with that of similarly treated control sections from cats which had received no diisopropylfluorophosphate or 2-PAM. Typical results are shown in the photomicrographs in Fig. 1.

In ganglia from control animals, staining for acetylcholinesterase is intense in the cell membranes and cytoplasm of the cholinergic neurons (ciliary ganglion) and considerably lighter in adrenergic neurons (majority in the stellate ganglion). Heavy staining for the enzyme is also seen in the terminations of the cholinergic preganglionic fibers, which are represented in the stellate ganglion chiefly by the protoplasmic tracts. Faint staining for nonspecific cholinesterase can be seen in the glial cells of both ganglia. In the intercostal muscle, acetylcholinesterase activity is concentrated in the subneural apparatus of the motor endplates.

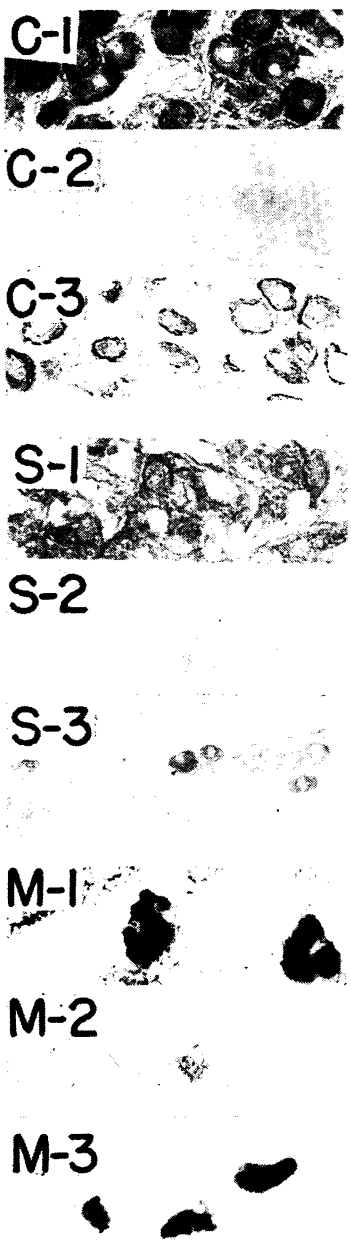

Fig. 1. Sections of ciliary ganglion (*C*), stellate ganglion (*S*) and intercostal motor endplates (*M*) of cats, stained for acetylcholinesterase. 1, Control; 2, diisopropylfluorophosphate (20 μmole/kg) 79 min prior to sacrifice; 3, diisopropylfluorophosphate 79 min and 2-PAM (40 μmole/kg) 15 min prior to sacrifice. Ganglia were incubated 80 min, magnification × 120; endplates were incubated 20 min, magnification × 350.

The acetylcholinesterase of the ganglia of the diisopropylfluorophosphate controls sacrificed at 35 minutes was practically completely inactivated. In the two controls sacrificed at approximately 80 minutes, there was faint staining of the cytoplasm of the cholinergic neurons, suggesting the synthesis of new acetylcholinesterase. This is in keeping with the aforementioned conclusion that synthesis of the enzyme may occur within the endoplasmic reticulum (*9*). Marked reduction occurred in the acetylcholinesterase activity of the motor endplates, which showed, however, considerable variation in intensity.

Findings in all six cats which received diisopropylfluorophosphate followed by 2-PAM were practically identical, despite the differences in doses and time intervals. The cholinergic neurons of the ciliary ganglia showed distinct peripheral staining, and faint but definite staining within the cytoplasm. The same was true of the cholinergic neurons of the stellate ganglion; in this tissue, staining could be seen also in the protoplasmic tracts. Staining of the motor endplates varied, but the average intensity was considerably greater than that of the controls.

When diisopropylfluorophosphate is injected intravenously, most of the agent probably reacts with esterases and other constituents of the tissues within a few minutes; after an hour, it is highly unlikely that any uncombined compound is present (*13*). Therefore, these results appear to demonstrate conclusively that 2-PAM can reactivate acetylcholinesterase of neurons and motor endplates *in vivo* after its inactivation by diisopropylfluorophosphate.

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New Mutation in Polioviruses

During the last several years our knowledge of the mutation of polioviruses has been much increased through studies in various *in vitro* systems. Mutants of reduced virulence for monkeys have been discovered (*1*). With regards the size of plaque on monolayers of monkey kidney cells, Dulbecco and Vogt (*2*) have obtained an *r* (rapid) mutant of Brunhilde, and Dubes (*3*) has isolated slow Mahoney, a genetic variant of Mahoney that produces relatively tiny plaques. Dubes (*4*) has also reported on the cold-adapted variants of polioviruses. Heat-resistant variants of polioviruses were also isolated (*5*).

It has been shown (*6*) that spontaneously occurring neutralizing substances for three types of poliovirus can be found in normal adult bovine serums. Although the data thus far accumulated indicate apparently the possible identity of the neutralizing substances with the antibody, it must be admitted that the evidences are not yet conclusive enough, and further experimental work is required before this can be accepted as proved. Accordingly, in this report we refer to these substances as normal bovine serum (NBS) inhibitors for the sake of brevity.

In the work reported here (*7*), mutants of the poliovirus strains, Mahoney and MEF-1, "resistant" to NBS inhibitors, have been obtained readily through serial passages of the parental viruses in HeLa cell cultures with a medium containing 20 to 40 percent inhibitory normal bovine serum. Our strain of HeLa cells has been propagated quite satisfactorily during the last 15 months in medium YLA (Earle, 0.5 percent lactalbumin hydrolyzate and 0.1 percent Difco yeast extract) (*8*), supplemented with only 20 percent normal bovine serum.

A simple method was devised that can

be used both for the demonstration of NBS inhibitors in bovine serums and for the detection of mutants of polioviruses resistant to NBS inhibitors. When monolayers of either HeLa cells or monkey kidney cells that had been inoculated with parental polioviruses were overlayered with YLA agar containing 10 to 20 percent inhibitory normal bovine serum, only small plaques (usually 0.5 to 1.5 mm) were produced 4 to 5 days after seeding. In contrast, larger plaques (up to 8 to 10 mm) were formed on control plates that were overlayered with agar containing 10 percent noninhibitory horse or calf serum. Furthermore, it was found that plaques produced by the resistant mutants are definitely larger than those produced by the parental polioviruses on inhibitory agar plates and approach the plaque sizes of the latter on the control plates. After the large mutant plaques have been replated on inhibitory agar plates, clones of the mutant virus can be readily obtained.

Both Mahoney and MEF-I strains were first twice plaque-purified on HeLa cell monolayers, and respective stocks, grown on HeLa cell cultures, were used to initiate serial passages. A bottle culture of HeLa cells (5 to 8×10^6 cells) with 9 ml of medium YLA supplemented with 40 percent inhibitory NBS-120 was inoculated with 1 ml of undiluted Mahoney stock (10^8 pfu/ml) and examined daily for cytopathogenic action. As soon as cellular degeneration was complete, culture fluid was harvested and 1 ml of the harvested fluid was inoculated again into a new HeLa cell bottle culture containing 40 percent NBS-120. A resistant mutant was obtained from the seventh passage of the series of serial passages.

After the mutant had been plaque-purified on HeLa cell monolayers, the plaque sizes of the mutant (Pl-289) and those of the parental Mahoney were compared on HeLa cell monolayers (Table 1.) It is clear that plaques of the mutant (Pl-289) are definitely larger than those of the original Mahoney on HeLa cell monolayers with 20 percent NBS-120 agar overlay and that the plaques show a distribution essentially similar to that of parental Mahoney on control plates.

In another experiment, resistant mutants were detected even after single passage of parental MEF-1 virus in HeLa cell bottle culture in the presence of 40 percent strongly inhibitory NBS-70. In this instance, cellular destruction was complete only 7 to 9 days after inoculation of 1 ml of undiluted MEF-1 stock. Plaque sizes of a mutant of MEF-1 (Pl-91) thus obtained are compared with those of parental MEF-1 on monolayers of monkey kidney cells in Fig. 1. It is clear that the mutant (Pl-91) produces larger plaques than those of the original MEF-1 on monkey kidney monolayers with NBS-70 agar overlay.

These mutants of both types of poliovirus isolated from different series of serial passages have been studied with respect to their sensitivity to NBS inhibitors. Undiluted normal bovine serum used for respective serial passages and virus (10^5 pfu/ml) were mixed and incubated at 37°C. At various subsequent times, a sample of the mixture was further diluted and assayed for active virus by the plaque method (*9*) on HeLa cell monolayers to determine the kinetic curve of inactivation (*10*) of the original and mutant viruses. In each case, it was clearly observed that the mutant viruses were inactivated definitely slower and to a lesser extent than the respective original viruses. Therefore it may be concluded at least that mutation to resistance to NBS inhibitors is involved in the formation of larger plaques on the NBS inhibitor plates, although it does not exclude the possibility that other types of mutation may also be involved. In contrast, since no apparent difference could be demonstrated between the kinetic curves of neutralization (*10*) of the original viruses and respective mutants with homologous antiserum, it is clear that the mutants are immunologically identical with the respective original viruses.

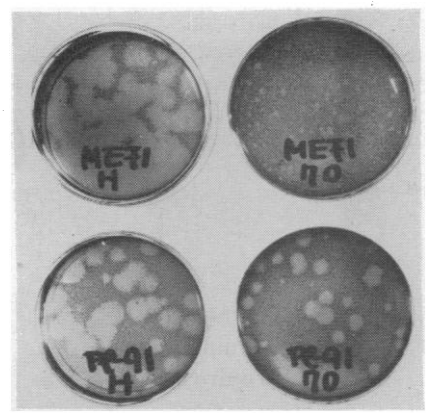

Fig. 1. Comparison of plaque sizes of plaque-purified MEF-1 stock and its resistant mutant (Pl-91), isolated and twice plaque-purified after single passage of parental MEF-1 in HeLa cell bottle culture with medium containing 40 percent NBS-70, on monolayers of monkey kidney cells. *H* and 70, agar overlay containing 10 percent horse serum and 20 percent NBS-70, respectively. The photograph was taken 3 days after inoculation (approximately one-half natural size).

The resistant mutants have been compared with the original viruses in virulence for mice by both the intracerebral and intraspinal routes of inoculation. The data available at this time show that the resistant mutants of both Mahoney and MEF-1 do not differ in virulence in mice from their respective progenitors. The mutants were found to be stable through ten rapid serial passages in tissue cultures of HeLa, monkey kidney cells, and embryonic human skin muscle in the absence of NBS inhibitors.

Table 1. Distribution of plaque sizes of parental Mahoney virus and its mutant "resistant" to normal bovine serum (NBS) inhibitor on monolayers of HeLa cells.

Virus		Content of agar overlay	Frequency distribution of plaque sizes (5%)*						
Strain	Diln.		Plaque diameter (mm)						
			< 0.5	1	2	3	4	5	6
Mahoney, plaque-purified wild type	10^{-5}	10% horse serum	7.6	18.6	15.7	32.0	15.7	7.9	2.4
Mahoney, plaque-purified wild type	10^{-5}	20% NBS-120	100	0	0	0	0	0	0
Pl-289, plaque-purified Mahoney mutant†	$(1/4)10^{-4}$	10% horse serum	19.3	30.0	37.9	9.3	2.9	0.7	0
Pl-289, plaque-purified Mahoney mutant†	$(1/4)10^{-4}$	20% NBS-120	12.2	25.2	18.3	25.2	13.0	4.6	1.5

* After 4 days' incubation at 37°C, 200 to 400 plaques were counted. All the determinations were made on the same lot of HeLa cell monolayers.
† Isolated from the seventh passage in HeLa cell bottle culture with a medium YLA containing 40 percent inhibitory NBS-120.

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