[CONTRIBUTION FROM THE DEPARTMENT OF ANIMAL AND PLANT PATHOLOGY OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Concentration and Purification of Tobacco Mosaic Virus by Means of the Sharples Super-centrifuge

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When the infectious juice pressed from frozen macerated mosaic-diseased Turkish tobacco plants is clarified and centrifuged at a sufficiently high speed, there may be recovered from the solid jelly-like sediment about 3 mg. of tobacco mosaic virus per cc. of juice.[1] Three or four successive sedimentations at high centrifugal force, each followed by clarification at low centrifugal force are required to yield a virus preparation sufficiently pure for most chemical work. The method is especially suitable for plant viruses, since in most cases practically all material of high molecular weight except the viruses is rendered insoluble by the freezing process.[2] The method should also be suitable for any virus that can be produced in a medium free of material possessing a sedimentation constant comparable to that of the virus. During the past five years, air-driven vacuum centrifuges of the type described by Bauer and Pickels[3] and by Biscoe, Pickels and Wyckoff[4] have been used to provide the high centrifugal force necessary to sediment medium and small sized viruses. In this laboratory duraluminum or magnesium–aluminum alloy heads from 6 to 8 inches in diameter and carrying from 6 to 14 Lusteroid tubes 0.75 × 3 inches in size have been spun at speeds from 24,000 to 36,000 r. p. m. to yield a centrifugal force of about 50,000 times gravity at the centers of the Lusteroid tubes. Since acceleration and deceleration each requires eight to ten minutes and the tobacco mosaic virus must be spun about an hour in order to secure complete sedimentation, about two working days with 2 centrifuges having a combined capacity of 350 cc. are required to prepare about 4 g. of purified tobacco mosaic virus. The equipment is admirably suited for the centrifugation of solutions up to a liter or so in volume and for work with unstable viruses, since the virus solution may be centrifuged near its freezing point.[5] However, for certain types of work amounts of tobacco mosaic virus of 100 g. or more are needed, and it is obvious that the centrifugation of the necessary 30 liters or so of infectious juice not only requires much time and effort, but also places a severe strain on the physical equipment. Consequently, during the past year other means for the centrifugation of large volumes of virus solutions have been sought.

In 1927 McKinney[6] attempted to use the commercially available Sharples Laboratory Super-centrifuge for the concentration of tobacco mosaic virus, but, because of the large volumes of liquid required, soon discarded it in favor of a small, specially constructed centrifuge with a closed bowl holding about 10 cc. of solution. However, by means of this centrifuge, which yielded a centrifugal force of about 50,000 times gravity, McKinney was able to demonstrate that tobacco mosaic virus could be concentrated by centrifugation. In 1933 Schlesinger[7] showed that a coli-bacteriophage could be concentrated and purified by means of a similar, specially constructed centrifuge having a closed hollow cylinder. The first real test with viruses of the Sharples Super-centrifuge equipped with a bowl for continuous flow, and hence useful for large volumes of fluid, appears to have been made by McIntosh and Selbie[8] in 1940. Using a considerably modified centrifuge, these workers demonstrated that the infectivity of large volumes of fluids containing bacteria, vaccine virus or either of two bacteriophages could be reduced at least 100 times by passage through the centrifuge. The infective particles that were removed from the effluent liquid were recovered in the sediment. In view of these results, it appeared desirable to make a thorough study of the usefulness of the Sharples Laboratory Super-centrifuge for the concentration and purification of tobacco mosaic virus.

Experimental

A regular model Sharples Laboratory Super-centrifuge with a compressed-air turbine drive and a monel metal

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(2) W. M. Stanley, *Ann. Rev. Biochem.*, **9**, 548 (1940).
(3) J. H. Bauer and E. G. Pickels, *J. Bact.*, **31**, 53 (1936); *J. Exp. Med.*, **64**, 503 (1936).
(4) J. Biscoe, E. G. Pickels and R. W. G. Wyckoff, *Rev. Scient. Instruments*, **7**, 246 (1936); *J. Exp. Med.*, **64**, 39 (1936).
(5) W. M. Stanley and R. W. G. Wyckoff, *Science*, **85**, 181 (1937).
(6) H. H. McKinney, *J. Agr. Res.*, **35**, 13 (1927).
(7) M. Schlesinger, *Biochem. Z.*, **264**, 6 (1933).
(8) J. McIntosh and F. R. Selbie, *Brit. J. Exp. Path.*, **21**, 153 (1940).

clarifier bowl was used. This model develops 62,000 times the force of gravity at the speed of 50,000 r. p. m. which was used in this work. A sheet of celluloid or of cellulose acetate, 14.5 × 19.8 cm. and about 0.4 mm. thick, was used as a liner for the bowl, and inside this was placed a second liner of filter paper. A cooling coil mounted between the rotating bowl and the outer shell of the centrifuge and composed of about 14 feet of 0.25-inch copper tubing, through which ice water was passed by means of a small electric centrifugal pump, was used to absorb heat generated by the rotating bowl. In preliminary experiments the smallest delivery jet supplied with the centrifuge was used with the infectious juice from mosaic-diseased Turkish tobacco plants or with purified preparations of tobacco mosaic virus in 0.1 *M* phosphate buffer to determine the effect of factors such as rate of flow, temperature, and *p*H of ingoing liquid on the yield of virus. Centrifugation at *p*H 5, 6, or 7 appeared to have no significant effect on the yield of virus. The temperature of the ingoing liquid also appeared to be of little importance for, regardless of this temperature, that of the bowl contents was usually 20–25° and that of the effluent liquid was usually about 17°. The temperature of the effluent liquid appeared to be governed by the evaporation caused by the rapid flow of air through the centrifuge. The evaporation was usually sufficient to reduce the volume of the effluent liquid by about 20%. The concentration of the effluent liquid caused by evaporation was neglected in the calculations of the yields of virus. The factor having the greatest influence on the yield of virus was found to be the rate of flow of the preparation. Flow rates of 30 cc. per minute or greater resulted in yields of 50% or less, whereas flow rates between 20 and 25 cc. per minute gave yields of 60 to 75%. In order to secure a uniform slow rate of flow, a short length of tubing from a No. 22 hypodermic needle was soldered into the original delivery jet. This gave a rate of flow of about 15 cc. per minute, which, as may be seen from the data presented in Table I, resulted in yields of 3 to 4 mg. of virus per cc. in the case of infectious juices and of 75 to 93% in the case of purified virus preparations.

TABLE I

REPRESENTATIVE DATA FOR SUPER-CENTRIFUGE RUNS IN WHICH A SATISFACTORY RECOVERY OF TOBACCO MOSAIC VIRUS WAS ACHIEVED

Starting material			Effluent	Yield of
Volume, cc.	Protein, mg./cc.	Rate of flow, cc./min.	protein, mg./cc.	virus, mg./cc. of juice
Infectious juice				
5000	9.0	14	5.37	3.4
3560	9.0	13.4	4.37	3.9
5000	10.0	13.2	6.65	3.7
2400	10.0	13.0	6.55	4.1
5000	8.75	13.2	5.63	3.2
4000	8.75	12.0	..	3.0
5000	9.1	14.0	6.0	2.7
5000	9.1	13.0	5.0	3.4
Purified virus 0.1 *M* PO_4 *p*H 7				
				%
995	3.1	15	0.34	89
5600	3.0	16.5	.75	75
4100	3.0	15.3	.69	77
1000	10.4	12.3	.69	93
1400	10.4	15	.94	91
1200	10.4	14	1.69	84
3000	9.3	15	0.69	93

In one experiment the virus in 200 cc. of infectious juice containing 9.0 mg. of protein per cc. was purified by 3 successive sedimentations in the vacuum centrifuge and the virus in 5 liters of the same juice was purified by means of the Sharples Super-centrifuge. The supernatant fluid from the first centrifugation in the vacuum centrifuge contained 2.4 mg. of protein per cc., and the yield of virus following 3 sedimentations was 733 mg., equivalent to 3.66 mg. per cc. of juice. The effluent from the Sharples Super-centrifuge, on the other hand, contained 5.37 mg. of protein per cc. In order to remove the last portions of the juice from the bowl, there was introduced, immediately following the juice and without stopping the centrifuge, 500 cc. of 0.4 *M* phosphate buffer at *p*H 7. This buffer had the same density as that of the juice. On stopping the centrifuge, the material on the liner was dissolved in the bowl contents consisting of about 250 cc. of the buffer. The celluloid and filter paper liners were well washed with sufficient water, which, when added to the bowl contents, gave a final solution containing 0.1 *M* phosphate buffer. This solution, which was found to contain 16.9 g. of protein, was diluted with 0.1 *M* phosphate buffer at *p*H 7, so that the final protein concentration was 3 mg. per cc. This solution was passed through the Sharples Super-centrifuge under the same conditions described above, and the effluent was found to contain 0.75 mg. of protein per cc. The liner and bowl contents which contained 12.4 g. of protein were diluted to a protein concentration of 3 mg. per cc. and again passed through the centrifuge. The effluent was found to contain 0.69 mg. of protein per cc. and the liner and bowl contained 9.6 g. of virus. Under the conditions of the experiment, the yield of virus was 52.5% of that obtainable by means of the vacuum centrifuge.

However, the two final sedimentations with the vacuum centrifuge were carried out at a protein concentration of about 10 mg. per cc., as is customary in our laboratory, whereas in the case of the Sharples Super-centrifuge the 2 final sedimentations were made at a protein concentration of only 3 mg. per cc. It seemed possible that this might account in part for the difference in yields; hence, a test run was made by passing a liter of a solution containing 10.4 mg. of virus per cc. in 0.1 *M* phosphate buffer at *p*H 7 through the Sharples Super-centrifuge. Although the same delivery jet was used, the rate of flow of this solution was reduced to 12.3 cc. per minute, presumably due to the increased viscosity. The effluent was found to contain but 0.69 mg. of protein per cc.; hence, 93% of the virus was removed. As may be seen from the data presented in the second part of Table I, yields of the same order of magnitude were obtained in several experiments in which purified virus at a concentration of about 10 mg. per cc. was used. The fact that the protein concentration of the ingoing liquid could be increased from 3 to 10 mg. per cc. without appreciably affecting the concentration of protein in the effluent demonstrates that a more efficient recovery of virus may be accomplished at the higher virus concentration. When protein concentrations of about 10 mg. per cc. were used for all runs after the original juice was sedimented, the over-all yield of virus was increased to about 65%.

Discussion

The need for easily available and relatively inexpensive equipment by means of which purified tobacco mosaic virus may be prepared readily in quantity is met by the Sharples Super-centrifuge equipped with a cooling coil. It is necessary only to solder a short piece of tubing from a No. 22 hypodermic needle into the original delivery jet and to use a double liner of celluloid and filter paper in the rotating clarifier bowl. It is possible during the course of ten hours to prepare 10 to 15 g. of tobacco mosaic virus, purified by 4 sedimentations by using a flow rate of about 15 cc. per minute for the infectious juice used as starting material, and a flow rate of 12–15 cc. per minute for the final 3 sedimentations of the virus at a concentration of about 10 mg. per cc. The fact that the over-all recovery of virus from the starting material is somewhat less than that obtainable with the vacuum type centrifuge is more than offset by the fact that during a given period of time the amount of purified virus obtainable with the Sharples Super-centrifuge is over ten times that obtainable with a vacuum type centrifuge.

The amount of virus directly isolable from the infectious juice may be increased somewhat if the effluent juice is immediately passed through the centrifuge a second time. Although this expedient is hardly worth while in the case of tobacco mosaic virus, due to the relatively high virus concentration and the ease with which large amounts of infectious juice may be obtained, Dr. Knight of this laboratory has found the second passage of effluent juice to be quite advantageous in the case of cucumber virus 4. The concentration of this virus in the infectious juice from cucumber plants is only a few tenths of a mg. per cc.,[9] and it is somewhat more difficult to obtain large quantities of the infectious juice. After an initial concentration of this virus to about 10 mg. per cc., the recovery on sedimentation should be equivalent to that obtainable with a similar concentration of tobacco mosaic virus, since the two viruses have approximately the same sedimentation constant. It seems likely that, in general, a second passage of infectious juice will be advantageous in those cases where the concentration of the virus is less than about 1 mg. per cc. and in those cases where the sedimentation constant of the virus is appreciably less than that of tobacco mosaic virus.

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Summary

The concentration and purification of tobacco mosaic virus have been accomplished efficiently by means of a Sharples Laboratory Super-centrifuge, equipped with a regular clarifier bowl operated at a speed of 50,000 r. p. m. by means of compressed air. It was found advantageous to use a cooling coil, a celluloid and a filter paper liner in the bowl, and a modified delivery jet constructed from a small hypodermic needle. Using the clarified juice from mosaic-diseased Turkish tobacco plants as starting material, it was possible to prepare 10 to 15 g. of tobacco mosaic virus, sufficiently pure for most purposes, during the course of ten hours by means of such equipment

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(9) C. A. Knight and W. M. Stanley, *J. Biol. Chem.*, **141**, 29 (1941).