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SEMIANNUAL REPORT

For Period January 1 to June 30, 1964

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Program Coordinator

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MARCH 1965

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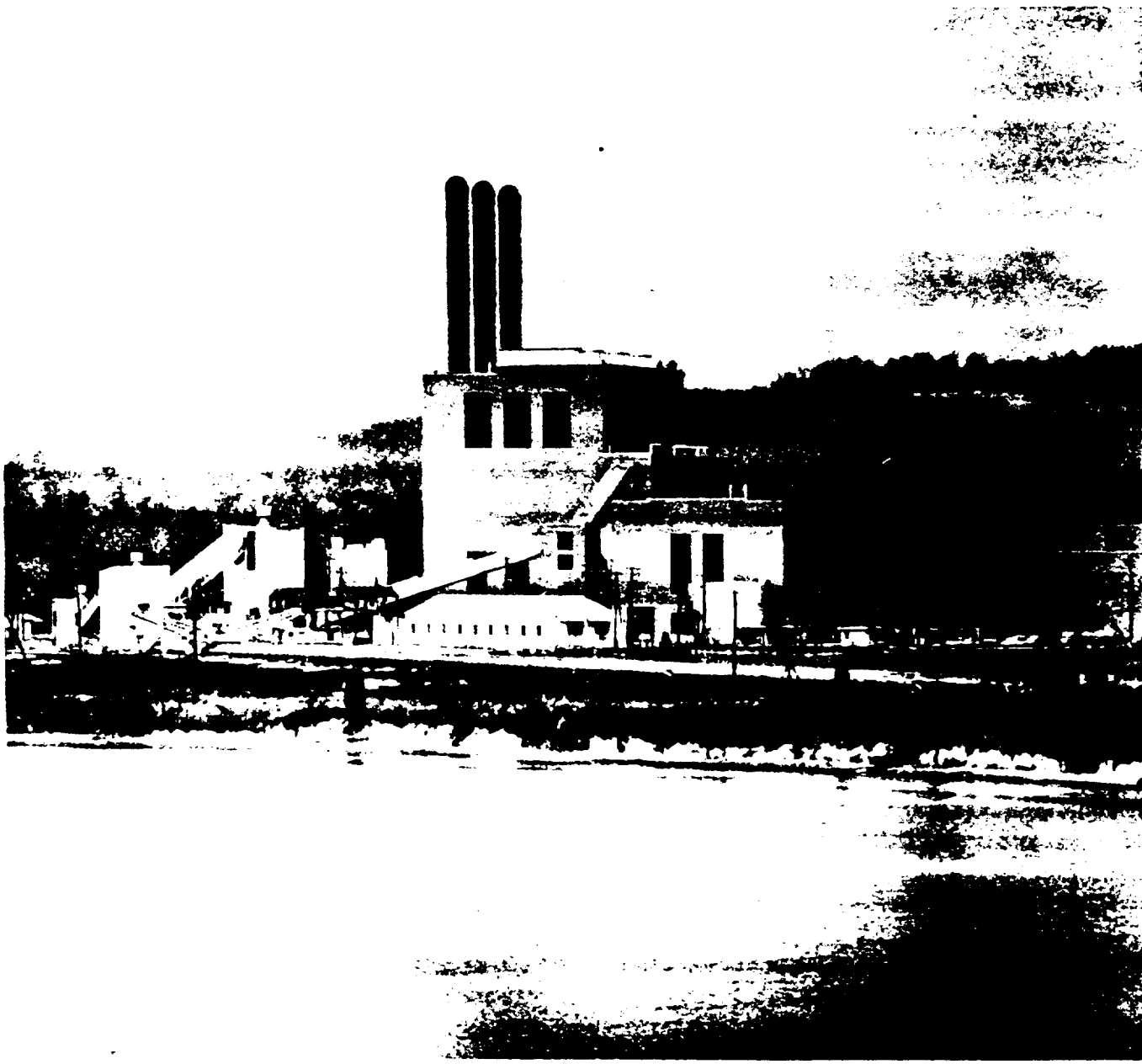

Electrical Power Generating Facilities of Oak Ridge Gaseous Diffusion Plant.

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Summary

This report describes work done under the Joint National Institutes of Health–Atomic Energy Commission Zonal Centrifuge Development Program on the application to difficult biophysical separation problems of techniques and instruments developed by the AEC. The objective of this program is to develop separation systems which will allow profiles of subcellular constituents to be constructed which include the maximum number of cell organelles and individual molecular species. Work on zonal centrifuge systems designed to separate particles ranging in size from whole cells to small molecules has progressed with the development of several new rotor systems. The B-IV rotor has been shown to effectively separate quantities of macroglobulin from rat and other animal sera, thereby extending the usefulness of this instrument down to 20S. By application of the two-dimensional S-ρ separation system previously described, a clear spectrum of rat liver glycogen particles has been obtained, and the molecular structure revealed by negative staining is under investigation. Virus-like particles have been recovered from a human breast tumor with the same technique. Further studies on centrifugal fast freezing show that a high percentage of yeast cells survive freezing, but that the lethal damage is done during warming, which must be done rapidly if a high survival rate is to be obtained. Theoretical studies on the particle capacity of gradients in zonal centrifuges are being carried to the point that experimental studies may be designed to examine anomalous sedimentation behavior and band spreading. The low-speed A-IX rotor has been redesigned and strengthened and is now designated A-XII. Scanning systems that would make this rotor an analytical centrifuge have been completed. Continuous-flow rotors in which the sedimenting particles are banded in a gradient during the continuous-flow process (rotor B-VIII) have been built and successfully tested.

A completely enclosed virus separation facility has been designed for large-scale virus separation. In addition to centrifuges, new countercurrent systems for liquid-liquid extractions and the flame ionization detector for nonvolatile samples are under development. A prototype automated system for carbohydrate analysis has been completed and tested with a variety of sugar-containing samples. With an experimental high-pressure capillary column nucleotide analyzer, it has been demonstrated that rapid analysis of very small samples can be performed with micro ion exchange beads, warm columns, a high flow rate, and columns of very small bore.

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I. Introduction

This report is the fourth prepared under the Joint NIH-AEC Zonal Centrifuge Development Program. The central theme remains the exploration of technologies and skills, originally developed for AEC programs, for application to basic biomedical problems. While swords are not easily beaten into scalpels, the possibility of new incisive edges for research tools would appear to justify the effort. The program is being carried out at three locations: the Biology Division laboratories (ORNL) at the Y-12 site, the Technical Division of the Oak Ridge Gaseous Diffusion Plant (ORGDP), and in an area of the ORGDP power generating plant. One floor of a section of this plant has been modified during this report period to house the centrifuge systems described in these reports. The zonal centrifuge program is, therefore, a small, hopefully prophetic attempt at the redirection of part of a vast existing enterprise. A photograph of the electrical power generating facilities of the Oak Ridge Gaseous Diffusion Plant which houses the Joint NIH-AEC Zonal Centrifuge Development Program is included as a frontispiece.

The development of zonal centrifuges is by no means complete; however, this report marks a turning point in their development. First, a program aimed at adapting the centrifuge systems thus far developed to the large-scale isolation of virus particles and viral subunits has been undertaken in collaboration with the Board for Vaccine Development of the National Institute of Allergy and Infectious Diseases. Second, drawings of the B-IV, B-V, B-VIII, A-IX, and A-XII rotors and cores have been released to industry through the AEC Division of Technical Information Extension at Oak Ridge. Specifications and drawings of new developments will continue to be released as rapidly as possible. Third, a seminar attended by 73 representatives from industry and from research laboratories across the country was held on April 30–May 1, 1964, under the auspices of the ORNL Office of Industrial Cooperation. Plans for a training course in zonal centrifuge operation are under way and will be held in Oak Ridge, September 16–18, 1964. Last, arrangements have been made to publish some of the results of this program as a supplement to the *Journal of the National Cancer Institute*. As detailed results appear in the open literature, the necessity for extensive reports for limited distribution is minimized. Future reports in this series will, therefore, be in summary form.

Since the problem of bringing zonal centrifuges into general use has been at least partially solved, the direction of future efforts must now be considered. If the past history of biophysical tools is indicative of the future history of new ones, then we should aim at once for simplification of components and operating technique. With this in mind, the B-X and B-XI rotors described in this report have been developed. These eliminate the high-speed upper bearing, and still allow operation at speeds sufficiently high to separate ribosomal subunits. Work on the development of other rotors designed for specific purposes will continue, together with studies on the containment of both centrifuges and ancillary virological research systems. For the present, the chief biological problem is the identification of the many fractions which can now be isolated. This work will progress slowly, as collaborative studies with interested investigators are arranged.

As is indicated by the contents of this and previous reports, several techniques in addition to those concerned with centrifugation are being explored. These include: the wire-fed flame ionization analyzer, the carbohydrate analyzer, the high-pressure nucleotide analyzer, digital chromatographic data reduction, countercurrent

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distribution, ultrafast freezing, and new methods for achieving electrophoretic separations. While these involve interesting and valuable new separation and analytical systems, it may be asked how these relate to the problem of cancer in man.

As previously reported, methods for isolating and identifying virus-like particles in plasma and in tissue breis have been developed and shown to be effective. The problem of demonstrating that these particles are both infectious and oncogenic is outside the present scope of this program. The isolation of viral antigens which have been shown to be present in many animal tumors, often in a nonparticulate form, offers an additional challenge to separation methods. If some human neoplasms are ultimately shown to be the end result of virus infection, then the problem of preparing suitable vaccines must be considered. Live-virus vaccines will be extremely difficult to develop and, indeed, may not be considered in view of the difficulty of demonstrating that human oncogenicity has been eliminated. Killed virus preparations may suffer from the same hazard. The possibility of isolating the effective antigens from virus-infected cells in a high state of purity must, therefore, be given close attention. This will involve better protein fractionation methods, together with improved analytical systems for the subunits of which the antigens may be composed.

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II. Biomedical Applications of Zonal Centrifuges Developed Under This Program

A. ISOLATION AND PHYSICOCHEMICAL PROPERTIES OF RAT MACROGLOBULINS

W. D. Fisher R. E. Canning

As experimental studies with the B-IV zonal ultracentrifuge have progressed, particles of smaller dimensions have been separated. In experiments with ribosomal subunits, particles having sedimentation coefficients as low as 30S were distinguished. To explore the lower limits of the separations which might be achieved with this system, the possibility of isolating plasma macroglobulins has been examined.

Of the various gamma globulins of plasma possessing antigenic activity, the so-called 7S gamma globulins have been extensively studied with respect to structure and biological activity; the 19S gamma globulins are more difficult to isolate in quantity and have been less extensively studied.[1] Since good analytical separations of 19S antibodies have been obtained in sucrose density gradients with swinging bucket rotors,[2] an attempt was made to isolate rat macroglobulin in the B-IV zonal centrifuge described by Anderson and coworkers.[3] This paper reports the isolation and physicochemical properties of an apparently homogeneous rat macroglobulin.

Preparation of Serum and Zonal Centrifugation

A globulin fraction from pooled rat serum was obtained by two precipitations with 40% ammonium sulfate followed by exhaustive dialysis against tap water. The resulting fraction was made up in 0.2 μ Miller-Golder buffer (pH = 7.5) to one-fifth the original plasma volume. This material was then separated with a 1-liter sucrose gradient (10–15% in 0.2 μ Miller-Golder buffer) using 400 ml of overlay. Centrifugation was to 350,000 × 10^6 $\omega^2 t$ (approximately 6 hr at 40,000 rpm).

Sedimentation measurements were made with a Spinco model E analytical ultracentrifuge with 12-mm cells.

Electrophoretic and diffusion measurements were performed with a Spinco model H unit using 11-ml cells.

Immunoelectrophoresis was done as described by Grabar and Williams.[4]

Carbohydrate was measured by the anthrone method.

The separation of rat macroglobulin by the B-IV rotor is shown in Fig. II-A-1. The starting sample represents 60 ml of rat plasma; the yield was 250 mg of macroglobulin. After concentration by pressure dialysis the leading peak was ultracentrifugally and immunoelectrophoretically homogeneous (Figs. II-A-2 and II-A-3). The physical-chemical properties of the material are listed in Table I. The carbohydrate content of the macroglobulin is 10%. Treatment with 0.1 *M* mercaptoethanol dissociated the macroglobulin into subunits of approximately 8S.

The data reported show that the B-IV zonal centrifuge can be used to prepare a homogeneous rat macroglobulin in preparative quantities. This extends the demonstrated usefulness of the zonal centrifuge into the size range of Sephadex separations. In conjunction with protein chromatographic

[1] R. R. Porter, *Brit. Med. Bull.* 19, 197 (1963).

[2] G. M. Edelman, H. G. Kunkel, and E. C. Franklin, *J. Exptl. Med.* 108, 105 (1958).

[3] N. G. Anderson *et al.*, *Life Sci.* 3, 667–71 (1964).

[4] P. Grabar and C. A. Williams, *Biochim. Biophys. Acta* 10, 193 (1953).

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methods, zonal centrifugation should be a useful tool in studying the immunological relationship of the various antibodies in plasma, especially the 20S antibodies, which by previous methods are the more difficult to isolate. The isolation of other macroglobulins (chicken, mouse, and fish) by zonal centrifugation is in progress.

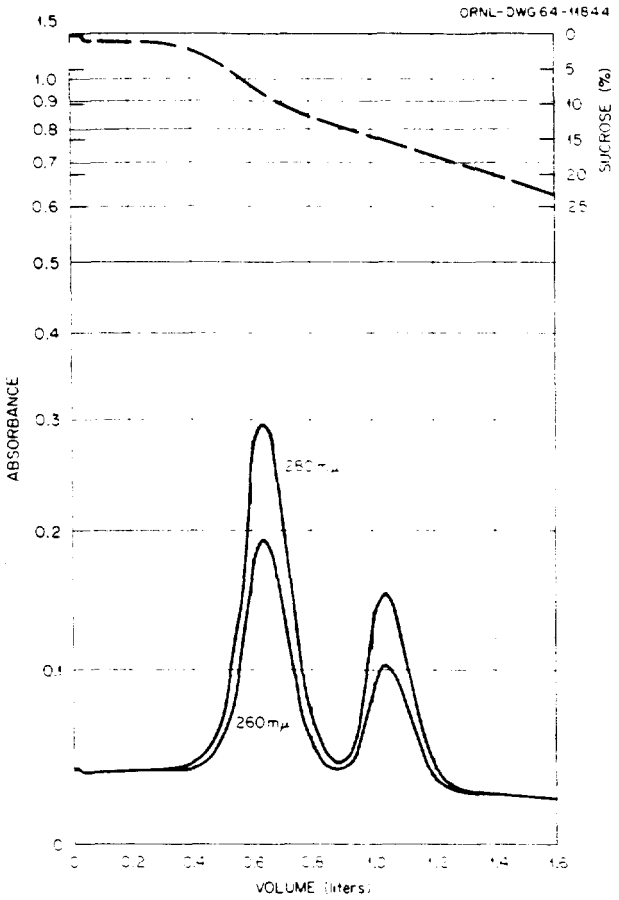

Fig. II-A-1. Separation of Rat Macroglobulin in B-IV Zonal Centrifuge. Upper curve, sucrose concentration; lower curves, absorbancy (0.2-cm light path).

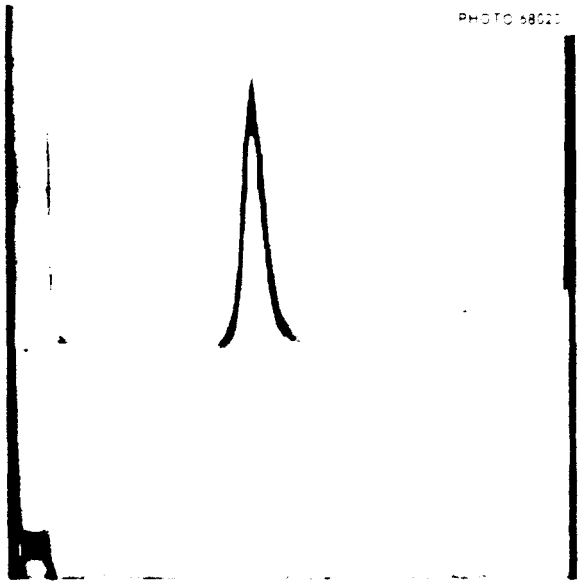

Fig. II-A-2. Ultracentrifuge Pattern for Rat Macroglobulin in 0.2 μ Miller-Golder Buffer After 20 Min at Speed (59,780 RPM).

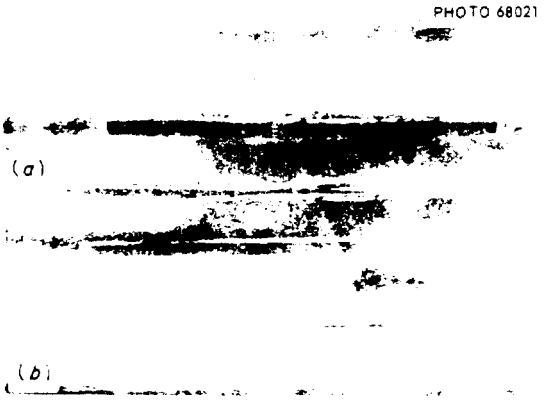

Fig. II-A-3. Immunoelectrophoretic Patterns of (a) Macroglobulin and (b) Rat Serum. In both cases the upper trough contains rabbit-anti-whole-rat serum and the bottom trough rabbit-anti-rat macroglobulin.

Table I. Physicochemical Properties of Rat Macroglobulin

Sedimentation constant, $S_{20,w}$	20.0S
Diffusion constant, $D_{20,w}$	2.22×10^{-7} cm^2/sec
Calculated particle specific volume, $\bar{V}$	0.744 ml/g
Molecular weight (s, D, $\bar{V}$)	859,400
Electrophoretic mobility at pH 6.2 phosphate buffer, $\mu = 0.1$	-4.22×10^{-5} cm^2 v^{-1} sec^{-1}
Isoelectric point	pH 4.38

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B. ISOLATION OF NATIVE GLYCOGEN

N. G. Anderson W. W. Harris
A. A. Barber

The subcellular components of tissues may be separated to give a two-dimensional pattern by using rate zonal centrifugation followed by isopycnic zonal centrifugation as previously described.[5] Experimentally, a number of bands are observed with both normal and malignant tissues, which must now be characterized in much greater detail. In this study we are interested in the band tentatively identified as glycogen in orienting experiments. In previous studies it has been shown that homogeneous virus particles sedimented as narrow, discrete zones in the presence of the components of a tissue brei, suggesting that the wide zones observed with microsomes, for example, are actually due to particle polydispersity. Glycogen is an excellent material for examining this point, since the available evidence suggests that it exists in tissues in a wide range of sizes, and, in addition, it has not been observed to behave as an osmometer.

While glycogen is the major energy and carbohydrate reserve of many human tissues, a number of problems concerning both its structure and mechanism of synthesis remain to be solved. The majority of physical studies have been carried out on material isolated by either cold 5–10% aqueous trichloroacetic acid extraction or by extraction with 30% KOH at 100°C as described by Pflüger.[6] With both methods the glycogen is precipitated from solution by ethanol. The sedimentation coefficients for material obtained by this procedure range from 20S to 300S (refs. 7–12) with some material as high as 2000S.[13] In all instances it is polydisperse. In freshly prepared liver homogenates, Lazarow showed that glycogen behaves as a much larger particle, with a sedimentation coefficient closer to several thousand for the major portion of the particles.[14,15] Electron micrographs of glycogen in tissue sections bear out Lazarow's conclusions.[16–21] The native structure of glycogen cannot be satisfactorily determined from sections, however, and it has not been possible to determine whether glycogen exists as a beaded chain structure, as a rosette, or as a tight cluster of subunits. Electron microscopy of the isolated particles using negative staining is therefore desirable to elucidate the tertiary structure of native glycogen.

Preparation of Rat Liver Breis

Sprague-Dawley rats were stunned and exsanguinated, and the liver from each was rapidly removed and weighed in a tared beaker of cold 8% sucrose. The volume was adjusted to give a 10% (w/v) brei, and the liver was homogenized in a cold coaxial hand homogenizer. After being strained through cheesecloth, 20 ml of the cold brei was introduced into a B-IV zonal centrifuge rotor operating at 5°C and at a speed of 3000 rpm. A 200-ml overlay of pH 7.5, μ = 0.1 Miller-Golder buffer was used to move the sample layer free of the core. The rotor was then accelerated to 20,000 rpm for 15 min. After deceleration to 5000 rpm, the rotor was unloaded as previously described.[5] The ultraviolet absorbance diagram and the computer S* plot are shown in Fig. II-B-1.

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[5] *Joint NIH-AEC Zonal Centrifuge Development Program Semiann. Rept. July 1–Dec. 31, 1963*, ORNL-3656.

[6] E. F. W. Pflüger, *Das Glycogen*, p. 530, Bonn, 1905.

[7] W. J. Polglase, D. M. Brown, and E. L. Smith, *J. Biol. Chem.* **199**, 105–12 (1952).

[8] D. J. Bell *et al.*, *Biochem. J.* **42**, 405–8 (1948).

[9] W. A. J. Bryce *et al.*, *J. Chem. Soc.* 711–15 (1958).

[10] W. A. J. Bryce *et al.*, *J. Chem. Soc.* 3845–51 (1958).

[11] F. Meyer and J. P. Zalta, *Compt. Rend.* **247**, 357–59 (1958).

[12] A. Calderbank *et al.*, *Biochem. J.* **74**, 223–29 (1960).

[13] S. A. Orrell, Jr., and E. Bueding, *J. Am. Chem. Soc.* **80**, 3800 (1958).

[14] A. Lazarow, *Anat. Record* **84**, 31–50 (1942).

[15] A. Lazarow, *Arch. Biochem. Biophys.* **7**, 337–43 (1945).

[16] J. P. Revel, L. Napolitano, and D. W. Fawcett, *J. Biophys. Biochem. Cytol.* **8**, 575–89 (1960).

[17] H. E. Karrer and J. Cox, *J. Ultrastruct. Res.* **4**, 191–212 (1960).

[18] M. H. Himes and A. W. Pollister, *J. Histochem. Cytochem.* **10**, 175–85 (1962).

[19] R. Laguens and N. Bianchi, *Exptl. Mol. Pathol.* **2**, 534–40 (1963).

[20] H. Sheldon, M. Silverberg, and I. Kerner, *J. Cell. Biol.* **13**, 468–73 (1962).

[21] H. Themann, *Naturwissenschaften* **47**, 155 (1960).

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The fractions between the starting layer and the mitochondrial layer were then banded, using CsCl in No. 30 rotor tubes. A band at a density of approximately 1.62 was observed extending from tube 7 past tube 31. Material recovered from these bands was examined in the electron microscope, with phosphotungstate being used as a negative stain. The results are shown in Fig. II-B-2. The sedimentation coefficients for the fractions shown are indicated in Fig. II-B-1. If the hydrodynamic volume of the particles is calculated from the density and sedimentation data, a value for the radius of the particles is obtained which is less than one-half that observed in cuts 12–31. This is in agreement with the actual observations, which indicate that the particles are not solid

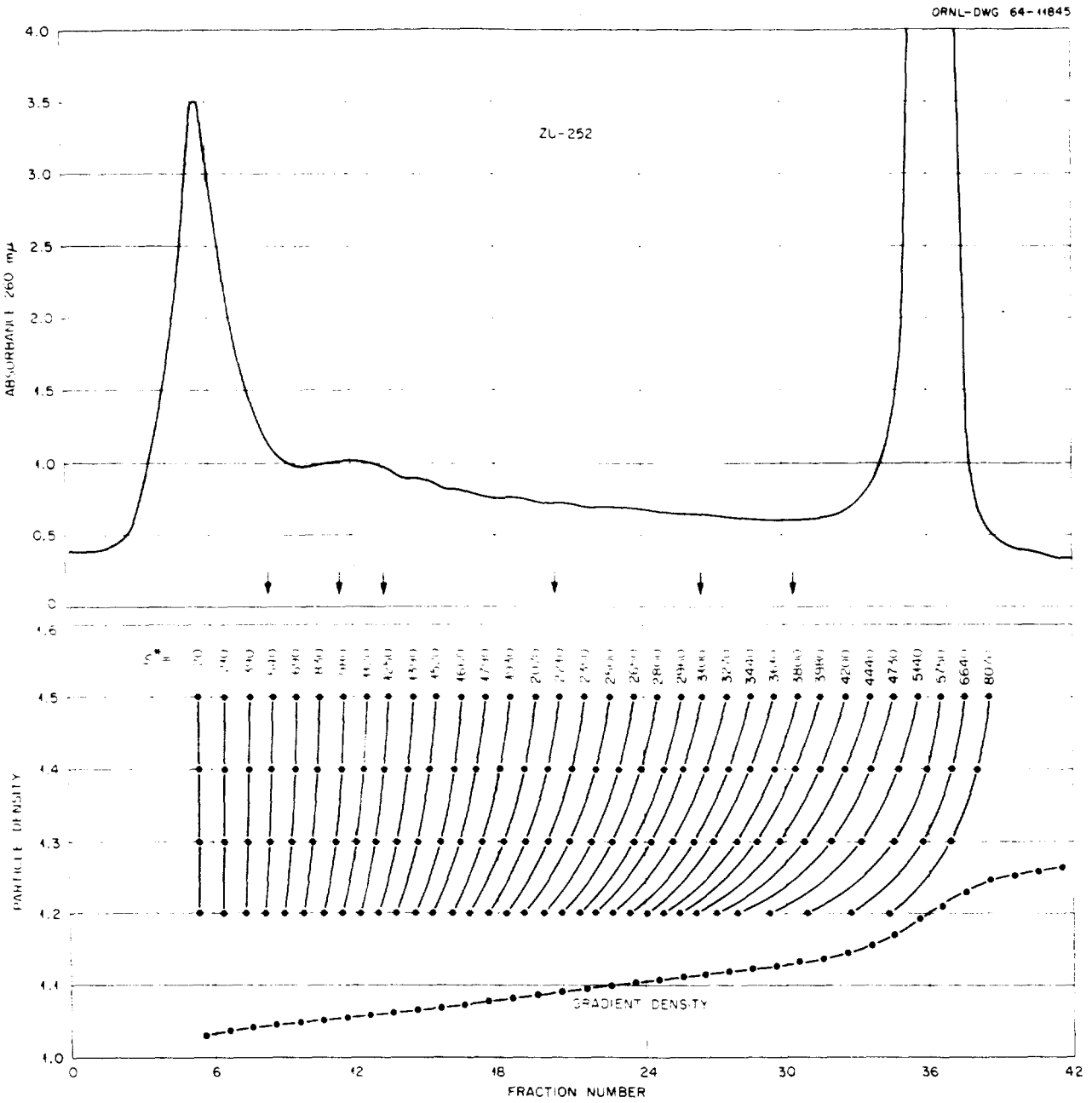

Fig. II-B-1. Rate Zonal Separation of Rat Liver Brei in B-IV Zonal Centrifuge. Details described in text.

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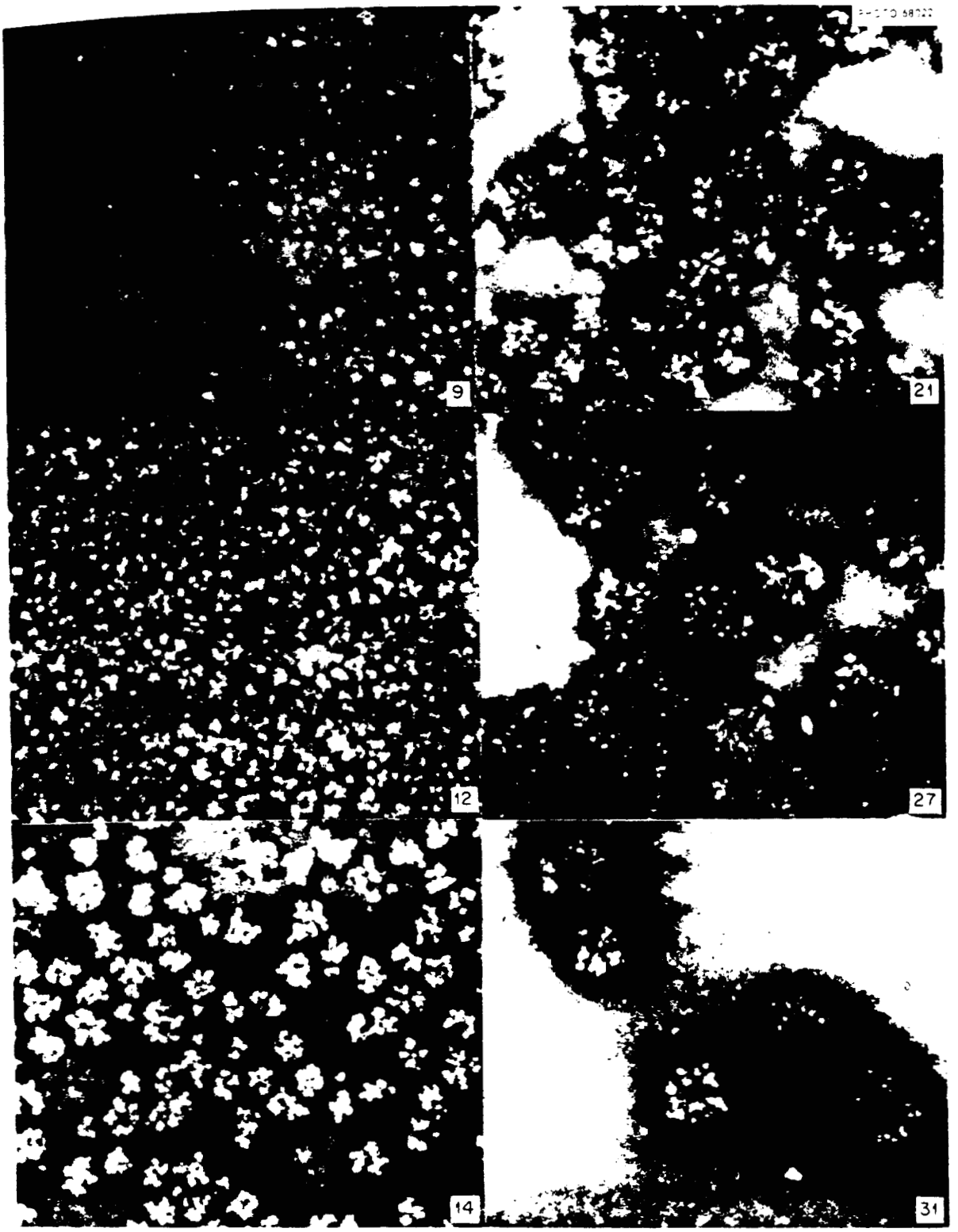

Fig. II-B-2. Electron Micrographs of Glycogen Isolated by Banding Fractions 9, 12, 14, 21, 27, and 31 in Fig. II-B-1 in CsCl. 72,000×.

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but are composed of an aggregate of substructures held together in a popcorn-ball configuration. The fine structure of these particles is now being intensively studied.

C. ISOLATION OF PARTICLES FROM HUMAN TUMORS

N. G. Anderson W. W. Harris

During this report period the usefulness of the S-ρ method for the isolation of virus-like particles from human tumors has been examined by using a few tissue samples. Four human leukemic spleens and two breast tumors have been studied. The results demonstrate that (1) virus-like particles can be found in human cancer tissue, (2) comparable studies on normal tissue must be performed, and (3) a thorough analysis of all the bands isolated is desirable to provide reference points. An example of the latter is the work on the glycogen band presented in the previous section.

The most promising results were obtained with a 49-g primary human breast tumor frozen immediately after surgical removal and kept at −60°C until ready for use. A 10% homogenate of the tumor was prepared in 0.05 *M* pH 6.7 sodium

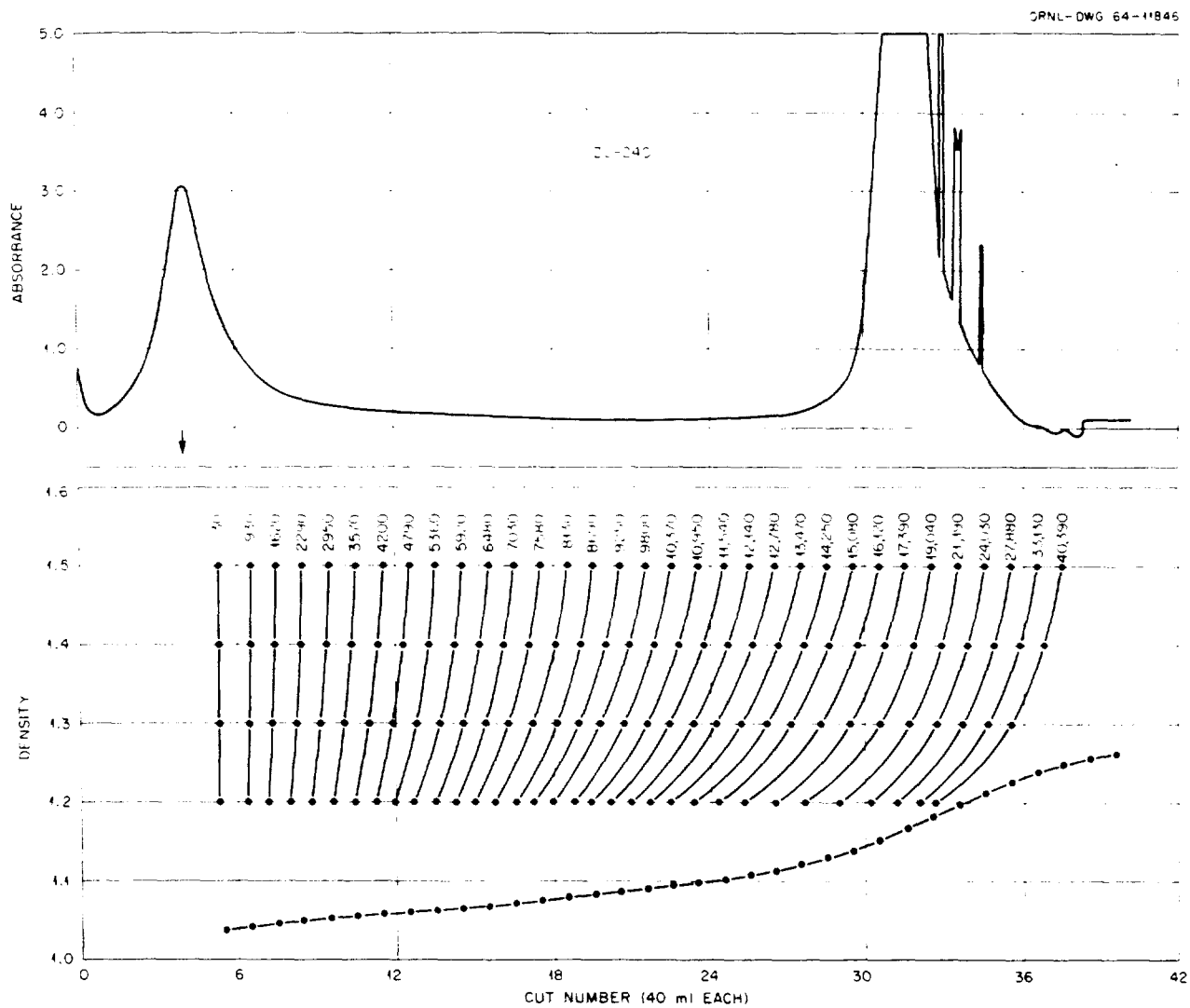

Fig. II-C-1. B-IV Zonal Centrifuge Run Diagram for Human Breast Tumor.

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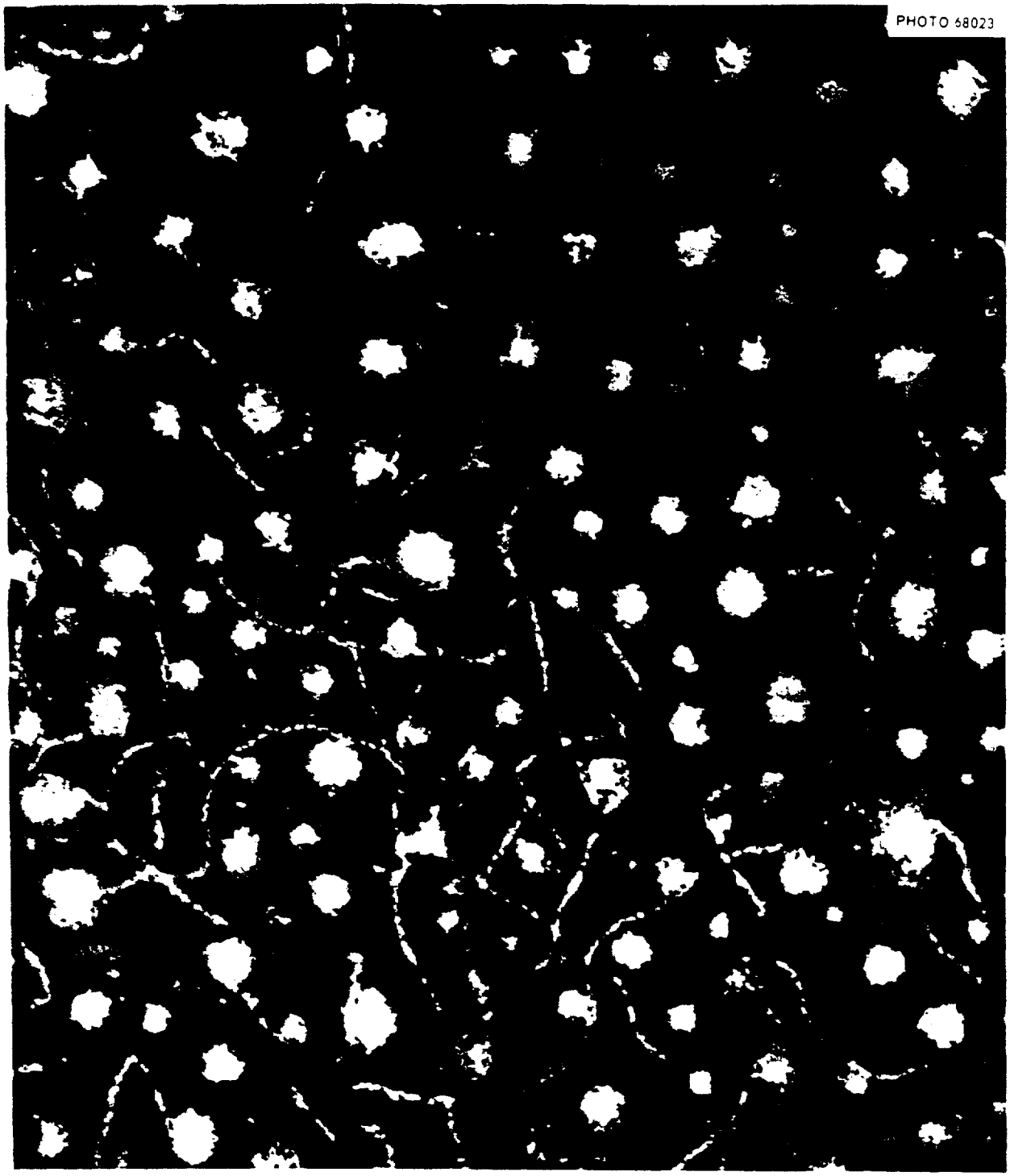

Fig. II-C-2. Virus-Like Particles Observed in Fraction Obtained by Rate Zonal Centrifugation and Isopycnic Banding of Human Breast Tumor. Obtained in cut No. 4 from run shown in Fig. II-C-1. 80,000×.

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citrate. The larger tissue fragments were removed by slow-speed centrifugation, after which particles ranging in size from mitochondria to small viruses were sedimented in the Spinco No. 30 angle-head rotor for 30 min at 24,000 rpm. The pellets were resuspended to a final volume of 28 ml and used as the starting sample in the B-IV zonal ultracentrifuge. The centrifuge was run until $\int \omega^2 \, dt = 1236 \times 10^7$. The recovered zonal centrifuge fractions were layered over 9 g of dry CsCl in No. 30 rotor tubes and centrifuged overnight at 24,000 rpm. The run diagram and computer plot are shown in Fig. II-C-1.

Under these conditions the virus-like particles would not be spread through more than a few tubes. Cuts 4–9 were examined in the electron microscope. The particles shown in Fig. II-C-2 were banded from cut 4, whose position is indicated in Fig. II-C-1. The particles averaged 125 μ in diameter, with a central denser portion having a diameter of approximately 100 μ. The strands also seen in the micrograph would occupy about 20% of the core if they originated from this source. The similarity between these particles and the particles which are associated with mammary tumors in mice is of more than passing interest.

With these and subsequent studies, techniques for examining tumors for virus particles have advanced to the point where a continuing search program may be considered.

D. EFFECT OF THE WARMING RATE ON THE SURVIVAL OF YEAST CELLS FROZEN CENTRIFUGALLY

J. G. Green Peter Mazur

The survival of yeast cells frozen at cooling rates of 1°C/min or above appears to depend largely on whether or not ice forms within the cells. Low cooling rates (~1°/min) favor high survivals because they cause the cells to dehydrate and, as a consequence, prevent intracellular crystallization. With higher cooling rates (> 50°/min), the cells do not have sufficient time to dehydrate, and so they freeze internally.[22–24]

However, another phenomenon appears in rapidly cooled cells: their survival becomes dependent on the rate of warming and thawing, with rapid warming yielding the higher survivals.[22] Crystal size is known to decrease with increasing cooling velocities, and it has been hypothesized[22] that rapid cooling produces smaller crystals within yeast cells and that these small crystals, because of their higher surface free energy, grow to damaging size during slow warming but not during rapid warming. The technique of centrifugal freezing described previously[25,26] provides a means of testing this hypothesis, since it permits reproducible, extremely rapid freezing of cell suspensions. Therefore, it has been applied to determine the effects of varied warming rates on the subsequent survival of rapidly frozen unicellular yeast (*Saccharomyces cerevisiae*).

The yeast was cultured in liquid media,[22] isolated by centrifugation, washed three times with sterile distilled water, and resuspended in sterile water to a final concentration of about 2×10^8 cells/ml. In previous experimentation, the cell suspension was injected from a hypodermic needle into the spinning rotor containing liquid nitrogen; however, in the present study, 4 ml of the cell suspension was introduced into the rotor as an aerosol from a glass atomizer designed to give minimal evaporation of water from the cells during delivery and to produce small droplets. The freezing of the suspended cells was conducted in a rotor spinning at 3600 rpm in an International PR-2 centrifuge, since earlier unreported studies yielded best cell survival from this treatment. The frozen suspension, still in liquid nitrogen, was transferred to a chilled, insulated cup to expedite transfer to the containers used in warming.

Two series of warming experiments were performed. In the first series, the frozen cells in a small amount of liquid nitrogen were poured into a beaker containing 1 liter of 0.08 *M* potassium phosphate plating diluent at temperatures of 5, 15, 25, 35, 45, and 55°C. To facilitate dispersion of the suspension during warming, the solution was stirred by a propeller agitator at a speed which caused sporadic vortex breakup.

[22] P. Mazur, *Biophys. J.* 1, 247 (1961).

[23] P. Mazur, *Biophys. J.* 3, 323 (1963).

[24] P. Mazur, *J. Gen. Physiol.* 47, 347 (1963).

[25] N. G. Anderson and J. G. Green, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p. 115.

[26] N. G. Anderson, J. G. Green, and P. Mazur, *Joint NIH-AEC Centrifuge Development Program Semiann. Rept. July 1–Dec. 31, 1962*, ORNL-3415, p. 60.

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