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Acetoxycycloheximide Enhances Audiogenic Seizures in DBA/2J Mice

Abstract. *Inborn errors of metabolism often cause epilepsy, as with certain strains of mice. Aggravating the metabolic defect with a protein synthesis inhibitor increases the symptoms. Mature animals that have "outgrown" their genetic susceptibility to audiogenic seizures are made susceptible again by acetoxycycloheximide. After a single small dose the incidence and severity of audiogenic seizures increases at 16 hours, reaches a maximum of 40 hours, and then declines gradually.*

Young mice of certain strains can be made to convulse by exposure to loud sound. Details of age-specificity in different strains have been reported (*1*, *2*). In different laboratories in different years, the incidence and severity of convulsions in DBA/2J mice have been different, but all agree that these animals "outgrow" this attribute. Attainment of anatomical and electroencephalographic maturity of cerebral cortex (*3*) coincides with the beginning of sensitivity to audiogenic seizures. Attainment of sexual maturity approximately coincides with decline in sensitivity.

Concerning the seizure susceptibility and the age-specificity, several authorities suggest that deficiency of phenylalanine hydroxylase predisposes mice to deficiency of a neurotransmitter (*4–7*). This has not been proven. Abnormal amounts of adenosine triphosphate (ATP) and adenosine triphosphatase (*6*, *8*) and low tolerance to anoxia (*1*) have been demonstrated in DBA mice, suggesting that metabolism of high energy phosphate may have a direct role in susceptibility to seizure.

Acetoxycycloheximide is a potent inhibitor of protein synthesis that interferes with the incorporation of labeled valine into protein in the mouse brain (*9*). We studied the effect of acetoxycycloheximide on audiogenic seizures in DBA/2J mice with two sets of experiments. One deals with the rates of age-specific attack and of mortality; the other deals with the time course of the drug effect in animals 40 days of age. We have extended the age of susceptibility to audiogenic seizures, have increased the severity of seizures, and have demonstrated that the time course of this effect is similar to that of other behavorial effects of inhibition of protein synthesis is by this drug.

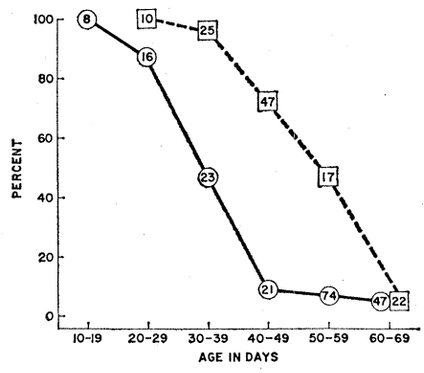

Fig. 1. Incidence of audiogenic seizure from age 17 to 69 days. Numerals in squares represent number of animals receiving acetoxycycloheximide (4 μg per gram of body weight) by injection 24 hours before testing. Numerals in circles represent number of animals receiving saline injection.

The DBA/2J mice were housed in groups on pine sawdust or cedar shavings and were fed Purina mouse chow and tap water. Only animals direct from the supplier (*10*) were used in the time course experiment. Animals direct from the supplier and offspring of unselected matings of original and first generation animals in our laboratory were used in the experiment on the relation of age to incidence.

An injection of sodium chloride solution (Cutter; U.S.P.) was used as control and diluent. The drug solution (*11*) was 400 μg of acetoxycycloheximide per milliliter of diluent. Both fluids were refrigerated until used. Each animal was caught by the tail, was assigned to drug or control group without regard to its ability to avoid capture, was weighed, and was given a dorsal subcutaneous injection (0.01 ml of solution per gram of body weight).

All animals were tested in a clear plastic cage (20 by 20 by 30 cm) with pine sawdust on the bottom and an electric doorbell under the top. The stimulus intensity was 112 db above 0.0002 dyne/cm^2 within the cage. Stimulus duration was either 1 minute or until tonic convulsion, whichever was first. Animals were observed for the several stages of audiogenic activation: stun, wild running and leaping, clonic convulsion, and death—but only clonic convulsion or death was used as criterion in this report.

In the age-specific incidence experiment, animals were tested in groups of one to four, 24 hours after injection, without regard to time of day. In the time course experiment, animals were tested singly, and all tests were done between 8 a.m. and 12 noon.

Because we were interested in the decline of sensitivity with age rather than the precise age of onset, we have few animals under 20 days of age. The susceptibility to clonic convulsion in mice, grouped by age in 10-day intervals, declines with age (Fig. 1). A parallel decline in susceptibility is seen in mide, but the curve is shifted to the animals treated with acetoxycycloheximide right by two 10-day intervals.

Figure 2 shows the incidence of death from convulsion among all animals stimulated, grouped by age in 10-day intervals. The incidence of death from convulsion in the control animals falls faster than the incidence of con-

vulsion (Figs. 1 and 2); that is, the convulsions are less severe in older animals. All animals less than 20 days of age (17 to 19 days) die, whereas the mortality rate approaches zero between 50 and 69 days. In contrast, the seizure mortality rate in the animals treated with acetoxycycloheximide is approximately 100 percent for all ages tested (77 convulsed, 76 died during convulsion).

These data are based on 189 control animals and 121 animals that were treated with acetoxycycloheximide. The differences in the incidence of convulsion and in the mortality rate due to seizure between the two groups in the intervals of 30 to 39, 40 to 49, and 50 to 59 days are statistically significant ($P < .01$, chi-square test).

Figure 3 shows the incidence of convulsion in animals treated at 40 days of age (tested at 40 to 43 days) with single injections of acetoxycycloheximide (4 μg per gram of body weight). Each animal was tested once. At 4 and 8 hours after acetoxycycloheximide treatment, the incidence of seizure does not differ significantly from that in saline control animals. By 16 hours the incidence of seizure is 62 percent, rising to 100 percent at 40 hours. The incidence of seizure then declines slowly to approach, but not reach, the control level by 92 hours after treatment. The mortality rate due to seizure in this series is zero in the first 8 hours, 60 percent at 16 hours, 100 percent at 30 and 40 hours, approximately 80 percent at 63 and 72 hours, and approximately 50 percent at 92 hours. These data are based on 117 treated animals. The differences from the controls, with respect to both incidence of seizure and mortality, are significant for all groups, later than 8 hours after treatment ($P < .01$, chi-square test). Further, the values at different times are significantly different from chance variation ($P < .01$, chi-square test).

There are several possibilities for the way in which injection of acetoxycycloheximide enhances the incidence and severity of audiogenic seizure in these animals. A direct stimulating effect may be operating. The time course of the effect we have observed is different from the early onset and gradual decline with time that we would expect with a direct stimulant. A nonspecific toxic effect or the production of electrolyte imbalance are other possibilities to consider. The animals have slight diarrhea for 12 to 24 hours and appear

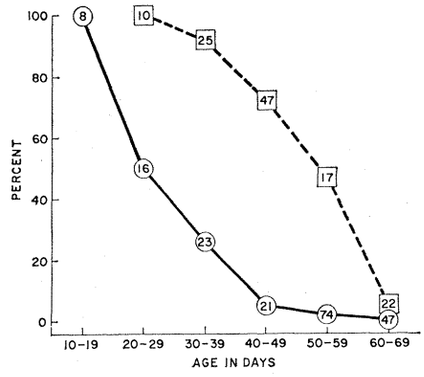

Fig. 2. Death from audiogenic seizure in mice aged 17 to 69 days. Symbols are the same as in Fig. 1.

less active in the 8- to 16-hour period, but they show no evidence of illness later than 24 hours after treatment. The parallel between the reduction of incidence of seizure with age in the saline control and in the animals treated with acetoxycycloheximide suggests that the same factors produce the maturation effect in both cases. Inhibition of structural maturation is unlikely in view of the development of structural and electrical maturity by 16 to 17 days of age (*3*). Impairment of maturation of a metabolic system that is important to brain function as the animals mature is an attractive hypothesis.

Deficiency of phenylalanine hydroxylase has been demonstrated in DBA/2J mice (*4*). This deficiency has been linked to dilute coat color and susceptibility to audiogenic seizure, although some investigators report that these are not all due to the same gene (*12, 13*). Dietary deprivation of pyridoxine, a cofactor in phenylalanine hydroxylase activity, accentuates and extends the age of susceptibility to audiogenic seizure (*5, 12*). Blockage of phenylalanine hydroxylase synthesis by acetoxycycloheximide, further reducing an already deficient supply, may be the mechanism of enhancement of audiogenic seizures in our experiments.

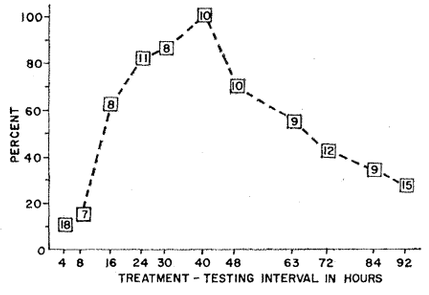

Fig. 3. Incidence of audiogenic seizure in mice at various times after receiving acetoxycycloheximide (4 μg per gram of body weight) by injection. All animals were 40 days old. Numerals in squares represent number of animals tested at each time.

Abnormalities of oxidative phosphorylation in mice that are susceptible to audiogenic seizure have been reported (*6*). The age of maximum abnormality with respect to adenosine triphosphatase corresponds to the age of maximum sensitivity to seizure (21 days in their animals). The ATP metabolism becomes more nearly normal in older animals (35 days). These data suggest that an age-dependent deficiency of a rate limiting enzyme in ATP metabolism may be present in the susceptible strains. Abnormality of oxidative phosphorylation may be the cause of the seizures, the low tolerance to anoxia, and the high death rate from convulsion in these mice. Further reduction of a deficient enzyme in the oxidative phosphorylation system by acetoxycycloheximide may be the mechanism of the increased incidence and severity of seizures in our treated animals.

The reported time course of the deleterious effect of acetoxycycloheximide on memory in mice (*14*) and memory in goldfish (*15*) is similar to the time course of our effect. By contrast, acetoxycycloheximide and cycloheximide impairment in the mouse of recovery from sciatic nerve conduction block induced by a local anesthetic lasts less than 2 hours (*16*). Likewise, inhibition of protein synthesis by cycloheximide in microsomes from mouse liver and from yeast occurs within a few minutes (*17*), and the block of protein synthesis in mouse brain by acetoxycycloheximide lasts only "for as long as 24 hours" (*9*). The last three studies seem to demonstrate that protein synthesis block begins promptly but lasts only a few hours after a single dose of cyclo- or acetoxycycloheximide. The behavioral effects, including increased susceptibility to convulsion, are most easily understood as a summation of the temporary reduction of protein synthesis and the natural disappearance of a necessary protein.

It seems likely that susceptibility to audiogenic seizure is due to a deficiency in an enzyme system which is important in brain function during adolescence but which normally becomes less important with maturity. Although phenylalanine hydroxylase and adeno-

sine triphosphatase have been suggested, we have no new information on the question "which enzyme?" Our experiments do demonstrate that there is a prominent and reversible accentuation of the incidence and severity of audiogenic seizure coincident with transitory inhibition of protein synthesis by acetoxycycloheximide.

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Erythrocytes: Pits and Vacuoles as Seen with Transmission and Scanning Electron Microscopy

Abstract. *Vacuoles containing inclusions were observed by transmission electron microscopy in erythrocytes of a splenectomized patient with hemoglobin Ann Arbor. The membranes of these vacuoles became fused with the surface membrane of the red cell, thus opening the vacuoles and exposing their contents to the outside. These vacuoles when they have become thus attached to the cell membrane of the erythrocyte are responsible for the pits observed with scanning electron microscopy.*

Holroyde and Gardner (*1*) have reported that crater-like indentations of the erythrocyte surface membrane when viewed by means of interference-contrast microscopy are largely optical illusions. These authors present evidence that many of these indentations actually reflect the presence of vacuoles of low optical density lying beneath the plasma membrane. Scanning electron microscopy, however, showed a number of membrane indentations to be true pits or craters. We now present evidence that the pits and craters in red blood cells observed by scanning electron microscopy represent vacuoles, or vacuoles containing inclusions, which have reached the surface plasma membrane of the red cell and fused with it, as seen by transmission electron microscopy.

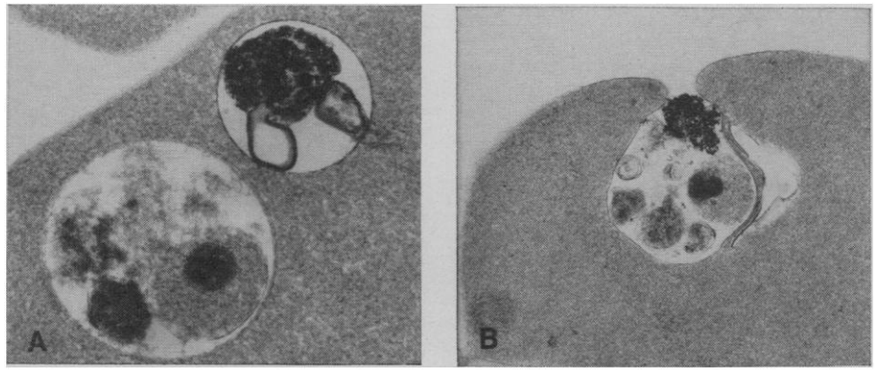

Fig. 1. A transmission electron micrograph of two inclusion-bearing vacuoles within red blood cell. Ferritin, hemoglobin, membranes, and remnants of mitochondria are present in the vacuoles (× 17,820). (B) Opening of the vacuole at the surface of the red cell (× 25,000).

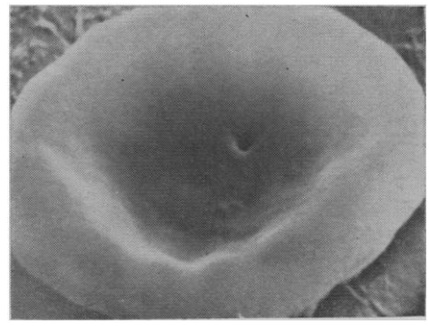

Fig. 2. A scanning electron micrograph of a red blood cell with a pit on the surface (× 9000).

We obtained red blood cells from a splenectomized patient. Blood for transmission electron microscopy was fixed at room temperature for 1 hour in 1.5 percent glutaraldehyde containing 2.5 percent sucrose and buffered with 0.05*M* sodium cacodylate (*p*H 7.4), treated with osmium tetroxide, dehydrated through a series of graded alcohols and propylene oxide, and embedded in Epon 812. Sections were cut with a Du Pont diamond knife, stained with uranyl acetate and lead citrate (*2*), and examined in a Siemens Elmiskop 101 or an RCA EMU-3H electron microscope. For scanning electron microscopy, the cells were washed three times in physiologic saline and then fixed for 1 hour in 1.5 percent glutaraldehyde containing 2.5 percent sucrose and buffered to a *p*H of 7.4 with 0.05*M* sodium cacodylate (300 milliosmoles). The red blood cells were washed three times in distilled water. A drop of the cell suspension was placed on an aluminum stub, freeze-dried, coated with palladium-gold, and examined in a scanning electron microscope (Materials Analysis Company, model 700).

The patient is the propositus of a family with hemoglobin Ann Arbor in which the 80th residue of the α-polypeptide chain, normally leucine, has been substituted by arginine (*3*). This abnormal hemoglobin is one of a number of heat-unstable inherited variants which cause hemolytic anemia. The pro-