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Microsomal Particles and Protein Synthesis

RICHARD B. ROBERTS, Editor

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FOREWORD

The formation of a new society provides an occasion for innovations in the forms of meetings and publications. The Program Committee of the Biophysical Society attempted to seize this opportunity in arranging the 1958 meeting at Cambridge. In addition to the usual short contributed papers, review papers were scheduled for the three afternoon sessions to inform the membership of progress and problems in selected areas of biophysics. The contributed papers in these areas were allocated ample time for presentation and discussion, resulting in what might be called "contributed symposia." Those working in the field had the advantages of a symposium type of meeting; those less familiar had an introduction provided by the review papers followed by an opportunity to participate in the detailed technical sessions.

It also appeared desirable to alter the usual publication procedures. Customarily the complete proceedings of "invited symposia" are published but only the abstracts of short contributions. The twenty papers dealing with microsomal particles normally would be published individually during the next two years, scattered through different issues of five or ten journals. Alternatively, it would be possible to publish a transcript of the symposium. This procedure would provide a more complete account than is given in the abstracts; the material would be in one volume; and it could be issued much more rapidly than individual contributions to journals. Both the Council of the Biophysical Society and a majority of the contributors favored publication of the symposium material.

Transcripts, however, require an enormous editorial effort. It therefore seemed preferable to request the contributors to provide their own edited transcripts. These transcripts have been accepted with the understanding that publication in this volume which records material presented at a meeting would not preclude later publication in the usual journals.

The purpose of this volume is not to present well established theories or reviews of well known work. Rather, it is to publish new facts and new data while they are still fresh, useful, and possibly wrong. The relative costs of research time and publication make such a book worth purchasing if it saves no more than an hour of research time. The entire publication costs are justified if the book can save a month in some research program.

The Washington Academy of Sciences has agreed to act as publisher in the hope of demonstrating that this type of book can be brought out rapidly and that it does serve a useful function.

We wish to thank the Office of Publications of Carnegie Institution of Washington for editorial help in seeing the project through the press.

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INTRODUCTION

The topic "Microsomal Particles and Protein Synthesis" seemed particularly appropriate for the first symposium of the Biophysical Society. The particles owe their recognition to the electron microscope and the ultracentrifuge. X-ray diffraction studies will undoubtedly contribute to the picture of the structure of the particles; radioactive tracers show the kinetics of formation of the particles and their products; radiation experiments can give other evidence about the role of the particles in protein synthesis. Thus many of the special areas of competence of biophysicists are involved in the study of the particles.

More important, however, is the timeliness of a symposium devoted to a discussion of these ubiquitous granules. For a number of years circumstantial evidence has accumulated which indicates that ribonucleic acid (RNA) is implicated in protein synthesis. More recently it has been recognized that a large part of the RNA occurs in the form of ribonucleoprotein (RNP) particles. Particles of roughly the same size and composition have been isolated from sources differing as widely as rat liver, pea seedlings, and microorganisms. Accordingly there has developed a widespread faith that the particles are an important part of the machinery for protein synthesis.

It must be admitted, however, that the evidence is entirely circumstantial. A number of arguments would come immediately to the mind of a lawyer defending the particles from the charge of protein synthesis. (1) In vivo experiments have shown incorporation of radioactive tracers which is initially higher in the microsome fraction than in the soluble proteins, but the kinetic data are not sufficiently complete to prove a precursor-product relationship. For example, it is possible that steady-state conditions do not prevail; there is no certainty that both components draw on the same pool of amino acids; only the average of the soluble proteins is measured, whereas individual components might behave quite differently. (2) There are many cases both in complete and in cell-free systems where RNAase has been observed to inhibit protein synthesis. In many of these the addition of RNA (*not* RNP) is sufficient to restore the synthetic activity. (3) Cell-free systems showing unequivocal protein synthesis use cells that are only partially disrupted, and the requirement for particles is not demonstrated. In those systems where the particles have been partly purified, the incorporation data are more suggestive of exchange than of true protein synthesis. (4) No mechanism has been suggested which shows how the structure of the particle is compatible with its function as the template for synthesis of long chains. It appears that the particles have not yet

been proved guilty beyond all reasonable doubt. In the last few years, however, there have been marked advances in the study of the particles which promise to resolve these lingering doubts. Thus a symposium dealing with the particles and their function in living cells could hardly fail to bring forth new and exciting information.

In this symposium a number of papers were concerned with methods of isolation, the size, the composition, and the stability of the particles. One striking observation was that particles of roughly 80 S are found in a wide variety of materials; another area of agreement was in the requirement for magnesium to stabilize the particles. There was a consensus of opinion that carefully purified particles have little enzymatic activity and that their RNA content is 40 per cent or more. Several reports showed that the protein moieties of nucleoprotein have certain distinctive properties. Other studies explored the reasons for the variations in particle sizes that are observed both in vivo and in vitro. New kinetic data were presented which indicate that the protein of the particles does not serve as precursor material for nonparticulate protein. The incorporation of adenylamino acids was demonstrated in one study which also illuminated the need for caution in the interpretation of incorporation studies. Other papers reported less direct methods of approach to the understanding of the particles and their role in protein synthesis, such as studies of radiation effects and studies of incorporation of amino acid analogs. All together these reports provide a number of new facts that must be taken into account by any theory of protein and nucleic acid synthesis.

During the course of the symposium a semantic difficulty became apparent. To some of the participants, microsomes mean the ribonucleoprotein particles of the microsome fraction contaminated by other protein and lipid material; to others, the microsomes consist of protein and lipid contaminated by particles. The phrase "microsomal particles" does not seem adequate, and "ribonucleoprotein particles of the microsome fraction" is much too awkward. During the meeting the word "ribosome" was suggested; this seems a very satisfactory name, and it has a pleasant sound. The present confusion would be eliminated if "ribosome" were adopted to designate ribonucleoprotein particles in the size range 20 to 100 S.

The symposium provided to the participants an opportunity for comparing notes on methods and techniques and for exchange of views on the status of various problems. It undoubtedly affected the immediate research plans of a number of the participants. This volume is being published in the hope that it will extend some of these benefits to those who did not attend.

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Isolation and Characterization of Bacterial Nucleoprotein Particles

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Fractionation of the particulate matter from broken cells has long excited the biochemist. Lilienfeld [1] prepared nuclear and cytoplasmic fractions and studied the properties of a deoxynucleoprotein (DNP). Huiskamp [2] noted the influence of buffer salts on isolated DNP. The possibility of differential extraction of subcellular structures was investigated by Bensley and Hoerr [3]. The technique of purifying subcellular components has advanced rapidly through the efforts of Claude [4], Hogeboom and Schneider [5], and Anderson [6].

The fractionation of subcellular components offers the possibility of integrating the fields of intracellular anatomy, cellular physiology, and biochemistry. Siekevitz [7] working with mitochondria and Palade [8] and Zamecnik [9] working with the microsomal fraction have begun this integration by equating isolated fractions to structures observed in the electron microscope. Our studies with the ribonucleoprotein of *Azotobacter vinelandii* have clearly demonstrated that progress in this integration of fields demands a detailed understanding of the properties and stability of the subcellular particles. Previous studies [10] on the protein synthesis in cell-free extracts of *A. vinelandii* must now be reinterpreted in the light of our current understanding of the stability of bacterial ribonucleoprotein.

In 1954 Palade and Porter [8*a*] demonstrated endoplasmic reticulum in animal cells. Hodge, Martin, and Morton [11] in 1957 demonstrated similar structures in plant cells, and Sacks [12] has found related structures in yeasts, higher molds, and algae, leaving, at the present time, only the bacteria without clearly demonstrated endoplasmic reticulum. Pochon [13] found structures in *A. vinelandii* which by staining and observation in the light microscope were identified as nuclei. We have observed granularity in regions of thin sections through

A. vinelandii examined in the electron microscope. Several speakers at this conference have referred to similar granularity in sections of *Escherichia coli* as ribonucleoprotein particles, but no clear identification of these granules as such has yet been accomplished. Ribonucleoprotein particles, similar in size and chemical composition to those from animal cells, yeast, and fungi [14], can, however, be prepared from bacteria [15]. Several papers in this volume describe the ribonucleoprotein of *E. coli*, and this paper will treat the preparation and properties of ribonucleoprotein particles from *A. vinelandii*.

RUPTURE OF BACTERIAL CELLS

One of the aims of this work is to prepare subcellular structures having a useful correspondence to structures that existed in the intact cell. We have no single criterion to indicate when such a preparation has been accomplished, but we use as supporting evidence reproducibility of the product when prepared by several varying methods and we also invoke all the information about the stability of isolated components. The stability observations are described in a later section of this paper. Three different methods of cell rupture have been found to permit isolation of indistinguishable particles, provided that the cultures used were harvested at a similar stage of growth.

The first method was physical grinding with number 320 mesh Carborundum which had been washed with hydrochloric acid and rinsed with distilled water until neutral. The cells had been previously washed with distilled water. The packed cell paste was ground with 4 parts by weight Carborundum for approximately 15 minutes or until moist. An additional 2 parts of Carborundum was added, and the cells were ground for approximately 5 minutes more. Visible microscopic examination of the mixture revealed that approximately 95 per cent of the cells were ruptured by the grinding method. The ground cells were diluted with 8 times the original cell volume of the following buffer: 1.6×10^{-3} *M* K_2HPO_4, 0.4×10^{-3} *M* KH_2PO_4, and 5×10^{-3} *M* $MgSO_4$. This will be referred to as the RNP buffer. The supernate from centrifuging this mixture at 500*g* for 30 minutes is referred to as the crude extract.

The second method of cell breakage employed cells grown in the presence of 2 *M* glycerol. The cells were collected by low-speed centrifugation, and the pellet was diluted into 8 volumes of the RNP buffer to rupture and produce the crude extract.

In the third method of cell breakage, *A. vinelandii* protoplasts were ruptured by osmotic shock. Weibull [16] showed that *Bacillus megatherium*, when treated with lysozyme in sucrose solutions of high osmotic pressure, changed to a spherical form which is readily ruptured by lowering the osmotic pressure. We have avoided the use of sucrose in view of our findings on the instability of the isolated particles in dilute sucrose solutions. The *A. vinelandii* cells were washed in the RNP buffer, and suspended in 1.5×10^{-3} *M* EDTA at a dilution such that the optical density at 660 mμ was approximately 0.75. These solutions had been previously osmotically adjusted with glycerol or with Carbo-

wax "4000" to maintain the protoplasts. The turbid solutions were brought to 13 μg/ml in crystalline egg white lysozyme, and the turbidity was observed until its rapid decrease ceased. The protoplasts were then collected by low-speed centrifugation, washed once in osmotically adjusted RNP buffer, collected again, and ruptured by osmotic shock upon dilution with 10 times the packed cell volume of RNP buffer to yield a crude extract. During the development of the protoplast procedure, both the formation of the protoplasts and their osmotic rupture upon dilution were followed in the visible and phase contrast microscope.

PURIFICATION OF RIBONUCLEOPROTEIN PARTICLES (fig. 1)

The crude extract derived from any of the above three procedures is centrifuged at 4900*g* for 30 minutes. The pellet that accumulates consists of cell debris

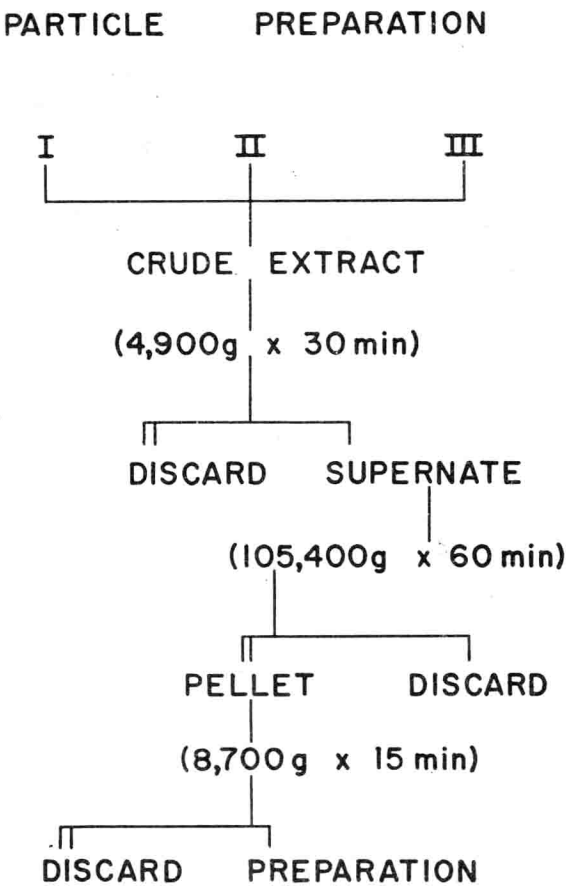

Fig. 1. Flow sheet for differential centrifugation of ribonucleoprotein from a crude extract of *A. vinelandii* prepared by I grinding, II osmotic shock, or III protoplastic osmotic shock.

and, with method I, some Carborundum that was not removed at lower speeds. The supernatant liquid from this step is now centrifuged at 105,000*g* for 60 minutes. The pellet so obtained is solubilized in RNP buffer for approximately 12 hours, and then centrifuged at 8700*g* for 15 minutes. The precipitate is discarded, and the supernatant liquid is examined in the analytical ultracentrifuge to determine the number of sedimenting components, their relative amounts, and their sedimentation coefficients. When the 86 S component is desired, the 105,000*g* and the 8700*g* cycle is repeated until over 90 per cent of the area in the schlieren pattern is under the appropriate peak.

PROPERTIES OF RIBONUCLEOPROTEIN PARTICLES

When a crude extract is processed to the stage labeled "preparation" in figure 1, and is examined in the analytical ultracentrifuge, it is found to sediment as a single peak of sedimentation coefficient 86 S. If, however, the same crude extract is carried to the same stage employing a buffer in which the magnesium concentration has been reduced to 10^{-3} *M*, the ultracentrifuge pattern now shows five significant components. Comparison of the schlieren and ultraviolet absorption photographs in the ultracentrifuge suggests that all these components contain nucleoprotein. The sedimentation coefficients extrapolated to zero concentration and corrected to 20° C are 86, 77, 58, 39, and 10 S. The 86, 58, and 39 S components are usually found in largest amount. Figures 2 and 3

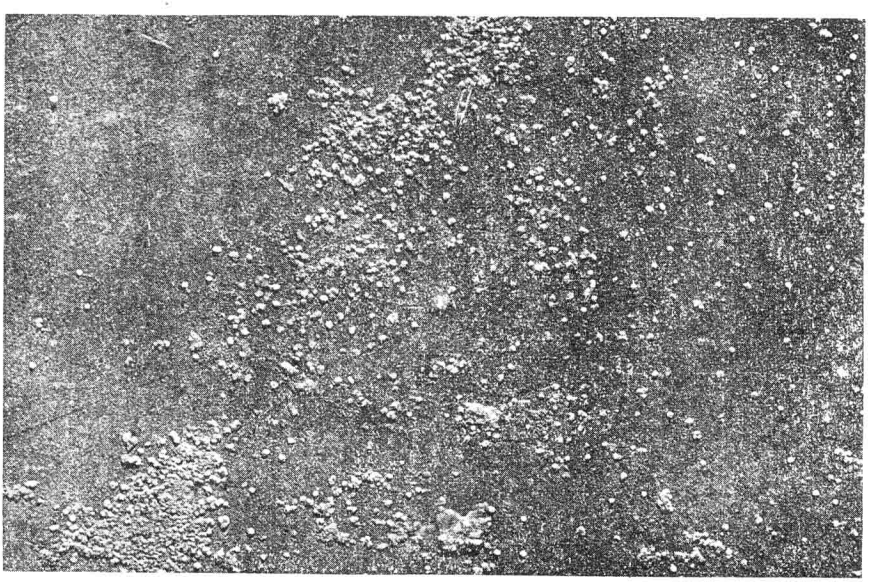

Fig. 2. An electron micrograph of the edge of a droplet of RNP particles sprayed onto a collodion membrane and shadowed with uranium. Magnified 34,000 times. Taken on a Siemens Elmiskop I by Professor Paul Kaesberg.

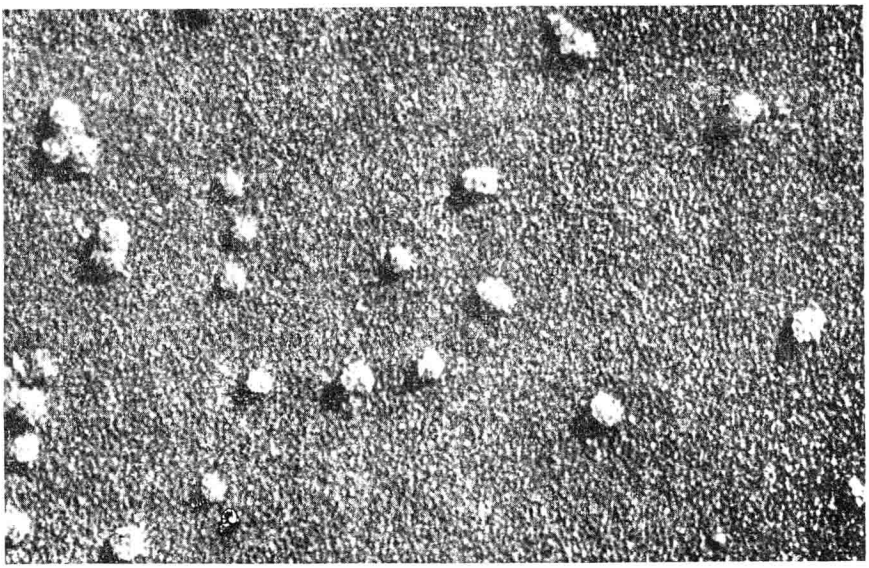

Fig. 3. An electron micrograph of a central portion of a sprayed droplet showing RNP particles magnified 170,000 times. The smallest particles are about 200 A in diameter, the largest about 250 A. From the shapes of their shadows it is estimated that their thicknesses are about 75 per cent as great as their diameters. Taken on a Siemens Elmiskop I by Professor Paul Kaesberg.

are electron micrographs of a purified preparation of the 86 S particles which was diluted 1000fold with distilled water and then quickly sprayed on a collodion membrane and air-dried. It is not yet known how the short exposure to distilled water will affect particle structure. The electron micrographs taken under these conditions show that at least two size classes are present, both of which are roughly spherical. The 86, 58, and 39 S particles all show small dependence of sedimentation coefficient on concentration, which also suggests that the particles are not markedly asymmetric.

The particles appear to contain ribonucleic acid and protein and to be free of lipid and deoxynucleic acid. The nucleic acid component has been separated and purified by detergent treatment [17], phenol [18], chloroform [19], and glacial acetic acid [20] extraction. The protein component when separated from the nucleic acid has been found to be insoluble in aqueous solutions unless prepared through a 67 per cent glacial acetic acid procedure. The protein shows an ultraviolet absorption typical of a protein rich in tyrosine. We have derived only one major protein from the particle at this point. The derived protein appears to have only one type of N-terminal amino acid, which we have very tentatively identified as glycine. The number of protein subunits per particle has not yet been quantitatively determined, but the experiments to date suggest a large number.

Ribose nucleic acid prepared from the particle by chloroform extraction of the protein shows markedly the hyperchromic effect characteristic of polymerized nucleic acids. Immediately upon addition of the alkali the optical density at 260 mμ increases 15 per cent. After incubation for 16 hours at 30° C with 0.5 *N* NaOH and adjusting the *p*H to 7.5, the final hyperchromic effect is found to be 39.1 per cent. The nucleotides (table 1) arising upon alkaline hydrolysis of the ribose nucleic acid have been chromatographed on Dowex-1-formate ion-exchange columns developed with gradient elution. The unknown nucleotide shows chromatographic behavior similar to that of the new ribonucleotide reported by Cohn, but its acid, alkaline, and neutral ultraviolet absorption spectra are not identical to those of the fifth nucleotide which we have isolated from yeast.

The 86 S particle has been examined for its stability as a function of salt, chelating agents, enzymatic attack, *p*H, and sucrose concentration (fig. 4). The results of these studies were fed back into improvements in the preparative procedure and are of utmost importance to the interpretation of labeled amino acid incorporation studies in the particulate fractions of *A. vinelandii*. If the 86 S particle is suspended in a *p*H 7.05 buffer of 2×10^{-3} *M* phosphate, 10^{-3} *M* $MgSO_4$, with NaCl added to a total ionic strength of 0.03, or is dialyzed against 2×10^{-3} *M* K_2HPO_4:KH_2PO_4 (4:1) buffer, it dissociates to yield 58 and 39 S components.

Our early studies showed that the 58 and 39 S components could be returned to 10^{-3} *M* Mg^{++}-containing solutions without re-forming the 86 S particle which had previously been stable to that environment. Encouraged by our discussions with Dr. Paul Ts'o at this conference, we explored further and found that in 5×10^{-3} *M* Mg^{++} the 58 and 39 S components recombined to form the 86 S particle, and that once formed this particle again was stable in 10^{-3} Mg^{++}. In all these studies the buffer also contained 2×10^{-3} *M* potassium phosphate buffer of *p*H 7.05.[1] We also confirm Ts'o's observation that the area of the 39 S peak is about one-half that of the 58 S peak, which suggests that one small 39 S and one larger 58 S particle combine to form the 86 S particle. Upon addition of 0.01 *M*, *p*H 7, ethylenediaminetetraacetic acid, the particles further dissociated to ribonucleoprotein of sedimentation coefficient less than 5 S (not extrapolated to zero concentration). The particles are also rapidly degraded to small fragments by ribonuclease but are not attacked by deoxyribonuclease. They are precipitated by *p*H below 6.5 or above 7.5.

Attempts to use sucrose for certain stages of the purification led to the observation that, if sucrose was added to the RNP buffer, the particles aggregated and were readily removed by low-speed centrifugation. Sucrose concentrations from 3 to 30 per cent were all found to have this effect. This finding necessi-

[1] *Note added in proof:* We recently reported at the 1958 meeting of the Federation of American Societies for Experimental Biology that a buffer 5×10^{-3} *M* in MgO and adjusted to *p*H 7.05 with cacodylic acid gives improved yield and excellent stability of the 80 S class of RNP from yeast, *E. coli*, and *A. vinelandii*.

TABLE 1. Analysis of Nucleic Acid Hydrolyzed with 0.5 *N* NaOH for 16 Hours at 37° C

Hydrolyzate was chromatographed on a Dowex-1-formate column with gradient elution.

	Nucleoprotein Particle, mole %	Whole Cells,* mole %
AMP	25	24
GMP	24	31
CMP	19	26
UMP	24	20
Unknown nucleotide	7	Not reported

* Whole cell data from Lombard and Chargaff [21].

tates re-evaluation of some studies [10] that have been carried out on *A. vinelandii* and raises the important question whether this phenomenon can occur in ribonucleoprotein from other sources.

The ribonucleoprotein has been assayed for a large number of enzymatic activities. It appears free of nucleotide phosphatase activity, glucose-1-phosphatase, oxidative phosphorylation enzymes, and electron-transport enzymes. A feeble glucose-6-phosphatase activity of 10 mM phosphate released per minute

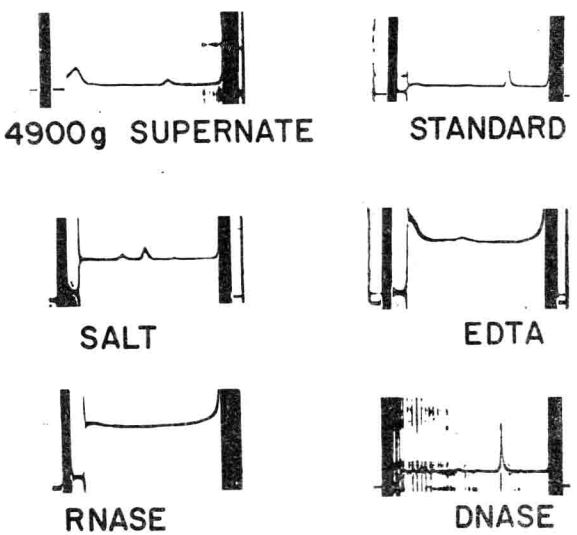

Fig. 4. The preparation and stability of *A. vinelandii* nucleoprotein. Each analytical ultracentrifuge pattern is labeled with the stage of preparation (top pair) or the treatment to which a pure 86 S product was subjected. The salt and EDTA treatment are described in the text. The RNAse action occurred in less than 5 minutes at 1 part of enzyme per 1000 parts of particle whereas the DNAse at the same concentration produced no change in 45 minutes.

per gram of particle was detected, but it is at most a few per cent of that found in equal weights of liver microsome fractions.

DISCUSSION

The study of *A. vinelandii* ribonucleoprotein has been more successful in posing interesting questions than in providing answers to previous questions. Can the sharp requirements of the nucleoprotein for divalent cations and for certain *p*H ranges be exploited to give information on the mode of combination of nucleic acid and protein or combination between nucleoprotein subunits? Does the marked difference in ribonuclease sensitivity of the plant ribonucleoprotein viruses and the bacterial ribonucleoprotein imply a different orientation or localization of nucleic acid and protein? The plant viruses now appear to have a protein coating with nucleic acid (or nucleoprotein) in an inner layer concentric with the protein coat and thus protected from ribonuclease attack. How different must the structure be to permit the rapid attack observed?

Will the small subunits derivable by salt and EDTA treatment also yield information on the mode of action or size of the functional nucleic acid?

The striking effect of divalent cations on the physical state of nucleoprotein is now becoming recognized as a phenomenon common to many systems. Huiskamp [2] in 1901 noted that thymus nucleoprotein was precipitated by 0.01 *M* calcium, barium, and magnesium salts and dissolved in excesses (0.1 *M*) of these same salts. He equated changes in physical properties of nucleoprotein solutions during dialysis to losses of divalent cations. He noted that heavy-metal divalent cations formed nucleoprotein precipitates that were difficult to dissolve. Korkes [22] and co-workers used a similar observation on manganese RNP to remove RNP from bacterial extracts. Carter and Hall [23] working in the laboratories of J. W. Williams noted that thymus nucleoprotein in sodium chloride solutions was a rodlike molecule but in calcium chloride solutions it became compact and showed no dependence of sedimentation rate on concentration. Wiberg and Neuman [24] have studied the binding of magnesium and calcium by RNA and DNA and find a region of concentration through which the number of equivalents bound changes rapidly. This concentration range is the same as that which we find critical for nucleoprotein structural changes.

The studies reported here, added to the work of Mazia [25] on the role of polyvalent cations in deoxynucleoprotein and nuclear structure and of Chao and Schachman [14*a*] in ribonucleoprotein stability, and to the many excellent contributions presented at the second annual Biophysics Conference, will help establish the basic rules for fractionation of subcellular particles in a reproducible manner.

The dependence of nucleoprotein structure upon divalent cation concentration is striking enough for these ions to become of interest in consideration of the variables which dictate when a nucleic acid will be in the double helix,

when it will split, when it is "soluble RNA," and when not. Re-evaluation of Brachet's [26] findings on the relation of ribonucleoprotein to growth phase of yeast will be warranted in the light of current concepts of the importance of buffer media.

Whereas the divalent cations of the buffer medium play an important role in nucleoprotein structure, there appear to be mineral elements which are influential in nucleic acid function and are not in free equilibrium with the buffer. Zittle [27] and Jungner [28] demonstrated that carefully isolated yeast RNA contained characteristic amounts of metal ions. Kihlman [29] and Mazia [25] have related structural integrity of chromosomes to metal content. Loring and Cooper [30] find that certain cations are important for nucleoprotein stability of tobacco mosaic virus and hence for infectivity. Racker and Krimsky [31] have evidence that metal ions are involved in an animal virus. These are but a few of the many nucleoprotein-metal systems cited in the literature.

Thus it appears that for viruses, as well as for subcellular particles, elucidation of the structural and functional role of cations will be a fertile and challenging frontier for those with pioneering instincts.

SUMMARY

Ribonucleoprotein particles of sedimentation coefficient $S_{20^\circ} = 86$ have been isolated from *A. vinelandii*. The particles are free of lipid and DNA. They are stable at neutral *p*H, in low-ionic-strength solution when divalent cations are present; they are unstable in sucrose, in concentrated salts, and in the presence of ribonuclease. Nucleic acid derived from the particles contains an unidentified fifth base. The 86 S unit reversibly dissociates to particles of 58 and 39 S when the Mg^{++} concentration is lowered.

ACKNOWLEDGMENTS

We are pleased to acknowledge the generous aid of our colleagues. Professor Paul Kaesberg conducted the electron-microscope studies, Miss Vatsala Thakur provided the N-terminal amino acid analyses, and Miss Fay Hoh collaborated in studies on the protein derived from the particles. Financial support from the National Institutes of Health and Wisconsin Alumni Research Foundation is gratefully acknowledged.

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