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Purification of the MM Poliomyelitis Virus.*

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In the numerous attempts to purify different strains of the poliomyelitis virus many methods have been employed including adsorption,[1] ultracentrifugation,[2,3,4] salting out and ultracentrifugation,[5,6,7] isoelectric precipitation, salting out, and ultracentrifugation,[8] isoelectric precipitation and ultracentrifugation,[9] freezing and thawing and ultracentrifugation,[10] and precipitation with acetone.[11]

In recent years the use of organic solvents as precipitating agents has found wide application in the purification of various proteins. It has been shown that methyl alcohol at low temperatures loses its denaturing effect on proteins[12] and that conditions of alcohol concentration, ionic strength, temperature, and protein concentration have to be controlled in order to achieve optimal separation.[13] For the purification of tetanus toxoid[14] and diphtheria toxoid[15] these conditions have been established. The successful purification of different strains of the influenza virus by alcohol precipitation at low temperatures has also been reported[16] and the same authors observed that essentially the same procedure may be used for the purification of other viral and rickettsial agents, regardless of whether the starting material was infected allantoic fluid, yolk sac, chick embryo, mouse or rabbit brain.

The present work deals with the purification of the rodent polioencephalomyelitis virus known as the MM strain,[17] by methods which use freezing and thawing, alcohol precipitation and dialysis as their main features. After numerous preliminary experiments the following method gave the purest preparation with the best yield.

A 33% mixture of infected mouse brain and cord in 0.1 M phosphate buffer pH 7.0 is prepared by homogenizing the previously frozen tissue in a Potter-Elvehjem glass homogenizer. The mixture is then frozen at −20°C for 24 hours. After thawing the tissue particles are centrifuged off at 4,800 RPM at 4°C for one hour. The clear supernatant fluid is shaken with an equal volume of ether and centrifuged at 4,800 RPM in the cold for 15 minutes. The gelatinous layer at the surface is pierced with a glass rod, the clear fluid underneath is collected in a beaker, the remaining ether is suctioned off in a desiccator connected with a water pump for 30 minutes and the clear solution is then transferred into centrifuge tubes and frozen at −20°C over night. After thawing to 0°C, the insoluble material is centrifuged off at 4,800 RPM in the cold for one hour and discarded.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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TABLE I.
Nitrogen Content, Virus Activity, and Virus Yield During Purification of MM Poliomyelitis Virus.

	G nitrogen/ml	LD_{50}/ml	G nitrogen/LD_{50}	Yield in %
Original solution	.00347	$10^{7.9}$	$3.4 \times 10^{-10.9}$	100
Fraction 1	.00270	$10^{7.8}$	$2.7 \times 10^{-10.8}$	82
" 2	.00063	$10^{9.2}$	$6.3 \times 10^{-13.2}$	74
" 3	.000397	$10^{9.0}$	$3.9 \times 10^{-13.0}$	66
" 4	.000207	$10^{9.2}$	$2.0 \times 10^{-13.2}$	67
" 5	.00014	$10^{9.1}$	$2.4 \times 10^{-13.1}$	65
" 6	.00008	$10^{8.8}$	$8.0 \times 10^{-13.8}$	65

The clear supernate (fraction 1) is diluted 15 times with isotonic saline solution, the pH is then adjusted with N/10 hydrochloric acid by means of glass electrodes to 5.9 and the temperature of the solution is lowered to –1°C by immersing the beaker in a constant temperature cold bath. Enough methyl alcohol chilled to –20°C and measured at that temperature is slowly added through a capillary to make up to 32% of the solution. The alcohol is left in contact with the solution for at least 6 hours and the precipitate is centrifuged off at 4,800 RPM in the cold for one hour. The clear supernatant fluid is discarded, the precipitate drained in the cold and washed once with a chilled mixture of 32% methyl alcohol in isotonic saline solution of pH 5.9. The precipitate is again drained in the cold and then thoroughly suspended in one-twentieth of the original volume of phosphate buffer at room temperature and stirred with an electric stirrer for one hour. The insoluble particles are centrifuged off at 4,800 RPM in the cold for one hour and discarded. The supernate (fraction 2) is frozen at –20°C for 24 hours. After thawing, but still at 0°C, the formed precipitate is centrifuged off at 4,800 RPM in the cold for 30 minutes and discarded. The clear opalescent supernate (fraction 3) is diluted 10 times with isotonic saline solution, the pH is adjusted to 5.5 with N/10 hydrochloric acid, the temperature is lowered to –1°C and enough chilled methyl alcohol is added to give a final concentration of 30%. After at least 6 hours, the precipitate is centrifuged off in the cold for 30 minutes and the supernate is discarded. The precipitate is drained in the cold and washed with a chilled solution of 30% methyl alcohol in isotonic saline pH 5.5, again drained in the cold and then thoroughly suspended in one-tenth of the original volume of phosphate buffer pH 7.0 at room temperature and stirred with an electric stirrer for one hour. The insoluble particles are centrifuged off for one hour in the cold at 4,800 RPM and discarded. The supernatant fluid (fraction 4) is frozen over night. After thawing, but still at 0°C, the precipitate formed is centrifuged off in the cold at 4,800 RPM for one hour and is discarded. The water-clear supernatant fluid (fraction 5) is transferred into cellophane tubing of 8 mm diameter and dialyzed against frequent changes of distilled water in the cold for one day. After centrifuging the solution in the cold at 4,800 RPM for one hour a small precipitate of water insoluble proteins can be separated and discarded. The water clear supernatant fluid (fraction 6) contains the purified virus.

The nitrogen content of all fractions was measured by the micro Kjeldahl method. The activity of each fraction was ascertained by intracerebral injection of 0.03 ml of the diluted fractions into 3- to 5-weeks-old mice of the ZBC strain obtained from the colony of Dr. John Bittner, University of Minnesota. The animals were observed for 2 weeks. Groups of 8 mice were used for each dilution and the final LD_{50} was calculated by the method of Reed and Muench.[18]

The results of a typical experiment and the effect of each step on the nitrogen content and the specific virus activity are shown in

[18] Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

Table I. In this experiment a 43-fold purification was achieved and after the deduction of the 35% loss of virus activity a purification factor of about 28 resulted.

Conclusion. A simple method requiring standard laboratory equipment only is described which by means of freezing and thawing, alcohol precipitation and dialysis accomplishes a marked purification of the MM poliomyelitis virus.

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Electronmicroscopy of the Purified MM Poliomyelitis Virus.*

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The purified virus used for electronmicroscopic studies was prepared in essentially the same way as described in detail in the preceding paper. During the preliminary studies the observation was made that highly concentrated brain suspensions in phosphate buffer yielded heavier precipitates after freezing and that a higher degree of purification could be achieved by keeping the protein concentration as high as possible.

A 25% mixture of infected mouse brain and cord in 0.1 M phosphate buffer pH 7.0 was prepared by homogenizing the previously frozen tissue in a Potter-Elvehjem glass homogenizer. The mixture was then frozen for 24 hours. After thawing the tissue particles were centrifuged off, the clear supernate was extracted with two-thirds its volume of ether, centrifuged and the gelatinous layer at the surface was discarded. The remaining ether was removed in a desiccator and the solution was frozen overnight. After thawing the precipitate was centrifuged off and discarded. The temperature was then lowered to $-1°C$ and enough chilled methyl alcohol was added to a final concentration of 30% methanol. After 6 hours the precipitate was centrifuged off, washed once with 30% methyl alcohol in buffer, suspended in one-tenth of the original volume, the insoluble particles were centrifuged off and the supernate was frozen overnight. After thawing the precipitate was centrifuged off and discarded. The supernatant fluid was dialysed against distilled water for 24 hours, the water insoluble proteins were centrifuged off and discarded. The supernatant fluid was frozen for 2 weeks, thawed and a final precipitate was centrifuged off and discarded.

By this procedure the nitrogen content was reduced from 2.72 mg per ml in the original clear solution to 0.0119 mg per ml in the final fraction. Thus, a 228 fold purification was achieved or 99.5% of the total nitrogen of the original solution was removed. The final yield amounted to about 10% of the total virus activity of the original solution and therefore a purification factor of about 22.8 resulted. The final fraction contained 1.19×10^{-12} g of "virus" nitrogen per ml and one inoculum contained 3.9×10^{-14} g of "virus" nitrogen.

The mounts for the electron micrographs were prepared by the usual technique of applying a small drop of the 10 times diluted final fraction (1.19×10^{-13} g nitrogen per ml) to a thin formvar membrane supported by a 1/8 inch diameter disc of 200 mesh copper screen and allowing it to dry. The gold-shadowed specimen was prepared by applying a small drop of the solution to a microscope slide. This was treated by the gold shadowing technique of Williams and Wyckoff[1] (8 AU film of gold at a shadow angle of 5 to 1) stripped with collodion and mounted on the copper screen. Measurement of the par-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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