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Purified Human Poliomyelitis Virus: Infectivity for Cynomolgus Monkeys.*

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(Introduced by M. B. Visscher.)

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In a series of studies on the purification of poliomyelitis viruses, it was of interest to determine the comparative activities of purified and unpurified human poliomyelitis virus. It was hoped that such an investigation would provide some information, however incomplete, on the approximate yield of active virus that might be expected from monkey spinal cord tissues by the methanol precipitation technique of purification.

It should be emphasized that the term *purified,* as used in this report, refers to preparations in which a relatively large proportion of protein has been removed, and by no means does it suggest that virus has been obtained which is completely free of non-viral protein.

Ultracentrifugal studies made by Loring and Schwerdt[1] showed that poliomyelitis-infected monkey tissues exhibited infectivity when diluted on a weight basis to 10^{-3}. After purification and concentration of the virus by ultracentrifugation methods, these workers demonstrated that preparations having 5 x 10^{-9} g of nitrogen per ml contained a minimal lethal dose of virus for rhesus monkeys.

In this investigation the comparative infectivity for cynomolgus monkeys of a recently isolated human poliomyelitis virus both before and after purification was studied. The virus was purified from nervous tissues by methanol precipitation according to a method described by Cox, *et al.*[2] for influenza viruses and modified by the authors for poliomyelitis viruses.[3]

During the 1946 poliomyelitis epidemic in Minnesota, spinal cords were collected from several human subjects who had exhibited spino-bulbar forms of the infection. After several months' storage in 50% buffered glycerine, 10% saline suspensions of these cords were inoculated intracerebrally into *Macacus rhesus* monkeys, which subsequently developed typical symptomatology and pathology of poliomyelitis. Spinal cord tissue from one of these monkeys was chosen for this study.

A second passage of the virus was made in a cynomolgus monkey by an intracerebral injection of 0.2 ml of a 10% saline suspension of spinal cord. This animal exhibited a marked temperature elevation and hyperexcitability on the sixth day after inoculation, and the next day developed extensive paralysis in all 4 limbs, at which time it was sacrificed. Histological examination of the brain and cervical and lumbar cord revealed typical lesions of a poliomyelitis infection. The entire spinal cord was removed aseptically and stored at –20°C until subjected to the purification procedures previously described.[3] Briefly, the technique used in the purification of infected monkey spinal cord necessitated preliminary grinding of the tissue, dilution in distilled water to form a 20% suspension, and two successive freezings and thawings with intermittent centrifugation of the heavy tissue sediments that formed. The slightly turbid supernate that resulted from the second centrifugation was arbitrarily called crude virus suspension. As shown in Table I, micro-Kjeldahl determination of the total nitrogen present in this suspension revealed

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1 Loring, H. S., and Schwerdt, C. E., *J. Exp. Med.*, 1942, **75**, 395.

2 Cox, H. R., Van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, **56**, 149.

3 Brumfield, H. P., Stulberg, C. S., and Halvorson, H. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 410.

TABLE I.
Infectivity of Purified and Crude Human Poliomyelitis Virus in Cynomolgus Monkeys.

	Mg N per ml	Dilutions				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Crude virus suspension	1.28	5/5*	3/3	2/3	0/3	1/3
Purified virus suspension	.0098	3/3	3/3	2/3	0/3	0/3

* Denominator indicates number of monkeys inoculated; numerator indicates number of infections.

1.28 mg of nitrogen per ml.

Twenty ml of the crude virus suspension was then treated with ether and centrifuged. The lipoidal layer was removed, the dissolved ether distilled off, and the aqueous suspension diluted to 200 ml with 0.1 molar phosphate buffer at pH 7. Absolute methanol cooled to –40°C was added to make a concentration of 30%, and the mixture was kept at –4°C for one hour. The resultant turbid suspension was centrifuged, the precipitate washed with 0.02 M phosphate buffer pH 5.6, and the undissolved precipitate redissolved in 20 ml of 0.1 M phosphate buffer pH 7. Subjection of the resultant water-clear suspension to micro-Kjeldahl procedure for total nitrogen determination revealed .0098 mg N per ml. Comparison of the nitrogen determinations on equal volumes of crude and purified suspensions showed that 99.2% of the nitrogen present in the crude suspension had been removed by methanol purification.

To determine the minimal infective dose of virus present in the crude suspension serial ten-fold dilutions to 10^{-5} were prepared, and each dilution was injected intracerebrally in dosages of 0.5 ml into cynomolgus monkeys. Since the animals were to be used subsequently in another contingent investigation, the conditions necessitated the use of five monkeys for the 1-10 dilution and groups of 3 monkeys for each of the higher dilutions.

Daily temperatures were obtained for each of the animals. The average rectal temperature of the monkeys, based on recordings made previous to the inoculations and during the first 3 days of the incubation period, was 103°F. A rise of 2 degrees was considered indicative of early symptoms. All infected animals exhibited at least a 3-degree rise before their temperatures dropped to a subnormal level upon the advent of paralytic symptoms. The incubation periods varied from 6 to 20 days.

Monkeys showing typical symptoms of poliomyelitis were sacrificed, and subsequent histological examination of their spinal cords confirmed the clinical diagnoses. All the animals inoculated with doses of crude virus below 10^{-3} developed infections, as did 2 of 3 in the 10^{-3} dilution group and one of 3 in the 10^{-5} dilution group. One of the animals of the 10^{-3} group recovered, although it exhibited residual paralysis in both legs. There were no indications of infection in the remaining monkeys. Although the error involved in monkey titration limits the calculation of an exact value for the minimal infective dose, it can be estimated from these results that approximately 1000 to 10,000 infectious units were present in the crude material. Protection against challenge inoculations or pathological evidence of infection could not be determined in the animals that did not show symptoms during this experiment. Results of the infectivity test and nitrogen determination are shown in Table I.

Titration of the purified product in cynomolgus monkeys disclosed a close correlation in the activity of crude and purified virus. In the latter titration two of the animals of the 10^{-3} dilution group developed typical symptoms and pathology of poliomyelitis, indicating that the estimated 1000 minimal infective doses in crude material were also present in the purified material.

During the course of a parallel investigation, an attempt was made to infect cynomolgus monkeys by the oral administration of crude and purified virus. Infectivity of strains of poliomyelitis viruses for several species of monkeys when administered by

the oral or gastro-intestinal route has been reviewed by Horstmann and her co-workers,[4] who report successful transmission of these viruses when virus in the form of infected spinal cord or feces was injected.

In our experiments gelatin capsules were filled with 1 ml of a 10^{-1} dilution of crude and purified viruses. The capsules were placed in a balling gun commonly used in veterinary practice and injected into the esophagus. The capsules were then swallowed by the animals.

Crude virus suspension was administered in this manner to seven cynomolgus monkeys, and purified virus similarly to three cynomolgus monkeys. Two of the animals fed the crude virus developed paralytic symptoms of poliomyelitis, one in 11 days after the inoculation and the other in 18 days. The fact that the first monkey, which died from the infection, had accidentally bitten the capsule at the time of inoculation, may have resulted in infection by another route. Evidence of infection by another was not available, however, since examination of the olfactory bulbs or nerves was not made. In the case of the second monkey, no virus was allowed to come in contact with the tissues of the oropharynx, and it was assumed that the intact capsule passed into the stomach and that the resultant infection of the CNS was accomplished via the alimentary route. Of the 3 animals fed purified virus, one developed paralytic symptoms of poliomyelitis after an incubation period of 23 days. Histological examination of the cords of the three monkeys exhibiting symptoms confirmed the diagnoses.

Conclusions. Methanol precipitation at low temperatures resulted in partial purification of human poliomyelitis virus, in which a significant amount of protein was eliminated from infected monkey cord. Intracerebral titration in cynomolgus monkeys revealed that there was little observable loss of infectious units of virus during the purification procedure. It was observed that approximately 5×10^{-9} g of N injected into monkeys provided a minimal lethal dose, although considerable error may be involved since this value was dependent on the highly variable monkey titration. However, this value indicates a remarkable correlation with that reported by Loring and Schwerdt,[1] although the nitrogen value for methanol-precipitated virus is lower than the values obtained when virus was purified by ultracentrifugation methods.

Infected monkey cord exhibited a rather low infectivity for cynomolgus monkeys when administered orally by capsule. It is interesting to note that in one instance the purified virus was infective and that apparently this infectivity did not differ appreciably from that of the crude virus when inoculated orally. Other investigators[5] have been unsuccessful in infecting cynomolgus monkeys by feeding virus contained in gelatin capsules.

Summary. Human poliomyelitis virus in the form of infected monkey cord was purified by methanol precipitation at low temperatures. Titration by intracerebral inoculation in cynomolgus monkeys indicated no appreciable loss in activity of the virus during the purification procedure. It was shown that purified virus was infective for cynomolgus monkeys when administered by the oral route.

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[4] Horstmann, D. M., Melnick, J. L., Ward, R., and Sa Fleitas, M. J., *J. Exp. Med.*, 1947, **86**, 309.

[5] Faber, H. K., Silverberg, R. J., and Dong, L., *J. Exp. Med.*, 1943, **78**, 499.