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BIOLOGY DIVISION

THE JOINT NATIONAL INSTITUTES OF HEALTH-ATOMIC ENERGY COMMISSION  
ZONAL CENTRIFUGE DEVELOPMENT PROGRAM  
SEMIANNUAL REPORT

For Period July 1-December 31, 1963

N. G. Anderson  
Program Coordinator

AUGUST 1964

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OAK RIDGE NATIONAL LABORATORY  
Oak Ridge, Tennessee  
operated by  
UNION CARBIDE CORPORATION  
for the  
U. S. ATOMIC ENERGY COMMISSION

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## Summary

This report, the third under the Joint National Institutes of Health-Atomic Energy Commission Zonal Centrifuge Development Program, describes work on methods for separating subcellular particles, including viruses, being carried out at the Biophysical Separations Laboratory of the Oak Ridge Gaseous Diffusion Plant and at the Biology Division of the Oak Ridge National Laboratory.

The major efforts during this period have been directed toward the development of integrated centrifugal systems for isolating trace quantities of virus from tissues, plasma, and culture fluids. It has been found that when the sedimentation rates of subcellular particles are plotted against the particle banding densities, a vacant area, or "window," appears in the area where most viruses would be plotted. To exploit this "virus window," it has been necessary to develop methods for separating masses of particulate material first on the basis of sedimentation rate and second on the basis of density, and to adapt analytical methods to the description of the separations. To translate these theoretical possibilities into reality, the B-IV zonal centrifuge has been developed, together with methods for multiple-fraction isopycnic banding. The resolution of the system has been studied using a variety of biological materials, and several types of virus particles have been recovered in a high state of purity from tissue breis. The applicability of this system to the problem of searching human tumors for virus-

like particles is now being explored. Before this can be effectively done, however, a survey of normal tissue components isolated by these systems is required. By use of a simplified system for particle isolation, virus-like particles have been consistently recovered from human leukemic plasma.

Isolation of virus particles by continuous-flow centrifugation has been examined both theoretically and experimentally. A rotor (B-V) capable of sedimenting particles as small as Echo 28 has been constructed and successfully tested. Since many viruses are inactivated by pelleting, the possibility of devising a high-speed continuous-flow centrifuge in which the virus particles are sedimented into a density gradient has also been examined. Two systems (B-VI and B-VII) designed and built during this period showed both the feasibility of this approach and the difficulties to be overcome. On the basis of this work, rotor B-VIII has been built and successfully tested.

The low-speed A-IX rotor has undergone extensive tests with cell fractions from plant and animal sources, and the high-speed C series rotors have been gradually moved from the level of experimental test systems to that of biological experimentation.

The program continues to be an interesting and a productive experiment in the adaptation of defense-oriented technologies to biomedical purposes.

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## I. Introduction

The Oak Ridge installation of the Atomic Energy Commission (AEC) was originally organized to achieve difficult separations. These included the separation of uranium isotopes by two large-scale processes, the separation of plutonium and other fission products, and the isolation of numerous radioisotopes from contaminating substances. It has been of interest to determine whether certain of the skills, techniques, facilities, and individuals associated with these AEC programs could be adapted to the solution of difficult separation problems encountered in biomedical research. The separation of subcellular organelles and viruses presents problems which are comparable in difficulty with some of the isotopic separations. If small virus particles existing at levels as low as, for example, one particle per cell are to be isolated, it will be necessary to achieve purifications ranging from  $10^6$  to  $10^8$  fold. The centriole, a key organelle in the process of cell division, has not been isolated from mammalian cells to date owing to the lack of high-capacity high-resolution separation systems. It is not unlikely that other cell components which occupy only a small fraction of the cell mass remain to be discovered.

The common denominator of our present interests is the virus particle. Evidence has accumulated suggesting that ionizing radiation may activate latent oncogenic viruses in animals. The mechanisms involved are obscure. However, virus infection is known to produce chromosome breakage in tissue culture and may conceivably cause the specific chromosomal abnormalities observed in human leukemic cells. The central experimental difficulty is that the definitive experiments which are possible in animals cannot be done in man. The problem of whether human leukemia, for example, may involve a virus released by radiation is not amenable to a simple solution.

The direct approach, that of looking for the virus by physical means, brings to one focus certain of

the interests existing in the AEC, the National Cancer Institute, and the Biophysical Separations Laboratory at Oak Ridge. Specifically, the development of virus-isolation techniques is germane to the problems of the cause of human cancer generally, the relation of radiation to human neoplastic disease, especially leukemia, and the physical problem of the separation of particles in centrifugal or other physical systems. From the biophysical viewpoint many virus particles are nearly ideal test objects for the study of separation methods. Viruses can be detected over an extremely wide range of concentrations by biological and physical methods, they exist in a size range which falls within the size spectrum of subcellular particles, and they are, in many instances, both nearly spherical and of uniform size and density. Thus the problem of isolating trace quantities of virus from cells and tissues is of interest from many points of view. The problems to be solved are, for the most part, concerned with the development of separation methods having the highest resolution that can be obtained. Only as the physical, colloid-chemical, mathematical, metallurgical, and engineering problems involved are solved can the parallel biological problems be attacked. Initially, therefore, this program must be concerned with the development of tools, their perfection, and their calibration. As this is accomplished, emphasis can be shifted to their application to pertinent biological problems.

It should be emphasized that concentration on the isolation of one type of subcellular particle — the virus — does not diminish the applicability of the systems developed to the isolation of other subcellular particles. On the contrary, the isolation of particles suspected to be heterogeneous in their physical properties can best be done after it has been demonstrated that the polydispersity observed is not due to low resolution of the isolation system.

## II. Biomedical Applications of Zonal Centrifuges

### A. ISOLATION OF TRACE AMOUNTS OF VIRUS FROM TISSUES

N. G. Anderson    C. L. Burger  
W. W. Harris

We shall address ourselves first to the problems of whether, in theory, trace amounts of various types of virus particles can be isolated in a high state of purity from tissues; second, to the various methods by which such separations might be accomplished; and third, to experimental studies with model systems.

#### 1. Viruses and Subcellular Particles

Initially the question must be asked: Are most virus particles sufficiently different from the particles found in broken cell suspensions to allow their isolation by physical methods in a reasonable state of purity? On purely theoretical grounds it is evident that particles having very different densities may have the same sedimentation coefficients 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 of the particle and the solvent. In the case of the uniform spherical viruses, we wish to isolate particles of one radius and one density. Separations based on sedimentation rate are sensitive to both parameters, while isopycnic separations depend, obviously, on density alone. To make separations of maximal resolution, therefore, it is necessary to perform both a rate-zonal and an equilibrium, or isopycnic, fractionation. The choice of the order of the separation depends on several considerations. Cell nuclei, which may have the same equilibrium density as certain vi-

ruses, are disrupted or extracted by the high salt concentrations employed in most equilibrium centrifugation procedures, including those using cesium chloride. Such extracted nucleoprotein would not behave as do nuclei in subsequent rate-zonal separations. Other cell components may also be altered by salt extraction. An additional disadvantage is that in equilibrium studies with presently available systems, sufficient centrifugal force to band soluble proteins sharply in a relatively short time is not generally available. Banded virus particles may therefore not be free of contamination by smaller particles which have not reached their isopycnic points. Further, the rate separations, which do not involve expensive materials, but which do require relatively large volume gradients, may be done at a lower speed than that usually required for isopycnic banding.

To maximize the particle radius dependence of the rate separations, the difference in density between the particle of interest and the solvent should be maximized, and the separations carried out at the lowest density practicable. This is evident from the fact that the effect of radius on sedimentation approaches zero as the term  $(\rho_p - \rho_0)$ , where  $\rho_p$  is the density of the particle and  $\rho_0$ , the density of the solvent, approaches zero (i.e., particles of any radius may band at the same density level if they have the same density). These considerations suggest that the maximal separation for scanning purposes may be obtained by first separating subcellular particles on the basis of sedimentation rate in a zonal centrifuge and then subfractionating the fractions obtained on the basis of density alone. Alternate methods adapted to the problem of isolating specific particles will be discussed in subsequent reports. Of interest here is the question: Will such separations yield relatively pure viruses?

### 2. Virus Window

Restating the above question more precisely: If all cell constituents, with the exception of viruses, are plotted with the sedimentation rate in water at 20°C along the abscissa and banding density along the ordinate, will there be a vacant space, or "window," in the area where most viruses would fall? If viruses are theoretically separable by such a combination of methods, at

what concentration in a tissue can they be detected using available physical detection methods?

Data on the sedimentation rates and densities of viruses and various cell components are given in Fig. II-A-1. The values for the sedimentation coefficients are corrected to water at 20°C. In practice, separations dependent on density alone are made in salt gradients. The isopycnic point of a given particle is not the same in all gradients; it will depend on the degree of hydration of the

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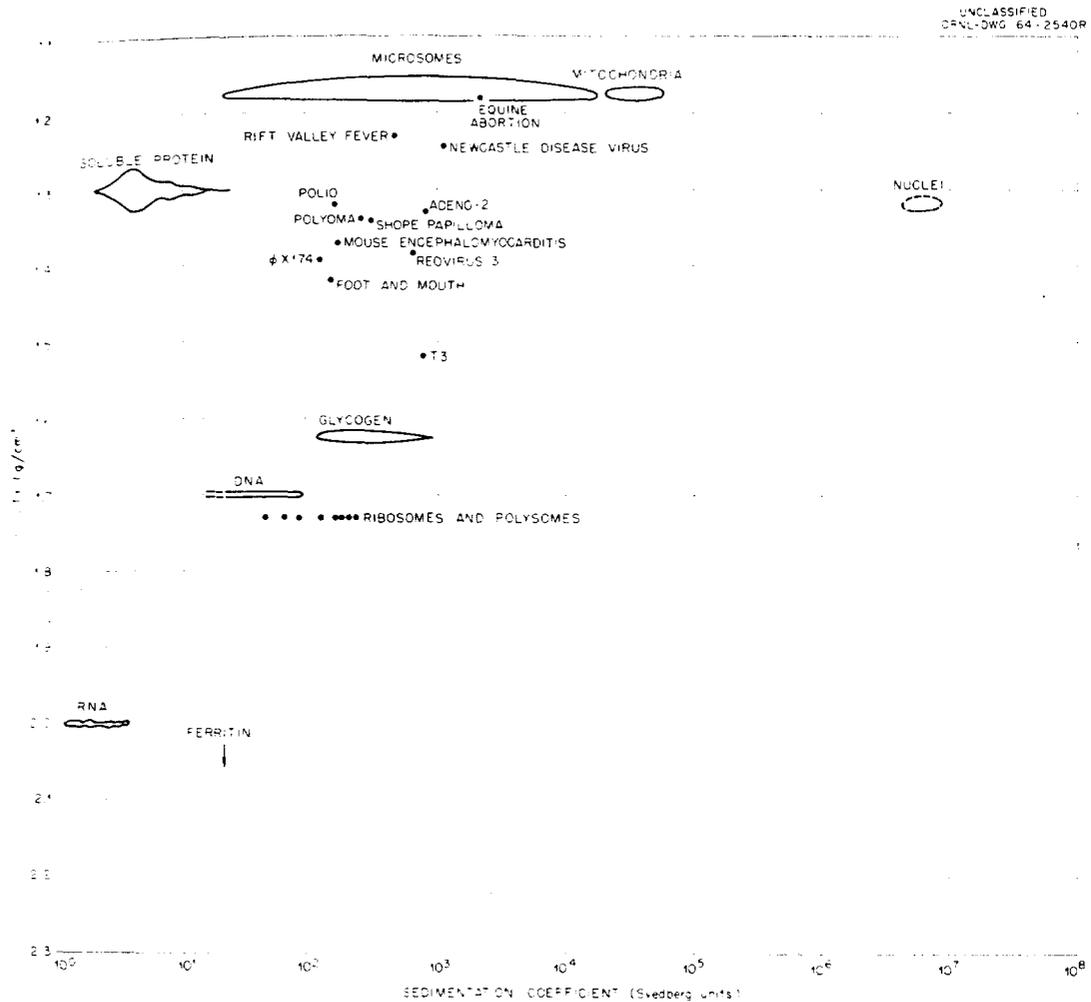


Fig. II-A-1. Sedimentation Coefficients and Banding Densities of Cell Components and Viruses. Sources of data given in text. Note that most virus particles fall in an area, here termed the "virus window," essentially free of cell constituents.

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particle in a given medium, the permeability of the particle to the solutes used to make the gradients, and the extent of the solute binding. The banding densities given, unless otherwise stated, are for cesium chloride. In other gradients the values may be numerically different, but the overall distribution of viral and cell component densities would tend to remain the same. The values for the sedimentation rates of proteins are those for rat liver (Anderson and Canning, 1959) which do not differ greatly from the values for rat brain, kidney, and testes. The density ranges for soluble tissue proteins have not been determined in CsCl; however, they probably do not vary greatly from the value of 1.295 reported for bovine serum albumin (BSA) and for human hemoglobin (Cox and Schumaker, 1961). Values for the sedimentation coefficients of ribosomes and polysomes are for rabbit reticulocytes (Gierer, 1963), and the densities are those extrapolated from *Escherichia coli* ribosome data (Tissieres *et al.*, 1959). It is assumed that polysome densities would not be very different. The range of sedimentation coefficients shown for "microsomes" is that observed with rat liver in the zonal ultracentrifuge, and the density range was obtained by banding in CsCl fractions separated in the zonal ultracentrifuge. The sedimentation rates for mitochondria are calculated from the data of de Duve *et al.* (1959) and of Thomson and Klipfel (1958). Nuclear sedimentation coefficients are calculated values assuming diameters ranging from 6 to 12  $\mu$  and an average density of 1.040 (Falzone *et al.*, 1962) in dilute buffer. The CsCl density is estimated on the assumption that nuclei contain 77% protein and 19% DNA (Hale and Kay, 1956) and that the isopycnic densities would be the sum of the density of each component times the percentage present. In practice, the nucleoprotein would dissociate, at least partially, and would probably yield several bands. The nuclear isopycnic density is therefore a calculated value given to indicate a region where separations of nuclear from nonnuclear materials would be difficult. The position of glycogen is taken from studies on the distribution of sedimentation coefficients of material isolated from liver (Meyer and Zalta, 1958; Bell *et al.*, 1948; Polglase, Brown, and Smith, 1952). The banding density in CsCl is taken from experimental studies carried out in this laboratory (Anderson, unpublished) using glycogen isolated from rat liver. The value is

slightly higher than that previously reported (Bell *et al.*, 1948; Bryce *et al.*, 1958). Ferritin was isolated from human leukemic spleen by zonal centrifugation followed by isopycnic banding.

Deoxyribonucleic acid (DNA), which would not ordinarily be found free in homogenates of mammalian cells, but would be encountered in bacterial homogenates, and might be found extracted from viruses during the preparation of pathological tissues, is also included in Fig. II-A-1. The banding density range is taken from Schildkraut, Marmur, and Doty (1962), and the range of sedimentation rates includes that of single DNA molecules from T2 phage (Hershey, Burgi, and Ingraham, 1962) 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 (Davern and Meselson, 1960) has therefore been used. The sedimentation rate range is taken from a number of sources (Cheng, 1962); (Cox and Amstein, 1963). The accurate positioning of cell components from various tissues in the sedimentation rate-banding density (hereafter termed  $S-\rho$ ) 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 (Green, 1962; Kohler, 1962); equine abortion virus (Darlington and Randall, 1963); foot-and-mouth disease virus (Breese, Trautman, and Bachrach, 1960; Trautman and Bachrach, personal communication); mouse encephalomyocarditis virus (Faulkner *et al.*, 1961; Hansen and Schäfer, 1962); Newcastle disease virus (Rott, Reda, and Schäfer, 1963; Stenback, 1963);  $\phi$ X 174 (Sinsheimer, 1959; Eigner, 1962); polio virus (Holland *et al.*, 1960; Polson and Levitt, 1963); mouse polyoma (Crawford and Crawford, 1963; Howatson and Crawford, 1963; Mayor, 1961; Melnick, 1962; Winocour, 1963); reovirus 3 (Gomatos and Tamm, 1963); Rift Valley fever virus (Naude, Madsen, and Polson, 1954; Polson and Levitt, 1963); Rous sarcoma (Crawford and Crawford, 1961; Epstein, 1956, 1958; Epstein and Holt, 1958); and Shope papilloma virus (Breedis, Berwick, and Anderson, 1962; Crawford and Crawford, 1963).

It is evident from an examination of Fig. II-A-1 that a "window" appears in the  $S-\rho$  plot which includes a minimum amount of nonviral material, and that a large fraction of known viruses would

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viruses would

fall in it. In those instances where a virus has  
the same banding density as a part of the micro-  
somal fraction, it is evident that the virus cannot  
be completely separated from contaminating mate-  
rial by centrifugal procedures. Chemical (Kovacs,  
1962) or enzymatic dissection may then be neces-  
sary. Since the microsomal particles have a  
wide range of sedimentation rates, however, it  
should be possible to isolate the virus from the  
majority of the microsomal mass. The amount of  
endoplasmic reticulum present which gives rise to  
microsomes varies widely in different cells and  
tissues but is low in many tumors and in some  
cells used for virus production.

These considerations have prompted us to ex-  
plore methods for large-scale separation of sub-  
cellular constituents which would be analogous  
to the separations shown diagrammatically in  
Fig. II-A-1.

### 3. Detection of Virus Particles

Infectivity in a suitable test system will remain  
the most sensitive method for detecting virus  
particles. The physical methods for detecting  
viruses isolated in a reasonable state of purity  
include absorbance in the ultraviolet, light  
scattering, electron microscopy, and measurement  
of fluorescence after conjugation with fluorescent  
antibodies. The usefulness of each of these  
methods depends on the concentration of virus  
achieved. By the use of negative staining tech-  
niques, particle counting methods, or sectioning  
of pellets, it appears that the identification of  
virus particles on a morphological basis is feasi-  
ble at the level of one particle per cell if the virus  
is obtained in a pure form in the same concentra-  
tion in which it existed in the source tissue. For  
example, if liver containing  $4 \times 10^8$  cells/g is  
used, then the final virus concentration ( $4 \times$   
 $10^8$ /ml) would be at the lower limits of detection  
using spray counting methods (Dourmashkin, 1962;  
Breese and Trautman, 1960) but well within the  
range of the Sharp counting technique (Sharp and  
Beard, 1952; Sharp and Overman, 1958; Smith and  
Benyesh-Melnick, 1961).

For detection of most viruses by ultraviolet  
absorbance measurements, somewhat larger  
amounts of virus will be required. The number of  
optical density units necessary will depend on  
the optical density 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 ab-  
sorbance unit, then  $13 \times 10^{12}$  particles of the  
small foot-and-mouth disease virus (Trautman,  
personal communication),  $1.2 \times 10^9$  physical  
particles of poxvirus (Joklik, 1962), or  $2.3 \times 10^6$   
vaccinia particles (Planterose, Nishimura, and  
Salzman, 1962) would be required. This limit  
may be lowered by the use of long light path cells  
(Reimer, personal communication), 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 is found in the starting tissue,  
assuming that only one virus particle is found per  
cell. For very large viruses, however, the direct  
absorbance measurements would probably suffice.

Light scattering as a method of detecting virus-  
sized particles in gradients is capable of much  
greater sensitivity than absorption measurements.  
As will be shown in subsequent sections, this  
method is sensitive below the level where elec-  
tron microscope counting is ordinarily done. At  
the arbitrary lower limit of interest here ( $\sim 10^8$   
particles/ml), light scattering appears to be the  
gradient monitoring method of choice.

It is concluded that if methods can be developed  
which will allow only those particles having a  
narrow range of sizes and densities characteristic  
of a given virus to be isolated, and if the final  
concentration is equal to or greater than the con-  
centration in the tissue of origin, then virus parti-  
cles which exist at a level as low as one particle  
per cell can be isolated and described. Since the  
mass of a virus varies from approximately  $10^{-6}$   
(Rubin, 1962) to  $10^{-8}$  of that of a cell, the purifi-  
cation (as distinguished from concentration) re-  
quired is from a million to 10 million fold.

### 4. Determination of Sedimentation Rates

A computer program (report ORNL-3502 and  
Chap. XIV of this report) is used to determine the  
equivalent sedimentation rates ( $S^*$ ), in water at  
20°C, which spherical particles which do not be-  
have osmotically would have if found at various  
points in the gradient after a given period of  
centrifugation. The basic equation is a modifica-  
tion of that used by Martin and Ames (1961). In a

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zonal centrifuge the position of a given particle is determined in the gradient stream recovered from the centrifuge at the end of the run. The term  $\alpha^2 t$  used in the calculations therefore refers to the centrifugation done during acceleration, at speed, and during deceleration. An electronic system is therefore used to compute  $\int \omega^2 dt$  continuously during the course of the run.

In experiments designed for searching tissue breis for virus particles, it is desirable to centrifuge long enough to spread known virus particles between the soluble protein zone and the mitochondrial zone. Rat liver mitochondria tend to

band isopycnicly in sucrose gradients at density level of  $1.203 \text{ g/cm}^3$  (44.5 w/w % sucrose). In practice, virus particles are not allowed to reach this density during rate-zonal centrifugation.

By use of the B-IV computer program, the positions which particles having a range of densities and sedimentation coefficients would have as function of equivalent centrifuge time at 20,000 rpm were calculated. These are plotted in Fig II-A-2. With the gradient used for these calculations, a suitable spread is obtained in 1 hr at 20,000 rpm. This speed and time have been used for the majority of the studies made to date.

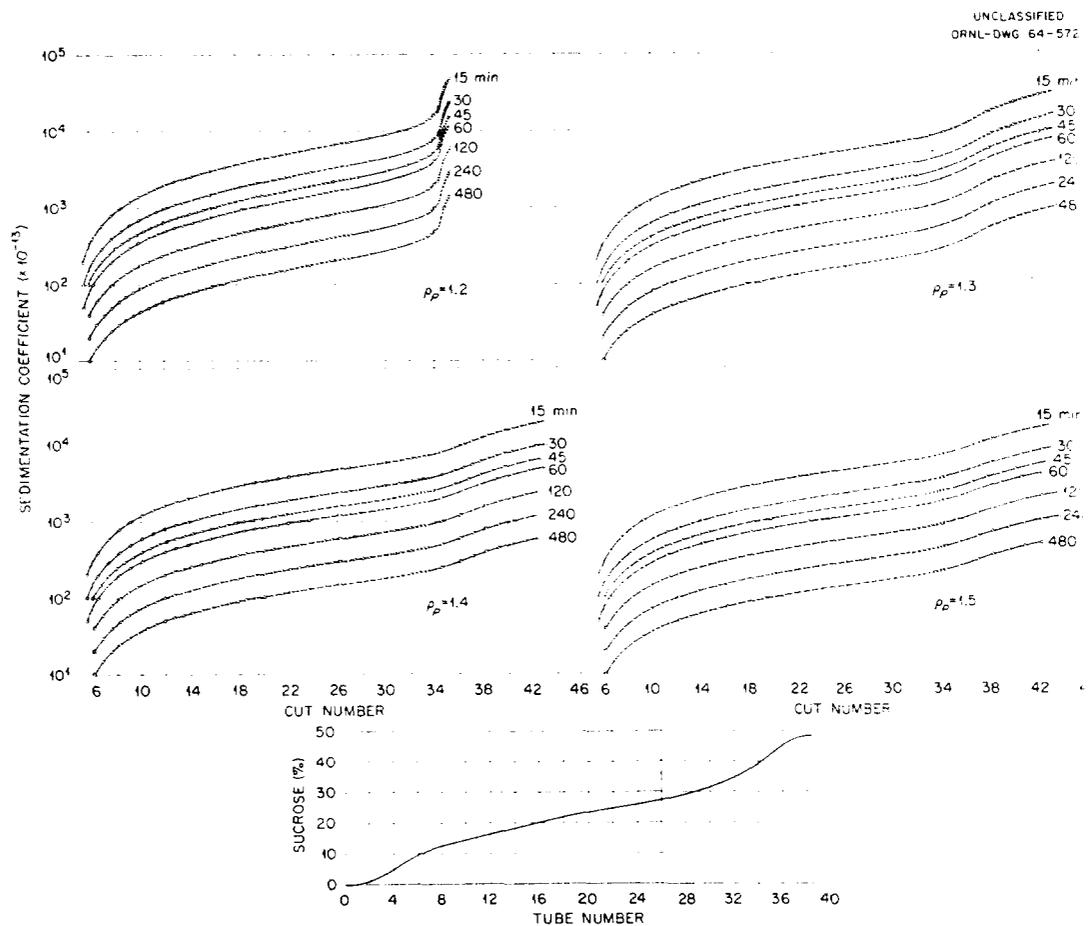
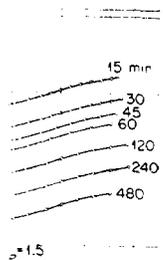
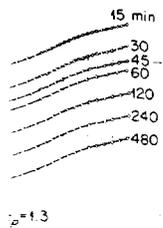


Fig. II-A-2. Positions in a Sucrose Density Gradient in the B-IV Rotor Which Particles Having Indicated Densities and Sedimentation Coefficients Would Have After Centrifugation at 20,000 rpm for Times Indicated (Run 208).

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gradients at a w/w % sucrose), not allowed to centrifugation. program, the position of densities could have as a time at 20,000 plotted in Fig. these calculated in 1 hr at have been used to date.

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indicated Densi-  
d (Run 208).

### 5. Rate-Zonal Separations

From early studies on the use of centrifugal force to stabilize density gradients as they are introduced into centrifuge tubes in a spinning rotor (Albright and Anderson, 1958), it became apparent that the operations of setting up the gradient, introducing the sample layer, separating the various particles in a centrifugal field, and recovering the separated fractions might well be accomplished in a suitable hollow centrifuge rotor *during rotation*. The first zonal rotor built in 1955 (Anderson, 1955, 1956) confirmed this view. Further work resulted in the development of the B-II rotor completed in 1960 (Anderson, 1962). Unfortunately, this rotor was destructively unstable in the region of 31,000 rpm. The steps necessary to redesign this system for operation at 40,000 rpm have resulted in the development of the B-IV system described in Chap. VI of this report. Detailed procedures for operation and calibration are presented in Chap. XIII. Since it is now possible to obtain highly purified and concentrated suspensions of virus particles from human and animal tissues, it has been considered of importance to develop methods for containing as much of the apparatus as possible in Freon-tight glove boxes (see Chap. XI). In addition, all solutions used must be sterile and, as far as possible, particle free (see Chap. XI). The gradient recovered at the end of the run is monitored for ultraviolet absorbance as it flows out of the centrifuge. In addition, total protein (determined by an automated method), refractive index, and physical density are determined on all the fractions collected.

### 6. Isopycnic Banding

The fractions obtained by rate-zonal centrifugation must now be banded isopycnically (Meselson, Stahl, and Vinograd, 1957) to determine whether particles having different banding densities are present. Since some viruses are known to be inactivated by pelleting, it is not desirable to centrifuge particulate material out of the sucrose solutions and then resuspend it in CsCl or other salt solutions and subsequently centrifuge the CsCl suspensions in swinging-bucket rotors. Rather it is important to band the unpelleted particles as rapidly as possible by a method which would allow as many as 24 samples to be run at

once. In previous studies (ORNL-3502) it was shown that the shearing which occurs during the transition from rotation to rest in a liquid density gradient in an angle-head rotor is small and that sharp banding of viruses and DNA could be obtained. Larger particles tend to clump under these conditions but will still band. To generate a large number of gradients rapidly, two methods have been employed. In the first the zonal centrifuge fractions (forty 40-ml tubes) are transferred to Spinco 30 rotor tubes containing 6 to 9 g of dry CsCl. During a 14- to 16-hr centrifugation at 24,000 rpm, the CsCl dissolves to form a steep gradient. In the second method the tubes were half filled with the particle-containing sucrose solutions, and then 10 to 15 ml of saturated CsCl was run to the bottom of the centrifuge tube through a narrow piece of tubing. The tubes were then completely filled with the particle suspension. The latter method requires more time but yields more reproducible gradients. The details of methods for preparing and scanning gradients are discussed in subsequent sections.

### 7. Virus-Isolation Studies

The amount of material in a tissue brei having the same sedimentation properties as a given virus or virus-like particle limits the purification which can be achieved by rate-zonal centrifugation. In the previous report (ORNL-3502) the results of studies on rat spleen and liver preparations were presented. These findings have now been extended to normal and leukemic mouse spleen, and to human spleen from patients with Hodgkin's disease.

### 8. S-ρ Plots with Tissue

The development of particle search methods has been guided by a variety of experiments (48 in all during this period with the B-II and B-IV zonal centrifuge rotors) using animal tissues and viruses, bacteriophage, and human autopsy tissue samples. The results confirm the presence of a "virus window" slightly beclouded by a small number of unidentified cell particles. Many hundred electron micrographs have been taken of the fractions obtained. Presentation of these is deferred until all the necessary control experiments have been done

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and the interpretations and comparisons are complete. Although the entire search system has been assembled only very recently, the studies to date bear out the early expectations. The results with one complete run using a sample of normal human

spleen are shown in Fig. II-A-3. A total of 37.8 g of tissue was homogenized in 151 g of pH 7.5 phosphate-NaCl buffer for 1 min using a Sorval motor-driven blade-homogenizer. The brei was then centrifuged in 250-ml sterile closed cups

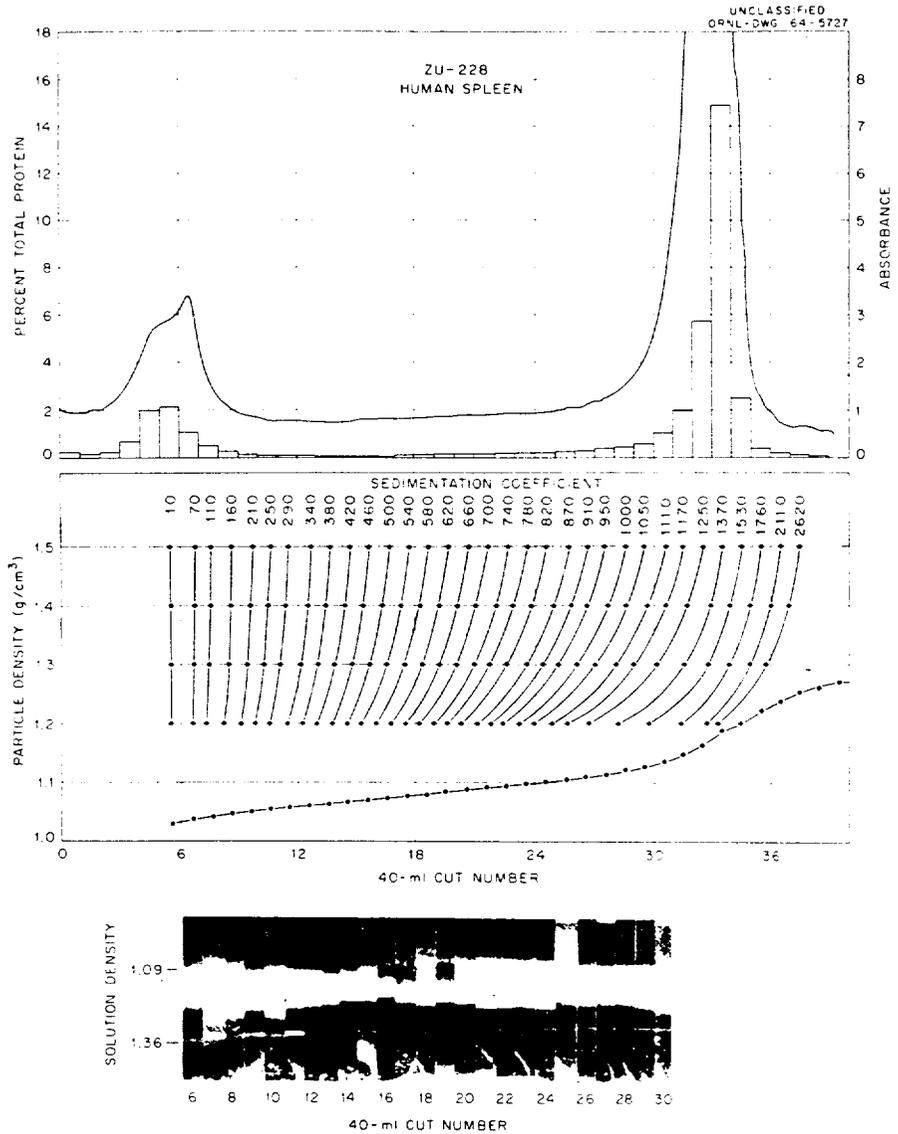


Fig. II-A-3. Fractionation of Normal Human Spleen. Upper plot shows rate zonal centrifugation separation, computer program plot is shown in center, and isopycnic separations in CsCl are shown at the bottom. Preparation of sample described in text.

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A total of 37.8 g of pH 7.5 using a Sorvall The brei was e closed cups



at 2300 rpm in the International PR-2. The supernatant was removed and centrifuged for 2 hr at 27,000 rpm in the Spinco 30 rotor. The pellets were resuspended and combined in a total volume of 21 ml and used as the sample in a rate-zonal separation run in the B-IV zonal ultracentrifuge using a 1-liter gradient extending from 10 to 30% (w/w) sucrose with a cushion of 55% sucrose (see operating procedure in Chap. XIII). The centrifuge was run 1 hr at 20,000 rpm and unloaded at 5000 rpm. Fractions 6 to 30 were banded at 24,000 rpm for 8 hr using 8 g of dry CsCl per rotor tube. The results are shown in Fig. II-A-3. It should be noted that only a small portion (0.1–0.2%) of the total sample per fraction collected is found in the virus sedimentation rate range. The material present in the lower sharp band in the isopycnic separations was present in an amount too small to be identified as yet by electron microscopy. Under these conditions ferritin is found in a very pure state at the bottom of the CsCl tubes, generally in fractions 6 to 9. These results must now be extended using normal tissues from a variety of sources combined with biochemical analysis to identify the various fractions separated.

### 9. Alternate Procedures

The method as described here for searching tissues is useful for up to about 4 g of tissue without precentrifugation. By removing nuclei and mitochondria by low-speed centrifugation and subsequently sedimenting virus and microsomes, the initial virus concentration can be increased and masses of tissue as large as 50 to 100 g can be used, depending on the nature of the tissues and the mass of microsomal material present. To process larger amounts of tissue, several other systems are being examined. Theoretical studies on continuous-flow centrifugation (Berman and Anderson, in preparation) suggest that serial or cascaded centrifuge systems can be constructed which will allow the microscopic elements of tissue breis to be removed from multiliter batches of brei, with subsequent sedimentation of the virus in higher-speed continuous-flow rotors such as the B-V recently described (Barringer, Anderson, and Nunley, in preparation; also Chap. V, Sect. A).

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In the B-V rotor the virus particles are pelleted onto a vertical surface and must be removed from the rotor at rest. Experiments now in progress demonstrate that continuous-flow centrifugation can be arranged so that the feed stream flows over a density gradient. Particles settling out of the stream are therefore also banded at their isopycnic position. The gradient is recovered from the rotor during rotation. The B-VIII rotor used to demonstrate this principle yields peaks with the viruses thus far studied. These are concentrated into volumes of 40 to 100 ml. The virus may be further reduced in volume by banding in high-speed rotors such as the experimental C-II rotor (141,000 rpm, 310,000  $\times$  g with 110 ml of CsCl solution with an average density of 1.7). The application of these alternate systems to tissues will be described in subsequent papers. To explore the resolution of each S-p separation system, a variety of virus-induced tumors and infected tissues must be examined.

## B. ANALYTICAL STUDIES ON RIBOSOMES ISOLATED WITH THE B-IV ZONAL ULTRACENTRIFUGE

Eric Schram      W. D. Fisher

Although much information is available concerning the role of ribosomes in protein synthesis, little is known about ribosome formation – especially about the synthesis of their structural proteins. The experiments reported here were performed to gain some insight into the specificity and the mechanism of synthesis of these proteins.

### 1. Separation of 30S and 50S Ribosomal Subunits by Zonal Centrifugation

Since ribosomes are generally contaminated with nascent proteins or with cytoplasmic proteins, the choice of the isolation procedure is a crucial step in the study of ribosomal proteins. Moreover, the quantities of material needed for such a study can be obtained only with large-volume rotors. For preparative separation, use was made of the B-IV zonal rotor described in Chap. VI. *Escherichia coli* were grown in the presence of  $^{35}\text{S}$  sulfate so that the proteins could be selectively traced by radioactivity.

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The purest ribosomal subunits were obtained by first isolating ribosomes with an *S* value equal to or higher than 70 in a sucrose gradient (see Fig. II-B-1), and by rerunning this fraction in the same gradient after dissociation at low magnesium concentration (see Fig. II-B-2). The optimum gradient for the separation of ribosomal subunits ranged from 13 to 20% (w/w) sucrose. The buffers used had the following compositions: (1) for the homogenization of *E. coli* in the French pressure cell and the isolation of 70S particles,  $10^{-2}$  M Tris and  $10^{-2}$  M  $Mg^{2+}$  (pH 7.4); (2) for the dissociation of the 70S particles and the isolation of their subunits,  $10^{-2}$  M sodium-potassium phosphate and  $10^{-3}$  M magnesium acetate (pH 7.4).

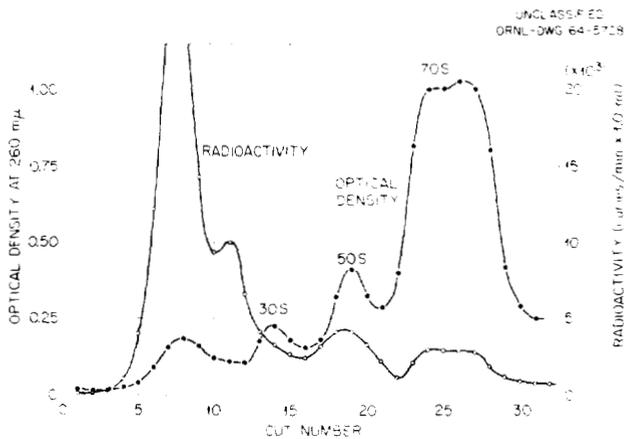


Fig. II-B-1. Zonal Centrifuge Pattern of a Partially Purified Ribosome Pellet from *E. coli*.

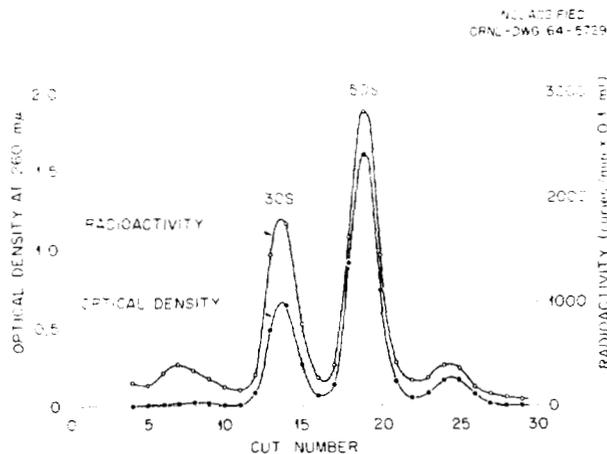


Fig. II-B-2. Zonal Centrifuge Pattern of Dissociated Ribosomes.

The ribosome suspensions obtained by zonal ultracentrifugation were first concentrated by centrifugation in a Spinco 30 rotor, and the proteins were extracted with hydrochloric acid. The efficiency of the separations achieved is indicated by the absence of ribonuclease in the 50S fraction. Previous studies indicate that this enzyme is associated with the 30S fraction.

## 2. Separation of Ribosomal Proteins by Disk Electrophoresis

In previous studies starch has generally been used for the separation of ribosomal proteins by electrophoresis. To increase the resolution of the separations and to reduce the size of the sample required, we have used polyacrylamide gel columns. As anticipated from the basic properties of ribosomal protein, the best results were obtained using the low-pH buffer system previously used in the separation of other basic proteins (Reisfeld *et al.*, 1962). Each of the patterns observed in the 30S and 50S subunits shows approximately a dozen peaks which appear consistently, but which differ for the two sizes of particles (see Figs. II-B-3 to II-B-5). Minor differences were observed between

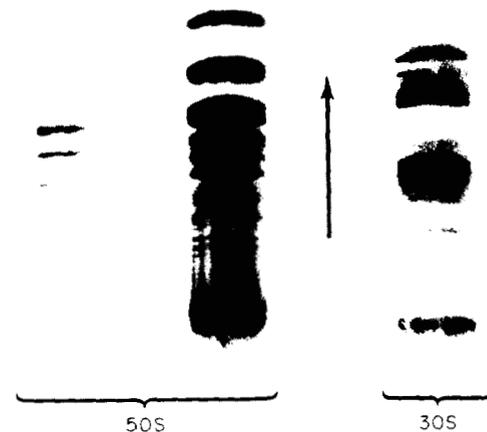


Fig. II-B-3. Disk-Electrophoresis Patterns of Ribosomal Proteins Extracted from 30S and 50S Ribosomal Subunits of *E. coli*.

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the patterns for "native" and "derived" 50S particles. No differences were observed between the ribosomes from cultures at rest and those from exponentially growing cultures. The amino acid analyses of these samples are incomplete at this writing.

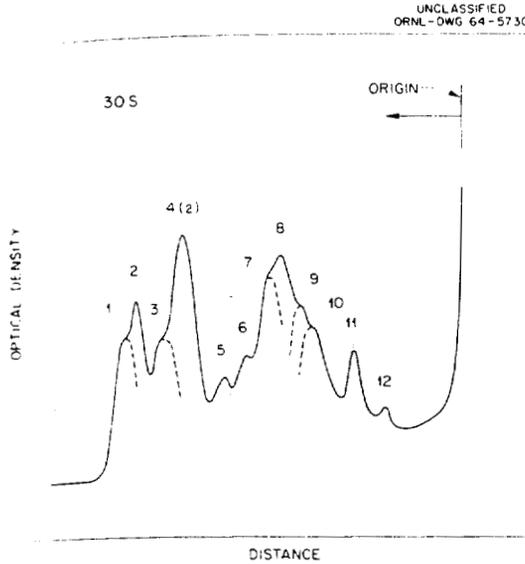


Fig. II-B-4. Absorbance Pattern of Disk-Electrophoresis Analysis of 30S Ribosomal Proteins.

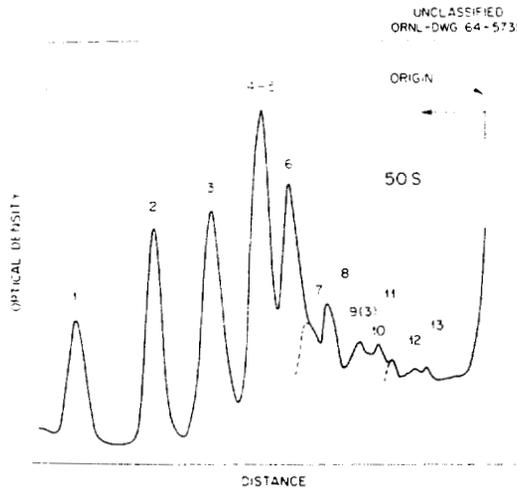


Fig. II-B-5. Absorbance Pattern of Disk-Electrophoresis Analysis of Proteins of 50S Ribosomal Subunits.

### 3. Radioactivity Determinations

Although not apparent from the ultraviolet absorbance scans of the preparative zonal centrifuge separations nor from the patterns obtained with the analytical ultracentrifuge, contamination of the 30S particles by the overlapping soluble protein peak can be inferred from the radioactivity curves (Fig. II-B-6). Such contamination occurred only when the particles had not been prepared from

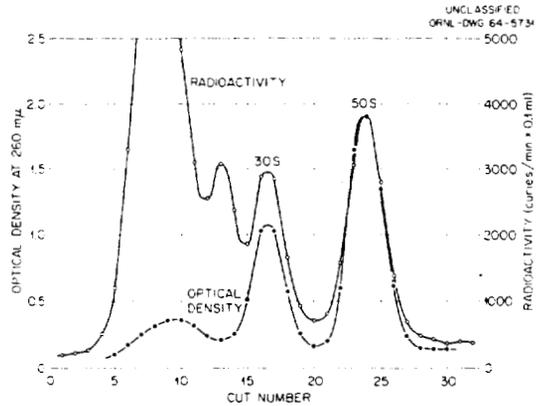


Fig. II-B-6. Zonal Centrifuge Pattern of Dissociated Ribosomal Subunits Prepared Directly from a Differential Centrifugation Pellet Without Prior Isolation of 70S Subunits.

Table II-B-1. Specific Radioactivity of the Ribosomal Subunits from *E. coli* Grown in the Presence of <sup>35</sup>S

	Before Chasing	After Chasing
Native 50S	113	42
Derived		
30S	145	26.5
50S	95	17.4
Ratio of native 50S to derived 50S	1.19	2.45
Ratio of derived 30S to derived 50S	1.53	1.52

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previously isolated 70S particles and was encountered even when the starting material was a ribosome pellet prepared by differential centrifugation. The importance and the necessity of using zonal centrifugation for preparative work are evident.

It appears from Table II-B-1 that the specific radioactivity (radioactivity vs RNA content) of the 30S subunits is 50% higher than that of the 50S. After chasing with nonradioactive sulfur, the same ratio is observed between the specific radioactivities of the derived subunits, but the specific radioactivity of the native 50S material is now found to be much higher than that of the derived ones. The radioactivity, before and after chasing, of the several fractions isolated by disk electrophoresis is now being studied.

#### 4. Discussion

Green and Hall (1961) have observed differences in the physiological activity and RNA composition of native and derived ribosomal subunits. Such differences do not appear clearly from the electrophoresis data in this study, but the radioactivity data would imply the absence of an equilibrium between the two types of particles (native and derived). Moreover, the higher specific activities found in native 50S after chasing would suggest that these particles are actually derived from the 70S particles. The higher specific radioactivities found in the 30S as compared to that of the 50S may be ascribed to a higher protein content if the proteins from both the 30S and 50S are found to contain the same percentage of cystine and methionine (Spahr, 1962). This view is further supported by the fact that a lower ratio of optical density at 260  $m\mu$  to optical density at 235  $m\mu$  is consistently found for the 30S particles. Radioactivity measurements now being made on the proteins separated by disk electrophoresis will aid in answering some of the questions raised above and will also indicate whether any of the proteins undergo a higher turnover rate than others.

#### C. VIRUS-LIKE PARTICLES IN HUMAN LEUKEMIC PLASMA

C. L. Burger            N. G. Anderson  
W. W. Harris            T. W. Bartlett  
R. M. Kniseley

During this report period, a total of 250 coded plasma samples from human subjects have been prepared for examination by electron microscopy. Of these, 40 to 50 were from nonleukemic individuals. Microscopy data are presently available on only 140 of these samples. The correlation of particle morphology with diagnosis, treatment, and other parameters will be made by computer analysis.

#### 1. Methods

Samples were prepared as described (Burger *et al.*, 1964). However, banding of the virus pellet has been explored by a number of techniques using greater volumes of plasma per patient to facilitate electron microscopy.

After the resuspended pellets are banded in CsCl, photographs are taken of the banding tubes, and fractions are then recovered by pumping the tube contents through a stainless steel needle and into a spectrophotometer modified with a wave-shifting mechanism to alternately read 260  $m\mu$  and 280  $m\mu$ . The sample then passes through a Waters refractometer (model 34H), the pump, and finally into a fraction collection tube. The fractions are then prepared for microscopy as reported (Burger *et al.*, 1964).

The gradient analyzer (designed and fabricated by ORGDP Instrument Development Group) used for recovery of banded samples is shown in Fig. II-C-1. The pump used to withdraw the fluid from the tubes is provided with a continuous-speed control (front left, below table holding fraction collector) which varies rate of withdrawal from 0 to 3.7 ml/min, when using  $\frac{1}{16}$ -in.-ID tubing. The total volume of fluid within the system, from tip of stainless steel needle to end of tubing above collection vessel, is 0.5 ml. The tube holder, an evacuated double-walled chamber which insulates the tube during pumpout, has a light

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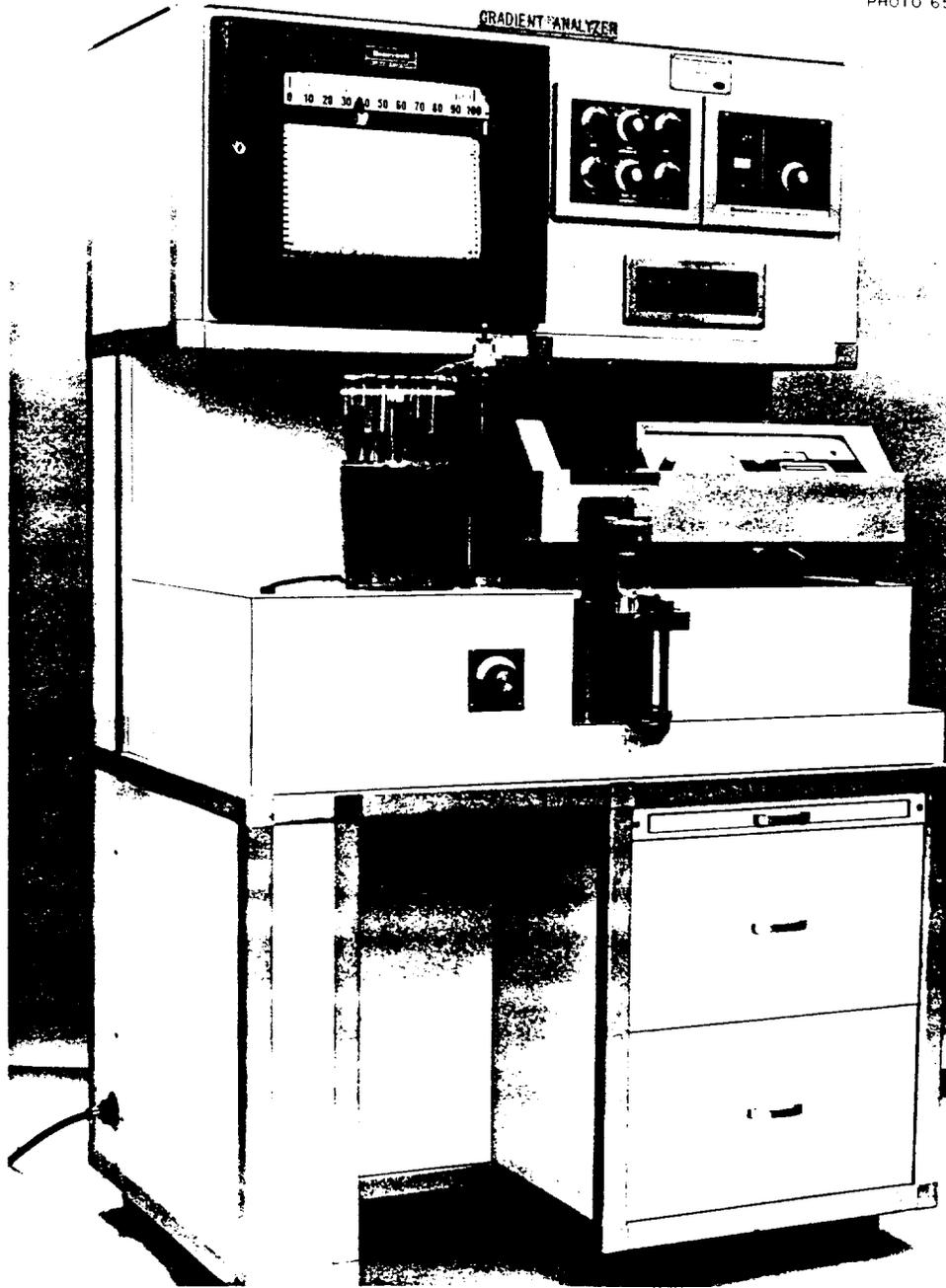


Fig. II-C-1. Gradient Analyzer Used to Recover Virus Banded in Salt Gradients. Absorbance at two wave-lengths and refractive index are recorded.

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below, permitting visual observation of bands in the tube. The screw is removed from the cap of the Spinco 30 rotor tube, and a plastic adapter is screwed into the opening. The rack holding the tube is then raised, after positioning the needle over the opening in the adapter. The needle is thus positioned at the bottom of the tube, with minimum disturbance of the gradient.

The reference cell of the spectrophotometer has been modified by the addition of a variable slit adjustment. The housing above the cell compartment of the DB contains the refractometer flow cell, modified to reduce the volume and hence the lag time of the instrument. The refractometer has been further altered by the addition of a fine adjustment which permits the operator to set the output voltage of the unit between the settings required through use of the scale and zero adjustment.

The fraction collector provides for eighteen 15-ml centrifuge tubes. An event-recording pen marks the base of the strip chart each time the fraction collector advances, either automatically on a timed basis or manually by depressing a control switch. A visual record of absorbancy at 260 and 280  $m\mu$ , refractive index, and tube number is thus obtained for each sample.

The tubing used throughout the system may be washed with formalin between samples, or new tubing may be inserted. The DB and refractometer cells are not at the moment expendable, but disposable DB cells are under study. The important feature is that no "memory" or cross contamination be detected, and none has been observed as checked by comparing leukemic and nonleukemic samples. The recorder is equipped with a 12-point print wheel; two points record refractive index and the others alternate between 260 and 280  $m\mu$ .

The panels above the DB contain the hydrogen lamp power supply, zero adjustments for both wavelengths, and the electronic controls for the refractometer. A series of switches include main power, fraction collector power, pump, and sample light. The correlation between virus-like particles observed in plasma and tissues of the same leukemic individuals is under investigation, and results will be reported in future communications. The occurrence of particles other than the "R" and "Q" type previously reported (Burger *et al.*, 1964) will also be described.

#### D. STUDIES ON THE A-IX ROTOR

R. E. Canning

As each new rotor system is developed, its performance is examined using test particles as a prelude to studies on the separation of more complex biological materials. The A-IX rotor is designed for the separation of cells and for studies on the distribution of drugs, chemical carcinogens, and enzymatic activities among cell fractions. Before this can be done, however, optimal gradients and operating techniques must be developed.

The performance of the A-IX rotor was studied using bovine serum albumin (BSA), rat red blood cells (RRBC), and ragweed pollen. In all cases a 1-liter gradient of 17 to 55% sucrose ( $\rho = 1.0719$  to 1.2663) was used with 55% sucrose as a cushion at the centrifugal end of the gradient. For the overlay, 160 ml of phosphate buffer (pH 7.5,  $\mu$  (ionic strength) = 0.1) was used.

The effects of movement of the sample zone into and out of the rotor and of disturbances occurring during acceleration and deceleration were studied using BSA (10 ml of 4% BSA in 8.5 w/w % sucrose) as the sample. The sample was introduced into the rotor at 1100 rpm, after which the rotor was accelerated to 4000 rpm ( $3000 \times g$  at  $R_{max}$ ) for 15 min. The rotor was then decelerated to 1100 rpm and unloaded into 40-ml centrifuge tubes through an ultraviolet absorbance monitor. The absorbance recorder chart is shown in Fig. II-D-1. The sample zone was calculated to be

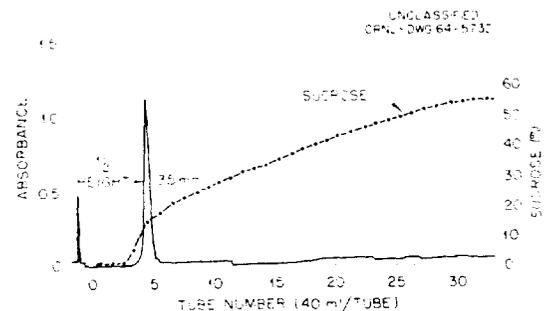


Fig. II-D-1. Run Diagram of Bovine Serum Albumin (BSA) in Rotor A-IX. Ten ml of 4% BSA in 8.5% sucrose; 4000 rpm ( $3000 \times g$  at  $R_{max}$ ), 15 min. Loading and unloading done at 1100 rpm. Scanned at 260  $m\mu$  in 0.2-cm light path flow cell.

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