Separation of Subcellular Components and Viruses by Combined Rate- and Isopycnic-Zonal Centrifugation [1]

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SUMMARY

The re-isolation of trace amounts of virus added to tissue homogenates has been taken as a model problem in the development of high-resolution methods for separating particles. From a review of data on these particles, it was found that, when sedimentation rates are plotted against their banding or buoyant densities, most viruses fall in a "window" which appears to be free of other particles. To examine this concept experimentally, a technique for sequential rate-zonal and isopycnic-zonal centrifugation has been developed which allows this two-dimensional separation to be made and visualized. By use of this so-called s-ρ system, trace quantities of T3 phage particles have been recovered and identified from rat liver, brain, and spleen homogenates. The method may be used to isolate trace quantities of virus-like particles that have occasionally been reported in human tumors.—Nat Cancer Inst Monogr 21: 253–283, 1966.

AMONG THE properties of subcellular components and viruses that may be exploited by physical separation methods are particle size and density. Two questions arise at once: 1) To what degree are functionally distinct particles physically separable in theory, *i.e.*, to what extent do they have unique size and density distributions, and 2) how well do separations obtainable in practice approximate those predicted on theoretical grounds? In many instances the two questions are inseparable since the physical properties of interest cannot be measured independent of the experimental conditions. Thus it is difficult to determine whether broad zones that may be seen in a zonal centrifuge result from poor centrifugal resolution or from particle heterogeneity. It is of interest to study the behavior of an internal standard of known size and density added to tissue homogenates. The resolution obtained with the standard may then be compared to separations obtained with cell particulates. Virus particles appeared to

[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] Department of Zoology, University of California, Los Angeles, Calif. Aided by AEC Contract No. AT(11-1)-34 Project 49.

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

794-527—66——28

be the most suitable internal standard in the size and density range of interest in connection with the development of intermediate-speed zonal centrifuges.

In this paper we discuss 1) whether, in theory, homogeneous particles such as certain viruses could be re-isolated in a high state of purity from tissue homogenates, 2) the development of methods for achieving high-resolution separations based on particle size and density, and 3) experimental studies using model systems. Since, in addition to serving as a model problem, the isolation of virus particles from tissues, including pathological samples and neoplasms (*1–3*), is of considerable practical interest, factors limiting the detection of separated virus particles by physical methods will be analyzed. The results may guide attempts to isolate minor cell constituents including, for example, centrioles. Although the major portion of the following discussion concerns viruses, it should be understood that they serve here chiefly as model particles.

SUBCELLULAR PARTICLES AND VIRUSES

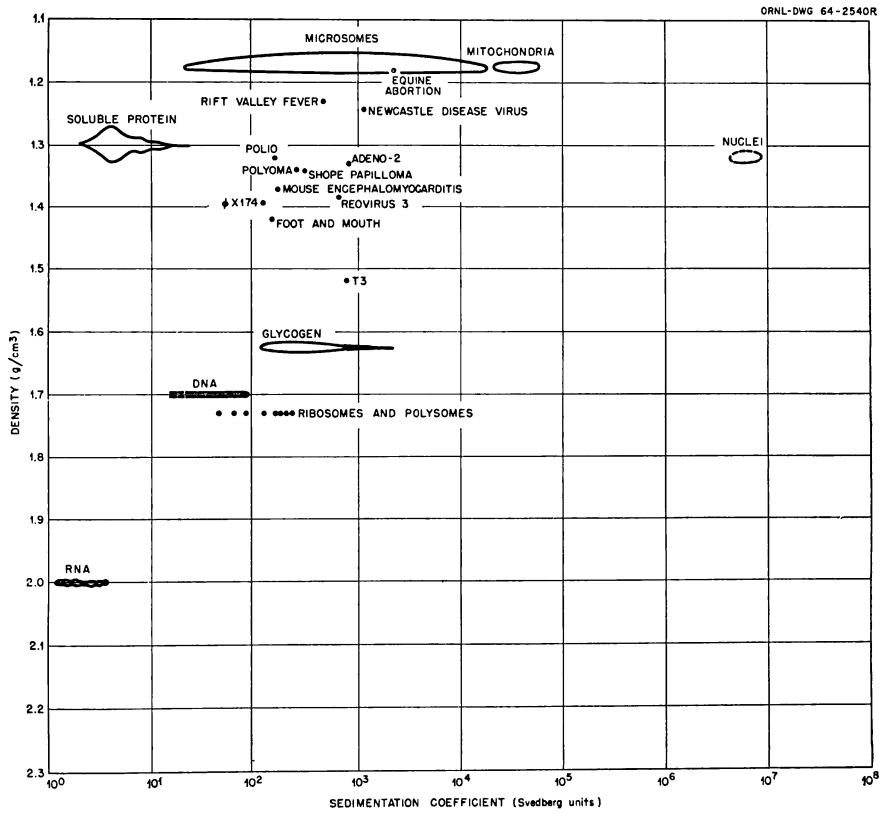
The question to be asked initially is: Are most viruses sufficiently different from the components of a broken cell suspension to allow their isolation in a reasonable state of purity? (Conversely, are most cell constituents sufficiently different from viruses . . . etc.) On purely theoretical grounds, it is evident that particles having quite different densities may have the same sedimentation rates in a given solvent. This follows from the fact that the sedimentation rate of a sphere is proportional to the product of the square of the particle radius and the difference in density between the particle and the suspending medium. Tissues probably contain a spectrum of particles having sedimentation coefficients in the viral range. It is less likely, however, that tissues contain many particles having both the same sedimentation coefficient and buoyant density as a particular virus. Viruses contain appreciable and constant amounts of nucleic acid, whereas very few cell particulates having the same size would be expected to have identical nucleic acid contents. Those few very small viruses that overlap ribosomes and polysomes may be separated by enzymatic digestion or dissociation of the latter structures. *It is concluded, however, that both rate-zonal and isopycnic-zonal separations must be used if maximal resolution is to be obtained.* For convenience, separations in which both of these methods are used have been termed s-ρ separations.

The Virus "Window" Concept

Available data on the sedimentation rates and densities of viruses and major cell components are given in text-figure 1. The values indicated for the sedimentation coefficients are corrected to water at 20° C. Ex-

perimentally the isopycnic separations were made in salt gradients, and the banding densities, unless otherwise stated, were for cesium chloride gradients. In gradients prepared from other solutes, the values would be numerically different, but the over-all distribution of viral and cellular components would tend to remain the same. The values used for the sedimentation coefficients for proteins are those for rat liver (*4*) which are similar to the values recorded for rat brain, kidney, and testis. While the banding densities for soluble tissue proteins have not been experimentally determined, they probably do not vary greatly from the value of 1.295 reported for bovine serum albumin and human hemoglobin (*5*). Values used for the sedimentation coefficients of ribosomes and polysomes were for rabbit reticulocytes (*6*), while the banding densities were those extrapolated from *Escherichia coli* (*7*). It is assumed that polysome densities would not be widely different. The range of sedimentation coefficients shown for "microsomes" was that observed experimentally with rat liver in the zonal centrifuge, while the density range was obtained by banding in neutral CsCl. The sedimentation rates for mitochondria are calculated from the data of de Duve *et al.* (*8*) and of Thomson and Klipfel (*9*). Nuclear sedimentation coefficients were calculated values for diameters ranging from 6 to 12 μ and an average density of 1.040 (*10*) in dilute buffer. The CsCl density was estimated on the assumption that nuclei contained 77 percent protein and 19 percent deoxyribonucleic acid (DNA) (*11*) and that the isopycnic densities would be the sum of the density of each component times the percentage present. In a salt gradient, nucleoprotein tends to dissociate, at least partially, and probably yields several bands. The nuclear isopycnic density was therefore a calculated value given to indicate a region where separations of nuclear from non-nuclear materials would be difficult. The position of glycogen is taken from studies on the distribution of sedimentation coefficients of material isolated from liver (*12–15*). The banding density in CsCl was taken from experimental studies carried out in this Laboratory (*15*) with glycogen isolated from rat liver. The value was slightly higher than that previously reported (*13*, *16*). The position of ferritin is not shown. Ultracentrifuge studies (*17*) suggest that these particles may be heterogeneous with respect to iron content and therefore would vary in both sedimentation rate and banding density level. Preliminary experiments with ferritin from both human and horse spleen confirm this view.

Also included in text-figure 1 are data on DNA, which would not ordinarily be found free in homogenates of mammalian cells, but would be encountered in bacterial homogenates, and might be extracted from viruses during the preparation of pathological tissues. The banding density range was taken from Schildkraut *et al.* (*18*), and the range of sedimentation rates includes that of single DNA molecules from T5 phage (*19*) and a variety of published values. Ribonucleic acid (RNA) has a density in CsCl at the limits of the density available with this salt. The density of 2.0 observed in cesium formate (*20*) has therefore been used. The sedimentation rate range is taken from a number of sources (*21*, *22*).



TEXT-FIGURE 1.—Sedimentation coefficients and banding densities of cell components and viruses. Sources of data are given in text. *Note* that most virus particles fall in an area, here termed the "virus window," essentially free of cell constituents.

The accurate positioning of cell components from various tissues in the s-ρ plot will require much additional experimental work, and some of the values used here must be treated with reservation.

Data on the following viruses are taken from published studies: adenovirus 2 (*23*, *24*); equine abortion virus (*25*); foot-and-mouth disease virus [(*26*); R. Trautman and H. L. Bachrach, personal communication]; mouse encephalomyocarditis virus (*27*, *28*); Newcastle disease virus (*29*, *30*); φX 174 (*31*, *32*); polio virus (*33*, *34*); mouse polyoma (*35–39*); reovirus 3 (*40*); Rift Valley fever virus (*34*, *41*); Rous sarcoma (*42–45*); and Shope papilloma virus (*42*, *46*).

It is evident from an examination of text-figure 1 that a large open area, or "window," appears in the center of the s-ρ plot which includes a small amount of nonviral material and a large fraction of known viruses. In those instances where a virus has the same banding density as a part of the microsomal fraction, it is evident that the virus cannot be completely separated from contaminating material by centrifugal procedures alone. Chemical (*47*) or enzymatic dissection may then be necessary.

Since the microsomal particles have a wide range of sedimentation rates, however, it should be possible to isolate the virus from most of the microsomal mass. The amount of endoplasmic reticulum giving rise to microsomes varies widely among cells and tissues but is low in many tumors and in some cells used for virus production.

These considerations have prompted us to explore methods for large-scale separation of subcellular constituents which would accomplish the theoretical separations shown diagrammatically in text-figure 1.

Detection of Virus Particles

Infectivity in a suitable test system is the most sensitive method for detecting virus particles. However, it gives no indication of the purity of the fractions assayed. The physical methods for detecting viruses isolated in a reasonable state of purity include absorbance in the ultraviolet range, light scattering, electron microscopy, and measurement of fluorescence after conjugation with fluorescent antibodies. The usefulness of these methods depends on their sensitivity. By the use of negative-staining techniques, particle-counting methods, or sectioning of pellets, it appears that the identification of virus particles on a morphological basis is feasible at the level of one particle per cell if the virus is obtained in a pure form and in the same concentration in which it existed in the source tissue. For example, if liver containing 4×10^8 cells per g is used, then the final virus concentration (4×10^8/ml) would be at the lower limits of detection with spray-counting methods (*48*, *49*) but well within the range of the Sharp counting technique (*50–52*).

For detection of most viruses by ultraviolet absorbance measurements, somewhat larger amounts of virus are required. The number of absorbance units necessary depends on the absorbancy of the gradient itself, the length of the light path, and the volume in which the virus is recovered. If the lower limit is 0.1 absorbance unit, then 13×10^{12} particles of the small foot-and-mouth disease virus (R. Trautman, personal communication), 1.2×10^9 physical particles of pox virus (*53*), or 2.3×10^6 vaccinia particles (*54*) would be required. This limit may be lowered by the use of long light path (*55*) cells, electronic methods for extending spectrophotometric ranges, or refined small-volume banding methods. The conclusion is, however, that, if small virus particles are to be detected by ultraviolet absorbance, the concentration per unit volume must be 10 to 100 times higher than that found in the starting tissue, assuming that only one virus particle per cell is found. For very large viruses, however, direct absorbance measurements would probably suffice.

Light scattering as a method of detecting virus-sized particles in gradients is theoretically more sensitive than absorption measurements. In practice, certain compromises must be made. For example, the photographic technique used in this paper involves the use of centrifuge tubes that are designed specifically for virus work, *i.e.*, not to leak or contaminate the operator. They are not as good optically as a glass vessel.

Further, photographic techniques as ordinarily used are not as sensitive as photomultipliers. Considerable improvement in detection sensitivity is therefore possible. The detection limits for virus particles by physical means will actually be set by the quantity of nonviral tissue material found at a given position in the s-ρ separation.

If methods can be developed to allow isolation of particles having the narrow range of sizes and densities characteristic of a given virus, and if the final particle concentration is equal to or greater than the concentration in the tissue of origin, then virus particles at concentrations possibly as low as one particle per cell could in theory be isolated and described. Since the mass of a virus varies from approximately 10^{-6} (*56*) to 10^{-8} of that of a cell, the purification (as distinguished from concentration) required is from a million-fold to 10 million-fold.

Available data on the composition and masses of viruses and the absorbance of solutions containing known amounts of particles have been tabulated (*57*). The latter are calculated values and do not include the increased absorbance caused by scattered light. The increased absorbance due to Tyndall scattering is a function of the refractive index of the suspending medium. Previous studies on light scattering of virus particles have been done in dilute solutions (*58*) and scattering would be less in dense-banding media. In the calculated absorbancies (*57*), the effect of scattering on observed total absorbance was neglected. Ultimately it will be necessary to determine the absorbance of suspensions having a known number of physical particles in solutions isopycnic with the particles.

As highly purified virus materials become available for investigation and medical use, it is necessary to consider the units in which concentrations of virus particles are expressed, the relation between these units, and the methods used for analyzing virus suspensions.

The following proposals are internally consistent and would help one to compare different viruses, to analyze given preparations, and to estimate concentration. The basic unit is the weight of an anhydrous virus particle in grams. Fractions of a gram have been given names corresponding to 10^{-3N} g, where N is a whole number. In keeping with this convention, 10^{-18} has been chosen as the weight unit and might well be termed a "virogram." The viruses thus far described range from approximately 11 to 3500 virograms. Thus whole numbers can be used. In the past, virus concentration has often been expressed as μg of virus per ml of suspension. If a unit of virus concentration containing 10^{12} virus particles per ml is chosen, then the μg of virus per unit concentration is found to be numerically equal to the weight of a single virus in virograms. Thus the weight of a single cowpox virus is 3500 virograms, and a solution containing 10^{12} cowpox virus particles per ml would contain 3500 μg of virus per ml. A polio virus with a weight of 12 virograms yields a suspension with 12 μg of virus per ml at a concentration of 10^{12} physical particles per ml. In this work, we consider the *physically defined* particle (PP) and not the *infectious unit* (IU). The absorbance at 260 mμ of virus suspensions at 10^{12} PP per ml is also a useful number. It spans the range of absorbancies usually measured with conventional spectrophotometers, except for the large pox viruses, where dilution by one order of magnitude (10^{11} PP/ml) is required to make reliable measurements. With these units there are simple correlations between number, particle weight, concentration, and absorbance.

Search for Viruses by Electron Microscopy

The electron microscope has been invaluable in identifying virus particles in cells. It is important to know whether centrifuge systems may approach, or possibly surpass, the sensitivity of the electron microscope in identifying virus particles in cells. For this discussion we will consider virus concentrations at the limiting level of one particle per cell.

An estimation of the number of photographs that must be examined to find one virus particle per cell is given by the approximation

$$N = \frac{4\pi r^3 M^2}{3TA},$$

where N is the number of photographs to be examined; r, the radius of the cell in cm; M, the electron microscope magnification; T, the thickness of the section in cm; and A, the area of the photograph in cm^2.

For a cell 15 μ in diameter, approximately six hundred 8 $\times$ 10-inch photographs at 20,000 $\times$, using 250 A sections, would be required (fig. 1). As the diameter of the cell or the magnification is increased, so is the number of photographs.

It is evident that a statistical study of the number of virus particles in a tissue at this level is inordinately tedious and involves thousands of photographs (6,000 for 10 cells).

Detection of Virus Particles in Gradients

For detection of virus particles by ultraviolet absorption, the lower limit with present systems is approximately 0.1 absorbance unit. With a small virus, such as polio, this represents approximately 1.3×10^{12} PP; with a large one, such as vaccinia, the same absorbancy is produced by about 1.4×10^{10} PP. If one particle per cell is to be detected, then the number of original cells must be equal to this number of particles. For rat liver as an example, 1 g of tissue contains 2 to 4 $\times$ 10^8 cells. The upper limit for tissue used directly in the B-IV rotor is about 10 g, giving about 3×10^9 cells. This is one order of magnitude below the ultraviolet absorption limits for vaccinia and three below that for polio.

However, if 10^8 virus particles can be isolated from 1 g of tissue free from other contamination and in a volume of 1 ml, then their detection through conventional counting techniques by electron microscopy is fairly simple. Further, if the isopycnic separations can be done in a much smaller volume, the limit of 0.1 absorbance unit may be considerably decreased.

Use of Negative Staining for Detecting Virus Particles

The negative staining technique of Brenner and Horne (*59*) is extremely useful for identifying certain viruses, especially where large numbers of samples are to be scanned. While the technique as ordinarily used is

not suitable for the purpose of obtaining accurate counts, it is important to have an estimation of the number of particles required to give the microscopist a reasonable chance of finding them. The following experiment indicates that this simple method, as routinely used in this laboratory, is useful for trace virus isolation.

Methods

A solution of T4 bacteriophage containing 1.14×10^{10} particles per ml was kindly prepared by Dr. Stanley Leibo of the Biology Division of the Oak Ridge National Laboratory. Serial tenfold dilutions were prepared with 0.9 percent NaCl.

A drop of the virus suspension was placed on a carbon-coated Formvar 400-mesh specimen screen. After standing for 2 minutes, the drop was removed by touching to a piece of filter paper, and a drop of 2 percent (w/v) phosphotungstic acid (*p*H 7.0) was added. Fifteen seconds later the phosphotungstic stain was removed and the screens were allowed to dry in air.

Results

When serial dilutions of the T4 bacteriophage suspension were examined, the following results were obtained.

10^{10} Particles per ml: estimated average, 12 particles per grid opening. All grid openings had at least 2 particles and some had as many as 50.

10^{9} Particles per ml: average of 5 particles per grid opening. No particles were found in some openings and the maximum found was slightly greater than 5.

10^{8} Particles per ml: 3 particles found in 10 grid openings.

10^{7} Particles per ml: no particles observed with the technique described above. (A very few were found when the drop of test solution was left in contact with the specimen screen for 5 minutes.)

It is concluded that if virus particles are present at the level of 1 per cell, and if all nonviral material could be removed, then the virus could be detected without concentration by this method.

RATE-ZONAL SEPARATIONS

Two gradients have been used for the separation of tissue components, depending largely on the results desired. By use of a gradient extending linearly from 17 to 55 percent w/w sucrose with a 66 percent sucrose underlay, mitochondria are banded isopycnically, while microsomes may be spread out through most of the gradient by a suitable choice of speed and centrifugation time. Ribosomes have been only partially sedimented out of the soluble materials zone, while part of the nuclei is retained by the 66 percent sucrose at the outer end of the gradient.

For the separation of smaller particles directly out of a tissue brei, a shallower gradient has been used which extends from 10 to 30 percent sucrose with either a 47 or 55 percent sucrose as the underlay. In this

gradient, mitochondria are banded at the interface between the dense end of the gradient and the sucrose underlay. In these studies no effort is made to recover nuclei. The centrifugation time and rotor speed could be chosen so that particles in the size range of the virus would be spread out through that portion of the gradient between the sample zone and the mitochondria, or sedimentation may be limited so that virus-like particles would be restricted to a smaller portion of the gradient. For maximal separation and for evaluation of the separations that may be obtained, the former procedure is useful, whereas the latter method is most useful in the search for viruses where very small numbers of particles are expected. In the majority of the studies recorded in this paper, 10 to 30 percent w/w sucrose gradients have been used with a 47 percent sucrose underlay. With this gradient, particles having a density of 1.3 and a sedimentation coefficient of 2000 S^* would appear in the neighborhood of tube 34 after 60 minutes at 20,000 rpm ($\int \omega^2 dt = 1580 \times 10^7$). This time and speed are useful when maximal resolution of virus or virus-like particles is desired. For survey studies, 15 to 30 minutes at the same speed ($\int \omega^2 dt = 395$ to 790×10^7) would restrict similar particles to the first 6 to 12 tubes in the gradient below the sample layer.

The choice of whether to use the rate separation or the isopycnic separation first depends on several considerations. Cell nuclei may have protein/nucleic acid ratios, and therefore banding densities, in the range observed for virus particles. If an isopycnic separation is done initially, nuclei may be disrupted and extracted by the high salt concentrations used. Such extracted nucleoprotein would be difficult to separate from smaller particles since it would not behave as intact nuclei do in subsequent rate-zonal separations. Other cell components including ribosomes may also be altered by salt extraction. An additional disadvantage in doing equilibrium studies first is that, with presently available systems, sufficient centrifugal force is not available to band soluble proteins sharply in a relatively short time. Banded virus and other small particles may therefore not be free of contamination by small soluble particles that have not reached their isopycnic points. In addition, isopycnic separations often require more expensive materials than those used in rate runs. The deciding factor, however, is that no way for making large numbers (up to 36) of rate-zonal separations has been devised, whereas this number of isopycnic zonal runs can now be made. In all experimental studies reported here, therefore, rate separations have been made first.

Protein Distribution in Gradients

It is of interest to know the general distribution of both ultraviolet-absorbing materials and protein through gradients from several tissues to obtain an indication of the amount of the material having sedimentation properties in the viral range.

Homogenates of rat liver, brain, and spleen were therefore examined using the B-IV zonal centrifuge system. The tissue sample preparation

is described in the legend of each figure. Protein was determined by the method of Lowry *et al.* (*60*). The results are shown in figures 2, 3, and 4.

Each 40 ml fraction in the virus sedimentation coefficient range contains on the average less than 1 percent of the total protein of the homogenate in the case of spleen and liver. In many instances, especially in plant extracts, rate-zonal centrifugation alone will yield a sufficient purification of viruses for many purposes.

Isopycnic Banding of Cell Components

To translate the theoretical separation shown in text-figure 1 into an experimental technique, it is necessary to make isopycnic zonal or banding separations of a large number of fractions. Rather high centrifugal forces are required to do this in a reasonable length of time. Unfortunately, the available high-speed, swinging-bucket rotors will spin only a small number of samples. Banding of small particles may be done in angle-head centrifuges (*61*). Thirty-six samples may therefore be run by use of 3 model L preparative ultracentrifuges.

Three different banding techniques have been used to explore s-ρ separations of tissue components. These are:

1) Wide density range banding. Cesium chloride gradients have been used for this purpose and will band nearly all known tissue components. Cesium formate is required if free RNA is to be banded; otherwise CsCl is the solute of choice.
2) For particles having buoyant densities less than 1.5 (this would include all viruses that might be expected to occur in animal tissues), potassium citrate has been used.
3) For studies on density differences in membrane components banding in sucrose itself is often useful (*62*). The viscosity of concentrated sucrose solutions at low temperatures is very high. Isopycnic banding of small particles in sucrose solutions therefore requires a longer centrifugation time than when salts are used.

In this paper only the results with wide-range banding are presented. Studies with potassium citrate and sucrose are in process.

Gradient preparation

Polycarbonate centrifuge tubes for the Spinco No. 30 rotor developed under this program (*63*) were filled with 10 to 15 ml of undialyzed fractions from the rate-zonal centrifuge runs. Twelve ml of the dense gradient material was then run in to the bottom of the centrifuge tube through a Pasteur pipette. The gradients were allowed to form during centrifugation by diffusion. The steep gradients formed by diffusion are useful for survey studies where particles having a wide range of densities are to be observed. When higher resolution separations of a few components are

required, a step gradient may be formed initially by introducing a series of solutions of increasing density through the pipette.

Band detection

As previously mentioned, light scattering is one of the most sensitive methods available for detecting the presence of particles banded in a centrifuge tube. The refractive index of the gradient in the region of an isopycnic zone approaches that of the particle and, in some instances, may match that of the particle. Particulate subcellular constituents and viruses are not internally homogeneous with respect to either the buoyant density or refractive index of their constituent molecules. This is one reason why viruses and the larger subcellular particles scatter appreciable amounts of light even at their isopycnic level. The number of particles required to give a band that may be identified photographically is a function of particle size, shape, permeability, and composition, the refractive index of the suspending medium, and the optical properties of the centrifuge tube and of the photographic system used for making the photograph or pycnograph.

Sensitivity

To obtain an indication of the amount of material required, T3 phage particles were banded in CsCl, as described, and photographed. The results are shown in figure 5. It is evident that homogeneous virus particles present in fairly small amounts, and having a banding density in the virus window, would not be observed at the level of one particle per cell under the experimental conditions used here, since 10^{11} particles were required to give a visible band. Similar experiments with *E. coli* cells are shown in figure 6, and suggest that particles in this size range would be seen at the one-per-cell level, since 10^{7} particles could easily be seen.

Buoyant Density Separations With Tissues

The separations obtained with CsCl banding of fractions initially isolated by rate-zonal centrifugation are shown in the lower part of figures 2, 3, and 4. From previous studies with density beads, the density of the endoplasmic reticulum and the density of glycogen are known (*15*, *64*) and are indicated in the figures.

In these experiments the absorbance of the recovered gradient from the B-IV rate-zonal rotor is shown along the upper portion of the diagram. The computer plot of equivalent sedimentation coefficients (S^*) is aligned immediately below the absorbance diagram and includes also a plot of the density of the recovered gradient. Along the bottom of the chart is a composite photograph of all of the isopycnic banding tubes, with the density indicated to the left of the photograph. It is evident at once, especially in brain tissue, that a wealth of information is to be gained from the careful examination of the observed bands. For the present purposes, however, the observation of a broad area between the level at

which membranous elements are found and the level of the glycogen band in liver and the absence of any well-defined bands below the membrane level in brain and spleen suggest that the distribution of particulate material seen in figure 1 does in fact occur. We may now be asked whether the so-called virus window area is sufficiently free of traces of particulate matter to allow small amounts of a test virus to be isolated and observed.

For this purpose suspensions containing known numbers of T3 bacteriophage were added to tissue homogenates prior to fractionation by the s-ρ system.

Purification of T3 Phage

A lysate of T3 phage was concentrated by continuous flow centrifugation in the B-V rotor (*65*) and further purified by banding in CsCl. On examination in the electron microscope, a few larger contaminating phage particles resembling T2 were observed. The sample was therefore purified by rate-zonal centrifugation (fig. 7). Two bands, indicated by arrows, were observed in CsCl. These were recovered and examined. The first band contained only T3, the second only contaminating T2 (fig. 8). The T3 sample was dialyzed against a dilute phosphate buffer and analyzed spectrophotometrically.

Recovery of T3 From Tissue Homogenates

Ten absorbance units (8.5×10^{12} physical particles) were added to 20 ml of a rat liver homogenate containing 4 g of tissue (approximately 1.6×10^9 cells) to give an average of 5×10^3 particles per cell. If the particles had pre-existed in the cell and been uniformly distributed, approximately 10 would have been visible on the average in an 8×10 inch electron micrograph at 20,000 $\times$ using 250 A thick sections. The results of s-ρ separation with this sample are shown in figure 9. A band absent from the control, but at the proper position for T3 was observed. Electron micrographs of the recovered band are shown in figure 10. In five 8×10 enlargements at 80,000 $\times$ an average of 4 virus particles per micrograph was seen. At the level of 5,000 particles per cell (one part in 3.3×10^3) the particles are grossly evident and can be easily identified in the banding tubes by light scattering and by electron microscopy. With an improved optical system for observing bands by light scattering, this number could probably be decreased at least an order of magnitude. Similar studies have been done with T2 bacteriophage.

Combined s-ρ separations of brain and spleen were also performed as shown in figures 11 and 12, using 8.5×10^{11} phage (one-tenth the amount used above). No well-defined bands were seen by scattered light. However, when samples were taken for electron microscopy at the levels indicated, virus particles were seen (fig. 10). It is evident that at the level of approximately 500 particles per cell virus particles can be detected easily. The lower limit reached in the present studies is 20 T3 phage

particles per average rat liver cell. Demonstration of the particles by negative staining required examination of several fields per particle found, however. If the entire virus window area is removed and examined by the improved method of Geister and Peters (*66*) which has a lower limit of 10^5 particles per ml, the level of one particle per cell could probably be reached. In additional experiments the location of virus particles in the s-ρ diagram has been confirmed by infectivity studies (unpublished observations).

The problem of whether phage particles sediment in a density gradient independent of the tissue particles has been examined by using P^{32}-labeled T3 phage added to a liver homogenate. The radioactivity distribution was very similar to that seen in figure 7 for free phage particles in the absence of tissue.

APPLICATION TO THE SEARCH FOR ONCOGENIC VIRUSES

The methods described here are applicable to the problem of trace virus isolation from tumors, especially if the particles sought have a high nucleic acid content. When the virus particles contain appreciable amounts of lipid and only a low percentage of nucleic acid, the virus particles would tend to band in, or close to, the endoplasmic reticulum band. The Bittner virus, for example, bands at a density level of 1.19 g per ml in potassium tartrate (*67*) but would probably band at a slightly higher density level in CsCl. However, the virus would generally be much less heterogeneous than the microsomes with respect to both sedimentation rate and density.

The problem of using larger amounts of starting material in the B-IV rotor may be solved by first performing differential centrifugation to isolate only particles in the virus sedimentation-rate range. Mitochondria and nuclei are removed first, and the microsome-virus fraction is then sedimented to remove it from soluble materials. With microsome-poor tissues such as spleen, concentrates from 100 g of tissue may thus be obtained in a sufficiently small volume for use as the starting sample in the rate run. A human breast tumor examined in an exploratory run yielded unidentified particles from the density level at approximately 1.18 (fig. 13). Similar particles have occasionally been seen in a control experiment with normal breast tissue. It is quite evident that no conclusions can be drawn as yet concerning the nature of the particles isolated, but it appears worthwhile to explore human tumors more thoroughly with this technique.

DISCUSSION

Rate-zonal separations of tissue homogenates followed by isopycnic-zonal centrifugation in angle-head centrifuge tubes have been used to obtain a two-dimensional separation of tissue components.

The gradients, centrifugation times, and the banding densities have been adjusted so that particles in the range of known viruses would be distributed through both gradients. The experimental question asked is: Are relatively homogeneous particles such as viruses separated into discrete bands or zones by this technique? This point is important in evaluating the behavior of particles known to be heterogeneous in size and, in some instances, banding density. The results indicate that good resolution is obtained in both the rate and isopycnic separations.

The absence of appreciable numbers of particles having the physical properties of the smaller viruses has been demonstrated in three tissues. In those instances where virus particles have densities bordering the microsome range, very considerable purification would still be expected on the basis of sedimentation rate alone from liver and spleen. Protein determinations showed that only a small part of the total tissue mass was recovered in each of the fractions in the virus range. In brain, however, a large mass of very light material was recovered in the middle of what has been considered the microsome region. In addition to demonstrating the resolution which may be obtained, these studies lay the groundwork for attempts to isolate viruses and virus-like particles from tissues suspected to contain them.

In the work described, rather steep CsCl gradients have been employed for the isopycnic studies. These covered the range from light membrane particles to glycogen. Careful inspection of these banding patterns reveals a number of bands not as yet identified. These deserve careful study in much shallower gradients capable of giving greater resolution and separation and in gradients prepared with other substances to minimize the possibility of artifacts.

The s-ρ separation methods appear to be a useful addition to our biophysical armamentarium for both separating and visualizing subcellular particles and viruses. The exploration of cells and tissues by these methods will require extended efforts which should include electron microscopy and chemical and enzymatic analyses.

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PLATES

794-527—66——29

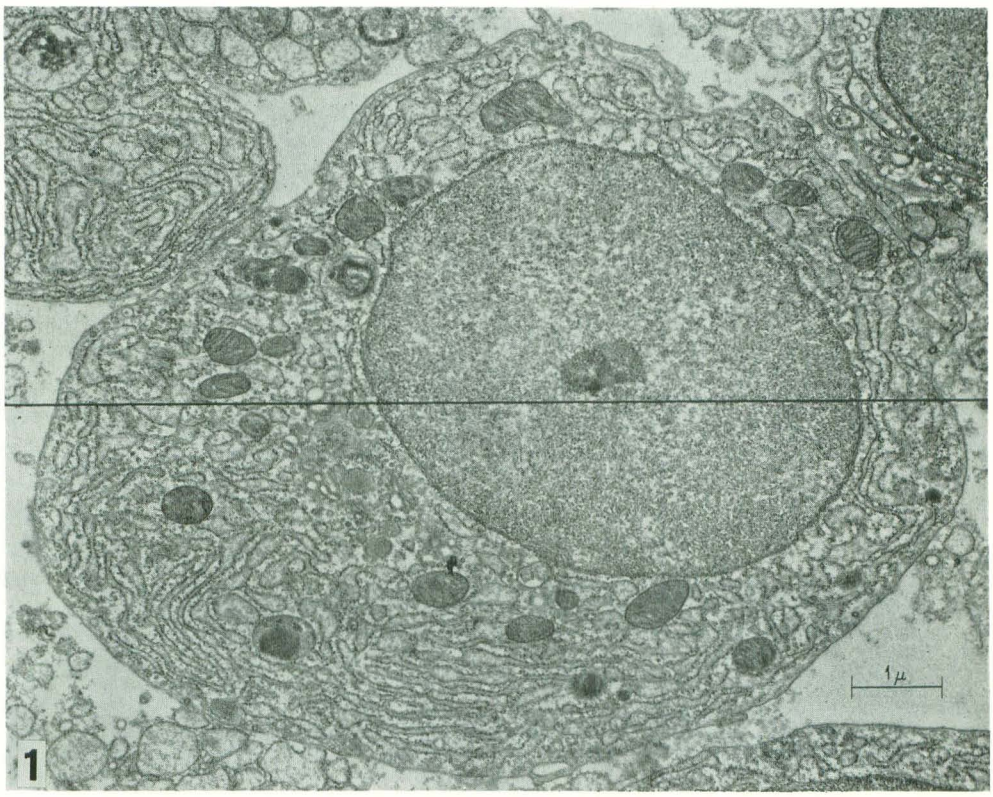

FIGURE 1.—Electron micrograph of section of cell from the lamina propria of the human intestine. The thickness of a 250 A section is shown as a *solid line*. (Electron micrograph courtesy of Dr. John L. Watson.)

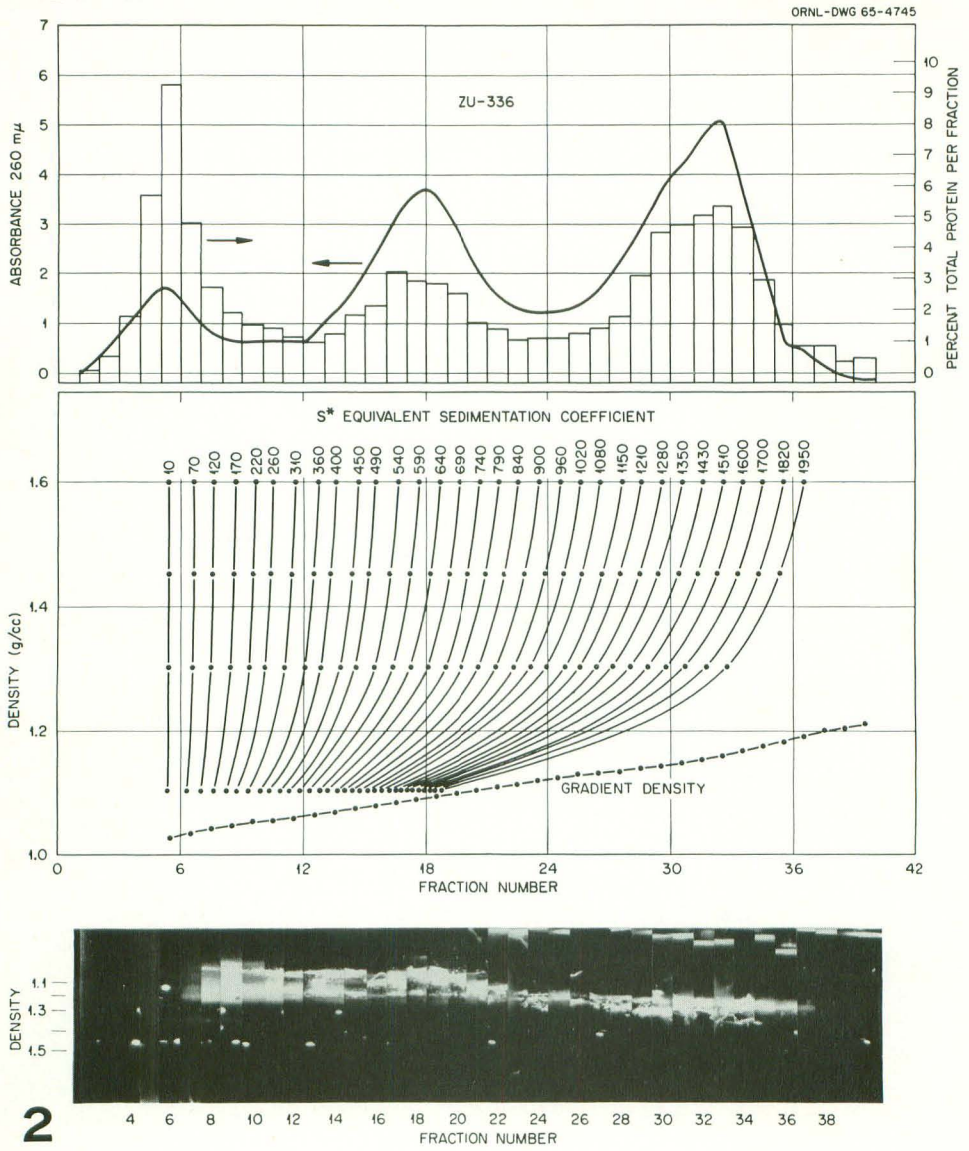

FIGURE 2.—Combined rate-zonal and isopycnic-zonal separation of particles found in a rat brain homogenate. The sample layer was 20 ml of a 20 percent w/v homogenate of fresh unperfused rat brain. The *upper, center,* and *lower* sections give, respectively, the results of rate-zonal centrifugation in the B-IV rotor, the computer output for determining equivalent sedimentation coefficients, and the results of isopycnic banding using cesium chloride. In the *upper diagram* the absorbance at 260 mμ was determined with a 0.2 cm light path cell and the observed absorbancies were multiplied by 5. The results of the protein analyses (*bar diagram*) indicate the percent of total homogenate protein found in each fraction. In the computer printout the positions of hypothetical particles having the densities indicated along the ordinate are plotted together with the density gradient curve. In the lower photograph the banding density pattern in cesium chloride of the fractions previously separated by rate-zonal centrifugation. *White dots* in this composite photograph are plastic density-indicator beads.

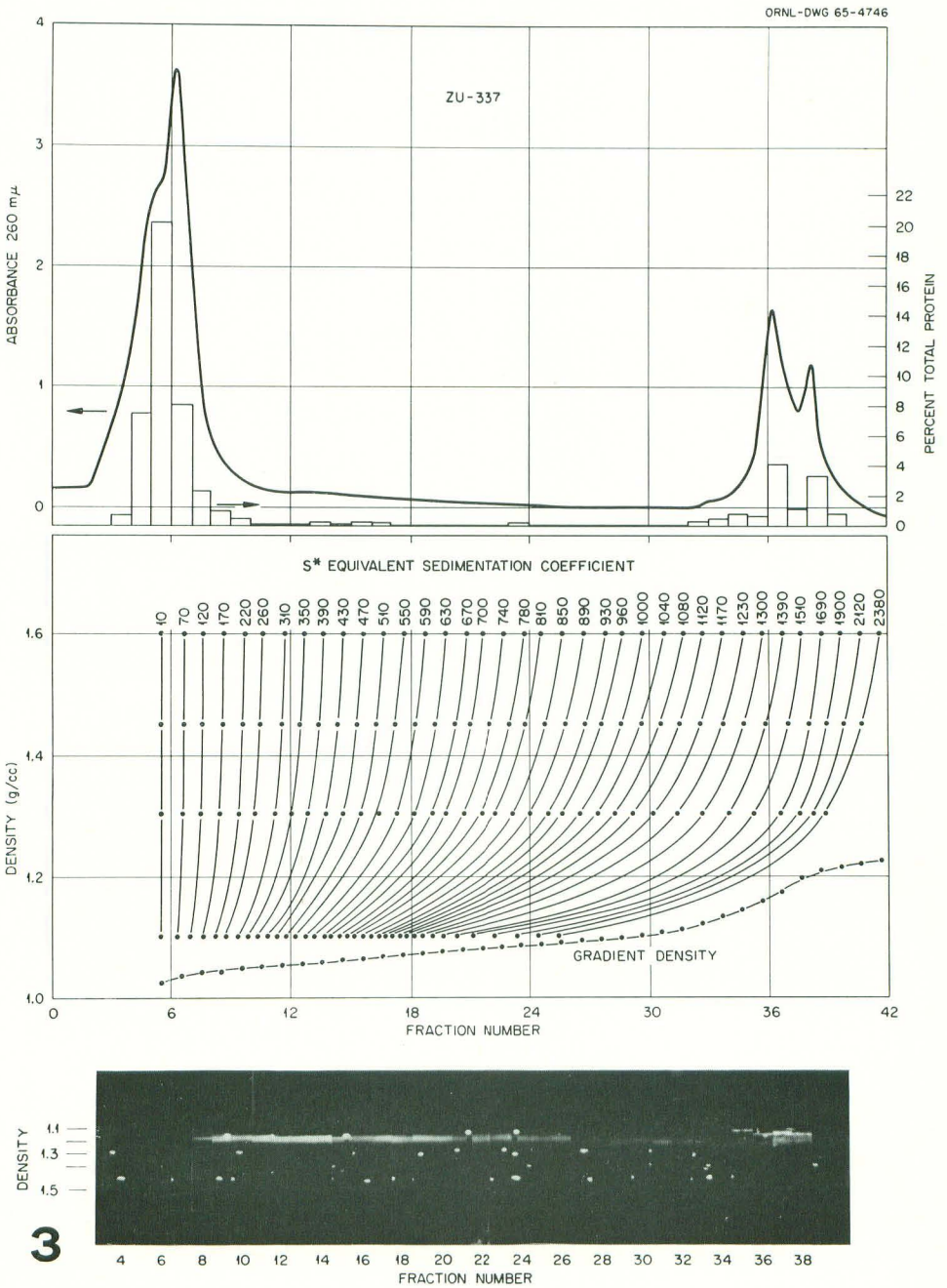

FIGURE 3.—Combined rate-zonal and isopycnic-zonal separation of particles found in a rat spleen homogenate. For details *see* legend of figure 2.

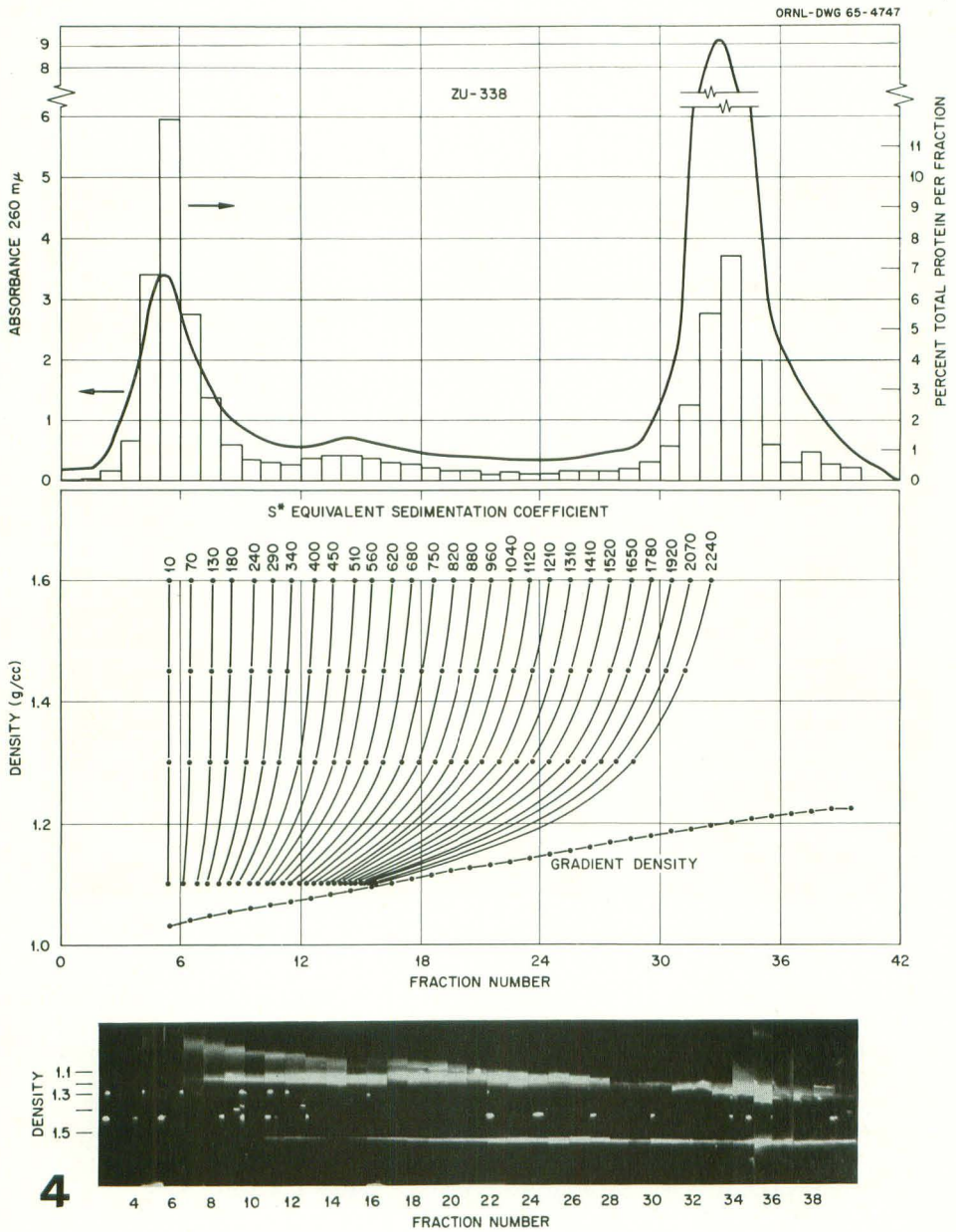

FIGURE 4.—Combined rate-zonal and isopycnic-zonal separation of particles found in a rat liver homogenate. For details *see* legend of figure 2.

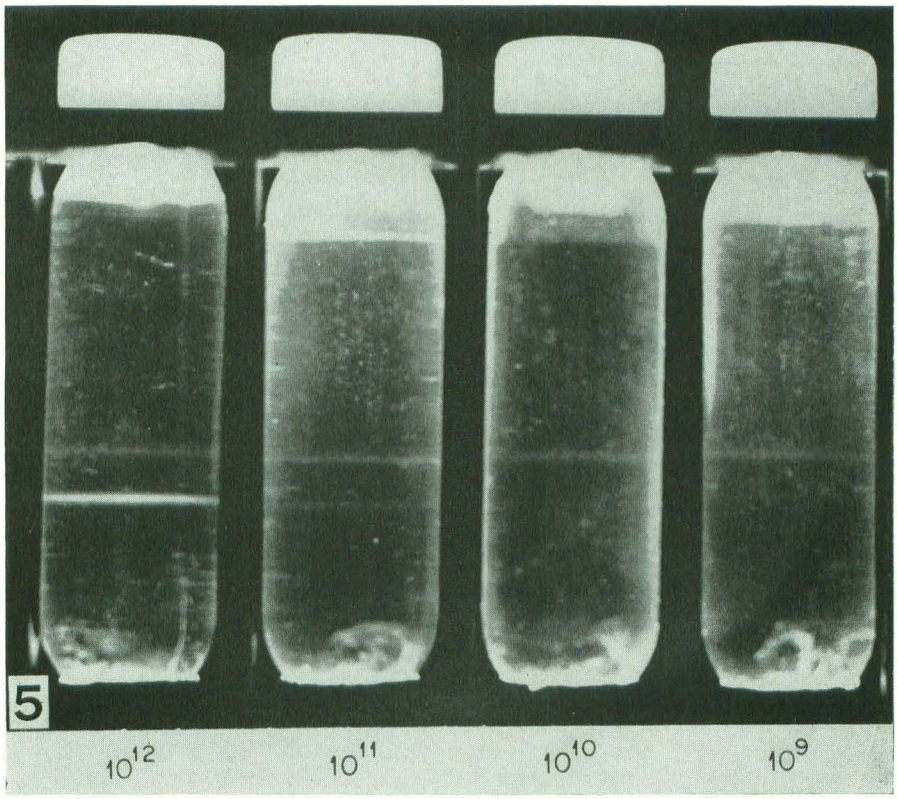

FIGURE 5.—T3 phage banded in CsCl and photographed by scattered light. The total number of particles in each tube is indicated. The *band in the center* of all 4 tubes is due to the presence of small numbers of bacteria and serves to indicate the reproducibility of the gradients. The *lower band* clearly seen in the 10^{12} tube is the phage band. It is also visible at 10^{11} but not when only 10^{10} or 10^{9} particles are present.

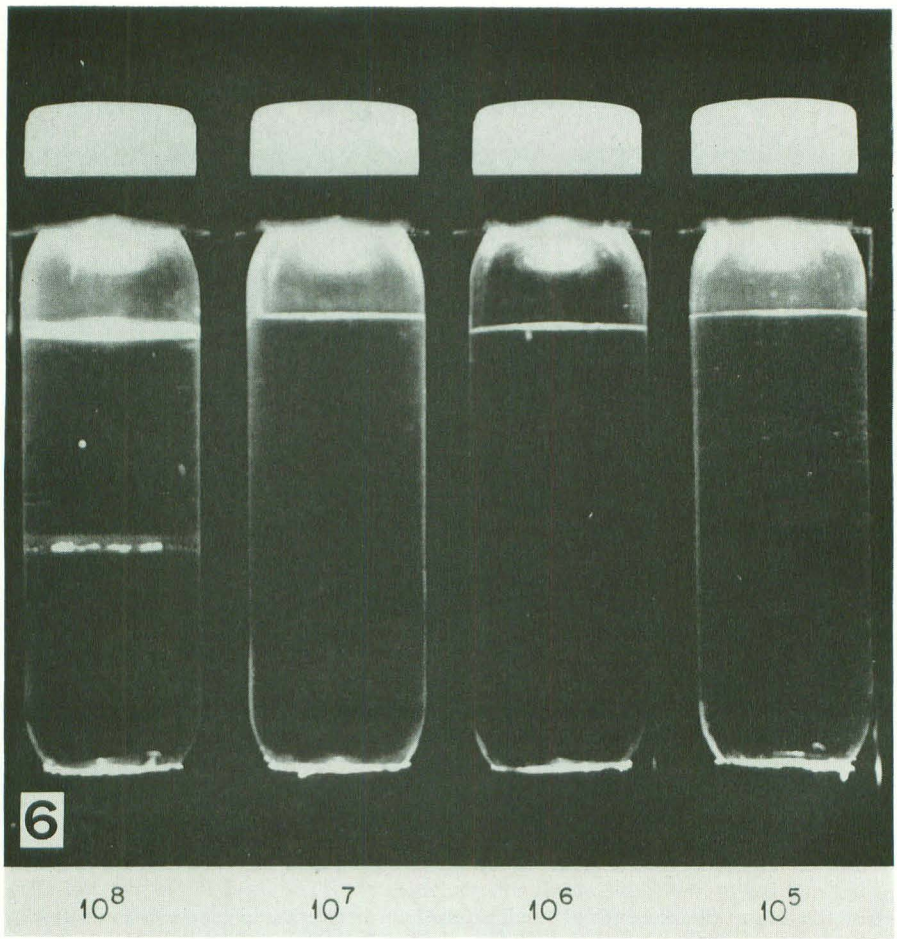

FIGURE 6.—Banding of *Escherichia coli* cells in a potassium citrate gradient. The total number of cells present is indicated. The lower limit of detection appears to be 10^7 cells.

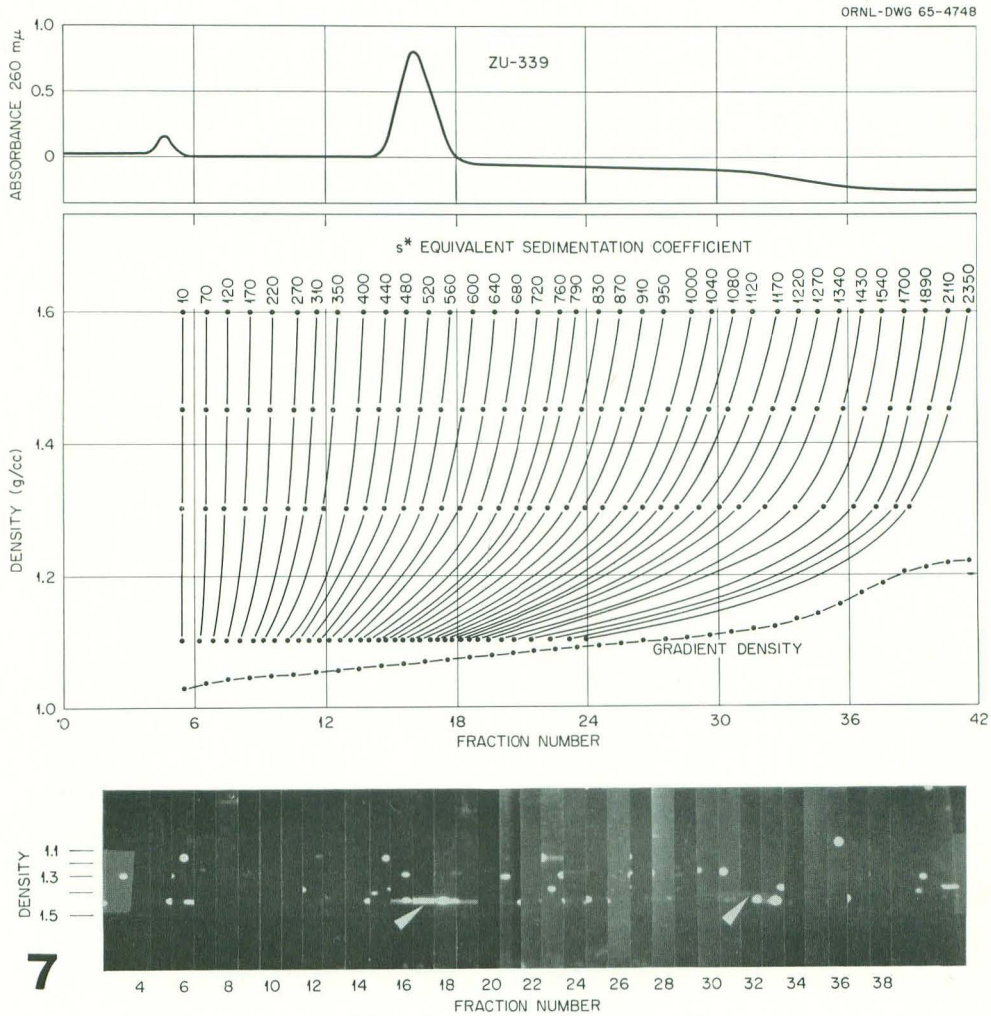

FIGURE 7.—Combined rate-zonal and isopycnic-zonal separation of T3 phage preparation contaminated with T2 phage. Position of the two virus bands indicated by *arrows* in the *lower pycnogram*.

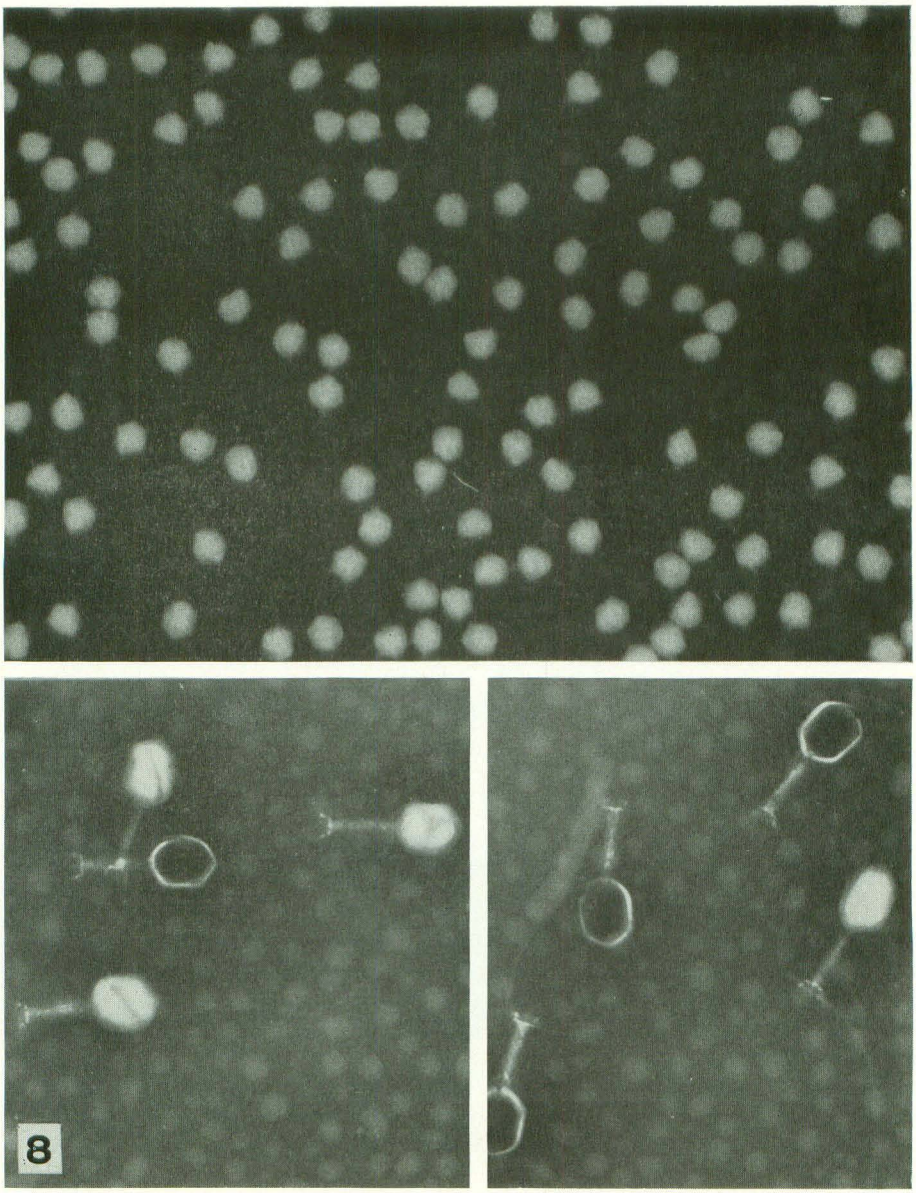

FIGURE 8.—T2 and T3 bacteriophage recovered from bands indicated in figure 7. *Upper micrograph* shows T3 particles from fraction 17; *lower two micrographs* show T2 particles recovered from fraction 32. × 80,000

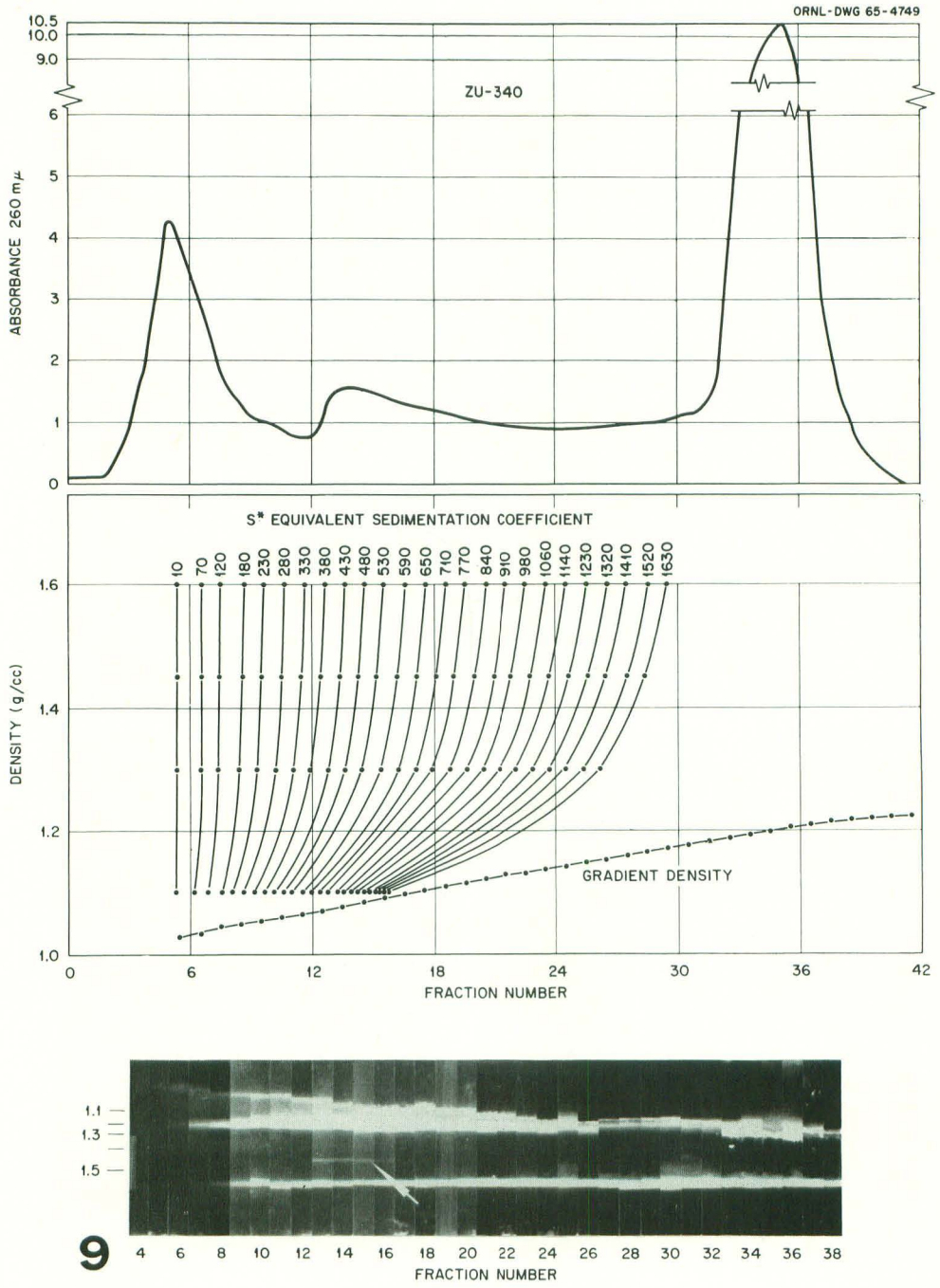

FIGURE 9.—Recovery of added T3 phage from a rat liver brei using the s-ρ technique. The particles from the band indicated in the *pycnogram at the bottom* were recovered and are shown in figure 10.

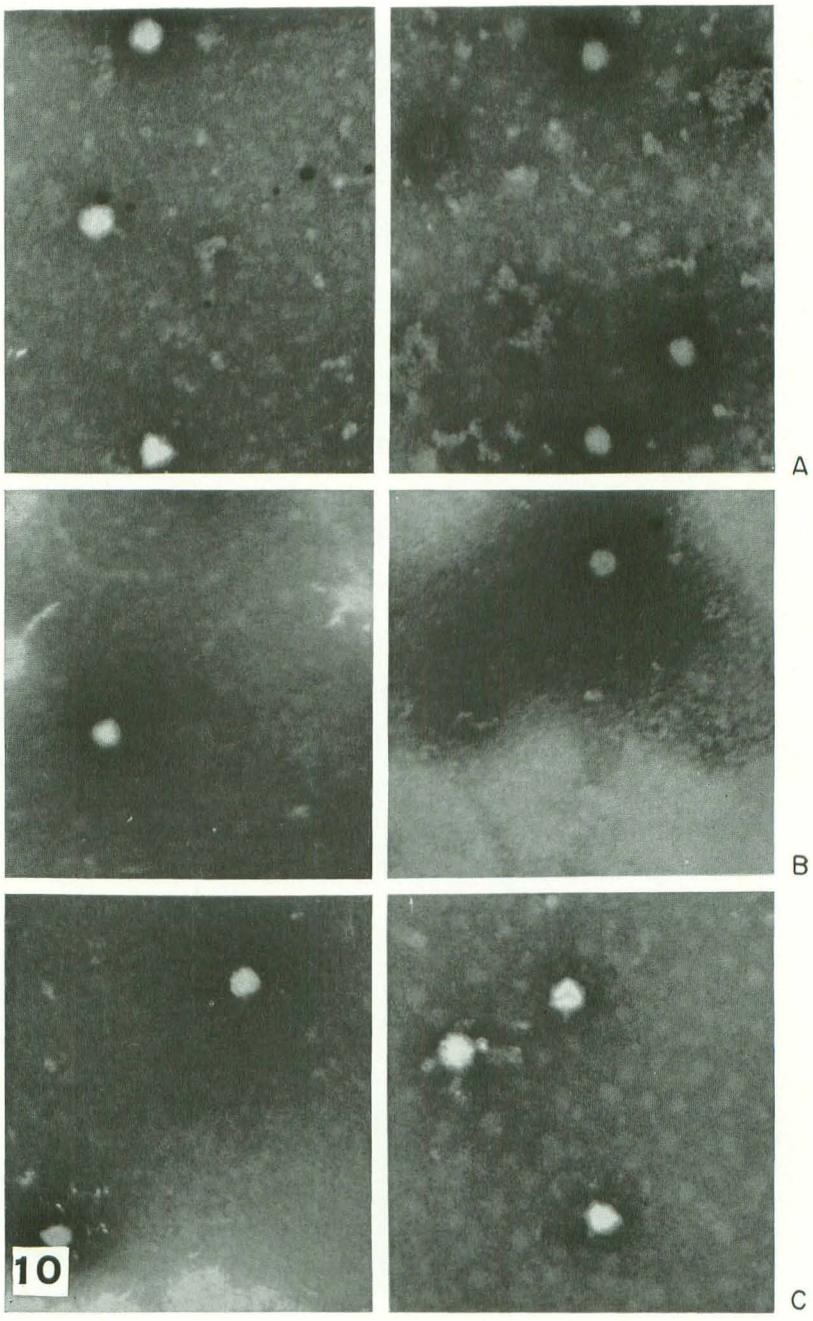

FIGURE 10.—Electron micrographs of T3 bacteriophage recovered from rat tissue homogenates. The positions from which the samples were recovered are indicated in figures 9, 11, and 12. A, liver; B, brain; and C, spleen.

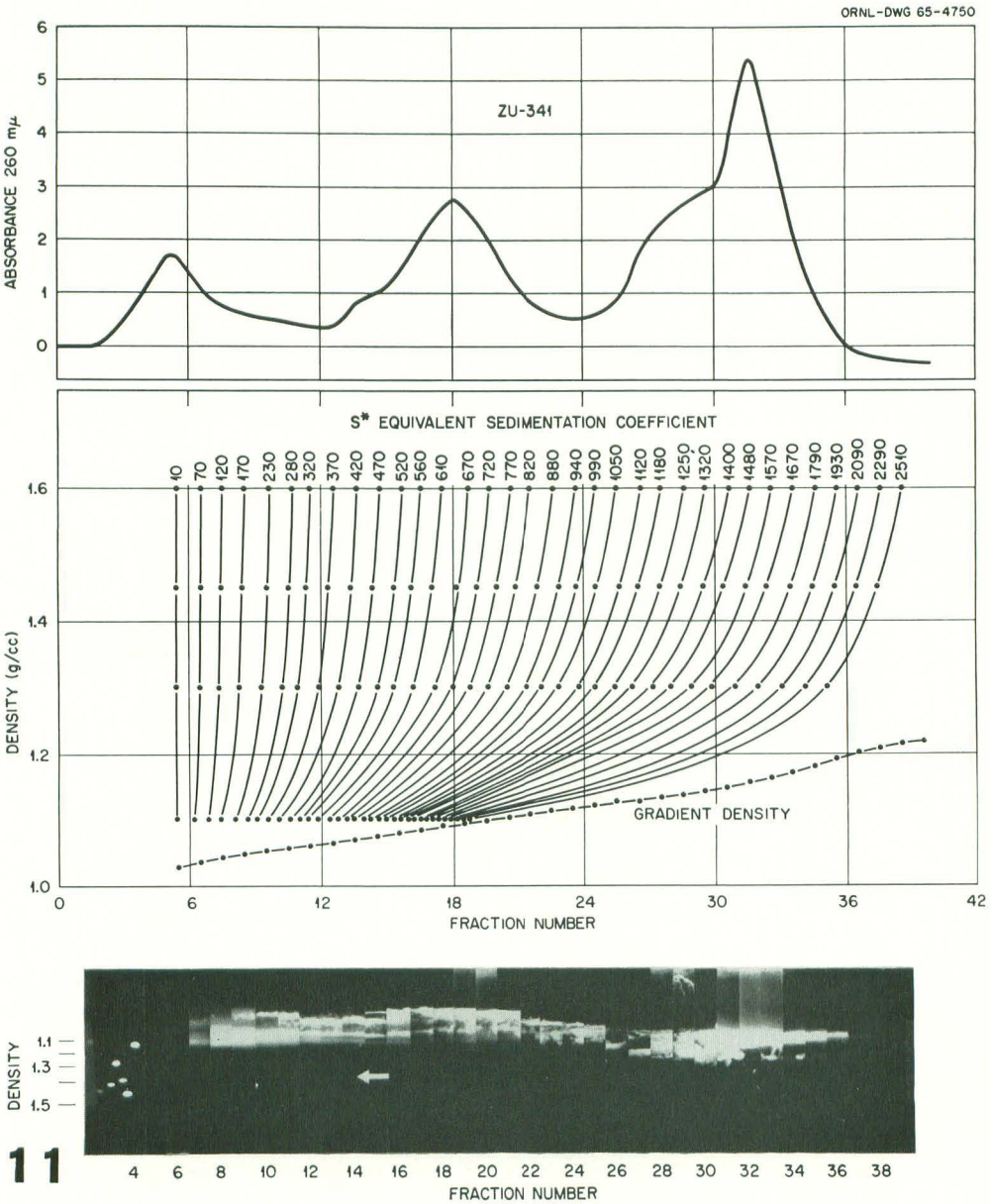

FIGURE 11.—Recovery of added T3 phage particles from rat brain brei using the s-ρ technique. Particles recovered from the density level indicated in the pycnogram were recovered, concentrated, and are shown in figure 10.

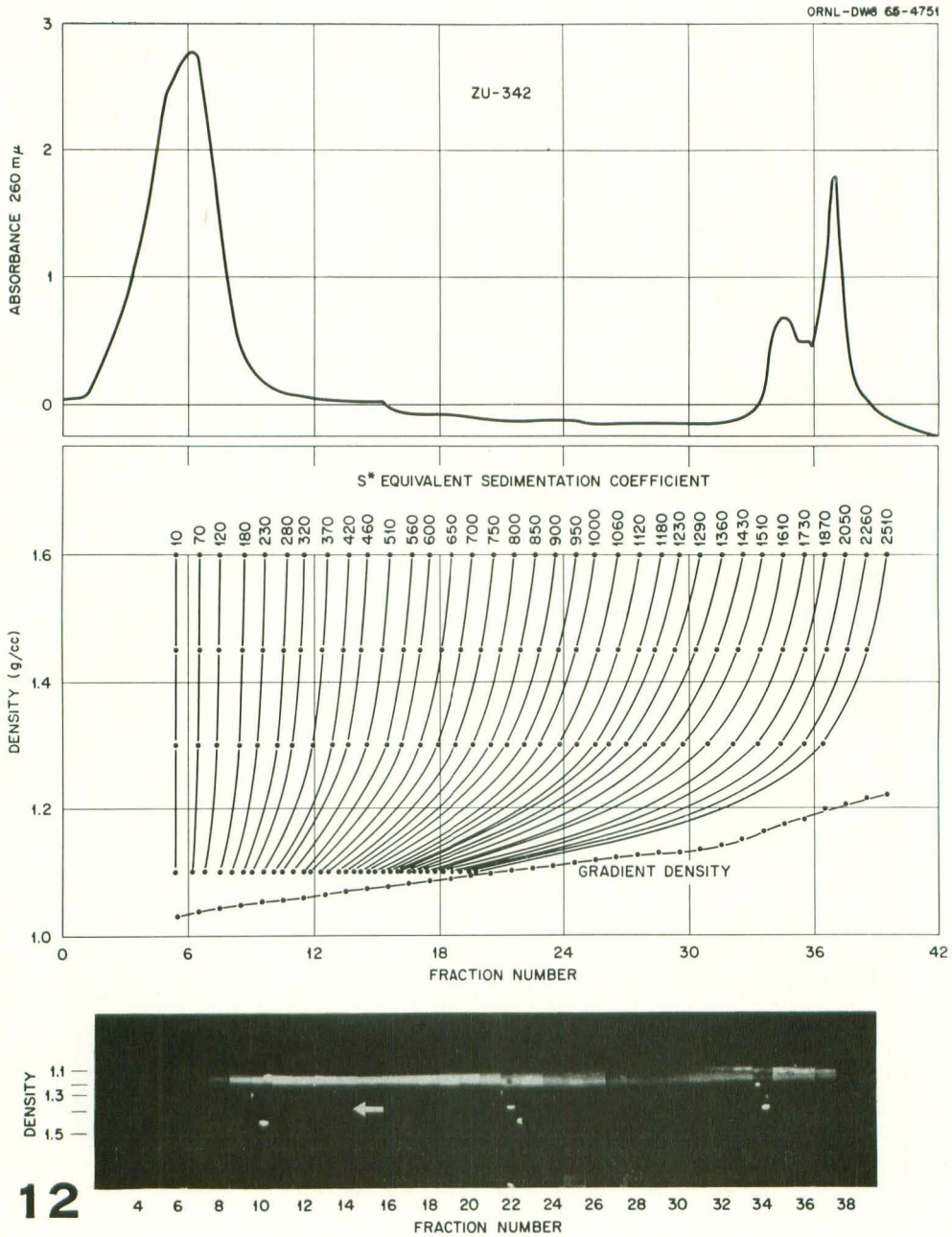

FIGURE 12.—Recovery of added T3 phage particles from a rat spleen homogenate using the s-ρ technique. Particles recovered at the density level indicated in the pycnogram were recovered, concentrated, and are shown in figure 10.

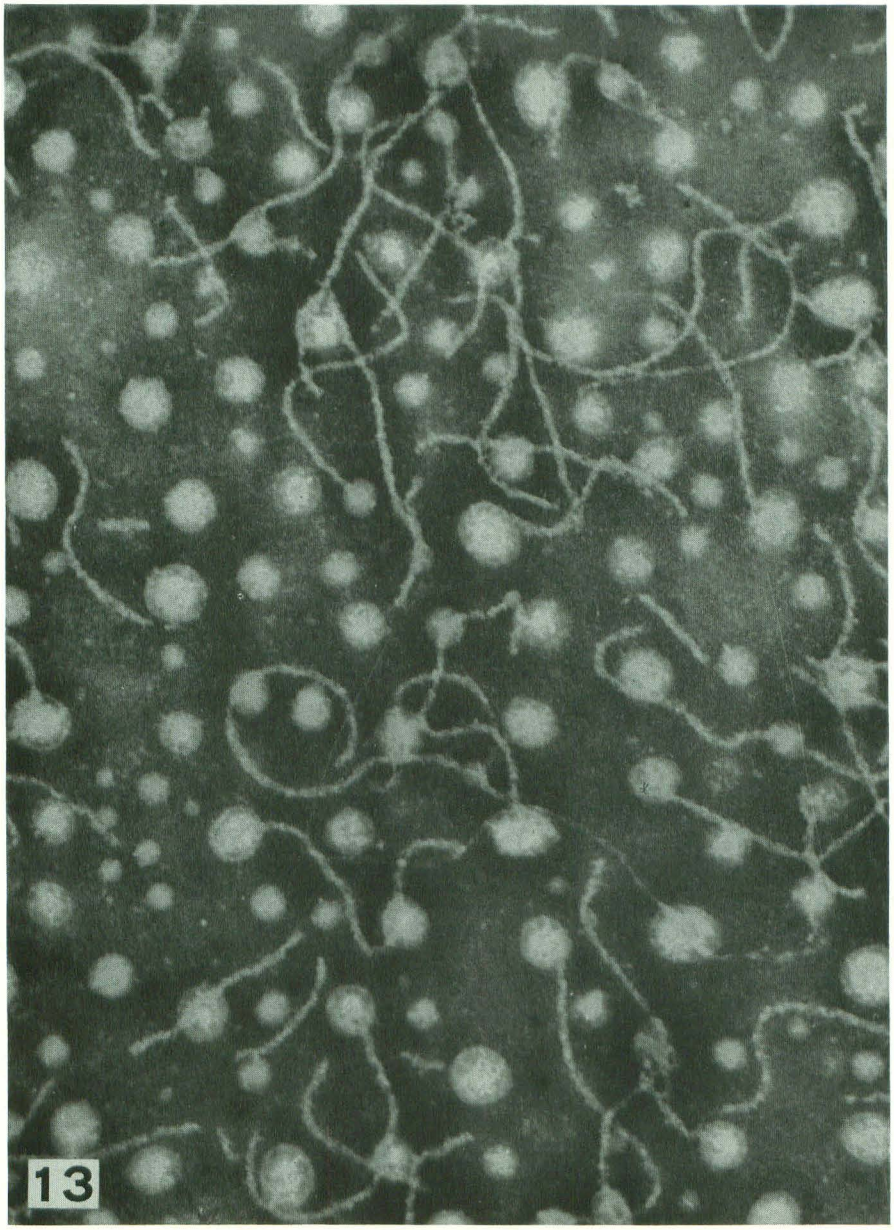

FIGURE 13.—Particles observed in a fraction obtained by rate-zonal centrifugation and isopycnic banding of human breast tumor. Negative staining. × 80,000