example] which promote recovery of the anticholinesterase-inhibited twitch (*1*) had no important effect on recovery of the tetanic response after poisoning with tabun (Fig. 1) or sarin. Another quaternary compound that was found (*1*) to speed recovery of the twitch response, N-benzyl atropinium chloride, had some efficacy in enhancing recovery of the tetanic response after sarin administration (Fig. 1). Figure 1 shows, however, that even with N-benzyl atropinium chloride, the tetanic response did not become reasonably normal within the observation period of 2 hours after the poisoning.

Our attention turned then to oximes, and especially to pyridine-2-aldoxime methiodide (2-PAM). This compound has been reported to be quite active *in vitro* in reactivating cholinesterase inhibited by TEPP, DFP, or sarin (*3*), in overcoming neuromuscular block induced by the same cholinesterase inhibitors in the isolated phrenic-diaphragm preparation of the rat (*4*), and in preventing mortality in mice that have been poisoned with paraoxon (*5*). Other oximes were found (*4*) to be effective *in vivo* in overcoming anticholinesterase-induced neuromuscular block in the gracilis muscle of the rat and in the tibialis of the cat.

Intravenous injection of 5 mg of 2-PAM per kilogram after intravenous administration of about 12 LD_{50} of either sarin or tabun produced comparatively rapid and complete recovery of the tetanic response (Fig. 1). This recovery

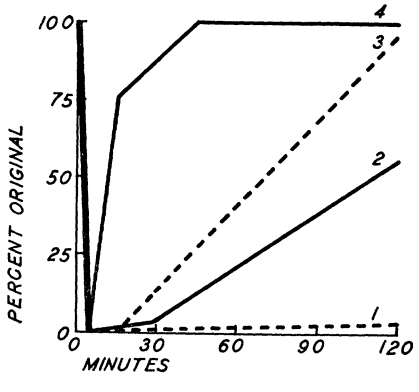

Fig. 1. Effects of various treatments on the recovery, by the gastrocnemius-soleus-tibialis anticus muscle group of the cat, of ability to maintain a tetanus for 30 seconds of indirect electric stimulation with just supramaximal square-ware stimuli. Atropine (0.5 mg/kg intravenously) was given just before the intravenous injection, at zero time, of tabun (0.8 mg/kg) (broken lines) or of sarin (0.22 mg/kg) (solid lines). Treatments, given intravenously at 5-minute intervals: (i) WIN 12306, 1 mg/kg; (ii) N-benzyl atropinium chloride, 5 mg/kg; (iii and iv) 2-PAM, 5 mg/kg.

is even faster and more complete within the duration of our experiments than that produced by N-benzyl atropinium chloride.

Administration of graded doses of anticholinesterase to groups of six rabbits, followed by treatment of the same rabbits by no other procedure than intravenous injection of either 5 mg of 2-PAM per kilogram, 2 mg of atropine sulfate per kilogram, a mixture of 2 mg of atropine sulfate and 5 mg of 2-PAM per kilogram, or nothing, has yielded approximations of the LD_{50} of sarin with various drug therapies. The LD_{50} of sarin for rabbits that are given no treatment is 16 μg/kg; for rabbits that are treated with 2-PAM alone, 24 μg/kg; for those treated with atropine alone, 51 μg/kg; and for those treated with both atropine and 2-PAM, 331 μg/kg. This last figure is based on results from 13 groups of rabbits poisoned with doses of sarin ranging from 64 μg/kg to 1.2 mg/kg.

Even with the highest dose of sarin, the combined treatment with atropine and 2-PAM saved one rabbit from the two groups of animals (12 rabbits) so poisoned and treated. None of the other treatments saved any animals at doses of sarin above 130 μg/kg, although combined treatment with atropine and 2-PAM saved 30 of 66 rabbits that were given doses of 160 or more μg/kg of sarin. Chemical treatment, to be effective after large doses of anticholinesterase, must be instituted within 1 minute after sarin injection.

It is apparent that 2-PAM alone, in the dose used, is a less effective therapeutic agent in anticholinesterase poisoning than atropine, but that it and atropine together make a very effective therapeutic combination. This conclusion supplements that reached by T. A. Loomis (*6*), namely, that 2-PAM is not significantly therapeutic in animals that are poisoned by sarin and given no other treatment.

If the effectiveness of 2-PAM as an adjunct to atropine in treating anticholinesterase poisoning is attributable primarily to its ability to overcome neuromuscular block elicited by the cholinesterase inhibitors, it is easy to see why 2-PAM is of little value alone. Death in animals that are not treated with atropine or other anticholinergic tertiary amines is caused, in large part, by central respiratory failure (*7*). Because 2-PAM is a quaternary amine, it would not be expected to pass the blood-brain barrier and to affect the activity of centers within the brain. Even though the 2-PAM were capable of overcoming all the other peripheral effects of anticholinesterase compounds, in addition to restoring neuromuscular transmission, it could not benefit the poisoned subjects in the face of continued central respiratory inhibition (*8*).

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8. WIN 12306 and a number of other oxamides were supplied by Maurice L. Tainter of the Sterling-Winthrop Research Laboratories. N-benzyl atropinium chloride was prepared by Lawrence J. Edberg of the pharmacology branch, Army Chemical Center. The 2-PAM was supplied by J. M. Steinberg and B. E. Hackley, Jr., of the Directorate of Research, Army Chemical Center, and was made by treatment of 2-pyridine aldehyde with hydroxylamine and quaternization of the aldoxime.

11 February 1957

Echo Virus Type 9 (New Member of Coxsackie Group Type A?) as a Cause of Epidemic Meningitis

Last summer, an extensive epidemic of aseptic meningitis occurred in many parts of Western Europe: as far as we know, in Belgium, the Netherlands, Germany, Switzerland, England, and Denmark. This disease was characterized by meningeal symptoms (sometimes accompanied by exanthem), always with a very marked pleocytosis (up to 5000 cells/mm^3) and by its highly contagious nature; often all the members of a family, children and adults, were affected.

From 399 patients with the typical syndrome, 190 virus strains were isolated from fecal specimens, 29 from spinal fluids, and 6 from throat swabs. Proof of the etiologic significance of these virus strains was obtained by direct isolation from cerebrospinal fluid and by showing a rise of fourfold or more in the titer of neutralizing and complement-fixing antibodies in all paired serums of the patients examined (to date, 112 have been examined for neutralizing antibodies and 37 for complement-fixing antibodies).

No antibodies were found in serums from patients suffering from nonparalytic poliomyelitis (13 patients), paralytic poliomyelitis (19 patients) or atypical

pneumonia (9 patients), and none were found in 72 serums from healthy individuals. These virus strains were found to be cytopathogenic in primary tissue cultures of various cell types. They caused complete destruction of cultures of human amniotic cells and about 50 percent destruction of monkey kidney cells, and they had a moderate cytopathogenic effect in cultures of fibroblasts from monkey testis and epithelial cells from monkey epididymus. No effect was seen on the following cell types in continuous culture: HeLa, two strains of human kidney epithelium (adult and fetal), human lung fibroblasts, strain KB (Eagle) (*1*), conjunctiva (*2*), human intestine (*2*), and human liver (*2*), mouse mammary carcinoma cells (strain Ma Ca, de Bruyn), and normal mouse kidney epithelium.

The cytopathogenic effect of all these strains was prevented by neutralization with rabbit antiserum ECHO type 9 (*3*). Conversely, two standard ECHO type 9 virus strains (*4*) were neutralized by several rabbit serums against Belgian strains and by serums of human convalescents.

Most of these strains, as infected tissue culture fluid, produced myositis in suckling mice at least up to the age of 10 days. Many caused paralysis and some even death. Several of these strains, however, did not cause histologically detectable myositis. The two aforementioned standard strains belonged to the last group.

When a mixture of infectious tissue culture fluid and anti-ECHO type 9 serum was injected into suckling mice, no myositis occurred, while controls without immune serum died or were paralyzed. In the complement-fixation test, antigen prepared from aqueous suspension of baby mice was even better antigen than tissue culture fluid.

On histological examination of infected baby mice, no lesions other than those of myositis were found. The virus content of 10-percent suspensions of different tissues was determined in tissue culture; muscle contained about 10^6 TCD_{50}/ml, while brain, liver, kidney, and spleen contained from 10 to 10^2 TCD_{50}/ml. Up to now, three strains tested were not pathogenic for adult mice.

Ten original fecal specimens from which virus was isolated in tissue culture and found pathogenic for suckling mice did not cause any clinical symptom on direct inoculation into these animals; lesions were, however, detectable, histologically. A Belgian prototype strain was not neutralized by Dalldorf's antiserums against Coxsackie types A_1 to A_{17} and A_{19} and B_1 to B_5.

Considering these facts, we feel justified in proposing that the viruses of ECHO type 9 should be removed from the ECHO group and reclassified, probably as a new member of the Coxsackie A group of viruses.

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Notes

1. From H. Eagle.
2. Supplied by Microbiological Associates.
3. Kindly sent by A. Sabin.
4. Received through the courtesy of H. v. Magnus and A. Svedmyr.

31 January 1957

Induced Copulation of Aedine Mosquitoes

Aedine mosquitoes that develop in floodwaters are difficult to maintain as colonies in cages because conditions conducive to copulation are rarely attained. An efficient procedure for stimulating copulation has been devised in our laboratory, and by this means viable eggs have been obtained from *Aedes stimulans* and *Aedes vexans*.

Copulation is induced by manipulating an immobilized female into contact with a decapitated male. From the literature we learned that decapitation of males of mantids removed the center for inhibition of the copulatory act (*1*). Female mantids often eat the heads from their suitors prior to or during copulation, yet the act goes forward normally. Roeder (*2*) has shown that the center of inhibition in mantids is in the subesophageal ganglion. Removal of the head of male mosquitoes removes the ganglion also.

Fig. 1. Ventral view of male and female of *Aedes stimulans* in act of copulation (×18).

Mosquitoes that had emerged for a time greater than 72 hours were prepared for mating as follows. Females were anesthetized by exposure to atmospheres of either carbon dioxide or chloroform. The dorsum of the thorax of each was cemented to the tip of a fine needle. Several were prepared at each time and held until ready for use. Males were decapitated without prior anesthesia. Each of them was immobilized by cementing the dorsum of the thorax to a piece of white cardboard. Several of these could likewise be prepared at once before mating the series.

Contact between two immobilized mosquitoes was accomplished manually under a stereomicroscope magnifying about 20 diameters (Fig. 1). Each female was placed in mating position with its ventral side up and its head directed away from the male. Apexes of the two abdomens were then brought together. The position thus was end to end and venters uppermost. Insemination was usually completed within a few seconds.

The females were not injured by the treatment. The kind of cement used to attach them to the needles is not critical as long as it sets within a minute or two and can be loosened after copulation.

Techniques employed here permit matings at a rate of about 25 females an hour. This rate is reasonable for maintenance of even a large colony of one species or small colonies of several species. Since the eggs may be kept for months, they may be accumulated for large-scale experiments.

It was found that males would copulate with several females but that sperms were transferred dependably to only the first two. A large series of single matings resulted in insemination of 90 percent of the females. A comparable series in which males were mated a second time resulted in transfer of sperms to 85 percent of the females. When the males were mated a third time, only about half of the females were inseminated.

Attempts to inseminate *Culex pipiens* and *Anopheles quadrimaculatus* in this manner met with little success. A contributing factor seems to be differences in genitalia. Proper contact is difficult to establish, and the sperm mass rarely enters the genital opening of the female.

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