An Evaluation of the B-V (Continuous-Flow) and B-IV (Density Gradient) Rotors by Use of Live Polio Virus [1]

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SUMMARY

The B-V (continuous-flow) and B-IV (density gradient) centrifuge rotors have been evaluated in the Spinco zonal centrifuge, using polio virus, type 3 Saukett strain. The B-V rotor can sediment live polio virus from raw tissue culture harvest fluids with 99 percent capture efficiency at flow rates up to 2.5 liters per hour. Fractions obtained had been purified more than 30-fold and concentrated more than 100-fold. Practical aspects, such as virus harvest without rotor disassembly and process interruption, were investigated. Five-fold further purification of B-V processed live polio virus was effected by rate-zonal sedimentation in the B-IV rotor. Virus recovered in high yield after centrifugation in the B-IV rotor contained additional impurities, including polysomes. Decontamination failure was observed when virus was allowed to dry in the presence of 20 percent (w/w) sucrose in medium 199.—Nat Cancer Inst Monogr 21: 375–388, 1966.

THIS JOINT EFFORT, at the Lilly Research and Biological Development Laboratories in collaboration with the Oak Ridge National Laboratory, was undertaken to evaluate new centrifuge rotors and systems being developed at the Oak Ridge National Laboratory.

We desired to determine the efficiency of these systems for separating small animal viruses from the relatively large amounts of impurities commonly found in such preparations. Polio virus was chosen as a model because of its small size, our prior experience, technical facilities, and readily available supplies of live virus material.

For the B-V rotor, the following information was desired:

1. Is virus capture adequate at useful flow rates?
2. Can one efficiently harvest the concentrated virus midway in a run without rotor disassembly?
3. Is it possible to stop a centrifugation for several hours and then successfully resume centrifugation without sacrifice?

[1] This work was performed under a contract between Union Carbide Corporation, Nuclear Division, and the Lilly Research Laboratories, and was also jointly sponsored by the National Institutes of Health and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

[2] Operated for the U.S. Atomic Energy Commission by Union Carbide Corporation.

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We also wished to compare the performance of the B-IV rotor in separating live polio virus to that of the B-II rotor in separating inactivated polio virus (*1*). Finally, we wanted to know if safety precautions for virus containment were adequate.

MATERIALS AND METHODS

Virus.—All experiments were performed with a single production lot of live, unfiltered, type 3 polio virus (Saukett strain), grown in Maitland tissue cultures of rhesus monkey kidney.

Virus titration.—Appropriate tenfold serial dilutions of each sample were prepared in medium 199 containing sodium bicarbonate buffer with neomycin the only antibiotic. One half ml of each dilution of virus, along with 1.5 ml of fresh medium, was inoculated into each of ten 8-day-old roller tube cultures of primary rhesus monkey kidney cells. Cultures were incubated at 36° C in roller drums, and examined for 4 + CPE on the 4th and 7th days. The TCID50 titer of each sample was calculated on the 7th day by the method of Reed and Muench (*2*).

Protein determinations.—Protein analysis was made by the method of Lowry *et al.* (*3*).

Instrumentation.—A prototype Spinco zonal centrifuge was used for all experiments (*1*, *4*). This instrument was modified for remote control operation by remounting the instrument panel controls and indicators in a chassis located in an adjacent room, cabling all connections back to the instrument (fig. 1). The mechanical tachometer-odometer was left in its original location. The vacuum control was relocated on the top panel for more convenient operation. This prototype instrument was further modified for 40,000 rpm operation by the temporary installation of an oil-dampened, water-cooled, upper bearing and improved seal assembly.[3] The B-V (continuous-flow) rotor (*5–8*) was an experimental rotor made at ORNL. The first B-IV (density gradient) (*8*, *9*) run was made with an ORNL developmental rotor, other B-IV runs with a rotor obtained from the Spinco Division of Beckman Instruments Company.

The upper-bearing cooling water was recycled from a refrigerated tank held at 4° C. Generally, unless otherwise stated in this report, the operational procedures adopted were those previously described (*6–8*, *10*). B-V runs were made at 40,000 rpm. The flow-rate of tissue culture infectious material was controlled by regulation of air pressure on the liquid surface in a sealed tank with dip tube.

A Spinco gradient-producing engine was used to form the linear density gradients used with B-IV fractionations. Specially purified, sterile solutions of 60 percent (w/w) sucrose, which had an absorbancy less than 0.25 at 260 mμ in a 10 cm cell, were used.[4] The 1200 ml gradient started

[3] These modifications are now standard for recent Spinco zonal centrifuges.

[4] Obtainable from Elanco Products Co., a Division of Eli Lilly and Company, Indianapolis, Ind., as Gradient Solution No. 1, Item number 1019.

with 10 percent (w/w) sucrose and ended with 30 percent (w/w) sucrose in phosphate-buffered $\left(\frac{\Gamma}{2} = 0.1\right)$ saline (1% NaCl) at *p*H 7.0 throughout. This gradient rested on an unbuffered 60 percent (w/w) sucrose underlay. Fifty ml of sample containing 3 percent (w/w) sucrose was placed on top of the gradient and overlaid by 200 ml of distilled water or phosphate-buffered saline. The rate-zonal B-IV fractionations were made either in 60 minutes at 40,000 rpm or in 90 minutes at 30,000 rpm, as indicated in the text-figures. Either 20 or 40 ml fractions were automatically collected in 50 ml vaccine vials.

The gradient was analyzed by refractometry either with a calibrated Milan Liquid Analyzer or by direct analysis of the fractions with a Bausch and Lomb Abbe Refractometer. A Cary Model 15 Recording Spectrophotometer was used to obtain absorbancy throughout the ultraviolet range with fused-silica cells up to 10 cm in path length.

Virus counts were made by a modification of Sharp's procedure, wherein virus was spun directly onto carbon films supported by 200 mesh grids, which, in turn, were supported by silicone rubber cushions in the Spinco SW-39 swinging bucket rotor. Appropriate tenfold serial dilutions of virus in 0.2 M ammonium acetate were sedimented onto these films in a stepwise procedure wherein a supernatant fluid from a prior sedimentation at a relatively slower speed of shorter duration was carefully removed and respun at a higher speed or for longer duration. Essentially all virus was collected by the following sequential centrifugations: 5000 rpm for 15 minutes; 39,000 rpm for 60 minutes; 39,000 rpm for 120 minutes. Electron micrographs (3¼ × 4 inches) on randomly selected fields of the platinum-shadowed films were made with a calibrated RCA-EMU-3E electron microscope with 30 μ objective aperture, using 32,000 (±2%) electronic magnification (*11*).

These micrographs were optically projected onto a ruled grid for virus counting.

Virus containment.—Salk polio virus immunization was required for all persons involved in this experiment. The centrifuge was sealed within a walk-in, stainless-steel, dry-box (7.5 × 6 × 8 ft) and operated by the previously mentioned remote controls and/or by rubber gloves sealed to ports (fig. 1). This dry-box contained six 30-watt germicidal ultraviolet lights. It was maintained at negative pressure (0.5 inch water) by means of a fan, which vented infectious aerosols to the outside through a flame (700° C). During fractionation, infectious samples could be introduced from or withdrawn to the outside through tubing. In the event of failure of any critical system, appropriate controls automatically seal the centrifuge room. The entire room and its contents could be sterilized overnight both before and after each run by raising the temperature in the room to 55° C and introducing 7.1 kg of a mixture of 12 percent ethylene oxide and 88 percent Freon 12 (Matheson) volatilized with a heat exchanger. The ethylene oxide concentration was monitored by infrared absorbancy at 3.3 μ by use of 10 cm cell with NaCl windows. Starting with 100 mg per

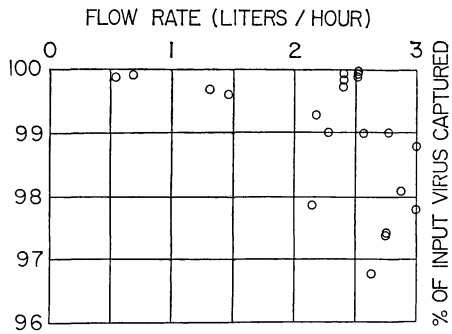
liter of ethylene oxide, the concentration generally dropped to 60 mg per liter in 20 hours.

The effectiveness of room decontamination procedures was tested for a virus spill that dried (dry spill) and for a spill which did not dry (wet spill). To test the wet spill, 5 ml aliquots of polio virus were pipetted into Falcon plastic tissue culture flasks and a loose cotton plug was inserted in the necks. The depth of fluid was 3 mm when a flask was placed on its side in the dry-box. To test the dried spill, 1 ml of polio virus in medium 199 with 20 percent sucrose was distributed as droplets in petri dishes and allowed to dry at 20° C for 20 hours before introduction to the dry-box. The room was then brought to the sterilizing condition described, and flasks or petri dishes were withdrawn at various times during sterilization. Material in flasks was titered directly and dried material in petri dishes was resuspended in 10 ml of medium 199 and titered.

RESULTS

B-V (Continuous-Flow) Rotor

In all, 60 liters of raw, unfiltered tissue culture polio-infected fluid were processed by this continuous-flow rotor. In text-figure 1 we have plotted the percent of input virus captured by the rotor as a function of flow rate through the system. These values are derived from titration data of randomly selected, 40 ml effluent samples. The loss of titer actually represents virus removed from the continuously flowing stream ("clean out"). The tacit assumption of this plot is that titer loss represents recoverable virus rather than virus destroyed by the process.



TEXT-FIGURE 1.—Efficiency of polio virus capture at various flow rates through the B-V (continuous-flow) rotor.

Table 1 gives the purification and concentration data of harvested fractions. The column "original virus" gives data relative to the starting material. Fraction 1 was obtained by rotor disassembly and rinse after 15 liters had been processed at a mean flow rate of 2.5 liters per hour. Fractions 2 through 7 were taken without rotor disassembly during the course of a 4-day run. During the 1st day, 10.6 liters of original virus were passed through the rotor at a mean flow rate of 3 liters per hour. Effluent titer was $10^{-3.5}$. At the end of the working day the sample flow

was stopped and the rotor was allowed to decelerate to zero rpm without dynamic breaking. The rotor and its contents were kept refrigerated *in situ* at 4° C overnight. The next morning the rotor was accelerated to full speed before the sample flow was resumed. The first 40 ml of effluent titered $10^{-6.4}$. By the time an additional 120 ml had flowed, virus concentration had dropped to $10^{-3.6}$ in the effluent. This transient loss was inconsequential. Effluent titer remained at a low level throughout the 2d day when an additional 16.7 liters of raw fluid were passed through the rotor at an average flow rate of 2.4 liters per hour. At the close of the 2d day the rotor was again decelerated to zero rpm in the manner just described.

At the start of the 3d day, an attempt was made to harvest the virus that had accumulated on the rotor wall. To wash virus from the wall, the rotor was accelerated to 10,000 rpm and immediately decelerated to zero rpm with the brake on and with the fluid flow stopped. This process was repeated 3 more times. Fractions 2, 3, and 4 (table 1) were taken successively by discharging the turbid rotor contents through the disconnected input tube by means of air forced into the output tube with a syringe. The rotor was at rest during this harvest. Virus yield of these 3 samples accounted for one half the total virus which had been accumulated at that time. The rotor was rinsed by filling with original virus suspension (titer $10^{-6.5}$) and back-flushing with air as above. Titers of 3 successive 40 ml rinses were $10^{-7.4}$, $10^{-6.8}$, and $10^{-6.5}$, representing less than 1 percent of the accumulated virus.

The rotor was again accelerated to 40,000 rpm and a mean flow rate of 2.7 liters per hour resumed throughout the 3d day until an additional 13.5 liters had been processed. During the 3d day, effluent titer gradually increased from $10^{-4.9}$ to $10^{-5.7}$, indicating less efficient virus retention. As before, the system was closed down at the end of the 3d day. During the 4th day, an additional 4.5 liters were processed at a mean flow rate of 3.2 liters per hour. Effluent had a mean titer of $10^{-5.0}$. The rotor was decelerated from 40,000 rpm to 1000 rpm with the brake on. It was then cycled between 15,000 and 1000 rpm 3 times and stopped. We were unable to pump out the rotor contents by forward flow of medium 199 at 10 lb/in^2 pressure because the rotor had become plugged. We successfully discharged fractions 5, 6, and 7 of table 1 by back-flush with air as previously described.

The rotor was removed from the centrifuge, opened, and the core withdrawn. We observed that three quarters of the available annular space was occluded with solid material, and that probably only one of the four radial fluid flow channels at the base of the core remained clear. This solid material was resuspended in medium 199 and constitutes fraction 8 of table 1.

Fractions 1 through 8 account for two thirds of the virus employed in this experiment.

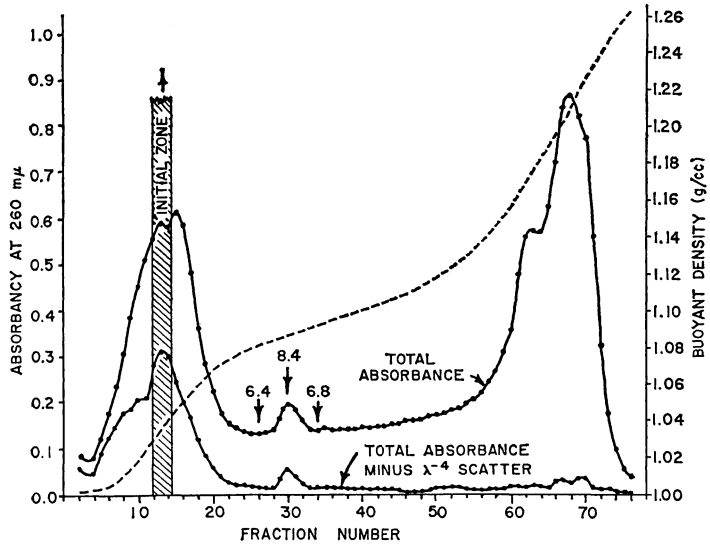
TABLE 1.—Harvested polio virus fractions from B-V centrifugation

Harvest fraction No.	Original virus	1	2	3	4	5	6	7	8
Volume (ml)	60, 000	150	30	55	40	40	40	40	100
Titer—(log 10) per ml	6. 5	8. 6	8. 4	8. 6	8. 4	8. 2	8. 4	8. 6	8. 2
Concentration factor (based on infectivity)	1×	100×	89×	120×	88×	56×	73×	126×	51×
Lowry protein (mg/ml)	0. 029	Not done	0. 211	0. 097	0. 208	0. 100	0. 089	0. 119	1. 044
Specific infectivity $\left(\frac{\text{infectious virus}}{\text{mg protein}}\right)$	2.13×10^8	—	2.60×10^9	7.68×10^9	2.6×10^9	3.48×10^9	5.3×10^9	6.55×10^9	3.05×10^8
Purification factor (based on specific infectivity)	1×	—	12×	36×	12×	16×	24×	31×	1. 4×

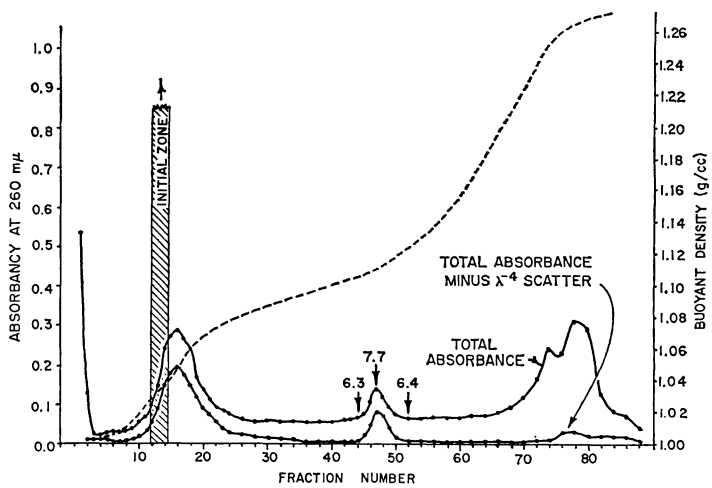
B-IV (Density Gradient) Rotor

Text-figure 2 shows the sedimentation profile of 50 ml of fraction 1 of table 1 when spun for 90 minutes at 30,000 rpm. The infectivity peak corresponds to the 260 mμ peak at fraction 30.

Text-figure 3 shows the sedimentation profile of 50 ml of pooled fractions 3 and 7 of table 1 when spun for 60 minutes at 40,000 rpm. This



TEXT-FIGURE 2.—Sedimentation profile for 50 ml of fraction 1 of table 1: B-IV rotor, 30,000 rpm for 90 minutes. Titers in $\log_{10}$ infectious particles per ml indicated for three 20 ml fractions.



TEXT-FIGURE 3.—Sedimentation profile for 50 ml of pooled fractions 3 and 7 of table 1: B-IV rotor, 40,000 rpm for 60 minutes. Titers in $\log_{10}$ infectious particles per ml indicated for 3 fractions.

50 ml represents essentially all the virus but only 3 percent of the protein present in 6150 ml of raw, infectious tissue culture fluid. The infectivity peak corresponds to the 260 mμ peak at fraction 47. The additional purification achieved by the B-IV rotor is obvious (in this case, fivefold with respect to protein). Figure 2 is an electron micrograph of the starting material of the B-IV run (pooled fractions 3 and 7 of table 1) and figure 3 is an electron micrograph of fraction 47. Both micrographs were made under identical conditions for particle counting (1/100 dilution, spun onto carbon films in SW-39 rotor at 39,000 rpm for 1 hour after preclarification at 5000 rpm for 15 minutes). The B-IV purification is strikingly self-evident in these micrographs. Figure 4 shows a polysome. These structures are found occasionally in microscopic fields from fraction 47.

Electron microscopic particle counts of pooled fractions (3 and 7 of table 1) gave the value of $10^{11.8}$ physical particles per ml. Fraction 47 had $10^{11.6}$ physical particles per ml. On the basis of our previous experience in counting this virus, the difference probably is not significant. Fraction 47 had 22 μg protein per ml. Virus titer of the pooled fractions (3 and 7 of table 1) was $10^{-8.6}$. Fraction 47 had a titer of $10^{-7.7}$, a statistically significant infectivity loss.

Room Decontaminations

For the case of a wet spill, flasks containing type 2 polio virus were withdrawn at 3, 4, 6, and 12 hours. Control titer was $10^{-7.3}$. We were unable to demonstrate live virus in any experimental flask. Similar sterilization was also obtained with T2 bacteriophage and SV2, a Coxsackie-like simian enterovirus.

Table 2 lists our findings where virus was allowed to dry in medium 199 containing 20 percent sucrose before sterilization.

TABLE 2.—Residual titer before and after drying and after exposure to ethylene oxide in the dry state. Polio virus type 3 dried in medium 199 containing 20 percent sucrose

Sample	$-\text{Log}_{10}$ titer
Undried virus	6. 7
Dried virus exposed to ETO for:	
0 hours	4. 6
2 hours	2. 2
4 hours	2. 0
8 hours	1. 9
20 hours	1. 0

DISCUSSION

The results with the B-V rotor, as listed in table 1, although limited, indicate that this device permits the processing of large amounts of unfiltered, relatively impure and dilute polio virus to give a concentrated and purified product in high yield. In some cases virus was concentrated more than 100-fold and purified more than 30-fold. The ability to

interrupt the process or to harvest by a simple procedure at any time during a run is a practical advantage. The process began to degenerate during the 3d day, as revealed by less efficient virus retention; this probably resulted from occlusion of the rotor with tissue debris. The occluding material (fraction 8) had a high titer but low specific infectivity, a result indicating that relatively large amounts of nonviral material were filling the rotor. This suggests the need for prefiltration of material processed by the device and possibly the need for greater hold-up volume capacity in future designs.

The two B-IV runs generally confirm earlier findings of Anderson (*1*) regarding the efficient purification of polio virus by zonal centrifugation. The sedimentation profile reveals the complexity of the starting material. The B-V starting material harvested by back-flush with air is relatively cleaner than is the B-V starting material harvested by terminal rotor disassembly and resuspension. This is revealed by a comparison of the profiles of our two B-IV runs. We do not know why infectious titer decreased in the second B-IV separation (text-fig. 3).

In contrast to this study, the starting material for Anderson's previous study was formalin-inactivated virus purified and concentrated by the nucleic acid method (*12*). The B-V purified and concentrated material had relatively less soluble, 260 mμ absorbing impurity than did the nucleic acid-purified material. The B-IV profile revealed relatively more large or dense light-scattering material than did the nucleic acid-purified vaccine.

Our inability to decontaminate the polio virus, dried in the presence of 20 percent sucrose in medium 199, with ethylene oxide is disturbing (table 2). It is probable that the virus was encapsulated within a sucrose crystal, which protected the virus from ethylene oxide. If so, it is likely that aerosolized droplets may also dry to produce infectious dusts. New equipment should be designed to permit heat sterilization of all contaminated parts.

NOTE: After the completion of this work, and as a result of it, the seal used for continuous-flow centrifugation was modified to include a bleed tube for removing air bubbles from the seal inlet line (*13*).

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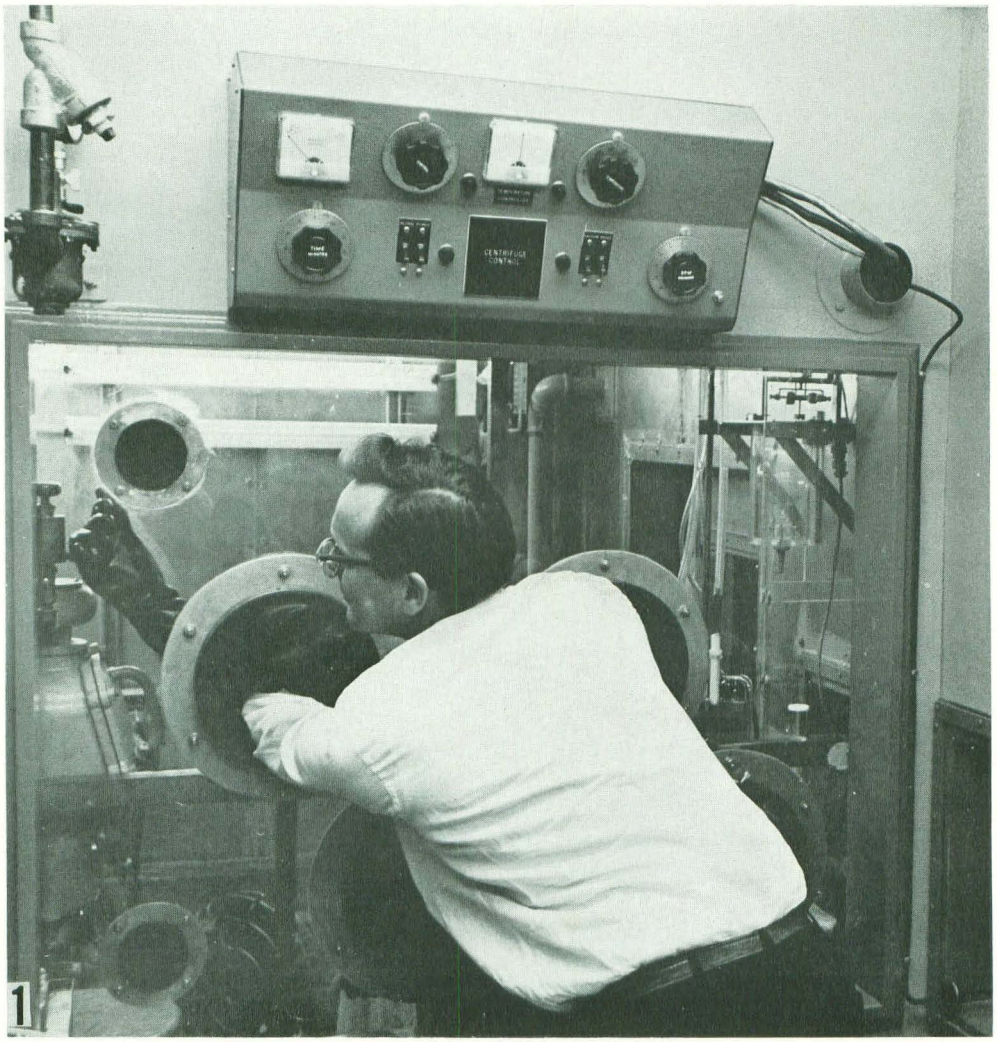

FIGURE 1.—Control room for remote operation of zonal centrifuge.

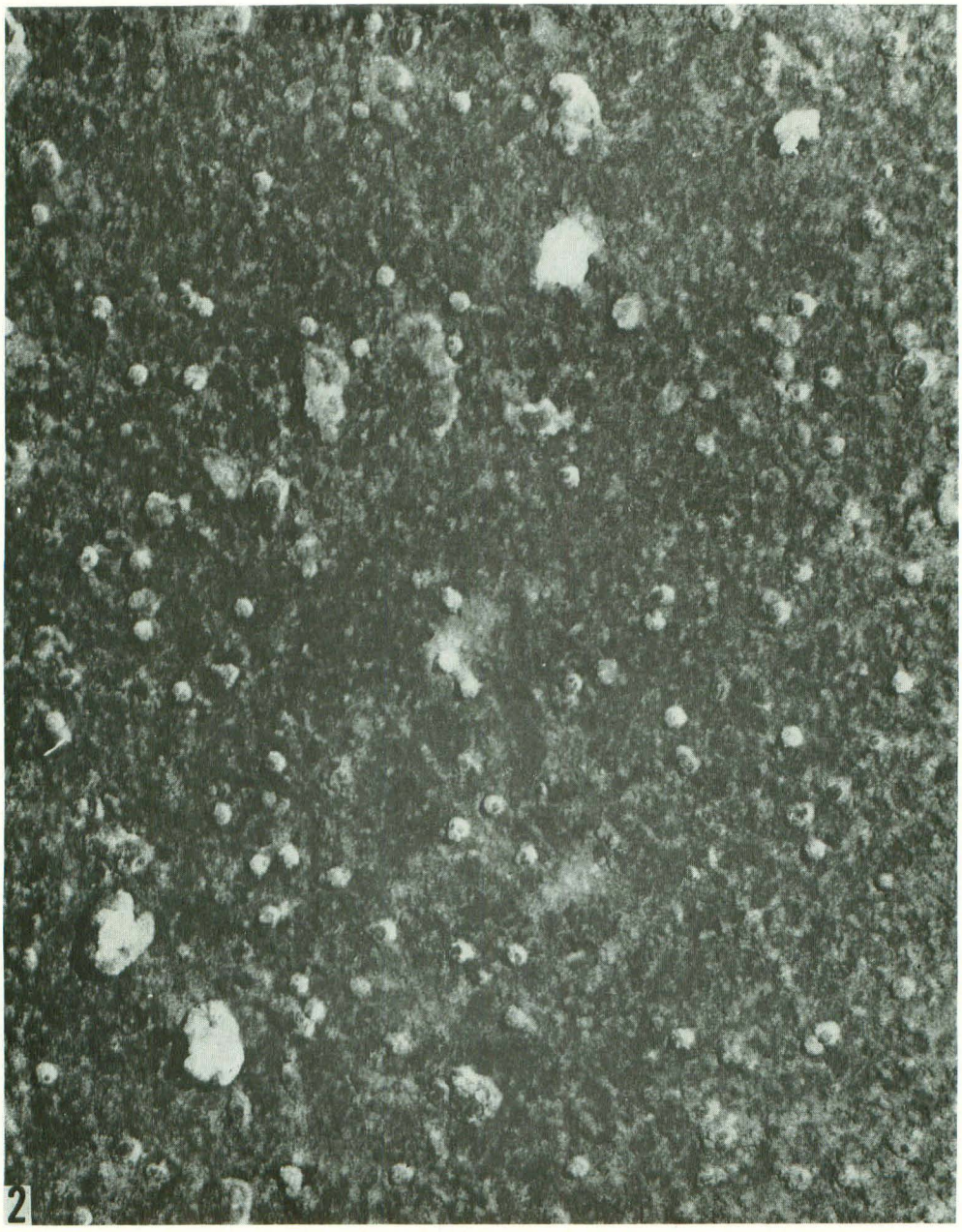

FIGURE 2.—Electron micrograph of starting material of B-IV run shown in text-figure 3. Magnification: 96,000, 1/100 dilution spun onto electron microscopic grids in SW-39 (swinging bucket) rotor at 39,000 rpm for 60 minutes, after preclarification at 5000 rpm for 15 minutes.

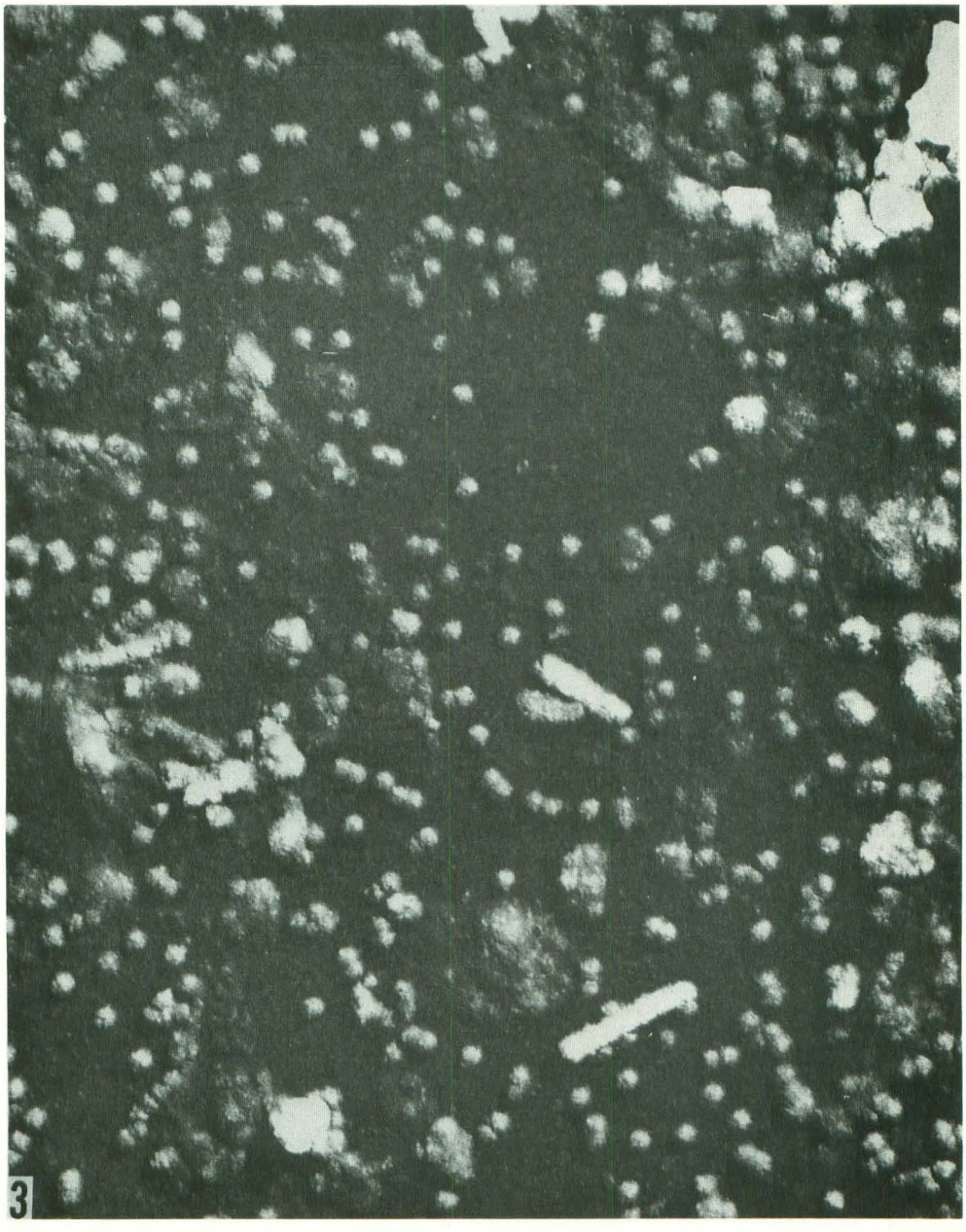

FIGURE 3.—Electron micrograph of the peak fraction 47 of the B-IV run shown in text-figure 3. × 96,000. Same treatment as in figure 2.

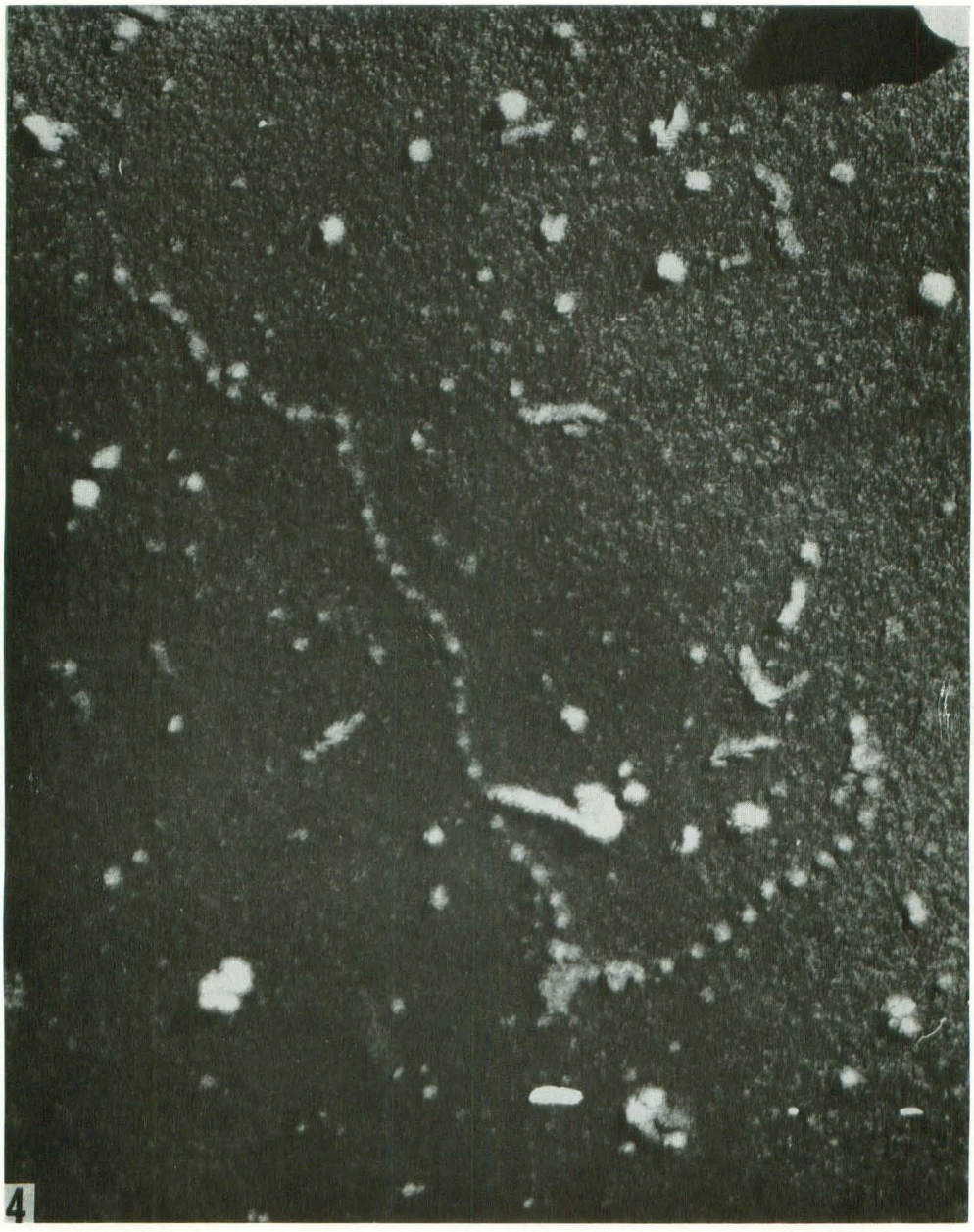

FIGURE 4.—Electron micrograph of a polysome, found in occasional fields from fraction 47. × 96,000