unaware of several studies on the toxicity of certain antibiotics to small animals. D. Hamre, G. Rake, C. McKee, and H. MacPhillamy [*Amer. J. Med. Sci.* **206**, 642 (1943)] described the toxic effect of penicillin in guinea pigs, and G. Rake and I published toxicity data on streptomycin and dihydrostreptomycin in mice [*J. Bacteriol.* **53**, 205 (1947)].

The authors were also apparently unaware of the need to measure therapeutic doses of any drug in terms of the body weight of the recipient. Thus they did not realize that 4 mg/20 g of mouse body weight means 200 mg/kg of body weight, a toxicity which certainly does not fit into the category of "extremely toxic" when measured against the therapeutic dose.

RICHARD DONOVICK
Department of Microbiology, Squibb Institute for Medical Research, New Brunswick, New Jersey 08903

19 April 1967

Donovick is, of course, quite correct in stating that we were apparently unaware of several studies of the toxicity of antibiotics to small animals. It is furthermore the case that many of our colleagues and professional contacts we made in pharmaceutical firms were also unaware of such toxicity. This is exactly the reason why we wrote calling attention to our "ignorance" in the hope that other investigators would be saved the unhappy experience which we had. It appears that persons not directly involved in the area of antibiotic research could be misled by simply following directions as indicated in the package inserts of some of these drugs.

We consider that, rather than wasting space by publishing our note, we have elicited two valuable literature references provided by Donovick.

V. A. A. KILLBY
P. H. SILVERMAN
Department of Zoology, University of Illinois, Urbana

21 June 1967