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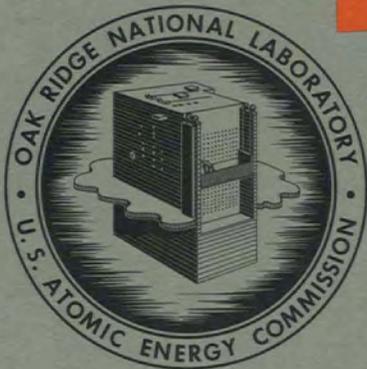
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TO THE NATIONAL INSTITUTE OF
ALLERGY AND INFECTIOUS DISEASES
MARCH 1 TO JULY 1, 1966

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SEMIANNUAL CONTRACT PROGRESS REPORT
TO THE
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

March 1 to July 1, 1966

N. G. Anderson and G. B. Cline
Biophysical Separations Laboratory, Biology Division,
Oak Ridge National Laboratory

Research jointly sponsored by the Vaccine Development Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, and the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

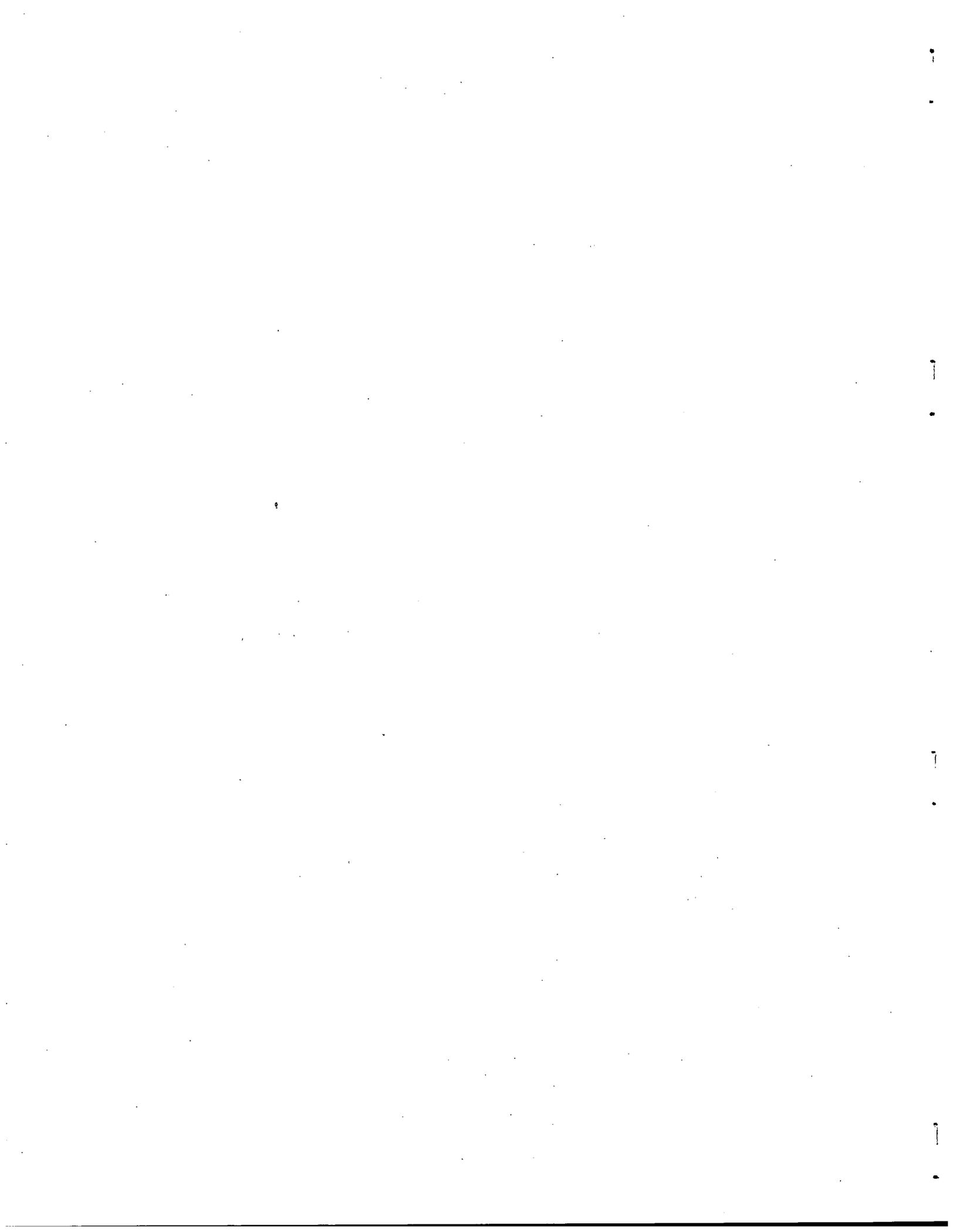
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SEMIANNUAL CONTRACT PROGRESS REPORT
TO THE
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Title of Project: Development of techniques for virus and viral subunit isolation from large volumes of tissue culture

For the Period: March 1, 1966, to July 1, 1966

Responsible Investigators: Norman G. Anderson
G. B. Cline

Name of Institution: Biophysical Separations Laboratory
Biology Division
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Address: Building K-703
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Interagency Agreement: 40-30-64

Supported by: Research jointly sponsored by the Vaccine Development Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, and the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

The new air-turbine-driven K-II continuous sample flow zonal centrifuge has been tested for rotational stability to 27,500 rpm with and without the B-XVI type of core but not with a density gradient. A modification of the lower damper assembly is required before gradient stability and recovery tests can be completed.

The B-VIII and B-IX rotors have been modified with a centrifugal operating valve which limits the direction of flow of gradient and/or displacing solution while at low operating speeds. This modification permits high-resolution separation of viruses from other cell culture components. The modified B-IX (called B-XVI) has been used for T3 phage and adenovirus 7 isolations.

A set of criteria has been formulated for use in determining the operational usefulness of the cylindrical (B-IV-B-IX types) zonal rotors. These criteria define the physical parameters which have been found to be most important for routine operation. These parameters concern runout and tolerances on the lower and upper end caps and the journal bearing and a test for gradient mixing during gradient recovery.

B-XVI Rotor

For the preparation of large quantities of virus for use in vaccines and/or analytical studies, methods for concentrating and purifying virus particles from large fluid volumes are required.

High-performance continuous-flow rotors which allow viruses as small as polio to be isolated from large fluid volumes have been described.^{1,2} As a further refinement of these continuous-flow rotors, the sample stream through the rotor has been arranged to flow across the surface of a liquid density gradient which is imprisoned in the rotor during high-speed operation.³ Particles in the flowing stream are sedimented into the gradient and are banded at or near their isopycnic density. Rotors of this type, designated B-VIII and B-IX, have been used to isolate adenovirus,³ ECHO 28 virus, the respiratory syncytial virus,⁴ and flu virus.⁵ The operation of the B-VIII and B-IX has been discussed in detail in previous semiannual reports. The upper feed line in the rotor core allows fluid to (1) flow in to the rotor edge during loading and unloading of the gradient, or (2) the virus-depleted stream to flow out during high-speed operation. The performance of this type of rotor has been approximately as expected, except that the resolution of recovered bands with some viruses has been low.

When the continuous-flow phase of an experiment has been completed, the rotor is decelerated to approximately 5000 rpm and the gradient recovered by displacement with a dense fluid pumped to the rotor edge through the upper edge line. During recent studies, dyes have been added to the displacing fluid to determine how much of this fluid mixes with the recovered gradient. The amount of mixing has been found to be considerable, with dye being found distributed throughout much of the gradient. The exact reasons for this mixing are unknown. However, one possibility could be a Bernoulli effect which might occur at the "T" between two lines in the top of the rotor core. This effect would draw part of the lighter portion of the gradient continuously into the displacement stream which flows to the edge of the rotor core. The core of the rotor has therefore been redesigned to eliminate the mixing problem. A simple valve which employs an O-ring as the moving member is used to bypass the "T" during

unloading of the gradient. The valve opens at a rotor speed between approximately 7000 and 12,000 rpm and is closed at the unloading speed of 5000 rpm. When the valve is open, the virus solution flows over the surface of the gradient and out the usual effluent holes. When the valve is closed at the low rotor rpm, the displacing solution flows over the top of the core to the edge of the rotor and cannot flow to or mix with the fluid at the surface of the gradient. This modified B-IX rotor has been designated B-XVI (see Fig. 1a and b).

Since the 750-ml gradient is only 1.1 cm deep in this rotor, it was not originally expected that several different particle zones would be completely resolved in this rotor, hence our acceptance of the poorer resolution obtained with cores B-VIII and B-IX. The resolution obtained in practice with B-XVI, however, quite exceeds our expectations.

The adenovirus T7 and phage T3 used in testing this rotor were chosen as model particles since their sedimentation characteristics and the banding densities in cesium chloride were known. The adenovirus was grown in monkey kidney cells. The T3 phage was grown on Escherichia coli B cells in a tryptone broth medium. The phage lysate was first processed in a refrigerated Spinco model K continuous-flow centrifuge to remove most of the unlysed cells and large debris. The partially clarified lysate was then used for this study.

Figure 2 shows the absorbance at 260 m μ and density values of the recovered CsCl density gradient fractions from the B-XVI continuous-flow rotor after processing 2 liters of adenovirus T7. The virus suspension was pumped through the rotor at 2.85 liters/hr and allowed to band for 1 hr at 36,000 rpm before being unloaded from the rotor. The rotor was unloaded at 5000 rpm using CsCl density 1.51 to displace the gradient. The large absorbance curve to the left in the profile is due primarily to the diffusion of phenol red (from the virus suspension) into the gradient. The small zone centered at density 1.3 in fraction 16 was found to be cell debris. The large zone centered in density 1.336 in fraction 19 is highly concentrated adenovirus as shown in Fig. 3. Note the complete absence of contaminating cell debris. Some fields showed the presence of adeno associated virus.

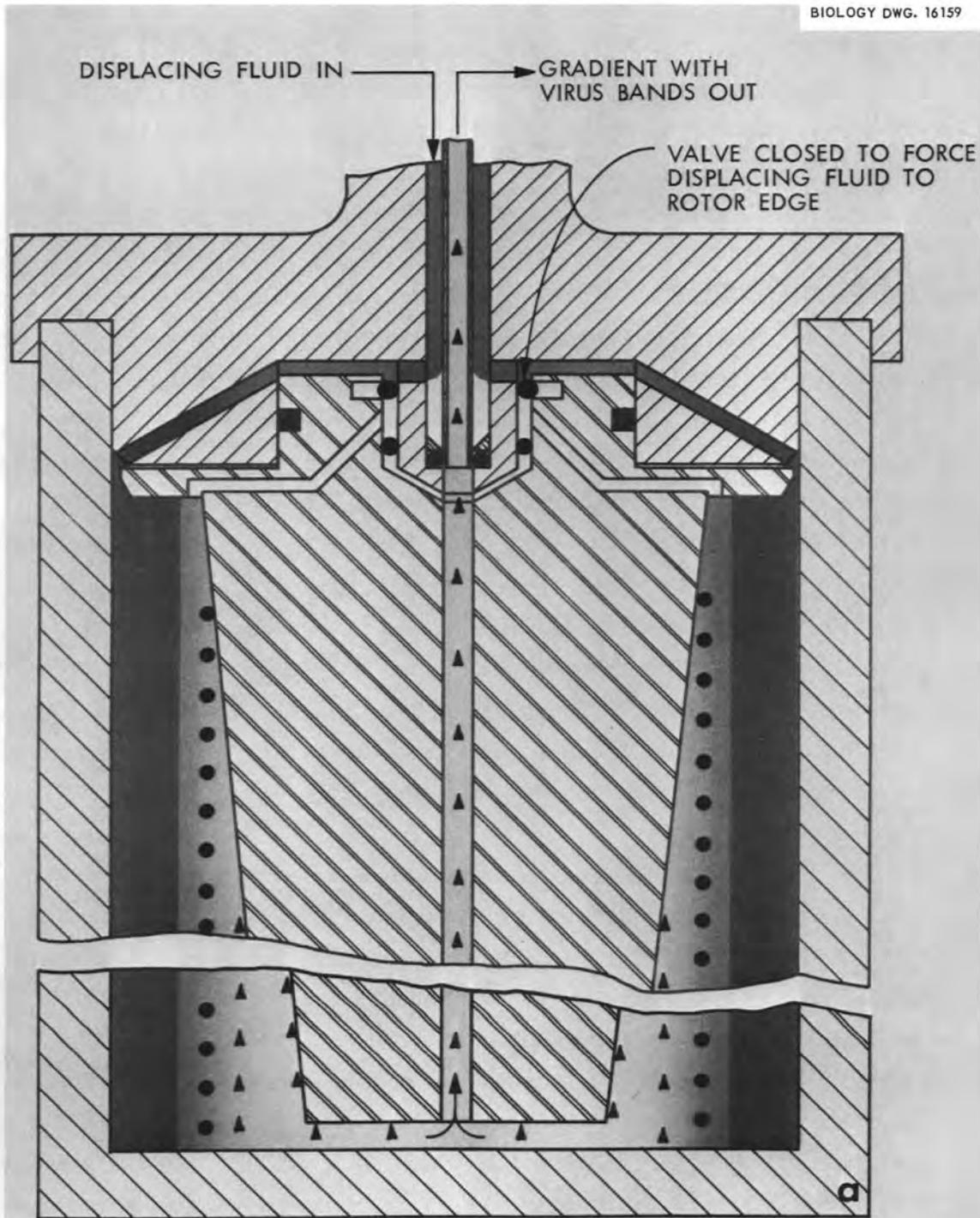
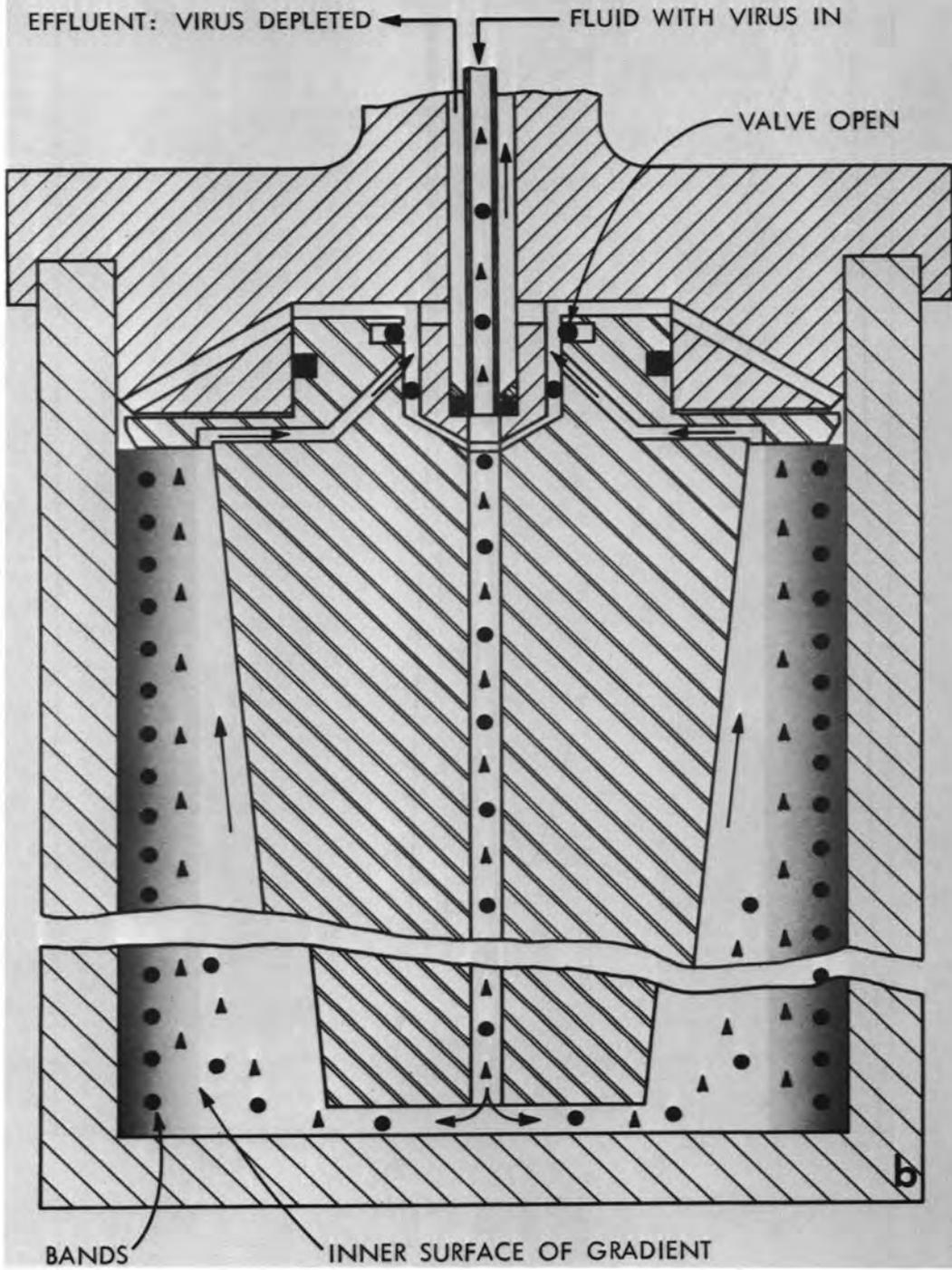


Fig. 1. Schematic Diagram of Operation of the B-XVI Rotor (Modified from B-IX). Part a shows the position of the O-ring valve during unloading of the gradient at low rotor speed. Part b shows the position of the O-ring valve during high rotor speed, when the virus-containing solution is pumped through the rotor. Virus particles and zones are denoted by the \blacktriangle and \bullet . The bottom of the rotor in parts a and b shows the completed flow path.



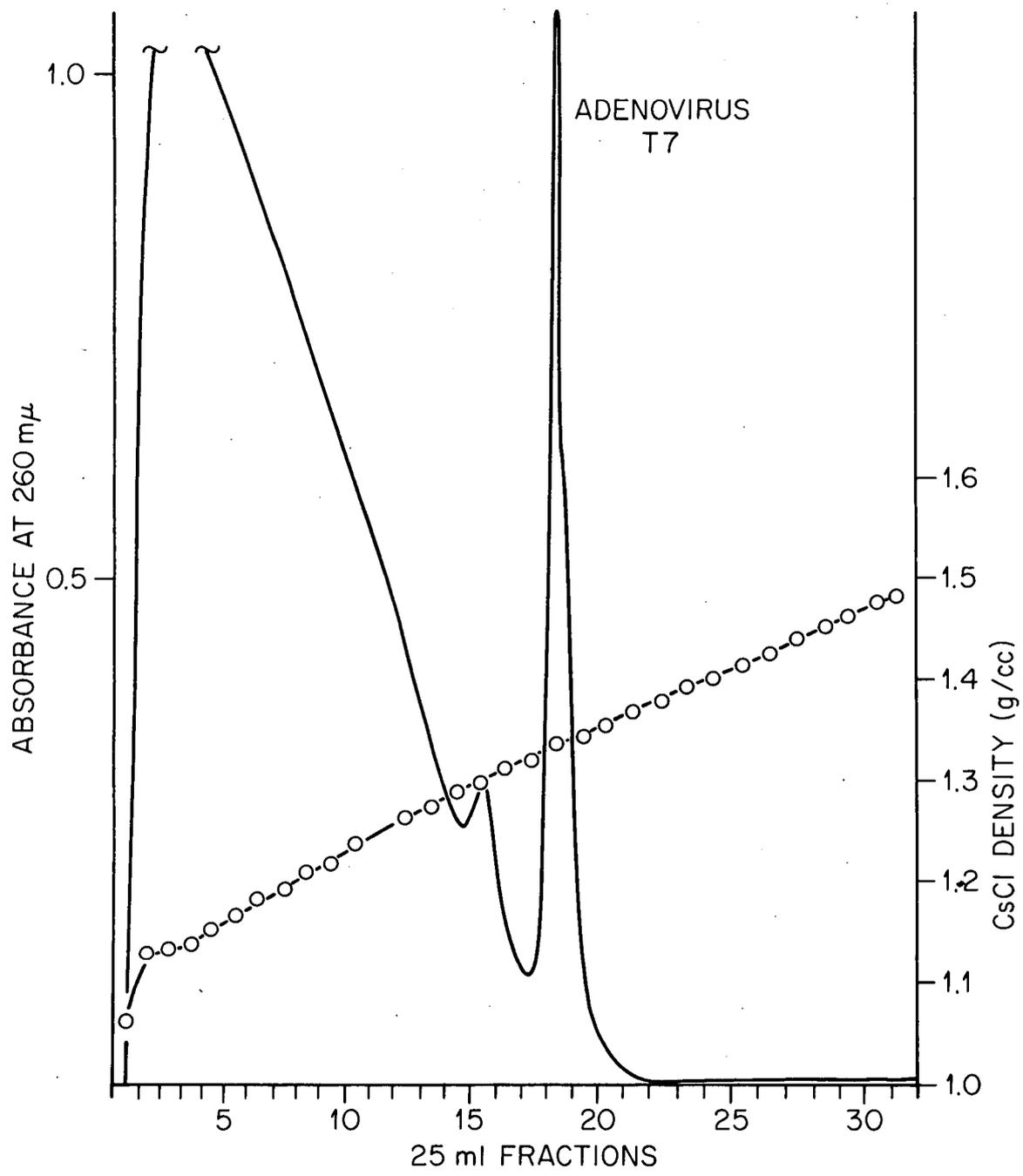


Fig. 2. Isolation of Adenovirus T7 in a Cesium Chloride Gradient in the B-XVI Rotor System.

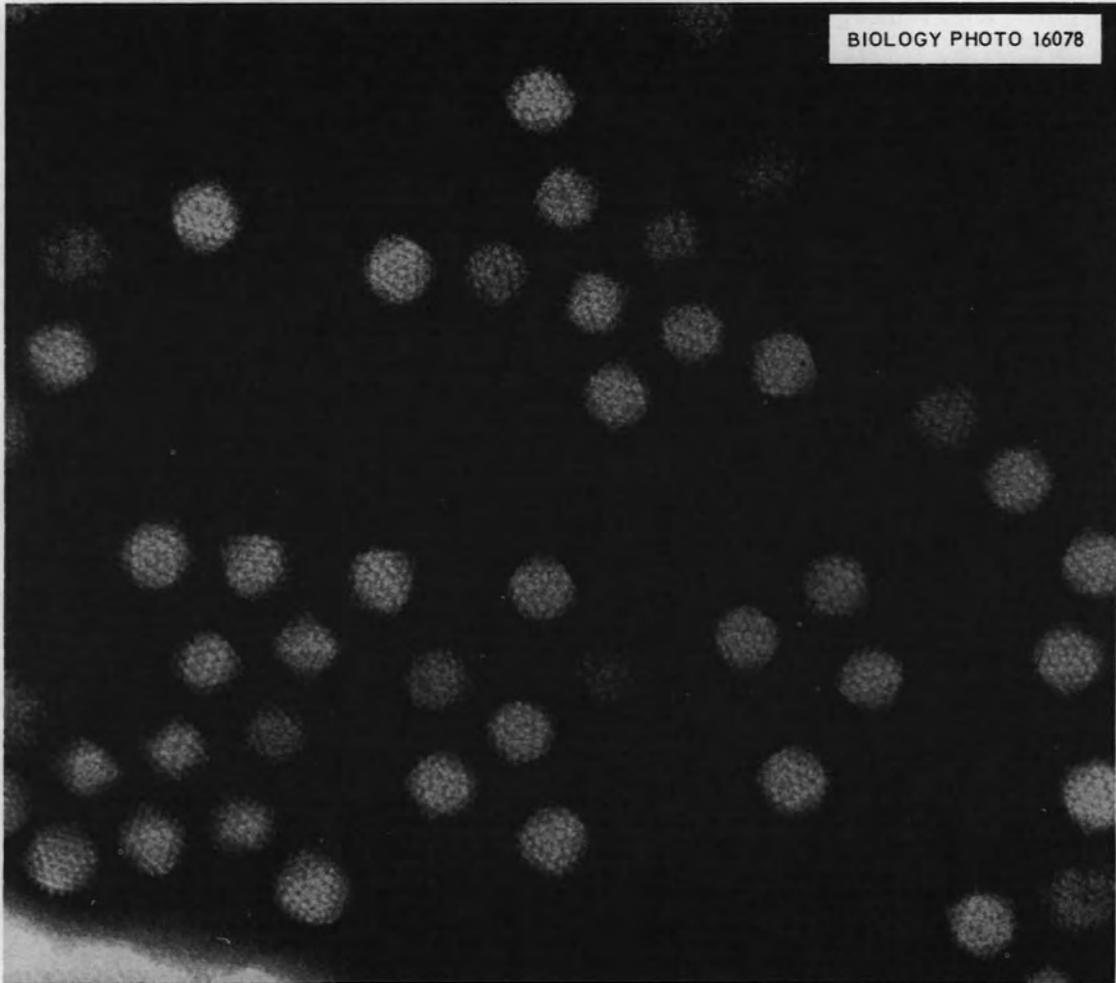


Fig. 3. Electron Micrograph of Adenovirus T7 Isolated in the B-XVI Rotor System.

The second test particle used in the B-XVI rotor was T3 bacteriophage. Eight liters of harvest fluid were passed through the rotor at a flow rate of 4 liters/hr at a rotor speed of 35,000 rpm. The collected virus was centrifuged at this rotor speed for 1 hr after continuation of the flow-through to permit the last virus particles trapped to sediment to near their isopycnic density. Figure 4 shows the absorbance profile of this experiment. The large zone to the left in the profile is due primarily to color from the tryptone broth solution. A zone of cell debris is partially obscured by the broth but is centered in fraction 13. The virus zone is centered in density 1.50 CsCl in fraction 29 and represents (theoretically) better than 99% of the T3 phage from 8 liters of solution. Electron microscopy verified the presence of phage in this zone. The material in a second zone at density 1.53 is unidentified. However, electron microscopy indicates the presence of many damaged phage particles.

Figure 5 is an absorbance profile of a mixture of the adenovirus and T3 phage particles isolated in the above experiments. Samples of each type of virus were mixed with 2 liters of Tris buffer, pH 7.5, and passed through the B-XVI rotor at an average flow rate of 3 liters/hr. The profile shows that both species of virus were trapped by the rotor and were sedimented into density zones characteristic of the viruses. Cross contamination of the virus zones could not be detected by electron microscopy.

Figure 6 shows the results of an experiment in which a previously concentrated T3 phage lysate was diluted to a volume of 2 liters with Tris buffer and pumped through the B-XVI rotor at 2.5 liters/hr. The T3 sample was a 100X concentrate prepared in the B-V zonal rotor. The five zones of material obtained were (from left to right): (1) phage ghosts and bacterial membranes, (2) bacterial cells, (3) bacterial cells and other debris, (4) pure T3 phage, and (5) damaged T3 phage.

Except where a sufficient concentration of light or heavy contaminating particulate material is present to clog the rotor lines or the seal system, the B-XVI core can be used with up to 100-liter batches of virus. When rapidly diffusing solutes such as CsCl are used for the gradient, a small amount of saturated gradient solution may be introduced to the rotor wall at intervals to replace solute which has been washed out by the flowing stream.

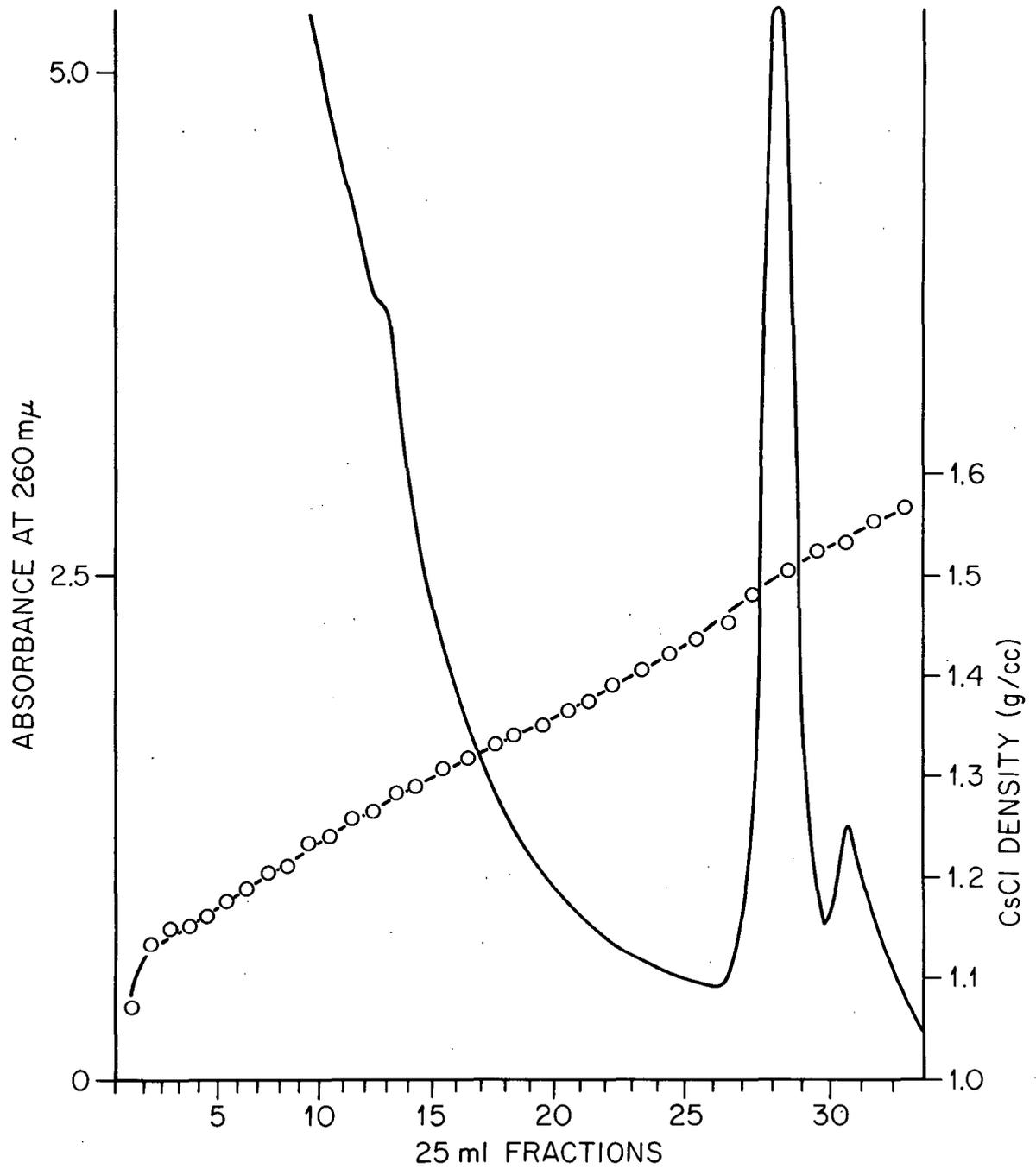


Fig. 4. Isolation of T3 Phage from 8 Liters of Harvest Fluid by Use of the B-XVI Rotor System.

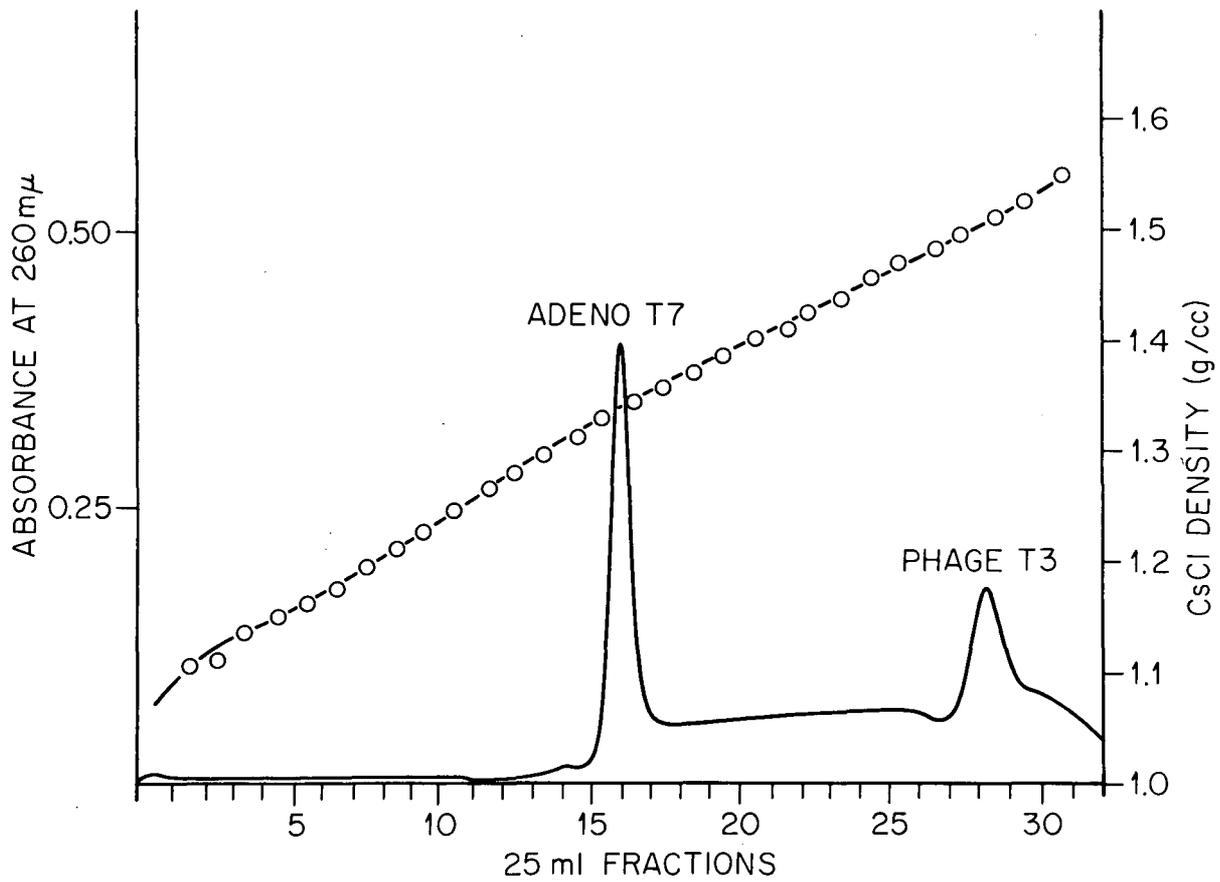


Fig. 5. Separation of a Mixture of Adenovirus T7 and T3 Phage in a 2-Liter Sample by Use of the B-XVI Rotor.

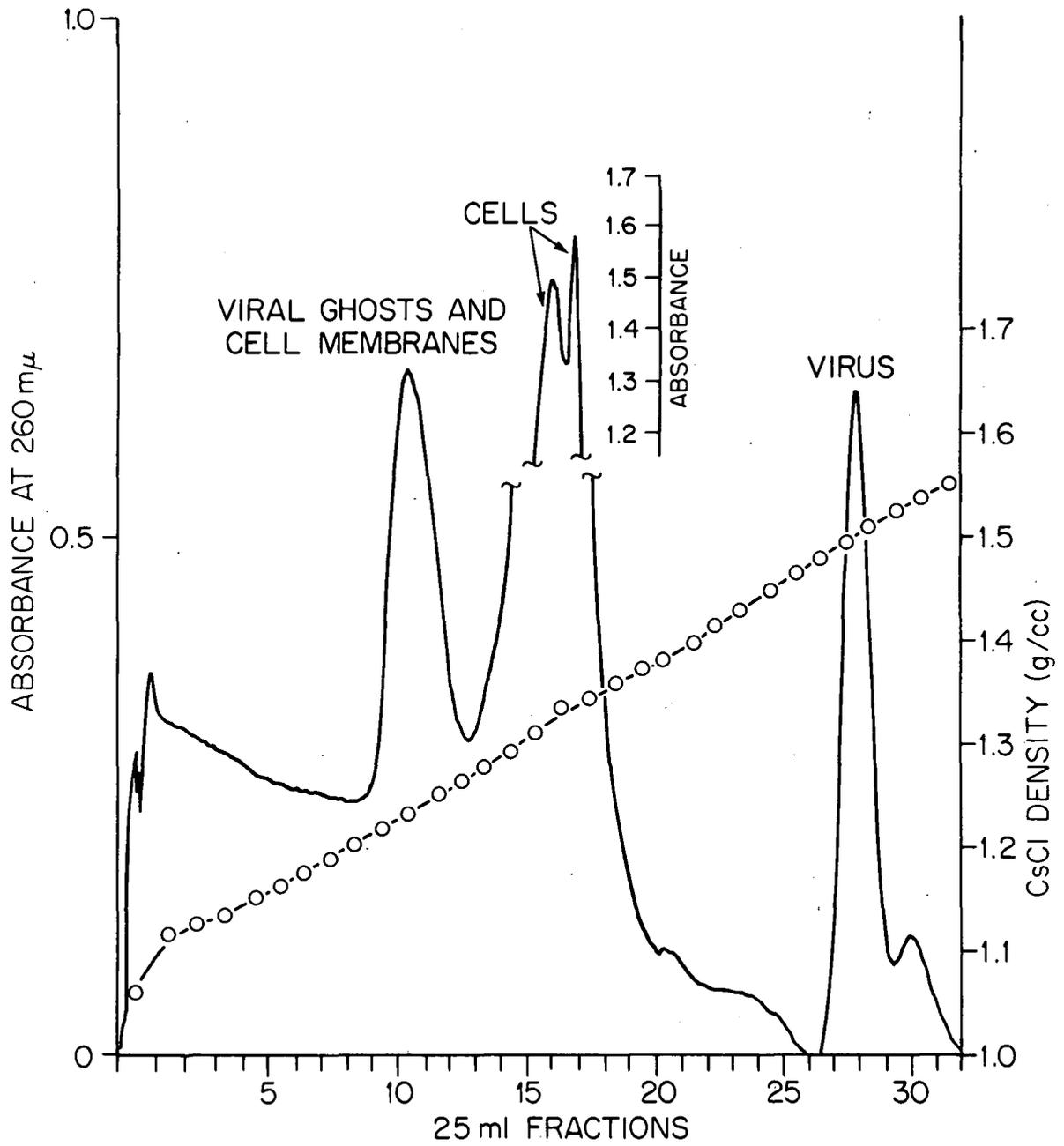


Fig. 6. Fractionation of a Concentrated T3 Escherichia coli Lysate by Use of the B-XVI Rotor System.

These studies with adenovirus T7 and T3 phage show that the B-XVI continuous-flow zonal rotor offers a rapid and efficient method for separating, concentrating, and purifying viruses from multiliter quantities of solution. The resolution possible with this technique approaches that obtained with swinging bucket rotors and other zonal rotors which utilize a small volume of concentrated starting sample, or in previous orienting studies with the B-II rotor.⁶ Virus isolation in the B-XVI continuous-flow rotor combines the technique of continuous-flow centrifugation with high-resolution isopycnic banding into a single step. This capability makes the rotor system useful for large volume applications, including the purification of viruses for biochemical study and for the preparation of vaccines.

The advantages of using highly purified antigens for the preparation of vaccines for human use have been previously emphasized.⁶ The B-XVI rotor core increases the resolution of the initial concentration step and removes a large portion of particles which have buoyant densities different from that of the virus desired.

While virus isolation has been stressed here, the same rotor system and operating procedures are useful for the isolation of any particles which can be readily banded isopycnically, including bacterial cell wall or membrane fractions (for vaccine and other purposes), subcellular particles (using cascaded centrifuges operating at increased speeds), and viruses and other particulate matter from natural waters.⁷

Evaluation of Performance of Continuous-Flow Rotors

Continuous-flow rotors of the B-IX type (B-VIII, B-XVI) have been shown to be useful for the isolation of viruses from multiliter volumes of solution. It has recently been shown that the B-VIII and B-IX rotors were not generally useful if good resolution sucrose gradient separations were to be made. These rotors gave particularly poor resolution when cesium chloride density gradients were used. The reason(s) for the low resolution of these two rotor cores are outlined above and involve the common pathway in the rotor channels at the top of the core. A modification of the rotor core to include the O-ring centrifugal valve has made

these rotors into high-resolution systems which are useful for any gradient material.

The B-IX type of core is now available commercially in a shape and configuration which is similar to but different from the Oak Ridge design. The commercially available B-IX (corresponds most closely to the original Oak Ridge B-VIII rotor) and B-IX-A rotors (similar to our B-IX) have been checked for gradient recovery and mixing by the same procedures used to evaluate the Oak Ridge rotors. The need to evaluate these rotors was evident after finding that density gradients of either sucrose or any other gradient material (CsCl or K citrate) could not be recovered without undue mixing and loss of resolution. In addition to the apparent malfunctioning of the operation of these commercially available rotors, it was evident that the rotors could not always be rotated to their top designed speed. Single reasons for this were not always apparent but mainly involved the usual problems of rotor instability, loose rotor shaft, and seal leakage. These problems also occur in rotors fabricated at Oak Ridge. A set of specifications have to be met by the Oak Ridge rotors before they are used for any isolation work. This report describes a method of determining the operational tolerances, what these tolerances are, and a method for determining gradient stability during recovery.

Materials and Methods

Any zonal rotor of the long upright type (B-IV type) is checked for alignment in a V-block using a dial reading measuring gage [Federal Products Corp., measures 0.1 mil per division (Fig. 7)]. Bottom and top end caps must have less than 1 mil lateral movement in the threads when tapped smartly with an 8-oz Plexiglas hammer. The stem of the top end cap must be centered within 1/2 mil of the center of the rotor shell and not be movable with a light tap with a 4-oz Plexiglas hammer midway in the shaft.

For checks of rotational stability, the rotor with and/or without a core is operated (accelerated, etc.) in the centrifuge in the normal manner. A proximity probe (wire-wound inductance type) is fixed 40 mils (or any calibrated distance) from (1) the bottom end cap, (2) the top end

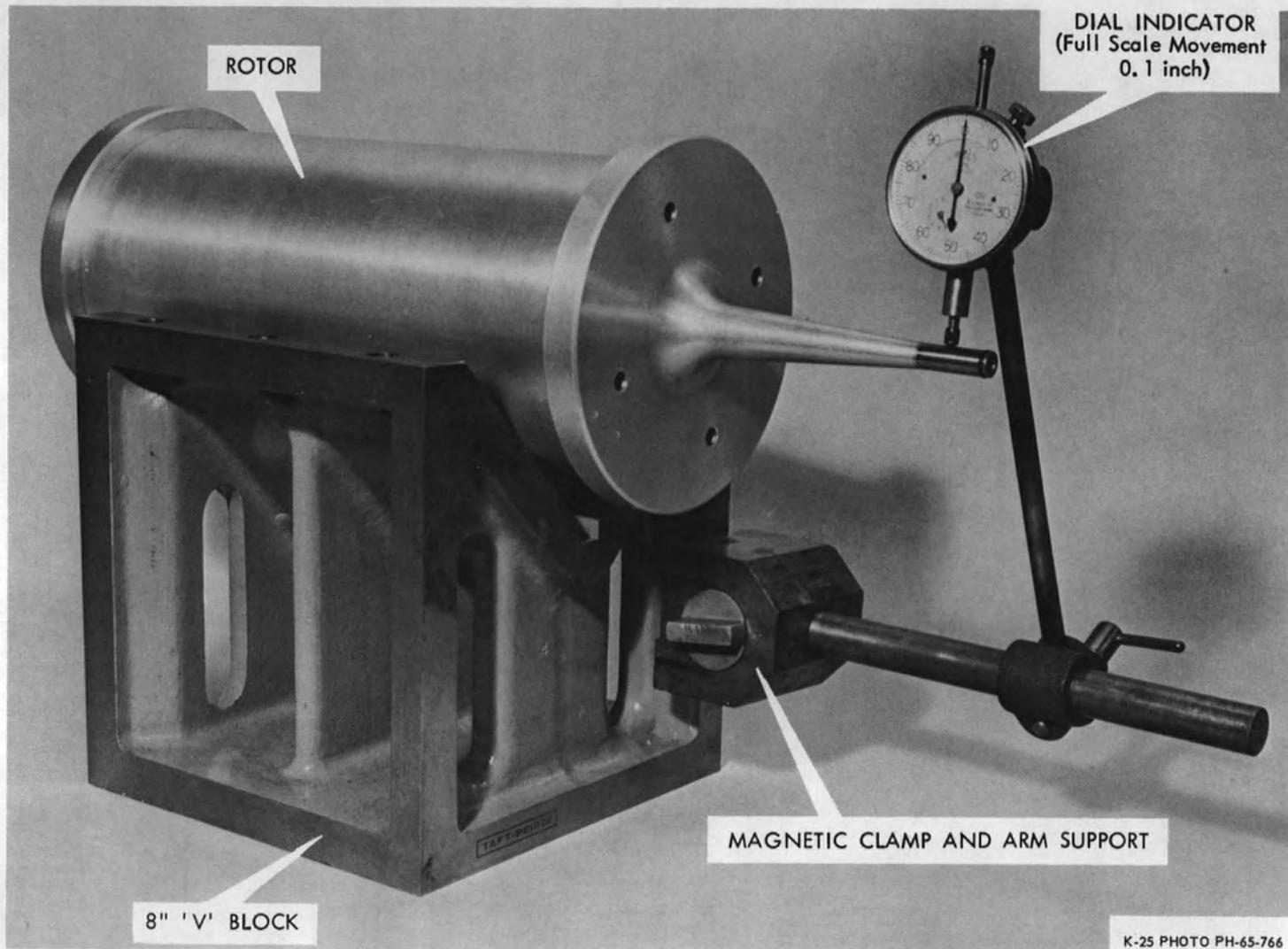


Fig. 7. Rotor Shaft Aligning Assembly.

cap, and (3) the journal of the upper damper bearing. These probes display on a multichannel oscilloscope the distance between the rotor and any given probe as a function of time. Lateral movement of any of the three parts of the rotor can be measured directly from the calibrated oscilloscope screen.

The tolerances which have been found to be practical for the operation to top operational speed (40,000 rpm) vary with the speed of the rotor but must be within certain limits in order to pass through precessions and criticals. These limits for several speeds are shown in Table 1. Since the dynamic balancing point changes with each different rotor core used, it is apparent that these tolerances must be checked using each rotor core. Variations between cores must be minimized by the addition or removal of weight to balance the rotor during rotation. It has been found that if the end caps and rotor stem are in alignment and within the prescribed tolerances, then the balancing of the rotor with any core is a relatively simple matter.

Additional indications of smooth operation of these zonal rotors can be obtained by palpation of the damper bearing and by listening for vibrations. Palpation is a particularly good method if electronic pickups are not used. The first point to be remembered is that this rotor with any core will have distinct criticals (i.e., rotational speeds at which the rotor is dynamically unstable). For the B-IV type of rotor, vibrations of the damper bearing can be expected at: (1) 7500 to 10,000 rpm, (2) 26,000 to 29,000 rpm, and (3) 36,000 to 37,000 rpm. Often these criticals cannot be palpated if the system is operating optimally. Experience has shown that in the 36,000-rpm critical the seal may begin to cavitate and cause an audible buzzing sound. This cavitation has been found to be due primarily to the first harmonic vibration of the stem of the rotating seal assembly, which runs from the dynamic seal to the manifold plug in the top end cap. This harmonic has been changed by the addition of a spacer halfway down the stem. An additional spacer has been inserted at the top of the stem at the point at which the stem inserts into the dynamic seal.

Table 1. Maximum Permissible Runout in Mils As Determined
at the Damper Bearing (Journal Portion), Top End Cap,
and Bottom End Cap as a Function of Rotor Speed

Speed (rpm)	Damper	Top End Cap	Bottom End Cap
× 1000			
4	0	2.0	1.0
6	0.25	3.0	1.0
8	2.5	5.0	2.0
10	1.25	4.0	2.0
12	1.0	2.0	2.0
14	1.0	1.0	1.5
16	1.0	1.0	1.5
18	1.0	1.5	1.25
20	1.0	0.75	1.25
22	0.75	0.75	1.25
24	0.75	0.75	1.25
26	0.5	0.75	1.25
28	0.5	0.75	1.25
30	0.75	0.75	1.25
32	0.75	0.75	1.25
34	0.75	0.75	1.25
36	0.5	0.75	1.25
38	0.5	0.75	1.25
40	0.5	0.75	1.25

One last point at which rotor malfunction can occur is in the contact of the seal assembly with the stem of the rotor. In the Oak Ridge rotors, the stem of the rotor must show at least $1/4$ in. above the journal bearing but not more than $3/8$ in. If the shaft is too low, the seal assembly will not be seated and will allow loss of cooling water. If it is too high, the seal assembly will not be held firmly in the journal housing.

The second problem in the operation of a zonal rotor and especially the continuous-flow rotors is in introducing and recovering the density gradient without losing resolution. This problem has been solved with the use of an O-ring centrifugal valve (described above) to direct the flow of solutions to the proper points in the rotor. However, as a safety check and as a check for any rotor containing a gradient which has to be displaced, it is best to displace the gradient with a heavier solution containing an indicator dye. The dye used most frequently in this laboratory is phenol red in an alkaline medium; however, any water-soluble dye will work. During unloading, if there is any mixing between the gradient and the displacing solution, then the color will turn up in the gradient. A limited amount of dye will appear in the portion of the gradient in contact with the displacing fluid. This amount depends upon the concentration of the dye, the temperature of the fluids, the length of time of unloading, and the surface area between the gradient and the displacing solution. Phenol red can be detected easily in the spectrophotometer at the $260\text{ m}\mu$ wavelength and less easily at $280\text{ m}\mu$. This permits a measurement of the amount of phenol red in any given sample of gradient. For the B-XVI rotor, the phenol red color can be detected first in fraction 25 out of 32 fractions. In studies carried out thus far, penetration of dye into this volume is nearly the same regardless of the gradient material. The usual unloading rate is about 25 ml/min , which results in an unloading duration of about 32 min. It is assumed that the dye in the outer portion of the density gradient is due to diffusion primarily, but it can also be due in part to a slight mixing of displacing fluid and gradient as the displacing solution slides downward between the rotor wall and the gradient at the beginning of the unloading phase.

The criteria outlined and discussed above must be met by rotors designed at Oak Ridge National Laboratory for virus and subcellular particle isolation studies and are used when necessary in modifying commercially available zonal rotors for routine operation. These criteria are considered to be minimal standards which must be met for successful routine operation of the long-cylindrical (B-IV type) zonal rotors.

Progress on the K-II Production Rotor

The K-II production rotor is a large-capacity continuous-sample type of rotor (B-XVI type) holding 3.6 liters of density gradient. This rotor is operated in a completely new centrifuge (Fig. 8). The rotor is designed primarily for myxovirus isolation (particularly influenza virus) and will be operated to 30,000 rpm with 10 to 20 liters/hr sample flow. Theory indicates that nearly 100% removal of influenza virus at 10 liters/hr can be expected.

Minor problems with the lower damper bearing have delayed gradient testing. The rotor has been tested to 27,500 rpm without a core or gradient and shown to operate within prescribed tolerances. The rotor has also been tested to 27,500 rpm (probable operational speed) with the B-IX type of core. The malfunction with the bottom bearing does not permit operating the rotor with 3.6 liters of sucrose density gradient at top operating speed at this time. The rotor stability problem will be corrected with a modification of the present design. Gradient stability and recovery tests will be carried out at the earliest possible time.

Work during FY 1967 under this interagency agreement will be restricted to the completion of the K-II rotor system.

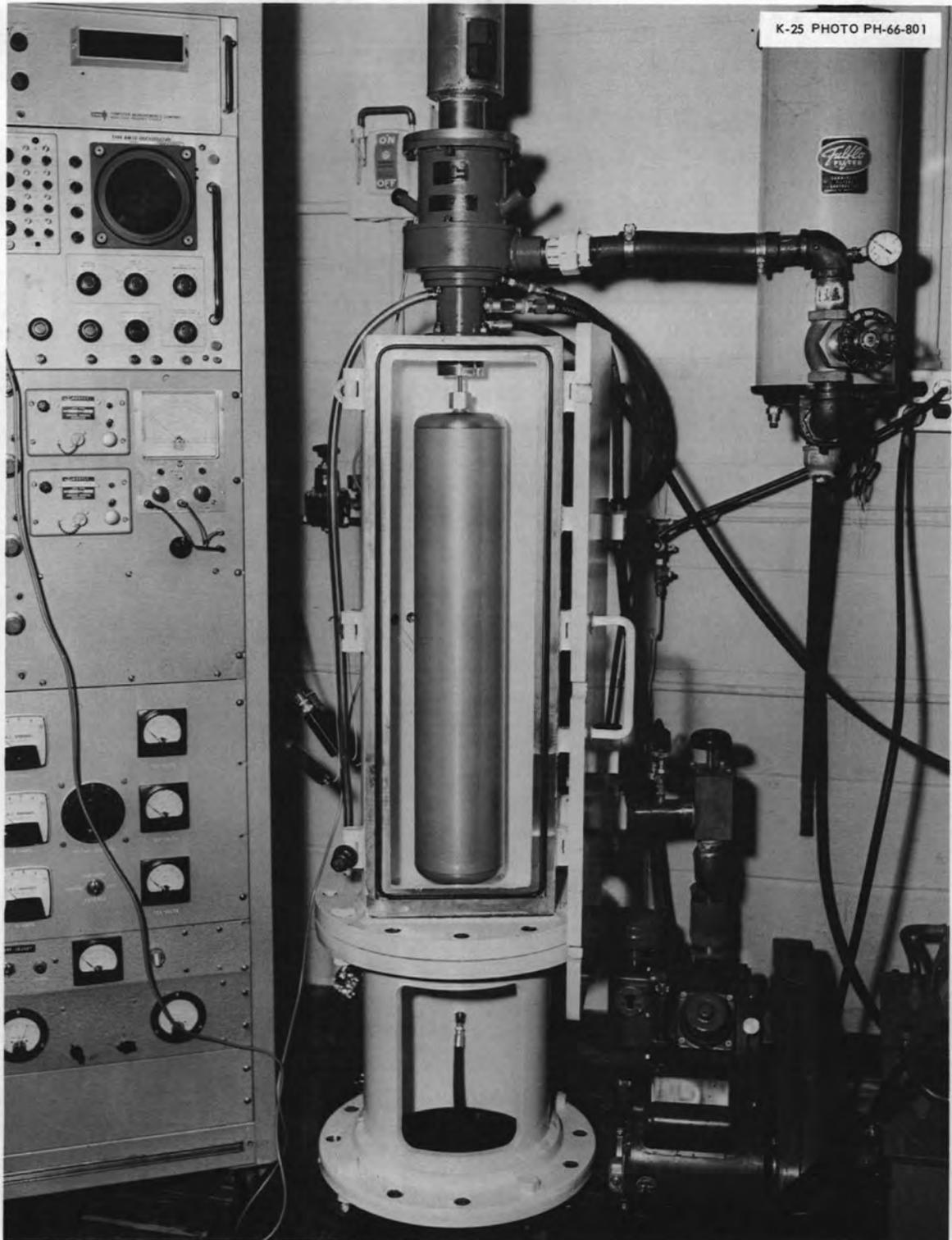


Fig. 8. K-II Experimental Centrifuge System. Controls are in the bank to the left and the armor shield and rotor in the center.

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