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The Effects of Plant Virus Purification Procedures on Extracts of Healthy Plants

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THE EFFECTS OF PLANT VIRUS PURIFICATION PROCEDURES ON EXTRACTS OF HEALTHY PLANTS

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INTRODUCTION

The purification of viruses from infected plants concerns mainly the removal of the viruses from the components of the host cell. To do this without destroying the virus particle, it is often necessary to use a denaturing or adsorbing agent that acts on the impurities but not on the virus. It is usually impossible to purify plant viruses simply by application of separation procedures such as centrifugation and electrophoresis.

Denaturation of plant components during purification of stable plant viruses may be accomplished simply by carrying out centrifugation or other procedures at room temperature for several days. Selection of a denaturation procedure is particularly important if the virus is relatively unstable, if it is present in low concentrations in the plant, or if the work is designed to study the initial state of the virus in the plant. Different viruses respond differently to the action of these agents. One virus may be successfully purified from its host by a particular treatment, but another virus in the same host may suffer a complete loss of infectivity as a result of the same treatment. The action of these agents upon the host cell components may be more uniform than it is upon viruses, since properties of these components should not differ too greatly among plants that are commonly used as virus hosts.

One of the commonly used techniques for separation of components in virus purification is density-gradient centrifugation, in which particles are centrifuged through a liquid column which has a density gradient resulting from concentration gradients of two other substances of different densities. If the particles are floated in a layer on top of the column, they will sediment as a zone through the column during centrifugation. The final position of the particles after centrifugation depends upon the time centrifuged and the material from which the column is prepared. Separation in sucrose density-gradient columns is usually dependent upon the size, shape, and density of the particles. Separation in columns prepared from cesium chloride is based on the density of the particles because centrifugation is usually continued until an equilibrium position has been reached.

This study was undertaken to devise an easy, accurate method to detect and separate various components in extracts of healthy bean, tobacco, and barley plants by using techniques common to virus purification and to determine the effect of some of the more common denaturing and adsorbing agents on these healthy plant components. Conditions for density-gradient centrifugation were selected to give the best separation of normal components, and these conditions were then used as the basis of an analytical method to evaluate the denaturation procedures.

LITERATURE REVIEW

Methods used for the clarification of virus-containing plant extracts have been summarized by Steere (1959) and by Markham (1959). Some of these will be described here briefly. These methods are generally followed by additional purification procedures, such as differential centrifugation, density-gradient centrifugation, electrophoresis, or precipitation of the virus with acid or salt. The selection of the method usually depends upon the purpose for which the virus is being purified.

Freezing infected tissues before grinding has long been advocated to improve the ease of grinding and to facilitate the release of virus from the host tissues. Takahashi (1951) reported getting more than twice the yield of tobacco mosaic virus (TMV) from frozen tissues than from unfrozen coarsely macerated tissue. Stanley (1940) purified tomato bushy-stunt virus by first freezing the plants to denature and render insoluble the normal proteins of high molecular weight that contaminate viruses purified by differential centrifugation. Freezing tissues is highly recommended unless it causes loss in virus activity.

Heating the infected sap is one of the two methods recommended by Markham (1959) and is probably one of the most widely used methods. The procedure usually consists of heating the sap to a temperature between 40° and 60° C and holding

it there for a few seconds to ten minutes or longer. The coagulum of denatured protoplasmic proteins and chloroplasts is then removed by low speed centrifugation. Steere and Williams (1948) and Steere (1952) used short periods at 55° C to purify tomato bushy-stunt virus and TMV, respectively, for electron microscope observations. Schlegel and Rawlins (1953) purified TMV for spectrophotometric assay by heating it for 15 minutes at 60° C, and obtained good reproducibility with little loss in virus activity. Brakke purified wheat streak mosaic virus (1958) and barley stripe mosaic virus (1959) by heating infected sap at 40° C for one hour.

Precipitation of normal components by ethanol is the other method recommended by Markham (1959). The strained sap is stirred vigorously while 300 ml of 90% ethanol are added to each liter of sap. The coagulum formed can be centrifuged off, leaving a slightly cloudy fluid containing the virus. However, some viruses may not tolerate this level of ethanol, and others may be precipitated by dilute ethanol at the pH of the sap.

Acidification of the crude sap to denature proteins was used by Commoner et al. (1950) in the study of TMV synthesis. Rice et al. (1955) purified squash mosaic virus by acidification to pH 5.0 and differential centrifugation. Lindberg et al. (1956) used acid treatment in the purification of a group of squash mosaic viruses, but this was not successful for a similar group of melon mosaic viruses.

Schneider (1953) used chloroform-water emulsions to denature noninfectious material in tobacco sap infected with TMV. The virus remained dispersed in the aqueous phase and remained infectious, while there was a relatively large accumulation of plant constituents at the interface between the chloroform and water. Chlorophyll and certain other constituents dissolved in the excess chloroform.

Tomlinson, Shepherd, and Walker (1959) purified cucumber mosaic virus successfully from cucumber corollas and from infected tobacco leaves with the aid of n-butanol. Heavy precipitates formed when the crude sap was mixed with 8 to 9% n-butanol and stirred for 30 minutes. The butanol seemed to keep the virus in solution, as well as to precipitate many of the normal components.

Steere (1956) combined these two procedures and used a mixture of equal parts of n-butanol and chloroform for the purification of tobacco ringspot virus. An emulsion was formed by slowly adding two volumes of this mixture to one volume of infected sap with rapid stirring. After the emulsion was broken by centrifugation, the bottom layer was a mixture of chloroform and butanol and contained all the chlorophyll. Above this was a solid layer of denatured protein and cellular debris. The top layer was an aqueous phase saturated with chloroform and butanol and contained nearly all of the virus and some of the noninfectious plant protein. When this layer

was allowed to stand overnight at 22° C most of the nonvirus protein was denatured and could be removed by centrifugation. The residual solvents seemed to stabilize the virus. This procedure has also been used in the purification of tomato bushy-stunt, southern bean mosaic, turnip yellows, alfalfa mosaic, and tobacco mosaic viruses.

Ether is another organic solvent which has been used in plant virus purification. Bagnall, Wetter, and Larson (1959) used ether for potato virus M, potato virus S, and carnation latent virus. Wetter (1960) used it with seven additional rod-shaped viruses. He shook crude sap with an equal volume of cooled ether for 5-15 minutes, and separated the emulsion by low speed centrifugation. The lower aqueous layer was then shaken with an equal volume of carbon tetrachloride to remove the dissolved ether.

Fulton (1957, 1959) removed much of the extraneous matter from freshly ground infected leaves by adsorption onto hydrated calcium phosphate, which had been freshly precipitated from a mixture of Na_2HPO_4 and $CaCl_2$. The ionic strength of the buffer was a critical factor in the adsorption of virus and green host material. This procedure has been used successfully for several prunus viruses, rose mosaic virus, and apple mosaic virus, but resulted in loss of infectivity with tobacco ringspot, cucumber mosaic, tomato spotted wilt, and peach yellow bud mosaic viruses.

Brakke (1959) used a detergent to disperse barley stripe mosaic virus in density-gradient columns after heating had caused its aggregation. The detergent was also quite effective in dispersing normal host components. These components reached a final depth after only a short period of centrifugation in density-gradient columns, suggesting that they were comparatively large particles of low density, and were colloidal in nature rather than being true molecules. As with other treatments, this did not succeed for some viruses.

In most of these reports, mention has been made of treatments applied to healthy plant extracts, usually stating that the material recovered is far smaller than that obtained from infected plants, or that it is present in insignificant amounts. The criteria of purity of virus preparations has often been the colorless appearance of the final product. When this has been achieved the virus has been classified as pure.

Pirie (1950) directed attention toward material that could be carried through purification procedures into a virus preparation made by ultracentrifugation of tobacco leaf extracts. He was able to isolate a nucleoprotein that had some similarity to plant viruses from sap of young uninfected tobacco leaves. This inhomogeneous material was characterized in electron microscope observations mainly as spheres 20-30 mμ

in diameter, with much amorphous material also present. The methods generally used for clarifying the sap of virus-infected plants as a preliminary to serological or chemical study of the virus usually coagulated the nucleoprotein to a varying extent, as well as removing chloroplasts and chloroplast fragments. The chromoproteins usually were aggregated by centrifugation, leaving a colorless or pale-green opalescent solution. Enzymes associated with the nucleoprotein made it quite unstable in sap when first isolated, which explains some changes in virus preparations during isolation. He pointed out that there were structures in the leaf extracts with many points of resemblance to the microsomes that could be separated by similar methods from animal tissues. Later, Holden and Pirie (1955) observed that the properties of this nucleoprotein were not constant in different preparations, and suggested this could be connected with the physiological state of the original tobacco plant from which the isolations were made.

Pirie (1956) studied components of TMV preparations made in different ways. When quick and gentle methods were used, the end product contained material that was not present in preparations made by methods that were rougher and more dilatory. This material was mostly normal leaf nucleoprotein, which could be removed by purification steps that may alter some properties of the virus. It was not determined

definitely if this nucleoprotein material was combined with the virus or merely mixed with it. The suggestion was made that the contaminants tended to concentrate at the ends of the virus rods preventing end-to-end aggregation, since treatments which removed the nucleoprotein contaminants usually resulted in increased aggregation.

More recently, microsomal nucleoprotein particles have been isolated and characterized from pea seedlings by T'so, Bonner, and Vinograd (1958). Sedimentation in the analytical ultracentrifuge detected four species of particles with sedimentation constants of 80s, 60s, 40s, and 26s at infinite dilution. When a certain amount of magnesium was removed from the system, the 80s particle dissociated into a 40s unit and a 60s unit. When more magnesium was removed, the 60s unit dissociated to a 40s unit and two 26s units, or to two 40s units, one of which dissociated to two 26s units. The particles contained 35-40% ribonucleic acid by weight, and appeared as spheres in the electron microscope.

Ribonucleoprotein particles from Escherichia coli have also been isolated and characterized by Tissieres et al. (1959). Magnesium stabilized the particles, and, on varying its concentration, four kinds of components were observed with sedimentation constants of 30s, 50s, 70s, and 100s. The 70s particles were formed from one 30s and one 50s; the 100s particles were formed from two 70s particles. Each type of

particle was isolated and found to contain 63% ribonucleic acid, and all had the same density. Electron microscope studies with shadow cast specimens (Hall and Slayter, 1959) and with negatively and positively stained specimens (Huxley, 1960) revealed small, roughly spherical particles. The 70s particles contained two subunits of unequal size which resembled the 50s and 30s particles, and in the 100s particles, the smaller subunits of the 70s particles were paired.

MATERIALS AND METHODS

Source material

Bean (*Phaseolis vulgaris* L. 'Great Northern UI 59'), tobacco (*Nicotiana tabacum* L. 'Havana 38'), and barley (*Hordeum vulgare* L. 'Moore ') plants were grown in four inch pots in the greenhouse. Fully expanded first trifoliate leaves were harvested 18-20 days after planting, usually at a stage when the second trifoliates were just beginning to expand. Tobacco leaves were harvested when the plants were in the 5-7 leaf stage, about four weeks after transplanting. Barley leaves were harvested from plants in the 4-5 leaf stage, 16-18 days after planting. The leaves were wrapped in damp paper toweling and waxed paper and refrigerated until use.

Preparation of extracts

Leaves were weighed and ground finely in a mortar and pestle with 1 ml of 0.01 M neutral phosphate buffer per gram of leaves. The large center midrib of tobacco was removed before grinding. The buffer had 0.005 M K_2HPO_4 and 0.005 M KH_2PO_4 in distilled water to give a pH value of 7.0. The extract was squeezed through a double thickness of cheese-cloth and centrifuged for 15 minutes at 10,000 rpm in the SS-34 rotor of the Servall Type RC-2 refrigerated centrifuge.

This supernatant fluid, the crude extract, was used for analysis or for further treatment.

High speed centrifugation was done in the No. 40 rotor of the Spinco Model L preparatory ultracentrifuge, either for 100 to 120 min at 38,000 rpm in 5/8 by 2⅛ inch tubes or for 60 min at 36,000 rpm in ½ by 1¼ inch tubes. Pellets were resuspended in 1 ml of 0.01 M neutral phosphate buffer per gram of leaves. This was designated as a 1:1 dilution of the extract. All concentrations reported as dilutions were calculated by dividing the volume of the sample in ml by the weight in gm of the tissue from which the sample was derived.

Treatments

The effect of several different denaturing or adsorbing agents on either the crude extract or the resuspended high speed pellet was studied. The treatments were applied in the following manners.

Heat. About 5 ml of the extract in a plastic or glass tube was placed in a large beaker of water at 55 ± 1° C for 10 min, or in a constant temperature water bath at 40 ± 0.5° C for one hour. Samples were cooled by holding the tube in running tap water and then centrifuged for 10 min at 10,000 rpm.

Organic solvents. Several different solvents and methods of treatment were applied, always in the cold at 4° C.

a) Ether. The sample was mixed with an equal volume of diethyl ether (Wetter, 1960), and shaken vigorously by hand for 3 min or gently by rotation on a dialysis wheel for 1 hr. The emulsion was broken by centrifugation for 10 min at 3000 rpm. The lower aqueous phase was removed into a syringe through a long needle and placed in an open petri dish in front of a fan in the cold for 30 min to evaporate the dissolved ether.

b) n-Butanol. According to the methods of Tomlinson et al. (1959), the sample was mixed with 10% by volume of n-butanol and shaken gently by rotation on a dialysis wheel for 1 hr. After centrifugation for 10 min at 10,000 rpm, the supernatant fluid was allowed to evaporate for 30 min to remove the dissolved n-butanol in the same manner as in the ether treatment.

c) Chloroform-butanol. The sample was first shaken vigorously for 3 min with an equal volume of chloroform and centrifuged 10 min at 3000 rpm. The upper aqueous phase was removed with a syringe and shaken for 3 min with an equal volume of n-butanol. After centrifugation for 10 min at 3000 rpm, the upper aqueous phase was placed in an open petri dish for 30 min in the cold to evaporate a portion of the dissolved solvents.

d) Ether-butanol. The sample was first shaken vigorously for 3 min with an equal volume of diethyl ether and

centrifuged for 10 min at 3000 rpm. The lower aqueous phase was removed and shaken for 3 min with an equal volume of n-butanol. After centrifugation for 10 min at 3000 rpm, the lower aqueous phase was allowed to evaporate in the same manner as in the other treatments.

e) Ethanol. Following the method described by Markham (1959), the extract was placed in a small beaker and stirred magnetically while 30% by volume of 90% ethanol was added dropwise from a pipette. After an additional stirring of 10 min, the mixture was centrifuged for 10 min at 10,000 rpm.

Calcium phosphate. The extract was placed in a small beaker and stirred magnetically while 1/4 volume of 0.2 M Na_2HPO_4 and 1/20 volume of 1.0 M $CaCl_2$ were added simultaneously from pipettes by drops. After stirring for an additional 20 min, the sample was centrifuged for 10 min at 10,000 rpm.

Acidification. The pH of the extract to be treated was determined. Sufficient 1.0 N HCl was added to lower the pH value about one unit. Either before or after centrifugation for 10 min at 10,000 rpm, sufficient 1.0 N NaOH was added to return the pH value to near neutrality.

Freezing. Freshly harvested leaves, crude extracts, and resuspended high speed pellets were frozen for various periods of time. Frozen leaves were thawed and extracts were prepared as usual. Frozen liquid extracts were thawed and centrifuged for 10 min at 10,000 rpm.

Igepon T-73. To test the effect of the detergent Igepon T-73 (sodium N-methyl N-oleoyl taurate), 0.1% was mixed in the 0.01 M neutral phosphate buffer used to prepare the density-gradient columns (Brakke, 1959). Extracts were layered on these columns and effects occurred during centrifugation.

Magnesium and EDTA. The effect of several different levels of magnesium upon the components of the resuspended high speed pellet was tested by using gradient columns with various concentrations of $MgCl_2$ in 0.005 M tris buffer (tris-(hydroxymethyl) amino methane) adjusted to pH 7.4 with HCl. The effect of removal of magnesium was tested by using gradient columns with 0.005 M EDTA (sodium ethylenediaminetetraacetate) adjusted to pH 7.0 with tris. As with Igepon, effects occurred during centrifugation.

Analysis of the extracts

Density-gradient centrifugation. Density-gradient centrifugation was used as an analytical method to assess the value of various denaturation procedures used for purification of plant viruses. Columns for rate zonal centrifugation (Brakke, 1960) were prepared by layering 4, 7, 7, and 7 ml of either 100, 200, 300, and 400 mg sucrose per ml or 50, 100, 150, and 200 mg sucrose per ml, respectively, in 1 by 3 inch tubes. The layered solutions were allowed to diffuse

overnight or longer in the cold. These were termed 10-40 or 5-20 rate columns. Unless stated otherwise, 0.01 M neutral phosphate buffer was used to dissolve the sucrose. Two-milliliter samples were layered carefully on the tops of the gradient columns immediately before centrifuging at 23,000 rpm in the SW 25.1 rotor of the Spinco Model L preparatory ultracentrifuge, usually for 4 hr at 4° C. Several variations of sucrose gradient columns were used and will be mentioned in the appropriate sections of the results.

Equilibrium zonal centrifugation (Brakke, 1960) in cesium chloride gradient columns (Meselson et al., 1957) was used to analyze the extracts. Columns were prepared by layering 1.3 ml of each solution of 0.01 M neutral phosphate buffer containing 1.0 M, 2.0 M, and 3.0 M CsCl, respectively, in 1/2 by 2 inch tubes. Eight-tenths-milliliter samples were layered on each tube before centrifuging at 30,000 rpm in the SW-39 rotor of the Spinco Model L ultracentrifuge.

Loading of the rotors and removal of the tubes after centrifugation was usually done at room temperature, but the centrifuge was refrigerated, and the temperature of the tube contents was 5-10° C after centrifugation.

Analysis of centrifuged gradient columns. Light-scattering materials in the centrifuged tubes were detected in a darkened room by directing a narrow beam of light down through the tube. The depth and description of the visible zones was recorded.

Ultraviolet absorption at 254 mμ was recorded automatically throughout the columns with the Instrumentation Specialties Company (ISCO) Model D Density Gradient Fractionator and Model U U. V. Flow Densitometer as described by Brakke (1962). For simplicity, this procedure will be called "scanning". Samples of the zones were collected manually as they came through the fractionator, and were used for further analysis.

Components in the plant extracts that had been separated during centrifugation appeared as distinct visible zones in the tubes, and as peaks corresponding to the ultraviolet absorption on the scanning pattern recorded by the densitometer. The area under the peaks corresponded to the concentration of the components in the tube, and the depth of the absorption peaks could be correlated with the depth of the visible zones.

Measurement of effectiveness of treatments. Effectiveness of the denaturing treatments was evaluated by scanning the tubes in which the treated extracts had been centrifuged, and measuring the area under the individual peaks or under the scanning pattern of the entire tube. The base line was determined by scanning an identical centrifuged gradient column which had been layered with 2 ml of buffer or distilled water. This was necessary because the material used to prepare the columns absorbed or scattered a small amount of light

depending upon its concentration. Measurements of scanning patterns of treated extracts were compared with those of untreated resuspended high speed pellets to determine the relative amount of absorbing material that each of the treatments removed.

Area measurements were made with a cathetometer and converted to ml x absorbance at 254 mμ.

Characterization of individual components

Ultraviolet absorption. Samples of zones collected from density-gradient columns were analyzed spectrophotometrically in a Beckmann Model DU spectrophotometer. Values of optical density were measured for wave lengths between 230 mμ and 300 mμ. Distilled water was used as a blank. Corrections for variation between quartz cells were made by determining the optical density of distilled water in each cell at each wave length used, and subtracting this value from the reading obtained for the sample.

Electron microscopy. Samples of zones collected from density-gradient columns were dialyzed for two days against several changes of distilled water, after which they were mixed with 10% glycerine, 0.01 to 0.1% bovine serum albumin, and 0.033% 264 mμ polystyrene latex spheres. A low pressure Vaponefrin atomizer was used to spray the samples onto a carbon backed collodion film supported on a 20-mesh copper

wire grid. They were shadowed with tungsten oxide, viewed in an RCA EMU-2 electron microscope, and photographed at a magnification of 20,000.

Dry weight. The pellet from high speed centrifugation of bean crude extract was resuspended at a 10:1 concentration. This was centrifuged for 1 hr on a 10-40 rate tube. A 10 ml sample from a depth of 2.5 to 4.5 cm was extracted into a syringe through a needle with a bent tip, mixed with 2 ml of distilled water, and floated on an equilibrium zonal centrifugation gradient column, prepared by layering 4 ml of each solution of 0.01 M neutral phosphate buffer containing 300, 400, 500, and 600 mg sucrose per ml, respectively, in 1 by 3 inch tubes. After centrifugation for 8 hours at 23,000 rpm, depths of the visible zones were recorded. One tube was scanned and samples of the zones were collected. From the other two tubes, three samples were collected into a syringe through a needle with a bent tip. Depths of the samples were 3.3 to 4.3 cm, 4.4 to 4.7cm, and 4.8 to 5.0 cm, corresponding to the zones. Ultraviolet absorption spectra of ten-fold dilutions of the samples were recorded, and the remainder of the samples were dialyzed against distilled water for 2½ days. They were concentrated by centrifuging for 1 hr at 38,000 rpm in the No. 40 rotor in 5/8 by 2½ inch tubes. Pellets were resuspended in 3 ml of distilled water, and two 1 ml samples of each were placed in previously dried weighing bottles and

dried at 105° C to a constant weight. Ultraviolet absorption spectra were again recorded on dilutions of the concentrated samples.

EXPERIMENTAL RESULTS

Characterization of components in plant extracts

Density-gradient centrifugation provided a very useful technique for the separation of components present in extracts of leaves of healthy plants. By varying the concentration of sucrose, the slope of the gradient, and the centrifugation time, the degree of separation and the components detected in phosphate buffer could be varied considerably. Figure 1 shows scanning patterns of three different types of gradient tubes each with a resuspended high speed pellet of one of the three plants used, and each after a different time of centrifugation. Extracts of all three plants gave similar results on each of the different types of gradient columns, except for slight differences in the concentration of the individual components.

Figure 1 A represents material derived from 1 gm of bean leaves centrifuged for 2 hr on a 10-40 rate column. The smaller components remained in the top 1.5 cm of the tube and were not separated sufficiently for the scanner to detect the individual zones. A broad band of larger, more rapidly sedimenting, low-absorbing material appeared between 2.5 and 3.5 cm. A small green pellet was formed at the bottom of the tube. Figure 1 B represents material from 1 gm of tobacco leaves centrifuged for 4 hr on a 5-20 rate column. The

smaller components sedimented as far as 2.5 cm and three definite zones were detected by the scanner. The larger material all sedimented to the pellet and almost no absorbing material remained below 3.0 cm. Fig. 1 C represents material from 1 gm of barley leaves centrifuged for 2 hr on a gradient column prepared by layering 3, 5, 5, 5, 5, and 4 ml of 50, 100, 150, 200, 400, and 600 mg sucrose per ml, respectively, in 1 by 3 inch tubes. The smaller components sedimented as far as 1.5 cm, but individual components were not detected as in the 5-20 rate tubes. The larger components were evenly distributed through the remainder of the tube, with no definite absorbance at a particular depth. Green material sedimented to a depth of about 5 cm, and a small pellet was formed.

This and similar experiments showed that density-gradient centrifugation of crude extracts separated components into three general classes: non-sedimenting, highly-absorbing, brown-colored, low molecular weight material; slowly-sedimenting, highly-absorbing, colorless material that separated into 3-5 distinct components; and fast-sedimenting, green-colored, low-absorbing, light scattering material.

The non-sedimenting material could be removed from the extracts by high speed centrifugation, and was assumed to consist of low molecular weight proteins and phenols.

The slowly-sedimenting material consisted of nucleoprotein-containing particles, presumably ribosomes. The

ribosomal components were small, dense, and poor light scatterers. Concentrations that gave pronounced peaks in UV scanning patterns were often not visible by scattered light.

The fast-sedimenting material consisted of green chloroplasts, or fragments thereof, and other comparatively large particles containing protein and probably carbohydrates and lipids, but little or no nucleic acid. These components, as found from 2-5 cm depth in Fig. 1 A, scattered much light and concentrations of these that gave zones readily detected by light scattering often were not detected by UV scanning. They were larger than the ribosomes and sedimented faster in rate zonal centrifugation, but were not as dense and did not sediment as far in equilibrium zonal centrifugation in cesium chloride. This fast-sedimenting material could be separated into three components by equilibrium zonal centrifugation in sucrose. The lightest and heaviest of the three components were white, but the intermediate one was green.

Since the presence of proteins and phenolic materials in a gradient tube often obscured some of the ribosomal components in scanning patterns, they were usually removed by high speed centrifugation before analysis of the samples on density-gradient columns. Scanning patterns of tubes containing crude extracts with no preceding high speed centrifugation are shown in Fig. 9 D, E, and F.

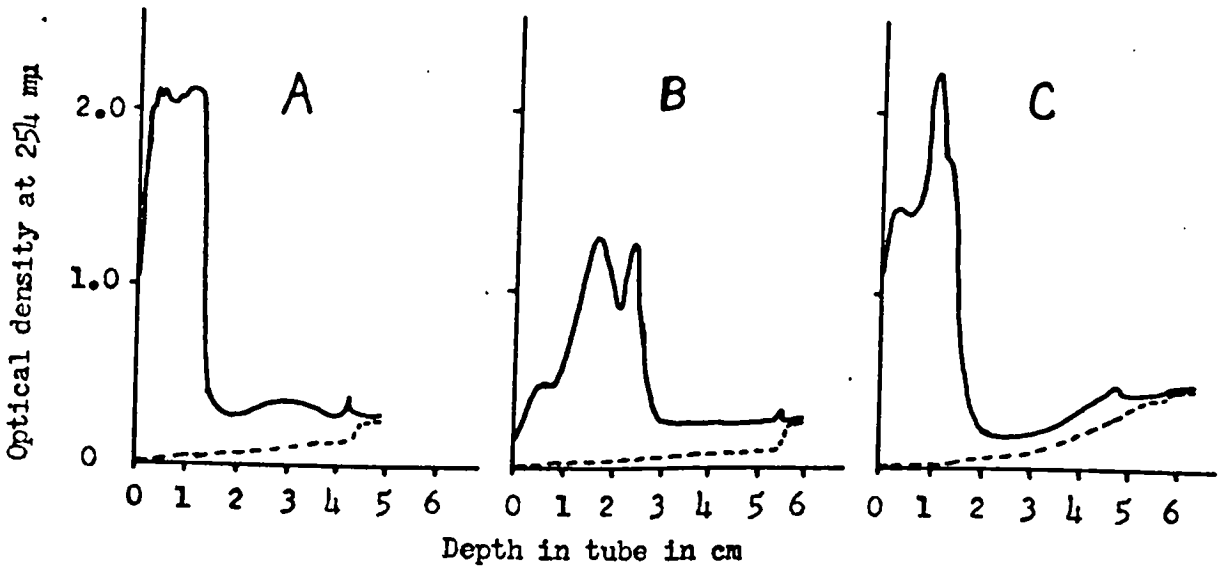


Figure 1. Scanning patterns of density-gradient columns prepared with 0.01 M neutral phosphate buffer, containing resuspended high speed pellets from healthy plants. The dotted line represents the base line, determined by scanning an identical centrifuged gradient column containing water. Differences in the height of the base line after the bottom of the column had passed through the absorption cell, at 4.5 cm in A and about 5.3 cm in B and C, were due to differences in absorption of the dense sucrose used to float the tube contents through the cell.

A - Gradient column of 100-400 mg sucrose per ml, centrifuged 2 hr at 23,000 rpm, containing material derived from 1 gm of bean leaves.

B - Gradient column of 50-200 mg sucrose per ml, centrifuged 4 hr at 23,000 rpm, containing material derived from 1 gm of tobacco leaves.

C - Gradient column of 3, 5, 5, 5, 5, and 4 ml of 50, 100, 150, 200, 400, and 600 mg sucrose per ml, respectively, containing material derived from 1 gm of barley leaves.

Properties of components

Components of bean sap were separated by density-gradient centrifugation of the resuspended high speed pellet on 5-20 rate columns. Samples of each component were collected as the tubes were scanned. Fig. 7 A shows a typical scanning pattern of a gradient tube from which samples were collected for studies of the properties. The four samples characterized are represented by the four peaks in the scanning pattern.

Ultraviolet absorption. Ultraviolet absorption curves of the four samples are shown in Fig. 2. The non-sedimenting material at the top of the tube (a) had a very slight peak at 260 mμ, with 260/280 mμ ratio of 1.2. Ribosome samples (b, c, d) all had a definite peak at 260 mμ, with 260/280 mμ ratios of 1.5, 1.8, and 1.8, respectively, indicating a high concentration of nucleic acid and some differences between the samples. Samples of ribosomes always showed this characteristic spectrum, even after dialysis for two days against distilled water, or treatment with organic solvents. However, the concentration of these components as measured by the absorbance varied considerably among preparations of bean extracts.

Ribosomal preparations from tobacco had similar absorption spectra.

Ultraviolet absorption of the three larger components is shown in Figure 3. Absorption values in Fig. 3 A were

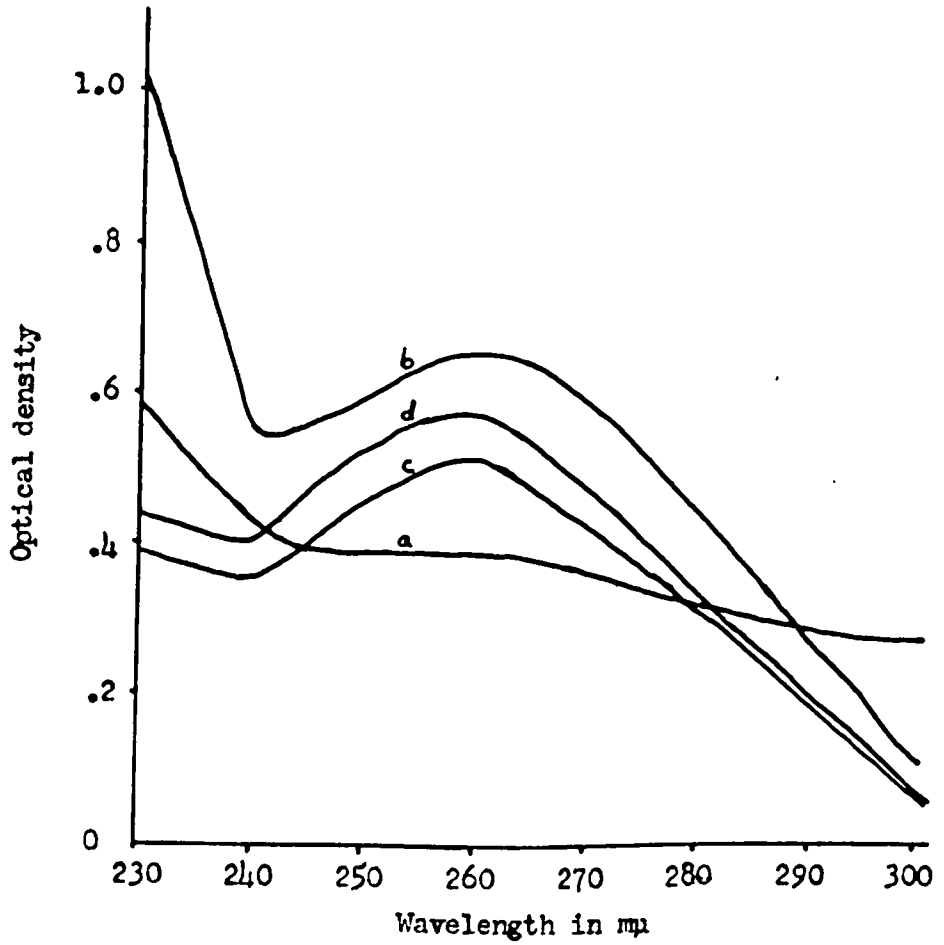


Figure 2. Ultraviolet absorption spectra of separated ribosomal components after dialysis versus water. Each sample represents material derived from approximately 1.5 gm of bean leaves in 5 ml of solution. Samples were taken from a gradient column similar to that in Fig. 7 A.

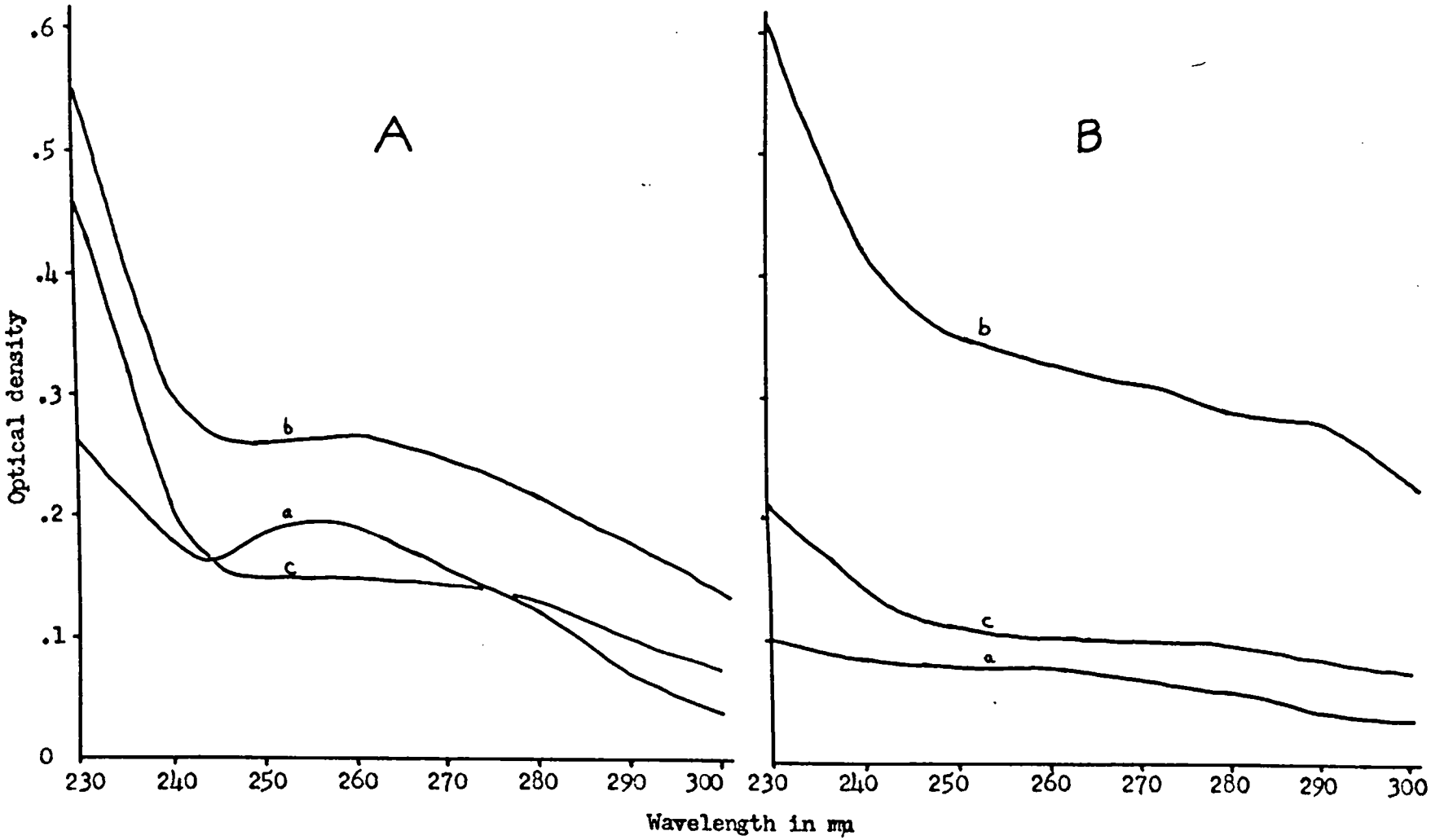


Figure 3. Ultraviolet absorption spectra of the three larger components.
A - Measured on samples from equilibrium gradient columns. Values represent material derived from (a) 0.2 gm, (b) 0.35 gm, and (c) 0.5 gm bean leaves per 5 ml solution.
B - Measured after dialysis and concentration. Values represent material derived from (a) 0.2 gm, (b) and (c) 0.4 gm bean leaves per 5 ml of solution.

measured on dilutions of samples from equilibrium gradient columns. Values for Fig. 3 B were measured on samples after dialysis against distilled water for 2½ days and concentration by high speed centrifugation. These components showed only a very slight peak at 260 mμ before dialysis and none afterwards, indicating a very low concentration of nucleic acid. Most of the measured absorbance was probably due to scattered light, since these particles were quite large compared to the ribosomes.

Dry weight. Yields of the three larger components in order of increasing density were 3.5 mg, 6.3 mg, and 2.8 mg per 100 gm of leaf tissue, respectively. Absorbance at 260 mμ for a 1 mg/ml concentration and a 1 cm light path was 5.9, 5.8, and 4.8, respectively, for the same components. These calculations were based on UV absorbance of the dialyzed and concentrated samples.

Sedimentation constants. Approximate sedimentation constants of the ribosomal components were determined by the methods of Brakke (1958). Density-gradient columns were prepared from 3, 7, 7, 7, and 4 ml of 0, 100, 200, 300, and 400 mg sucrose per ml, respectively. The sedimentation constant is approximately equal to the ratio of the time required for TMV to sediment to a measured depth to the time required for the various components to sediment to the same depth multiplied by 190s, the sedimentation constant of TMV.

Freshly prepared resuspended high speed pellets from beans gave three visible ribosome zones in gradient columns prepared with neutral phosphate buffer. When material derived from 2 gm of leaves was used, particles with sedimentation rates of 14s, 28s, and 66 s were observed, with the major component being 66s. The same preparation at a 3-fold higher concentration gave particles with 14s, 66s, and 82s values. The major component was the 82s one, and the 28s unit was not recognizable as a visible zone. Day-old preparations representing material derived from 2 gm of leaves on phosphate buffer columns gave particles with sedimentation rates of 15s, 38s, and 51s, but gave 15s, 38s, 53s, and 76s values on gradient columns prepared with 0.005 M tris-HCl buffer at pH 7.4.

Electron microscopy. Plate 1 shows particles present in samples taken from gradient columns such as that in Fig. 7 A. Particles from all four depths appeared similar and were characterized mainly by the presence of many sizes of spheres. Samples of the non-sedimenting material (A) and of the upper ribosome zone (B) also had many very small particles. In the latter sample, particles appeared nearly amorphous and flattened, and some were joined together in short chains. Particles in the second ribosome sample (C) appeared more rigid and uniform in size than did the previous samples. Particles in the most rapidly sedimenting ribosome zone (D)

appeared smaller and less rigid and a greater percentage of the particles were aggregated into short rods.

Plate 2 A shows particles from the top centimeter of a gradient tube made with 0.005 M EDTA buffered at pH 7.0 with tris (Fig. 7 C). The particles appeared spherical and rather uniform, and not too much different from those observed in Plate 1 A.

Plate 2 B shows particles from the major zone obtained at a 4 cm depth when 0.002 M $MgCl_2$ was included in the gradient column (Fig. 7 B). These were quite similar to the ones observed in the most rapidly sedimenting ribosome zone in phosphate buffer. However, an even greater percentage of the particles were aggregated into short rods of various lengths.

Plate 3 shows particles present in the three samples of larger components. The upper white zone (A) appeared to consist of several sizes of spherical particles and a few large flattened structures. The bright green zone (B) consisted of large flattened bodies which were probably grana. A few smaller particles were also present. The lower white zone (C) had a much lower concentration of particles, which appeared to be primarily short rods or spheres aggregated to form rods. In both the upper and lower samples, large flattened structures were observed in a low concentration. These were probably due to contamination in the sampling procedure.

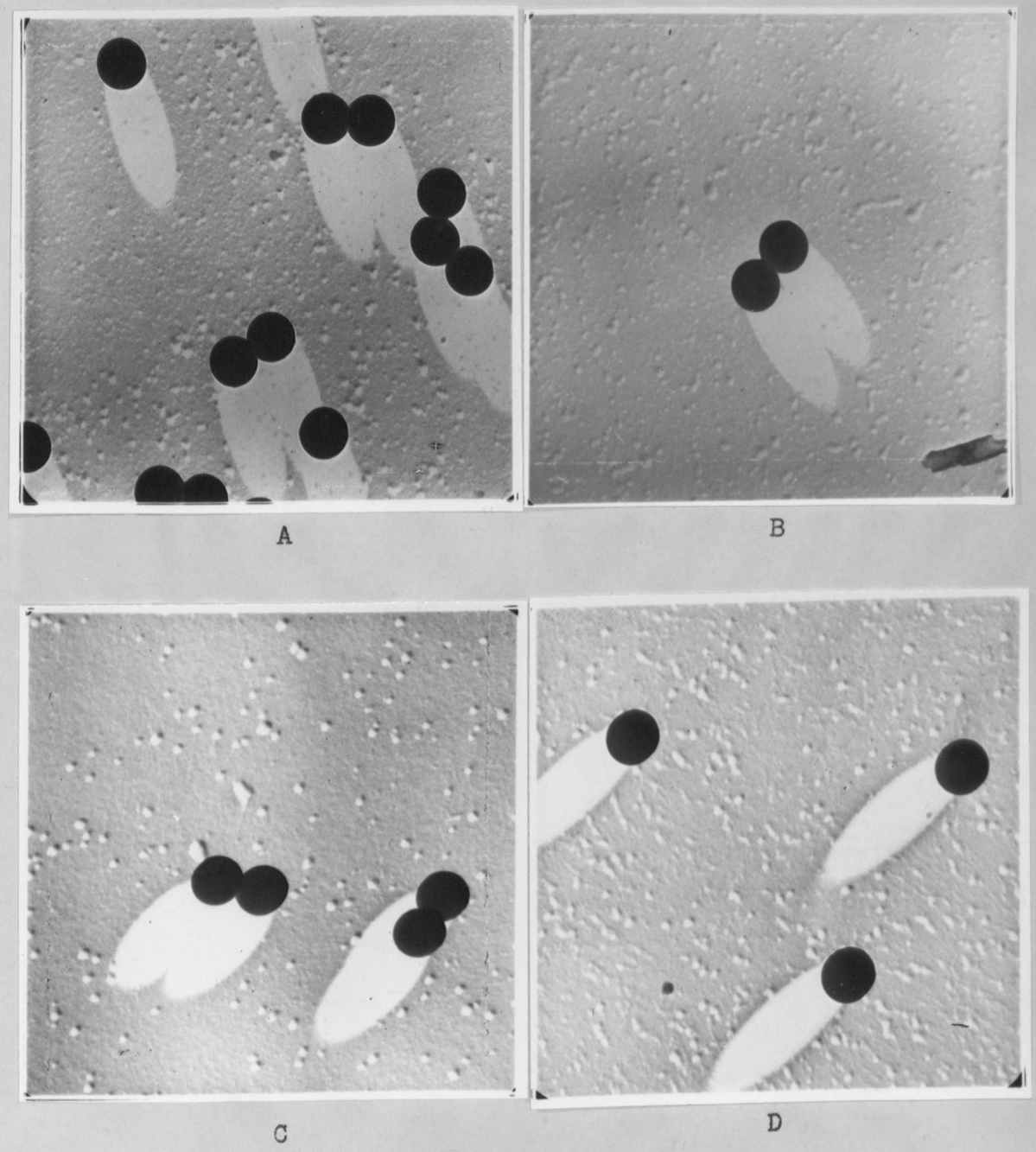
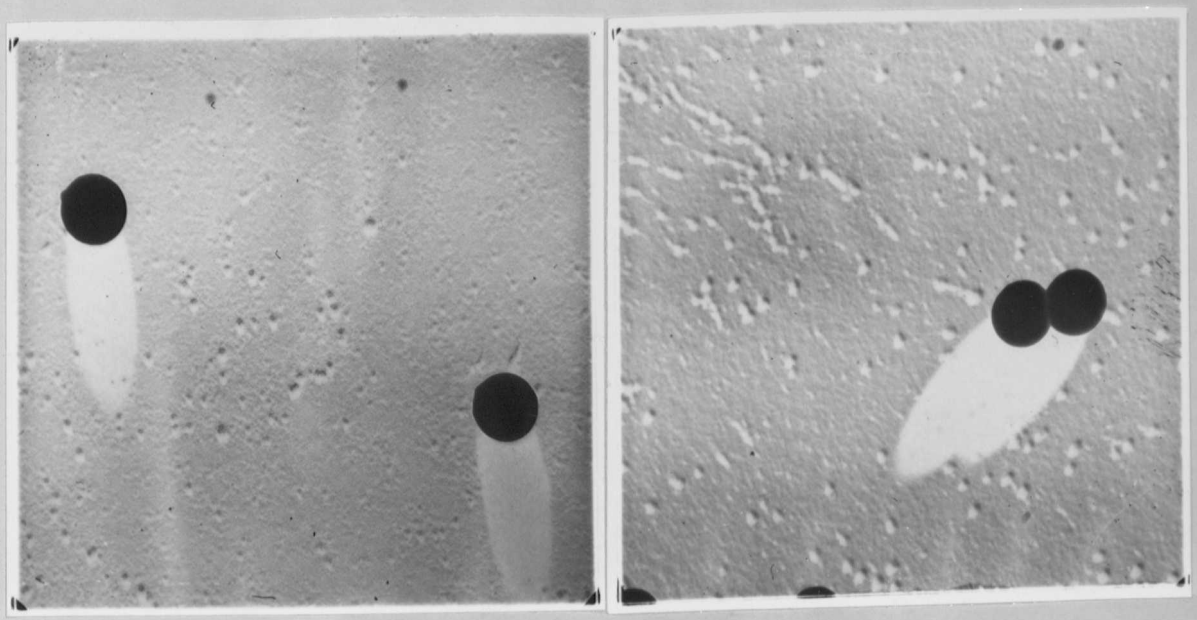


Plate 1. Electron micrographs of bean extract samples from 5-20 rate columns similar to that in Fig. 7 A. Shadowed with tungsten oxide. Magnification is about X30,000. Latex particles have a diameter of 264 mμ.

A - Non-sedimenting material from the upper 0.5 cm.
B - Upper zone of slowly-sedimenting ribosomes, about 1.3 cm.
C - Middle zone of slowly-sedimenting ribosomes, about 1.9 cm.
D - Lower zone of faster-sedimenting ribosomes, about 2.4 cm.

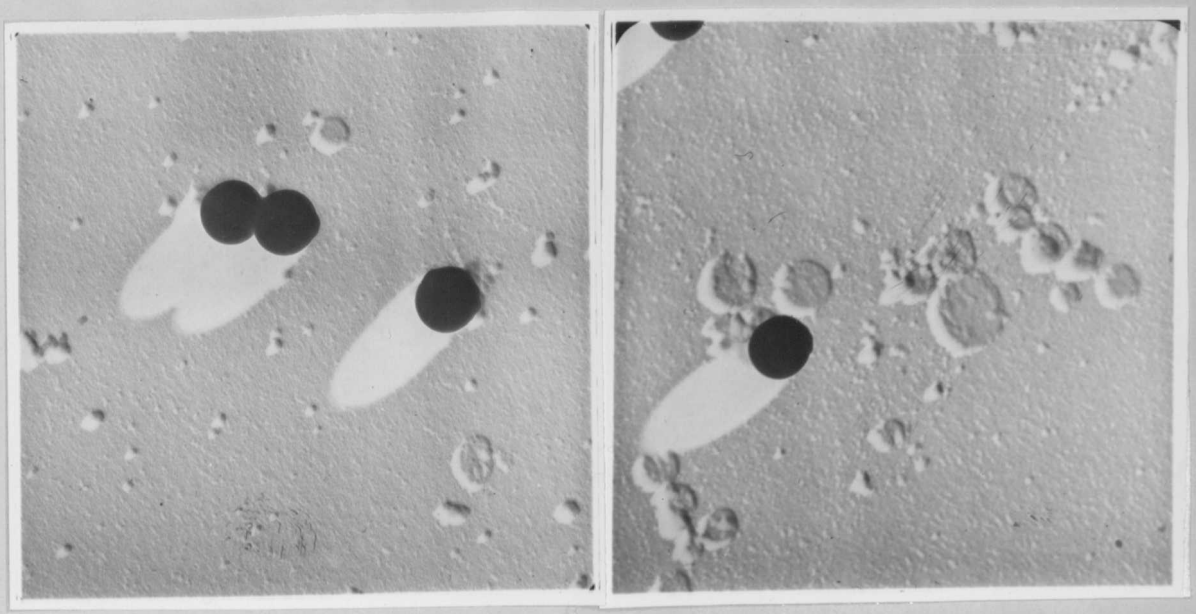


A B

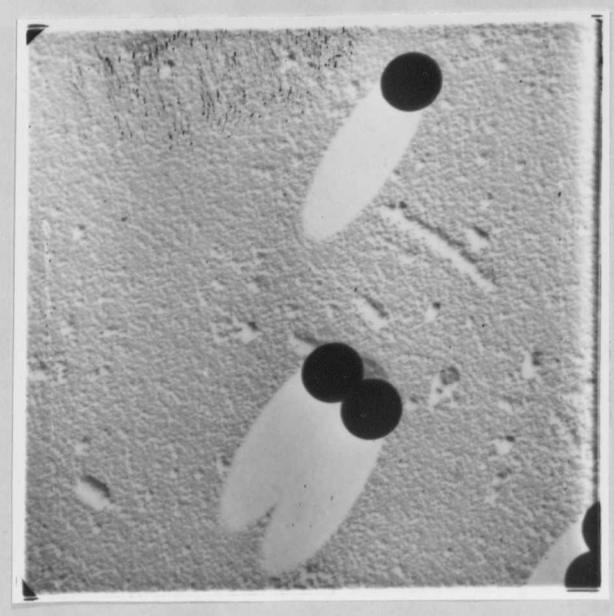
Plate 2. Electron micrographs of bean extract samples from 5-20 rate columns shadowed with tungsten oxide. Magnification is about X30,000. Latex particles have a diameter of 264 mμ.

A - Particles from the top cm of a column made with 0.005 M EDTA buffered at pH 7.0 with tris, similar to Fig. 7 C.

B - Particles from the 4 cm zone in a column made with 0.002 M $MgCl_2$, similar to Fig. 7 B.



A B



C

Plate 3. Electron micrographs of the larger components in bean extracts, separated by equilibrium zonal centrifugation, diayzed, concentrated, and shadowed with tungsten oxide. Magnification is about X30,000. Latex particles have a diameter of 264 mμ.

A - Particles from the upper white zone.
B - Particles from the middle bright green zone.
C - Particles from the lower white zone.

Dependence of concentration of components on the physiological state of the plants

As a general rule, older leaves yielded less material than did younger leaves. Extracts from primary leaves of beans had less green material than extracts from trifoliate leaves, but ribosomal components were still observed. The age of bean plants caused a variation in detectable components shown in Fig. 4. Trifoliate leaves harvested 15 days after planting (A) were not yet fully expanded. First trifoliate leaves were fully expanded and second trifoliates were just beginning to expand 18 days after planting (B). Both first and second trifoliate leaves were used when harvested 22 days after planting (C), when the plants were beginning to vine. All three gradient columns contained material derived from 0.67 gm of leaves. Total absorbance decreased with increasing age, but separation of the ribosomal components was most complete with the intermediate age of leaves.

Detectable components also varied with time of day of harvest, and to avoid this difference, most of the work was done with leaves harvested just before noon. Very little variation in extracts was observed between plants grown during different seasons of the year.

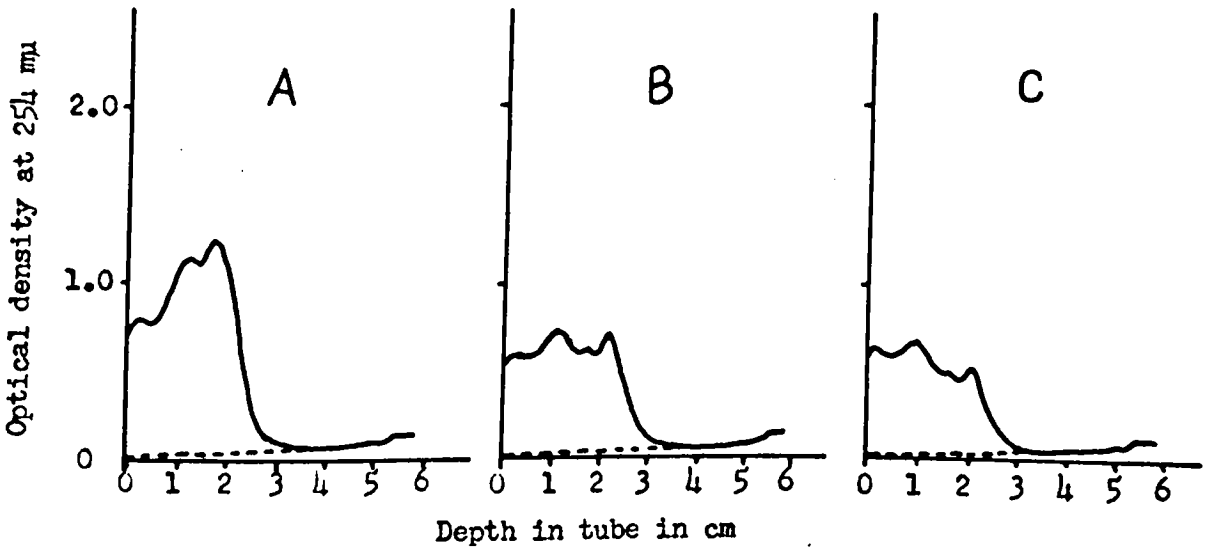


Figure 4. Scanning patterns of 50-200 mg sucrose per ml phosphate buffer gradient columns centrifuged 4 hrs at 23,000 rpm. Each tube contained an equivalent of 0.67 gm of bean leaves in the form of resuspended high speed pellets. A, B, and C were harvested 15, 18, and 22 days, respectively, after planting.

Effects of conditions of density-gradient centrifugation

The degree of separation and the components detected in extracts of healthy plants varied considerably with the materials used for preparation of the gradient columns.

Centrifugation in Igepon T-73. Density-gradient centrifugation of healthy plant extracts in columns containing phosphate buffer and 0.1% Igepon T-73 caused a dispersion of the components into many sharp zones of various colors -- shades of red, yellow, green, and brown -- in the top 2 cm of the columns. As shown in Fig. 5 A, the scanner did not detect these zones by UV absorption, even at concentrations as low as 0.2 gm leaves per tube. As observed by Brakke (1959), these components reached a final depth after only a short period of centrifugation, and sedimented no further in 5-20 rate columns than in 10-40 rate columns. Longer periods of centrifugation did not cause further sedimentation. This suggested that the colored particles had a low density equal to that of the sucrose solution at 0 to 2 cm. However, when extracts were dissolved in 150 mg sucrose per ml, layered within the gradient column at 3.0 cm, and centrifuged, the particles responsible for the colored zones sedimented further down into the tube, as did the UV absorbing particles (Fig. 5 B). Only a very small amount of material floated toward the top of the tube during centrifugation, as should have

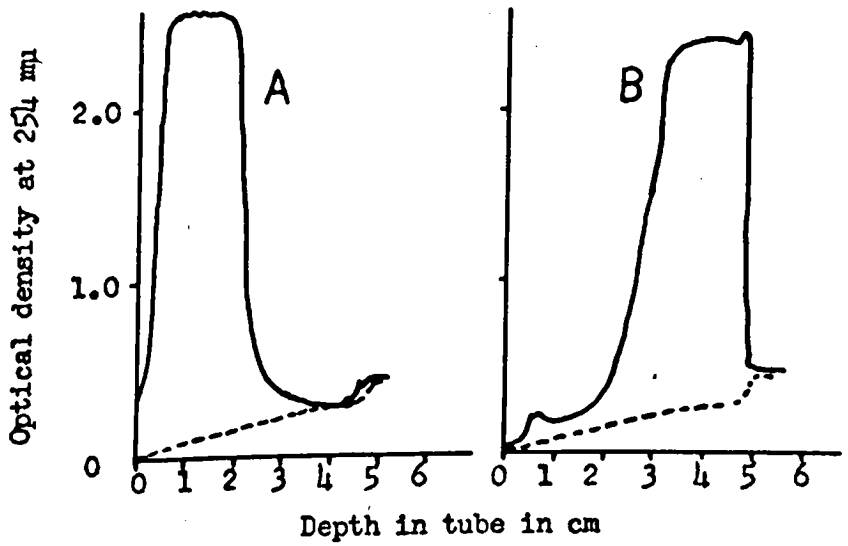


Figure 5. Scanning patterns of gradient columns of 50-200 mg sucrose per ml of phosphate buffer and 0.1% Igepon T-73, containing material derived from 1 gm of bean leaves, in the form of the resuspended high speed pellet.

A - Sample layered on top of the tube.

B - Sample dissolved in 150 mg sucrose per ml, and layered within the gradient column at 3 cm.

occurred if the particles were less dense than the sucrose in which they were layered. This suggests that the high concentration of sucrose had some effect on the particles.

Centrifugation in cesium chloride. Cesium chloride gradient columns had a range of density from 1.12 to 1.37. When an extract equivalent to 0.4 gm of bean leaves was layered on columns which were centrifuged 5 hr at 30,000 rpm, visible zones occurred only at 1.0 cm and at 2.0 cm. The latter zone was a thin layer of green material apparently precipitated at the high salt concentration. Scanning patterns revealed zones of highly absorbing material at about 3.0 cm and at the bottom of the tube, about 3.8 cm.

A similar effect occurred with tobacco extracts. Zones were visible at 1.0 cm, and 1.5 to 2.0 cm. The latter band was slightly green. Scanning patterns revealed low absorbance around 2.0 cm, but very high absorbance around 3.0 cm and at the bottom of the tube.

The ribosomal particles that sedimented to these lower depths were evidently too small to be visible by scattered light, but were easily detected by scanning. The density of the CsCl in this region of the tube was 1.3 to 1.4, which would indicate that the ribosomes had a high density, and were more dense than the green material that sedimented rapidly in sucrose gradient columns.

Recycling of ribosomal components. Recycling the ribosomal components by collecting samples from gradient columns during scanning and layering them on more gradient columns was largely unsuccessful. Samples were either diluted or dialyzed to reduce the sucrose concentration to give a density less than that of the top of the gradient column. Samples collected from phosphate buffer tubes and layered on Igepon tubes or on phosphate buffer tubes, or from Igepon tubes and layered on phosphate buffer tubes did not sediment to the depth to which they sedimented during the original centrifugation. If four ribosomal samples were collected and recombined to layer on another gradient column, centrifugation did not cause sedimentation to the original depths. This effect may indicate that the particles were unstable when separated and did not retain their sedimentation properties, even though they retained other properties such as ultraviolet absorption.

Recycling from sucrose columns to CsCl columns and separating on the basis of density was attempted with tobacco extracts. The scanning pattern for the equivalent of 1 gm of tobacco centrifuged 5 hr on 5-20 rate columns is shown in Fig. 6 A. Particularly good separation was obtained with this preparation, and five samples were collected at positions indicated on the scanning pattern. These samples were layered on CsCl gradient columns and centrifuged 5 hrs.

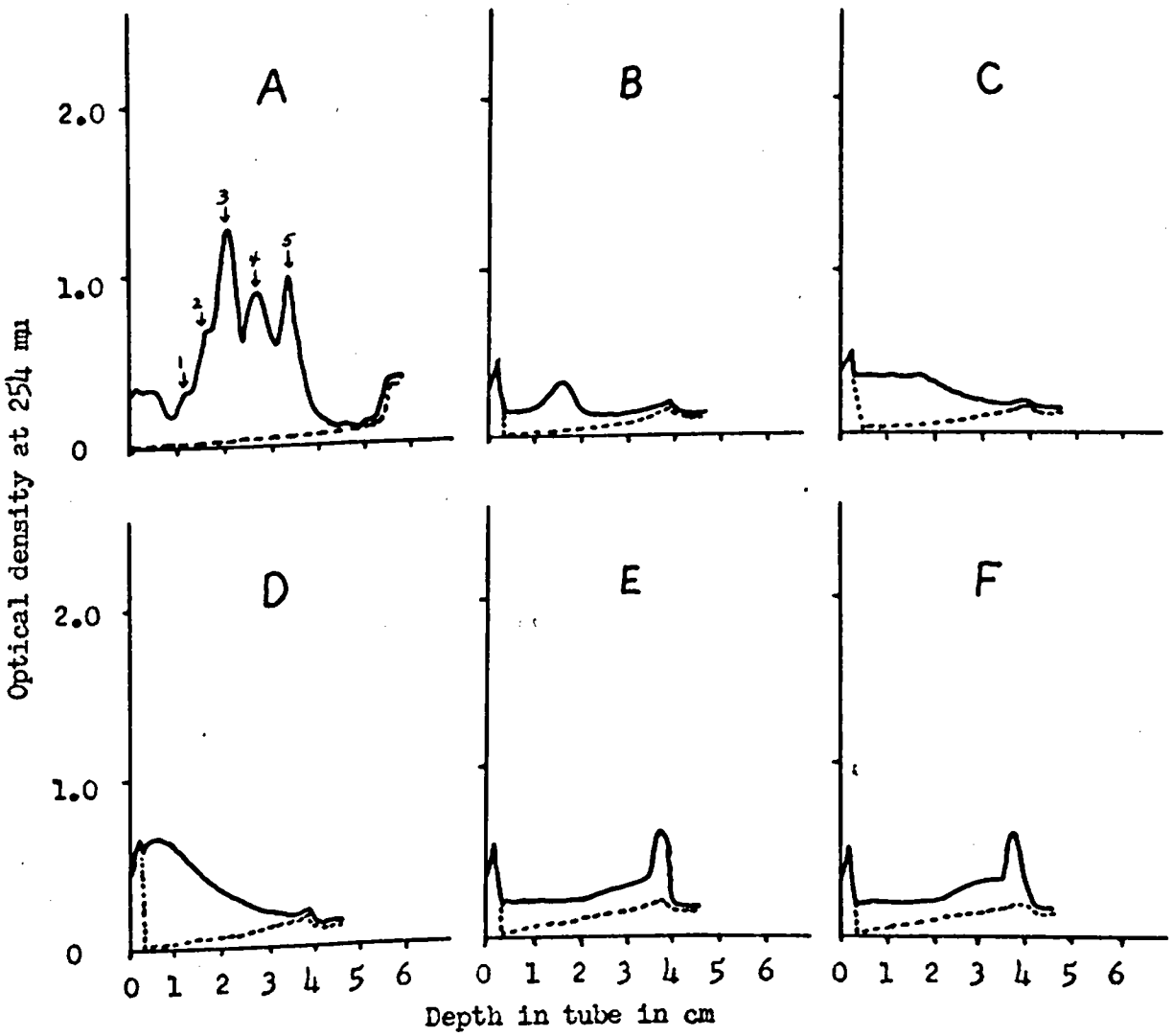


Figure 6. Scanning patterns of gradient columns containing the resuspended high speed pellet derived from 1 gm of tobacco leaves.

A - Gradient column of 50-200 mg sucrose per ml of phosphate buffer, centrifuged 5 hr at 23,000 rpm.

B - F - Gradient columns of 1.0 M, 2.0 M, and 3.0 M CsCl in phosphate buffer, centrifuged 5 hr at 30,000 rpm, and containing, respectively, the 5 samples from the sucrose column in A.

Scanning patterns of these tubes are shown in Figure 6. Sample 1 (B) sedimented to about 1.5 cm. Samples 2 (C) and 3 (D) had no particular zone of absorbance, and did not seem to sediment much. Samples 4 (E) and 5 (F) sedimented nearly to the bottom of the tube, with sample 5 showing some absorbance between 2.0 and 3.0 cm. Apparently the ribosomal components are of different densities and these densities do not correspond directly to their sedimentation rate in sucrose density-gradient columns.

Centrifugation in various magnesium concentrations. The effect of magnesium on the ribosomal components was tested by using gradient columns containing various concentrations of magnesium chloride. Figure 7 shows scanning patterns of the resuspended high speed pellet of bean centrifuged on columns with phosphate buffer (A), 0.002 M $MgCl_2$ (B), and 0.005 M EDTA buffered at pH 7.0 with tris (C). Magnesium caused a marked change in the sedimentation of the ribosomes, with the major peak occurring at 4.0 cm in 0.01 M $MgCl_2$. The chelating agent, EDTA, which binds magnesium, caused a breakdown of the ribosomes. Nearly all of the absorbance was within the top 1.5 cm of the tube where free nucleic acid should be. Sedimentation in 0.005 M tris-HCl at pH 7.4 (Fig. 8 B) resulted in a change similar to that caused by magnesium, with peaks at 3.0 and 4.0 cm that were not present in phosphate buffer columns. Figure 8 also shows sedimentation patterns in tris

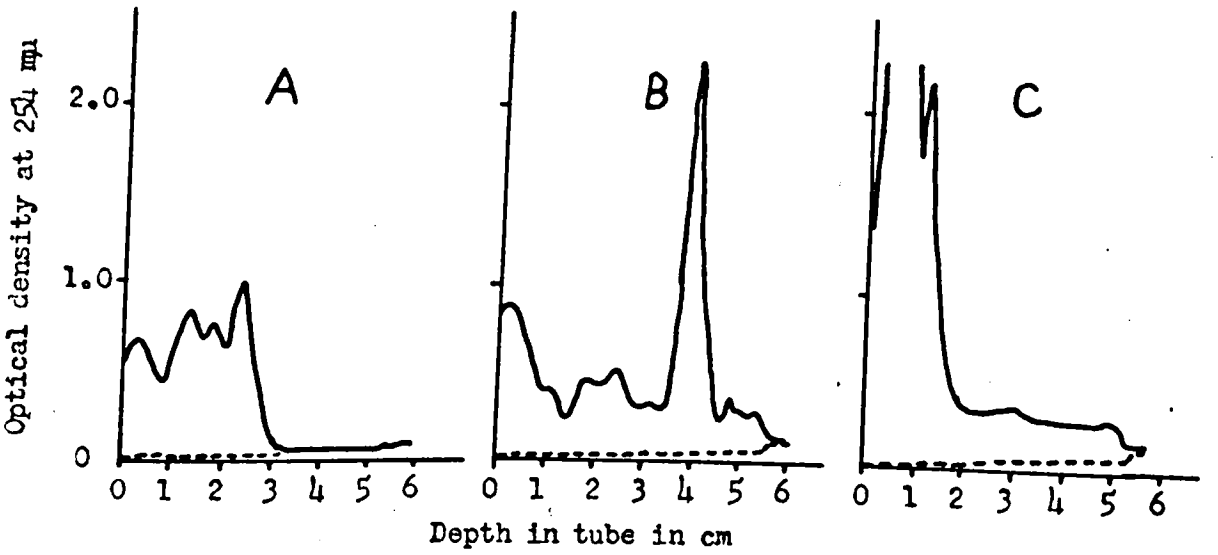


Figure 7. Scanning patterns of 50-200 mg sucrose per ml gradient columns, centrifuged 4 hr at 23,000 rpm, containing the resuspended high speed pellet derived from 0.67 gm of bean leaves.

A - Sucrose dissolved in 0.01 M neutral phosphate buffer.

B - Sucrose dissolved in 0.002 M $MgCl_2$.

C - Sucrose dissolved in 0.005 M EDTA, adjusted to pH 7.0 with tris.

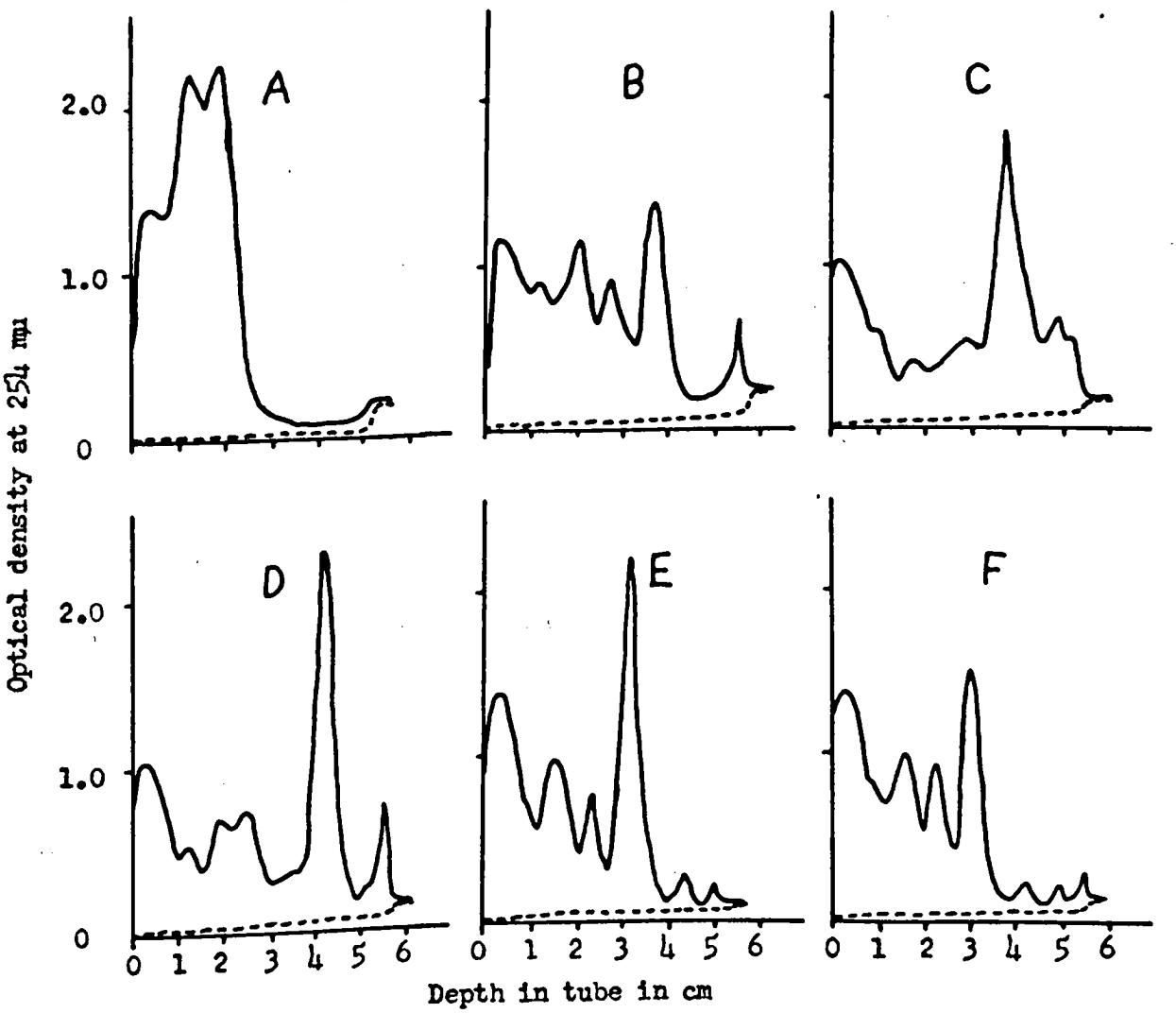


Figure 8. Scanning patterns of 50-200 mg sucrose per ml gradient columns, centrifuged 4 hr at 23,000 rpm, containing the resuspended high speed pellet derived from 0.67 gm of bean leaves.

A - 0.01 M neutral phosphate buffer
B - 0.005 M tris-HCl buffer pH 7.4
C - 0.005 M tris-HCl + 0.01 M $MgCl_2$
D - 0.005 M tris-HCl + 0.001 M $MgCl_2$
E - 0.005 M tris-HCl + 0.0001 M $MgCl_2$
F - 0.005 M tris-HCl + 0.00001 M $MgCl_2$

Gradient columns were prepared by dissolving the sucrose in the above materials. Tubes E and F were centrifuged at a lower temperature, so components did not sediment as rapidly.

buffer containing four different concentrations of magnesium chloride, ranging from 10^{-2} to 10^{-5} M (C-E). The major component reached a maximum concentration at 10^{-3} M, and decreased with further decrease in magnesium. It should be noted that this change to higher sedimentation rates occurred in $MgCl_2$ alone, in tris alone, but not in tris with EDTA.

Tobacco extracts behaved in a similar manner in the presence of magnesium and EDTA.

Effects of denaturing and adsorbing agents

The effect of treating plant extracts with various denaturing or adsorbing agents was analyzed by centrifugation for 4 hr on 5-20 rate columns in 0.01 M phosphate buffer, and recording the scanning patterns in the tubes. Controls were the untreated extracts from the same preparations of leaves centrifuged at the same time.

Tables I - V contain results of representative experiments of treatments of healthy plant extracts. Concentrations are expressed as ml x absorbance at 254 mμ measured from the scanning patterns. Values represent the material in each of the fractions of the gradient column that survived the particular treatment. Peaks in the scanning patterns usually overlapped so that values may not be a true measure of the concentration of the individual components. Scanning patterns were separated into non-sedimenting material in the

top centimenter, two ribosome zones with different sedimentation rates as shown in Fig. 10 A for beans and Fig. 1 B for tobacco, and larger components usually present below about 3 cm. The experiment represented in Table II gave separation of ribosomes into three zones with different sedimentation rates, as shown in Fig. 7 A. Ribosomal components from barley usually separated into two zones. Measurements of these two, as well as non-sedimenting material and the larger components, were made.

Treatments are listed in the tables in the order of relative effectiveness based on the percentage of the total amount of absorbance removed. However, the relative effectiveness varied from one component to another.

No visible zones were observed in many of the gradient columns centrifuged with treated extracts. Photometric scanning showed the presence of small amounts of absorbing material which otherwise would not have been detected.

Stage of the extract at time of treatment. In the purification of viruses, extracts are usually treated after grinding the tissue or after low speed centrifugation. Tests were conducted to determine if the effectiveness of the treatments varied with the time of application. Figure 9 shows scanning patterns of gradient columns of bean extracts, with treatments applied to the crude sap or to the resuspended high speed pellet. Fig. 9 A shows the untreated high speed

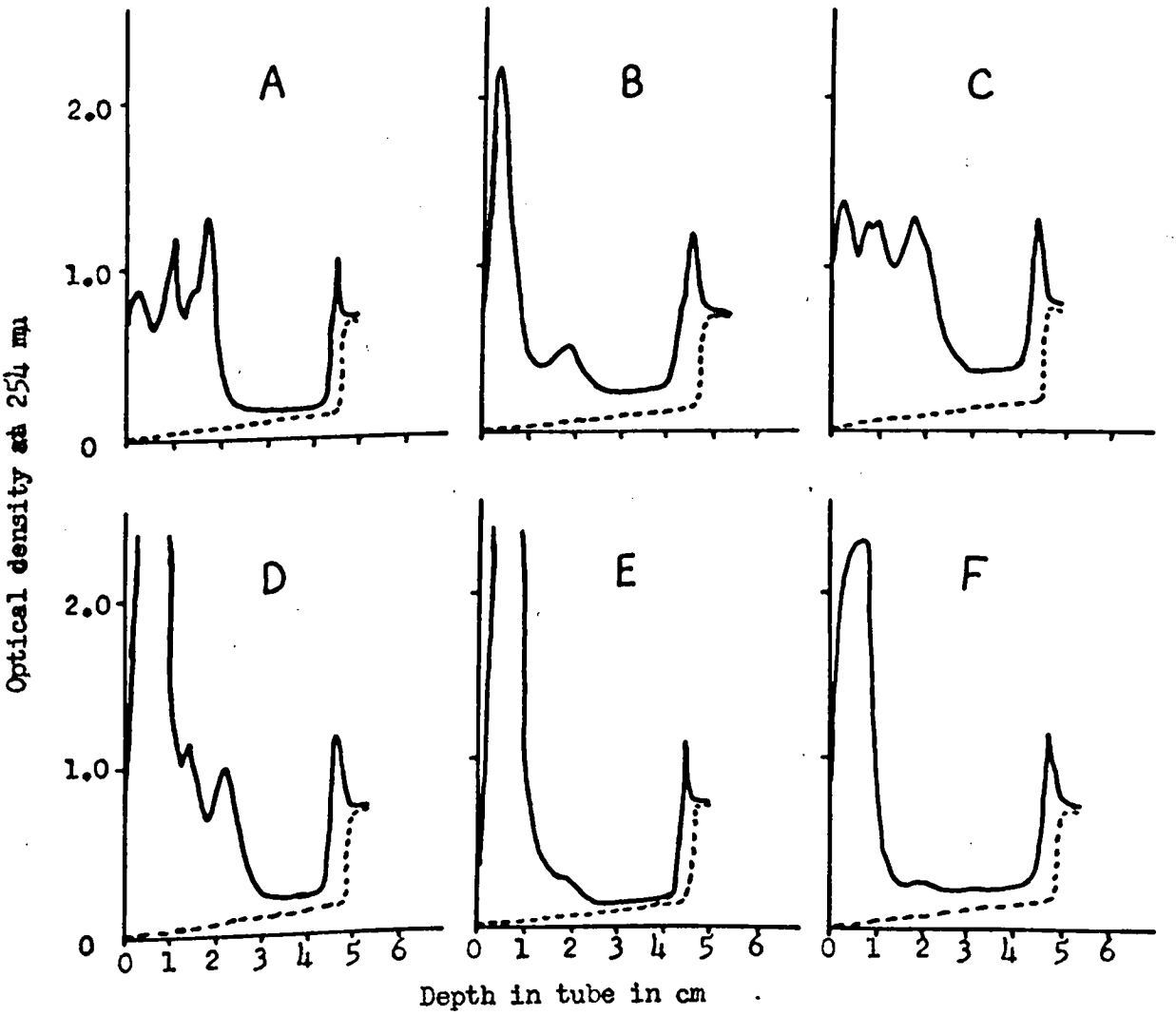


Figure 9. Scanning patterns of 50-200 mg sucrose per ml gradient columns centrifuged 4 hr at 23,000 rpm.

A - Untreated bean resuspended high speed pellet, representing 0.4 gm of leaves.

B - Bean resuspended high speed pellet treated 1 hr at 40°, representing 0.4 gm leaves.

C - Bean resuspended high speed pellet treated with $CaHPO_4$, representing 1 gm of leaves.

D - Untreated bean crude sap, representing 0.56 gm leaves.

E - Bean crude sap treated 1 hr at 40°, representing 0.56 gm leaves.

F - Bean crude sap treated with $CaHPO_4$, representing 1.3 gm leaves.

pellet, with the characteristic ribosome peaks. When the crude sap was centrifuged on gradient columns, the non-sedimenting phenolic compounds caused a high absorbance in the top 1 cm of the tube and obscured some of the ribosome peaks (D) in the untreated control. Disregarding the absorbance from 0 to 1 cm in E and F, it can be seen that both 1 hr at 40° and $CaHPO_4$ were more effective in removing components when applied to the crude sap than when applied to the resuspended high speed pellet (B and C). This was particularly true with $CaHPO_4$ adsorption, and with several of the other treatments. Because of this difference, treatments were applied to the crude sap and followed by high speed centrifugation before density-gradient centrifugation.

Effects of freezing. The effect of short- and long-term freezing on bean leaves, crude sap, and resuspended high speed pellet was tested. Freezing intact leaves for one month caused very little change in the material extracted. Freezing for 10½ months caused some breakdown of the ribosomal components, but little reduction in total absorbance in the tube (Fig. 10 D) and some removal of the green material. One month of freezing either of the crude sap or the resuspended high speed pellet (Fig. 10 B) caused little change from the fresh extract (Fig. 10 A). When the crude sap was frozen 10½ months, all of the green color was removed, nearly all of of the components were precipitated from solution, and little

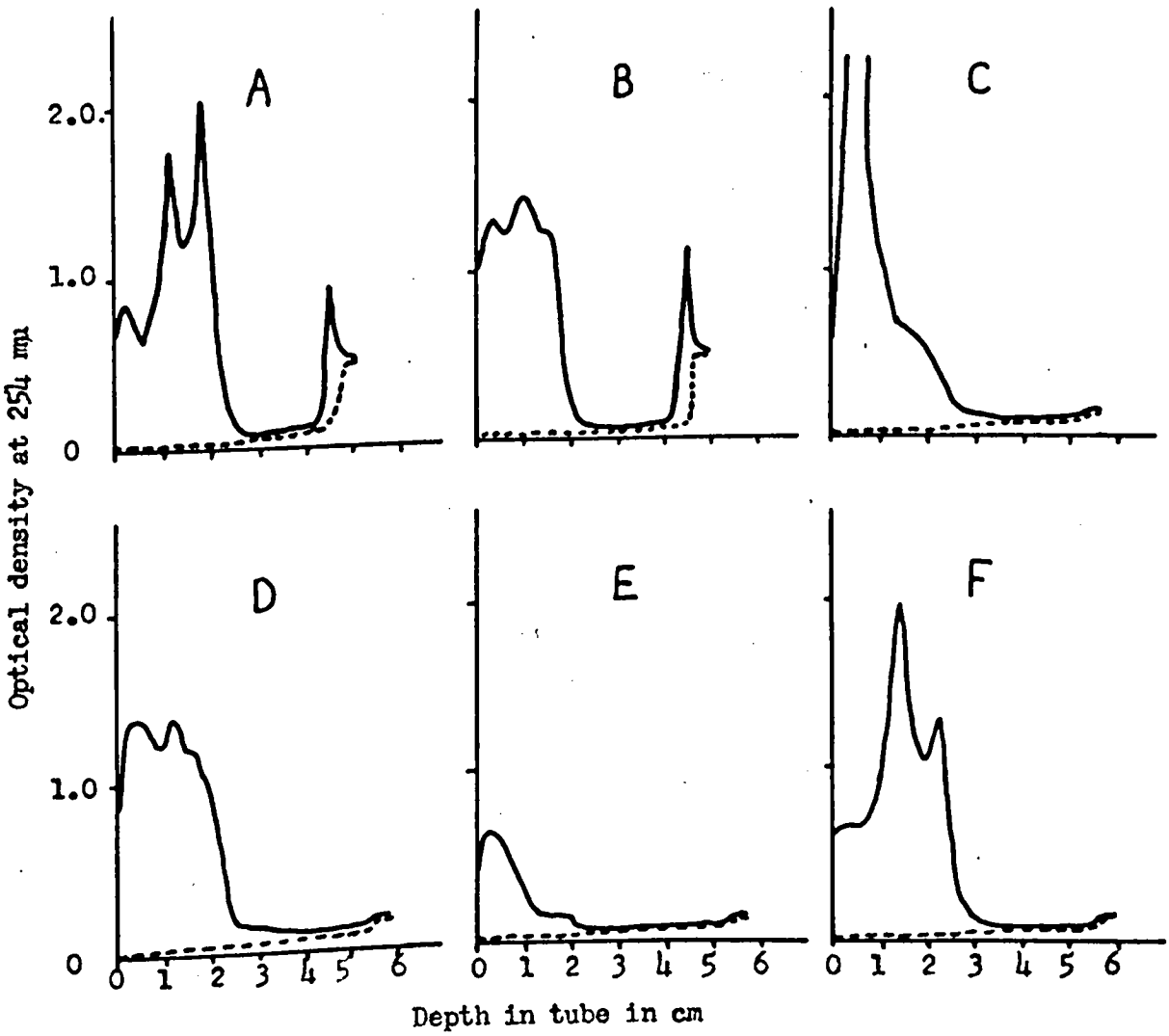


Figure 10. Scanning patterns of 50-200 mg sucrose per ml gradient columns centrifuged 4 hr at 23,000 rpm. A-E contain material from 0.67 gm of bean leaves.

A - Untreated, freshly prepared resuspended high speed pellet.

B - Resuspended high speed pellet frozen one month.

C - Resuspended high speed pellet frozen 10½ months.

D - Resuspended high speed pellet prepared from bean leaves frozen 10½ months.

E - Resuspended high speed pellet prepared from bean crude sap frozen 10½ months.

F - Resuspended high speed pellet prepared from tobacco leaves frozen 10½ months.

absorbance remained in the gradient tube (Fig. 10 E). Long term freezing of the high speed pellet caused a breakdown of the ribosomal components, resulting in high absorbance in the top of the tube (Fig. 10 C). This sample retained some green color. Freezing tobacco leaves did not appear to be effective in removing absorbing components, although most of the green color was removed (Fig. 10 F).

Treatments of bean extracts. The amount of material surviving various treatments of crude sap of beans is shown in Table I. Several controls were required because treatments were applied to different preparations, and the untreated extracts varied. In general, heat treatment, calcium phosphate, and ethanol were more effective than the other organic solvents. The treatments also varied in the degree to which they removed the individual components.

Heat treatments, calcium phosphate, ethanol, and chloroform-butanol removed the green components. The latter two also turned the sap brown. Neither ether treatment removed all of the green, but the longer time was more effective in removing the ribosomal components. Acid treatment to pH 5.5 was not very effective on either the crude sap or the resuspended high speed pellet. Low speed centrifugation at pH 5.2 and adjustment to neutrality removed all of the green color. No zones were visible, but light absorption was detected near the top of the tube and around 2.0 cm.

Results of treatments of the high speed pellet with three organic solvents are shown in Table II. The ether and n-butanol treated samples were still quite green, but the chloroform-butanol treated sample was nearly clear. The three treatments were about equal in effectiveness of removal of ribosomal components, and were far less effective than when applied to the crude sap.

Gradient columns with untreated extracts always had a pellet at the bottom after centrifugation. Extracts treated with ether, n-butanol, 1 hr at 40°, and $CaHPO_4$ often, but not always, had pellets in the gradient tubes. Other treatments usually had no pellet.

Treatments of tobacco extracts. Ten different treatments of tobacco crude extract were applied to the same preparation. The amount of material surviving each of these treatments is summarized in Table III. Calcium phosphate was most effective, followed by heating for 10 min at 55°. Heating for 1 hr at 40° was least effective. Acid treatment removed only those materials that were irreversibly precipitated at pH 5.0, since the sap was adjusted to neutrality before low speed centrifugation. One hour shaking with ether was more effective than 3 min shaking with the same solvent. Butanol in combination with chloroform or ether was more effective than by itself. Treatments which removed nearly all of the green color were calcium phosphate, 10 min at 55°,

chloroform-butanol, ether-butanol, and 10% n-butanol. Heating 1 hr at 40° and shaking 3 min with ether removed very little of the green color. Treatments which had pellets after density-gradient centrifugation were 1 hr at 40°, 3 min ether, 90% ethanol, and acid, indicating that these treatments were less effective in removing the larger components. As with bean, the treatments varied in the relative amounts of each of the components removed.

Treatments of barley extracts. Effect of treatments of barley crude sap was analyzed on two types of gradient tubes. Table IV shows material surviving treatments and analyzed on 5-20 rate columns. In experiments of Table V, effectiveness of treatments was determined by using 50-600 mg sucrose per ml tubes, which would give a better measurement of the faster-sedimenting, larger components. With barley, both heat treatments were very effective. As with bean and tobacco, 1 hr shaking with ether was more effective than 3 min shaking with the same solvent. Calcium phosphate removed far less material from barley than it did from bean and tobacco extracts. Ethanol, which was quite effective on beans, did not remove nearly as much material from barley.

Of the treatments analyzed on 5-20 rate columns, only the 3 min ether treated extract was green and formed a pellet in the bottom of the tube. Those treatments analyzed on 50-600 mg sucrose per ml columns all showed a gradually

increased light scattering below about 3.5 cm, but were relatively free of any green color. This indicates that there were materials present in the larger components that were colorless and did not absorb very strongly.

TABLE I

AMOUNT OF MATERIAL SURVIVING TREATMENTS OF BEAN CRUDE SAP

Treatments[a]	Fraction from gradient column[b] 1	2	3	4	Total	% of control
90% EtOH	1.5[c]	0.8	0.6	1.0	3.9	7
$CaHPO_4$	1.9	3.1	1.1	0.8	6.9	12
Control	7.6	21.2	24.1	3.4	56.3	--
$CaHPO_4$	1.4	1.3	0.8	1.1	4.6	13
Control	5.6	16.3	13.7	0.8	36.4	--
10 Min 55° C	1.6	0.7	0.5	0.0	2.8	6
1 Hr 40° C	2.6	1.4	2.6	0.5	7.1	15
Control	6.8	20.8	17.8	1.4	46.8	--
10% n-Butanol	1.5	2.8	6.1	0.4	10.9	26
Control	4.6	19.4	16.6	1.3	41.9	--
$CHCl_3$-butanol	2.7	2.1	3.2	0.5	8.5	20
10% n-Butanol	3.8	5.4	6.4	0.5	16.1	39
1 Hr ether	4.4	7.3	7.1	0.0	18.8	45
Control	8.4	12.3	19.9	1.2	41.8	--

[a] Treatments were applied to crude sap and followed by high speed centrifugation. Pellet was resuspended at 1:1 dilution and analyzed by density-gradient centrifugation.

[b] Gradient columns were 50-200 mg sucrose per ml of phosphate buffer. Centrifuged 4 hr at 23,000 rpm.
Fraction 1 = non-sedimenting material
Fraction 2 = slowly-sedimenting ribosomes
Fraction 3 = fast-sedimenting ribosomes
Fraction 4 = rapidly-sedimenting larger material

[c] Expressed as ml x absorbance at 254 mμ measured from scanning patterns. Each value represents material derived from 2 gm leaves.

TABLE II

AMOUNT OF MATERIAL SURVIVING TREATMENTS OF BEAN RESUSPENDED HIGH SPEED PELLET

Treatment[a]	Fraction from gradient column[b] 1	2	3	4	5	Total	% of control
$CHCl_3$-butanol	3.9[c]	2.2	1.5	4.1	0.2	11.9	66
1 Hr ether	3.4	5.1	1.1	4.2	0.2	14.0	78
10% n-Butanol	4.3	3.5	1.5	4.7	1.0	15.0	84
Control	3.6	6.1	1.9	5.8	0.5	17.9	--

[a] Treatments were applied to a 1:1 dilution of the resuspended high speed pellet, then diluted 1:3 for density-gradient centrifugation.

[b] Gradient columns were 50-200 mg sucrose per ml of phosphate buffer. Centrifuged 4 hr at 23,000 rpm.
Fraction 1 = non-sedimenting material
Fraction 2 = top zone of ribosome material
Fraction 3 = middle zone of ribosome material
Fraction 4 = bottom zone of ribosome material
Fraction 5 = rapidly-sedimenting larger material

[c] Expressed as ml x absorbance at 254 mμ measured from scanning patterns. Each value represents material derived from 0.67 gm of leaves.

TABLE III

AMOUNT OF MATERIAL SURVIVING TREATMENTS OF TOBACCO CRUDE SAP

Treatment[a]	Fraction from gradient column[b] 1	2	3	4	Total	% of control
$CaHPO_4$	0.2[c]	0.1	0.0	0.0	0.3	1
10 Min 55° C	0.4	0.1	0.0	0.0	0.5	2
$CHCl_3$-butanol	0.7	1.0	1.1	0.1	2.9	11
Ether-butanol	0.4	0.9	1.5	1.4	4.2	16
Acid	0.8	3.0	0.8	0.0	4.6	17
1 Hr ether	0.7	1.7	1.9	0.7	5.0	19
10% n-Butanol	1.0	2.4	2.7	1.6	7.7	29
3 Min ether	1.0	3.0	3.2	1.2	8.4	31
90% EtOH	1.4	4.7	1.8	0.9	8.8	33
1 Hr 40° C	1.0	3.4	3.1	1.6	9.1	34
Control	2.4	14.3	6.8	3.2	26.7	--

[a] Treatment was applied to crude sap and followed by high speed centrifugation. Pellet was resuspended at 1:1 dilution and analyzed by density-gradient centrifugation.

[b] Gradient columns were 50-200 mg sucrose per ml of phosphate buffer. Centrifuged 4 hr at 23,000 rpm.
Fraction 1 = non-sedimenting material
Fraction 2 = slowly-sedimenting ribosomes
Fraction 3 = fast-sedimenting ribosomes
Fraction 4 = rapidly-sedimenting larger material

[c] Expressed as ml x absorbance at 254 mμ measured from scanning patterns. Each value represents material derived from 2 gm of leaves.

TABLE IV

AMOUNT OF MATERIAL SURVIVING TREATMENTS OF BARLEY CRUDE SAP

Treatment[a]	Fraction from gradient column[b] 1	2	3	4	Total	% of control
10 Min 55° C	0.4[c]	0.0	0.0	0.0	0.4	2
1 Hr 40° C	0.3	0.2	0.0	0.0	0.5	3
10% n-Butanol	1.2	0.8	1.0	0.04	3.0	18
1 Hr ether	1.1	1.0	0.8	0.03	2.9	17
3 Min ether	2.5	2.5	3.0	0.4	8.4	50
Control	2.6	8.5	4.6	0.1	16.7	--

[a] Treatments were applied to crude sap and followed by high speed centrifugation. Pellet was resuspended at 1:1 dilution and analyzed by density-gradient centrifugation.

[b] Gradient columns were 50-200 mg sucrose per ml of phosphate buffer. Centrifuged 4 hr at 23,000 rpm.
Fraction 1 = non-sedimenting material
Fraction 2 = slowly-sedimenting ribosomes
Fraction 3 = fast-sedimenting ribosomes
Fraction 4 = rapidly-sedimenting larger material

[c] Expressed as ml x absorbance at 254 mμ measured from scanning patterns. Each value represents material derived from 2 gm of leaves.

TABLE V

AMOUNT OF MATERIAL SURVIVING TREATMENTS OF BARLEY CRUDE SAP

Treatment[a]	Fraction from gradient column[b] 1	2	3	4	Total	% of control
$CaHPO_4$	3.3[c]	1.7	0.2	0.4	5.6	22
$CHCl_3$-butanol	1.6	3.3	1.9	0.4	7.2	29
Ether-butanol	1.8	3.4	2.7	0.3	8.2	33
90% EtOH	4.0	4.1	3.0	3.9	15.0	60
Control	6.7	11.0	4.6	2.6	24.9	--

[a] Treatments were applied to crude sap and followed by high speed centrifugation. Pellet was resuspended at 1:1 dilution and analyzed by density-gradient centrifugation.

[b] Gradient columns were 3, 5, 5, 5, 5, and 4 ml of 50, 100, 150, 200, 400, and 600 mg sucrose respectively, per ml of phosphate buffer. Centrifuged 2 hr at 23,000 rpm.
Fraction 1 = non-sedimenting material
Fraction 2 = slowly-sedimenting ribosomes
Fraction 3 = fast-sedimenting ribosomes
Fraction 4 = rapidly-sedimenting larger material

[c] Expressed as ml x absorbance at 254 mμ measured from scanning patterns. Each value represents material derived from 2 gm of leaves.

DISCUSSION

The use of photometric scanning for the detection of components in density-gradient columns was quite useful as a rapid means of evaluating the effectiveness of various treatments of extracts of healthy plants. Observation of centrifuged gradient columns by passing a ray of light down through the tube was also useful, but was of value only for detecting particles that were large enough or present in a high enough concentration to scatter sufficient light to make the zone visible. An accurate estimation of concentration of the separate components was very difficult by this method. When untreated plant extracts were centrifuged, light-scattering materials were usually present throughout the gradient column, making it almost impossible to accurately detect the separate zones. Dilution of the extracts to decrease light-scattering also decreased the components that sedimented as discrete zones to a point where they were hardly visible. Photometric scanning was considerably more sensitive in detecting ribosomal components which absorbed highly at 254 mμ but scattered little light. As long as the concentration of these materials was below the maximum recording capacity of the scanner, separate peaks were detected even in the presence of many light-scattering materials.

Measurement of the area under scanning pattern peaks gave an estimation of the concentration of the various

components. These peaks were often quite close together, so divisions for measurement could not be made accurately enough to avoid contamination from adjacent peaks. Procedures were standardized as much as possible to make any errors consistent between the various measurements.

Depths of the tops of the visible zones were usually 1 to 2 millimeters above the depth of the maximum height of the peak on the scanning pattern. As a general rule, any visible zone in a gradient column was expressed as a peak on the scanning pattern. A notable exception to this was the failure of the scanner to separate the many colored zones produced during centrifugation of plant extracts through Igepon-containing gradient columns. In contrast, scanning often detected peaks where no visible zones were observed. A concentration of ribosomes of 3 to 5 ml x absorbance seemed necessary before a zone was visible, depending upon light-scattering in the background. Centrifugation in cesium chloride gradient columns particularly resulted in detection of high concentrations of absorbing material at depths where no zones were visible. Regions of considerable light-scattering, such as often found in the lower half of rate gradient columns, were apparently due to the larger components which absorbed very little UV light.

Ribosome particles with approximately the same properties were isolated from bean, tobacco, and barley leaves.

Properties were also similar to those of ribonucleoprotein particles from pea seedlings (Tso et al., 1958) and from E. coli (Tissieres et al., 1959), and to properties of the normal leaf nucleoprotein material isolated from tobacco leaves by Pirie (1950, 1956). Sedimentation constants determined in this study were only approximate, but their values fell into the range of values reported for ribosome particles from other sources. Alteration of sedimentation patterns in density-gradient tubes containing different concentrations of magnesium (Fig. 8) was consistent with the results reported for other ribosome particles. Electron microscopy of ribosome particles from bean leaves showed small, nearly spherical particles that seemed to be more aggregated in samples sedimenting at a faster rate, which agrees with observations of E. coli particles (Hall and Slayter, 1959; Huxley, 1960).

Instability of these particles with increased purification, as indicated by the failure of recycling attempts, may have been due in part to the removal of magnesium from the system. Complete removal of magnesium with EDTA caused breakdown of the particles into still smaller units, which also occurred with particles from E. coli and pea seedlings. The extracts were continually exposed to 0.01 M phosphate buffer, which also removed magnesium from the system. Sedimentation through gradient columns containing tris buffer or various concentrations of magnesium resulted in the formation

of larger particles than were present in the extract when sedimented through phosphate buffer or water gradient columns. Higher concentrations of plant extracts, such as used in the determination of sedimentation constants, resulted in the formation of faster-sedimenting particles than found in lower concentrations, even when both were sedimented through phosphate buffer. The smaller amount of aggregation at higher dilutions could have been caused by the lower concentration of magnesium or by the lower concentration of small ribosomes. The result suggests a readily reversible reaction.

Recycling of the various ribosomal components on cesium chloride gradient columns indicated that there may be some differences in density between the particles. This is different from the situation reported for E. coli ribosomes, which apparently all have about the same density.

Ultraviolet absorption spectra of ribosomal samples from gradient columns indicated the particles contained a high concentration of nucleic acid. Slight differences between the particles were observed in the different absorption spectra and in their appearance in electron micrographs.

These differences in density, absorption spectra, appearance, and the fact that a portion of the slowly-sedimenting ribosomal fractions remained even at a high magnesium concentrations suggests that something other than, or in addition to an aggregation of basically similar components

is involved in the ribosomal pattern in extracts of healthy plant leaves.

The various denaturing and adsorbing agents that are commonly used for plant virus purification varied somewhat in their action on the components in healthy plant extracts. Differences were evident among the three species of plants tested, and between the action on the ribosome components and on the faster-sedimenting protein components. Treatments of the resuspended high speed pellet with organic solvents and other agents removed some of the green color of the extract, but removed very little of the ribosomal components. The same treatments applied to the crude sap were much more effective in removing the ribosomes, indicating something that labilized the ribosomes was removed from the system during high speed centrifugation.

In general, the treatments applied in this study were quite effective in removing normal-plant components from the system. Nearly any treatment which does not denature the virus in question should be a useful means of removing some of these components. However, the action of these agents may be different in plant extracts containing a virus, or the normal-plant components contained in infected plant extracts may be different from those contained in healthy plant extracts.

Heating for 10 min at 55° C was the most consistently effective treatment for removing ribosomal components, as well as the green color.

Freezing has often been used to facilitate the release of viruses from host tissue and has been claimed to denature many of the host materials. In the present experiments, short-term freezing of one month did not remove many materials, and long-term freezing was most effective when applied to crude sap. Freezing of intact leaves did not alter the host material to any great extent.

One of the simplest ways to avoid contamination in virus preparations is to use older leaves containing a lower concentration of ribosomal components and extractable green material. If it is necessary to use younger leaves, the best method for removing ribosomes is probably by aggregation with high magnesium concentrations or dispersion with EDTA. This would, of course, depend on the properties of the virus in question.

The isolation and detection of these normal-plant components is very important to the successful purification of plant viruses. This fact was recognized by Pirie (1950, 1956), who detected normal-plant nucleoprotein particles in purified preparations of TMV. Contamination is usually not a problem with stable viruses that may be purified over a period of several days by rather rough methods. For unstable

viruses or those that are present in low concentrations in plant extracts, contamination could be quite important. Ribonucleoprotein particles were easily isolated from healthy plants by methods often used in the purification of plant viruses. Methods which may remove the contaminating material may also cause aggregation or denaturation of the virus and cannot be used. Even density-gradient centrifugation may not be sufficient for separating ribonucleoprotein particles from some viruses, since under certain conditions these particles have sedimentation constants in the range of several spherical viruses.

Ultraviolet absorption at 260 mμ is often used as a measure of virus concentration. With partially purified preparations, many contaminating materials such as ribosomal components or the larger components may interfere with this measurement. Dry weight analysis of the larger components indicated that although the yield of such particles per weight of tissue is smaller than for some viruses, absorbance of a 1 mg/ml concentration is greater than for many plant viruses, such as TMV which has a value of 3.24. The ribosomal components absorb much more strongly at 260 mμ than do the larger conponents. Tissieres et al. (1959) has reported values from 14 to 18 for ribonucleoprotein particles from E. coli. Even a small amount of contamination by these particles could cause a sizable error in estimation of virus

concentration. Ultraviolet absorption spectra have been used as a criterion of virus purity, but spectra for ribosomal particles closely resemble those of some viruses.

Further study of the ribosomal particles may be very useful in connection with the study of non-infectious nucleoprotein particles that have been associated with some viruses. Contaminating normal-plant components could also be very important in plant virus serology. Study of the action of these particles and enzyme systems associated with them may reveal some important facts concerning synthesis of viruses within plant cells.

SUMMARY

Protoplasmic components of healthy bean, tobacco, and barley plants were isolated and studied by use of techniques common in plant virus purification. Three major types of components were separated by density-gradient centrifugation in columns prepared with 0.01 M neutral phosphate buffer. Material that did not sediment in high speed centrifugation or through gradient columns consisted mainly of phenols and low molecular weight proteins. Ribosomal components sedimented through density-gradient columns at rates similar to some small spherical plant viruses and to previously reported sedimentation rates for ribosomes from E. coli and pea seedlings. These particles appeared spherical in the electron microscope, and strongly absorbed ultraviolet light. The integrity of the particles depended upon the concentration of magnesium in the system, with larger particles predominating in higher concentrations of magnesium. Three to five distinct ribosomal components differing in sedimentation rate, density, and ultraviolet absorption spectra were observed. Material that sedimented more rapidly through rate density-gradient columns could be separated into three components by equilibrium zonal centrifugation in sucrose gradient columns. These components were larger, less dense, and absorbed less ultraviolet light than the ribosomes. The lightest and heaviest of these components were white, but the intermediate one was green.

Older leaves usually yielded a lower concentration of components. Different conditions of density-gradient centrifugation varied the number and relative concentration of the components detected. The pattern of sedimentation was markedly changed by centrifugation in gradient columns containing phosphate buffer and Igepon, cesium chloride, tris-HCl buffer, tris-HCl and EDTA, or various concentrations of magnesium. Instability of the ribosomal components was indicated by the failure of recycling attempts.

Centrifugation in density-gradient columns containing phosphate buffer was used as an analytical method to evaluate the effectiveness of different denaturation procedures on extracts of healthy plants. Concentration of the components was measured by photometric scanning of the centrifuged gradient columns.

Denaturation treatments tested included heating, various organic solvents, calcium phosphate, acidification, and freezing. Scanning patterns of treated extracts were compared to those of untreated resuspended high speed pellet from the same preparation of leaves. Effectiveness varied among the plant species tested, but in general, heating, long-term freezing, and calcium phosphate adsorption removed more of the normal plant components than did other treatments. Treatments of the crude sap followed by high speed centrifugation were more effective than the same treatments on

solutions of the resuspended high speed pellet. Components were also effectively removed from extracts by aggregation with high magnesium concentrations and dispersion with EDTA.

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