Ultracentrifuge Concentration of Poliovirus and Effect of Calf Serum and Gelatin. (23356)

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A primary objective in establishment of a poliomyelitis vaccine safety testing program has been to develop methods for detection of the possible presence of live poliovirus in large volumes of poliomyelitis vaccine. Testing of a concentrated poliomyelitis vaccine offers the possibility of combining the facility inherent in manipulating small volumes with the poliovirus sensitivity of the monkey kidney tissue culture system. Although a number of physical and chemical methods have been used for concentrating poliovirus, ultracentrifugation offers a practical and simple procedure involving minimal manipulation or additions of compounds foreign to a biological product. An investigation of the effectiveness of poliovirus concentration in the ultracentrifuge was undertaken because of paucity of data on ultracentrifuge concentration of infectious tissue culture fluids in contrast to the more carefully studied behavior of infected tissue extracts (1,2,10). Results of the present study indicate that poliovirus sedimented from experimental vaccine or Medium 199 has the undesirable quality of forming a loose aggregate easily disturbed by fluid movement. Addition of small amounts of calf serum or gelatin to the system results in formation of a firm pellet which contains most of the virus.

Material and methods. Type I (Mahoney), Type II (MEF_1), and Type III (Saukett) strains were obtained in monkey kidney tissue culture fluid from Dr. Fred Stimpert, Parke, Davis and Co., Detroit, Mich. They were passaged 4 or 5 times in monkey kidney tissue culture and stored at –20°C or in a solid CO_2 chest. Trypsinized monkey kidney tissue cultures were used. These were grown in roller tubes, 2-ounce prescription bottles or 1-liter bottles with either lactalbumin hydrolysate medium or Medium 199(3,4), each containing 2% calf serum. These sera were determined to be free of poliovirus inhibitors by roller tube titration of virus in the presence of 4% serum. Maintenance medium was the same as the growth medium except that only 1% calf serum was used. Virus titers were determined initially with roller tubes using 5 tubes per either 3.2 fold or 10 fold dilutions in medium. Endpoints were calculated as the number of 50% tissue culture infectious doses ($TCID_{50}$) per 0.5 ml of inoculum(5). Subsequently plaque titrations in stoppered 2-ounce tissue culture bottles were used(6,7) and the results expressed as plaque-forming units (PFU) per 0.2 ml. To simulate a residually infected vaccine whose behavior in the ultracentrifuge could be studied, commercially prepared poliomyelitis vaccine or Medium 199 was used to dilute infectious virus from original titers of $10^{7.0}$ $TCID_{50}$ per 0.5 ml to as low as $10^{0.5}$ $TCID_{50}$ per 0.5 ml. These fluids, at pH 7.0 to pH 7.6, were centrifuged in a Spinco model "L" Ultracentrifuge. A No. 30 rotor was used. Thirty-three ml stainless steel centrifuge cups with aluminum caps were filled to capacity with fluid containing virus and centrifuged at 30,000 rpm (78,000 times gravity). To minimize convection currents, the chamber temperature was set to –20°C in order to maintain the fluid at 4°C. Deceleration was allowed to proceed without application of the brake. Various fractions of supernatant were collected by gentle aspiration through the center hole in the centrifuge tube cap with the use of a syringe and side hole 14 gauge needle submerged to a predetermined depth. Any material remaining attached to the cup wall was worked free with a rubber policeman and recovered with three 1.0 ml rinses of medium. This suspension was designated as a sediment. In later studies, a pipette was found to be more convenient for resuspension of the sediment than the rubber policeman and gave equal virus recovery. Fractions, without

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TABLE I. Distribution of Type II Poliovirus, Added to Poliomyelitis Vaccine, after Ultracentrifugation.

Exp. No.	Time centrifuged (hr)	% poliovirus* in Supernatant Upper 31 ml	Supernatant Lower 2 ml	Sediment	Total $TCID_{50}$ Recovered from contents of centrifuge cup	Present in control held in refrigerator	Calculated from dilution
1	2	26	60	14	$10^{2.4}$	$10^{2.4}$	$10^{2.9}$
2	2	25	62	13	$10^{6.7}$	$10^{7.1}$	$10^{7.8}$
3	2	40	52	8	$10^{2.7}$	$10^{2.9}$	$10^{3.0}$
3	3	0	77	23	$10^{2.6}$	$10^{2.9}$	$10^{3.0}$
4	4.3	5	90	5	$10^{2.4}$	$10^{2.3}$	$10^{1.9}$

* Expressed as % of total $TCID_{50}$ recovered from contents of centrifuge cup.

clarification, were assayed for virus content immediately or after storage at 4°C overnight. Gelatin was prepared as a 6% stock solution, heated to 80°C for 20 minutes and filtered through an S-1 Seitz-type filter pad.

Results. Sedimentation of virus from vaccine or culture medium. Experiments were conducted to determine effectiveness of ultracentrifugation for concentrating MEF_1 poliovirus in serum-free Medium 199 and in commercially prepared vaccine. Diluted virus suspensions, designed to simulate an incompletely inactivated vaccine, were centrifuged for 2 to 4.3 hours at 78,000 times gravity. The upper 31 ml of supernatant, lower 2 ml of supernatant, and sediment were removed separately and assayed simultaneously with an uncentrifuged control aliquot kept in a centrifuge cup at 4°C during the experiment. Results of these titrations, expressed as percentage of the virus recovered in supernatant fractions and sediment, and as total virus content, are shown in Table I. The yield of the virus in supernatant plus pellet was compared with the total virus derived from the control and with that calculated from the initial virus dilution. Virus recovery in experiments with small and large amounts of virus closely approximated the expected result. This indicates that there was no loss or inactivation of virus in the concentration procedure. Although most of the added virus was concentrated in the lower 2 ml portion of the supernatant, it was not adherent to the cup wall and thus was not collected with the sediment. Increased time of sedimentation did not significantly increase virus content of the sediment but did increase the fraction of virus in the lower supernatant. In experiments in which virus was diluted in Medium 199 without serum, rather than vaccine, in only 2 of 12 instances was the virus concentrated satisfactorily in the sediment. It was concluded that poliovirus added to vaccine or Medium 199 could not be satisfactorily concentrated in the sediment with the existing method.

Effect of calf serum on concentration of virus from vaccine or medium. In previous work done in connection with virus stability, it had been noted that when calf serum was present in the suspension a firm small brown pellet appeared following centrifugation, but did not form in the absence of serum. Thus it appeared to be due to the serum. It was considered possible that a "calf serum pellet" might enmesh sedimented virus and hold it to the cup wall. On this basis the virus suspension was centrifuged for 5 hours in the presence of 5% calf serum. Analysis revealed 96% of the virus in the pellet, no virus in the upper 31 ml supernatant, and 4% of the virus in the lower 2 ml supernatant. In this instance, addition of calf serum resulted in a pellet which contained almost all the poliovirus. In other experiments similar results were obtained whether the serum was heated at 56°C for 30 minutes or was unheated. To determine the minimal amount of serum required to cause virus adherence to the cup wall, 3 or 4 hour centrifugations were done with 0% to 4% calf serum. Results shown in Table II indicate that a minimum of 1% calf serum in 33 ml of fluid results in adequate concentration. This is correlated with the observation that a definite pellet was not observed in cups with less than 1% calf se-

TABLE II. Effect of Varying Concentrations of Calf Serum on Type II Poliovirus Distribution after Ultracentrifugation for 3 or 4 Hours.

% calf serum	% poliovirus* in Supernatant	Sediment
.0	36	64
.0	37	63
.0	75	25
.015	19	81
.06	43	57
.25	39	61
1.0	0	100
1.0	1	99
2.0	0	100
4.0	0	100

* Expressed as % PFU of total recovered from contents of centrifuge cup.

rum.

Studies described above were done using MEF_1 strain of Type II poliovirus. It was found that Types I and III could be concentrated under similar conditions. The time required to concentrate the virus in the serum pellet was determined by centrifugation for varying periods. The results of experiments with all 3 types of poliovirus are illustrated in Table III. They indicate that maximal concentration in the pellet occurred between 2 and 3 hours of sedimentation when at least 1% calf serum was present.

In previously studied systems some remixing of partially sedimented material usually occurred during deceleration(8). This was caused by the tendency of the fluid to continue rotation. More rapid deceleration increased the tendency. To minimize this effect, all early centrifugation experiments were conducted without application of the brake. To determine whether braking would interfere with concentration of poliovirus in the serum pellet, aliquots of virus in Medium 199

TABLE III. Effect of Time of Ultracentrifugation on Sedimentation of Poliovirus in the Presence of Calf Serum.

Time (hr) centrifuged	No. of experiments averaged	% poliovirus* in Supernatant	Pellet
2	4	23	77
3	5	.5	99.5
4	8	2	98
5	1	4	96
8	1	3	97

* Expressed as % of total $TCID_{50}$ recovered from contents of centrifuge cup.

TABLE IV. Effect of Calf Sera, Containing Inhibitors to Poliovirus, on Concentration by Ultracentrifugation.

Calf serum	Total virus* in Supernatant	Sediment	Total virus* recovered from contents of centrifuge cup
None	4.2	3.8	4.3
2% No. 6	2.3	3.8	3.8
2% " 7	<2.2	3.8	3.8
2% " 4	2.6	2.7	3.0

* Expressed as $\log_{10}$ PFU.

with 1% calf serum were centrifuged for 3 hours with and without rapid deceleration through application of the brake. In repeated experiments over 95% of the virus was recovered from the pellet under both conditions. This indicated that virus once incorporated into the calf serum pellet is not readily resuspended by disturbances due to braking. It was also determined that decantation of supernatant gave the same good virus recovery from the pellet as did aspiration of supernatant.

Other materials which result in satisfactory concentration into a pellet. The presence of inhibitors to poliovirus in occasional lots of calf serum would be expected to decrease the quantity of virus which could be recovered when these sera were used. Such an effect occurred with the 3 calf sera shown in Table IV. This led to a search for a material which would result in satisfactory concentration and also be free of inhibitors. So that a rational choice of possible substitutes could be made, a study was undertaken to determine which component of calf serum was responsible for the effect. It was found that dialyzed serum retained its effectiveness, indicating that the activity was associated with the protein portion. Paper electrophoretic analysis of calf serum, serum supernatant, and serum pellet,† indicated that various globulin components were concentrated into the pellet. This suggested that large protein molecules might have the same effect as calf serum. Some of the materials studied for this property are

† Done by Dr. Aaron Cooper of the Division of Biologics Standards, N.I.H., Public Health Service, U. S. Dept. of Health, Education and Welfare.

TABLE V. Effect of Various Additives on Concentration of Poliovirus by Ultracentrifugation.

Additive	Total virus* in Supernatant	Total virus* in Sediment	Total virus* recovered from contents of centrifuge cup
None	4.7	3.1	4.7
Bovine fibrinogen .3%	2.3	3.7	3.7
" γ-globulin .3%	2.5	3.8	3.8
Bacto hemoglobin .3%	3.3	4.1	4.1
Gelatin .3%	2.5	4.5	4.5
Horse serum 3%	4.1	3.1	4.1
Rabbit " 3%	4.2	3.7	4.3
Control titer			4.4

* Expressed as $\log_{10}$ PFU.

shown in Table V. Gelatin (Nutritional Biochemicals Corp.) was chosen for further study because it resulted in satisfactory concentration into a discrete pellet and was free of inhibitors to poliovirus. The other materials were eliminated either because they did not result in satisfactory concentration or because they were inhibitory to poliovirus. Table VI summarizes an experiment designed to determine minimal concentration of gelatin required satisfactorily to concentrate poliovirus into a pellet. It can be seen that 0.015% concentration was minimal. It should be noted from the total virus recovered that gelatin is free of inhibitors to poliovirus.

Concentration of virus preparations partly inactivated by formalin. Under conditions described above live poliomyelitis virus can be effectively concentrated from medium or vaccine to which it has been added. To investigate the possibility that virus incompletely inactivated by formaldehyde might behave differently, experimental poliomyelitis vaccines containing residual live virus were sedimented for 3 hours in the presence of calf serum or gelatin. In repeated experiments, effective concentration of virus was obtained. This indicates similar sedimentation behavior of untreated and formalin treated poliovirus and suitability for use in a vaccine safety test.

Discussion. It has been shown that a large fraction of poliovirus sedimented from serum-free fluids generally is not collected with the sediment but rather with the supernatant. In the presence of added calf serum or gelatin the virus is concentrated in a pellet. It seems unlikely that calf serum exerts this effect through an increased rate of virus sedimentation, because comparison of Tables I and III indicates that virus is as readily sedimented in the absence as in the presence of serum. Another possible interpretation is that the effect of calf serum is mediated through increased viscosity as has been demonstrated with sucrose(8). This seems unlikely because the calculated change in viscosity induced by 1% calf serum is very small. An explanation which seems to be supported by the data is that a calf serum or gelatin pellet is able to associate with sedimented poliovirus and prevent dispersion by firmly binding it to the cup wall. This would explain the observation that sedimented virus collected at the base of the centrifuge cup, but was firmly adherent to the cup wall only in the presence of calf serum or gelatin. The interpretation assumes that virus sedimented from serum-free fluids forms a loose sediment which is easily disrupted as the supernatant is collected. Additional support for this view comes from experiments in which diminishing amounts of calf serum were used. When the quantity of serum became too small to form a visible pellet, the system simultaneously lost the ability to satisfactorily concentrate virus into the pellet.

Silverberg(9) observed that a 20% mouse brain suspension of Lansing Type II poliovirus was concentrated satisfactorily by ultracentrifugation while a 1% suspension was poorly concentrated. When 10% normal

TABLE VI. Effect of Diminishing Amounts of Gelatin on Concentration of Poliovirus.

% gelatin (final conc.)	% virus* in Supernatant	% virus* in Sediment	Total virus† recovered from contents of centrifuge cup
.06	4	96	4.2
.06	1	99	4.3
.06	12	88	4.4
.015	3	97	4.5
.004	57	43	4.3
.001	88	12	4.2
.00025	81	19	4.4
.0	77	23	4.3
.0	67	33	4.0

* Expressed as % of total PFU recovered from contents of centrifuge cup.
† Expressed as $\log_{10}$ PFU.

mouse serum was added to the 1% suspension, good concentration occurred and was associated with the appearance of a discrete pellet. The results of this study did not permit conclusions as to the mechanism involved and the variable factors in the system were not further studied.

A disadvantage of calf serum for concentration is the too frequent presence of inhibitors to poliovirus. One alternative was to substitute sera from other species. Results with horse and rabbit sera indicated that these sera at 3% concentration lacked the ability to form a similar enmeshing pellet. Electrophoretic studies of calf serum pellets showed that they were mainly composed of the larger serum molecules. This suggested that other large protein molecules would form such a pellet. A study of some readily available proteins showed that gelatin (Nutritional Biochemicals Corp.) could form a poliovirus-enmeshing pellet and was free of inhibitors.

The data presented demonstrate that reproducible concentration of poliovirus can be accomplished by sedimentation at 78,000 times gravity in the presence of 2% inhibitor-free calf serum or 0.06% gelatin. Such concentrated virus can be used for chemical, physical and serological studies as well as safety testing of larger volumes of poliomyelitis vaccines in a simplified, practical manner.

Summary. Studies were undertaken to determine the efficiency of concentration of poliovirus from tissue culture fluids in the ultracentrifuge. Under suitable sedimentation conditions most of the virus was concentrated to the lower portion of the centrifuge cup, but in the absence of calf serum was not present in a sediment adherent to the cup wall. Addition of calf serum resulted in formation of a firm, discrete pellet attached to the centrifuge cup wall which contained more than 95% of the virus. Further experiments indicated that the activity was associated with the globulin protein fraction of calf serum and that gelatin had a similar effect. Studies varying the amounts of calf serum and gelatin and duration of ultracentrifugation indicated that 2% calf serum or 0.06% gelatin and 3 hours centrifugation are minimal for effective concentration of poliovirus when sedimented at 78,000 times gravity in the number 30 rotor of the Spinco model "L" preparative Ultracentrifuge.

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Postirradiation Creatinuria in *Macaca mulatta*.* (23357)

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The authors have observed a profound creatinuria in rats immediately following large doses of radiation(1). Subsequently it was found that 2 dogs irradiated with 400 and 500 rep of X-ray, respectively, did not excrete ex-

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