Problems in Biocontainment [1]

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SUMMARY

Rather large amounts of virus materials may be isolated by use of the rotor systems described in this volume. We have reduced the possibility of operator infection by redesigning an angle-head centrifuge rotor tube to prevent gross leakage of fluid, arranging to recover virus bands in disposable sterile syringes, and developing dialysis methods which minimize handling. Photographic means for locating bands are also described. For work with highly infectious agents, a completely contained laboratory has been built in which purification by continuous-flow centrifugation, rate-zonal centrifugation, and isopycnic banding may be carried out. The unit is completely sterilized with ethylene oxide, has an automatically programed sterilization cycle, provides for steam sterilization of all wastes, and has an incinerator on the exhaust air line. If contained systems are to come into general use, research instrumentation and procedures must be redesigned so that experiments may be as easily done with containment as without it.—Nat Cancer Inst Monogr 21: 485-502, 1966.

WITHOUT THE development of new techniques for biocontainment, requiring elimination of all possible hazards associated with infectious agents would effectively stop a large fraction of research now in progress. This follows from the fact that only total containment can remove all conceivable means by which contamination of the environment could occur. For this reason the concept of grading the biocontainment requirements to the level of potential hazard has been adopted in most virology and microbiology laboratories. This concept has been reduced to a system of hazard classification by the Biohazards Committee of the Oak Ridge National Laboratory (*1*).

While no incidence level is considered acceptable for biological accidents, the rate reported without cessation of activities (*i.e.*, the level tacitly accepted) is quite high. Considering data available up until 1962, a total of 107 fatalities and 2,241 illnesses from laboratory-acquired

[1] This research performed under the Joint National Institutes of Health-Atomic Energy Commission Zonal Centrifuge Development Program which is supported by the National Cancer Institute, the National Institute of Allergy and Infectious Diseases, and the U.S. Atomic Energy Commission.

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

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infections was recorded. This is considered to be only a modest fraction of the number that actually occurred (*2*).

The centrifuge systems described in this volume allow very large quantities of purified virus materials to be prepared. In addition, particles widely dispersed in tissues can be isolated in a high state of purity. Agents that are relatively innocuous or rarely infectious in very low concentrations may behave quite differently when introduced into animal or human recipients in concentrated or purified form.

Neither space, funds, nor human factors will allow all the biophysical and biochemical tools now used in virological and microbiological research laboratories to be enclosed in sealed chambers and manipulated through glove ports or by remote servomechanisms. In attempting to deal with this very difficult problem, we have directed our studies along three major lines.

First, efforts have been made to reduce (but not necessarily eliminate) hazards associated with existing procedures by relatively small changes. Second, complete containment units for existing separations and analytical systems, including zonal centrifuges, have been constructed to allow experimental studies to proceed. Third, the problem of redesigning procedures or equipment, specifically for convenient hazard-free operation, has been examined. The first line of endeavor seeks to decrease quickly hazards associated with existing procedures, the second allows new procedures and systems to be evaluated safely, and the third approaches the problem of contained research facilities that would be widely used, *i.e.*, systems allowing experiments to be performed more quickly and conveniently with containment than without it.

IMPROVEMENT OF EXISTING METHODS

Attention has been directed initially to improvements in techniques that often involve physical contact between the operator and virus suspensions.

Redesign of Angle-Head Centrifuge Tubes

Metal closures for high-speed centrifugation have been used with only slight modification for over 25 years. Contact with wet centrifuge tubes is probably the readiest source of gross contamination in a virological laboratory.

A series of polycarbonate [3] centrifuge tubes for the Spinco No. 30 rotor were machined to determine whether the plastic tubes and closures would withstand the centrifugal force at maximum rotor speed. No failures were observed. However, it was evident that machined tubes would be too expensive. Several alternate designs suitable for blow-molding were prepared and, in collaboration with a plastics fabrication firm,[4] one

[3] General Electric Company.
[4] Nalge Company, Rochester, N.Y.

basic design (fig. 1) was chosen for further study.[5] Two versions were fabricated and tested: Oak Ridge type 30 A has a ¼-inch diameter opening, while the Oak Ridge type 30 B has a ½-inch diameter aperture. Type A can hold 27 ml when spun without the cap in place. If this or a smaller volume is used no fluid presses against the plastic tube cap (text-fig. 1). Leakage will occur only if the tube ruptures. The polypropylene caps are inexpensive and do not deform during centrifugation. Type A tubes are specifically designed for banding infectious agents by angle-head, density-gradient centrifugation (*3*) where the separated bands are withdrawn with a needle. For separations in which a pellet is to be recovered mechanically, type B tubes (capacity 25 ml if the suspension does not reach the cap during centrifugation) are preferable. The tubes can also be run empty or partly filled. No water is needed in the rotor holes if the tubes are fairly new. Both the tubes and closures may be steam-sterilized. For work with highly infectious agents, new tubes should be used, since failure in operation has been observed only in tubes that have been in service for some time. Recently, tubes of a similar design have been fabricated for the Spinco No. 40 rotor.

A thin cap, which may be cemented into position, has recently been developed that allows storage of sterile, sealed type A tubes at liquid nitrogen temperatures.

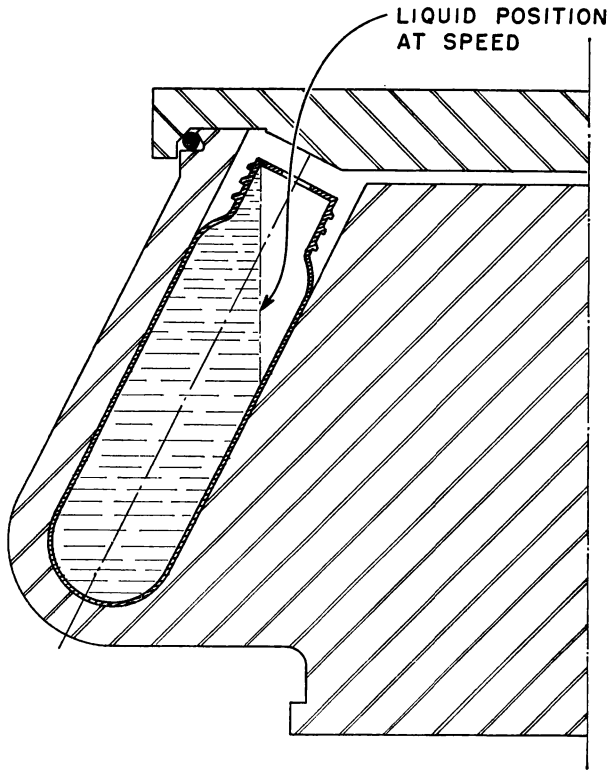
Density Beads for Liquid Gradients

The physical density of fractions recovered from liquid gradients used in centrifuges is generally determined by measuring the refractive index. This is usually done manually, and ample opportunity for contact with the sample exists during preparation for measurement and when the optical surfaces are cleaned. It appeared desirable, therefore, to explore means for determining the buoyant density of observed bands in the centrifuge tubes. Plastic beads have been employed previously (*4*); however, a series of color-coded beads, whose densities covered the buoyant density range of the virus, have not been previously available.

During centrifugation, density-indicating beads are suspended in the gradient at their isopycnic positions as are viruses or other particles present in the sample. The use of the beads provides a simple means of estimating the density of the particles at their isopycnic positions without manipulation of the specimens.

The basic requirements to be met in developing density-indicating plastic beads are low cost and density homogeneity. Attempts to vary the density of a resin, such as polystyrene, by blending with a filler met with limited success because of inhomogeneity produced by incomplete mixing or entrapped air bubbles. The percentage of water uptake by the density beads should be low.

[5] These tubes are now available from International Equipment Company, Needham Heights, Mass., and the Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.



TEXT-FIGURE 1.—Position of fluid in type A tube during rotation. *Note* fluid does not touch the tube cap.

By use of commercially available resins,[6] beads of four different densities were prepared by a controlled process to obtain a porous-free starting material. The plastic was then extruded [7] in continuous strands, cooled, and chopped into short rods. The rods were then ball-milled [8] into $\frac{1}{16}$-inch diameter beads.

Density variations in each of 4 batches of beads were established by banding samples isopycnically in both shallow and steep density gradients. Sample populations ranging from 24 to 60 beads were used for each determination. Density distributions to within ± 0.005 g per cm^3 were established from these data. The results are shown in table 1.

Photography of Particle Bands in Density Gradients

Where sufficient virus material has been banded to be seen by scattered light, the location of plastic density indicator beads and virus particles may be determined photographically (fig. 2).

The design allows the box to be incorporated into a contained enclosure with only the camera and electrical components on the noncontained side. Methyl methacrylate light pipes conduct light to each tube. Twelve No. 30 rotor plastic tubes may be photographed at one time.

[6] Purchased from Eastman Kodak Co.
[7] Extruded by the Extron Corporation, Knoxville, Tenn.
[8] Milled by the United Plastic Company, Maywood, N.J.

TABLE 1.—Classification of density-indicating plastic beads

Plastic	Batch color	Range of densities (g/cm³)	Density distribution: Density ± 0.005 g/cm³	Density distribution: Percent of total sample
Polystyrene	Yellow	1. 065–1. 105	1. 070	3. 4
			1. 080	43. 3
			1. 090	43. 3
			1. 100	10. 0
Cellulose acetate	Green	1. 272–1. 278	1. 275	100
Polyvinylchloride (rigid)	Tan	1. 361–1. 369	1. 365	100
Polyvinylchloride (high temperature)	Gray	1. 518–1. 534	1. 520	10
			1. 530	90

Recovery of Samples From Isopycnic Bands

The techniques for the recovery of gradients from plastic centrifuge tubes offer additional opportunities for exposure, especially where drops are collected through holes punched in the bottom of the tube. Where quantitative recovery of bands is not necessary, bands can be recovered by use of a motor-driven disposable plastic syringe (2.5 ml) and a disposable spinal needle (3.5 inches). These are mounted in the device shown in figure 3 and may be manually lowered to remove liquid from any level in the centrifuge tube. Variable illumination is provided from below. The syringe may be driven in either direction at the rate of 0.2 ml per minute.

Combined Camera and Band Recovery Apparatus

We have also developed a compact system for photographing single tubes against a dark background. By use of Polaroid film, photographs may be obtained in short periods of time and used as a guide in removing samples with the recovery apparatus mounted in the camera.

Dialysis of Samples

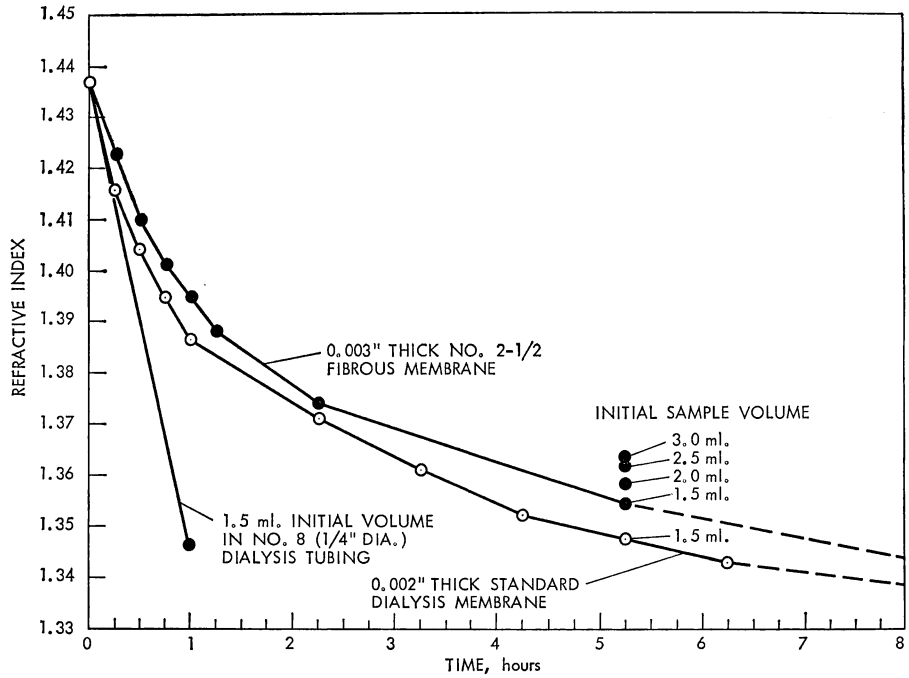
Dialysis of infectious or contaminated samples is an additional "wet" procedure with opportunity for infection. A variety of methods for the dialysis of samples removed with the band recovery apparatus have been examined. Of these, the two methods appearing to offer greatest promise are 1) a dialyzer board containing compartments for the simultaneous dialysis of up to twelve 3 ml fractions and 2) a plastic floater head, with serum cap and storage cover, used at one end of preclipped cellulose tubing for the pressure dialysis of individual pathogenic fractions up to 5 ml volume. The dialyzer board (fig. 4) consists of a 12-hole plastic base into which the fractions are collected or placed prior to dialysis, a sheet of fibrous cellulose dialysis membrane, and a clamped-on cover plate to seal the membrane around the lip of each ¾-inch diameter hole. After assembly, the board is placed in a circulating water bath and inverted to rest on

the 4 plastic legs. In this position, the fraction is supported by the membrane and desalting proceeds as the fresh water is circulated underneath each dialysis "window." After the dialysis is completed, the board is removed from the bath and placed in the filling position. The dialyzates can then be removed with a syringe by piercing each window with a needle. The bottoms of the holes are coned to permit removal of all material. The experimental model is of a nonautoclavable plastic (methacrylate). If the method comes into general use, a sterilizable material will be used.

The membrane, cut from flattened cellulose tubing,[9] is similar to standard dialysis tubing, except that a fibrous cellulose web is embedded in the tubing for strength. The web is only exposed on one side of the membrane sheet, and the "rough side" is turned away from the base containing the fractions during assembly to assure a satisfactory seal. This type of membrane is used solely because of its increased tensile strength, and is necessary for safety since the osmotic pressure developed within the fraction compartments is frequently sufficient to rupture conventional membranes when concentrated solutions are dialyzed, *e.g.*, 5 M potassium citrate. The relatively small membrane area exposed to the solution in this method results in a considerably longer dialysis period than experienced with ¼-inch diameter tubing for a given fraction volume; however, this may be desirable in certain cases, such as in the dialysis of fractions containing a myxovirus, since too rapid changes in salt concentration may result in inactivation. Text-figure 2 is a graph of the dialysis times required for 1.5 ml of 5 M potassium citrate with the use of both ¼-inch (No. 8 size) standard dialysis tubing in the conventional manner and the dialysis board with sheet membranes of both standard and fibrous materials. Note that the tubing contents reached a refractive index of 1.348 in 1 hour and that the board fractions reached the same point in 5.25 and 7 hours for the standard and fibrous membranes, respectively. The difference in the latter two time periods is attributed to differences in initial membrane thicknesses and excessive bulging and stretching of the standard sheet, with a resultant increase in its pore size. The thickness measurement of the fibrous membrane includes the fiber-mat contribution, the effective dialyzing layer thickness is therefore unknown, but somewhat less than the total value. The three single points plotted vertically along the 5.25-hour ordinate represent the refractive indexes attained after this time in the dialysis of other 2, 2.5, and 3 ml samples with the use of a fibrous membrane in the same board. Standard membrane can be used to reduce the dialysis time required for these larger volumes if the initial salt concentration of the fraction is considerably less than 5 M. It cannot be safely used with over 1.5 ml of 5 M potassium citrate solutions because of the pressure developed within the fraction compartment.

The design of the dialyzing board simplifies the handling problems associated with the processing of up to 12 fractions in a glove box, since

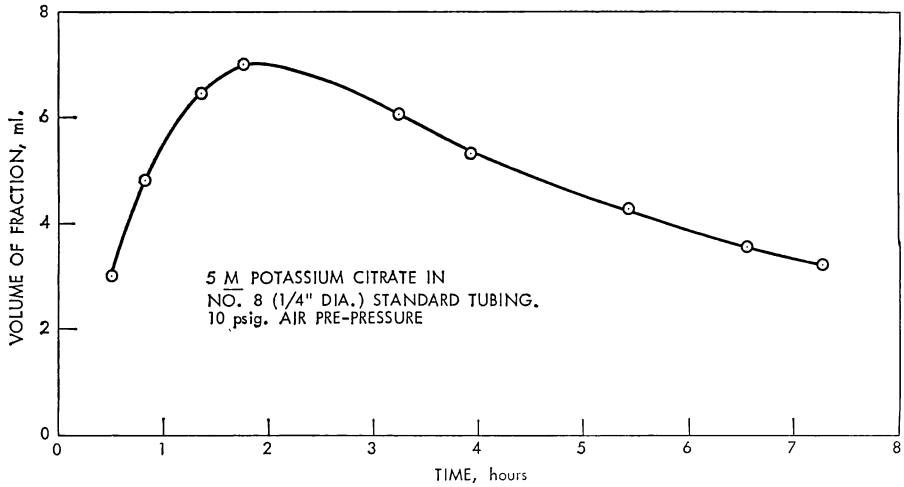
[9] Food Products Division, Union Carbide Corporation, Chicago, Ill., Fibrous Cellulose Casing, No. 2½, 3.8 inches flat width, 3.45 mils nominal thickness.



TEXT-FIGURE 2.—Dialysis times required for 1.5 ml fractions of 5 M potassium citrate to reach a refractive index of 1.348 in ¼-inch dialysis tubing. Standard and fibrous membranes with the 12-fraction dialysis board were used.

its rigid parts and the large cellulose sheet are considerably easier to handle with rubber gloves than wet dialysis tubing.

The floating plastic head (figs. 5 and 6) simplifies handling, containment, and storage in the dialysis of individual fractions whose volumes are up to about two thirds the volume of the attached tubing. A hollow float, having a serum cap septum access on the upper end and an O-ring to clamp the standard ¼-inch diameter dialysis tubing on the lower end, is intended for prior assembly and gas sterilization in the dry state. When needed, the assembly can be floated in a deep container of water to remove the glycerin from the tubing (if required), and the sample can then be introduced through the septum. Additional air can also be injected into the float through the septum to prepressurize the system and reduce the sample volume after the dialysis by forcing some of the water back through the tubing membrane. The results of the dialysis of a 3 ml fraction of 5 M potassium citrate, prepressurized to 10 psig, is shown in text-figure 3. In this case, the air volume contained in the head and tubing was about 12 ml at the start of the run. (A smaller head volume of 5 ml is planned, which should reduce the postdialysis time required to reach the original volume by reducing the air cushion above the liquid and thus raising the air pressure during the run.) The results illustrate the value of a pressurized floater head that has sufficient volume to allow expansion of the



TEXT-FIGURE 3.—Change in volume with time during the dialysis of a 3 ml fraction of 5 M potassium citrate under an initial pressure of 10 psig in a floating dialysis head.

fluid volume during the early phase of dialysis, and which then provides sufficient pressure to return the sample to its original volume.

The tubing used below the floater heads has thus far been standard ¼-inch diameter dialysis tubing preclipped at 24-inch intervals[10] by an aluminum wire crimp identical to that used commercially for closing large cellulose tubing. A pair of clips placed three fourths of an inch apart and spaced at 24-inch intervals provide two 12-inch-long, "pre-tied" dialysis tubes. The present O-ring seal for the tubing at the base of the float will hold without slippage of the wet tubing to about 13 psig with Buna-N rings. Other O-ring materials and seal designs are being evaluated to increase the holding power of the tubing seal to about 18 psig, since the burst pressure of the standard dialysis tubing is about 23 psig. An improved seal will allow a higher air pressure in the head and reduce the dialysis time required to return to the original fraction volume or less.

In addition to the advantages of preassembly and dry presterilization afforded by this design, the injection of liquid fractions into the tubing by a syringe provides better contamination control and allows a partial withdrawal of the dialyzate during the desalting period. Another advantage is that, after the dialysis is completed, the liquid can be drained back into the floater head, the tubing rolled up, and the cap (fig. 6) screwed on for sealing and safe storage of the fraction in a refrigerator.

CONTAINMENT SYSTEMS FOR ZONAL CENTRIFUGATION

Virus isolation in the zonal centrifuge has required that the centrifuge and its accessory equipment be sterilized and operated in a sterile envi-

[10] Supplied on an experimental basis by the Food Products Division, Union Carbide Corporation, Chicago, Ill.

ronment. The centrifuge and analytical equipment have been housed in a leak-proof Plexiglas glove box that can be sterilized before use with an ethylene oxide-Freon gas mixture and flushed with filtered air. This containment system has been used for the large-scale isolation of respiratory syncytial virus and ECHO-28 virus from liter volumes of tissue culture fluid and for the separation of subunits of adenovirus types 3, 4, and 7 from intact virus and cell debris.

The containment system (fig. 7) is essentially a complete laboratory, housing not only a zonal ultracentrifuge but also supporting analytical equipment, such as an ultraviolet spectrophotometer, density-gradient engine, sample pumps, water coolant system, refrigerators, a low-speed and high-speed centrifuge, zone fractionator, a modified Westphal balance, glassware, and such assorted items as syringes, pipettes, tubing, and vials. This equipment is manipulated through arm-length rubber gloves. The entire glove box can be sterilized by ethylene oxide by an automatic control system. Filtered air is pumped through the glove box to carry aerosols through an incinerator where the air is burned at 700° F. Aerosols are also exposed to ultraviolet light within the glove box. The temperature and humidity can be controlled to make the box equivalent to a cold room. This is of particular value when samples must be concentrated by pressure dialysis or pressure filtration.

Access to the interior of the glove box is by a double-door, pass-through, combination steam-gas autoclave on one end or by a double-door exchange compartment on the opposite end. The autoclave is used to steam-sterilize glassware and other autoclavable items needed inside the box and to sterilize contaminated glassware and tubing after completion of a run. It is also used as a gas sterilizer to decontaminate nonautoclavable items, such as centrifuge rotors, before or after use. Virus stocks are passed into the glove box after being wiped with formalin or 70 percent alcohol and are again wiped or dipped immediately inside the glove box. Virus samples produced during the separation procedures are capped in sterile vials or in screw-top test tubes and passed to the outside after being wiped or dipped in formalin.

Spilled materials in the glove box, dialysates, wash solutions, and waste from the steam sterilizer are dumped through valved ports in the floor of the glove box to a drain sterilizer where wastes are autoclaved before being run into the sewage lines.

This system has found ready application in continuous-flow centrifugation or in isopycnic centrifugation. Several runs on the same virus can be made without resterilizing the entire glove box each time. However, the rotor and accessory items are washed and gas is sterilized in the small autoclave before being reused. In experimental studies, up to 50 liters or more per week may be processed.

Corrosion of standard laboratory equipment, centrifuge equipment, drive systems, and electronic components after repeated exposure to ethylene oxide is being studied. For installations where the hazards are low but where containment is indicated, metal-supported plastic

enclosures that are attached to the vacuum chamber of a zonal centrifuge have been developed (fig. 8). These are sterilized by ethylene oxide, are provided with an air incinerator on the exhaust line, and are operated under negative pressure.

DEVELOPMENT OF INTEGRATED, CONTAINED LABORATORY SYSTEMS

Increased study of infectious agents will require more widespread use of contained systems. Large units, such as those described in the preceding section, will probably not find widespread application in virology laboratories throughout the country. However, the isolation of larger and larger quantities of infectious viral agents of more different types in an increasing number of laboratories is certain to take place. For contained systems to be economically available, standardization must occur. However, if designs are frozen which have not been sufficiently well developed to be generally acceptable, they will not be used during actual experimental studies. We conclude that a series of experimental systems must be designed and evaluated in practice in several different laboratories. Only those designs which find general acceptance and meet established minimum requirements should reach the stage of commercial production.

It is unlikely that research and development in the field of biocontainment can be successfully carried out except as part of a continuing research program in biophysical separation techniques with maximum participation by the research community. This is especially true if containment procedures are to be formally adopted and their use required.

The chief uses of large-scale virus preparations are in vaccine production and for structural and chemical analysis. The first step, therefore, in the development of integrated, contained systems would appear to be the design of a fairly compact system including a zonal or continuous-flow centrifuge with mechanisms for sterilizing the rotor, chamber, lower drive seals, upper shaft seals, and an upper chamber including space for the fluid line seals, samples, and sample collection vessels. A small rate of air flow should be provided for and effluent air and vacuum pump exhaust should be incinerated. Such a unit need not be large, and costs can be reduced by making extensive use of existing components. The redesign of other laboratory procedures and analytical devices into systems that may easily be used in containment probably will require prolonged study and evaluation.

In the present study, emphasis has been placed on procedures associated with centrifugation. For the development of integrated, contained laboratory systems, other factors should be considered—initial isolation procedures, tissue culture production of infectious agents, and methods of biological assay.

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PLATES

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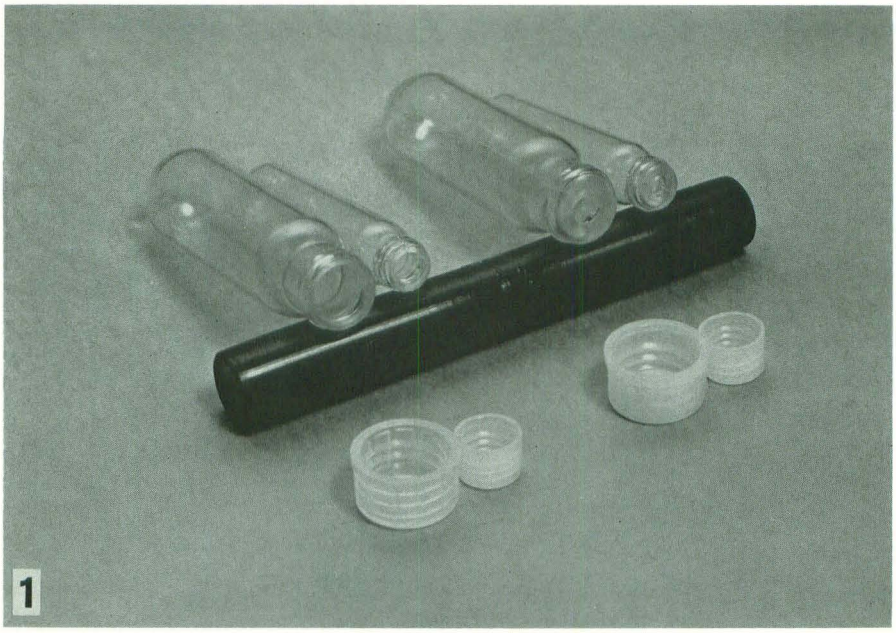

FIGURE 1.—Plastic ultracentrifuge tubes. Type A (small opening for virus isolation) shown on *right*, type B (large opening) shown on *left*.

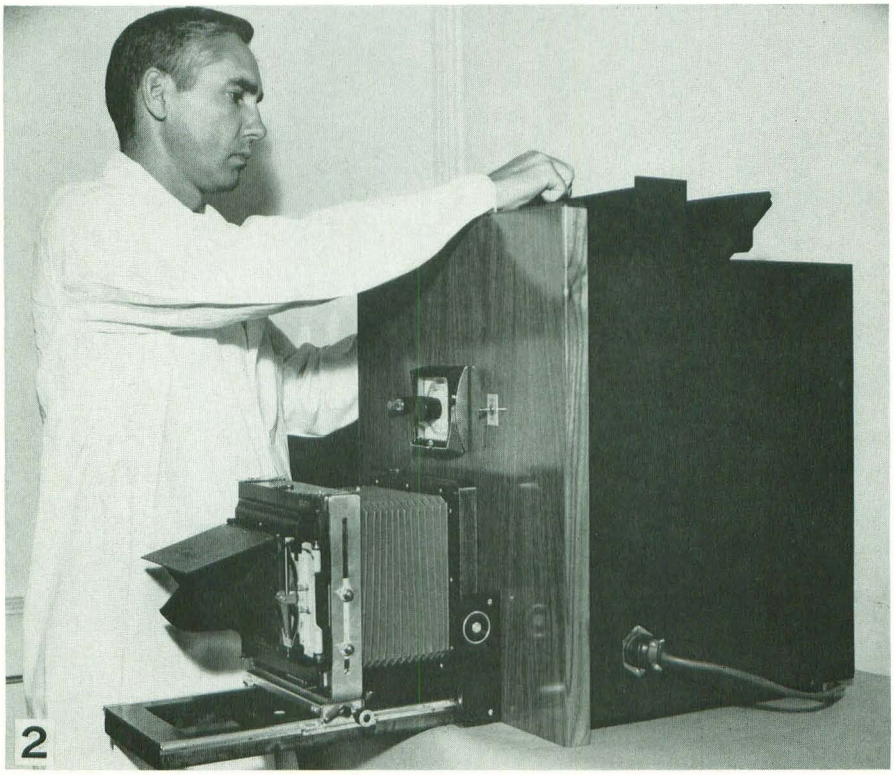

FIGURE 2.—Camera for photographing isopycnically banded particles in 12 angle-head centrifuge tubes by scattered light.

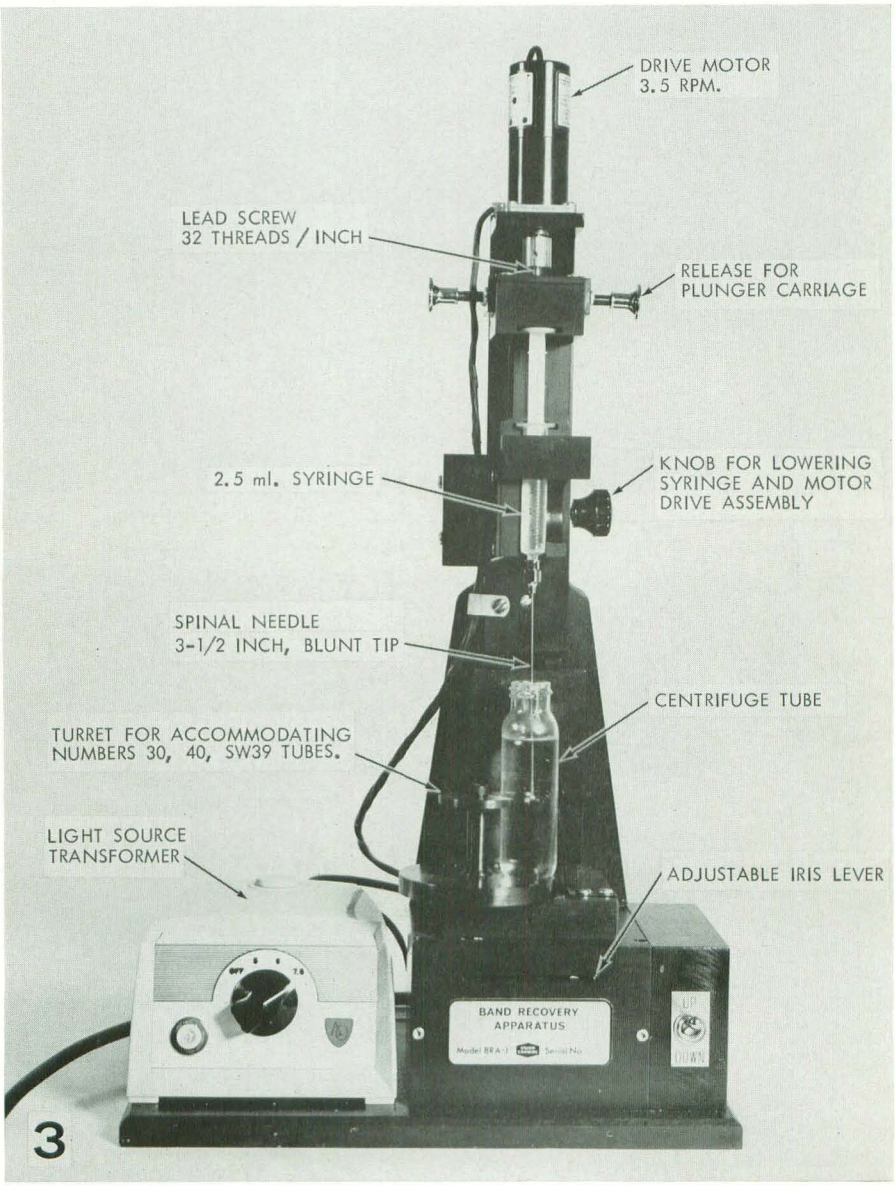

FIGURE 3.—Apparatus for recovering isopycnically banded particles from centrifuge tubes.

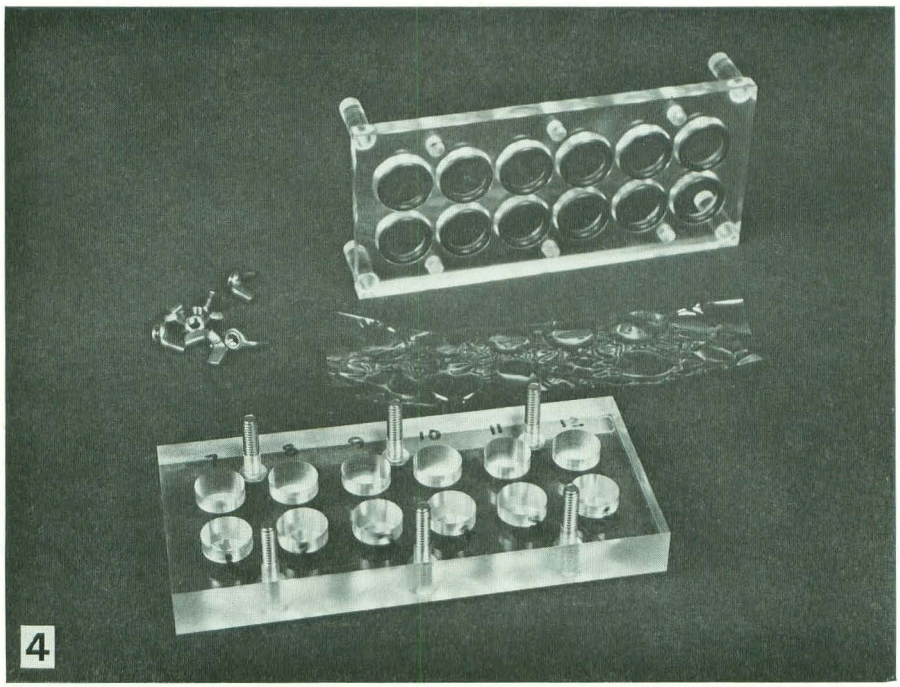

FIGURE 4.—Board for the aseptic dialysis of twelve 3 ml samples. The O-rings in the *upper section* seal the cellulose membrane sheet around the lip of each hole in the *lower section* when the 2 sections are clamped together.

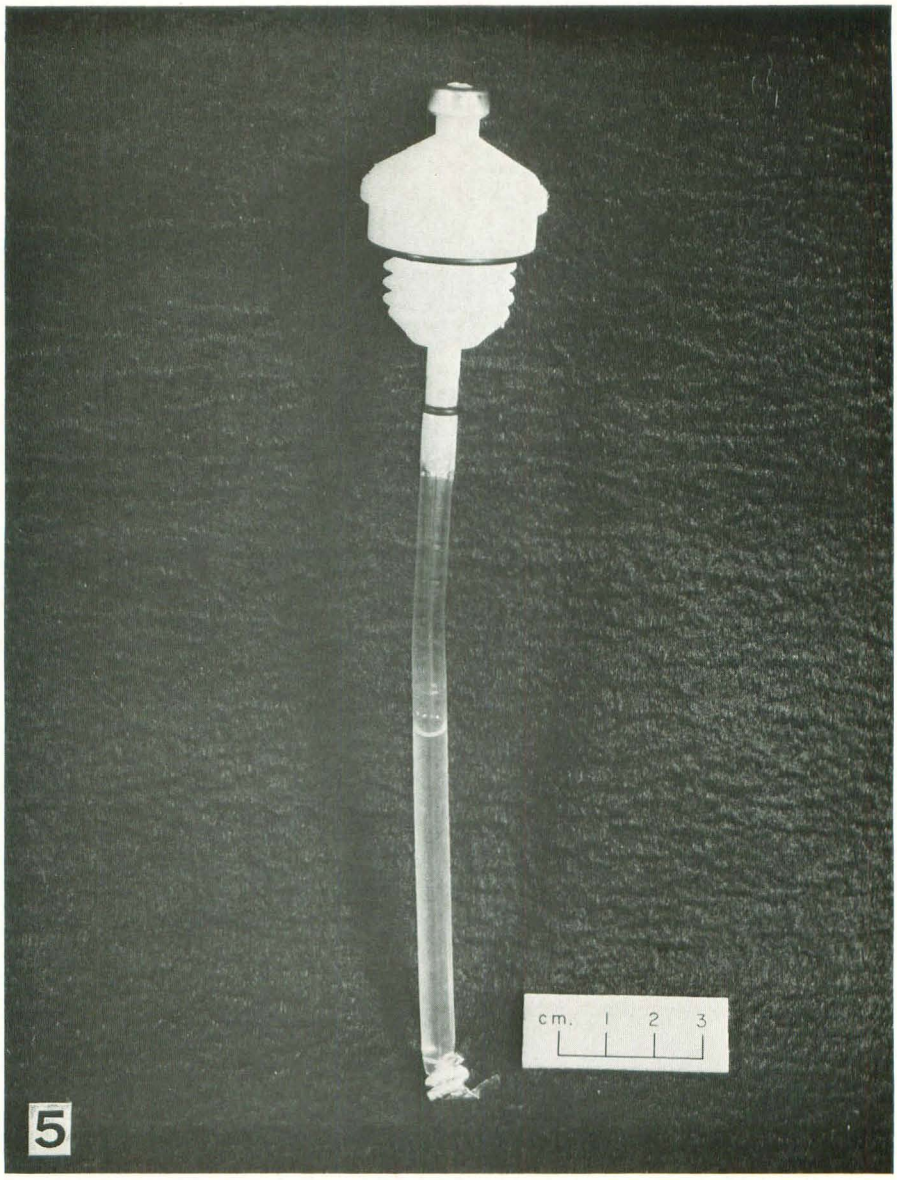

FIGURE 5.—Plastic floater head for use with preclipped dialysis tubing for the dialysis, containment, and storage of pathogenic fractions. The assembly may be gas-sterilized and stored dry before being filled through the cap septum.

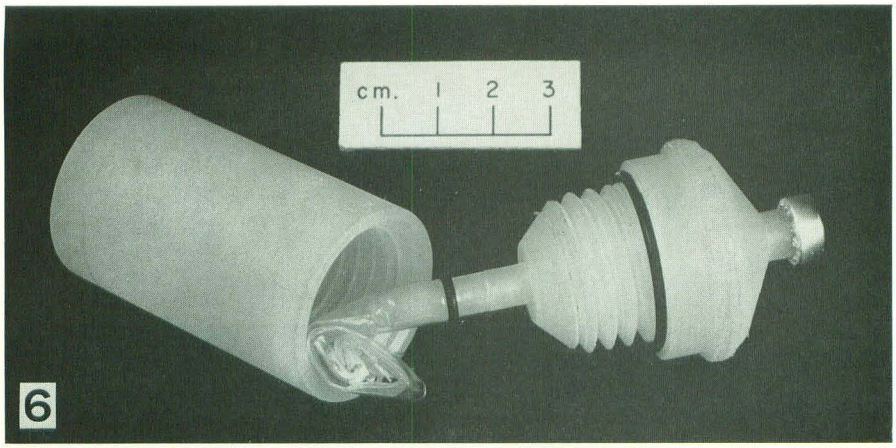

FIGURE 6.—Plastic dialysis floater head showing method of sealing the dialysis tubing and the use of a self-sealing cover for storing fractions after all liquid has been drained from the tubing into the hollow-head section.

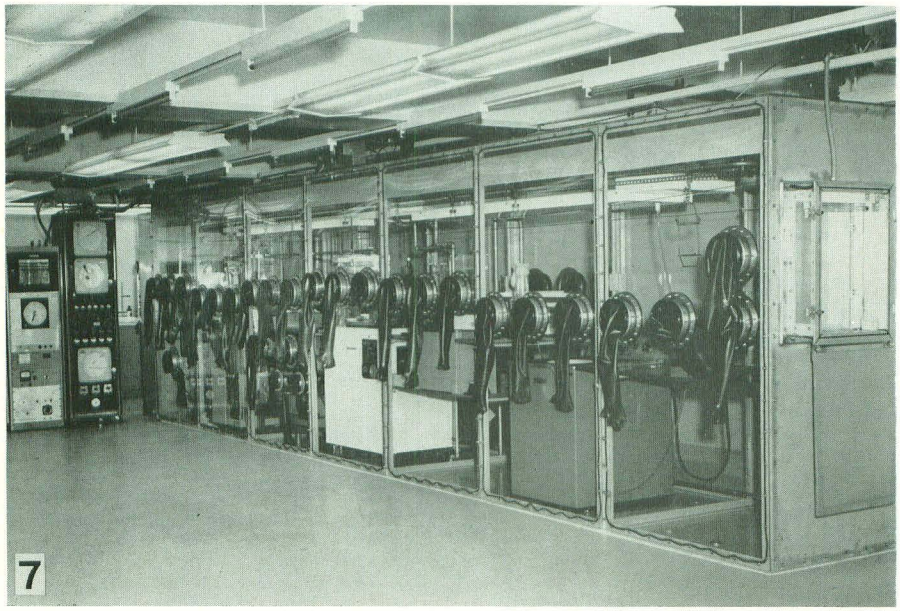

FIGURE 7.—Controls and complete containment system used for virus isolation. A combination steam and gas autoclave opens into the far end of the glove box. Centrifuge controls and automatic system for the glove-box sterilization cycle are shown at *left*. Air incinerators for decontaminating exhaust air are not shown.

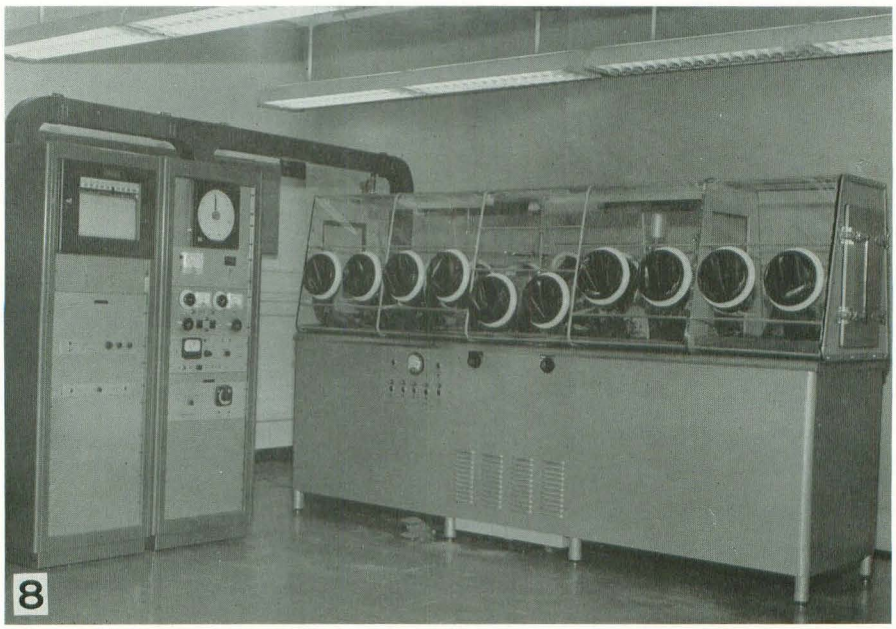

FIGURE 8.—Containment system for zonal centrifuge (installed at plant of Charles Pfizer and Co., Maywood, N.J.).