CENTRIFUGE EXPERIMENTS WITH THE VIRUS OF VACCINIA

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The nature of filtrable viruses is still a controversial subject. Gordon (1) suggested that rabbit vaccinia virus is associated in particles big enough to be thrown down by centrifugal force and even thought that the virus might come down under the action of gravity. MacCallum and Oppenheimer (2) stated that vaccine granules can be freed from lymph by differential centrifugation. Bedson (3) supported his theory that the non-passage of herpes virus through a colloidal membrane was due to the size of the virus by the fact that the virus in suspension can be concentrated by high-speed centrifugation. More recently Bland (4) reported similar results in his filtration and centrifuge experiments with vaccinia virus. These results have been criticized, however, on the ground that the experiments were conducted with emulsions or diffusates, where the virus might have been adsorbed by contaminating microorganisms or by cellular structures, and that consequently, when the adsorbing material was centrifuged down, it carried the virus with it.

In a previous communication (5) we have reported that if tissue containing the virus of herpes or vaccinia was suspended in hormone broth instead of saline solution, active filtrates could be constantly obtained. We suggested that if careful centrifuge experiments were carried out with cell-free filtrates, it might be possible to throw some light upon the real nature of the virus. The work recorded here is the result of such an investigation carried out with vaccinia virus.

Preparation and Testing of the Virus Filtrates

Active filtrates of vaccinia virus were prepared according to the method previously described, except that ordinary powdered Japanese glass was used instead

of sand. Fresh virus containing tissue was thoroughly ground in a mortar with the glass powder, and a 5 per cent suspension was made in hormone broth. It was then centrifuged for half an hour at about 2000 r.p.m. and the supernatant fluid poured off for filtration. A Berkefeld V filter was employed after testing its water flow, and a new filter was used for each filtration. The filtration was carried out as soon as possible after the tissue was taken from the animal and always under a negative pressure of 50 cm. of mercury. The filter was further controlled by adding a little of a culture of *B. prodigiosus* to the suspension before filtration was commenced. Big, albino rabbits were used for titrating the activity of the filtrates. The back of the animals was shaved the previous day and the points of injection

TABLE I

Dilution	Unfiltered control			Filtrate		
	3rd day	5th day	7th day	3rd day	5th day	7th day
Undiluted	Not tested	Not tested	Not tested	++	+++	++++
1:10	++	*++++	++++	+	++	+++
1:50	++	++++	++++	−	++	++
1:100	+	++++	++++	−	++	++
1:300	+	++++	++++	−	++	++
1:500	+	++++	++++	−	++	++
1:800	±	++++	++++	−	+	++
1:1000	−	++++	++++	−	−	+
1:1500	−	++++	++++	−	−	−
1:2000	−	+++	+++	−	−	−
1:3000	−	+++	+++	−	−	−
1:5000	−	++	++	−	−	−

* In this and the following tables ++++ represents a maximum reaction and ± a minute papule.

were marked out with a circular ink stamp. The injections were made intradermally, and 0.05 cc. of each dilution was injected. The titration of one such preparation prepared from virus pulp S. M. C., Source 683, is given in Table I.

Centrifuge Experiments

5 or 10 cc. of the filtrate was centrifuged in a conical centrifuge tube in an International size 1, Type S. B. centrifuge for varying lengths of time at the maximum speed—about 4000 r.p.m. Then 0.1 cc. of the fluid was carefully pipetted from the surface and the remaining fluid discarded until a similar amount was left in the bottom of the tube. Dilutions were made in hormone broth of both upper and lower fractions as well as of the uncentrifuged filtrate which served as the control. The dilutions were then injected intradermally into a rabbit. The

result of a 2 hour centrifuge experiment conducted with a green virus filtrate is shown in Table II.

As fresh calf pulp was not always easily obtained, an attempt was made to use rabbits as a source of supply. The preparation of rabbit green virus was very simple; the animals were inoculated on the skin

TABLE II

Dilution	Bottom fluid	Surface fluid	Control
Undiluted	++++	±	++++
1:10	++++	−	+++
1:50	+++	−	++
1:100	++++	−	++
1:300	++	−	++
1:500	+++	−	++
1:800	++	−	++
1:1000	++	−	±
1:1500	++	−	−

In this and the following tables readings were taken on the 5th day, unless stated otherwise.

TABLE III

Dilution	Surface fluid	Bottom fluid
Undiluted	+	++++
1:10	−	++++
1:100	−	++++
1:200	−	+++
1:400	−	+++
1:600	−	++
1:800	−	++
1:1000	−	++
1:1200	−	++

and the virus collected on the fifth day. Table III shows the result of a centrifuge experiment with a filtrate prepared from rabbit pulp. The end-point of the activity of the uncentrifuged filtrate was a dilution of 1:800.

Three strains of neurovaccine derived from intracerebral inoculation of different filtrates were obtained. From these 3 neurotropic strains

a number of active filtrates were prepared. Table IV gives the result of one of the centrifuge experiments made with a filtrate prepared from a neurovaccine brain.

All these experiments show a definite concentration of the virus in the lowermost layer of the centrifuged fluid, leaving the supernatant portion practically inactive. Evidence then seemed to be in favor of the theory that the virus itself or at least its aggregates might really be associated in particles big enough to be spun down by centrifugal force because of the fact that the filtrates contained no microorganisms or other cellular elements upon which the virus might be adsorbed, and so carried down along with this adventitious matter. It has been suggested (6), however, that inert particles present in the filtrates

TABLE IV

Dilution	Surface fluid	Bottom fluid	Control
1:50	−	+++	++
1:100	−	++	++
1:300	−	++	++
1:500	−	++	++
1:800	−	+	+
1:1000	−	++	±

might act as adsorbents, and carry down the virus. In order to meet this criticism, some further experiments were carried out.

The separation of coarser from finer particles by fractional centrifugation is an ordinary laboratory procedure. Since the inert particles assumed to be present in the broth filtrates must be bigger and heavier than the virus, their separation by fractional centrifugation should be possible.

In order to carry out such a separation, more active filtrates are necessary. Such filtrates have been prepared by Ward (7). The only difference in his technique from that already described in this paper is the substitution of pyrex glass powder for ordinary glass powder or sand as a grinding material. Table V gives the titration of a calf green virus filtrate prepared by this modified technique from virus pulp S. M. C., Source 723, and a neurovaccine Filtrate 084 obtained from a rabbit brain originally obtained from vaccine lymph, Lot 97, National Epidemic Prevention Bureau.

TABLE V

Dilution	Green virus 723		Neurovaccine 084	
	Filtrate	Unfiltered	Filtrate	Unfiltered
1:10	+++	++++	++++	++++
1:50	+++	++++	+++	++++
1:100	+++	+++	+++	++++
1:300	+++	+++	+++	++++
1:500	+++	+++	+++	++++
1:800	++	++	+++	+++
1:1000	+	++	++	+++
1:1500	++	++	++	+++
1:3000	++	+++	++	++
1:6000	++	++	++	++
1:12000	++	++	++	++
1:24000	++	++	+	++
1:48000	++	+		
1:120000	±	+		
1:600000		+		
1:1200000		+		

TABLE VI

Dilution	Control	Surface fluid	Bottom fluid
1:10	+++	+++	++++
1:50	++	+++	++++
1:100	++	++	+++
1:300	+++	+++	++++
1:500	+++	++	++++
1:800	++	++	++++
1:1000	++	++	+++
1:1500	++	+	+++
1:3000	+	+	+++
1:6000	+	+	++
1:12000	±	−	++
1:24000	±	−	++
1:48000	−	−	++
1:120000	−	−	++
1:600000	−	−	+
1:1200000	−	−	+

Fractional Centrifuge Experiments

10 cc. of calf green virus Filtrate 723 was centrifuged for 1 hour at 3500 r.p.m. in a centrifuge tube stoppered with a rubber cap. Then 5 cc. from the surface was carefully pipetted off without disturbing the lower portion and transferred to a second tube which was centrifuged for 4 hours at the same speed. Surface and bottom specimens were collected and then the various layers were mixed and a third specimen taken to serve as control. All 3 specimens were then titrated on the same rabbit. The readings were taken on the third day. Table VI shows the result of this titration.

Another experiment was then done in which Filtrate 723 was centrifuged for 4 hours at 3500 r.p.m., the upper half of the fluid removed and placed in the refrigerator over night. Next morning this was centrifuged for 4 hours at the same speed. Table VII shows the result of the titration of the upper and lower layers as well as of the whole fluid as control. Readings were taken on the third day.

TABLE VII

Dilution	Surface fluid	Bottom fluid	Control
1:10	+	++	++
1:50	−	++	+
1:100	−	++	+
1:300	−	++	±
1:500	−	+	+
1:800	−	+	−
1:1000	−	+	−
1:1500	−	+	−
1:3000	−	−	−
1:6000	−	−	−

From the results of Tables VI and VII, especially from Table VII, it seems improbable that the concentration of the virus was due to the adsorption of the virus by the inert particles in the filtrates, because such particles must have been thrown down by the preliminary centrifugation. The results shown in Table VII were confirmed by several other experiments in which the first centrifugation was carried out at 3500 r.p.m. for 4 hours.

DISCUSSION

The ease of filtration of vaccine virus through diatomaceous filters has been demonstrated once more. The success of filtration depends

of course upon many conditions, but the material used for grinding is evidently an important one. Ward's observation (7) that pyrex glass powder serves this purpose better than anything else has been confirmed, and it has been noted that ordinary glass powder is no better than sand. The superiority of pyrex fragments for this purpose is probably due to their not adsorbing the virus rather than to their sharper edges.

Another important condition necessary is the use of very fresh tissue containing the virus. Filtrates prepared from tissue removed from the animal for some time before being filtered are often quite inactive or only slightly active. It is possible that the size of the virus may be altered somehow by some unknown change taking place in the cellular substance of the tissue after death. Ward (7) has shown that if oxygen is excluded from the tissue after removal from the animal, very active filtrates can be obtained after some days, whereas the filtrates of tissue exposed to the air for the same length of time are inactive.

With regard to the success of the centrifuge experiments in concentrating the virus, in the author's opinion it is due to the corpuscular nature of the virus or its aggregates rather than to the presence of inert particles adsorbing and carrying down the virus, for these particles should have been removed by the prolonged preliminary centrifugation.

SUMMARY

Centrifuge experiments have been carried out with cell-free, active filtrates of vaccinia virus. The experiments have shown that the virus can be concentrated by this method, even in filtrates which have been subjected to prolonged preliminary centrifugation to throw down any inert particles which may have been present in the original filtrate. This fact, together with the knowledge that the virus can be almost completely held back by the Berkefeld N filter, as reported previously (5) indicates that the virus may be of considerable size.

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