a generalized diminution of sphingomyelin-cleaving enzyme activity in various tissues of patients with Niemann-Pick disease.

Summary.—The level of the enzyme which catalyzes the hydrolysis of sphingomyelin has been determined in tissue samples from patients with Niemann-Pick disease and compared with tissue specimens from other human sources.

It appears that the metabolic lesion in the classic infantile form of Niemann-Pick disease is attributable to a drastic attenuation or loss of activity of the enzyme which catalyzes the cleavage of sphingomyelin.

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*EFFECT OF ACETOXYCYCLOHEXIMIDE AND OF AN ACETOXYCYCLOHEXIMIDE-PUROMYCIN MIXTURE ON CEREBRAL PROTEIN SYNTHESIS AND MEMORY IN MICE**

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Puromycin affects memory in mice[1] and, as shown by Agranoff *et al.*,[2] in goldfish. It has been tentatively proposed that the destruction of memory (simple maze learning) in mice by puromycin depends upon the degree and duration of inhibition of protein synthesis produced by this antibiotic.[3–5] There is the possibility, however, that some other action of puromycin might be responsible for loss of memory. For this reason, analogues of puromycin have been tested but they have been found to be without effect on memory.[4] Acetoxycycloheximide has been used in the experiments reported here. This antibiotic has been observed to produce profound inhibition of protein synthesis *in vivo*[6] by inhibiting transfer of amino acid from sRNA to polypeptide.[7, 8] Puromycin has a different mode of action, being incorporated into the carboxyl ends of growing polypeptide chains and causing their premature release.[9, 10]

We have found (1) that acetoxycycloheximide, in contrast to puromycin, does not destroy memory of simple maze learning in mice in spite of deep and prolonged inhibition of cerebral protein synthesis, and (2) that amounts of puromycin which obliterate memory are without effect when injected simultaneously with acetoxycycloheximide. A possible explanation of our findings will be given in the *Discussion.*

Materials and Methods.—We are indebted to Dr. T. J. McBride of the John L. Smith Memorial for Cancer Research, Charles Pfizer & Co., for our supply of acetoxycycloheximide. Uniformly labeled L-C^{14} valine with a specific radioactivity of 50 μc/0.03 mg, and young adult albino mice of 28–32 gm were used.

Details of the behavioral and biochemical procedures have been given.[1, 4] Tests of the effect of acetoxycycloheximide on recent memory of the discriminative avoidance response in a Y-maze were made with bilateral temporal injections or with bilateral combined temporal plus ventricular plus frontal injections[1] given 1 day after the learning experience; tests of its effect on longer-term memory were made with the combined injections given 12–35 days after the learning experience. Each intracerebral injection had a volume of 0.012 ml. Retention tests for evaluation of memory of the training experience were given several days after treatment to allow ample time for recovery of the animal. The same procedures were followed with intracerebral injections containing both acetoxycycloheximide and puromycin. Radiovaline was again used to measure the rate of protein synthesis in the hippocampus, amygdala, thalamus, corpus striatum, and temporal, parietal, and frontal cortices. Mice were killed 40 min after subcutaneous injection of the radiovaline. In order to calculate the degree of inhibition of protein synthesis, the ratio of radiovaline incorporated into the protein of experimental and control animals was, as in previous work, corrected for changes in specific radioactivity of the free amino acid pool which followed treatment. Measurements were made of the concentration of total pool amino acids by the ninhydrin method of Rosen.[11]

Acetoxycycloheximide was quite toxic for 2 or 3 days following its intracerebral injection. During this period all mice had diarrhea, failed to clean themselves, or eat pellets. Their survival depended upon keeping them clean and feeding them milk and a sugar solution from a medicine dropper.

Results.—(1) *Behavioral studies: Mice injected intracerebrally with acetoxycycloheximide:* Most of the behavioral studies on recent memory were made with bilateral temporal injections of 60 or 120 μg of acetoxycycloheximide, and on longer-term memory with bilateral combined temporal plus ventricular plus frontal injections of 15 or 30 μg (Table 1). These observations were completed before the biochemical measurements were started. As will be shown (Fig. 2), the behavioral studies consequently include experiments made with larger amounts of the heximide than were necessary to obtain the degree and duration of inhibition of protein synthesis produced by puromycin. Table 1 shows that both recent (1-day) and longer-term (12–35 days) memory were essentially unaffected by injection procedures which regularly gave loss of memory when used with puromycin.

Mice injected intracerebrally with a mixture of acetoxycycloheximide and puromycin: It has been reported[1] that bilateral temporal injections, each of 90 μg of puromycin, consistently destroyed recent memory; and that bilateral combined temporal plus ventricular plus frontal injections, each of 30 μg, consistently destroyed longer-

TABLE 1

LACK OF EFFECT OF ACETOXYCYCLOHEXIMIDE (A) AND OF A MIXTURE OF (A) AND PUROMYCIN (P) ON RECENT (1-DAY) AND LONGER-TERM (12–35 DAYS) MEMORY

Substance	Injection site	μg/ Injection	Days after learning	No. of Mice in Which Memory Was: Lost	Impaired	Retained
A	T	60	1	0	1	8
A	T	120	1	0	1	3
A	T + V + F	15–30	1	0	0	2
A	T + V + F	15–30	12–35	0	0	5
A + P	T	120 + 120	1	0	1	6
A + P	T + V + F	8 or 15 + 30	14	0	0	6

T, V, and F refer, respectively, to temporal, ventricular, and frontal injections, all given bilaterally. For the 30 mice with retention of memory, the means and standard deviations for percentages of savings of trials and errors were, respectively, 90 ± 15 and 92 ± 10; for the three mice with impaired memory, the corresponding means were 45 and 68.

term memory.[1, 4] Table 1 shows that bilateral temporal injections of 120 μg each of puromycin and acetoxycycloheximide were, with one minor exception, without effect on recent memory in seven mice. The table also shows that the bilateral combined injections, each containing 30 μg of puromycin, plus either 8 or 15 μg of heximide, failed to destroy longer-term memory in six mice.

(2) *Biochemical studies: Effect of acetoxycycloheximide on cerebral protein synthesis:* Each of the bilateral temporal injections contained 60 or 120 μg of the heximide. In untreated, normal mice the radioactivities of the pools of the different areas of the brain were so nearly alike that a single mean of 345 counts/10 min/mg protein of the tissue was used.[3] Pool radioactivities were usually substantially increased in all cerebral areas for as long as 24 hr after the intracerebral injections as shown by their relationship to the normal mean in Figure 1. Figure 1 gives the pool values found in the hippocampus and the extent of variation around these values observed in the other six areas of the brain which were studied. It is evident that there was substantial variation in the radioactivities of the pools from one mouse to another.

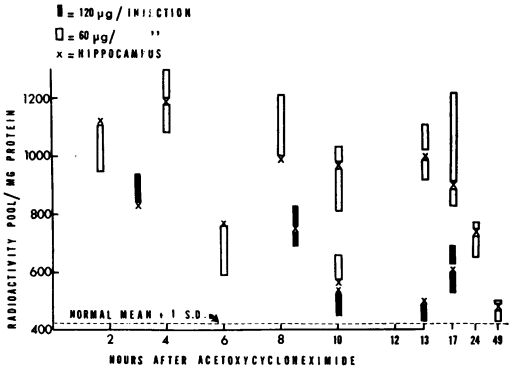

FIG. 1.—Radioactivity of the pools of the hippocampus 40 min after subcutaneous injection of radiovaline in counts/10 min/mg protein as a function of time after bilateral temporal injections of acetoxycycloheximide. The extent of variation of the pools of the other six areas of the brain is shown by the bars. Values obtained from seven of the mice of Fig. 2 are not included in Fig. 1 because of overlap with those which are plotted. *1 S.D.* = one standard deviation.

No change of consequence has been found in the concentration of the total free amino acids of cerebral pools after intracerebral injection of acetoxycycloheximide in agreement with measurements reported for the liver after intraperitoneal injection.[6] Since we needed to compare the inhibitory effect of the heximide with that of puromycin, the concentration of total pool amino acids was measured in a control group of five mice and in two groups of seven mice, each treated, respectively, with bilateral temporal injections of 90 μg of puromycin or 120 μg of acetoxycycloheximide. In each experimental group, three mice were killed at 4 hr and four at 8 hr after treatment. As measured by ninhydrin, the mean concentration

of amino acids ± its standard deviation in μg/mg protein of the temporal cortex was 68 ± 8.4 for the controls; 75 ± 10 for the puromycin group; and 76 ± 9.1 for the group with the heximide. Values for the hippocampus were practically identical with those of the temporal cortex. As in the past,[3] we have consequently taken the change in radioactivity of the pool after treatment with acetoxycycloheximide as equal to its change in specific radioactivity.

The degree of inhibition of protein synthesis in the hippocampus as a function of time after bilateral temporal injections of 60 or 120 μg of acetoxycycloheximide is given in Figure 2. Except in the following instances, the degrees of inhibition in the other six areas of the brain were within 5 per cent of those found in the hippocampus. With bilateral temporal injections of 60 μg, inhibition in the amygdala was less than that in the hippocampus by 7 per cent, both at 8 hr and in one experiment at 10 hr; and by 14 per cent at 13 hr. All the cerebral areas except the temporal cortex showed inhibition up to 20 per cent less than the hippocampus at 17 and 24 hr. There was clearly much less localization of the effect of bilateral temporal injections of the heximide than observed with puromycin.[3] Figure 2 also gives the results on the hippocampus of two mice about 11 hr following bilateral combined temporal plus ventricular plus frontal injections each of 30 μg and on two with injections each of 15 μg of acetoxycycloheximide. Again all parts of the brain were inhibited within 5 per cent of the value of the hippocampus except for the amygdala in the mice with 30 μg. In these two instances, inhibition in the amygdala was 7 per cent less than in the corresponding hippocampus.

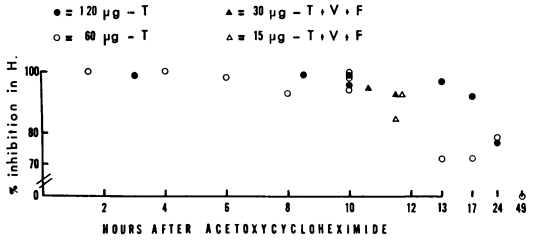

FIG. 2.—Changes with time in the inhibition of incorporation of radiovaline into protein of the hippocampus (*H*) after bilateral temporal (*T*) or bilateral combined temporal plus ventricular plus frontal (*T* + *V* + *F*) injections of acetoxycycloheximide. As discussed in the text, inhibition in other cerebral areas, with few exceptions, were within 5% of that of the hippocampus.

Effect of a mixture of acetoxycycloheximide and puromycin on cerebral protein synthesis: It was possible that a mixture of the two inhibitors failed to affect memory, as shown above, because they were lost from the brain more rapidly than when injected singly. For this reason, inhibition of protein synthesis was measured in the hippocampus, temporal cortex, and amygdala of two mice 10 hr after bilateral temporal injections of 120 μg of acetoxycycloheximide plus 120 μg of puromycin. The percentage inhibition in all areas of both mice exceeded 92 per cent and averaged 97 per cent.

Discussion.—The degree and duration of inhibition of protein synthesis in the hippocampus and temporal cortex following temporal injections of acetoxycycloheximide at least equal those following puromycin.[3] All of the other areas of the brain, including the amygdala, not previously studied, were inhibited to about the same degree as the hippocampus for 11 hr following injection of the heximide. Combined temporal plus ventricular plus frontal injections of acetoxycycloheximide also caused inhibition of protein synthesis in all cerebral areas at least equal to that produced by puromycin.[4] In contrast to puromycin, however, the heximide was

without effect on either recent or longer-term memory. These results make necessary a reappraisal of our tentative view that destruction of memory in mice by puromycin is to be explained by its inhibition of protein synthesis.

Furthermore, acetoxycycloheximide protected memory against the destructive effects of puromycin, although mixtures of the two antibiotics produced profound inhibition of protein synthesis. In experiments to be amplified and to be reported fully at a later time, it has been found that memory is also protected from puromycin by cycloheximide and chloramphenicol.

Acetoxycycloheximide and cycloheximide have a common mode of action which leads to a decreased rate of peptide bond formation with consequent preservation of mRNA.[12, 13] In cell-free systems, Trakatellis *et al.*[14] found liver polysomes isolated from cycloheximide-treated mice to synthesize protein at a normal rate, and Williamson and Schweet[12] found that cycloheximide protected polysomes from the disaggregation caused by puromycin. In *in vivo* experiments, cycloheximide, when injected with actinomycin or ethionine, inhibited the breakdown of mouse liver polysomes and their RNA which followed use of these agents alone.[14] We have also been much interested in the finding by Nathans[10] that there is a great decrease in incorporation of puromycin into peptide chains in the presence of chloramphenicol.

These several lines of evidence have led to a modification of our working hypothesis. We now take the view that the initial macromolecular change underlying maintenance of memory involves a change in the quantity of one or more species of messenger RNA, in conformity with important aspects of the proposals and reports of Hydén and his collaborators.[15, 16] These species of mRNA alter the synthetic rate of one or more proteins which are essential for the expression of memory, perhaps because of their effects on synaptic transmission. In turn, these proteins or their products act as inducers of their related mRNA; in this way the concentration of the inducer proteins is maintained. In this view, expression of memory depends upon changes in proteins initiated and sustained by quantitative changes in mRNA produced by a learning experience. Loss of this mRNA would lead to loss of essential protein with consequent loss of memory. In the presence of an inhibitor of protein synthesis, the concentration of essential protein could fall to levels too low for expression of memory, but loss of memory would be temporary if mRNA were conserved to continue its function when the inhibitor of protein synthesis had disappeared.

We now assume that puromycin destroys memory because of its effects on inducer protein and/or on synthesis of mRNA. It has been concluded from studies on bacterial[17] and vertebrate[18] cells that mRNA decays at a normal rate in the presence of puromycin. It has also been found that puromycin in a concentration such as we have used can markedly inhibit synthesis of RNA in vertebrate cells.[19] We assume that memory is destroyed by puromycin because the rate of renewal of essential mRNA is inadequate to compensate for its loss, possibly because the concentration of inducer protein falls below an effective level during the period of inhibition of protein synthesis and/or possibly as a result of direct inhibition of RNA synthesis.

The protection afforded polysomes and their mRNA by cycloheximide either when present alone or in combination with other substances would, in the view

given here, account for the lack of effect of acetoxycycloheximide on memory and the protection which it affords memory in the presence of puromycin.

Summary.—Intracerebral injections of acetoxycycloheximide caused profound inhibition of cerebral protein synthesis but, unlike injections of puromycin, were without effect on memory of simple maze learning in mice. Intracerebral injection of a mixture of the two antibiotics, which also caused profound inhibition of cerebral protein synthesis, protected memory against the destructive effects of puromycin when injected alone. An effort is made to explain these observations in terms of the preservation of quantitative changes in messenger RNA which may accompany a learning experience.

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