THE CYTOPLASMIC PROTEINS OF GREEN LEAVES

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ABSTRACT

The soluble cytoplasmic proteins of the leaves of a number of dicotyledonous plant species have been characterized as to their chemical and physical chemical properties. Electrophoretically, the proteins migrate as a single major component and from one to six minor components, the number depending upon the species. The principal component has a mobility of ca. -5.0 to -5.5 x 10^{-5} cm^2 sec^{-1} $volt^{-1}$ in 0.1 μ K-maleate buffer at pH 7.0, and constitutes ca. 60 to 80 per cent of the total protein. In the analytical ultracentrifuge, the proteins are resolved into two components; a high-molecular weight, apparently homogeneous component with a corrected sedimentation constant of 19S which constitutes ca. 30 to 50 per cent of the total protein, and a low-molecular weight, heterogeneous fraction which contains the enzymatic activity.

Chemically, the cytoplasmic proteins contain 13 to 15 per cent nitrogen and from 0.1 to 0.8 per cent phosphorus. This protein-bound P was found to be associated with the 19S component of cytoplasm in the form of ribonucleic acid. The principal component of cytoplasm is, therefore, a nucleoprotein.

The nucleotide composition of the ribonucleic acid in the cytoplasms of leaves of different plant species and in leaves of different physiological ages but from the same plant species were studied. The composition varies with the plant species but not with physiological age.

The effects of pH, of temperatures from 0° C. to 37° C., of dialysis and of storage at -20° C. upon the stability of whole

cytoplasm preparations was studied by chemical and ultracentrifugal analysis. Acidity greater than pH 6.5 and storage at -20° C. for extended periods preferentially denatures the protein moiety of the nucleoprotein component, while dialysis or incubation at room temperatures for short periods of time causes the loss, apparently by enzymatic degradation, of the ribonucleic acid moiety. No method of entirely preventing the loss of nucleic acid was found, although maintenance of a low temperature partially suppresses it.

A differential sedimentation procedure capable of preparing high-molecular weight fractions of cytoplasm (Fraction I protein preparations) containing only 5 to 10 per cent of low-molecular weight contaminants was developed when classical methods of chemical fractionation proved unsuitable. Such preparations were used to determine the physical properties of the nucleoprotein component.

The nucleoprotein components of spinach and tobacco have been shown to be closely similar, although certain physical properties of the nucleoprotein, such as partial specific volume, sedimentation constant and molecular weight, are dependent on the ribonucleic acid content.

The molecular weight of the nucleoprotein component in a particular preparation containing 11 per cent nucleic acid is estimated to be 360,000, based on determination of the partial specific volume and the sedimentation constant of this preparation together with an estimation of the frictional coefficient from a deduced shape factor and an assumed hydration value.

Fraction I protein preparations always contain high-molecular weight components that are not initially present in whole cytoplasm. It is shown that these components probably have their origin in aggregation of the nucleoprotein component.

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PART I
THE SOLUBLE CYTOPLASMIC PROTEINS

I. INTRODUCTION

The cytoplasmic proteins of green leaves are of broad and basic biological concern. These soluble proteins contain, for instance, a wide spectrum of enzymes which mediate many of the important metabolic processes of the cell. In recent years a number of these enzymes have been purified, characterized to some extent, and their comparative biochemistry studied in detail. The soluble proteins as a whole have also been shown by Menigenhi and Delwiche (1951) to be in a dynamic state, as might be expected by analogy with work on animals and microorganisms, and to act to some extent as a protein pool. But unlike many animal proteins which have been examined by the galaxy of physical and chemical techniques available to the protein chemist today, comparatively little is known about individual plant proteins other than those with enzymatic activity.

This lack of knowledge is in sharp contrast to the situation of 20 - 30 years ago during the reign of the nutritional biochemist when "plant proteins" were as well known as "animal proteins". During this period, elemental analyses and such amino acid analyses as had been perfected were performed on a large number of proteins of diverse origin. Because of the nutritional importance of plants, the plant proteins were examined as extensively as the animal

proteins. Since the emphasis was on nutritional value and the methods of separation then existent were poor, analyses were invariably of mixtures of proteins. The "bulk proteins" of commercially important seeds and of forage crops have been especially well examined.

The plant proteins have been reviewed a number of times recently [Chibnall (1939), Vickery (1945), Wood (1945), Lugg (1949), McCalla (1949), McKee (1949), Steward and Thompson (1950), Bonner (1950), and Wildman and Jagendorf (1952)]. The reviews of Lugg, McCalla, McKee, and several chapters of Chibnall are little more than catalogues of analyses of numerous "proteins" from plant material - predominantly seeds. Since many of these analyses are by methods now known to be grossly inaccurate, they are probably of little value except from a nutritional standpoint. Lugg (1949) particularly, is representative of a school of thought which is rapidly vanishing. For years he has analyzed the "bulk proteins" (author's nomenclature) of various species of plants for amino acid content and for nitrogen content by laborious chemical and microbiological methods, looking for differences between species, genera, etc. When refined analytical techniques were available, the precision of such analyses was increased but the basic material to be analyzed, the "bulk protein" remained the same variable, undefined mixture as before. It is only fair to say, however, that in all these reviews the lack of knowledge concerning all aspects of the leaf proteins has been emphasized.

Several vigorous trends are to be found in the more recent reviews. The first of these trends is the use of chromatographic methods to replace purely chemical fractionation of the amino acids. This has been a very important advance because it is now possible to perform accurate analyses on milligram quantities of supposedly pure proteins or their hydrolysis products, in order to establish the degree of similarity in composition or, in some instances, in the amino acid sequence of different preparations (Sanger and Porter, 1948; Edman, 1950). A second trend is the growing tendency to recognize, with Lugg (1949), that the classic concept of the division of proteins into classes, such as albumins, globulins, etc., based on solubility is not applicable to many of the plant proteins. This has necessitated the introduction of other methods of fractionation and examination. Thirdly, less emphasis is being placed on refinement of analyses of crude protein mixtures and more attention is being given to the isolation of single protein species prior to detailed chemical studies. This has led to application of the methods of physical protein chemistry - electrophoresis, ultracentrifugation, light scattering, diffusion and viscosity - as a means of following fractionation, as criteria of purity and as a supplement to chemical analyses.

The study of plant proteins by physical methods has lagged behind similar studies of animal proteins. This has been due in part to the relative ease with which "pure" proteins such as ovalbumin, serum albumin and β-lactoglobulin may be secured for

study as models by physical chemists, and in part to the ready financial support available for the study of animal proteins from groups interested in possible medical applications of physical chemical techniques. Until very recently, physical studies of plant proteins have been limited to the seed proteins. Svedberg and Pedersen (1940) have summarized the ultracentrifuge studies carried out on several proteins isolated from wheat and corn seed in their laboratory, and Johnson (1948) has reported a similar investigation of conarachin and arachin. Electrophoretic studies of several proteins from cereal seeds have been reviewed by McCalla (1949). Fontaine, et al. (1945) also used this method to study the peanut proteins.

Because of the quantity of material required, physical studies of the proteins from individual vegetative tissues of higher plants are almost of necessity limited to either tubers or leaves. In all plant tissues, the location of the protein relative to the architecture of the cell presents a difficult extraction problem. The cells of leaves are especially troublesome. A typical parenchyma cell has a tough outer cell wall which encloses a thin layer of cytoplasm which in turn surrounds a large vacuole. The particulate entities, the nucleus, chloroplasts, mitochrondia and perhaps microsomes, are embedded in the cytoplasmic layer. Each of these entities is differentiated from the cytoplasm by a membrane and the cytoplasm itself is further differentiated from the vacuole and from the cell wall by semipermeable membranes which prevent free passage of water and solutes between the vacuole and the outside

environment. The vacuolar fluid contains little if any protein but may be quite acid due to accumulation of organic acids. The problem in extraction is to apply sufficient force to disrupt the cell wall without rupturing the particulate bodies, which would contaminate the cytoplasmic proteins with proteins originally particulate, and to prevent dilution of the cytoplasm with vacuolar fluid, since this may cause denaturation of the cytoplasmic proteins.

The first extensive studies concerned with extraction of the cytoplasmic proteins were those of Chibnall (1939), whose procedures have been widely adopted. In Chibnall's method, the permeability of the cellular membrane is destroyed with ether-water and the vacuolar fluid squeezed out in a press. This effectively eliminates dilution of the cytoplasmic constituents with vacuolar fluids. The press residue is resuspended in a dilute buffer solution and the cell walls ruptured by grinding. The Waring blender or a modification of it, has been commonly employed for this purpose in recent years. The cellular debris and particulate matter are removed by a low speed (ca. 2000 x g.) centrifugation. Yields by this method are rather poor, varying from 20 to 60% of the total protein in the material extracted, according to Chibnall (1939). The effects of the acid vacuolar fluid and ether-water on the cytoplasmic proteins have not been carefully studied, but microscopic examination of cells following cytolysis shows the protoplast to be shriveled into one end of the cell, surrounded by ether-water and vacuolar material. The presence of a green color in the centrifuged

extract also indicates some solubilization of the chloroplast pigments.

The first physical studies of the leaf proteins were the electrophoretic analyses of soluble cytoplasmic proteins, prepared according to Chibnall's method, of healthy and tobacco mosaic infected tobacco leaves by Frampton and Takahashi (1944, 1946). Since the work of Frampton and Takahashi, there have been numerous electrophoretic studies of viruses but relatively few studies of the normal cytoplasmic proteins. With one exception (Commoner, et al. 1952), all published electrophoretic studies of the soluble cytoplasmic proteins of normal leaves have originated in the laboratory of Wildman and Bonner [Wildman and Bonner (1947), Wildman, et al. (1949b), Wildman and Bonner (1950), Campbell (1951), Singer, et al. (1952)]. These studies have shown that although the number of "components" which may be resolved in solutions of cytoplasmic proteins is variable, depending upon the plant species and the buffer and pH used for electrophoresis, at least 60 - 70% of the proteins migrate as a single peak. The remaining proteins are represented by from one to six minor peaks which are more or less incompletely resolved from the major peak. It was also found that the mobilities of the respective peaks are substantially constant between species.

The early studies of Frampton and Takahashi suggested that electrophoretic observation of the level of normal protein

associated with virus protein formation might well be feasible. Chibnall, however, had expressed doubt that the material extracted by his method was representative of the cytoplasmic proteins as a whole. This doubt, coupled with the poor and variable extraction yields, made quantative interpretation difficult. Better extraction procedures were needed before the electrophoretic approach to the study of the normal soluble proteins or to virus protein formation could be explored.

An improved extraction procedure which made possible quantitative studies of normal and virus proteins was developed by Wildman and Bonner (1947). Using a procedure which would rupture almost all of the cells, together with a strong buffer to control the pH of the vacuolar material, they quantitatively extracted material which appeared to be representative of the whole. Chibnall (1939) had previously shown that the "cytoplasmic proteins" which he extracted had an isoelectric range from ca. pH 5.5 to 4.0 and that once flocculated in this range their solubility was greatly decreased. He had further shown that the expressed sap from the cytolyzed leaves of many species has a pH of about 5.5 to 6.5. To eliminate the unknown effects of ether-water, Wildman and Bonner omitted the cytolysis step prior to disruption of the cells. The leaves, suspended in M/2 maleate buffer of pH 7.0 were ground in an Eppenbach colloid mill cooled by circulation of ice water around the shearing surfaces. The cellular debris and unbroken cells were removed from the slurry by filtration through sharkskin paper in a

basket centrifuge and the clarified extract used to grind more material. In this manner, the adverse effects of acid from the vacuole could be controlled, but dilution of the cytoplasm by the vacuolar volume and by the initial volume of buffer could not be prevented. The dilution by buffer was minimized, however, by using a fairly concentrated buffer solution and by recycling the initial extracts through the mill for extraction of more material.

Particulate matter was removed from the final extract by centrifugation at 20,000 x g. for one hour. The clear, light brown to amber colored supernatant solution of cytoplasm so obtained contained about 1.0 to 1.5% protein. There was no Tyndall effect nor evidence of any green color, so characteristic of Chibnall's preparations, in the final supernatant solutions. During colloid milling, the majority of the chloroplasts and presumably the nuclei were fragmented, releasing stroma, grana and nuclear material into the cytoplasm. Prolonged grinding may further disrupt a portion of the grana. If so, the fragments so formed do not appear to be solubilized, since the final solution lacks the green color that would be expected from the liberation of the chlorophyll-protein complex of the grana. The composition of stroma from chloroplasts is unknown, but if it includes protein, there will be a small and variable contribution of protein from this source to the "soluble cytoplasmic proteins" as prepared by Wildman's method. Similarly, fragmentation of nuclei may contribute a small quantity of nuclear protein to the cytoplasmic fraction.

With slight modification, this method has been successfully applied to extraction of leaves of a number of species (Wildman, et al. 1949; Campbell, 1951; Singer, et al. 1952).

The first ultracentrifugal studies of the cytoplasmic proteins were also made by the Wildman group (Singer, et al. 1951). These preliminary studies indicated that the soluble proteins of spinach contained a rapidly sedimentating, apparently homogeneous component which comprised approximately 30 - 50% of the total protein and a low-molecular weight, heterogeneous fraction which could not be resolved. Further investigation has shown that this high molecular weight protein is characteristic of the leaf cytoplasm of dicotyledenous species in general (Singer, et al. 1952).

The studies reported in this thesis constitute a continuation of the work discussed above, together with the physical chemical and chemical characterization of the high-molecular weight protein component of leaf cytoplasm.

II. MATERIALS AND METHODS

A. Plant Materials: All plants, with the exception of spinach (Spinacia sp.) which was procured from a local market, were grown under green house conditions. Nicotiana glutinosa, tomato (Lycopersicon esculentum) and cocklebur (Xanthium pennsylvanicum) were grown in sand culture supplied with complete nutrient solution. Cuba white, Maryland Mammoth and Turkish tobacco (all varieties of N. tabacum), pea (Pisum sativum), and gherkin (Cucumis anguria) were grown in the Earhart air-conditioned laboratory under a temperature regime previously found optimal for growth of the particular species.

In the case of Maryland Mammoth and Turkish tobacco, which were most extensively used, the aerial portions were harvested periodically, the root stock being retained for growth of more material. Large harvests of mature leaves were possible about every 30 days during the summer months, but because of lower light intensities during the winter months, harvests could be made only every 60 - 75 days.

All material used was in the vegetative stage, i.e., non-flowering. For most species this was accomplished by using fully matured leaves from young plants which had not reached the flowering stage. When necessary, as during the winter months, Maryland Mammoth and Turkish tobacco were kept vegetative by application of 8 hours of artificial light in addition to 8 hours of natural daylight.

Xanthium was grown at the Orlando greenhouse under 20 hour day-lengths.

Only mature, well expanded leaves which exhibited no evidence of pathological symptoms were used.

B. Preparation of Cytoplasmic Proteins. Soluble cytoplasmic protein solutions were generally prepared by a modification of the Wildman technique (Wildman and Bonner, 1947; Singer, et al. 1952). Several variations in the buffer used as a dispersing medium and in the disruption technique were explored. These will be described in section III-B. The procedure adopted was as follows:

The petioles, midribs and larger lateral veins were removed from the leaves immediately after harvesting, and the remaining tissue sliced into sections approximately 1 cm. square. An appropriate volume of ice-cold M/2 KOH-K-maleate buffer, pH 7.0 - 7.1, to be used as a dispersion medium, was measured out and introduced into a Eppenbach colloid mill. Usually 1.0 ml. of buffer was used for each 2 gm. tissue. The distance between the rotor and stator of the mill was adjusted to an arbitrary setting of "68" units (Eppenbach calibration) and leaf tissue added until a thick slurry formed. The mill clearance was reduced to "40" units and grinding continued for about 30 - 60 seconds before the slurry was drained from the still-operating mill. Cellular debris and unbroken cells were removed from the slurry in a basket centrifuge lined with sharkskin filter paper. This clarified protoplasmic

juice was returned to the mill and used to grind more leaf tissue, the filtration being repeated as frequently as necessary for efficient operation of the mill. All grinding and filtering operations were performed in a cold room at 2° C.

During the grinding, considerable air is dispersed into the grinding medium. That this causes some surface denaturation is evident from the amount of foaming which occurs. This effect is minimized by using a volume of solution large enough to prevent formation of a vortex in the hopper and by grinding as short a time as possible.

Local heating, caused by excessive generation of heat between the rotor and stator of the mill when narrow clearances or thick slurries are used, may cause a temperature rise of 10 - 15° C. This temperature rise is undesirable because of the accelerated enzymatic destruction of cytoplasmic components that occurs at high temperatures. To miminize the amount of heating, ice water was circulated through the chamber which surrounds the stator. Initial grinding was accomplished at as wide a clearance as consistent with good dispersal and the slurry was filtered frequently. The solutions were further handled in ice baths except during the actual milling and filtering operations.

The clarified extract from the last filtration step, containing the soluble cytoplasmic proteins, particulate matter and vacuolar material, will be referred to as "whole protoplasm". The particulate matter was removed by centrifugation at 25,000 r.p.m.

in the no. 30 head of a Spinco Model L Ultracentrifuge for 60 minutes. Maximum refrigeration conditions(rotor chamber at -22° to -24° C.) were used to keep the solution as cold as possible. The supernatant solution from this centrifugation, to be known as "cytoplasm" or "cytoplasmic protein extract", is always light amber to brown in color and is completely clear by either transmitted or reflected light. The pH of this solution varies from approximately 6.7 to 6.9, and the protein content ranges from ca. 0.7 to 1.3%, depending on the age and species of leaves extracted.

A flow-sheet of the extraction process is shown in Figure 1.

C. Estimation of Protein Concentration: The amount of protein in solution was estimated by weighing the material precipitated by 2 volumes of 1.0 N TCA. The precipitation mixture was maintained at 0° C. for 24 - 30 hours, the precipitate centrifuged down, washed twice with 0.5 N TCA, then dried to constant weight at 105° C. Justification of conditions of temperature, TCA concentration and time of precipitation used are given in Appendix I. Duplicate or triplicate aliquots containing 5 - 20 mg. protein were used. The precision of weighing was ± 0.05 mg. Precipitates were dried until two successive weighings of the same residue agreed to 0.1 mg. The drying time required to reach this state of dryness ranged from 2 to 8 or 10 days, depending upon the amount of residue.

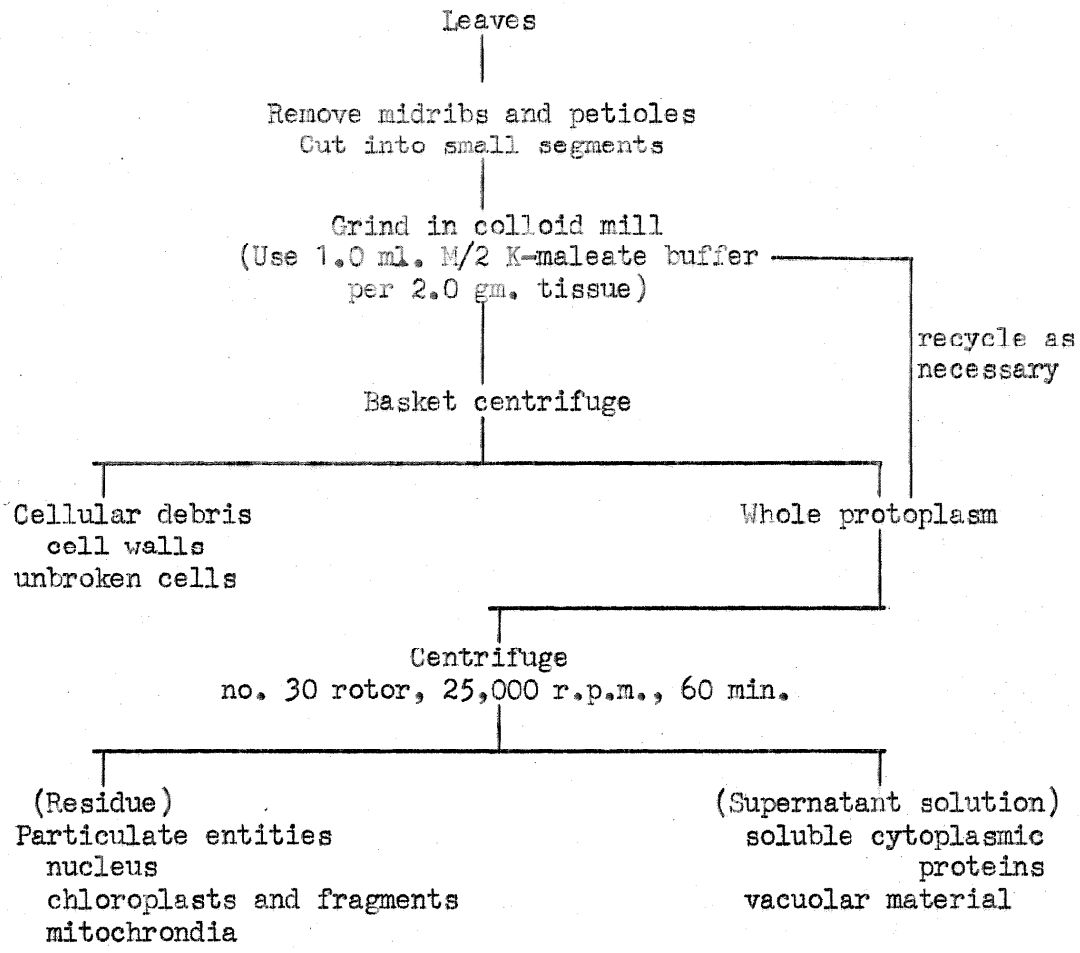

Figure 1. Flow-sheet for extraction of cytoplasmic proteins from leaf tissue.

With samples of approximately 10 mg., a loss of about 0.1 mg. per day after 3 or 4 days was commonly encountered. Whether this loss was due to slow loss of water or to actual decomposition of the protein itself is not known. When these circumstances were encountered, the 4 day dry weight was usually taken as the correct weight.

Replicate aliquots usually agreed to within $\pm$ 2 - 3%, which is then the precision of the determination. The accuracy may be somewhat less than this.

For more rapid estimation of protein, as for making dilutions, protein nitrogen was determined. Suitable aliquots were precipitated with cold TCA, as above, centrifuged and washed after about two hours. The precipitate was dissolved in 2 N NaOH, diluted to an appropriate volume and aliquots digested for total N, as described below. Precipitation with TCA is necessary because even in thoroughly dialyzed Fraction I protein preparations, which should contain only high-molecular weight material, there is a non-dialyzable, non-precipitatable fraction which amounts to about 10 - 15% of the total N in solution.

D. Estimation of Nitrogen: Samples for nitrogen analyses, either total nitrogen aliquots or TCA precipitates, were digested in 50 ml. Folin-Wu tubes with 2.0 ml. concentrated H_2SO_4 and about 5 mg. of a Na_2SO_4, $HgSO_4$ and selenium catalyst. The tubes were placed in a heavy brass rack which jacketed the lower one-half inch of the tubes, affording even heat distribution, and the digestion mixture gently refluxed

about two hours, or until light yellow. After cooling, two drops of 30°/o H_2O_2 were added to each tube and digestion was continued about 20 minutes. The superoxol treatment and subsequent heating was repeated twice. Based on the results of Miller and Miller (1948), the conversion to ammonia nitrogen should be about 98°/o complete with this digestion regime.

Ammonia nitrogen was determined by Nesslerization with the reagent of Koch and McMeekin (1924) according to the procedure of Lanni, et al. (1945). This reagent in which the potassium mercuric iodate complex and the base are combined in a single solution, keeps well and has a greater range of concentration and temperature tolerance than the "improved" two-solution reagent recommended by Thompson and Morrison (1951). The intensity of the color developed in 20 minutes was determined in a Klett-Summerson colorimeter with a no. 42 filter. The color does not obey Beer's law at low concentrations (< ca. 40 γ N) and is not reproducible enough from day to day to give results of high precision from a single calibration curve. A calibration curve was, therefore, constructed each time. If such a curve is made each time, the precision is limited only by the accuracy of the Klett readings. In the range of 20 to 100 γ N per sample, this is in the order of ± 2 - 3°/o.

E. Estimation of Phosphorus Content: Total phosphorus content was determined according to the method of Allen (1940) after incineration with 60°/o perchloric acid. The phosphomolybdate

color developed in 5 minutes was read in a Klett-Summerson colorimeter with a no. 66 filter. In the range of 5 - 40% P/sample, the method has a precision in the order of ± 1%.

Trichloroacetic acid precipitable phosphorus (TCA-P) was determined on aliquots which had been precipitated with cold TCA, then centrifuged and washed twice with cold TCA after four hours at 0° C. This short precipitation time was necessary because of the solubility of the TCA-P in either hot or cold TCA. A full discussion of this aspect of TCA-P estimation is presented in Appendix I.

F. Electrophoretic Analyses: Electrophoresis analyses were conducted in the Tiselius moving boundary electrophoresis apparatus as modified by Swingle (1947). Standard 11 ml. analytical cells were used. Photographic records of dn/dx vs. x were obtained by the Longsworth scanning technique. Before analysis, the solutions were dialyzed for at least 18 hours against either cacodylate or maleate buffer at pH 6.93 or 7.00, respectively. Both buffers were used at 0.1 ionic strength (0.1 μ). The composition of the cacodylate buffer was 0.0233 M cacodylic acid, 0.02 M NaOH and 0.08 M NaCl; that of the maleate buffer was 0.033 M maleic acid (as the anhydride) and 0.066 M KOH. Dialysis was carried out at 2 to 4° C., electrophoresis at 1.80° ± 0.02° C.

Mobilities were computed from measurements made on 4x enlargements of the dn/dx, x patterns according to the formula:

$$\mu = \frac{d \cdot A \cdot K}{t \cdot I \cdot R_x \cdot M} = cm^2\ volt^{-1}\ sec^{-1}, \qquad \text{(Eqn. 1)}$$

where

d = distance boundary moved in time t

A = cross-sectional area through cell

K = conductivity cell constant

t = duration of run in seconds

I = current in amperes

R_x = specific resistance of buffer in ohms

M = total magnification factor.

G. Ultracentrifuge Analyses: Analytical ultracentrifuge (UC) analyses were performed in the ultracentrifuge designed and built at this Institute. A brief description of the instrument is given in Appendix II.

For survey work, UC analyses were frequently made on whole cytoplasm solutions without preliminary dialysis. The medium was thus undefined with respect to density and viscosity; hence, sedimentation constants computed from these analyses could not be corrected to standard conditions. For UC analyses from which corrected sedimentation constants were desired, the material was first dialyzed against either the cacodylate or maleate buffer mentioned in connection with electrophoresis experiments.

For measurement of boundary positions during a run, the photographic records of dn/dx vs. x were enlarged 13.05 diameters and the peaks traced on millimeter coordinate paper. The centers

of the peaks were located to 0.05 mm. by averaging the values of x for several values of dn/dx. Since the peaks were nearly always symmetrical, variations in x with height (dn/dx) were due to errors in tracing. The "number average" procedure used is therefore valid. Also, since the Fraction I protein in which we are most interested does not exhibit any concentration dependence, correction for dilution as the concentration gradient moves through the cell is not necessary (see Appendix II for a full discussion of this dilution phenomenon).

Data taken for each exposure during the course of a sedimentation velocity run included: speed and temperature of the rotor and the time and duration of the exposure. From these data and the position of the gradient, computed from measurements of the plate, the sedimentation constant is calculated.

The sedimentation constant, $\underline{s}$, is defined as (Svedberg and Pedersen, 1940):

$$\underline{s} = \frac{dx/dt}{\omega^2 x} \qquad \text{(Eqn. 2)}$$

where dx is the actual distance which the boundaries moved (cm.) in time dt (seconds), ω is the angular velocity (radians/second) and x is the distance from the axis of rotation (cm.). The sedimentation rate is measured in units of 10^{-13} cm./sec./unit gravitational field, the Svedberg unit, S.

For comparison purposes, the value of $\underline{s}$, which may be

determined for any set of experimental conditions, has been corrected to a standard reference state. This reference state was arbitrarily chosen as water at 20° C. by Svedberg (Svedberg and Pedersen, 1940). To correct experimental values of $\underline{s}$ at any temperature and in any solvent to these standard conditions, the following corrections were applied:

$$\underline{s}_{w,20} = \underline{s}_{B,t} \times \frac{\eta_{B,t}}{\eta_{w,t}} \times \frac{\eta_{w,t}}{\eta_{w,20}} \times \frac{1 - \bar{V}\rho_{w,20}}{1 - \bar{V}\rho_{B,t}} \qquad \text{(Eqn. 3)}$$

The first factor, $\eta_{B,t} / \eta_{w,t}$, corrects the viscosity of the experimental solvent at temperature t to that of water at the same temperature. The second factor, $\eta_{w,t} / \eta_{w,20}$, relates the viscosity of water at the experimental temperature to that of water at 20°C. The last factor, $1 - \bar{V}\rho_{w,20} / 1 - \bar{V}\rho_{B,t}$, corrects for the difference in buoyancy due to the density of the solvent at t°, $\rho_{B,t}$, as compared to the density of water at 20°, $\rho_{w,20}$. $\bar{V}$ is the partial specific volume of the sedimenting component(s).

Two methods were used to compute the value of $\underline{s}$. Both are based on an integrated form of Eqn. 2:

$$\underline{s} = \frac{1}{\omega^2} \int_{t_1}^{t_2} \int_{x_1}^{x_2} \frac{dx}{x} \frac{1}{dt} = \frac{\ln \frac{x_2}{x_1}}{\omega^2 (t_2 - t_1)} \qquad \text{(Eqn. 4)}$$

In this equation, x_1 is the position of the center of the boundary at time t_1 and x_2 is the position at time t_2, where t_1 and t_2 may be for the first exposure and any subsequent exposure, or may be for successive exposures.

In the first method of computation, t_1 is used as a fixed reference exposure (chosen from one of the early exposures) and t_2 is the cumulated time from t_1 to each of the succeeding exposures. Eqn. 4 may be rearranged to the general form $y = mx + b$:

$$\ln \frac{x_2}{x_1} = \omega^2 \underline{s}(t_2 - t_1) \qquad \text{(Eqn. 5)}$$

Thus, if $\ln x_2/x_1$ is plotted against $(t_2 - t_1)$, the slope of the line is $\omega^2 s$. The slope may be determined graphically or may be computed by the method of least squares. Where all factors, speed, temperature, and $\underline{s}$, are constant throughout a run, this method of computation is very satisfactory. If, however, $\underline{s}$ varies with concentration, this method will be unsuitable since undue emphasis is placed on the first values of $\underline{s}$. Similarly, if either the speed or the temperature changes radically during the course of a run, corrections cannot be made.

The second method of computation used is more laborious but has the advantage that appropriate corrections can be made to each value of $\underline{s}$, and that equal weight is placed on all values. This method involves calculation of $\underline{s}$ values from Eqn. 4, treating successive exposures as pairs. The individual values of $\underline{s}$ are corrected for temperature variations according to Eqn. 3 if the temperature during the run varied more than $\pm$ 0.2°. If the temperature variation was less than this amount, the number average values of $\underline{s}$ and for temperature were computed and $\underline{s}_{w,20}$ calculated from Eqn. 3.

III. GENERAL CHARACTERIZATION OF THE SOLUBLE CYTOPLASMIC PROTEINS

A. Introduction

Prior to initiation of the work reported here a long range program on the biochemistry of plant proteins had been undertaken in the Wildman and Bonner laboratory. This program included a comprehensive study of the chemical and physical properties of the soluble protein fraction. The initial chemical studies were designed primarily for finding a means of fractionating the mixture of proteins known to be present in plant cytoplasm and to find, if possible, some chemical attribute or attributes for assessing the amount of fractionation achieved. Physical chemical studies, originally designed only to supplement chemical analyses and fractionations, proved to be of greater usefulness than the chemical approach.

The work reported in this section constitutes a further study, primarily by physical chemical methods, of the complex cytoplasmic protein mixture. By the use of physical methods, it has been possible to achieve better characterization of the soluble proteins as a whole and to further investigate the effects of extraction procedures, pH, salt concentration and enzymatic activity of the extract upon particular fractions of the cytoplasmic proteins.

No analyses are, however, of any great significance unless the material analyzed may be defined - either as representative of a mixture or as a specific part of a mixture. Fontaine (1945), for

example, extracted more than 90°/o of the protein from peanuts with salt solutions of varying concentrations, yet concluded that the extracted material was not representative of the total protein. Dispersal and solution of the proteins, which constitutes the major problem in the case of seed proteins, is not a primary problem in preparation of leaf proteins. Instead, the problem is to prevent the precipitation of material which is already in a dissolved state in the cytoplasm. If this can be accomplished, and if a reasonable proportion of the cells can be disrupted, then the material extracted may be considered representative of the whole. Regarding the significance of cytoplasmic extracts, Vickery has stated (1945, p. 368):

> "... although one can doubtless learn to isolate preparations that represent protein components of the cytoplasm, there is no reason whatever to expect such preparations to be homogeneous ....Because of the instability of these proteins it is improbable that the preparations secured... will resemble the original material of the cell at all closely in its physical and chemical properties."

These objections voiced in 1945 still stand, indeed are even emphasized by the lability exhibited by the nucleoprotein discussed in Part II. There can be no doubt, however, that the extracted cytoplasmic components play an integral and important role in the economy of the cell. If preparations which are truly representative may be obtained, study of them should yield important information with regard to this role.

Accordingly, the methods of extraction were reexamined to ascertain the degree of extraction accomplished and whether more efficient procedures could be developed. Some of the general physical and chemical properties of the cytoplasmic extracts were then studied in more detail. Fractionation with $(NH_4)_2\ SO_4$ was also reexamined with better analytical tools than had previously been available.

B. Efficiency of Extraction

During development of the colloid milling extraction procedure described in Section II-B above, Wildman and Bonner (1947) determined that from 66 - 72°/o of the total nitrogen in the leaf was extracted by the grinding regime used, and that this corresponded roughly to the proportion of ruptured cells as determined microscopically. Under prolonged grinding conditions, it was found that up to 90°/o of the total N could be extracted in the form of cell-free protoplasm. As the degree of extraction increased, the percentage of N in the extracted material remained constant. This strongly suggested that the composition of the extracted material was constant, and that the efficiency of extraction depended on the number of cells disrupted. These findings were in agreement with those of Chibnall (1939).

No rigorous total extraction studies were performed on material extracted by the current procedure, but from both soluble N and soluble P analyses it appears that not less than 90°/o of the cells are disrupted during extraction. This value was determined as follows:

Leaf punches were taken from the leaf tissue prior to grinding, quick-frozen and lyophilized from the frozen state. The balance of the leaf tissue was ground. The particulate matter in the protoplasm was separated from the soluble proteins by centrifugation and lyophilized, and the volume of the supernatant cytoplasm measured. Appropriate wet and dry weights were obtained for all fractions. All dried material was ground in a micro Wiley mill to pass a 60 mesh screen. Total N, total P, and soluble non-protein nitrogen and phosphorus were determined on the dried whole leaf tissue, the particulate residue, and on the solution of cytoplasmic proteins. Non-protein N and non-TCA-P were determined on the dried residues by extraction with cold 0.5 N TCA on fritted glass filters, and on cytoplasmic extracts after precipitation of protein with TCA.

From an appropriate balance sheet, it was found that approximately 85 - 90°/o of the soluble non-protein N and P computed to be in the amount of leaf material ground could be accounted for in the cell-free protoplasmic extract and about 80 - 85°/o in the cytoplasm. The remaining 5°/o appeared in the particulate fraction, which had not been washed free of occluded cytoplasm before drying. Recovery of total N or total P was not nearly as complete. Much of the N and P not recovered in the extract was included in the unbroken cells and cellular debris removed by filtration through sharkskin paper. Inasmuch as there was undoubtedly some soluble non-protein N and P also in this unanalyzed

fraction (from the protoplasmic juice with which the filter pads are saturated), recovery in the protoplasm of 90°/o of the theoretical quantity is indicative of rupture and dispersal of the contents of at least 90°/o of the cells.

It is not quite as simple to determine whether dispersal of the protein material of the cells is as complete as that of the non-protein N and P, for the total protein balance sheets are less accurate. It was found, however, by comparing the yields of protein extracted in several buffers at several pH's that the grinding conditions utilized provide the maximum yield of soluble protein.

For example, 1385 gms. of Turkish tobacco leaf tissue were sliced into approximately 1 cm. squares and randomized. Four aliquots of 485, 300, 300 and 300 gms. each were weighed out and ground in the following buffers:

1. Na-maleate, pH 7.02, M/2. Prepared by addition of solid NaOH to a solution of maleic acid to pH 7.0 at 25° C. and dilution to 0.5 M with respect to maleate ion.

2. Na-maleate, pH 7.56, M/2. Prepared as in 1 except adjusted to pH 7.56 at 25° C. before dilution.

3. Na-phosphate, pH 7.48, saturated at 0° C. Prepared by adding M/2 NaH_2PO_4 to M/2 Na_2HPO_4 to pH 8.0. When cooled to 0° C., much of the buffer salt crystallized out. The pH of this saturated solution was 7.48 at 25° C.

4. Na-borate-HCl, pH 8.31, saturated at 0° C. Prepared

by adding HCl to M/2 sodium borate to pH 8.5 at 25° C. Slight crystallization of the buffer salts occurred at 0° C. The pH of the saturated solution was 8.31 at 25° C.

One ml. of buffer was used for each two grams of tissue. Grinding and filtering operations were made as uniform as possible. From measurement of the final volume of buffer plus cytoplasm extracted, it was found that the yields of protoplasm per gram of tissue extracted were comparable in all cases. The cytoplasms were prepared by centrifugation and their protein concentrations determined.

The results, shown in Table I, indicate that nearly the same amount of protein was extracted in either maleate or phosphate buffer at pH 7.0 - 7.5. The phosphate buffer used permitted too great a pH shift (pH 7.48 to 6.52) and is therefore unsatisfactory in this regard. The amount of protein extracted in borate buffer was very low in comparison to extraction in either maleate or phosphate buffers. This is apparently caused by the borate ion rather than the alkaline pH since, as will be shown in Section III-E, the proteins are inherently stable at pH 8.5. Maximum extraction was obtained with maleate buffers at pH 7.0 - 7.5. It is possible that extraction in the range of pH 7.5 to 9.0 might be satisfactory, but at present no suitable buffers are available for this region. Phosphate buffers are undesirable because of interference with P analyses; organic buffers depend upon amine groups for buffering capacity in this range and thus interfere with nitrogen determinations.

TABLE I

COMPARISON OF SEVERAL BUFFERS FOR EXTRACTION OF THE CYTOPLASMIC PROTEINS*

	Buffer			
	Maleate	Maleate	Phosphate	Borate
Initial pH**	7.02	7.56	7.48	8.31
Molarity	0.5	0.5	Saturated at 0° C.	
pH cytoplasmic extract	6.78	6.91	6.52	7.42
Protein concentration mg./ml.	11.8	11.8	11.3	6.3
Relative amount extracted	100	100	96	53

* Extraction ratio of 1 ml. buffer/2gm. tissue.

** Measured at 25° C.

Other disruption methods were briefly tested. For grinding less than approximately 250 gms. of leaf tissue, the colloid mill is unsuitable because approximately 125 ml. of liquid are required for circulation. The "Omni-mixer", marketed by Ivan Sorvall, Inc., was found to be very good for 20 - 500 gms. of tissue. The operating principle of this unit is similar to that of a Waring blender. The blades are, however, mounted from the top instead of through the bottom, thereby completely eliminating the heating effects and leakage through the rotor bearing which makes conventional types of blenders unsuitable for this work. The degree of extraction is probably comparable to that of the colloid mill.

Several attempts were made to break up cells in a mortar in order to eliminate the local heating and surface denaturation that occurs during the milling operation. Leaf tissue was quick-frozen between cakes of dry ice and crushed to a fine powder. A mixture of this crushed material and powdered dry ice was ground in a large mortar for about 15 minutes. Powdered dry ice was added as necessary. The ground material was extracted with M/2 maleate buffer for one hour at 0° C. with continuous agitation. Subsequent analyses showed that more than 90°/o of the soluble P was found in the cytoplasmic extract, indicating nearly complete disruption of the cells. The protein concentration of the extract, however, was very low (approximately 2 - 3 mg./ml.) because of the quantity of buffer (2 ml./gm. tissue) necessary to soak up the spongy debris from

grinding. Because the deleterious effects on the protein caused by procedures available for concentrating the cytoplasm to a useful protein content are more serious than the effects brought about by grinding in the colloid mill, this method was abandoned.

C. Chemical Analyses of Whole Cytoplasm

In agreement with the work of other investigators (cf. Lugg, 1949; Chibnall, 1939) the soluble cytoplasmic proteins of a number of species were found to contain approximately 13 to 15.5% nitrogen. Variations within a species were as great as between species. These variations therefore probably reflect differences in techniques, rather than real differences in the starting material.

Wildman, Campbell, and Bonner (1949), Chibnall (1939), and Pirie (Bawden and Pirie, 1937; Pirie, 1950) have reported phosphorus to be associated with the cytoplasmic proteins, but there is no agreement among these authors as to how much phosphorus is present, or as to its form. Wildman, et al., also report adenine and ribose to be present in TCA precipitates of a cytoplasmic protein fraction prepared by precipitation with 0.35 saturated $(NH_4)_2SO_4$. These materials were present in the approximate ratio of 1 purine: 1 ribose: 2 phosphorus. Approximately 50% of the P was acid labile (30 min. in 1 N HCl at 100°). The similarity of this material to yeast adenylic acid was suggested. Holden (1952) recently reported separation of the P-containing compounds of leaf tissue into cold-acid-soluble-P, lipid-P, RNA and DNA. Due, however, to

the manner of fractionation of the leaf tissue (maceration in a food grinder followed by division into "sap" and "fiber" by squeezing the "sap" through madapollam), the correct distribution of these P-containing compounds within the cell cannot be determined from the data presented.

Although considerable lipid material has been found to be present in cytoplasmic extracts of spinach leaves, it is doubtful whether either phospholipids or DNA are true components of the soluble cytoplasmic proteins. The lipid material is found as a light yellowish-green, turbid layer floating on top of the clear amber cytoplasm solution in the centrifuge tube following sedimentation of the particulate matter. It is probable that this lipid material has its origin in ruptured grana.* Chloroplasts are known to be high in lipid content, and the chloroplasts and grana of spinach are especially easy to disrupt. Preparations made from tobacco leaves, whose chloroplasts and grana are much harder to disrupt, do not yield such a lipid layer. To avoid contamination of spinach cytoplasm solutions with lipids, the cytoplasm was carefully removed from between the over-lying lipid layer and the pellet with a syringe.

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* Menke (1938) reported a number of cytoplasmic protein preparations of spinach contained 0.4 to 0.7% total lipid material as compared with 30 - 33% in chloroplasts. He interprets this to indicate that the cytoplasm contains only trace amounts of lipids. Chibnall (1939, pp. 128-133) reports substantially the same analytical results but differs with Menke in their interpretation. Chibnall considers protoplasm to be a "lipid in protein" emulsion which is destroyed when the cell is disrupted. The two phases then separate, the lipid coalescing into droplets which are separated analytically with the chloroplast fraction.

During the present study, the presence of adenine, ribose and phosphorus (TCA-P) firmly bound to the protein was verified and in addition, guanine, cytidylic acid and uridylic acid were also identified in 1 N acid hydrolysates of hot-acid extracts of precipitated cytoplasmic proteins. These facts indicate that at least part of the true TCA-P, as distinguished from cold-acid soluble organic-P, is in the form of ribonucleic acid. The evidence for this will be fully discussed in Section IV. Furthermore, since all of the TCA-P may be removed from the protein moiety by hot acid extraction (3 - 5 min. in 1 N acid at 90 - 100° C.), phosphoproteins, which are extracted only with strong bases, cannot be present. This strongly suggests, then, that all the P associated with the cytoplasmic proteins is in the form of nucleic acid.

All the P-containing compounds of the cell except those associated with particulate entities are present in the cytoplasmic extract. Since the phosphorus content of the leaves varies markedly with their physiological status and the phosphorus nutritional status of the plant as a whole, the total-P content of these extracts varied markedly between experiments and between plant species, as may be seen in Table II. The distribution of the total-P among TCA-P, non-TCA-precipitable organic-P and inorganic-P illustrated by the data in this Table is typical. It should be emphasized, however, that because of the intense enzymatic activity of the preparations, the exact amount of organically-bound phosphorus is a function of

TABLE II

DISTRIBUTION OF PHOSPHORUS IN THE CYTOPLASMIC EXTRACTS OF TURKISH AND MARYLAND MAMMOTH TOBACCO

Sample no.	E-11		V-51-70		V-52		E-1	
Mg. protein/ml.	10.4		12.0		3.66		7.16	
	γ/ml.	% of total	γ/ml.	% of total	γ/ml.	% of total	γ/ml.	% of total
Total-P	185	100	344	100	70.0	100	69.5	100
TCA-P	30	16.2	70	20.4	17.9	25.6	21.9	31.7
Non-TCA-precipitable organic-P	16	8.6	274	79.6	52.1	74.4	4.3	6.2
Inorganic-P	139	75.2					43.3	62.3

the age of the extract and the care taken to minimize enzymatic activity during preparation. This is especially true of the level of non-TCA-precipitable organic-P, which is readily destroyed by phosphatase.

D. Analysis of Whole Cytoplasm by Physical Methods

Two methods of physical analysis were used to investigate the cytoplasmic proteins. These were electrophoresis, which characterizes molecules on the basis of net surface charge, and analytical ultracentrifugation, which characterizes molecules on the basis of mass and shape. The theory and technique of each of these methods has been the subject of a number of recent reviews (cf. Longsworth and McInnes, 1942, Briggs, 1950, and Alberty, 1948 a, b, for electrophoresis; Pickels, 1942, 1950, for ultracentrifugation). and a monograph (Abramson, Moyer and Gorin, 1942, and Svedberg and Pedersen, 1940). The work described in this thesis constitutes the first extensive use of the ultracentrifuge for examination of the cytoplasmic proteins of plants. The technical difficulties encountered, some of which are peculiar to the UC employed and some of which are peculiar to the protein system examined, are therefore discussed in detail in Appendix II.

Electrophoretic Analyses

Electrophoretic analyses of the cytoplasmic proteins of tobacco have been reported by Frampton and Takahashi (1944, 1946),

Wildman and Bonner (1947), Wildman et al., (1949), Singer et al., (1952) and Commoner, et al. (1952); Wildman and Bonner (1947) have also investigated the electrokinetic properties of the proteins from spinach cytoplasm. These investigations indicate that the proteins may be resolved into a major component which makes up 60 - 80°/o of the total protein, and one or more minor components. The major component was designated by Wildman and Bonner (1947) as"Fraction I", and the minor components were collectively designated as "Fraction II".

Campbell (1951) found, in an investigation which included ten species representing seven families of dicotyledonous plants, that the protein spectra of the various species examined were quite similar. In each case, a major component constituted 50°/o or more of the total protein, while from 1 - 6 minor components accounted for the remainder. The mobilities of the various components were found to vary between species and between preparations from the same species, but it was not determined whether this variation was due to differences in protein species, to artifacts of preparation, or to differences in the buffers and hydrogen-ion concentrations used for electrophoresis.

The electrophoretic behavior of whole plant cytoplasm observed during the present work was generally similar to that described by earlier workers. Typical scanning diagrams obtained with cytoplasms of Turkish tobacco and of pea are illustrated in Figure 2. In the case of pea, both ascending (asc) and descending (desc) limbs are shown after 120 minutes migration. The descending

pattern clearly shows a major component, Fraction I, and four minor components. With tobacco cytoplasm, on the other hand, only two minor components are resolved. The mobility of the major component in tobacco cytoplasm was - 5.25 x 10^{-5} $cm.^2$ sec^{-1} $volt^{-1}$ in 0.1 ionic strength K-maleate buffer, pH 7.0. Data are not available for computation of the mobility of Fraction I in pea, but Campbell (1951, p. 28) reported the mobility to be -3.9 x 10^{-5} $cm.^2$ sec^{-1} $volt^{-1}$ in the same buffer as employed for the experiments here.

Ultracentrifugal Analyses

In a preliminary investigation of the ultracentrifugal behavior of the cytoplasmic proteins of tobacco, Singer and Wildman (unpublished) found that the preparations were resolved into two components; one with a sedimentation constant of 18 - 19S which constituted approximately 30 - 50% of the total refractive increment measured on the photographic record, and a second, much more slowly sedimenting, heterogeneous component which could not be completely resolved from the meniscus and which had no definite sedimentation constant.

The heterogeniety of the low-molecular weight fraction is not unexpected, for the cytoplasm contains many enzymes whose molecular weights vary from 13,000 to 200,000 or higher. Of more significance was the demonstration that as in electrophoresis, an apparently homogeneous component constituted a large part of the total protein. To determine whether the presence of such a large

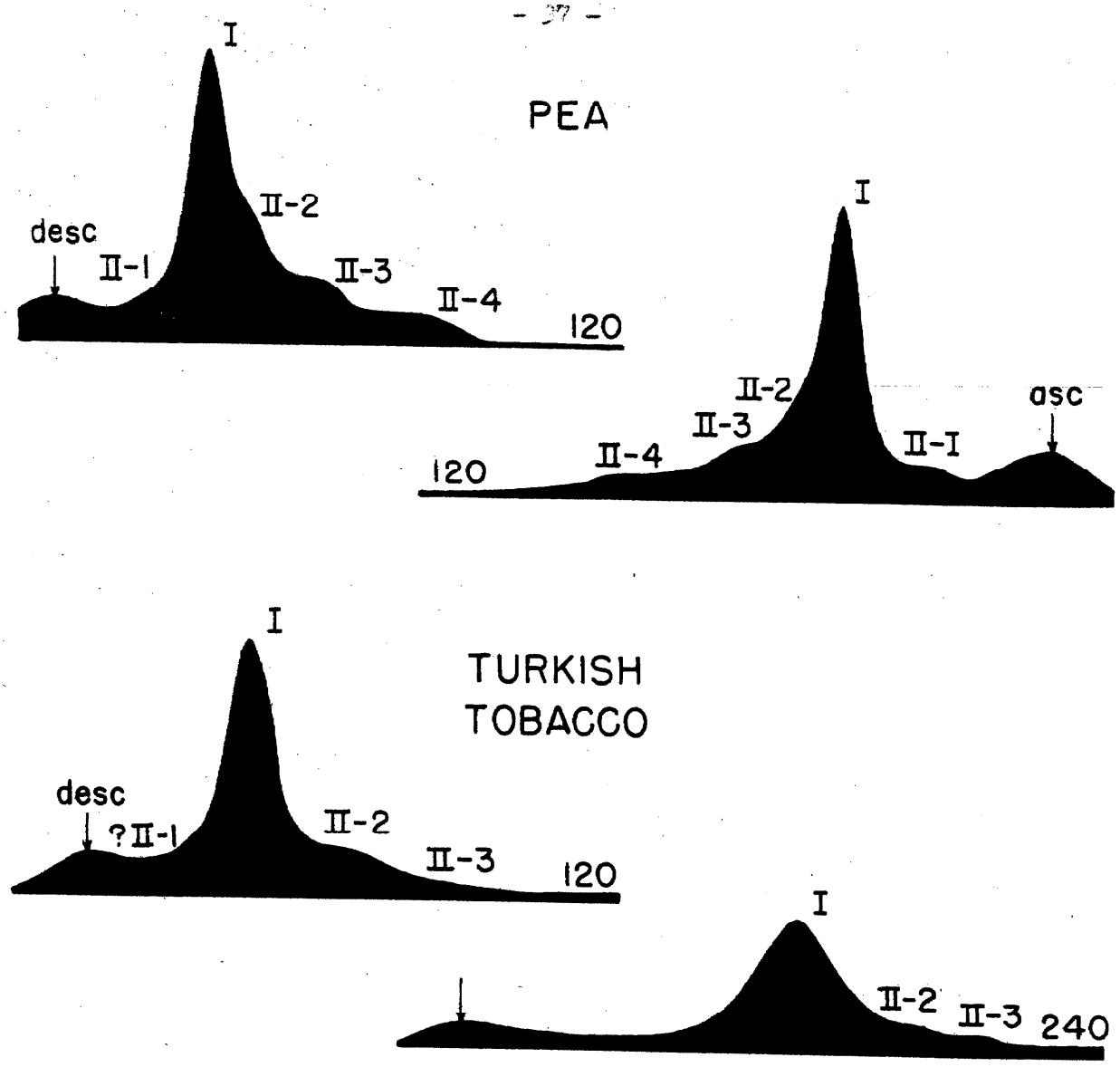


Figure 2. Representative electrophoretic diagrams of the cytoplasmic proteins of pea and of N. tabacum (var. Turkish). Analyses in 0.1 ionic strength K-maleate buffer, pH 7.0, Pea: ascending (asc) and descending (desc) limbs after 120 minutes migration at a potential gradient of 4.26 volts/cm. Turkish tobacco: descending limb only after 120 and 240 minutes migration at a potential gradient of 3.67 volts/cm. Protein concentration: 0.71°/o for pea, 0.55°/o for tobacco.

Fraction I (I) and the several minor components making up Fraction II (II) are shown.

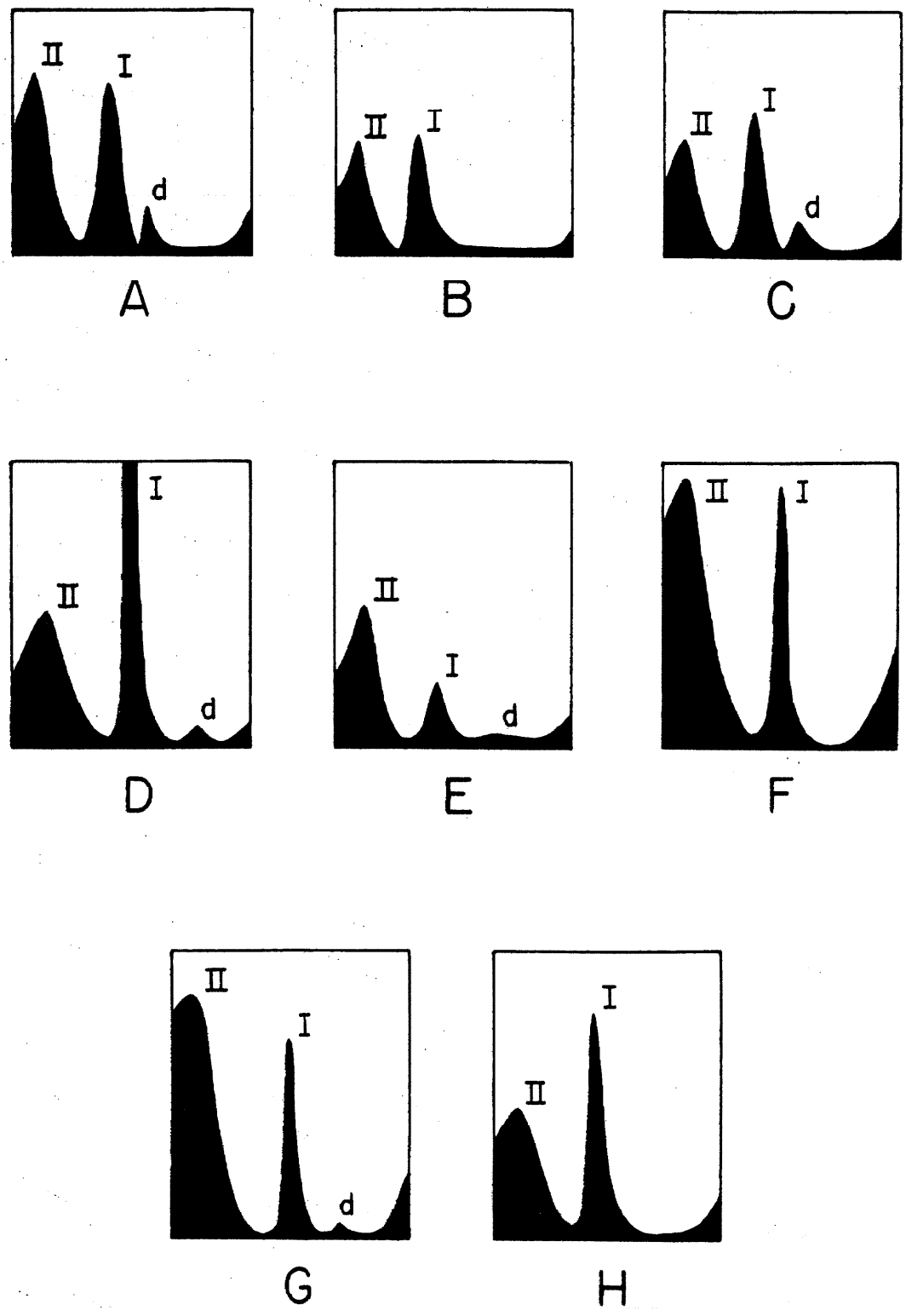
amount of a high-molecular weight protein was common to dicotyledonous species in general, a survey of representative species was made, with the assistance of Dr. J.M. Campbell and Dr. S.J. Singer.

Eight species (tomato, N. glutinosa, N. tabacum, vars. Cuba White, Maryland Mammoth and Turkish, gherkin, Xanthium sp., Spinacia sp., pea and sugar beet), representing five families were examined. The protein spectra of these several species were found to be strikingly similar, as is illustrated by the representative diagrams in Figure 3. Two peaks, analogous to the slowly-sedimenting, heterogeneous fraction and the homogeneous 18 - 19S component of tobacco, were found in all species. And as in tobacco, the 18 - 19S protein constitutes a large proportion of the total protein. The relative amount of this component in the cytoplasmic protein preparations from a number of species examined is given in Table III.

A small quantity of a third, more rapidly sedimenting component was found in four of the species examined. This fast component, with a sedimentation constant of 25 - 28S, is not a species or variety characteristic, for although it was never found in some species, such as spinach, it was only irregularly present in Turkish and Maryland Mammoth tobacco preparations. It is believed on the basis of work with Maryland Mammoth tobacco and with spinach that the 25 - 28S component results from dimerization of the 19S component and should be included in quantitative estimations of the latter.

Figure 3. Representative analytical ultracentrifuge patterns of the cytoplasmic proteins from various green leaves. A, L. esculentum (tomato); B, N. glutinosa; C, N. tabacum var. Turkish; D, C. anguria (gherkin); E, Xanthium sp.; F, Spinacia sp. (spinach); G, N. tabacum var. Cuba white; H, P. sativum (pea). The concentrations of proteins are in the range 0.7 to 2.0 per cent. These diagrams would typically be obtained about 1200 seconds after the rotor speed of 850 r.p.s. was attained. Sedimentation proceeds to the right. "d" is the 25 - 28S component, a dimer of the 18 - 19S component, I. II is the low-molecular weight heterogeneous component.

Figure 3



A single protein component thus predominates in both electrophoretic and ultracentrifuge analyses of cytoplasm from a variety of species. Although the proportion of the major component as determined by UC analysis is much less than that found by electrophoretic analysis, it was nevertheless tempting to assume during the early phases of the present work that the same protein is concerned. The term "Fraction I", was, therefore, carried over from the electrophoretic investigations as an alternate designation for the 19S component. It was soon shown by fractionation studies, however, that although the 19S protein constitutes the major portion of the principal electrophoretic component of cytoplasm, the latter is a mixture of proteins with closely similar charges rather than a single protein species. Hence the term "Fraction I" as originally defined on the basis of ammonium sulfate solubility and electrophoretic behavior (Wildman and Bonner, 1947) is not synonymous with "19S component". Accordingly, the term "Fraction I" has been replaced by the term "Fraction I protein" and more rigorously defined as the nucleoprotein component of cytoplasm having a corrected sedimentation constant of 18 - 19S. This definition is in accordance with current concepts and usage (Singer, et al. 1952; Eggman, et al. 1953).

It is apparent that ultracentrifugal analysis is the only method (other than those dependent on biological specificity) now available which permits assay of a single protein species in the complex cytoplasmic mixture. This method of assay is possible

TABLE III

CONTENT OF 19S COMPONENT IN CYTOPLASMIC EXTRACTS OF GREEN LEAVES

Plant	Per cent of Refractive Increment
N. tabacum var. Turkish	39*
N. tabacum var. Cuba White	30
N. glutinosa	40
Tomato	33
Spinach	28
Pea	43
Gherkin	50
Xanthium sp.	23

* Average of 20 independent experiments.

only because of the unique properties of the 19S component -- its high molecular weight and density relative to those of the remaining cytoplasmic proteins, and because it constitutes such a large part of the total proteins.

Use of the ultracentrifuge as an analytical tool has made possible the development of a fractionation procedure for isolation of the high-molecular weight component of cytoplasm. In conjunction with chemical analyses, use of the UC has also made it possible to determine, for the first time, whether the several factors which are concerned with instability of the cytoplasmic proteins predominantly affect the high- or low-molecular weight fractions.

The investigations regarding stability of the proteins in cytoplasmic extracts will next be considered.

E. Stability of Cytoplasmic Proteins in Cytoplasmic Extracts

Cytoplasmic protein preparations were found to undergo a variety of chemical and physical changes during storage. This instability is manifested in changes in the amount and chemical composition of the proteins and in changes which cause opalescence, turbidity, precipitation or sedimentation in a lower centrifugal field. Chemical changes result from enzymatic degradation, physical changes from aggregation or denaturation.

The primary factors which affect the stability of the cytoplasmic proteins are the same as those which affect the stability of most proteins, to wit, temperature, pH and salt-concentration.

Temperature changes exert a two-fold effect. An increase in temperature increases the rate of denaturation of the proteins by non-enzymatic mechanisms as well as the rate of enzymatic alteration of the extract. The predominant effect of pH and of salt-concentration is upon solubility of the proteins, although pH also has a marked effect upon the enzymatic activity of the extracts. The effects of these factors are, of course, qualitatively the same for all proteins. Among the cytoplasmic proteins, however, sharp quantatitive differences exist between the 19S component and the low-molecular weight fraction.

The factors which affect stability are all interrelated to some extent and it is frequently difficult to control all variables except the one under study. Consequently, all possible permutations of the important factors have not been examined. The material extracted by grinding leaf tissue in M/2 maleate buffer, pH 7.0-7.5, was accepted as "standard" and the studies were limited to determination of the influence on this material of: (a) pH, (b) temperature, (c) salt-concentration and dialysis and (d) storage at -20° C. Effort was made to determine the "useful life" of extracts prepared in this manner, and how best to preserve extracts in their initial state. Stability towards neutral salt solutions of high concentration will be considered in connection with fractionation studies reported in Section I-A, Part II; the influence of the other factors is discussed in detail in this section.

pH Stability of Cytoplasm

Even slightly acid conditions produce quite marked effects on the cytoplasmic proteins. Many of the proteins have an isoelectric point in the region of pH 5.5 to 6.5. A sample maintained at pH 6.6 may not display immediate visible changes, but in such a sample opalescence develops much more quickly at 0° C. than in a similar sample maintained at pH 7.0. Within the isoelectric range, denaturation of the proteins occurs concurrently with or prior to precipitation, and once precipitation has occurred, the protein cannot be redissolved on either the acid side of the isoelectric point or in a neutral buffer. The extent of this isoelectric precipitation is illustrated by the following experiment:

A sample of Turkish tobacco whole cytoplasm at pH 6.75 was dialyzed against 0.1 μ K-maleate buffer at pH 6.7 for 12 hours to reduce the salt-concentration and buffer strength, then centrifuged at 20,000 g. for one hour to remove any traces of particulate matter and aggregated protein. Seven 20.0 ml. aliquots of this dialyzed cytoplasm, containing 12.85 mg. protein/ml., were measured into sections of cellulose Visking tubing and dialyzed, with external agitation, against two 1,000 ml. volumes of 0.1 μ Na acetate-acetic acid buffers at pH's of 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 and 0.1 μ K-maleate buffer at pH 6.7. After 24 hours dialysis, the aliquot at pH 6.7 was completely clear while that at pH 6.0 was very cloudy. At all

other pH's, a heavy, well packed precipitate had formed. The contents of the dialysis bags were recovered in toto and centrifuged at 20,000 x g. for 30 minutes. The volume and pH of each supernatant solution was measured and appropriate aliquots precipitated with TCA for protein determinations. The hard-packed pellets were recovered and dried to constant weight. Total recovery of protein amounted to 94 - 98°/o. The amount of protein remaining in solution, computed on the basis of total protein recovered, was found to range from 100°/o at pH 6.7 to 8 or 9°/o at pH 3.5 to 4.5, as shown in Table IV. (The pH values cited are the final pH's of the cytoplasms, rather than those of the buffers.)

To determine whether a particular component of cytoplasm was preferentially denatured at acid pH's, the residual soluble proteins prepared in an experiment similar to that above were examined in the ultracentrifuge. Turkish tobacco cytoplasm was first dialyzed against 0.1 μ K-maleate buffer at pH 7.0 and 10.0 ml. aliquots were then further dialyzed for 24 hours against 0.1 μ buffers at pH 6.0, 6.9 (K-maleate) and 8.5 (Na-barbital). A copious precipitate formed in the sample at pH 6.0. At pH's 6.9 and 8.5, however, the solutions remained clear, indicating inherent stability of the proteins in this pH range. All three samples were centrifuged at 20,000 x g. for one hour to remove precipitated or aggregated protein and the supernatant solutions examined in the ultracentrifuge. The patterns obtained are reproduced in Figure 4. Since the solutions were comparable on

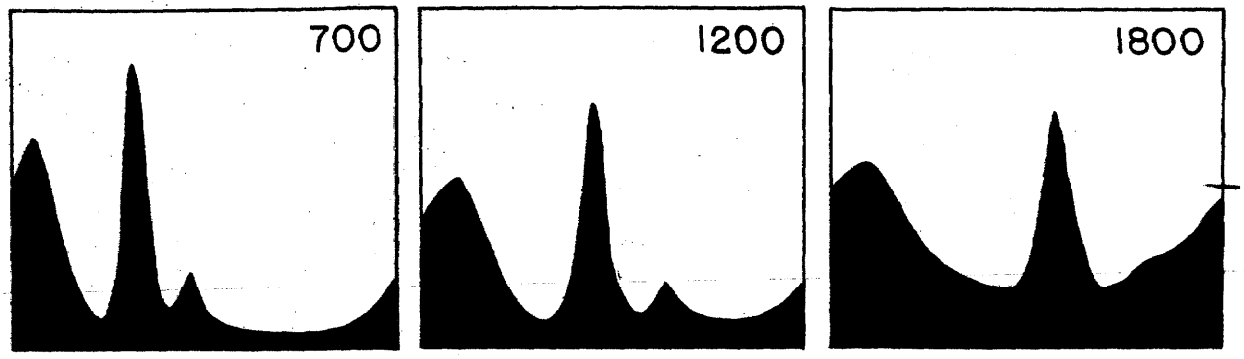
TABLE IV

PER CENT OF TOTAL CYTOPLASMIC PROTEIN REMAINING IN SOLUTION AFTER 24 HOURS DIALYSIS AT SEVERAL pH's

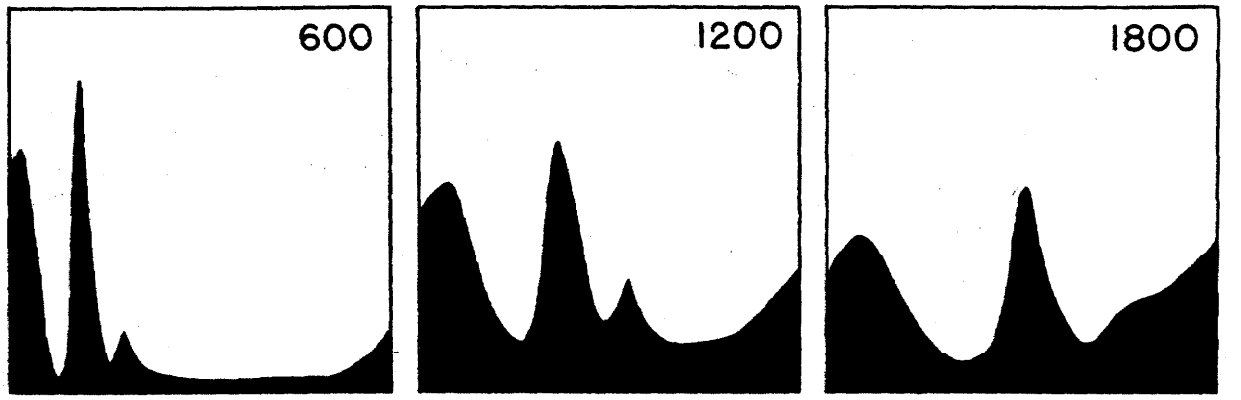
pH of cytoplasm*	Per cent of protein remaining in solution
6.7	100
6.2	59
5.6	30
5.1	14
4.6	9
4.1	8
3.6	9

* pH established by equilibrium dialysis of cytoplasmic protein solutions initially at pH 6.7. See text for buffers used. All pH's measured at 25° C. Dialysis performed at 2° C.

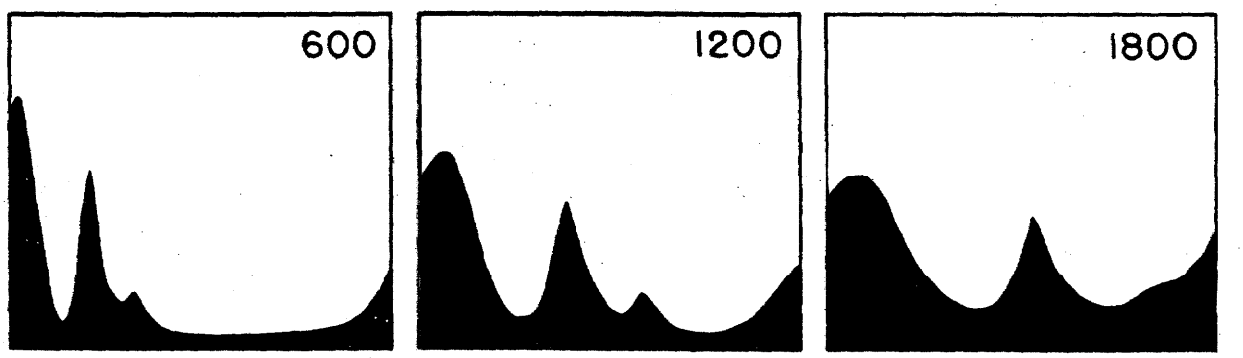
Figure 4. Analytical ultracentrifuge patterns showing the effect of pH on the composition of Turkish tobacco cytoplasmic proteins. The time in seconds after reaching 850 r.p.s. is indicated on each diagram. Sedimentation proceeds to the right.



pH=8.5



pH=6.9



pH=6.0

Figure 4

a volume basis, loss of area under the dn/dx, x curves represents a corresponding loss of protein material represented by the respective peaks. These experiments were performed before a technique for accurate area measurement had been perfected, so quantitative estimations of the concentration of the several components are not available. It is evident, however, from inspection of the photographic records that the area under the low-molecular weight peak is very nearly the same in each instance, and that the area under the Fraction I protein peak is the same at pH 6.9 and 8.5, where no precipitation occurred, but is considerably diminished at pH 6.0. This indicates that the 19S component is more susceptible to acid denaturation than are the low-molecular weight constituents of cytoplasm. The true relative susceptibility can be determined only by accurate estimation of the concentration of both components.

Influence of Temperature upon Stability of the Cytoplasmic Extract

Influence of Temperature upon Denaturation. The effects of temperature and of pH upon the rate of denaturation are interrelated. The present studies have been limited to observations made at 0°, 30° or 37° C. on extracts at pH 6.8 to 7.0.

Whole cytoplasm solutions at pH 6.8 to 7.0 do not exhibit visible changes suggestive of a change in protein solubility during 10 to 20 days storage in an ice bath. This behavior at 0° is in

marked contract to the behavior at 30° or 37° C. At 30° C., whole cytoplasm solutions usually show no visible change during two hours incubation, or during subsequent storage for several days at 0° C. If incubation is continued for six hours, opalescence develops and protein precipitates from solution during subsequent storage at 0° C. A slight opalescence sometimes forms during two hours incubation at 37° C., but after six hours, marked turbidity develops and 20 - 35% of the total protein becomes sedimentable at 20,000 x g. for 30 minutes. Denaturation, then, is greatly accelerated by a 7° rise in temperature. To avoid the complications of denaturation, incubation periods no greater than two hours at 30° or 37° C. were used for the enzymatic degradation studies discussed below.

Physical chemical studies designed to determine whether Fraction I protein is preferentially denatured under the influence of heat as well as by acid pH's were inconclusive.

Influence of Temperature upon Enzymatic Degradation of Cytoplasmic Extracts. Phosphatases, ribonuclease, several dehydrogenases, invertase, catalase, hexokinase, IAA oxidase, peroxidase and pectin methyl esterase have been demonstrated to be present in cytoplasmic extracts. Proteolytic enzymes may also be presumed to be present. Of all the chemical changes which might be expected to result from enzymatic activity in cytoplasmic extracts during incubation at elevated temperature (or more slowly at 0°), loss of TCA-P was the only change associated with the proteins which

could be readily detected analytically. The changes in TCA precipitable weight or TCA-N content which occurred during a reasonable experimental period were small and insignificant. Loss of TCA-P, however, is marked and occurs independently of denaturation. Hence, loss of TCA-P may be used as an index of the effectiveness of procedures designed to suppress general enzymatic activity.

To determine the mechanism of TCA-P loss, the TCA-P content of whole cytoplasm was followed as a function of time and temperature and of analytical procedures employed. These investigations demonstrated conclusively that the loss of TCA-P is real, not an artifact of analysis, and that the temperature dependence is similar to that expected of an enzymatic reaction. The experimental basis for these conclusions is summarized below.

A number of whole cytoplasm samples of widely different protein concentrations and TCA-P content were studied. Typical results are illustrated by the data of Table V. Aliquots for total-P and TCA-precipitable weight were taken before incubation and again after the desired incubation periods (generally two hours) at 30° or 37° C. Control analyses, from aliquots kept at 0°, were also analyzed until they were proved to be unnecessary. Such controls showed no detectable loss of TCA-precipitable weight (beyond the $\pm$ 3% accuracy of the determination) in 48 - 72 hours at 0°. The loss of TCA-P in two hours at 0° was of the order of 2 - 3%. From total-P analyses, which should be the same in all aliquots of

a given sample, the precision of analysis was found to be ± 1°/o, so the significance of the 2 - 3°/o loss of TCA-P, though reproducible, is doubtful.

Control aliquots were precipitated at 0° with cold TCA and incubated aliquots were precipitated at 30° or 37° with TCA at the same temperature, then cooled and stored at 0° for 18 - 24 hours before they were analyzed. To show that the observed difference in TCA-P content between control and incubated aliquots results from a difference in protein-bound P rather than from variation in the amount of P co-precipitated with the protein at the different temperatures, the following experiment was performed:

Aliquots of whole cytoplasm which had been incubated at 30° for two hours were: (a) precipitated at 30° with TCA at 30°, then kept at 30° for four hours; (b) precipitated as in (a), then immediately cooled and kept at 0° for four hours; (c) cooled to 0°, precipitated with cold TCA and kept at 0° for four hours; (d) precipitated as in (c), then kept at 30° for four hours. The TCA-P contents of (b) and (c) were identical; (a) and (d) were also identical but, were as would be expected from the solubility of TCA-P in TCA (see Appendix I), somewhat lower than that of (b) and (c). Thus, the temperature at which the initial precipitation is carried out is not of importance and the observed differences in TCA-P content between incubated and control samples are not artifacts of analysis.

The temperature dependence of TCA-P loss indicates that

TABLE V

THE EFFECT OF TEMPERATURE ON THE LOSS OF TCA-P CONTAINED IN WHOLE CYTOPLASM

Preparation No.	γTCA-P/ml. solution		Incubation conditions	Per cent Loss
	initial solution	after incubation		
V-51-70	80.4	76.0	2 hr.-0° C.	5
	80.4	41.9	96 hr.-0° C.	48
	80.4	26.6	2 hr.-30° C.	67
V-51-70 (after dialysis)	26.2	11.7	2 hr.-30° C.	55
E-6	46.6	27.8	2 hr.-37° C.	40
E-11	30.1	18.1	2 hr.-37° C.	40
	30.1	12.0	6 hr.-37° C.	60

the loss is probably enzymatic. At 0°, however, the loss of TCA-P was many-fold greater than loss of total protein, as measured by either TCA-N or TCA-precipitable weight. Therefore, the enzymatic mechanism which destroys TCA-P is much more active than are other enzymatic mechanisms at 0°. Since it was desired to study the TCA-P containing protein more extensively, some method was sought for controlling the loss of TCA-P other than by means of low temperature. Several attemps to determine the form of binding of the P which is released during loss of TCA-P were unsuccessful because of (a) the large amount of non-TCA-precipitable organic-P already present in the extract and (b) the intense phosphatase activity of the extract. It seemed possible that loss of TCA-P might involve concurrent loss of nucleotides from the protein and might thus be due to ribonuclease action. The possibility that loss of apparent TCA-P might be due to phosphatase activity alone was not excluded, however, since this loss approached a final limit, leaving a "TCA-P residue" resistant to further hydrolysis. Efforts to differentiate between these two possibilities by use of inhibitors were unsuccessful. Phosphatase inhibitors (zinc, arsenate and fluoride ions) denatured the proteins even at concentrations which caused only 10 - 20% inhibition of phosphatase activity.

Semi-quantitative chromatographic experiments revealed that loss of TCA-P and loss of nucleotides occur simultaneously. This favors the view that TCA-P loss is the result of ribonuclease activity. Since no good nuclease inhibitors are known, further

attempts to control enzymatic loss of TCA-P other than by means of low temperature were abandoned.

Effects of Dialysis

Use of physical methods of analysis requires dialysis against buffer solutions to establish a solvent of known characteristics. In view of the lability of the cytoplasmic proteins, the effects of dialysis were next investigated. It was desired to establish, if possible, the minimum ionic strength necessary to achieve maximum solubility of the proteins and to determine the effect of dialysis upon TCA-P content.

Loss of Protein During Water Dialysis. To determine whether the presence of salt is required for solubility of the cytoplasmic proteins, an aliquot of Cuba White tobacco cytoplasm was dialyzed against several changes of a large volume of distilled water adjusted to pH 7.0. A marked turbidity, followed by copious precipitation developed after approximately 3 - 4 hours. The contents of the dialysis bag were recovered *in toto* after 48 hours dialysis and separated into water-soluble and water-insoluble fractions by centrifugation. Recovery of total protein, on a TCA-precipitable weight basis, was 99°/o of the initial input; 40°/o as water-insoluble and 60°/o as water-soluble protein. The water-insoluble fraction failed to redissolve in 0.1 M buffer solutions at a neutral pH. The ratio of soluble:insoluble protein obtained by water dialysis is proportional to the protein concentration, as

is shown in Table VI. The significance of this concentration relationship is unknown. Solutions of cytoplasmic proteins dialyzed against 0.1 μ buffer at pH 7.0 were found to remain completely clear at 0° for 5 - 6 days after dialysis whereas similar solutions dialyzed against 0.05 μ buffer developed marked turbidity in 2 - 3 days. Hence, the cytoplasmic proteins require a salt-concentration greater than 0.05 μ for maximum solubility. In practice, a 0.1 μ (0.1 M for mono-valent ions) buffer was always used.

Loss of TCA-P During Dialysis Against 0.1 μ Buffer Solutions. The loss of TCA-P from several different cytoplasmic extracts during dialysis against 0.1 μ Na-maleate buffer for 24 hours is shown in Table VII. These data are expressed as γ TCA-P/mg. protein to correct for the dilution which occurred during dialysis. Control analyses on undialyzed samples were not performed, However, from a comparison of loss of TCA-P during storage at 0° [48% in 96 hours (Sample V-51-70, Table V)] with the loss during dialysis at 2° C [67.5% in 24 hours (Sample V-51-70, Table VII)], it is evident that TCA-P loss is greatly increased by dialysis. The loss is less than that caused by incubation at 30°. The cause of TCA-P loss during dialysis in unknown, but it may be suggested that the loss is enzymatic, and that the rate is increased by removal of hydrolysis products so the system does not approach equilibrium as closely as do extracts examined after storage at 0°.

Accurate physical analyses require that samples be dialyzed before analysis. It is unfortunate that this is necessary because changes in the composition of the extract during dialysis interject an element of doubt as to the relationship of the product

TABLE VI

FRACTIONATION OF CUBA WHITE TOBACCO CYTOPLASM BY DIALYSIS AGAINST DISTILLED WATER AT 0° C.

Protein conc.	protein recovered	% of recovered as		insoluble / soluble
		insoluble	soluble	
mg./ml.	per cent			
2.75	97.4	25.8	74.2	0.35
5.53	102	29.0	71.0	0.41
10.70	97.8	34.1	65.9	0.52
13.75	99.9	37.6	62.4	0.60

TABLE VII

LOSS OF TCA-P FROM WHOLE CYTOPLASM DURING 24 HOURS DIALYSIS AGAINST 0.1 μ BUFFERS

Sample	Buffer	Initial TCA wt.	TCA-P/mg. TCA wt.		Loss
			before dial.	after dial.	
		mg./ml.	γ	γ	per cent
V-51-70	Na-maleate pH 7.0	12.52	6.41	2.36	67.5
E-12	Na-maleate pH 7.6	13.64	8.38	3.92	53.2
E-13	Na-maleate pH 7.6	7.80	3.72	1.77	52.4

characterized to the material initially extracted from the cytoplasm. The effect of the loss of TCA-P during dialysis or storage at 0° C. upon the physical properties of the protein to which the TCA-P is attached are discussed in Part II. The procedures adopted for the characterization studies reported here are a compromise. Further work to define an area in which the requirements for physical analyses are compatible with stability of the protein mixture is highly desirable.

Effect of Storage at -20° C.

The final aspect of protein stability for which data were obtained concerns changes which occur in cytoplasm during storage at -20° C. This problem is of importance because after it was discovered that cytoplasmic extracts were not stable as solutions at 0° C., it became common practice in this laboratory to grind the tissue as it became available, filter off the cellular debris, quick-freeze the protoplasm and store it in a deep-freeze at -20° C. for subsequent use. The validity of this procedure was based on a report by Singer, et al.(1952) that they could find no qualitative differences in the UC patterns of unfrozen cytoplasmic extracts and extracts which had been frozen for 3 - 10 days. The results reported here cover much longer periods of storage.

Evidence for Denaturation During Storage. No changes in the character of the particulate residue were observed provided frozen protoplasm (stored at -20° C.) was worked up within about

15 to 20 days. It was noticed, however, that if solutions of cytoplasmic proteins were quick-frozen and stored under similar conditions for the same period, the solution was frequently opalescent when thawed and that some of the protein was susceptible to sedimentation at the relatively low centrifugal forces used to separate the particulate matter from the cytoplasm. Thus, it appears that either the particulate matter in the protoplasm prevents denaturation of the cytoplasmic proteins or, more probably, denaturation occurs but the denatured protein is not recognized in the mass of particulate, green residue. That the latter explanation is correct is apparent from observation of protoplasm stored for 30 - 45 days before separation of the particulate matter. During centrifugation of such samples, a light layer of brown protein is deposited on top of the particulate matter. The amount of protein deposited and the length of storage time tolerated before denaturation becomes evident is dependent upon pH. At pH 6.75 - 6.9, very little denatured protein is apparent even after 60 days storage, whereas considerable protein is deposited by a force of 50,000 x g. for 45 minutes from protoplasm stored for only 20 - 30 days at pH 6.4 - 6.6.

Chemical Changes Which Occur During Storage. Data to illustrate the extent of denaturation during storage have been obtained from analyses performed during the course of other experiments. The data are presented here not only to show that the phenomenon exists but also because of the interesting TCA-P

relationships they reveal. Analyses of four different samples of Maryland Mammoth tobacco are given in Table VIII. In each instance, initial determinations were performed either on unfrozen material or on material frozen only 24 to 72 hours. The cytoplasmic proteins were in all cases prepared by centrifugation of the protoplasm at 25,000 r.p.m. for 60 minutes in the no. 30 Spinco rotor. Results expressed on a volume basis are, therefore, comparable for a given sample. Centrifugation was carried out as quickly as possible after the stored samples were thawed and aliquots for analysis were taken immediately, to minimize emzymatic loss of TCA-P.

The data of Table VIII indicate that during storage substantial quantities of proteins are converted to a form which may be removed by a centrifugation regime which failed to remove the same protein prior to storage. The extent of this denaturation is roughly proportional to the time of storage. Also, it may be seen that, within experimental error, the TCA-P content on a volume basis has remained constant while the protein content decreased. This results in a rather large apparent increase in TCA-P/mg. protein for all of the solutions during storage.

Ultracentrifugal Examination of Whole Cytoplasm Before and After Extended Storage at -20° C. Since TCA-P is not lost during prolonged storage of cytoplasmic extracts at -20° C. and since all of the TCA-P in cytoplasm is initially attached to the Fraction I protein component (see Section II, Part II), one might expect Fraction I protein to be stable during storage. Ultra-

TABLE VIII

EFFECT OF STORAGE AT -20° C. UPON WHOLE CYTOPLASM EXTRACTS

Sample No.	Initial Analysis			Analysis after Storage			Change in			Days Stored
	TCA wt.	TCA-P		TCA wt.	TCA-P		TCA wt.	TCA-P		
								Vol. basis	wt. basis	
	mg./ml.	γ/ml.	γ/mg.wt.	mg./ml.	γ/ml.	γ/mg.wt.	°/c	°/o	°/o	
E-6	8.60	26.2	3.05	5.50	26.1	4.75	-36.	0	+55.0	335
E-11	10.40	40.1	3.86	7.80	39.7	5.09	-25.	-1.0	+32.0	255
E-12	13.65	102	7.47	12.65	103.	8.15	- 7.3	+1.0	+ 9.0	48
E-13	7.80	31.7	4.06	7.66	32.4	4.23	- 1.8	+2.2	+ 4.0	48

centrifugal examination of the four samples listed in Table VIII and of a fifth one, E-8, revealed, however, that the 19S component is denatured during storage. The pattern of behavior was qualitatively similar in all instances. The data obtained from examination of E-6 and E-8 may be considered as typical.

In order to avoid changes due to dialysis and dilution, initial and final analyses were performed on the cytoplasmic protein solutions directly in the buffer used for grinding the leaves. Changes in the initial and final UC patterns are, therefore, due solely to changes which occur during storage. A precise determination of the relative rates of denaturation of Fraction I protein and the low-molecular weight fraction requires knowledge of the concentration of one or both of these components in the initial and final solutions. This information is not available because, as indicated earlier, these experiments were performed before a technique for accurate measurement of the area under the peaks was perfected. It may be seen, however, from plates presented in Figures 5 and 6 that although there is no qualitative difference as a result of storage, a significant reduction in the area under the Fraction I protein peak occurs. It even appears probable from Figure 5 that the decrease in Fraction I protein is relatively much greater than the decrease in the low-molecular weight group of proteins. If this is true, it emphasizes again the great lability of the 19S component of cytoplasm.

The significant result of these studies is the demonstration that substantial quantities of Fraction I protein are lost during

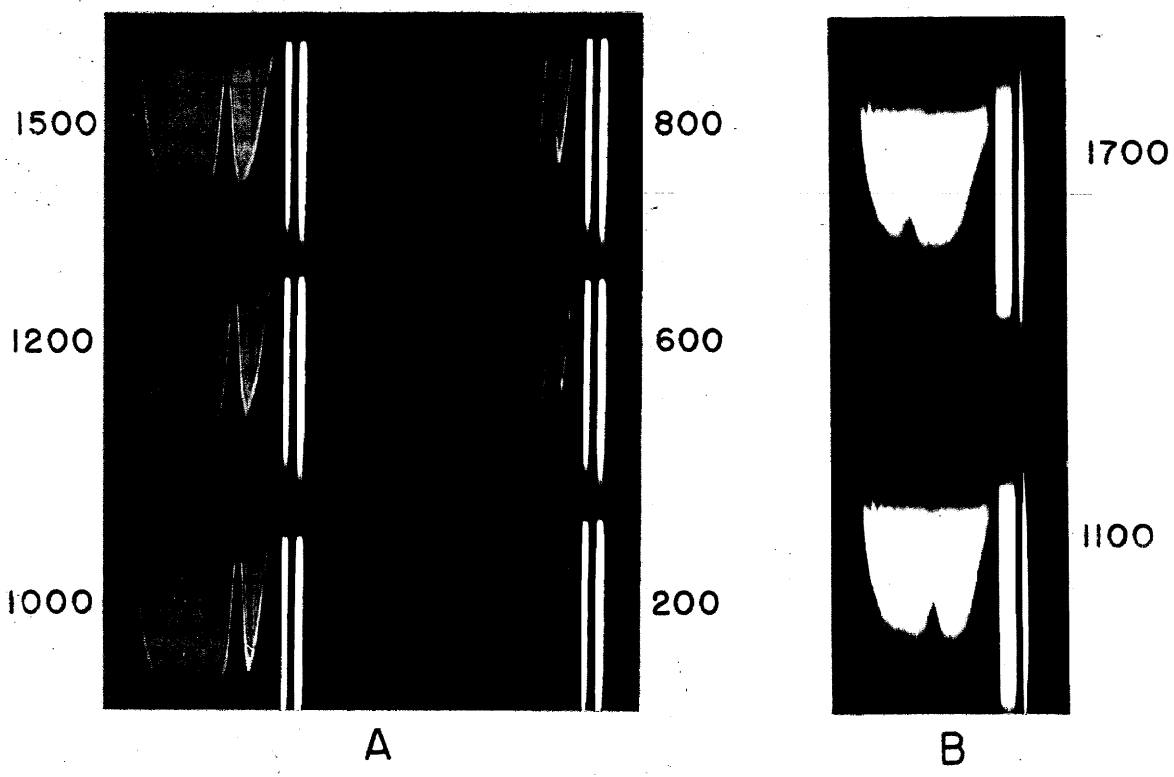


Figure 5. Contact prints of photographic records obtained during UC analysis of Turkish tobacco cytoplasm (E-6) before storage (A) and after 309 days storage at -20° C. (B). Sedimentation proceeds to the left. Time in seconds after attaining a rotor speed of 850 r.p.s. is indicated in the margin by each picture. Samples were run as prepared in grinding buffer without prior dialysis or dilution; therefore the diagrams are directly comparable. Initial concentration, 8.6 mg./ml.; concentration after storage, 5.5 mg./ml.

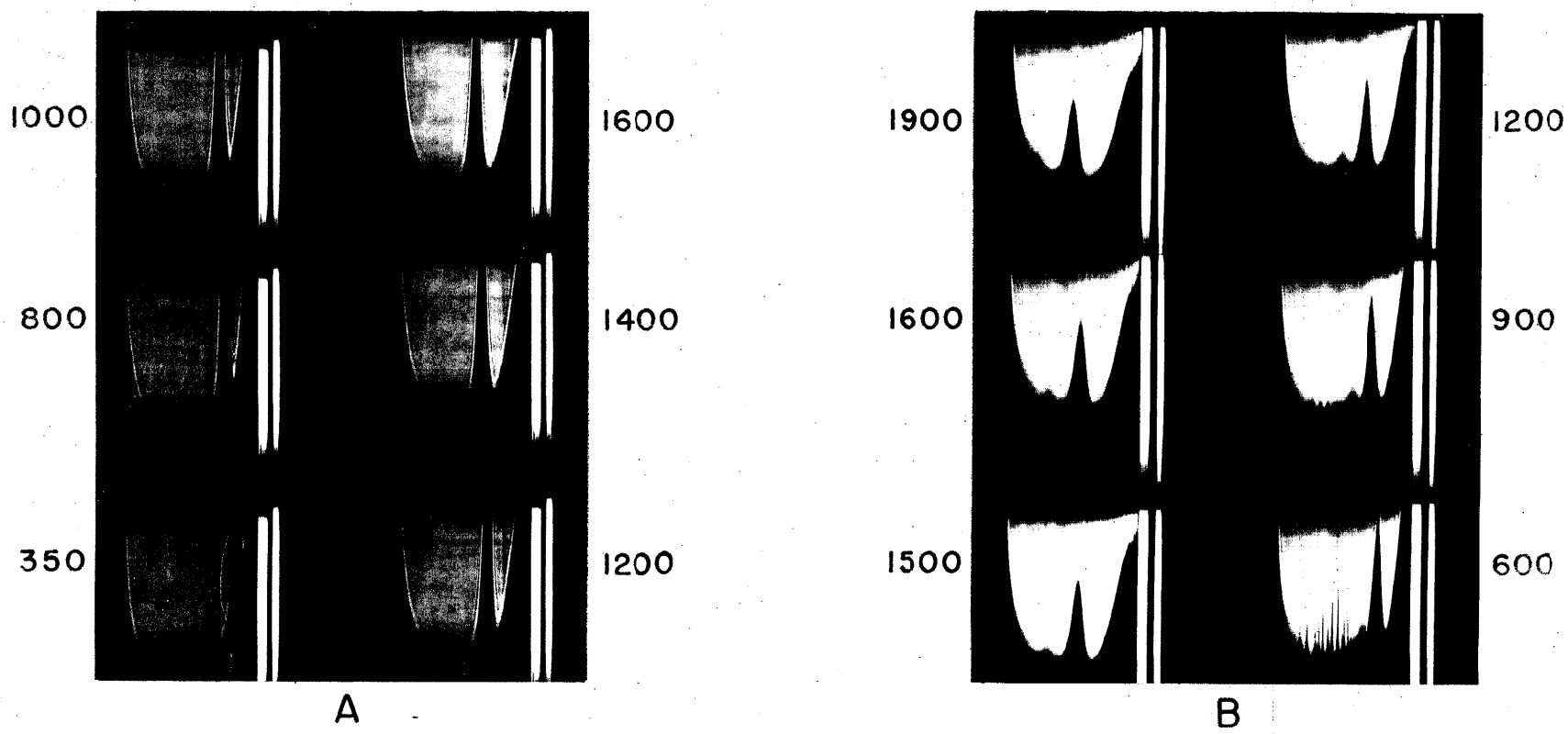


Figure 6. Contact prints of photographic records obtained during UC analysis of Turkish tobacco cytoplasm (E-8) before storage (A) and after 184 days storage at -20° C. (B). Sedimentation proceeds to the left. Time in seconds after attaining a rotor speed of 850 r.p.s. is indicated in the margin by each picture. Samples were run as in Figure 5. Initial concentration, 10.9 mg./ml. concentration after storage, 9.6 mg./ml.

storage even though the TCA-P attached to it is not lost.

Discussion. Any explanation for the apparent increase in TCA-P/mg. protein during storage must be consistent with the following facts:

(1) The amount of TCA-P/ml. solution remains constant during storage but the total protein content of the solutions decreases.

(2) Substantial quantities of the 19S component as well as the low-molecular weight proteins, are denatured.

(3) The TCA-P, in the form of ribonucleic acid, is initially attached to the 19S component.

Thus, any hypothesis must allow both for non-specific denaturation of protein material and for firm binding of TCA-P to undenatured protein.

Although several hypotheses are possible within the framework of these facts, the following one is believed to be the most probable:

During storage, the TCA-P is split from that portion of Fraction I protein which is denatured. Thus, no TCA-P becomes insoluble or remains attached to protein which becomes insoluble. When the solution is thawed, free RNA becomes reattached to protein to form a new, firm protein-RNA bond. This postulated reattachment might involve only the residual, undenatured portion of the 19S component or it might involve other, quite different proteins. The data at hand do not distinguish between these two alternatives.

Discussion of Factors Affecting Stability

The cytoplasmic proteins are readily denatured by heat, by slightly acid pH's, and by storage at -20° C. They are also autolyzed by enzymes present in the extract. No method of entirely preventing these changes has been discovered. Cytoplasmic extracts cannot be stored at 0° for more than a few days at the most, and freezing, though considerably better than storage at 0°, also causes deleterious effects. The only known manner in which these difficulties can be circumvented is to use fresh material in so far as possible for all critical studies.

The importance of controlling enzymatic activity and, particularily, pH cannot be emphasized too strongly. It may be suggested that the failure of earlier investigators to detect the 19S component in cytoplasm, in spite of the fact that it constitutes a large proportion of the total soluble protein, may be due to the acid lability of the protein moiety itself as well as to enzymatic destruction of the TCA-P moiety during extraction. All investigators other than those associated with the Wildman-Bonner group have either used cytolyzing agents, which cause protein denaturation, or have failed to control the pH of the extracted material. It is well established (cf. Chibnall, 1939) that the pH of many cytoplasmic extracts prepared by expressing the sap may vary from pH 6.5 to 5.5 or lower. The data of Table IV and Figure 4 show that exposure to these pH's causes precipitation

of a large part of the cytoplasmic proteins and a preferential denaturation of the Fraction I protein.

It is believed that sufficient knowledge of the cytoplasmic proteins in general has been gained to permit independent biochemical studies of the particulate fraction, the high-molecular weight fraction and the low-molecular weight fraction. Suppression of the enzymatic destruction of RNA, however, is a serious problem that must be overcome before any trustworthy turnover studies of the protein and RNA components of Fraction I protein may be performed. Furthermore, enzymatic loss of TCA-P during dialysis, which is variable, depending on the pH of the buffer used, the enzymatic activity of the extract and the temperature and duration of dialysis, casts doubt upon the validity of certain types of physical analyses, such as electrophoresis. This is so because of the heavy contribution to the charge of the molecule that is made by the phosphate groups of RNA. It is quite possible, for instance, that part of the variation in mobility of the cytoplasmic components reported among plant species and among different preparations of the same species may actually be an artifact caused by use of partially degraded material. The release of TCA-P from the protein also creates difficulties in the isolation of the 19S component and in assay for RNA in both cytoplasmic extracts and in Fraction I protein preparations. In these two instances, methods of minimizing the loss of TCA-P have been developed. No method of decreasing the loss of TCA-P during dialysis has been discovered.

IV. IDENTIFICATION AND COMPOSITION OF A CYTOPLASMIC RIBONUCLEIC ACID

The presence of a TCA-precipitable, phosphorus-containing moiety in cytoplasm, and the ease with which this moiety can be separated from protein by warm 1.0 N acid was discussed in Section III-C above. Wildman and Bonner had previously noted the presence of a material with a similar behavior in spinach cytoplasm. They identified pentose, adenine and phosphate in hydrolysates of a protein fraction which was rich in TCA-P (Wildman and Bonner, 1947; Wildman, et al. 1949) and, on the basis of these incomplete analyses and the rate of release of inorganic phosphate during hydrolysis, suggested that the TCA-P moiety might be a ribonucleic acid. During the present investigation, it has been proven rigorously that the soluble TCA-P found in both spinach and in tobacco cytoplasm is in the form of ribonucleic acid. The composition of the RNA has been established in several instances and the protein to which the RNA is firmly attached has been identified and characterized with respect to its physical chemical properties.

A. Identification of the TCA-P Moiety

The TCA-P containing moiety was extracted from the protein and identified in the following manner:

Aliquots of Turkish tobacco cytoplasm were precipitated and washed as described for TCA-P determinations in Section II-E. After the final wash, approximately 0.5 ml. of 1.0 N HCl was added to the wet precipitate in the centrifuge tube. The tube was sealed

and the contents hydrolyzed in a boiling water bath for 60 minutes. Following hydrolysis, the cooled hydrolysate, which was light yellow in color due to formation of humin from protein during hydrolysis, was spotted on Whatman no. 1 filter paper and the hydrolysis products resolved according to the paper chromatographic method of Smith and Markhan (1950), using a tertiary-butanol-HCl-water solvent system. The developed chromatograms were dried at room temperature and examined under an ultraviolet lamp ("Mineralite", Model V-41, Ultraviolet Products, Inc., South Pasadena, Calif.) in order to locate compounds which absorb in the ultraviolet region. Four ultraviolet absorbing spots, one also possessing a light blue fluorescence, were present. These were tentatively identified from their R_f values as guanine, adenine, cytidylic acid and uridylic acid. Positive identification of these compounds was made by (a) co-chromatography with similar hydrolysates of highly purified yeast RNA and with authentic samples of the individual compounds, and (b) by elution of the separated compounds from sections of the chromatograms in 1.0 N HCl, followed by comparison of the entire absorption spectrum of each compound with the spectrum of an authentic sample. These comparisons were made in a Cary Recording Spectrophotometer*. A yellow, non-UV-absorbing spot which remained at the origin was also present on these chromatograms. This was presumably from

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* Use of this instrument was made possible through the courtesy of Dr. Norman Simmons at the AEC project, Univ. of Calif. at Los Angeles.

the humin formed during hydrolysis. A reducing sugar with an R_f midway between that of cytidylic and uridylic acids was detected by spraying chromatograms similar to those discussed above with 4% benzidine in glacial acetic acid. This sugar was identified as a pentose by means of the orcinol reaction and later identified as ribose by co-chromatography with authentic samples of ribose in two different solvent systems.

The presence of inorganic phosphate (R_f 0.90 - 0.95) was demonstrated by spraying developed chromatograms with the Hanes-Isherwood spray reagent (Hanes and Isherwood, 1949). Following differential color development according to the method of Bandurski and Axelrod (1951), two additional phosphate spots with R_f values identical to those of cytidylic acid and uridylic acid were found.

Since no phosphate spot with an R_f identical to that of ribose was found, the ribose was present as free ribose and not as ribose bound in a nucleotide or phosphate linkage. The presence of ribose in the nucleotides could not be demonstrated with the benzidine reagent used, probably because insufficient water to hydrolyze the purine and phosphate linkages is present in the reagent.

All of the low-molecular weight, nucleotide-containing compounds are removed during precipitation and washing of the protein; hence, the presence of cytidylic and uridylic acids and of adenylic and guanylic acids (as the free bases, ribose and inorganic phosphate) in the hydrolysate, rigorously establishes, for the first time, the

presence of a polynucleotide -- ribonucleic acid -- in the TCA precipitate. That all of the TCA-P can be accounted for as RNA was shown by (a) failure to find more than trace amounts of P in the washed protein residue following dissociation of the TCA-P protein bond in 1.0 N HCl at 90° C. for 5 minutes, and (b) demonstration that all of the TCA-P initially present can be accounted for as nucleotide-P on the assumption of one mole of P for each mole of purine or pyrimidine base in the eluate of the protein residue described in (a). This latter point will be further discussed in the next section. Inasmuch as no free ribonucleic acid has been demonstrated in cytoplasm by independent physical methods, the ribonucleic acid must be present in whole cytoplasm as a nucleoprotein. Further evidence on this point will be presented in Part II.

B. Composition of the Cytoplasmic Ribonucleic Acid

To establish the quantity of each nucleotide component in the hydrolsate, appropriate areas of developed chromatograms containing the resolved components were cut out and eluted in 4.0 ml. of 1.0 N HCl for 16 - 20 hours at room temperature. The background contribution of the filter paper was determined by similar treatment of corresponding blanks cut from adjacent strips of the chromatogram. Generally, three samples and three blanks were run on the same chromatogram. Because of variation in the paper, it was necessary to run blanks for every chromatogram. The optical density of the eluates at an appropriate wave length was determined in a Beckman Model DU

spectrophotometer and the amount of nucleotide in each eluate computed from the molar extinction coefficients derived by Markham and Smith (1951):

guanine	$\varepsilon_{250} = 11.0 \times 10^3$
adenine	$\varepsilon_{260} = 13.0 \times 10^3$
cytidylic acid	$\varepsilon_{280} = 12.3 \times 10^3$
uridylic acid	$\varepsilon_{260} = 9.45 \times 10^3$

These extinction coefficients were derived with 0.1 N HCl as the solvent but were also found to be correct within the accuracy of the Beckman spectrophotometer for 1.0 N HCl as the solvent. 1.0 N HCl was chosen for eluting the nucleotides rather than 0.1 N acid because preliminary experiments indicated more complete elution of guanine in the stronger acid.

Several modifications of the procedure prior to the hydrolysis step were found to be necessary for quantitative work. First, the RNA must be removed from the protein prior to hydrolysis. This is necessary because the amino acids which would otherwise be formed from partial hydrolysis of the protein interfere with estimation of the nucleotides. Secondly, in order to determine the total quantity of nucleotide in a precipitate, it is necessary to know the exact volume of hot acid used to extract the RNA from the protein. To do this, the precipitate must be dried <u>in vacuo</u>. Drying, however, causes the precipitated protein to shrivel and become similar to sponge-rubber in texture. This texture makes removal of all the TCA-P and hydrolysate from the protein mass difficult. To circumvent this difficulty, it is necessary to hydrolyze a known aliquot

of the acid extract of the protein and to determine the amount of P in the remainder of the extraction acid and in the protein residue. From these values, the total recovery in terms of P may be estimated. It is also assumed that the composition of the portion of the RNA which is eluted from the protein is representative of the whole. Because of the difficulties which attend drying the precipitate prior to extraction of TCA-P, the drying step may be advantageously omitted if only relative amounts of each nucleotide are desired.

Quantitative nucleotide analysis has been applied to the following specific points:

(1) Demonstration that all the TCA-P in a protein precipitate may be accounted for as nucleotide-P on the assumption of one mole of P for each mole of purine or pyrimidine base.

(2) Comparison of the nucleotide composition of the cytoplasmic RNA'S from mature leaves of several plant species, and of leaves of the same species but of different physiological ages.

How much of the TCA-P in Cytoplasm is Ribonucleic Acid? In a typical experiment to determine how much of the TCA-P may be accounted for as nucleotide-P, four aliquots of spinach whole cytoplasm, each containing 5.91 μ M (183 γ) TCA-P were precipitated and washed as for TCA-P determinations. Two of the washed precipitates were suspended in 1.30 ml. of 1.0 N HCl and heated to 90° C. for five minutes to extract the TCA-P from the protein. The mixtures were immediately cooled to 0° C. to prevent further degradation, then centrifuged briefly at 2° C.

and the supernatant extracts decanted. The extracted protein residues were washed three times with 2.0 ml. portions of ice-cold 1.0 N HCl and analyzed for total-P. Of the 183 γ of TCA-P present initially, only traces (< 2 γ) of P were found in the residue after extraction with hot acid. Therefore, since all of the TCA-P is labile in hot acid, none of the TCA-P is present as a phosphoprotein.

The two remaining precipitates were dried *in vacuo* for 48 hours and the RNA extracted in 1.30 ml. of 1.0 N HCl, as above. The extracted, sponge-like protein residues were washed as completely as possible and analyzed for total-P. An average of 33.6 γ of total-P per residue was found. 1.00 ml. aliquots of the 1.30 ml. portions of acid used to extract the TCA-P from the protein were sealed into tubes and hydrolyzed for one hour at 100° C. Six aliquots from each hydrolysate were chromatographed, the resolved components eluted in 4.0 ml. of 1.0 N HCl, and the optical densities of the eluates determined, as described above.

From the optical densities, the extinction coefficients, the volume of hydrolysate chromatographed and the volume of acid used to extract the TCA-P from the protein, it was calculated that 1.99 μ M of pyrimidine nucleotides and 2.72 μ M of free purine bases were extracted from the protein precipitate. The free bases would then account for a further 2.72 μ M of P if it is assumed that they are initially associated with ribose and phosphorus in nucleotides. The total recovery of P, 1.08 μ M in the extracted residue and 4.71 μ M in the hydrolysate, was 98°/o. Similar observations and

recoveries have been made with several samples of Maryland Mammoth tobacco cytoplasm. The evidence, therefore, indicates that within the limits of analytical errors, all of the TCA-P present in whole cytoplasm may be accounted for as a component of ribonucleic acid.

Composition of the Cytoplasmic Ribonucleic Acid. The relative compositions of the RNA from samples of spinach, Maryland Mammoth tobacco and Turkish tobacco cytoplasms have been determined. Undried precipitates were always used for composition studies because of their convenience*. Analyses were made of the RNA's from cytoplasmic preparations of (a) mature spinach leaves, (b) very young Maryland Mammoth tobacco leaves (<2" long), and (c) mature leaves from the same plants as (b). In these three cases, aliquots of cytoplasm were precipitated with ice-cold TCA within two hours of the time extraction of the protoplasm was commenced. Throughout the period of extraction and centrifugation, the temperature of the extract did not exceed 2 - 3° C. The precipitated protein was centrifuged and washed about an hour after precipitation. In this manner, enzymatic degradation of TCA-P and loss of TCA-P due to solubility in TCA was reduced to a minimum. The samples should be, therefore, as nearly representative as can be obtained with the procedures and knowledge presently available.

The compositions of the RNA's extracted from these samples are summarized in Table IX. These values are the averages

* This is justified because it was found that the relative amounts of each hydrolysis product in the hot acid extract of dried and undried protein precipitates are the same.

TABLE IX

COMPOSITION OF RNA EXTRACTED FROM WHOLE CYTOPLASM OF SPINACH AND MARYLAND MAMMOTH TOBACCO LEAVES

	Plant Species		
	Spinach	Maryland Mammoth Tobacco	
	Mature Leaves	Immature Leaves	Mature Leaves
A. μM TCA-P/mg. protein	0.194	0.367	0.200
B. Component	μM in an arbitrary aliquot		
guanine	1.55	3.32	0.79
adenine	1.11	3.05	0.71
total purines	2.66	6.37	1.50
cytidylic acid	1.16	2.18	0.50
uridylic acid	0.92	1.86	0.44
total pyrimidines	2.08	4.04	0.94
C. Ratio of			
$\frac{\text{guanine}}{\text{adenine}}$	1.40	1.09	1.11
$\frac{\text{cytidylic acid}}{\text{uridylic acid}}$	1.26	1.17	1.14
$\frac{\text{total purines}}{\text{total pyrimidines}}$	1.28	1.58	1.59

obtained from chromatography of six aliquots from each of two hydrolysates of each sample. In general, the amount of any component in an aliquot agreed to within ± 3°/o of the average amount of the same component found in all of the aliquots. A replicate series of analyses, beginning with the initial TCA precipitation, produced ratios within 2°/o of those in Table IX-B. Thus, the values cited are considered to be quantitatively reliable.

C. Discussion

The analyses reported in section C of Table IX, indicate that the RNA's extracted from young and from mature leaves of tobacco are quite similar in composition, but that the RNA of tobacco has a different composition than that of spinach. To determine the validity and accuracy of these results, it is necessary to examine closely the difficulties attendant to such analyses.

There are two distinct problems involved in determination of the components of nucleic acid. First, the composition of the sample must be representative of the nucleic acid in the native state. Secondly, the method of hydrolysis must bring about adequate cleaveage of the polynucleotide without partial destruction or interconversion of any of the components.

Three rather different techinques are now in use for degrading pentose nucleic acids (PNA) to monobasic units. Markham and Smith (Smith and Markham, 1950; Markham and Smith, 1951) advocate use of 1.0 N HCl (100° C. for 60 minutes) to quantitatively degrade

polynucleotides to the free purine bases and pyrimidine nucleotides. Abrams (1951), however, has shown by isotope dilution experiments that 7 - 8% of the purine bases are destroyed by this hydrolysis procedure, probably by deamination to the corresponding hydroxypurines. Markham and Smith have performed rather extensive recovery studies to verify their hydrolysis procedure without finding any evidence for such loss of purines, nor has the presence of hydroxypurines been noted during the present studies. Markham and Smith have found, however, that about 5% of the pyrimidine nucleotides are hydrolyzed under the conditions employed. The presence of a small amount of both cytosine and uracil has been noted on a number of the chromatograms made during the current studies, and appropriate corrections (routinely, 5%) have been applied. To avoid the hydrolytic losses which occur in 1.0 N HCl, Marshak and Vogel (1951) propose the use of 12N perchloric acid (40 minutes at 100° C.) to cleave all nucleotides to the corresponding free bases. Davidson and Smellie (1952) report considerable loss of uracil under these conditions and propose instead, the use of 0.3 N KOH at 37° C. for 18 hours to degrade pentose polynucleotides to mononucleotides. The mononucleotides, which are indefinitely stable in 0.3 N KOH solution, are separated by ionophoresis, rather than by chromatography. These authors also found that if the concentration of KOH was increased to 1.0 N, about 10% of the cytidylic acid was deaminated to uridylic acid at 37° C., though not at room temperatures.

Thus, there is no agreement as to the most appropriate

hydrolytic procedure for cleavage of the polynucleotides. The method of Markham and Smith used during these studies is, however, certainly as good as any method presently available if the appropriate correction factors for the known, reproducible losses are applied. Extraction of PNA* from tissue of any kind presents two difficulties in connection with composition studies, aside from the separation of PNA and DNA. The first of these involves the possibility of different molecular species of PNA in different parts of the cell or in different tissues. There is some evidence, especially in the case of DNA, that this is not important, for the DNA's from all tissues of a species appear to have a constant composition, as have the PNA's from the subcellular particulate entities (Davidson and Smellie, 1952). The second, and momentarily much more important, problem is the probable partial degradation of all nucleic acids by nucleases during extraction from the tissue. Bacher and Allen (1950), for instance, have shown that the high guanine content which has always been associated with pancreas RNA is due in part to degradation by ribonuclease during extraction of RNA from the tissue and that if the ribonuclease is extracted prior to isolation of the RNA, the relative guanine content of the RNA is much lower than otherwise found.

The full significance of such an enzymatic degradation in the work reported here is not certain. It has been shown that TCA-P, in the form of nucleotides, is lost slowly at 0° C. but more rapidly at elevated temperatures. To minimize this loss, material

*The terminology PNA (pentose nucleic acid) is used in a generic sense or where the pentose has not been conclusively shown to be ribose.

intended for nucleic acid composition studies was always worked up as rapidly as possible, taking the utmost precautions to maintain a low temperature (< 2 - 3° C.) during all operations. For the analyses reported in Table IX, the cytoplasm from which the RNA was extracted was precipitated with TCA within two hours after commencement of extraction. It is well established that nucleotides released by RNA'ase are predominately pyrimidine nucleotides. If a maximum loss of 5°/o of the TCA-P initially present occurs during extraction, and if this is entirely due to loss of pyrimidine nucleotides, it would account for 10°/o apparent excess of purine residues, at the most, in the RNA subjected to analysis. Actually the loss of pyrimidine residues prior to precipitation of the nucleoprotein for extraction of RNA is believed to be much less than this.

The RNA which has been extracted from the cytoplasmic nucleoprotein may therefore be considered to be representative of the native nucleic acid in composition. Furthermore, since there seems to be but one nucleoprotein in the soluble cytoplasmic proteins, the extracted RNA probably represents but one molecular species of RNA. Since the hydrolytic procedure used to degrade the RNA and the chromatographic method used to separate the hydrolysis products are both among the best available today, the analyses obtained must be considered as representative and accurate.

For comparison purposes, the composition of RNA samples from a number of sources, together with the most reliable analyses

for two cytoplasmic RNA's, are presented in Table X. These analyses are believed to be the best available from an analytical point of view, though not necessarily from the standpoint of being representative of the PNA in the tissue from which they were extracted. All results have been recomputed to an arbitrary total of 40.0 μ M of total nucleotides. The analyses of three different yeast RNA's by three different methods of hydrolysis, shown in columns I, II, and III, serve to illustrate the diversity of composition of this material as commercially available. PNA's extracted from plant viruses are usually considered to be the most nearly representative of PNA in the native state because they are tightly bound to the virus protein and thus can be readily extracted, and also because it is presumed that there is only one molecular species associated with a given virus protein. Analyses of three RNA's from representative plant viruses are shown in Columns V, VI, and VII. Column IV contains the analysis of a RNA sample from rat liver. Although it is not certain how much enzymatic degradation of this sample may have occurred during preparation, the great excess of guanine and cytosine, and of total purine in this sample is remarkable -- and characteristic of a number of PNA's of animal origin.

In comparison with yeast RNA or PNA's of plant viruses, the RNA from spinach and Maryland Mammoth tobacco leaves show several distinct differences in composition. Spinach RNA contains a greater relative excess of guanine over adenine than any of the other plant RNA's. And, with the exception of the PNA from turnip yellow virus,

TABLE X

COMPOSITION OF REPRESENTATIVE PENTOSE NUCLEIC ACIDS

	I	II	III	IV	V	VI	VII	VIII	IX
Source	Yeast	Yeast	Yeast	Rat Liver	"type" tobacco mosaic virus	Turnip yellow virus	Tomato bushy stunt virus	Spinach cytoplasm	Maryland Mammoth tobacco cytoplasm
Reference*	1	2	3	1	2	4	4		
Hydrolytic method #	1	2	3	1	2	2	2	2	2
RELATIVE COMPOSITION* (μ M/40 μ M total nucleotides)									
Guanine	11.9	12.5	12.6	14.1	11.7	6.9	11.2	13.1	12.7
Adenine	9.6	10.3	11.2	8.1	12.4	9.1	9.9	9.4	11.7
Total purine	21.5	22.8	23.8	22.2	24.1	16.0	21.1	22.5	24.4
Cytidylic acid	8.5	8.0	7.9	11.2	6.2	15.3	8.7	9.8	8.4
Uridylic acid	10.0	9.3	8.2	6.5	9.6	8.9	10.1	7.8	7.2
Total pyrimidine	18.5	17.3	16.1	17.7	15.8	24.2	18.8	17.6	15.6
Adenine/Guanine	1.24	1.21	1.13	1.74	.94	.76	1.13	1.40	1.09
Cyti. A/Urid. A	.85	.86	.96	1.72	.65	1.72	.86	1.26	1.17
Total pur/Total pyr	1.16	1.32	1.47	1.25	1.53	.66	1.12	1.28	1.58

TABLE X (continued)

* References:

(1) Davidson, J.N. and Smellie, R.M.S., Biochem. J., 52, 594 (1952).

(2) Markham, R. and Smith, J.D., Biochem. J., 46, 513 (1950).

(3) Marshak, A. and Vogel, H.J., J. Biol. Chem., 189, 597 (1951).

(4) Markham, R. and Smith, J.D., Biochem. J., 49, 401 (1951).

Hydrolytic methods

(1) 0.3 N KOH for 18 hours at 37° C.

(2) 1.0 N HCl for 1 hour at 100° C.

(3) 12 N $HClO_4$ for 40 minutes at 100° C.

the RNA of both spinach and tobacco are the only ones which contain more cytidylic acid than uridylic acid. The total purine content of Maryland Mammoth tobacco RNA is also higher than found in any RNA other than that of "type TMV".

V. APPLICATION OF THE ANALYTICAL TECHNIQUES DEVELOPED TO A SPECIFIC PHYSIOLOGICAL PROBLEM

A specific problem bearing on the physiological role of Fraction I protein in the cell -- a study of the effect of physiological age upon the amount and composition of the cytoplasmic nucleoprotein component -- will be considered as an illustration of the application of the analytical techniques which have been developed.

A. Experimental and Results

Two samples of leaves were selected from a group of Maryland Mammoth tobacco plants grown in the Earhart Laboratory. One sample, E-12, was composed entirely of very small leaves (< 2" long) from the growing points. The other sample, E-13, was made up of an equal number of mature leaves (18 - 24" long) from the same plants. Both samples were ground (2 grams wet weight of leaves/ml. buffer) and the cytoplasms prepared as described previously.

Appropriate aliquots of the whole cytoplasm were analyzed for TCA-precipitable weight and TCA-P. Another aliquot of each cytoplasm was dialyzed against 0.1 μ K-maleate buffer pH 7.6. After 36 hours dialysis, both aliquots were diluted, with the same buffer as used for dialysis, to equal total nitrogen contents and subjected to UC analysis. The analytical data are presented in Table XI and the UC schlieren diagrams in Figure 7.

If it is assumed that the percentage of protein extracted was the same for the two samples, then the immature leaves contained

TABLE XI

CHEMICAL ANALYSES OF THE CYTOPLASMIC PROTEINS FROM YOUNG AND MATURE LEAVES OF MARYLAND MAMMOTH TOBACCO

	E-12 (young leaves)		E-13 (mature leaves)	
	Initial	After dialysis and dilution	Initial	After dialysis and dilution
TCA-precipitable weight (mg./ml.)	13.65	6.6	7.8	6.6
Total N (mg./ml.)		1.156		1.123
TCA-N (mg./ml.)	1.686	0.888	1.017	0.876
% N in protein	12.35	13.46	13.00	13.30
TCA-P (γ/mg. protein)	6.98	3.92	3.72	1.77

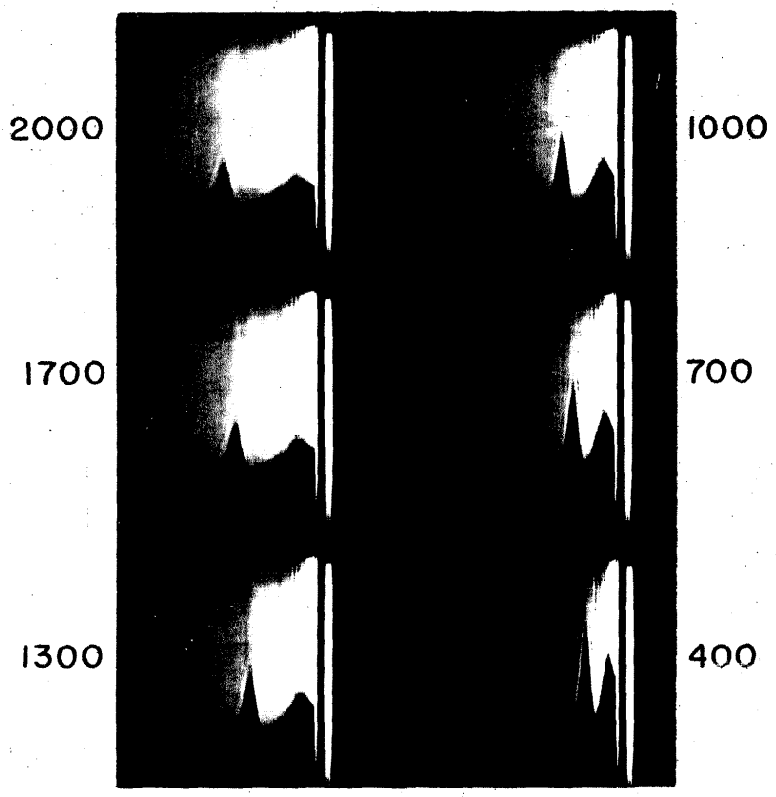


Figure 7. Contact prints of photographic records obtained during UC analysis of cytoplasm from young (A) and mature (B) leaves of Maryland Mammoth tobacco. Sedimentation proceeds to left. Time in seconds after attaining a rotor speed of 850 r.p.s.$^{15}_{4}$ indicated in margin by each picture. Samples were dialyzed against 0.1 μ K-maleate buffer, pH 7.6, prior to analysis. Concentration 6.6 mg. protein/ml. in both cases.

nearly twice as much protein on a weight basis as did the mature leaves. Furthermore, the young leaves contained nearly twice as much TCA-P/mg. protein as did the mature leaves. The protein and nitrogen contents of the dialyzed aliquots subjected to UC analysis were identical, hence the schlieren diagrams are directly comparable. In these diagrams, the poorly defined baseline, which makes accurate measurement of the areas under the peaks difficult, is clearly evident. It is apparent, however, that there is much less of the 19S component in the sample from immature leaves than in the sample from mature leaves. This fact, together with the greater RNA content/mg. protein found in immature leaves, indicates that although the relative amount of the nucleoprotein in these rapidly growing leaves is less than that in mature leaves, the RNA content of the nucleoprotein itself is much greater in growing leaves than in mature leaves. This is further evidence that the cytoplasmic nucleoprotein of plants has a variable RNA content rather than a definite RNA content, as do the plant viruses. The data of Table IX indicate, however, that although the amount of RNA associated with the protein may vary, the composition of the RNA from both immature and mature leaves of the same species is identical.

B. Discussion

Further investigation of the influence of environmental and physiological factors upon the 19S component of cytoplasm has not been possible, although it is conceivable that such studies

might help to elucidate the role of this major nucleoprotein component in the economy of the cell. One suggested role for nucleoproteins in the cell is in relation to protein synthesis. In growing yeast cells and in regenerating liver tissue, for instance, it has been found that rapid growth is accompanied by a relatively high content of RNA as compared to resting cells or to normal tissue. In many cases, cytoplasmic and nuclear RNA have not been separately determined, but where this has been done, the increase in cytoplasmic RNA, which has been interpreted to mean an increase in nucleoprotein, has always been much greater than that of nuclear RNA. It is this increase in cytoplasmic RNA during rapid growth which caused the suggestion of a possible role of nucleoprotein in the synthesis of new protein material [cf. Theorell (1947)], a view recently substantiated by Holloway and Ripley (1952), who demonstrated a close correlation between the RNA content of rabbit reticulocytes and incorporation of radioactive L-leucine into the total cellular proteins. In contrast, the cytoplasmic nucleoprotein content of rapidly expanding leaves, which are certainly synthesizing new material at a rapid rate even though the total number of cells is not increasing, is less, on either a relative or a total protein basis, than in mature leaves. What is the significance of this apparent contradiction? Is the cytoplasmic nucleoprotein of leaves concerned with protein synthesis, or is it concerned with some other, as yet unknown, function? Why is there so much more nucleoprotein

in mature leaves than in rapidly growing leaves? Much more work will be necessary before these, and all the other questions concerning the role of nucleoproteins in general, and of Fraction I protein in particular, can be answered. The various possible roles will be further discussed in Part III.

PART II

THE ISOLATION AND CHARACTERIZATION OF A HIGH-MOLECULAR WEIGHT CYTOPLASMIC NUCLEOPROTEIN

I. FRACTIONATION OF THE SOLUBLE CYTOPLASMIC PROTEINS

The classical methods for fractionation of protein mixtures have been fractional precipitation by high salt concentrations (ammonium or sodium sulfate), isoelectric precipitation and precipitation with large, positively charged molecules such as histones or nucleic acids. These methods have been successfully applied to mixtures containing only a few protein components. Difficulties are encountered, however, unless the protein species are of widely different characteristics. These classical methods have for example, achieved separation of the total globulin fraction from the albumin fraction of serum but have failed to achieve resolution of the closely related globulins. Dissociation and denaturation frequently attend use of these classical procedures.

Two fractionation techniques which minimize the extent of denaturation have been developed during the past ten years. Cohn and his group at Harvard have developed a method for fractionation of the blood serum complex by differential precipitation with cold alcohol (-8° to -20° C.) at precisely controlled pH's and ionic strengths. Cold acetone has been used in a similar manner. Though it is more complicated and time consuming than any of the other techniques now available, the electrophoresis-convection technique perfected by Kirkwood, et al. (Cann, et al., 1949; Cann and Kirkwood, 1950), is capable of achieving the most complete resolution of mixtures yet obtained. This technique, which utilizes a combination

of electrophoretic and convective transport to separate molecular species on the basis of their differences in mobility, requires that the proteins be soluble on both sides of the isoelectric point and that denaturation not occur concurrently with isoelectric precipitation. These requirements are necessary because the several components in a mixture are successively immobilized at the isoelectric point during the fractionation.

Certain of the fractionation methods described above are obviously unsuited for application to the cytoplasmic proteins of leaves. Electrophoresis-convection, for example, can not be used because these cytoplasmic proteins are unstable at their isoelectric points, and on the alkaline side of the isoelectric point the mobility distribution is too narrow to permit separation before autolysis of the extract becomes significant. Isoelectric precipitation is not feasible for similar reasons. Fractional precipitation with ammonium sulfate has therefore been utilized in most of the earlier work on leaf proteins. The application of this method will be discussed in detail in the following section. Fractionation by cold acetone or alcohol is undoubtedly feasible but because development of a suitable procedure would be laborious and require more running time on the ultracentrifuge than has been available, this possibility has not yet been investigated. Instead, effort was first centered on salt fractionation and, when this proved inadequate, upon physical methods of fractionation.

Ammonium Sulfate Fractionation

Historically, fractionation of the soluble cytoplasmic proteins, prepared in a manner acceptable to the writer as representative of the proteins as a whole, begins with the ammonium sulfate fractionations reported by Wildman and Bonner in 1947. Subsequent work has also been limited to the group associated with this laboratory. Extraction by the techniques commonly employed by other groups working in this general field (Takahashi; Commoner; Pirie) actually results in an initial unrecognized and undesirable fractionation due to conditions which cause denaturation during extraction. That this is the case is self-evident from the stability studies discussed in Section III E, Part I.

Wildman and Bonner (1947) separated the cytoplasmic proteins into two fractions, "Fraction I" and "Fraction II", insoluble and soluble, respectively in 0.35 saturated ammonium sulfate at pH 7.0. The precipitated fraction was readily and completely soluble in cold phosphate buffer at pH 6.8 or in veronal buffer at pH 8.5. When examined electrophoretically, this fraction appeared to be homogeneous in either buffer and to have the same mobility as the major component of cytoplasm. All the minor components of cytoplasm as well as a small amount of a protein component with the same electrophoretic mobility as the principal component, were present in Fraction II. Thus it appeared that by a simple salt fractionation, an electrophoretically homogeneous protein

had been separated from the complex mixture of cytoplasmic proteins, and that this homogeneous protein constituted the major portion, the "bulk protein" of cytoplasm. The term "Fraction I", therefore, was used by these authors to designate either the fraction of cytoplasm insoluble in 0.35 saturated ammonium sulfate or the principal component of cytoplasm as determined by electrophoresis.

Data obtained by Wildman and Bonner indicated that approximately 50 - 53°/o of the total protein of spinach cytoplasm was precipitated by 0.35 saturated ammonium sulfate, and that another 22 - 25°/o was precipitated by a series of three operations which involved concentration, water dialysis and electrodialysis. Each protein fraction precipitated by these operations was shown to be electrophoretically homogeneous and to have the same mobility as the fraction precipitated by 0.35 saturated salt. On this basis, all of these fractions were presumed to be identical. Thus, 75°/o of the total protein in whole cytoplasm was thought to be a single protein species, a conclusion in agreement with area measurements of the scanning patterns obtained by electrophoresis of whole cytoplasm preparations.

The techniques available to Wildman and Bonner at the time this fractionation was developed did not readily permit further testing of the homogeniety of Fraction I. But with the finding early in the present work that the only principal component of cytoplasm which was homogeneous in the ultracentrifuge comprised but 25 - 50°/o of the total protein in most instances (Table IX),

it became probable that Fraction I as prepared by salt precipitation might contain more than a single molecular species. That this is the case was shown by examination in the ultracentrifuge of a Turkish tobacco Fraction I sample prepared by precipitation with 0.38 saturated ammonium sulfate at pH 7.0 and re-solution in 0.1μ phosphate buffer of pH 6.8. This sample appeared to be electrophoretically homogeneous, but its UC pattern was similar to that of the initial whole cytoplasm from which it was prepared except for a slight enrichment of the 19S component. A similar fractionation at pH 8.5 produced substantially the same results, and repeated reprecipitation at either pH 7.0 or 8.5 failed to improve the separation. This finding serves to emphasize the importance of the ultracentrifuge as an analytical tool and the inadequacy of the salt fractionation procedure that had been used.

The possibility that a lower salt concentration, a concentration which, for instance, precipitated only 25 - 30°/o of the total protein, might preferentially precipitate the high-molecular weight, 19S component was therefore investigated. Since there was no information then available as to the amount of protein precipitated at various degrees of salt saturation, a complete series of fractionations from 25 to 50°/o saturation in steps of 5°/o was carried out as follows:

200 ml. of Turkish tobacco whole cytoplasm (sample V-51-75, pH 6.91 in K-maleate buffer) which had been quick-frozen and stored

at -20° C. for four days was thawed and centrifuged at 57,000 x g. for one hour. The supernatant solution (whole cytoplasm) was dialyzed at 2° C. for 18 hours against three, 1-liter changes of 0.1 μ Na-barbital buffer at pH 8.50. No detectable opalescence appeared during dialysis, and 30 minutes centrifugation at 50,000 x g. failed to produce a pellet. Six 20.0 ml. aliquots of this dialyzed cytoplasm, containing 11.65 mg. protein and 42.7% TCA-P per ml., were fractionated at 0° by dropwise addition of saturated ammonium sulfate at pH 8.5 (glass electrode at 10° C.) to 25, 30, 35, 40, 45 and 50% saturation. A volume of water such that the final volume of protein, salt and water mixture would be 40.0 ml. in each case was added prior to addition of the salt solution. The salt solution-protein mixtures were permitted to stand at 0° for one hour before the precipitated protein was removed by centrifugation (at 2° C.) at 20,000 x g. for 30 minutes. The precipitates were redissolved at 0° C. in 0.1μ Na-barbital buffer at pH 8.5. The precipitate formed at 25% saturation was dissolved in 10.0 ml. buffer, all others in 20.0 ml. Thus, in all cases except that at 25% saturation, each redissolved sample contained the protein precipitated from an equivalent volume of cytoplasm. The sample at 25% saturation contained twice the equivalent amount. Suitable aliquots were analyzed for TCA-P and TCA precipitable weight.

The results of these analyses (Table XI and Figure 8) indicate that no sharp separation of proteins occurred at any salt

TABLE XI

AMMONIUM SULFATE FRACTIONATION OF WHOLE CYTOPLASM AT pH 8.5

Salt concentration	Protein precipitated		TCA-P precipitated	
per cent of saturation at 0° C.	mg./ml.	per cent	γ/ml.	per cent
0	11.65	-	42.7	-
25	1.9	8	3.5	8
30	6.5	52	8.5	20
35	8.6	74	13.2	31
40	9.6	82	16.8	39
45	9.6	82	21.4	50
50	10.1	87	25.5	60

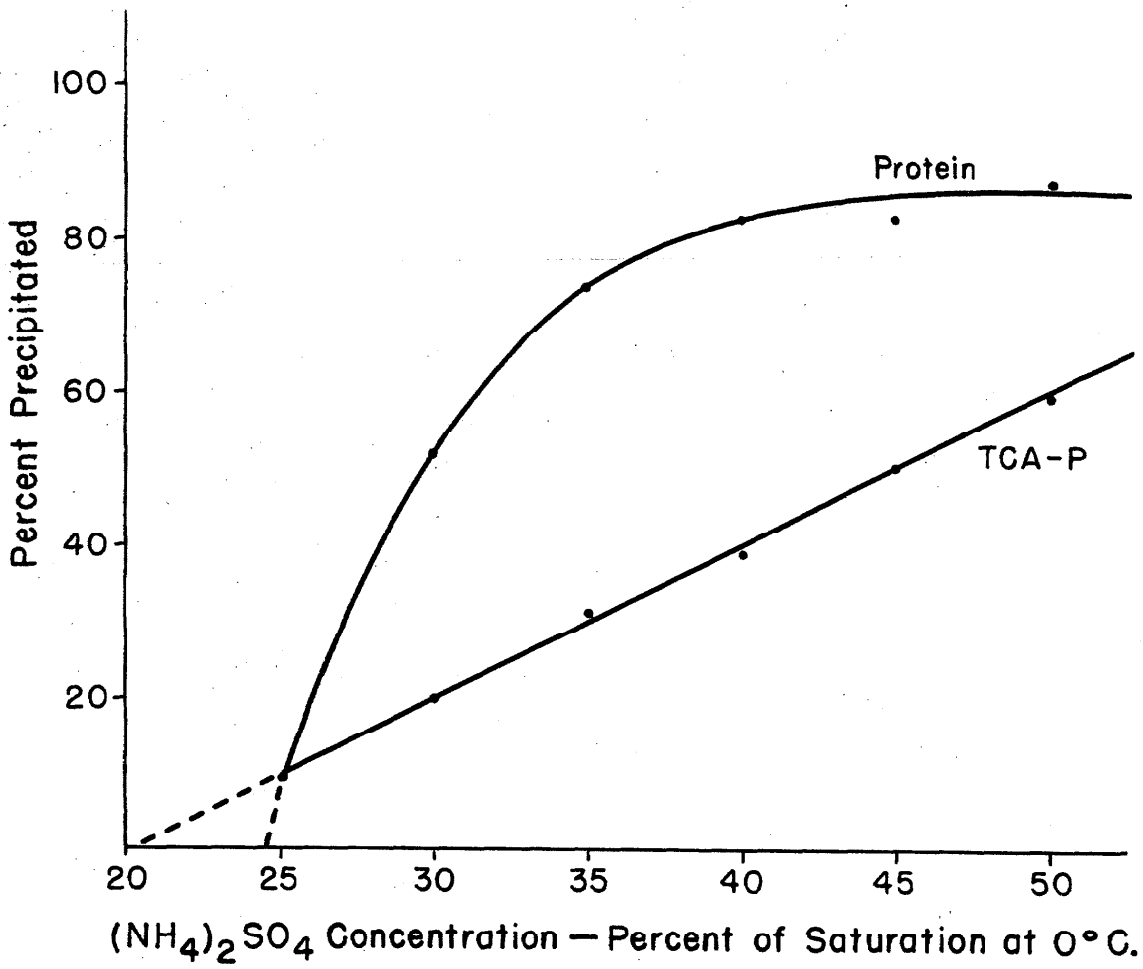


Figure 8. Percentage of initial amount of protein and TCA-P precipitated at pH 8.5 from a sample of Turkish tobacco cytoplasm by various concentrations of ammonium sulfate.

concentration. Furthermore, the linear increase in amount of TCA-P precipitated at increasing salt concentrations, in contrast to the asymptotic protein precipitation curve, implies that the TCA-P moiety may be split from the protein by the salt and independently precipitated. A linear increase in the amount of a single protein precipitated by increasing salt concentration over this range would be inconsistent with present knowledge concerning salting out of proteins.

Examination of several of the precipitated and supernatant fractions in the UC verified the non-specificity of salt precipitation. Typical patterns obtained with the initial whole cytoplasm, the fractions precipitated at 0.25, 0.30, and 0.40 saturation and the supernatant solution from 0.40 saturation are illustrated in Figure 9. It may be seen that although all of the 19S component is precipitated at 40°/o saturation, where only 18°/o of the total protein remains in solution (Figure 9E), the spectra of the proteins precipitated at both 25°/o (Figure 9B) and at 40°/o saturation (Figure 9D), where 8°/o and 82°/o, respectively, of the protein is precipitated, are qualitatively similar. This indicates that the 19S component is not preferentially precipitated at any salt concentration.

The concentration of ammonium sulfate required to precipitate all of the 19S component is not known since the only supernatant solution examined in the UC was the one from the 40°/o saturation precipitation. However, since the TCA-P is initially

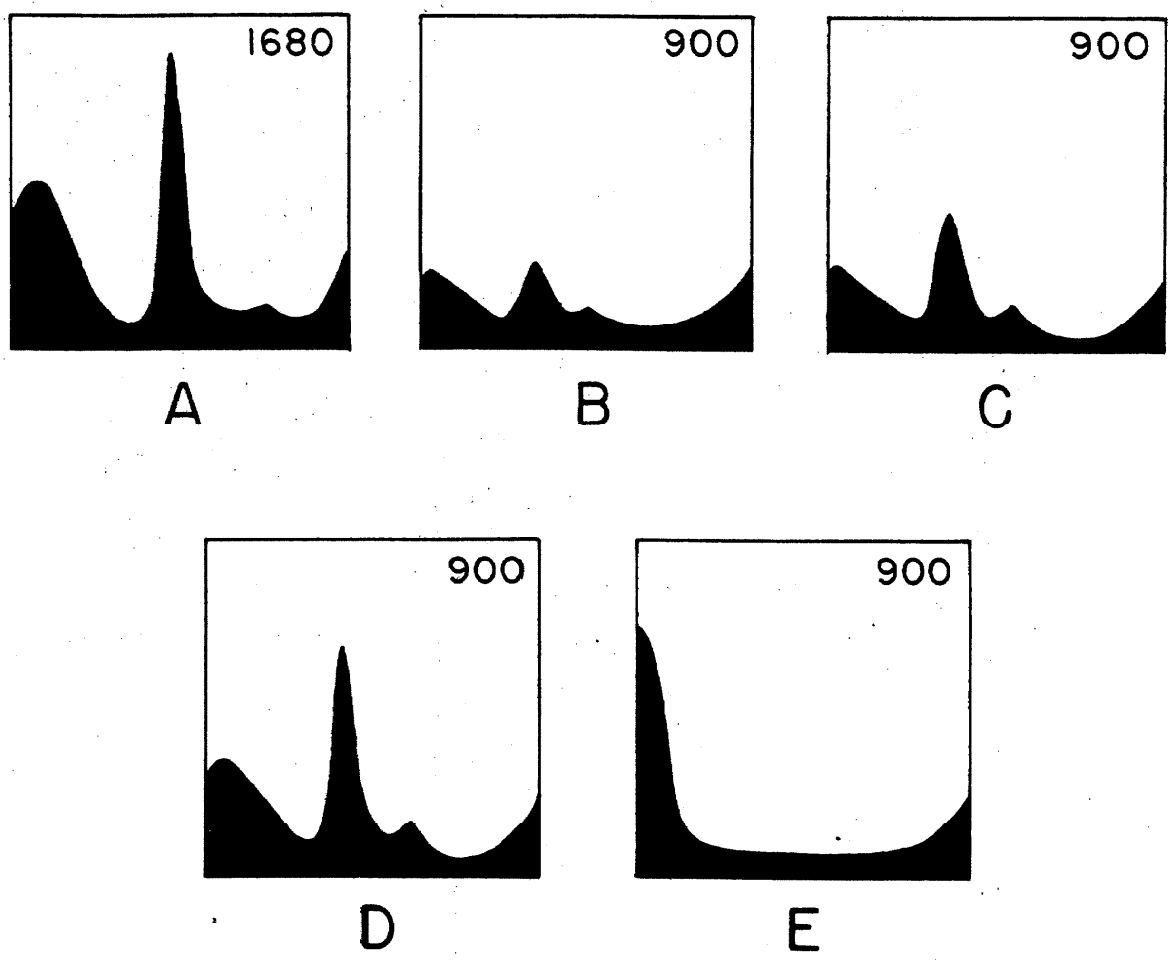


Figure 9. Sedimentation diagrams of the precipitated and soluble fractions of Turkish tobacco cytoplasm at several different ammonium sulfate concentrations. Time in second after attaining a rotor speed of 850 r.p.s. indicated in corner of each diagram. Sedimentation from left to right. Solvent was 0.1 μ Na-barbital buffer, pH 8.5. Each sample contains an amount of protein equivalent to that precipitated from an equal volume of whole cytoplasm except B, which contains twice the equivalent concentration. A, initial whole cytoplasm, 11.65 mg./ml. B, C and D, fractions precipitated at 25, 30 and 40% saturation, respectively; concentrations are 3.8, 6.5, 8.6 mg./ml. E, supernatant solution from 40% saturation, 2.5 mg./ml.

associated with the 19S component, the increased precipitation of TCA-P (from 39°/o at 40°/o salt saturation to 60°/o at 50°/o salt saturation) following complete precipitation of the 19S component at 40°/o salt saturation is further evidence that the TCA-P is dissociated from the protein and independently precipitated by high salt concentrations.

Because of the dissociation of the TCA-P moiety from its carrier protein and because of the non-specificity of the precipitation, attempts to fractionate the cytoplasmic proteins with salt were discontinued. Instead, a differential centrifugation method, which is suitable for preparing solutions containing 90 - 95°/o of Fraction I protein and its higher molecular weight aggregates, was developed.

Purification of Fraction I Protein by Preparative Ultracentrifugation.

Since Fraction I protein constitutes a large part of the total cytoplasmic proteins and possesses a sedimentation constant considerably greater than those of the other proteins of cytoplasm, it is possible to achieve substantial purification of Fraction I protein by the application of very high centrifugal forces. In an idealized case, the relative composition of a pellet collected by sedimentation in a gravitational field is a function of the relative concentrations and sedimentation rates of the individual components

in the mixture. In the cytoplasmic protein mixture, the composition of the first material sedimented may be calculated as follows, assuming for illustrative purposes that the mixture consists of 50°/o Fraction I protein and that the "average sedimentation rate" of the low-molecular weight fraction is 4.75*:

$$\frac{\text{°/o Fraction I protein}}{\text{°/o low-mol. wt. fraction}} \times \frac{S_{\text{Fr I protein}}}{S_{\text{low-mol. wt.}}}$$

$$= \frac{50}{50} \times \frac{19.0}{4.75} = \frac{4}{1} .$$

Thus, the pellet will consist of 4 parts of 19S protein to 1 part of low-molecular weight protein. If this pellet is redissolved and the solution centrifuged again, the first material sedimented will consist of 16 parts 19S protein to 1 part of low-molecular weight material. In practice, however, this ideal fractionation is not achieved because: (1) Fraction I protein seldom constitutes 50°/o of the original mixture, (2) a non-ideal, convective sedimentation system is used for centrifuging large lots of material, and (3) as sedimentation proceeds, the relative amount of Fraction I protein in the supernatant solution steadily decreases, leading to a reduction in the proportion of 19S protein deposited in the pellet during the last stages of sedimentation.

* The "initial sedimentation rate" for the low-molecular weight group of proteins is approximately 4-5S, as determined in the synthetic boundary cell. As sedimentation proceeds, the apparent sedimentation rate decreases due to loss of the higher-molecular weight components.

The procedure summarized in Figure 10 has been found to produce Fraction I protein preparations of 90 - 95°/o purity, as judged by analysis in the ultracentrifuge, from the cytoplasms of spinach, pea and tobacco. Approximately 1°/o solutions of cytoplasmic proteins (WC) are spun at 40,000 r.p.m. for 45 minutes in the no. 40 rotor of a Spinco Model L Preparative Ultracentrifuge. The rotor is pre-cooled to 0° C., or lower, and the centrifuge refrigerator set to maintain the temperature of the rotor chamber at about -20° C. The small pellet (R), which constitutes less than 5°/o of the total cytoplasmic proteins and often contains slight amounts of green matter, formed during this initial centrifugation is discarded and the supernatant solution (S) centrifuged for 2.5 hours at 40,000 r.p.m. An appreciable pellet, representing 20 - 40°/o of the total cytoplasmic proteins, is deposited during this second centrifugation. The supernatant solution (Fr I_1 - S) consisting of 50 - 70°/o of the Fraction I protein and almost all of the low-molecular weight material initially present in the whole cytoplasm, is discarded and each pellet dissolved in 1 to 2 ml. of 0.1 μ K-maleate buffer, pH 7.0, at 0° C. The pellet is allowed to stand in contact with buffer for 12 - 24 hours with occasional maceration and stirring to effect re-solution. The small amount of protein which fails to redissolve (Fr I_1 - R) is removed by a low-speed centrifugation and discarded. This completes the first fractionation cycle. The redissolved protein solution (Fr I_1), containing about 80°/o Fraction I protein, is again centrifuged for

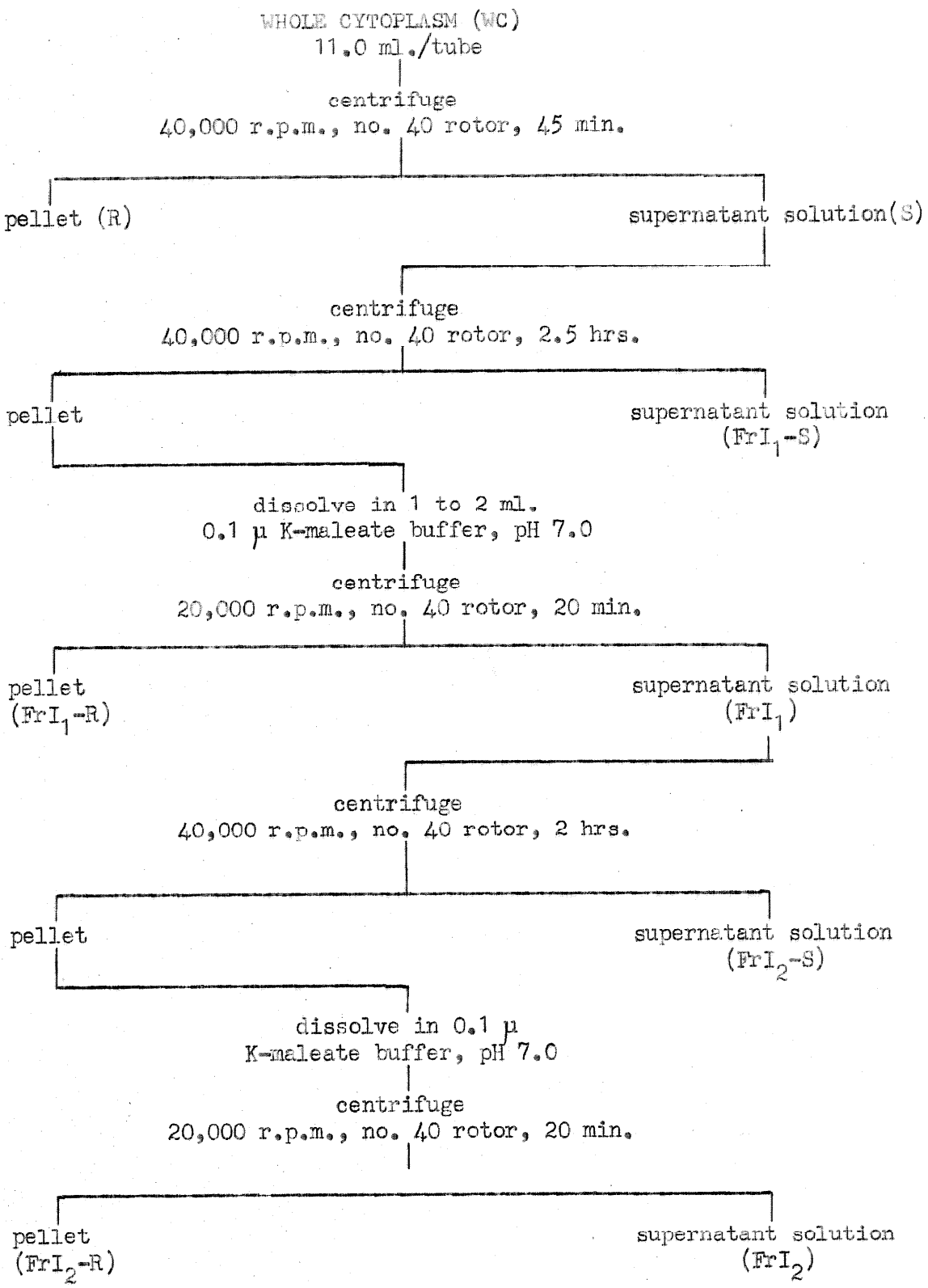


Figure 10. Scheme for preparation of Fraction I protein solutions by differential centrifugation.

2 hours at 40,000 r.p.m. The supernatant solution (Fr I_2 - S) is discarded and the pellet redissolved in 0.1 μ buffer as described above. The small amount of protein (Fr I_2 - R) which fails to redissolve is removed by a low-speed centrifugation and discarded, as during the first fractionation cycle. The clear, amber-to-brown, non-opalescent Fraction I protein solution (Fr I_2) which remains contains about 70 - 80% of the protein which was present in Fr I_2. Two fractionation cycles have been found to be the practical limit for large scale preparations. The Fraction I protein solutions so prepared contain only about 5 - 10% of low-molecular weight contaminants. The ultracentrifuge pattern of a solution of Fraction I protein (Fr I_2) prepared from spinach cytoplasm by this method is illustrated in Figure 11.

Analyses of Fractions Obtained by Differential Centrifugation

The fractionation of several typical samples of Turkish tobacco cytoplasms will be considered in detail. For one sample, E-5, a detailed chemical "balance sheet" is available. For sample E-8, chemical, UC and electrophoretical analyses of each fraction will be discussed, and for sample E-11, chemical and enzymatic analyses will be presented. The fractionation of E-8 will be considered first.

Physical Chemical Analyses. Sample E-8 was fractionated according to the scheme presented in Figure 10 and suitable aliquots

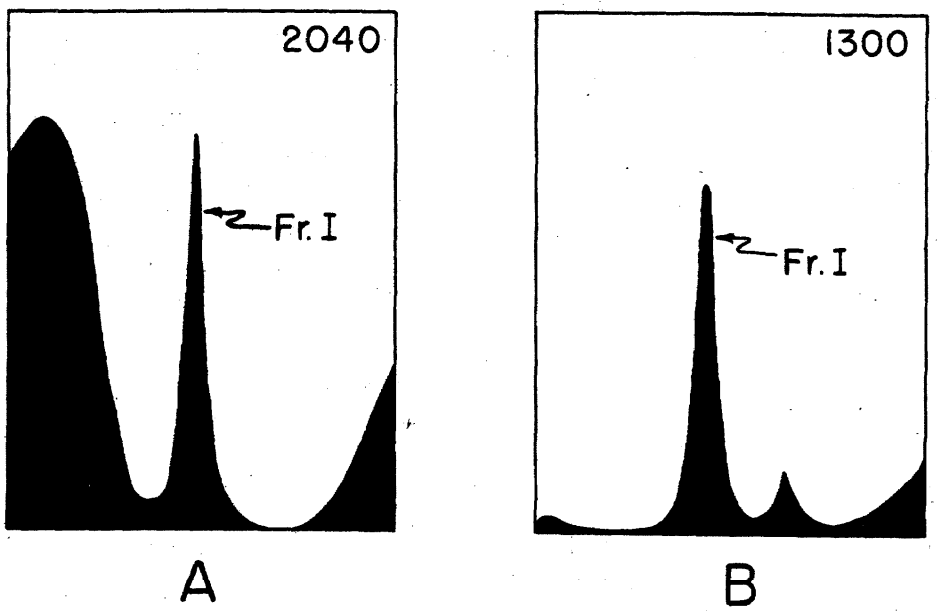


Figure 11. Analytical ultracentrifuge patterns of a typical spinach whole cytoplasm (A) and the Fraction I protein preparation made from it by two preparative UC cycles, (B). Arrows indicate the 19S, Fraction I protein component. Direction of sedimentation is from left to right. Time in seconds after reaching rotor speed of 850 r.p.s. is indicated in upper right corner of each diagram. A, in 0.3 M K-maleate buffer, pH 6.9; 13.0 mg. protein/ml. B, in 0. 1 μ K-maleate buffer, pH 7.5; 4.0 mg. protein/ml.

of each fraction, including those normally discarded, were analyzed for TCA-precipitable weight, TCA-P, and total-P. Other aliquots were also analyzed in the UC and by electrophoresis. The chemical analyses are summarized in Table XII.

For UC analysis, the various fractions were dialyzed to equilibrium at 2° C. against 0.1 μ K-maleate buffer, pH 7.0, then all diluted to the same concentration with buffer from the dialysis. The schlieren diagrams obtained during analysis of the original whole cytoplasm, and of fractions Fr I_1 - S and Fr I_2 are shown in Figure 12. The pattern obtained with whole cytoplasm is presented in Figure 12A and that obtained with the supernatant solution from the first cycle of fractionation (Fr I_1 - S) in Figure 12B. Since these solutions, which initially contained 10.95 and 7.58 mg. protein/ml., respectively, were dialyzed and diluted to a common concentration before analysis, diminution of the amount of Fraction I protein in the supernatant solution is not readily apparent from direct comparison of the size of the 19S peak in each diagram. It is immediately obvious, however, that the area under the peak representing low-molecular weight components is much greater in the supernatant solution Fr I_1 - S than in the unfractionated cytoplasmic protein solution. Therefore the relative amount of the 19S component in Fr I_1 - S must be less than in whole cytoplasm. The product of the second fractionation cycle (Fr I_2) is shown in Figure 12C. Only about 5% of the total area is due to

TABLE XII

CHEMICAL ANALYSES OF THE SEVERAL FRACTIONS OBTAINED DURING CENTRIFUGAL FRACTIONATION OF A TURKISH TOBACCO CYTOPLASM PREPARATION (E-8).

	Fraction			% sedimented during 1st cycle
	WC.	Fr I_1 - S	Fr I_2	
mg. protein/ml.	10.95	7.58	13.2	31
γ TCA-P/ml.	11.4	4.37	20.7	61
γ TCA-P/mg. protein	1.04	0.57	1.57	
γ Total-P/ml.	179	143		21

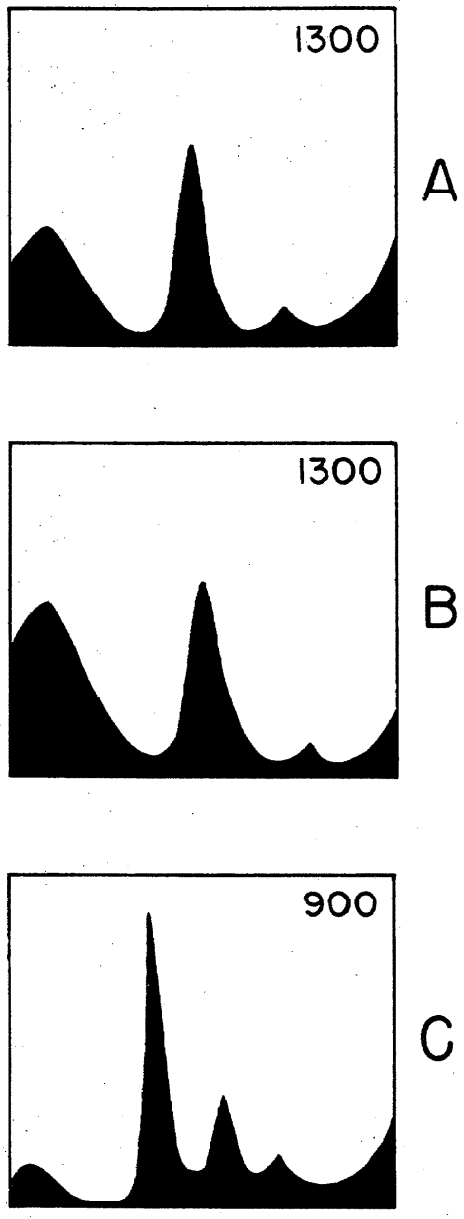


Figure 12.

A typical series of analytical ultracentrifuge patterns illustrating the course of fractionation of tobacco cytoplasmic proteins by high-speedcentrifugation (E-8). Rotor speed 850 r.p.s. A, whole cytoplasm before centrifugation; NaCl-Na cacodylate buffer, 0.1 μ, pH 7.1; 5.0 mg. protein/ml. B, supernatant solution after first centrifugation cycle; same buffer as A; 5.6 mg.protein/ml. C, twice-cycled Fraction I protein solution; K-maleate buffer, pH 7.5, 0.1 μ; 5.0 mg. protein/ml.

components with sedimentation constants lower than those of Fraction I. However, in addition to the 19S peak, other faster-sedimenting peaks are also present. Evidence to be presented later indicates that these are aggregates of the 19S component.

The scanning patterns obtained during electrophoresis of these same fractions after appropriate dilution and equilibrium dialysis at 2° C. against 0.1 μ NaCl-Na-cacodylate buffer, pH 7.12, are shown in Figure 13. The patterns show that under the electrophoresis conditions used, there is little separation of the proteins present in whole cytoplasm (Figure 13A). This is in accordance with the observations discussed in Section III-D, Part I. After removal of 31% of the total protein from whole cytoplasm by sedimentation, there was no evident change in the electrophoretic pattern except for the more pronounced shoulder at the base of the trailing edge of the main peak (Figure 13B). The curve is also somewhat broader in relation to its height, indicating either that the average diffusion rate of the remaining proteins is greater than the average diffusion rate of the unfractionated cytoplasmic proteins or that they are more heterogeneous with respect to electrophoretic mobility. Twice recycled Fr I_2 (Figure 13C), on the other hand, has a sharp, symmetrical peak with the same mobility as the principal component of cytoplasm. The calculated mobility of the principal component in each fraction is presented in Table XIII.

From these data and the UC and electrophoresis analyses

TABLE XIII

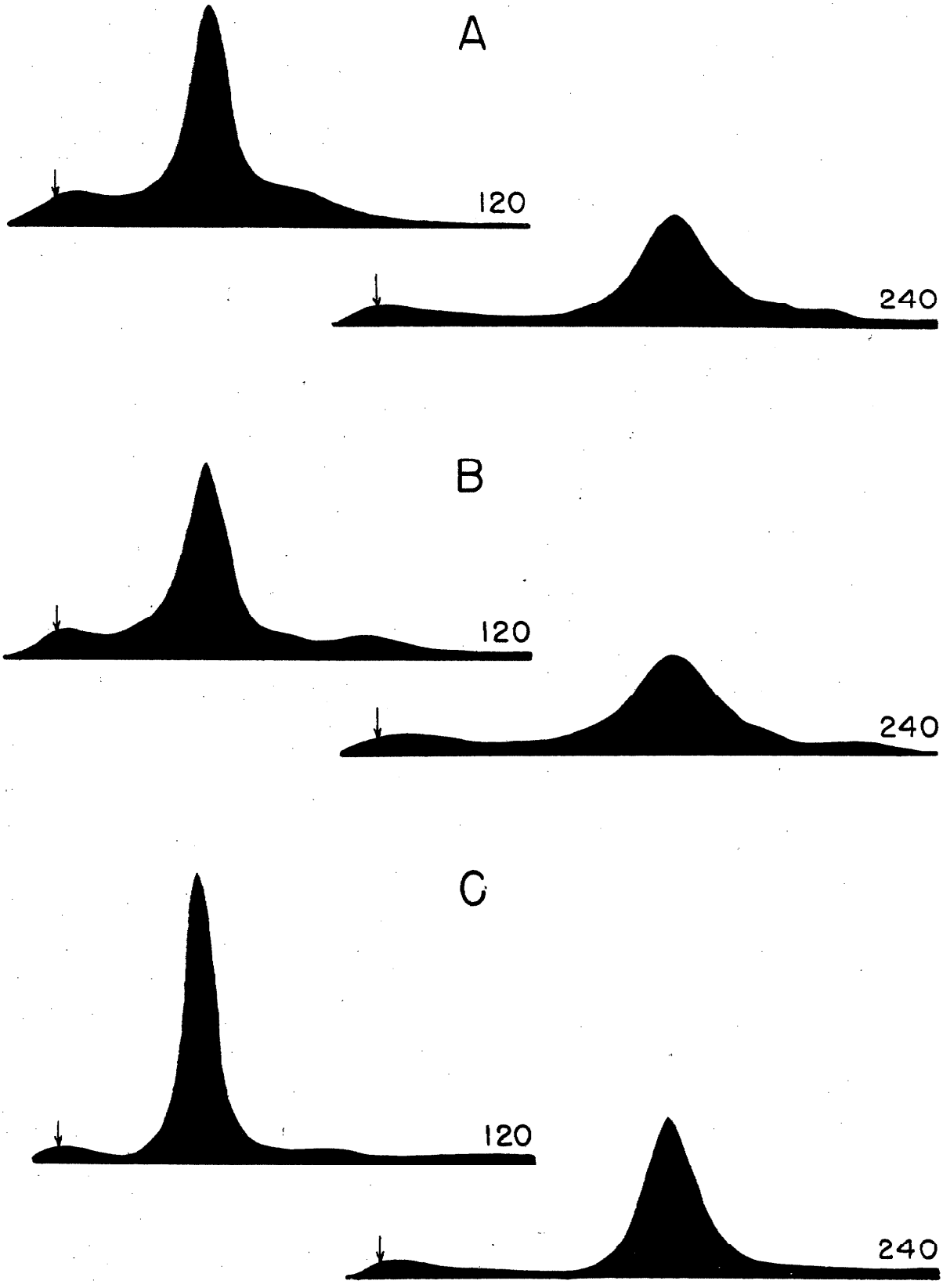
ELECTROPHORETIC MOBILITY OF THE PRINCIPAL COMPONENT OF VARIOUS FRACTIONS PREPARED BY HIGH-SPEED CENTRIFUGATION OF TURKISH TOBACCO CYTOPLASM (E-8)

Preparation	Mobility* (cm^2 $volt^{-1}$ sec^{-1})
A. Whole cytoplasm	-5.25×10^{-5}
B. Whole cytoplasm supernatant solution after first centrifugation cycle	-5.19×10^{-5}
C. Fraction I prepared by two centrifugation cycles	-5.15×10^{-5}

* Calculated in descending limb. Buffer composition: 0.08 M NaCl, 0.0033 M cacodylic acid, 0.02 Na cacodylate. Ionic strength 0.1; pH 7.12 at 27° C.

Figure 13. Electrophoresis diagrams showing the course of fractionation of tobacco cytoplasmic proteins by high-speed centrifugation (E-8). A, B, and C are the same fractions as analyzed ultracentrifugically in Figure 12. All are in NaCl-Na cacodylate buffer, pH 7.12, 0.1 μ. Protein concentration 5.50, 5.65 and 4.85 mg./ml. in A, B, and C, respectively. Only descending boundaries are shown. Time of migration is given to the right of each diagram; arrows indicate starting boundary.

Figure 13



presented in Figures 12 and 13, it is clear that the 19S component of cytoplasm has the same electrophoretic mobility as many of the other cytoplasmic proteins under the conditions of these observations and that all the components of Fraction I protein preparations which sediment more rapidly than 19S have the same electrophoretic mobility as does the 19S component. Of more significance is the demonstration that differential centrifugation provides a means of preparing the 19S component comparatively free of low-molecular weight material. This, of course, is of prime importance for any chemical, physical chemical or biochemical studies of this nucleoprotein.

Chemical analyses. To provide material for detailed chemical analyses, Sample E-5 was fractionated according to the scheme presented in Figure 10 and suitable aliquots of each fraction were analyzed for TCA-precipitable weight, TCA-P and total-P. The results of these analyses are presented in the form of a "balance sheet" in Table XIV. Several important facts are immediately apparent from these data. During the first fractionation cycle, 38°/o of the protein, 63°/o of the TCA-P and 21°/o of the total-P were sedimented. Thus the proteins which were sedimented contained more TCA-P/unit weight than did the proteins as a whole in the unfractionated cytoplasm. Recovery of 98.2°/o of the total-P in the several fractions is indicative of the overall experimental errors in P analyses and volume measurements; hence recovery of only 87°/o of the TCA-P indicates some loss of TCA-P other than

TABLE XIV

ANALYSES OF THE FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUCATION OF A TYPICAL TURKISH TOBACCO CYTOPLASM SAMPLE (E-5)

Fraction*	S	1st Fractionation cycle			recovery	$Fr\ I_1$	2nd Fractionation cycle			recovery
		$Fr\ I_1$ - S	$Fr\ I_1$ - R	$Fr\ I_1$			$Fr\ I_2$ - S	$Fr\ I_2$-R	$Fr\ I_2$	
Volume/fraction	132 ml.	130		20.0		11.0	10.5			
mg. TCA protein/ml.	7.90	4.67		12,45		12.45	4.97		8.07	
mg. TCA protein/fraction	1,042	607	148	249	1,004	137	52.2	6.7	72.6	131.5
% of S		58.2	14.2	23.8	96.2					
% of $Fr\ I_1$							38.1	4.9	53.0	96.0
% TCA-P/ml.	22.2	5.5		52.5		52.5	14.8		39.3	
% TCA-P/fraction	2,930	715	789	1,050	2,854	578	155	13	354	522
% of S		24.4	27.0	35.6	87.0					
% of $Fr\ I_1$							26.9	2.3	61.2	90.4
% Total-P/ml.	120.2	94.2		91.2		91.2	45.6		56.2	
% Total-P/fraction	15,866	12,246	1,489	1,824	15,557	1,003	479	18	506	1,003
% of S		77.3	9.4	11.5	98.2					
% of $Fr\ I_1$							47.8	1.8	50.4	100

* see Figure 10 for designation of fractions.

through sedimentation from solution. This loss was probably due to enzymatic activity. During the second fractionation cycle, 58°/o of the protein, 63.5°/o of the TCA-P and 52.2°/o of the total-P were sedimented, resulting in a further enrichment of TCA-P/mg. protein in the pellet.

Similar analyses, expressed on a volume basis, of 5 other tobacco samples are presented in Table XV. In each case, regardless of the initial TCA-P concentration, the proportion of the total TCA-P sedimented from solution (based on analyses of the sedimented material) is greatly in excess of the proportion of protein sedimented (columns 7 and 8). This results in a 2 to 5-fold enrichment of TCA-P/mg. protein in Fraction I protein preparations as compared with the initial whole cytoplasms and a corresponding decrease in TCA-P content/mg. protein in fraction Fr I_1 - S (Table XVI). The further increase in TCA-P obtained during the second fractionation cycle, corresponding to the increased enrichment of the 19S component, is well illustrated by the data presented for sample B-5 in this latter table.

Enzymatic Activity. As one method for following contamination of Fraction I protein preparations by low-molecular weight proteins, and in order to determine whether the 19S component possesses enzymatic properties, the several fractions obtained from fractionation of a number of Turkish tobacco cytoplasms were assayed for phosphatase activity. Phosphatase was chosen for three reasons: (1) This enzyme has a rather low molecular weight (ca. 50,000);

TABLE XV

LOSSES OF PROTEIN, TCA-P AND TOTAL-P FROM SEVERAL CYTOPLASMIC PROTEIN SOLUTIONS DURING THE FIRST FRACTIONATION CYCLE

Variety of *N. tabacum*	Sample No.	Whole Cytoplasm			Supernatant solution from 1st Fract. cycle (Fr I_1 - S)			Per cent Sedimented during 1st Fract. cycle		
		Protein	TCA-P	Total-P	Protein	TCA-P	Total-P	Protein	TCA-P	Total-P
		(1) mg./ml.	(2) γ/ml.	(3) γ/ml.	(4) mg./ml.	(5) γ/ml.	(6) γ/ml.	(7)	(8)	(9)
Turkish	V-51-70	12.0	70.3	344	8.7	33.5	317	27	52	8
Turkish	E-5	7.9	22.2	120	4.7	5.5	94.2	41	75	21
Turkish	E-6	8.6	23.3	204	6.7	8.5	178	22	64	13
Turkish	E-8	10.9	11.4	179	7.6	4.4	143	31	61	20
Turkish	E-11	10.4	30.1	186	7.4	7.3	149	28	75	20
Maryland Mammoth	E-20	14.1	54.9	-	11.7	35.0	-	17	36	-

TABLE XVI

COMPARISON OF TCA-P CONTENT OF SEVERAL WHOLE CYTOPLASM AND FRACTION I PROTEIN PREPARATIONS

Preparation Number	γTCA-P/mg. protein		
	Fraction		
	WC	Fr I	Fr I_1 - S
V-51-70	5.86	8.80 *	3.85
E-5	2.81	4.21 *	1.20
		4.86 **	
E-6	2.74	13.7 **	1.26
E-8	1.04	1.51 **	0.57
E-11	2.89	7.14 **	0.98
E-20	3.89	10.4 **	2.99

* Product of first fractionation cycle.

** Product of second fractionation cycle.

(2) Phosphatase is very stable toward both acid pH's and high temperature, and would hence be resistant to denaturation during the mild fractionation scheme employed; and (3) Wildman and Bonner (1947) had shown that the major portion of the phosphatase activity in spinach cytoplasm is concentrated in the fraction precipitated by 35°/o saturated ammonium sulfate. It was shown in Section III-D, Part I, that the principal component of this salt-precipitated fraction is the 19S component. Therefore if the 19S component is associated with phosphatase activity, phosphatase should be concentrated in Fraction I protein solutions prepared by centrifugation.

The following system was employed for assay of phosphatase activity: 0.1 ml. of the solution to be assayed was added to 2.0 ml. of nitrophenyl phosphate (2.0 mg./ml. in 0.1 M sodium acetate-acetic acid buffer, pH 5.0) and the mixture incubated at 25° to 37° C. At appropriate time intervals, generally 0, 5, 10 and 20 minutes, 0.5 ml. aliquots were removed and added to 9.5 ml. of 0.2 N NaOH. The intensity of the resulting stable yellow color, due to liberated nitrophenol, was determined in a Klett-Summerson colorimeter with a 42 filter. The specific activity of the protein (activity in arbitrary units/mg. protein/minute) was determined from the straight-line portion of curves obtained from a plot of the Klett readings, corrected for reagent blank values, against time.

The results obtained from the assay of sample E-11 fractions are presented in Figure 14. In this case, the portions of the curves from 0 - 10 minutes were used to compute specific activities, and

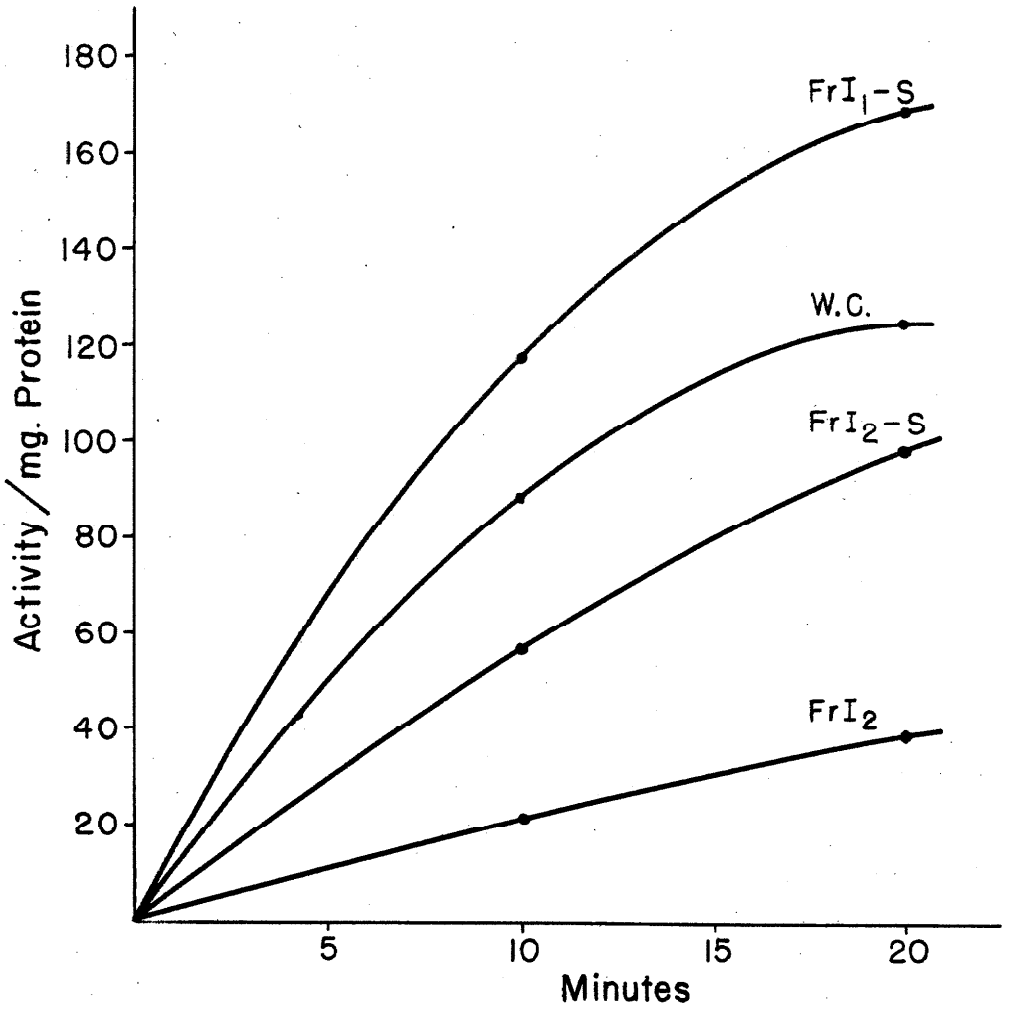


Figure 14. Phosphatase activity of several fractions of a Turkish tobacco cytoplasm preparation (sample E-11). Substrate was nitrophenylphosphate in 0.1 M Na acetate buffer at pH 5.0. Activity is expressed in arbitrary units. Fractions are designated as in Figure 10.

from these activity values the activities/mg. protein relative to that of the whole cytoplasm were calculated. These activities are given in Table XVII. Since the slopes of the curves for fractions Fr I_1 - S and WC in Figure 14 decrease with time, an appreciable error is introduced by not using initial slopes. This error lowers the apparent activity of these two fractions and thus increases the relative activity of fraction Fr I_2 somewhat. It is clear, however, that phosphatase is separated from Fraction I protein by the preparative procedure used.

Similar fractions of sample E-8, for which the chemical and physical analyses were presented above, were assayed for phosphatase and peroxidase activity*. The results obtained, presented in Table XVIII, indicate that in each case the activity is similarly partitioned between the various fractions. The level of activity observed for fraction Fr I_2 in this sample, i.e. 5°/o of the activity found in whole cytoplasm, is more in accord with the values normally obtained than is the 23°/o observed in fraction Fr I_2 of sample E-11 (Table XVII). In other instances, only trace amounts of phosphatase activity have been found in Fraction I protein preparations. In general, however, relative activities of 5-10°/o, roughly correspodning to the amount of low-molecular weight material present, have been found in these solutions.

Two facts are evident from these data. First, the

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* The peroxidase assays were kindly performed by Dr. Howard Boroughs.

TABLE XVII

PHOSPHATASE ACTIVITY OF SEVERAL FRACTIONS OF TURKISH TOBACCO CYTOPLASM SAMPLE E-11

Fraction	Activity per mg. protein	Relative activity
WC	890	100
Fr I_1 - S	1180	132
Fr I_2 - S	550	62
Fr I_2	210	23

TABLE XVIII

PHOSPHATASE AND PEROXIDOSE ACTIVITY OF SEVERAL FRACTIONS OF TURKISH TOBACCO CYTOPLASM SAMPLE E-8

	Peroxidose Activity		Phosphatase Activity	
Fraction	per mg. protein	relative	per mg. protein	relative
WC	23.7	100	29.2	100
Fr I_1 - S	34.3	145	41.0	141
Fr I_2	1.3	5.6	1.5	5.2

specific activity of phosphatase and of other low-molecular weight enzymes decreases as the relative amount of 19S protein in a preparation increases. Secondly, the relative activity of phosphatase in each fraction is similar to that of other stable enzymes of approximately the same molecular weight. This indicates that, contrary to the earlier suggestion of Wildman and Bonner (1947), Fraction I protein is not itself a phosphatase. Further evidence in support of this conclusion is the fact that 70 - 85% of the initial phosphatase activity is recovered from solutions in which all of the 19S component has been precipitated by acidification to pH 4 to 5. This is indicative that the 19S component is not necessary for phosphatase activity but it does not eliminate the possibility that at least part of the phosphatase activity may be associated with it in situ.

II. CHEMICAL CHARACTERIZATION OF FRACTION I PROTEIN PREPARATIONS

Chemical Analyses

Chemical analyses of Fraction I protein preparations have been limited to TCA- and total-N and P analyses. Protein preparations made by two fractionation cycles have approximately the same nitrogen content per mg. of protein as do the cytoplasmic proteins as a whole (i.e., 14 - 15°/o). Only about 70 - 85°/o of the total nitrogen present in such preparations is precipitable by TCA. This is surprising, since it would be expected that low-molecular weight nitrogen-containing compounds would be almost completely removed during two fractionation cycles. The nature of the compounds containing the non-precipitable portion of the nitrogen is unknown. Probably, they are not of very low molecular weight, however, for even in thoroughly dialyzed preparations only 85 - 90°/o of the total-N is TCA precipitable.

TCA-P and total-P analyses of sample E-5 are included in Table XIV. The TCA-P content per mg. of protein in Fraction I protein preparations is always much greater than that of the cytoplasmic proteins as a whole. In general, 80 - 90°/o of the total-P is precipitable with TCA, although in sample E-5 only 70°/o was TCA-precipitable. The non-TCA-P is believed to arise from enzymatic degradation of TCA-P during the last re-solution of the protein. In Fraction I protein preparations which have very low ribonuclease and phosphatase activities, as much as 90°/o of the

total-P is in the form of TCA-P; however, in preparations, such as that from sample E-5, which have high phosphatase and ribonuclease activities, only 60 to 70^{o}/o of the total-P may be TCA-P, the remainder being in the form of inorganic-P.

Identification of the TCA-P Moiety

By experiments similar to those outlined earlier for whole cytoplasm, the TCA-P moiety in Fraction I protein preparations has been identified as ribonucleic acid. Indeed, this finding necessarily followed the demonstration that all of the TCA-P present in whole cytoplasm is present in the form of RNA. The nucleotide composition of the RNA in Fraction I protein preparations was found to be similar, except for a somewhat higher purine/pyrimidine ratio, to that of the RNA in the whole cytoplasm from which the preparations were made. This finding is consistent with the results generally obtained when RNA's are partially hydrolyzed with ribonuclease. It therefore tends to support the supposition that TCA-P is lost from whole cytoplasm because of ribonuclease activity.

As with whole cytoplasm, all of the TCA-P in fraction Fr I_2 is accounted for in the form of nucleotide-P.

To What is the Ribonucleic Acid Attached?

It is evident from the data presented in Tables XIV, XV,

and XVI that the RNA moiety is concentrated in the high molecular weight fraction. It may be attached to a rapidly sedimenting protein, or it may itself have a high molecular weight. Protein free preparations of RNA with a molecular weight greater than ca. 100,000 have seldom been obtained. This is too low to account for the 2 - to 5- fold increase in RNA/mg. protein observed during the purification of Fraction I protein from whole cytoplasm. Furthermore, it has not been possible to demonstrate free RNA in such preparations even when, as in fraction Fr I_2 of sample E-6 (Table XVI), RNA constituted as much as 15°/o of the total solute in a 2°/o solution of Fraction I protein. Thus it is logical to assume that the RNA is attached to a protein.

That the protein to which the RNA is attached is the 19S component of cytoplasm is suggested from the partition of TCA-P and protein which occurs during the fractionation. In every case, the TCA-P content/mg. of protein increased directly as the purity of the 19S component (or aggregates of it) increased. In sample E-5, for instance, the TCA-P content/mg. of protein increased 10°/o between the first and second fractionation cycles despite the fact that only 63°/o of the TCA-P was sedimented from solution and approximately 10°/o more was destroyed (only 90°/o recovery - Table XIV). This increase of 10°/o in TCA-P is similar to the expected 10 - 15°/o increase in the relative amount of 19S protein in fraction Fr I_2 as compared with fraction Fr I_1.

The RNA cannot be attached to a protein sedimenting faster

than the 19S component (e.g. the 25 - 28S component) because preparations which do not possess this faster sedimenting component also contain TCA-P and show the same TCA-P protein partition that is typical of preparations with a fast component present.

Because of this correlation between the amount of 19S component and the TCA-P content, it is possible to estimate the amount of TCA-P to be expected in the final Fraction I protein preparation. Such an estimate may be made from the TCA-P content/mg. protein and the percentage of the 19S protein in whole cytoplasm, as illustrated by the following data obtained from analyses of sample E-8:

The whole cyoplasm contained 11.4 γ TCA-P and 10.95 mg. protein/ml. (Table XII). From UC schlieren diagrams similar to those presented in Figure 12A, 53°/o of the total area was found to be due to the 19S and 26 - 28S components*. If it is assumed that all of the TCA-P is associated with 53°/o of the protein, then the TCA-P content is 1.96 γ/mg. of 19S protein. Fraction Fr I_2, which may be assumed to have not more than 10°/o low-moleuclar weight components in it, contained 13.2 mg. total protein per ml. Thus, 23.4 γ TCA-P/ml. [(13.2)(0.90)(1.96)] would be expected in this fraction. Actually, 20.7 γ , or 89°/o of the expected amount, was found. This is excellent agreement in view of the errors inherent in the method used to measure the relative amount of the 19S component and the enzymatic loss of TCA-P that occurs during the isolation of Fraction I protein.

* These two components are treated together because, as will be shown later, the faster sedimenting component is a dimer of the 19S component.

III. PHYSICAL CHEMICAL CHARACTERIZATION

A number of experiments have been performed to define some of the physical chemical properties of the 19S component of cytoplasm. The presence of more rapidly sedimenting components in preparations obtained by two sedimentation cycles, could cause serious complications in interpretation of results obtained from viscosity, diffusion and light scattering measurements. Physical studies have, therefore, been limited to determination of the partial specific volume and to examination by electrophoresis, analytical ultracentrifugation and, in a very preliminary manner, electron microscopy.

Electrophoretic Analyses

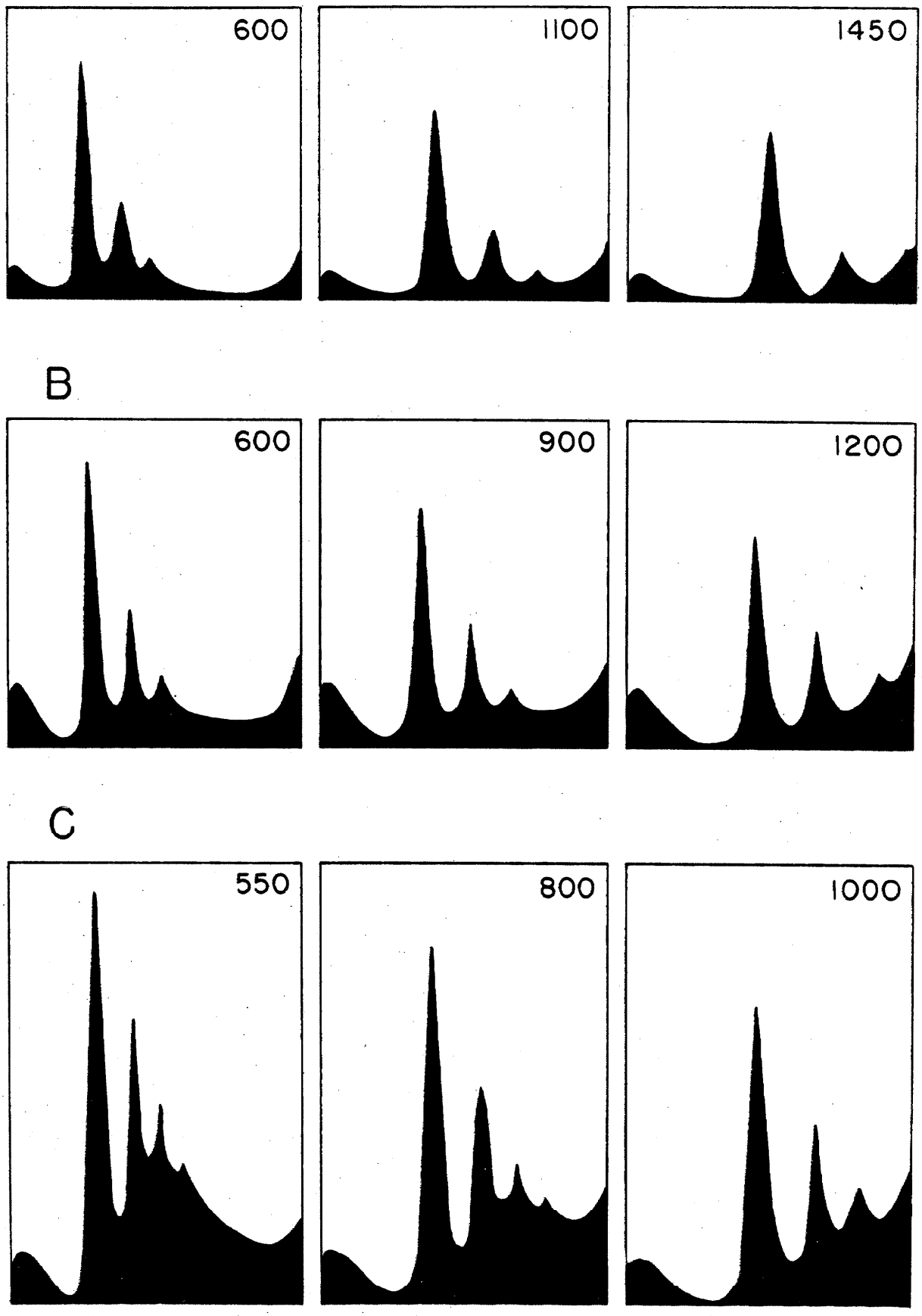
Two preparations of Fraction I protein from Turkish tobacco cytoplasm were subjected to electrophoresis in 0.1 μ Na cacodylate buffer at pH 7.12. Both preparations were characterized by a high degree of homogeniety under these conditions. The mobility of the principal component in each sample was, within experimental, error, the same (-5.15×10^{-5} cm^2 $volt^{-1}$ sec^{-1}). For illustration, the patterns obtained with sample E-8 are reproduced in Figure 13C.

Determination of Sedimentation Constants

Sedimentation diagrams of several typical Fraction I protein preparations are reproduced in Figure 15. A similar

Figure 15. Sedimentation behavior of several typical Fraction I protein preparations from Turkish (A,B) and Maryland Mammoth (C) tobacco leaves (samples E-8, E-11 and E-20,respectively). Rotor speed 850 r.p.s. Solvent, 0.1 μ K-maleate buffer, pH 7.0. The 19S component is the major component in each case. In A and B, there are clearly two faster moving aggregates of Fraction I protein, and in C there are three.

Figure 15



preparation from spinach is illustrated in Figure 11B. In spinach, an appreciable amount of a component of 26 - 28S was observed. In tobacco, two or more components with a sedimentation constant greater than 19S were observed. The nature and origin of these rapidly sedimenting components will be discussed later.

The presence of higher molecular weight components does not affect the sedimentation rate of Fraction I protein, which is the same in highly purified preparations as it is in whole cytoplasm. The sedimentation constants of Fraction I protein prepared from spinach and tobacco are essentially the same, as illustrated by the data in Table XIX. These data also indicate that over a 10-fold concentration range, the sedimentation constant of the Fraction I protein component exhibits virtually no dependence on concentration. This means that the low degree of spreading of the 19S peak during sedimentation (cf. Figures 17 and 23) is not to be attributed to the boundary sharpening that occurs with substances which exhibit a large dependence of s upon concentration. It is indicative of the homogeniety of the protein with respect to molecular weight.

Since in general the concentration dependence of s increases with increasing asymmetry of the sedimenting material (Singer, 1947), the fact that, for Fraction I protein, s is essentially independent of concentration strongly suggests that the molecule is nearly spherical. The importance of this in determination of the probable molecular weight will be considered shortly.

TABLE XIX

SEDIMENTATION CONSTANTS OF THE HIGH-MOLECULAR WEIGHT COMPONENTS IN SEVERAL FRACTIONS OF WHOLE CYTOPLASM AND FRACTION I PROTEIN PREPARATIONS

Species, Sample and Fraction	Concentration	Buffer	$s^{W}_{20} \times 10^{13}$** Component I	Component II	Component III
	mg./ml.				
A. Spinach Fr I_2	4.0	(1)	18.1	26.0	
	2.0	(1)	18.6	26.4	
	1.0	(1)	17.9	--	
	0.5	(1)	18.4	--	
B. Turkish tobacco (E-6) Fr I_2	21.6	(1)	18.0	25.7	30.7
C. Turkish tobacco (E-8) a) Whole cytoplasm	5.0	(1)	18.8		
b) Whole cytoplasm supernatant after 1st centrifugation cycle (Fr I_1 - S)	5.6	(1)	18.7		
c) Fr I_2	5.1	(1)	18.7		
	1.3	(1)	18.6		
D. Turkish tobacco (E-11) a) Whole cytoplasm	5.0	(2)	19.1		
b) Whole cytoplasm supernatant after 1st centrifugation cycle (Fr I_1 - S)	5.5	(2)	18.9		
c) Fr I_2	9.85	(2)	19.5	27.4	33.9
	4.92	(2)	19.1	27.9	34.8
E. Maryland Mammoth tobacco (E-20) Fr I_2	9.90	(1)	19.2	28.4	36.8
	4.95	(1)	19.1	28.0	--
	2.47	(1)	19.0	28.7	
	1.23	(1)	19.0	--	--

TABLE XIX continued

Species, Sample and Fraction	Concentration	Buffer	$s^{W}_{20} \times 10^{-13}$** Component I	II	III
	mg./ml.				
F. Various spinach and tobacoo whole cytoplasms	3 - 15	(1) or (3)	18.9 ≠		

** Values corrected to a solvent having the density and viscosity of water at 20° C.

≠ Average of 20 independent experiments.

(1) K-maleate buffer, pH 7.0, 0.1 μ.

(2) K-maleate buffer, pH 7.5, 0.1 μ.

(3) Na cacodylate - cacodylic acid - NaCl buffer, pH 6.9, 0.1 μ.

Determination of Partial Specific Volume

The partial specific volume ($\bar{V}$) of the total protein contained in fraction Fr I_2 from sample E-20, for which data are given in Section E, Table XIX was determined in the usual manner. This fraction contained approximately 5% low-molecular weight material and 3 components which sedimented more rapidly than 19S (see Figure 15C). The concentration of the low-molecular weight material is too small to be significant (since its partial specific volume is undoubtedly close to that of most proteins). The faster-sedimenting components are aggregates of the 19S component, as will be shown shortly; hence they presumably have the same density as the 19S component. Therefore, the partial specific volume of the total protein in this solution is very close to the true partial specific volume of the 19S component alone.

The densities of a dialyzed solution containing 4.95 mg. TCA-precipitable weight per ml. and of the buffer solution against which it was dialyzed were determined at 25.00° C. in a pycnometer of approximately 15 ml. capacity. From these data, the value of $\bar{V}$, calculated according to the method of Koenig (1950), was found to be 0.69_1.

This value of $\bar{V}$ is low for a protein but is in the range expected for a nucleoprotein since nucleic acids possess high densities. The particular preparation used for this determination contained 11.2% nucleic acid, as determined either by direct

nucleotide analysis or computed from TCA-P content. Using the value 0.52 for the partial specific volume of ribonucleic acid (Koenig, Larkin and Perrings, 1952), the partial specific volume of the protein moiety alone is computed to be 0.72. This value is lower than that of most proteins but is not unprecedented (cf. Cohn and Edsall, 1943, p. 428).

Probable Molecular Weight of the 19S Component

The molecular weight of proteins may be determined from the expression

$$M = \frac{\underline{f}\ \underline{s}_{20,w}}{(1-\bar{V}\rho)} \qquad \text{(Eqn. 1)}$$

where M is the anhydrous molecular weight, $\underline{f}$ the molar frictional coefficient, $\underline{s}_{20,w}$ the sedimentation constant extrapolated to zero concentration and corrected to the viscosity and density of water at 20° C., $\bar{V}$ the partial specific volume of the anhydrous protein, and ρ the density of the solvent. The use of this equation requires experimental determination of $\bar{V}$, $\underline{s}$ and $\underline{f}$.

Determination of $\bar{V}$ was described above.

The sedimentation constant, $\underline{s}$, was determined for a number of preparations and is essentially constant over a wide range of concentrations (Table XIX). Thus the value at zero concentration may be taken as the average value for a sample at several concentrations. Since $\bar{V}$ was determined for sample E-20

and this value, as well as the sedimentation constant itself, will depend to some extent upon the RNA content of the sample characterized, the value of $\underline{s}$ obtained with this same sample will be used. From Section E, Table XIX, this is seen to be 19.0S.

Normally, in order to determine $\underline{f}$, independent diffusion experiments are performed and $\underline{f}$ is obtained from the Einstein relation:

$$\underline{f} = \frac{RT}{D} \qquad \text{(Eqn. 2)}$$

where D is the diffusion constant extrapolated to zero concentration, R is the gas constant and T the absolutetemperature. For Fraction I protein solutions, diffusion measurements are at present impractical for two reasons: (1) the aggregates of Fraction I protein usually present in these preparations seriously complicate diffusion constant determinations (whereas they do not materially affect measurements of sedimentation constants); and (2) the instability of solutions of Fraction I protein (which is similar to the instability of the 19S component in solutions of whole cytoplasm) makes lengthy diffusion experiments impossible. The Fraction I protein molecules, however, are essentially spherical in shape. For spherical molecules, or molecules of known axial ratio, $\underline{f}$ may be evaluated without recourse to diffusion experiments.

In the case of spherical, unhydrated molecules, $\underline{f}$ is equal to the molar frictional coefficient, $\underline{f}_o$, calculated from Stokes'

equation:

$$\underline{f}_o = 6\pi\eta N \left(\frac{3M\bar{V}}{4\pi N}\right)^{1/3} \qquad \text{(Eqn. 3.)}$$

where η is the viscosity of the solvent in poise and N is Avogadro's number. Combined, equations 1 and 3 simplify to:

$$M_o = \frac{\underline{f}_o \, \underline{s}_{20,w}}{1 - \bar{V}\rho} = \left[\frac{6\pi\eta N \left(\frac{3\bar{V}}{4\pi N}\right)^{1/3} \underline{s}_{20,w}}{1 - \bar{V}\rho}\right]^{3/2} \qquad \text{(Eqn. 4)}$$

where M_o is the anhydrous molecular weight. Using $\bar{V} = 0.69$ and $\underline{s} = 19.0$S, the molecular weight of Fraction I protein calculated from this equation is 315,000.

In arriving at this molecular weight, the effect of hydration, which will increase the frictional coefficient, was ignored. The ratio of the frictional coefficient of ahydrated molecule of any shape to the frictional coefficient of an equivalent unhydrated spherical molecule may be written as $\underline{f}/\underline{f}_o$. Eqn. 1 may be rewritten as:

$$M = \frac{(\underline{f}/\underline{f}_o)\, \underline{f}_o \, \underline{s}_{20,w}}{1 - \bar{V}\rho} = (\underline{f}/\underline{f}_o)\, M_o \qquad \text{(Eqn. 5)}$$

The degree of hydration of Fraction I protein is unknown. If it is assumed to be hydrated to the extent of 40% by weight, the value of $\underline{f}/\underline{f}_o$ is 1.15 (Oncley, 1941). The molecular weight, from Eqn. 5, is 360,000. This should be regarded as a good first

approximation to the true molecular weight of Fraction I protein, and refinements of this value must wait upon the preparation of stable solutions containing no aggregates.

Electron Microscopy of Fraction I Protein Solutions

On the basis of the density and molecular weight determinations reported above, the diameter of an anhydrous molecule of Fraction I protein was computed to be 90Å. This basic diameter is little affected by changes in hydration, for a 40% increase in molecular volume only raises the diameter to 100Å. Since the ultimate resolution of most electron microscopes is in the order of 20Å (Williams, 1952), little detail will be seen in an object of 90Å diameter. Examination of Fraction I protein solutions in the electron microscope should, however, give information about the general size and shape of the molecule.

For examination, approximately 1% solutions of Fraction I protein were diluted 1:10,000 with 0.05 M K-maleate buffer, pH 7.0, and sprayed onto collodion coated screens.* The screens were air-dried and shadowed with Cr. Examination of these air-dried screens showed a large number of particles *ca.* 200Å in diameter. Only occasionally were particles somewhat less than 100Å in diameter seen. It was obvious, though, that excessive clumping had occurred during drying and there were strong indications that the 200Å particles were clusters of smaller subunits.

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* The screens were prepared and examined by Dr. E. Henderson of the Chemistry Division of the Institute.

To determine if this were so, screens sprayed with a 1:500 dilution of the protein solution and freeze-dried according to the Williams technique (1953) were shadowed with a gold alloy and examined. The results were disappointing in that less than 1°/o of the grids had any material whatever on them, apparently as a result of losses during lyophilization of all except the initial layer of sprayed material. With such dilute solutions, the initial layer is almost entirely water, with only an occasional protein molecule. The few particles seen seemed to be about evenly distributed between spheres of 200Å and spheres of ca. 90 - 100Å diameter. Further work has not been possible, but it is believed that with the proper techniques, electron microscopy will verify the size and shape factors arrived at from other physical measurements.*

Components of Higher Molecular Weight Than Fraction I Protein

In the general survey of the soluble cytoplasmic proteins which was discussed in Part I, a small percentage of a component sedimenting faster than Fraction I protein was found in four species (tomato, Turkish tobacco, gherkin and pea) out of the eight species

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* Dr. Morris Cohen and Dr. Sam G. Wildman of the Botany Department of U.C.L.A. have also found (personal communication) a number of spherical particles of ca. 90 - 100 Å diameter in Fraction I protein preparations.

examined (see Figure 3). No component with a sedimentation constant greater than 19S was observed in spinach whole cytoplasm.

The absence of a component with a higher molecular weight than Fraction I protein in spinach cytoplasm, even when examined in the ultracentrifuge at concentrations as high as 1.5% total protein, has been confirmed several times. As may be seen, however, in Figures 11 and 23 and from Table XIX, there is a small amount of a 26S component contained in Fraction I protein solutions prepared from spinach. The work performed with spinach cytoplasmic proteins strongly suggests that not only in spinach but also in other species where a 26S component is initially present in whole cytoplasm, these faster-sedimenting components are aggregates of the Fraction I protein. That this may be so is suggested by the following argument:

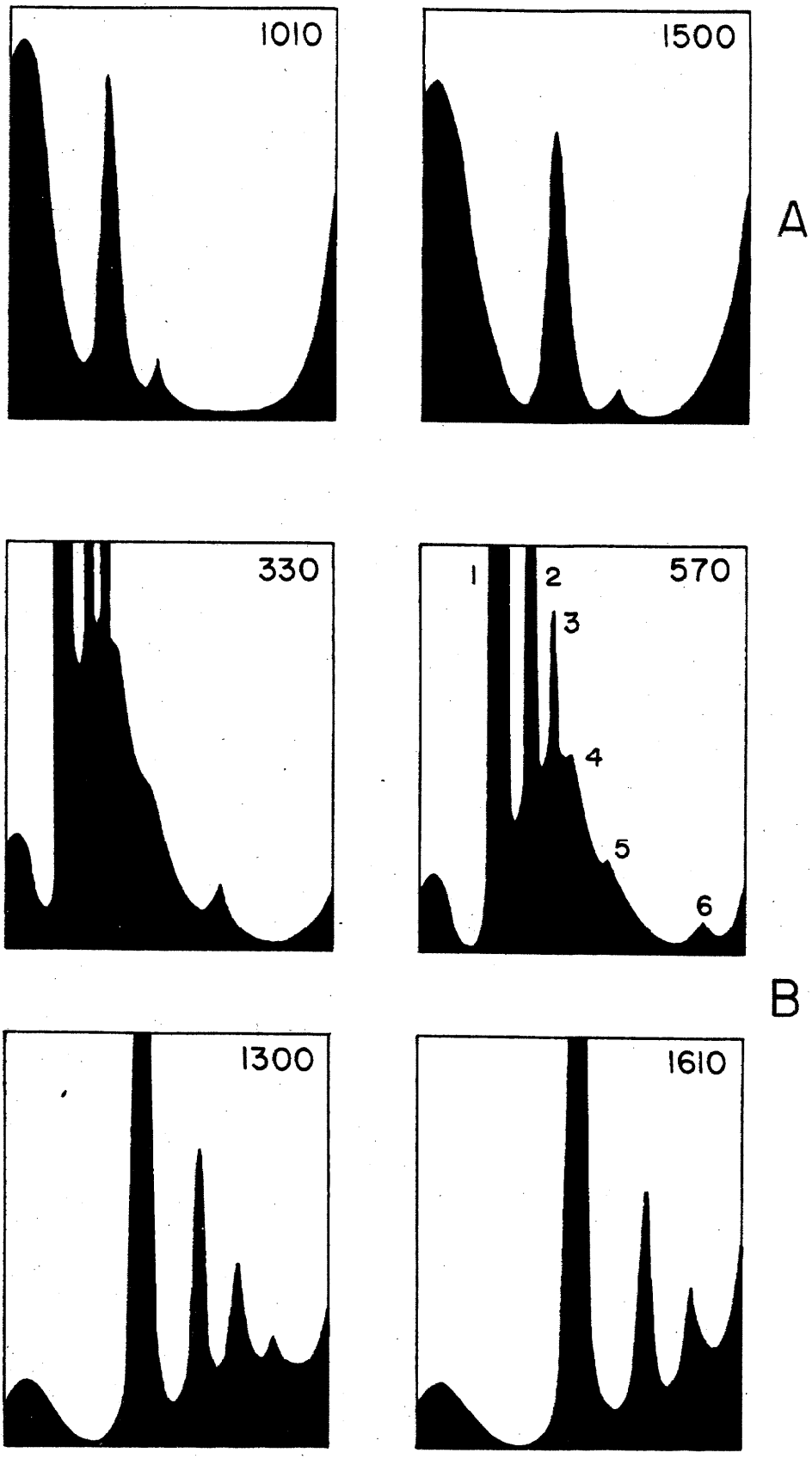
As little as 0.1 mg./ml. of the 26S component may be observed in Fraction I preparations. Failure to find the 26S component in spinach cytoplasmic protein solutions at 15 mg./ml. total protein suggests that it must constitute less than this minimal amount, or less than one part in 150 in such preparations. This means that the ratio of 26S to 19S component must be less than 1:75 in whole cytoplasm solutions containing roughly 50% Fraction I protein. Simple enrichment of the 26S component during two cycles of centrifugation should result in a product containing only 1 part of 26S protein to 59 parts of 19S protein. Area measurements

of twice sedimented Fraction I protein preparations show, however, the presence of approximately 1 part of 26S to 10 parts of 19S protein. It is evident, therefore, that the 26S component must have been formed during the isolation procedure and has not merely been enriched.

All preparations of Turkish and Maryland Mammoth tobacco cytoplasm examined have had a 26 - 28S component. In Figure 16 is reproduced the UC pattern of a Turkish tobacco Fraction I protein preparation and also that of the solution of whole cytoplasm (F-6) from which it was prepared. Although the whole cytoplasm contained Fraction I protein and one faster sedimenting component, the purified Fraction I protein preparation contained not only these two components, but also four others which sedimented even more rapidly. The corrected sedimentation constants of Fraction I protein and the first two of the components with a higher molecular weight are 18.0, 25.7 and 30.7, respectively. The sedimentation constants of the other three components could not be determined accurately from the material at hand. Of these six components, only two, those of 18S and 26S, were present in the whole cytoplasm. The other four were formed during the fractionation procedure. This particular preparation represents the most extreme aggregation yet observed, but all Fraction I protein preparations made from any variety of N. tabacum have had at least two components sedimenting faster than does Fraction I protein itself.

Figure 16. Sedimentation behavior of a Maryland Mammoth whole cytoplasm preparation (A) and the Fraction I protein preparation (B) made from it, illustrating the extreme aggregation sometimes observed. In the whole cytoplasm there was only a small amount of one component moving faster than 19S; in the Fraction I preparation there are five components moving faster than 19S. The resolution of these is shown in B. Numbers by the peaks indicate the 19S component (1) and higher molecular weight aggregates (2,3,4,5,6) which are multiples of the 19S units. Rotor speed 850 r.p.s. A, in 0.3 M K-maleate buffer, pH 6.9; 8.6 mg. protein/ml. B, in K-maleate buffer, pH 7.5, 0.1 μ; 21.6 mg. protein/ml.

Figure 16



If we consider the three components characterized by sedimentation constants of 18 - 19S, 26 - 28S and 31 - 35S, the values of these constants are consistent with the hypothesis that these components are related as monomer, dimer and trimer, respectively. Furthermore, it is noteworthy that the latter two components, while exhibiting more variation in sedimentation constant from preparation to preparation than does Fraction I protein, nevertheless show but little more dependence of sedimentation constant upon total protein concentration than does Fraction I protein itself. This is also consistent, since dimers and trimers of spherical molecules are relatively symmetrical molecules and should show little dependence of their sedimentation constants on concentration.

The only factor so far correlated with the degree of aggregation is the TCA-P content of the Fraction I protein preparations. Fraction I preparations made from a whole cytoplasm which contain less than about 1% TCA-P/mg. protein, as is generally the case with spinach, also have a low TCA-P content, and contain only a small amount of the 26S component. Tobacco cytoplasmic protein preparations from greenhouse grown plants, on the other hand, generally have a much higher TCA-P content. The Turkish tobacco cytoplasms used to make the preparations of Fraction I protein, for which data are given in Sections C, D and E, Table XIX, contained 2.74% ,2.89% and 3.89% TCA-P/mg. protein, respectively, (Table XVI). The corresponding Fraction I

preparations were highly aggregated (Figures 16B, 15B and 15C, respectively).

Further evidence that extensive aggregation, such as that shown in Figure 16B, is caused during centrifugal fractionation, and that for analytical purposes such aggregates should be treated as Fraction I protein, is illustrated by the UC runs shown in Figure 17. These runs were made in the synthetic boundary cell in order to estimate the amount of the low-molecular weight material present; hence the resolution of the high-molecular weight components is too poor to permit their quantitative estimation, but rough visual comparisons among the several runs may be made.

The original whole cytoplasm preparation (Turkish tobacco) contains a large amount of the 19S component and a small amount of the 26 - 28S component (Figure 17A). No component sedimenting faster than 26-28S is visible. The product at the end of the first fractionation cycle (Figure 17B) contains, in addition to 15% low-molecular weight material, approximately the same amount of 19S protein as in the original whole cytoplasm (as seen from the similarlity in areas under the 19S component peak in the two fractions). The relative amount of the 26 - 28S component has been increased over that present in the original cytoplasm, but there is no evidence of a discrete peak sedimenting faster than 26 - 28S in this fraction. In the final product of the second fractionation cycle (Figure 17C), the low-molecular weight material

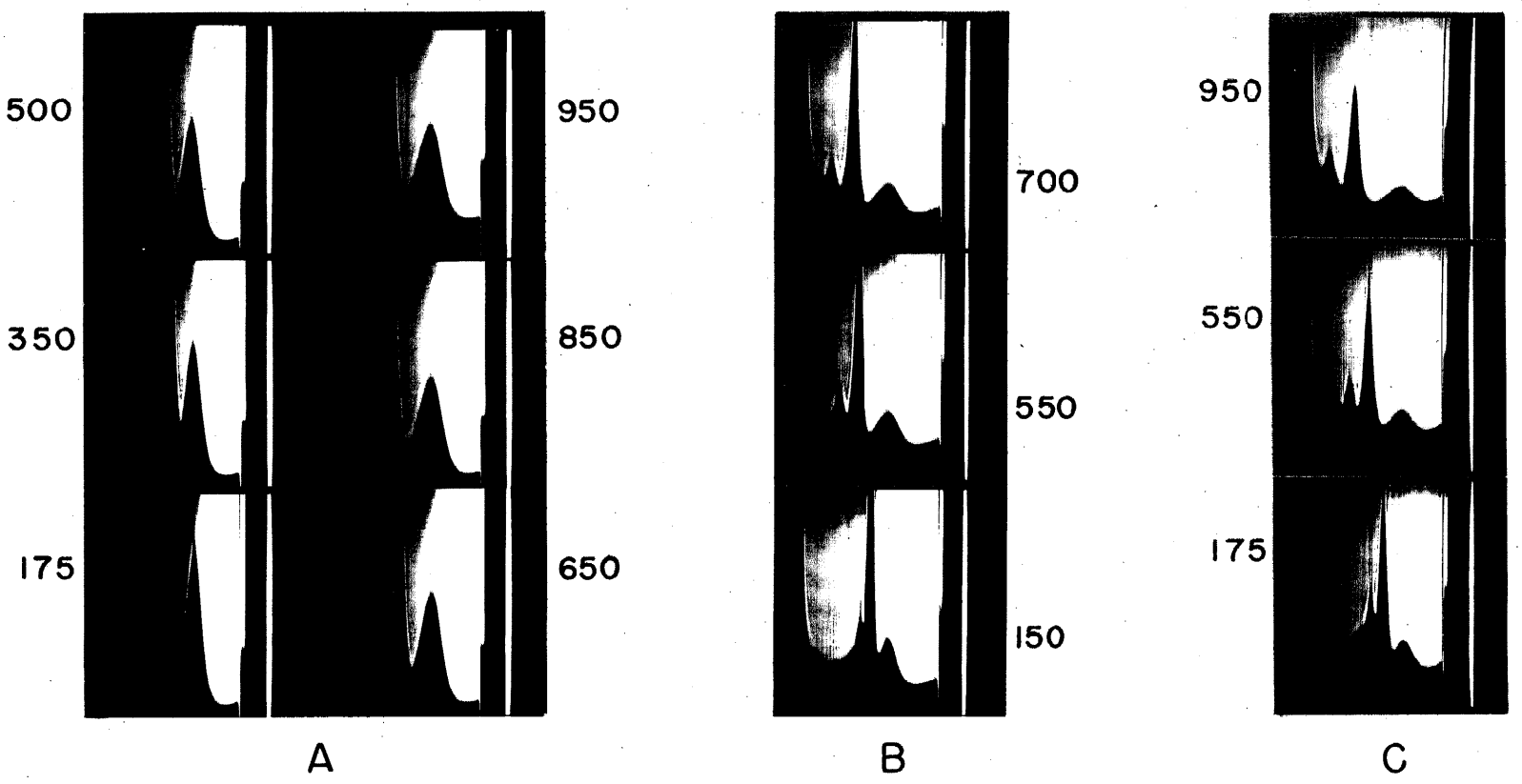


Figure 17. Sedimentation behavior of a typical preparation of Turkish tobacco whole cytoplasm and the one- and two-cycle Fraction I protein preparations made from it. Sample run in synthetic boundary cell. Solvent, K-maleate buffer, pH 7.0, 0.1 μ. Sedimentation proceeds from right to left. A. Whole cytoplasm; protein concentration, 10.2 mg./ml.; rotor speed, 851 r.p.s. B. Product of first fractionation cycle; 8.1 mg./ml.; 851 r.p.s. C. Product of second fractionation cycle; 7.3 mg./ml.; 854 r.p.s.

is decreased to only ca. 10 - 11°/o of the total protein. The relative amount of the 26 - 28S component is considerably greater than it was in the one-cycle product and, for the first time, a discrete peak sedimenting at ca. 34 - 36S is visible. The appearance of this new component and the increase in amount of 26 - 28S component in this fraction can be interpreted only as aggregation phenomena.

The evidence available at this time indicates that this aggregation results from the close packing of the nucleoprotein molecules during the second fractionation cycle. Once formed the aggregates apparently are stable to the extent that they do not break down even over several days in dilute solutions kept at 0° C.

IV. DISCUSSION

Fraction I protein preparations have been made from spinach, pea and tobacco leaves and as far as can be ascertained at present, there is a striking similarity in this protein in all three species. Variations in the TCA-P content and sedimentation constant are no greater among preparations made from three genera of plants than among different preparations made from the same species. If generic or species differences exist, their detection will depend on more refined techniques, such as detailed nucleic acid and amino acid analyses, rather than from the gross chemical and physical chemical analyses of the type utilized in the studies reported here.

Variation in TCA-P Content

Preparations of Fraction I protein exhibit some variation in the amount of TCA-P associated with the protein moiety. Such preparations generally contain 0.4 - 0.7% TCA-P, with 1.4% the maximum amount found as yet. Evidence has been presented in Part I of this dissertation to show that all of the TCA-P in whole cytoplasm is contained in RNA and evidence presented in Section III above strongly suggests that all of the RNA is properly associated with Fraction I protein. It follows then that the RNA content of the nucleoprotein preparations is primarily dependent on the amount of TCA-P initially present in whole cytoplasm. The latter has

been found to vary with the phosphorus nutrition of the plant and, as indicated in Section V, Part I, with the physiological age of the material, but the influence of these factors has not yet been fully assessed.

An additional source of variation in the RNA content of the nucleoprotein is enzymatic destruction of the RNA initially present in whole cytoplasm. Cytoplasmic protein solutions were found to contain phosphatases and ribonuclease. These two classes of enzymes are capable between them of rapidly degrading nucleic acid to a mixture of ribosides, inorganic phosphate and resistant polynucleotides. These same enzymes readily destroy the TCA-P present in whole cytoplasm solutions. Some destruction occurs even at 0° C. The RNA content of the isolated nucleoprotein, therefore, depends somewhat upon the rapidity with which the preparation is made and the care taken to keep it at a low temperature at all times.

What Has Been Characterized in the Ultracentrifuge?

Considerable emphasis has been placed upon the lability of the RNA moiety of the Fraction I protein molecule. Because of this lability, which is greatly aggravated at the temperatures maintained during analysis in the ultracentrifuge, the question arises as to what has been characterized in the ultracentrifuge -- a nucleoprotein, or the protein moiety alone? The TCA-P content of whole cytoplasm samples which have been dialyzed is very low due to loss of TCA-P during dialysis (cf. Table VII). Analysis of

dialyzed cytoplasms following incubation at 25° C. for 90 minutes (the time required to set up and complete a normal centrifuge run) indicates a loss under these conditions of only 10 - 20°/o of the TCA-P present. Thus, in the case of dialyzed cytoplasms it is fairly certain that at the end of a UC run the 19S component still contains nearly all of the RNA initially present. This amount, however, is so low (usually in the order of < 1°/o) that the physical properties of the 19S component are essentially those of the protein moiety alone rather than those of a nucleoprotein containing a high percentage of nucleic acid. It would seem, therefore, that in dialyzed whole cytoplasms, the protein moiety alone has been characterized.

Experiments on the lability of the TCA-P in twice-sedimented Fraction I protein preparations indicate that in general the TCA-P in these preparations is much more stable than in whole cytoplasm preparations. For example, only 5 - 10°/o loss of TCA-P occurs in solutions kept at 0° C. for 24 hours, and the loss during 24 hours dialysis at 0° C. is only 20 - 30°/o of that initially present. Considerable variation in the amount of TCA-P lost during incubation at 25° to 37° C. has been observed. This variation is roughly correlated with the enzymatic activity of each particular preparation. Thus, fraction Fr I_2 from sample E-20, which contained a high phosphatase activity (and presumably a high ribonuclease activity as well) lost ca. 40°/o of the TCA-P initially present during two hours incubation at 30° C. whereas a similar

fraction from sample E-8, which had a very low enzymatic activity, lost only 5 - 10°/o of its initial TCA-P under the same conditions.

In Fraction I protein preparations which were not dialyzed prior to UC analysis, the only loss of TCA-P which occurred was that due to enzymatic activity during the actual run at 20 - 25° C. For most samples, this is in the order of 5 - 20°/o of that initially present. The RNA content of the 19S component in such preparations is, therefore, much greater than the RNA content of the 19S component in dialyzed whole cytoplasm solutions. This difference in RNA content of the nucleoprotein molecule should be reflected in a difference in the density and perhaps in the size of the 19S component. These latter differences should in turn be reflected in a different sedimentation rate for the 19S component in the two preparations.

The magnitude of the change to be expected in $\underline{s}$ may be estimated from the basic equation for molecular weight cited in Section III above:

$$M = \frac{\underline{f}\ \underline{s}}{(1 - \bar{V}\rho)} \qquad \text{(Eqn. 1)}$$

If $\underline{f}$ and M are assumed to be constant, $\underline{s}$ should decrease $\underline{ca}$. 10°/o between the extreme cases where the nucleoprotein contains 11.2°/o RNA and the protein moiety alone, for the corresponding values of $\bar{V}$ are 0.69 and 0.72, respectively. Loss of TCA-P, however, must necessarily decrease not only the density of the nucleoprotein moiety but also its molecular weight. The decrease in $\underline{s}$ will

thus be even greater than the 10°/o computed on the assumption that both f and M are constant. The data in Table XIX indicate, however, that the sedimentation rates of the 19S component in whole cytoplasm and in the corresponding Fraction I protein preparations are, within ± 2 - 3°/o experimental error, the same for all preparations regardless of the RNA content.

In order for s to remain constant while both M and $\bar{V}$ change, the value of f must decrease. Returning to our previous example of a nucleoprotein containing about 11°/o RNA, loss of the RNA moiety will decrease the molecular weight roughly 10°/o. On the basis of $\bar{V}$ = 0.69 and M = 1 for the nucleoprotein and $\bar{V}$ = 0.72 and M = 0.9 for the protein moiety alone, the value of f for the protein without any nucleic acid attached must be only 81.5°/o of that of the nucleoprotein if s is constant. If the interpretation of Singer (1947) that concentration dependence of s is a function solely of the asymmetry of the molecule is correct, then the shape factor contribution to f is minimal and remains constant. This necessarily follows from the observation that s is independent of concentration regardless of the RNA content. A decrease in f, therefore, may be caused only by a decrease in the effective particle volume. Such a decrease in volume may be due to a decrease in the size of the molecule, as though removal of RNA occurred from the surface of the molecule or caused a shrinkage in some other manner, or it may be due to a change in hydration. Undoubtedly both of these factors enter, for a 18.5°/o decrease in f is equivalent

to decreasing the hydration from 130°/o by weight to 40°/o by weight (Oncley, 1941).

The changes which occur in the size and shape of the nucleoprotein as the nucleic acid is lost are amenable to attack by light scattering techniques provided the preparations can be "cleaned up" and the aggregates eliminated. Certainly elucidation of these changes is an interesting aspect of the characterization of Fraction I protein which should be further investigated.

On the basis of the present work, however, it appears that in purified Fraction I protein preparations, the nucleoprotein itself, though admittedly of somewhat variable nucleic acid content, has been characterized in the UC. This variability in nucleic acid content is relatively unimportant for the determination of $\underline{s}$. For the determination of the molecular weight of any particular sample, however, it is necessary to know the value of both $\underline{s}$ and of $\overline{V}$ for that sample. Thus, the anhydrous molecular weight, 315,000, determined without regard to hydration effects, is not the molecular weight of any sample of Fraction I protein but is the molecular weight of a particular sample which contains 11.2°/o nucleic acid.

PART III

THE BIOLOGICAL ROLE OF THE NUCLEOPROTEIN COMPONENT

It is reasonable to expect that a nucleoprotein which constitutes from 30 to 50 per cent of the total soluble protein in so many plant species should have an important role in the economy of the cell. The exact nature of this role is unknown at the present time, but one may make a few suggestions, based in part on analogy with other systems. Some evidence is also available as to what the role of the leaf nucleoprotein cannot be.

Wildman and Bonner (1947) showed that phosphatase activity was the only enzymatic activity associated with a salt precipitated fraction, a fraction which has been found during the present studies to be largely the 19S nucleoprotein component. But on the basis of experimental work outlined above in Section I-C, Part II, the phosphatase activity must be assumed to be separable from the 19S component. Therefore, the nucleoprotein does not possess any of the common enzymatic activities. Indeed, that any one enzyme should constitute so preponderant a share of the total protein is a radical departure from current concepts of enzymatic catalysis.

That the nucleoprotein component may under some conditions function as a "protein pool" is suggested by two different types of experiments. Axelrod and Jagendorf (1951) found that during a period

when detached leaves under starvation conditions lost 50 per cent of the total soluble proteins, the content of several enzymes (catalase, invertase and phosphatase) remained constant. This suggests that the level of protein, the enzymes, directly concerned with metabolic activity is maintained at the expense of other proteins. Although these authors did not specifically estimate the amount of 19S protein in their preparations, it is apparent that the nucleoprotein is the only component or fraction which may be reduced sufficiently to decrease the total protein content 50 per cent without affecting enzymatic activity. Thus, the nucleoprotein may act as a "bulk protein" or a "protein pool" in the sense that during times of stress it is more labile than the enzymatically active proteins.

Further evidence for such a possible function comes from investigations with virus infected plants. It has been established by Bawden and Kassanis (1950) and by others that during the course of infection of tobacco leaves by tobacco mosaic virus, the amount of non-virus protein decreases. Wildman, Cheo and Bonner (1949) showed that this decrease was primarily in the soluble cytoplasmic protein fraction, and that as the amount of virus increased the principal electrophoretic component of cytoplasm showed a proportionate decrease. Since the major portion of the principal electrophoretic component is the nucleoprotein moiety, these results of Wildman, et al. are again indicative that under stress the nucleoprotein is more labile than are the other protein components.

Experiments by Meneghini and Delwiche (1951), by Wildman and Jagendorf (1952b), and by others, in which the incorporation of an N^{15} label from ammonia into virus protein and into several other fractions of the normal soluble proteins was followed, indicate that the label is incorporated into virus protein more rapidly than into any fraction of the normal proteins. Thus, if virus is synthesized at the expense of the nucleoprotein, it appears most likely that the nucleoprotein acts as a "protein source" and is degraded before being converted into virus protein. The interpretation of the N^{15} experiments is still controversial, however, and further investigation is necessary to establish the mechanism of the transformation.

That the nucleoprotein may if necessary act as a "protein pool", thus seems reasonably well established. Starvation and virus infection are, however, abnormal occurrences in the normal life of a cell. We may, therefore, expect that normally this major protein component has a more fundamental role in the cell and that its functioning as a "protein pool" during times of stress is incidental.

A commonly proposed role of cytoplasmic nucleoproteins per se is in protein synthesis. This aspect of Fraction I protein was discussed in Section V, Part I. Evidence presented there indicated, however, that in rapidly expanding leaves where the rate of protein synthesis should be unusually rapid, the amount of nucleoprotein, on either an absolute or on a relative basis, is less than in mature leaves. Similar results were obtained when the experi-

ment was repeated with a different set of plants. The significance of these results, which apparently contradict analogous experiments with liver tissue and microbial cells, is not fully understood, but one possible interpretation is that this particular nucleoprotein is not involved in protein synthesis.

No further experimental evidence on which to base hypotheses is available from work with Fraction I protein directly. On the basis of recent work with muscle, nerve and protozoa cells, however, it is very tempting to speculate on a possible role of the 19S component in one of the most important and fundamental of all processes in the cell -- that of ion-uptake.

In two recent provocative reviews, Goldacre (1952) and Ling (1952) have independently advanced the speculative hypothesis that reversible, energy-requiring changes in the configuration of protein molecules are responsible for the binding and release of ions, and have cited experimental data obtained with muscle, nerve and amoeba cells in support of their arguments. The arguments of Ling are particularly convincing, for his hypothesis, which is based on apparently sound physical chemical data, is capable of explaining all observed experimental facts in connection with ion-uptake and accumulation in muscle or nerve cells without recourse to postulating a selectively-permeable membrane, a lipid-protein mosaic membrane, or an "activated ion-complex" to account for the specificity of both anion and cation accumulation. Attractive as the hypothesis of Ling is, it does not provide for accumulation of higher con-

centrations of solutes within the vacuoles of protozoan and plant cells than in the external medium or in cytoplasm. Indeed, Ling does not even consider accumulation within vacuoles. That vacuolar accumulation does occur is evident from dye accumulation studies cited by Goldacre (1952). To explain this phenomenon, Goldacre retains the concept of "relative impermeability" of the vacuole-protoplasm interface.

There is nothing in the hypotheses of either Goldacre or Ling to implicate nucleic acid or a nucleoprotein with ion accumulation. A protein moiety alone is all that is required to qualitatively explain the observed specificity. Lansing and Rosenthal (1952) have, however, indicated that a nucleoprotein may be involved in ion-uptake. These authors demonstrated rather convincingly that in Elodea leaves, the uptake of calcium or strontium ions, as measured by formation of oxalate crystals intracellularly, is greatly inhibited by brief exposure of the leaves to the action of ribonuclease. By histochemical and cytological techniques, these authors were able to demonstrate a peripheral layer of basic-staining material immediately below the hyaline layer in Arbacia eggs. This basophilic layer was presumed to be ribonucleic acid or a ribonucleoprotein, for it was not present following treatment with ribonuclease. Ribonucleic acid was, therefore, implicated in ion-uptake by extrapolation from a demonstrable loss of ribonucleic acid in Arbacia eggs to a presumed loss of ribonucleic acid in ribonuclease treated Elodea cells which would no

longer accumulate ions.

If ion-uptake and accumulation in the cell does in fact occur in accordance with either of these hypotheses, it is quite possible that the 19S component characterized herein is responsible for ion accumulation activity in plant cells. This hypothesis is especially attractive since both nucleic acid chelating properties and protein-ion binding capacity are combined in a single moiety. With our present knowledge concerning preparation of Fraction I protein solutions and the techniques available for studying ion-binding, the hypothesis that Fraction I protein is implicated in ion accumulation should be subject to experimental study.

APPENDIX I

DETERMINATION OF THE CONCENTRATION OF TRICHLORACETIC ACID PRECIPITABLE PHOSPHORUS (TCA-P) AND TOTAL PROTEIN IN CYTOPLASMIC PROTEIN SOLUTIONS

The choice of a protein precipitating agent for quantitative work is a difficult one. The ultimate choice depends largely on: (1) the method used to determine the amount of protein subsequent to precipitation (e.g., direct weighing, or analysis, as for a component group or for total nitrogen), (2) the molecular size distribution of the proteins in solution and how low a molecular weight is considered to be a protein, and (3) the non-amino acid constituents of the proteins and whether or not analyses for them are desired. At best, any procedure and any precipitant used is largely arbitrary, and although the method may be capable of a high degree of precision the absolute accuracy is equivocal.

Kirk (1947) has recently reviewed the chemical methods for determination of proteins and concluded there is no adequate comparison of methods and procedures in the literature. If the "deproteinization" methods which are designed to eliminate protein and perhaps other interfering substances in a particular analysis for non-protein material are excluded, the quantitative precipitants of choice seem to be trichloracetic acid (TCA), metaphosphate, or possibly perchloric acid [Briggs (1940), Neuberg and Strauss (1945), Neuberg, et al. (1944)]. Since it was desired to analyze

the precipitated material for phosphorus, the use of metaphosphate, which is tightly bound to the protein in an amount approximating the charge on the protein molecule (Briggs, 1940), is eliminated. Because it was thought that weighing of the precipitate would be more accurate and convenient on a routine basis than any other method of determining the amount of precipitated protein, the precipitant had to be volatile at 105° C., the drying temperature used. Both TCA and perchloric acid fulfill this requirement but due to the difficulty in handling and the corrosive properties of perchloric acid, TCA was the precipitant of choice.

Strangely enough, there are no good comparisons in the literature of the effectiveness of TCA as a protein precipitant as a function of final TCA concentration and temperature of precipitation. Hiller and Van Slyke (1922), in a study of a number of acidic precipitating agents found 2.5°/o TCA to be superior to other concentrations of TCA for precipitating a mixture of ox blood proteins and "Witte's peptone". Precipitations were made at room temperature and the precipitates were filtered off immediately. The superiority of 2.5°/o TCA lay in how little of the "peptones" were precipitated in comparison with other concentrations of TCA and with other reagents. No study of time and temperature as variables was made.

It was early observed in the studies reported here that the rapidity of precipitation, and hence completeness of precipitation in a given time, was a function of protein concentration

and the temperature at which the mixture was kept, although no precise data were taken. Because of the solubility of TCA-P in warm TCA, all precipitations were performed at 0° C. and the mixtures of protein and TCA kept at that temperature until precipitation was complete. If the protein concentration was fairly low (< 0.15%), flocculation was slow in comparison with more concentrated solutions. Two criteria were used to judge completeness of precipitation. Both were based on formation of additional precipitate after removal of the initial precipitate. One method consisted of centrifuging down the precipitate (at about 2° C. in a conical 12 ml. centrifuge tube) formed in 1 hour, then allowing the clear supernatant solution, still in contact with the precipitate, to remain at 0° C. for an additional period of time. Periodically this solution was observed for formation of additional precipitate, which would be deposited on the conical sides of the centrifuge tube and as a loose flocculum on top of the previous precipitate. The other method consisted of removing by centrifugation the precipitates formed at the end of several time periods, decanting the supernatant solutions and heating them to 80° C. for 10 minutes. This hastens formation of any additional precipitate.

When solutions of tobacco cytoplasmic proteins at about 0.7 - 1.0% protein concentration are precipitated with 2 volumes of 1.0 N TCA (0.67 N final concentration) at 0° C. and held at that temperature, precipitation appears to be complete in 6 to 8 hours,

as judged by failure to form additional precipitate at 0° C. If, however, the supernatant solution after 8 hours at 0° C. is heated to 80° C., a slight additional precipitate is formed. When the initial TCA-protein mixture is kept at 0° C. for 24 hours before centrifugation, heat treatment of the supernatant solution will not cause additional precipitation, indicating "complete" precipitation. If on the other hand, the TCA-protein mixture is centrifuged immediately upon mixing, the supernatant solution heated to 80° C. for 10 minutes, then cooled and centrifuged again to remove the flocculum formed during heating, no additional precipitate is formed upon standing for several days at 0° C. This indicates that precipitation is complete in a few minutes at elevated temperatures. Such heat treatment to hasten complete precipitation, which is widely used in some routine procedures, is unsuitable for the present study because of the solubility of TCA-P in warm TCA. This latter factor will be discussed below.

When the initial protein concentration is below about 0.15%, precipitation is much slower but also appears to be complete in 24 hours, at least in the case of tobacco cytoplasmic proteins. With the proteins from crown gall tissue cultures, however, the formation of additional precipitate after as long as 7 days at 0° C. has been observed. Thus, even though the precipitation of "total protein" is arbitrary, preliminary tests are necessary to insure that precipitation conditions are such as to obtain as complete precipitation as possible with the reagent employed and the protein system under study.

As detailed in the body of this thesis, there is a phosphorus-containing compound, identified as ribonucleic acid (RNA), which is precipitated with the protein. It is unfortunate that those conditions which tend to give the most complete precipitation of the proteins, e.g., prolonged treatment with cold TCA or heating to 80 - 90° C. for a few minutes, split the RNA from the protein moiety. Similar conditions of precipitation cannot, therefore, be used for both total protein and TCA-P analyses. This splitting of the RNA-protein linkage by acid is not completely unexpected. For example, Kaplan and Greenberg (1944) reported that cold TCA split rat liver nucleoprotein after long exposure (7 days) and Schneider (1945) proposed a procedure for quantitatively separating RNA and desoxyribonucleic acid (DNA) from protein moieties by heating with 5°/o TCA for 15 minutes. The latter author reported that splitting of RNA and DNA from proteins of rat liver and calf thymus tissue was complete in 8 minutes at 90° C. with 3 - 4°/o TCA. Steele, et al. (1949) modified the Schneider technique somewhat for the extraction of RNA and DNA from pollen grains in microquantities and Ogur and Rosen (1950) reported quantitative extraction of RNA and DNA from corn root tips and rabbit liver tissue by successive extractions with cold and hot perchloric acid. They reported complete extraction of all RNA by 1.0 N perchloric acid in 3 - 4 hours at 4° C. from a homogenate of corn root tips, and from rabbit liver homogenate in 6 hours. From one of their figures, though, it may be seen that 75°/o of the RNA

is removed from corn root tips in 1 hour and that extraction is virtually complete in two hours. Similar results were reported with 1.0 N TCA.

In the early part of the work reported here, the requirements for low precipitation temperature and a long period of contact with TCA to completely precipitate the protein and to obtain a fine, readily redispersed precipitate which could be easily washed were appreciated and complied with but it was assumed that the TCA-P was tightly bound to the protein and could be removed only by TCA, HCl or perchloric acid at elevated temperature (Wildman and Bonner, 1947). Accordingly, precipitates were frequently allowed to remain in contact with cold 0.67 N TCA for 48 - 60 hours, then centrifuged and washed at room temperature. TCA-P analyses of such samples were always consistent within themselves but were variable from replicate to replicate on different days. This variation was finally correlated with the time of contact with TCA and conditions of centrifugation. In fact, if the results of Ogur and Rosen were equally applicable to the nucleoprotein component studied here, it was fortunate that any TCA-P was ever found in the precipitate.

To test the applicability of the results of Ogur and Rosen to the cytoplasmic protein system, twenty seven, 2.00 ml. aliquots of a whole cytoplasm solution were pipetted into centrifuge tubes in an ice bath and precipitated with two volumes of ice-cold 1.0 N TCA. At various times after precipitation, sets of triplicates

were centrifuged and the precipitates washed twice with 4.0 ml. of ice-cold 0.5 N TCA. Two sets were also taken for TCA-weight, one at "0" time and one at 24 hours. Centrifugation was of five minutes duration in a centrifuge kept in a 2° C. cold room. Temperature rise during centrifugation was negligible. Except for the period of centrifugation, the mixtures were kept in an ice bath until after the last wash and transfer to digestion tubes. One set of triplicates at ca. 28 hours was also centrifuged and washed at room temperature to test the necessity of a cold room or a refrigerated centrifuge at this step. The results are presented in Table XX, from which it is evident that for determination of TCA precipitable weight, precipitation at 0° C. should proceed for at least 24 hours. By this time, however, about 10°/o loss of TCA-P had occurred. This loss of TCA-P also represents loss of TCA precipitable weight, but if it is assumed that a total of 15 mgs. of "protein" is precipitated and that 6°/o of this is RNA, 10°/o of which is lost in 24 hours, then the loss in weight is only 0.09 mg. This is much less than the experimental errors due to other causes and may be neglected.

In a somewhat similar experiment using perchloric acid at a final concentration of 0.67 N as the precipitant, the results were similar. The variation within a set of replicates, however, was greater than with TCA. This was thought to be due to the very definite granular character of the precipitate, which could not be redispersed and washed readily. The particles were quite spongy

TABLE XX

EFFECT OF PRECIPITATION TIME UPON WEIGHT AND TCA-P CONTENT OF CYTOPLASMIC PROTEIN PRECIPITATES

A. Weight of TCA precipitates kept in contact with 0.67 N TCA for various periods of time at 0° C.

Time of contact*	mg./aliquot	°/o increase
30 minutes	12.17	-
26 hours	12.67	4.0

B. Phosphorus content of precipitates kept in contact with 0.67 N TCA for various periods of time at 0° C.

Time of contact*	γTCA-P per aliquot	°/o loss
1 hour	57.0	-
3 hours	57.0	0
5 hours	56.4	1.0
10 hours	55.7	2.3
26 hours	51.7	9.3
28 hours**	48.8	14.4
49 hours	46.8	21.0

* Time of contact computed from addition of TCA during initial precipitation until completion of final wash.

** Centrifuged and washed at ca. 25° C., all other precipitates centrifuged and washed at 2° C.

and variable in size and undoubtedly contained varying amounts of non-TCA-P mechanically trapped within. Thus, it is apparent that the findings of Ogur and Rosen are not applicable to the cytoplasmic proteins studied here insofar as loss of RNA in a few hours is concerned. There is, though, a very marked loss of TCA-P over longer intervals. Hence in all the early work reported herein the results within any one experiment where the precipitates remained in contact with TCA for the same length of time are comparable, but comparison among different experiments may not be justified. Mention of this is made when necessary.

On the basis of the above findings, the procedure finally adopted is:

1. Add two volumes of ice-cold 1.0 N TCA to the samples and keep the mixture in an ice bath.
2. For TCA-P analyses, centrifuge at 2° C. and wash twice after 3 - 6 hours, using two volumes of ice-cold 0.5 N TCA for each wash.
3. For TCA precipitable weights, centrifuge and wash as above after 24 - 30 hours.

APPENDIX II

A BRIEF DESCRIPTION OF THE ANALYTICAL ULTRACENTRIFUGE AND A DISCUSSION OF THE PROBLEMS AND TECHNIQUES INVOLVED IN ESTIMATING THE CONCENTRATION OF COMPONENTS IN A POLYDISPERSE SYSTEM

A. Description of the instrument
- Rotor and cells
- Power supply
- Optical system
- Temperature measurement and control

B. Observation of concentration gradients within the cell
- The dn/dx, x diagram
- Calculation of area under dn/dx, x diagram from concentration data
- Relation of area to concentration
  - Dilution effect
  - Johnston-Ogston effect

C. Measurement of the area under the dn/dx, x curve
- Factors affecting base line position and shape
  - Compressibility of solvent
  - Window distortion
  - Sedimentation of buffer ions
  - Cecil-Ogston effect
  - Dilution effect
  - Backward diffusion
- Fixing the correct base line position

D. General discussion of estimation of concentration from area measurements

Systems in which resolution from the meniscus is complete

Systems in which resolution from the meniscus is incomplete

First method used--uncorrected relative areas

An absolute method

Direct calibration of machine from optical constants

Experimental determination of calibration factor

The synthetic boundary cell

Comparison of methods--experimental data

Recommended procedures

The analytical ultracentrifuge (UC) utilized for these studies was designed and built at the California Institute of Technology. Inasmuch as no description of it has been published, a brief synoposis of the mechanical and optical features employed will be presented here. A discussion of the principles involved in determination of concentration gradients within the cell and an evaluation of the factors which affect the base line position and apparent concentration of a component will also be presented because this material is not conveniently available in the literature. Lastly, several methods used for determining the concentration of a component in a mixture will be discussed and evaluated, and a procedure recommended for analysis of the cytoplasmic protein mixture.

A. DESCRIPTION OF THE INSTRUMENT

The rotor and cells were manufactured by the Specialized Instrument Corporation, Belmont, California, and are identical with those employed in the Spinco Model E Ultracentrifuge. The distance from the center of the elliptical-shaped rotor to the top edge of the compartment in which the sample is placed is 5.74 cm. in the conventional cell. In order to eliminate convection currents caused by material reflecting from the walls, the sample compartment is sector shaped, the angle subtended being 4°. A "dummy" cell is used in the opposite side of the rotor as a balance.

The rotor is suspended inside an armored and insulated chamber on a 0.10" steel piano wire which is directly coupled to a synchronously driven electric motor. The power supply consists of an audio oscillator, a phase splitter and two power amplifiers, together with appropriate regulating equipment. Acceleration and deceleration are obtained by manually adjusting the frequency of the oscillator. The speed at any time may be determined by matching the frequency of the oscillator with the speed of the rotor, using an oscilloscope to detect synchronism. Since the oscillator is accurately calibrated, the speed of the rotor may be determined to a high degree of accuracy.

The optical system employs conventional schlieren optics, except that parabolic mirrors, as in the Swingle electrophoresis apparatus (Swingle, 1947), are used in place of lenses. The knife edge is permanently fixed at 45°, but the entry slit may be rotated to achieve the same result as is usually obtained by rotating the knife edge in other schlieren optical systems. The cylindrical lens of Philpot (Philpot, 1938) is utilized to give a dn/dx, x diagram directly from the concentration gradients in the cell. The schlieren diagram is visualized on a viewing screen which is also equipped with a camera back that accepts a standard 3 1/4" and 4 1/4" Graflex plate holder. The incident beam is suitably masked so that six pictures may be taken on each plate. An unfiltered AH-6 high pressure mercury lamp is used as the light source.

The temperature of the rotor is measured continuously during a run by a thermocouple system that indicates the temperature differential between an external reference junction and an internal junction which is rigidly positioned in the rotor slip-stream. The precision of this system is of the order of $\pm$ 0.02° C. and the overall accuracy probably of the order of $\pm$ 0.05° C. when calibrated against diphenylether in an atmosphere of 2 mm hydrogen. Temperature control is accomplished by regulating the temperature and rate of flow of water circulating through copper cooling coils which surround the inside wall of the rotor chamber. Conduction of heat from the periphery of the rotor to the cooling coils is by means of the hydrogen atmosphere (2 mm pressure) maintained inside the chamber. With practice, temperature during a run may be kept constant to within $\pm$ 0.05° C.

B. OBSERVATION OF CONCENTRATION GRADIENTS WITHIN THE CELL

During the course of a velocity sedimentation run, molecules are separated from one another on the basis of their effective hydrodynamic mass. In a system composed of two molecular species, A and B, with sedimentation rates of S_A and S_B, respectively, the concentration of each of the species at two different times during a run may be represented as shown in the upper two diagrams of Figure 18. (The treatment given here is for a cell with parallel sides but is equally applicable to the conventional

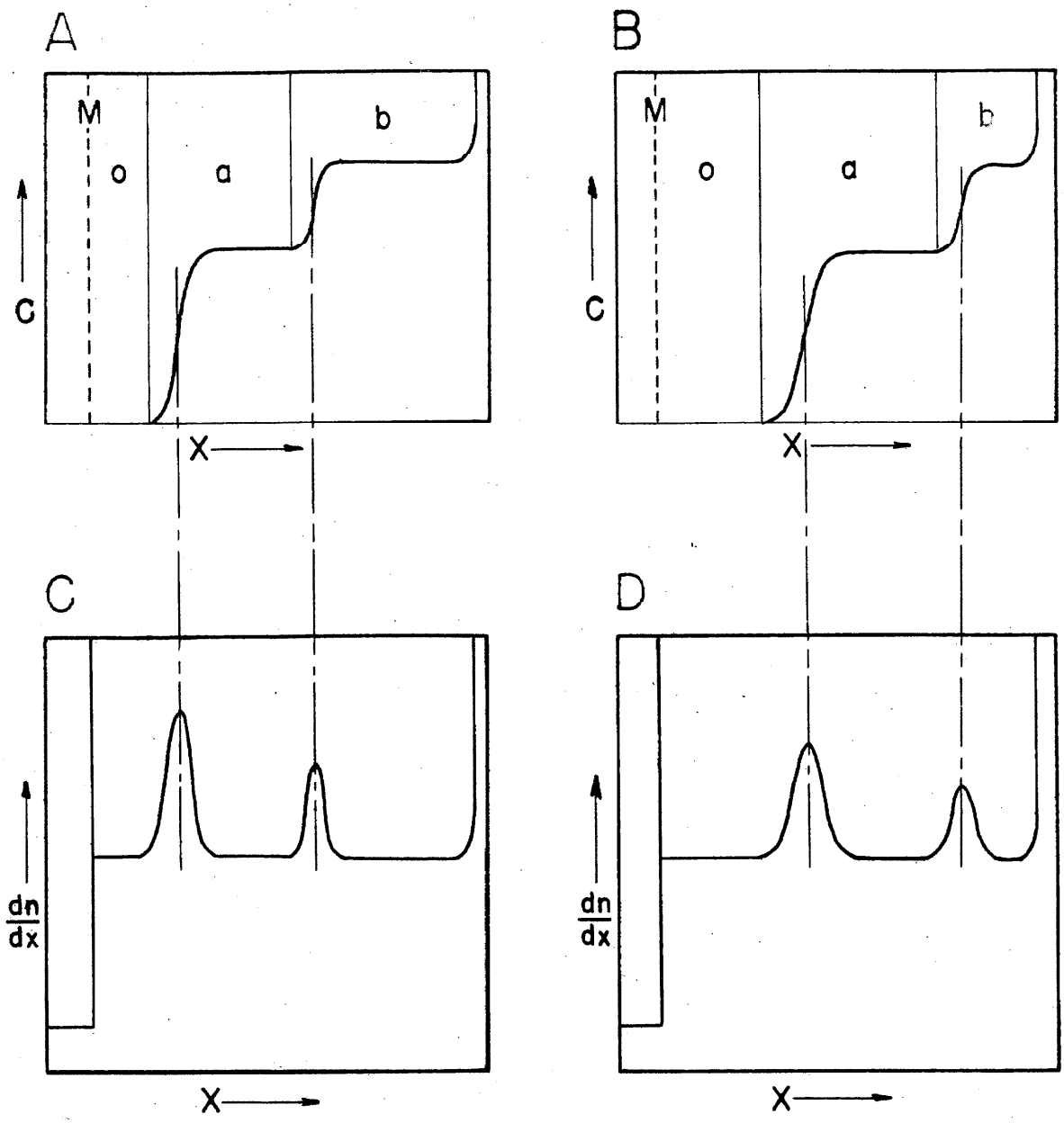


Figure 18. Relationship between C vs. X curves and dn/dx vs. X curves for a two component system. Diagrams A and B represent concentration gradients in the cell at two different times during sedimentation. Diagrams C and D are the corresponding derivative curves such as are observed with the schlieren optical system. See text for full explanation of symbols.

sector-shaped cell when the dilution effect due to the sector shape is taken into consideration, as discussed below.) In these diagrams concentration is shown as a function of the distance, X, from the axis of rotation. Superimposed on the X-axis is the position of the meniscus, M, and the end of the cell, E, farthest from the axis of rotation. Initially the cell is filled with a uniform mixture of molecular species A and B but as sedimentation proceeds both species sediment from the meniscus, leaving a region, o, of pure solvent behind the moving boundary of the slowest component. Since the rates of sedimentation are different, the two molecular species also become separated from one another and there exists a region, a, where only species A is found and a region, b, where both species A and B are present. The concentration of species A, α, may be readily determined in region a, but the concentration in region b, β, is the sum of the concentrations of A and B. Thus the concentration of B is $\beta - \alpha$.

The two general methods used to observe sedimentation are based on either the absorption characteristics or the refractive index properties of the molecules. At the present time only refractive index methods are extensively used, largely as a result of the "scale method" introduced by Lamm [Lamm (1937), Svedberg and Pedersen (1940)], the schlieren lens optics of Tiselius, et al. (1937), and the Philpot-Svennson cylindrical lens modification of the latter [Philpot (1938), Svennson (1939, 1940, 1946)]. These methods as applied to the ultracentrifuge

have been described in detail by Svedberg (1940), Pickels (1942) and by Lundgren and Ward (1951). With the schlieren optical system and a cylindrical lens, a plot of dn/dx vs. x is obtained directly on the photographic record. Such curves (cf. Figure 18 c, d) may be employed to follow the rate of sedimentation and to evaluate, subject to the limitations discussed below, the concentration of the components.

The dn/dx, x diagram: Figure 18 c and d are the dn/dx, x plots for the situations depicted in Figure 18 a and b, respectively. The maxima of the dn/dx curves correspond to the inflection points of the c vs. x plots and the limits on the X-axis correspond to the points where the concentration does not change with distance in the cell. That is, where dn/dx = 0 the curve returns to the base line. Thus any elevation of the dn/dx, x curve above the base line due to solvent alone is an indication that the dn/dx gradient in that portion of the cell where the base line is elevated is greater than expected from the solvent alone. Provided the "Cecil-Ogston effect" [Cecil and Ogston (1948)] is negligible and the true position and shape of the base line may be determined, this elevation of the dn/dx, x curve is a sensitive means of determining whether heterogeneous material which does not form definite peaks is present. In the idealized example presented in Figure 18, the total area under both dn/dx peaks is a function of the total concentration of A and B and is

the same in the two lower diagrams. However, since diffusion occurs simultaneously with sedimentation, the concentration gradients decrease with time. For this reason the c vs. x gradients in Figure 18 b are not as sharp as in Figure 18 a and the height of the corresponding dn/dx peaks is not as great in Figure 18 d as in Figure 18 c, although the area under corresponding peaks is the same. If the refractive index increment (dn/unit weight of material) is the same for both species A and B, then the ratio of the areas under the respective peaks is the ratio of the concentration of each component.

Calculation of the Area to be Expected under a dn/dx, x Peak: The refractive increment, N, for each molecular species in a mixture is the product of the specific refractive increment (a) (refractive increment/gm./100 ml. solution) and the concentration (C) of each species.

$$N = aC \qquad \text{(Eqn. 1.)}$$

The total refractive increment arising from the solute species is the sum of the refractive increments of the individual species. Svedberg (1940) first derived the relationship relating the area under the dn/dx, x curve obtained by scale optics to concentration. For schlieren optics, a useful form of Svedberg's basic equation, adapted from Cecil and Ogston (1948), is:

$$c = \frac{N}{a} = \frac{A}{aM_E^2 M_S M_C \text{ ab tan } \Psi}, \qquad \text{(Eqn. 2.)}$$

where N is the refractive increment; A the area of the peak measured in cm.^{2}; M_E the enlarger magnification; M_S the magnification of the schlieren lens; M_C the magnification of the cylinderical lens; a, the focal length of the focusing lens; b, the thickness of the fluid layer in the cell, and Ψ the angle of the diagonal knife edge from the vertical (or the angle of the entrance slit from horizontal in our instrument). From Eqn. 2 it is possible to compute the area, A, to be expected in an enlargement of the dn/dx, x diagram due to a given concentration of a component of known specific refractive index, or, conversely, if the area of the peak is known the concentration of the component may be computed.

Some of the considerations involved in determining the true area under the dn/dx, x curve will now be considered.

Relation of the measured Area under a dn/dx, x curve to True Concentration in a Sectorial Cell: The considerations above apply to an idealized case where the sides of the cell in which sedimentation occurs are parallel. In practice, a 4° sector-shaped cell is used to eliminate convection due to sedimenting particles striking the sides of the section. This means that when the material contained in a volume element of thickness dx at X_o is moved to X_n by sedimentation, the same quantity of material is now spread through a larger volume of solvent (Figure 19). The

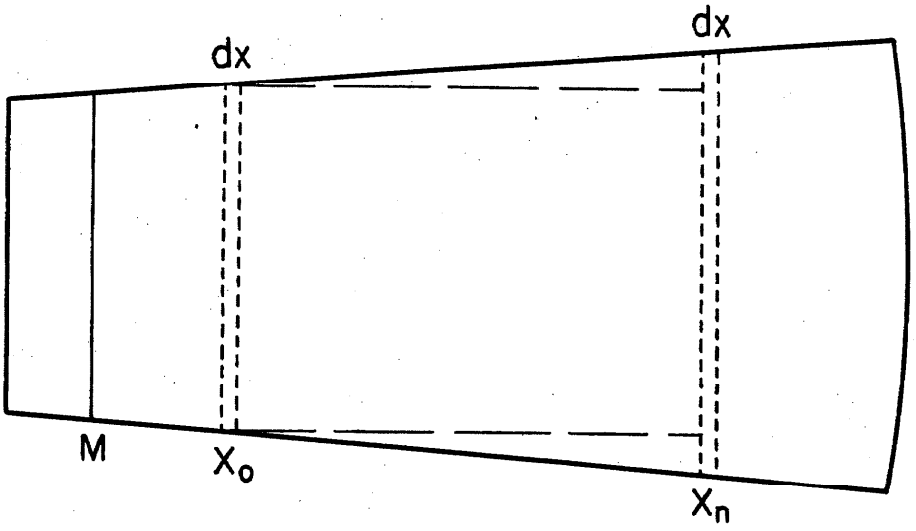


Figure 19. Dilution Effect from X_o to X_n in a Sectorial Cell.

volume in each element of thickness dx is proportional to $(X_o)^2$ and $(X_n)^2$, respectively. Hence the dilution factor is given by $(X_o/X_n)^2$, and if the concentration at X_o is C_o, then the concentration, C_n, at X_n is given by:

$$C_n = C_o \left[\frac{X_o}{X_n}\right]^2 \qquad \text{(Eqn. 3.)}$$

The concentration at the meniscus at the beginning of a sedimentation run is the total concentration of the material placed in the cell. This is the only point at which an analytically determined concentration exists and is usually taken as C_o. The position of the meniscus with reference to the axis of rotation is X_o, and the centers of the dn/dx, x peaks as the components sediments through the cell are X_1, X_2, ... , X_n. From measurement of the area under each peak, a series of values, A_1, A_2, ... , A_n is obtained. After correction for the dilution factor these measured values should all be equal to a constant, A_o, which is proportional to C_o. In a monodisperse system this dilution effect is the only factor which should decrease the concentration of the sedimenting component, and hence the concentration gradient, dc/dx, as it moves through the cell. If the base line may be accurately located, the constancy of A_o (A_1, A_2, ... , A_n after correction for dilution) may be used as a test for loss of faster or more slowly sedimenting components which are not present in high enough concentration to be discernible as a discrete peak.

The magnitude of this dilution effect under the conditions prevailing in the rotor used in these experiments is shown in Figure 20. In curve A of this Figure, the reciprocal, $(X_n/X_o)^2$, of the dilution factor has been plotted for X values from 5.74 cm. to 7.21cm., the distance of the top and bottom, respectively, of the sector of the normal cell from the axis of rotation. The appropriate correction factors for the synthetic boundary cell, in which the distance to the top reference line (the bottom of the insert cup) in the cell is 5.87 cm., are shown in curve B. These two curves are given only to illustrate the magnitude of correction necessary and not as calibration curves since the meniscus is very seldom at the top of the sector due to formation of an air bubble during filling of the cell.

The Johnston-Ogston Effect: In monodisperse systems, or in polydisperse systems where the sedimentation constant of each component is independent of concentration and where resolution of components is complete, the considerations above with regard to determination of concentration from area analyses apply without reservation. If, however, the sedimentation rate of the more slowly sedimenting component in a mixture is strongly concentration dependent, the trailing side of its sedimenting boundary, which is in a region where the total concentration is less than at the front of the boundary, will sediment more rapidly than the front edge of the same boundary. Johnston and Ogston (1946) examined the

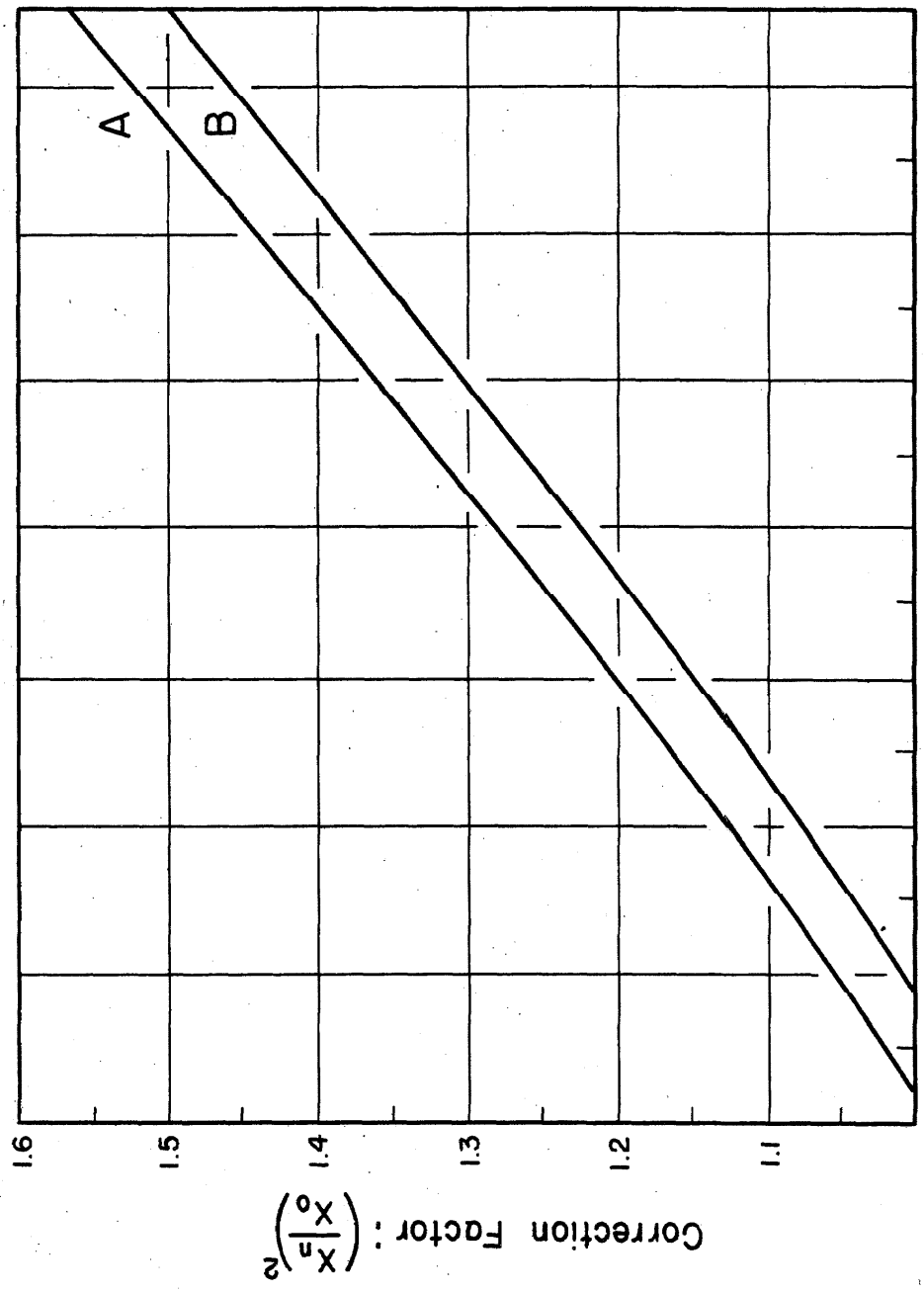


Figure 20. Correction Necessary to Compensate for the Dilution Effect in a Sectorial Cell.

quantitative aspects of this phenomenon and have shown that under these conditions the concentration of the slow component is always greater at its boundary than it is in the initial mixture. This causes the dn/dx, x curve to register an enhancement of the area under the slow peak. An apparent decrease of the area under the fast peak occurs due to a negative dn/dx gradient of the slow component across the boundary of the fast component.

Fortunately, since the 19S protein characterized here does not exhibit any concentration dependence, the Johnston-Ogston effect need not enter into area analyses. It is important, though, in determining the concentration of a component from area measurements made on a mixture, that the possibility of such interference be examined.

C. MEASUREMENT OF THE AREA UNDER A dn/dx, x CURVE

The theoretical considerations involved in determining the concentration of a component from the area under the dn/dx, x curve, discussed above, presuppose that the correct area under the curve may be measured accurately. In practice, the accurate measurement of this area poses several complications. The discussion of the complications will be divided into those factors which affect the position of the base line, and those factors which affect the shape of the base line. Methods for partially overcoming these difficulties have been developed and will also be discussed.

Factors Affecting Base Line Position: The shape of the dn/dx, x curve is determined by changes in dc/dx or dn/dx with X, but the position of the curve with respect to some fixed point in the optical axis is a function of the magnitude of dn/dx. For example, if an empty cell is placed in the static rotor and aligned in the optical path, the shadow of the knife edge is a straight line, for there is no change in dn/dx with X. The position relative to the optical axis of this knife edge shadow through air remains the same so long as the position and angle of the knife edge itself remains unchanged with respect to the optical axis. Thus, the position of the knife edge through an air bubble at the top of the cell may be used as a fixed reference point common to reference runs with solvent and to sedimentation runs. Similarly, if the cell is filled with liquid and placed in a static rotor, dn/dx will again be zero and the knife edge shadow position will be identical with its position as seen through air. Such an effect is shown in line a, Figure 21.

As soon as the liquid column in the cell is subjected to a gravitational field, foreign concentration gradients or extraneous dn disturbances, both of which register as changes in the dn/dx, x pattern, are created. Such effects may be separated into those due to the solvent and cell employed, and those due to sedimenting material.

Disturbances due to the solvent and cell, which may be

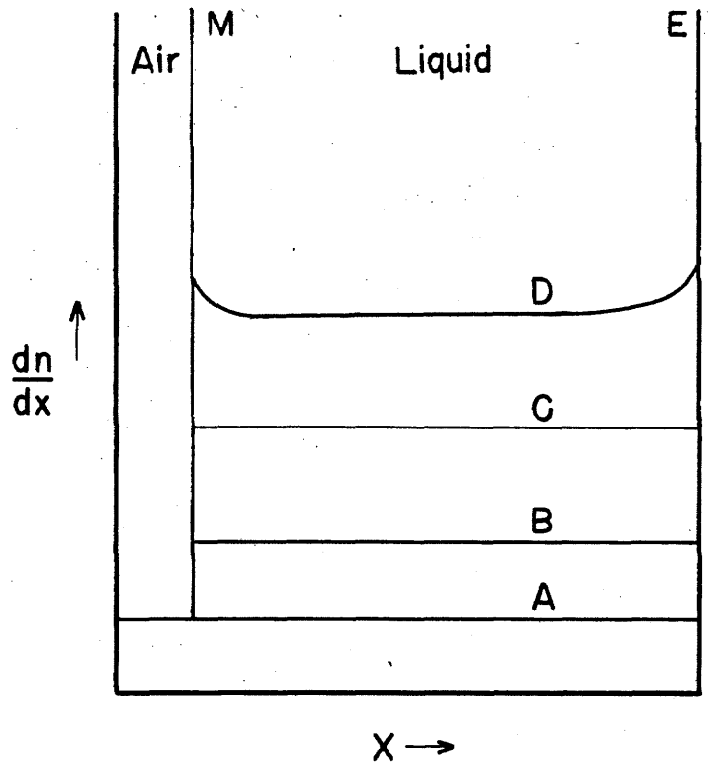


Figure 21. Position of Knife Edge Shadow (Base Line) on the dn/dx, x Diagram under Different Conditions.

Line A - Position through air or through liquid in a static rotor.

Lines B and C - Relative elevation of base line due to change in gravitational field. B, at 500 r.p.s.; C at 800 r.p.s.

Line D - Relative elevation and curvature of base line due to window distortion at 1000 r.p.s.

determined by means of a suitable "reference" run with solvent alone, are:

1. Compressibility of the solvent. All liquids are compressible to some extent. This accounts for the finite value of dn/dx in a gravitational field and the subsequent elevation of the base line position through the liquid layer relative to the position throughair. The magnitude of dn/dx and the effect of compressibility may be determined as follows:

$$\frac{dn}{dx} = \frac{dn}{dp} \cdot \frac{dp}{dx} \qquad \text{(Eqn. 4.)}$$

From the equation for pressure [pressure = (mass)·(gravity)·(height), or dp/dh = (m)·(g)],

$$\frac{dp}{dx} = \rho \omega^2 x \qquad \text{(Eqn. 5.)}$$

where ρ = density, ω the angular velocity, x the distance from the axis of rotation and $\omega^2 x$ is the gravitational field.

The refractive index, n, is proportional to the density of the liquid, or

$$n = K\rho \qquad \text{(Eqn. 6a)}$$

and

$$n_o = K \rho_o, \qquad \text{(Eqn. 6b)}$$

where K is a proportionality constant. Differentiating Eqn. 6a and substituting the value of K from Eqn. 6b,

$$dn = \frac{n_o}{\rho_o} d\rho \qquad \text{(Eqn. 7)}$$

But since dn/dp is the function desired, both sides may be divided by dp:

$$\frac{dn}{dp} = \frac{n_o}{\rho_o} \cdot \frac{d\rho}{dp} \qquad \text{(Eqn. 8)}$$

The term $\frac{d\rho}{dp}$, the change in density with pressure, may be defined in terms of density alone by introduction of the coefficient of compressibility, β, which is defined as

$$\beta = -\frac{\frac{dV}{dp}}{V} = \frac{\frac{d\rho}{dp}}{\rho} \qquad \text{(Eqn. 9)}$$

$$\therefore \qquad \frac{d\rho}{dp} = \beta\rho \qquad \text{(Eqn. 10)}$$

Substituting Eqns. 5, 8 and 10 in Eqn. 1,

$$\frac{dn}{dx} = \frac{n_o}{\rho_o} \cdot \beta\rho \cdot \omega^2 x \rho = \frac{n_o \beta \rho^2 \omega^2 x}{\rho_o} \qquad \text{(Eqn. 11)}$$

If we consider β to be constant and the variation of ρ to be small over the pressure range, then $\frac{dn}{dx}$ is a linear function of ω and x. Thus the value of $\frac{dn}{dx}$ for a constant position in the cell, (x), is proportional to the second power of the angular velocity, and at a constant angular velocity, it is proportional to x. In the Spinco rotor, the cell limits are at 5.74 and 7.20 cm., hence the change in $\frac{dn}{dx}$ due to change in the gravitational field over the length of the cell is 72/57 of the value of dn/dx at 5.74 cm.

The magnitude of this change (12.7°/o) may be seen in proper perspective when it is realized that the change from a static rotor to a speed of 900 r.p.s., which changes the value of dn/dx approximately 32,000,000 fold (from $\omega^2 = 0$ to $\omega^2 = 3.2 \times 10^6$), produces only about 2 cm. rise of the base line above the reference level through air. In comparison, a change of 12.7°/o in dn/dx through the cell is too small to be detected.

Thus, compressibility of the solvent has the effect of raising the base line relative to some fixed reference point in the optical path, as shown in lines b and c, Figure 21, and Figure 22, but does not impart a slope to it.

2. Window distortion. At speeds from about 500 r.p.s. upward, the quartz discs which cover the ends of the center sector in the cell become distorted in their mounting and bulge outward at the far end, producing a lens effect which acts as a non-uniform dn/dx gradient. The magnitude of the effect is variable, depending on the speed, the particular quartz discs, their mounting, and the cell employed. The effects, though, are manifested as an additional slope at both ends of the cell, as shown in line D, Figure 21.

3. Sedimentation of buffer salts. Some large inorganic ions used in the solvent to maintain pH or to suppress charge effects may be slowly sedimented in high gravitational fields. Sedimentation of the salt ions may be detected by making a reference run with solvent alone under conditions identical to those used for a normal run. If the shape of the base line changes with time, sedimentation

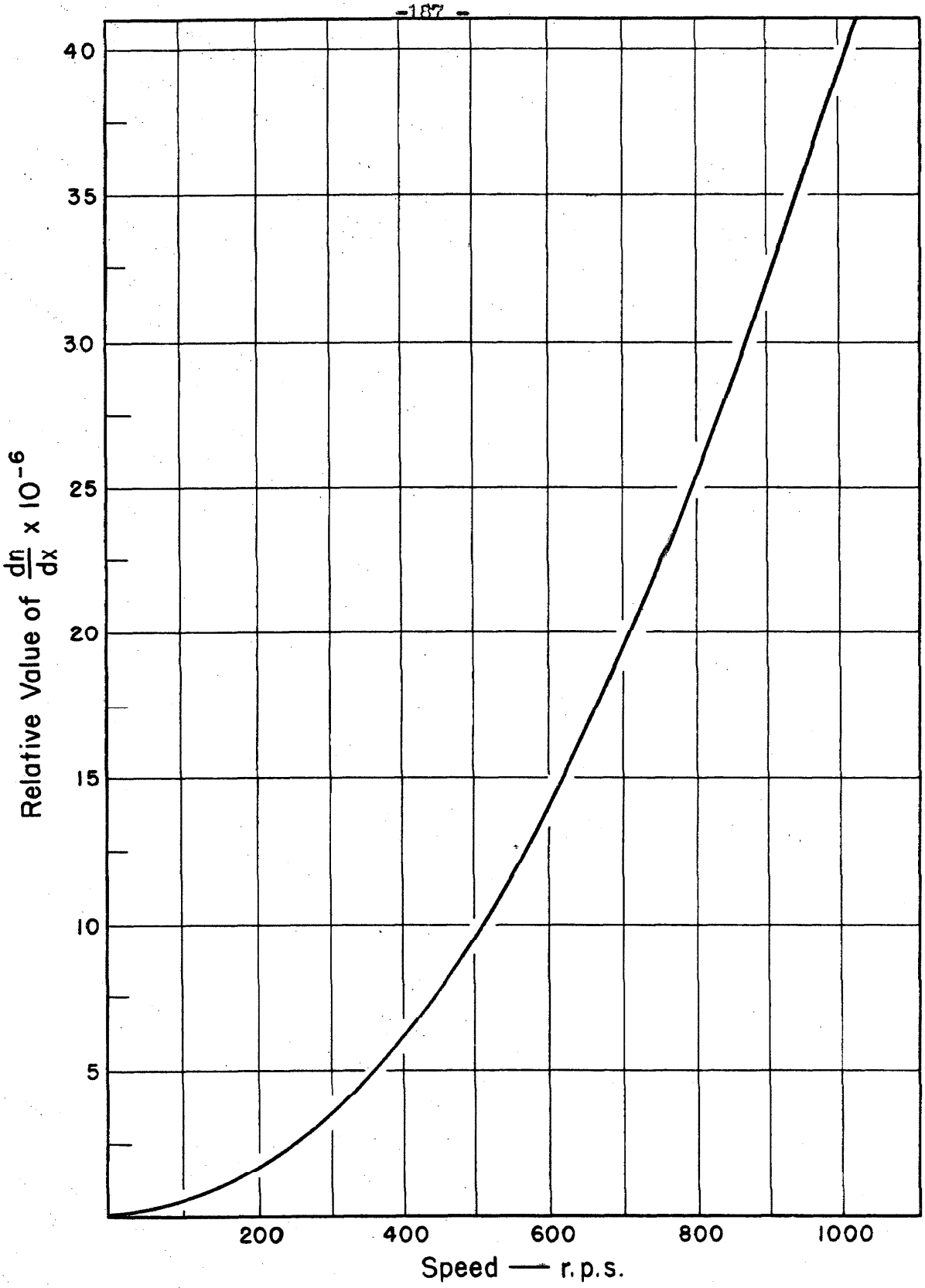


Figure 22. Relative Values of dn/dx as a Function of Speed of Rotation.

is occurring and it will be necessary to make a reference exposure under the same conditions and at the same time as each of the exposures in a normal run. No evidence for the sedimentation of maleate ion in the buffer used was found in these studies, and Cecil and Ogston (1948) reported no sedimentation of acetate ions at 1000 r.p.s.

When a sedimenting component(s) is introduced into the system, several additional factors affect the shape of the dn/dx, x diagram:

1. The first of these factors is the dilution effect discussed above. This simply lowers the dc/dx gradient, and hence the dn/dx gradient, as a component moves through the cell from X_o to X_n.

2. The second effect due to the presence of a sedimenting component, a change in slope of the base line ahead of the sedimenting boundary, was first described by Cecil and Ogston (1948) in a β-lactoglobulin system. They attributed this change in slope to the difference in the absolute refractive indices of the solvent and the solution acting in conjunction with the distorted cell windows (which form a lens) to alter the focal length of the entire optical system and cause an increase in slope of the base line above that expected on the basis of a reference run with solvent alone. The magnitude of this effect, if this is indeed the true explanation for it, should be dependent on the concentration of solute (which determines the differential between the indices of

refraction of solution and solvent) and the amount of cell window distortion. Under such circumstances, it may not always be possible to discern the presence of the Cecil-Ogston effect.

With β-lactoglobulin of very high homogeneity, Cecil and Ogston, using a "base line fitting" procedure which they describe, were able to measure in sedimentation diagrams, about 99°/o of the refractive index increment expected on the basis of differential refractometer measurements of the solutions before centrifugation.

3. Another possible disturbance of the position of the base line in front of the sedimenting component is backward diffusion of the solute from the end of the cell. This phenomenon will occur with a slowly sedimenting and rapidly diffusing material and will be most marked late in the run, for at this time the concentration gradient at the extreme end of the cell (due to piling up of solute by sedimentation) and the time for diffusion will both be greatest. The result will be a change in slope of the base line ahead of the sedimenting boundary, just as in the Cecil-Ogston effect above. Under certain conditions, in fact, the two effects may be indistinguishable. This backward diffusion effect has not been discussed in the literature and is probably unimportant for most systems studied in the ultracentrifuge. Certainly it was of no importance in the studies on cytoplasmic proteins because in these systems the protein is actually packed as an insoluble pellet at the end of the cell.

Fixing the Base Line Position: According to Cecil and Ogston (1948) the correct base line may be determined by fitting a tracing of the reference base line made with solvent alone to the base line ahead and behind the dn/dx peak on an experimental curve, tilting the reference base line ahead of the peak to fit the increased slope due to the protein solution (Cecil-Ogston effect, above) so that the two parts of the reference base line intersect at the center of the peak. Only experimental curves in which there was considerable length of base line on both sides of the peak were used. By their technique, the authors were able to measure and compute corrected areas to about $\pm$ 2%.

With the schlieren diagrams obtained from the ultracentrifuge employed for the studies reported here, however, it is necessary to use a fixed reference point in addition to the method of Cecil and Ogston, to locate the base line. This is necessary because of an optical imperfection, as yet undefined, which makes the knife edge shadow fuzzy. As a result, its apparent position on a photographic plate is somewhat dependent on the exposure given; the longer the exposure, the lower the position of the knife edge. To obviate the exposure effect, a series of reference exposures was made at each of the speeds used in normal operation of the instrument, using a fixed slit angle and mechanical stops on the knife edge to define a reproducible position with respect to the optical axis. Calibration

at each speed was necessary because the apparent knife edge position, when fixed at a given point relative to the optical axis, is a function of dn/dx through the cell, which in turn depends on the gravitational field acting on the liquid column. These reference base lines, therefore, were rigidly defined with reference to the optical system and, most important, with respect to an edge of the mask which limits the area of the photographic plate exposed to the incident beam. This mask is, of course, permanently fixed in relation to the optical axis. Thus, all variables were reproducible and the effects caused by poor definition of the knife edge could be minimized by using fixed reference points instead of a hazy shadow. As a result, the location of the base line was better defined than by use of the Cecil-Ogston technique alone. This is important, for small errors in location of the base line greatly affect the area under a broad peak.

Even with this modification, however, the accuracy obtainable is not as great as is desirable, probably due to failure to properly orient the base line. Some of the results obtained are shown in Table XXI and are discussed below in the section on calibration.

D. GENERAL DISCUSSION

In polydisperse systems where all components have the same specific refractive index and may be completely resolved, the

TABLE XXI

MEASURED AND CORRECTED AREAS UNDER THE dn/dx, x CURVES OBTAINED DURING SEDIMENTATION OF BOVINE SERUM ALBUMIN

Protein Conc. mg./ml.*	$(X_n/X_o)^2$	Measured Area**	Computed Area**	Average A_o
3.80	1.090	634	690	
	1.136	610	694	
	1.169	582	678	
	1.201	534	640	
	1.237	497	615	
				661
7.55	1.145	1177	1347	
	1.160	1166	1351	
	1.177	1132	1334	
				1344

* Determined by precipitation with TCA and drying to constant weight.

** In arbitrary planimeter units at an enlarger magnification of 13.05 diameters.

proportion of each component in a mixture may be simply determined by correcting measurements of the area under the dn/dx, x curve of each component to a common level in the cell (usually the meniscus) and for the Johnston-Ogston effect. From the proportions of each component and the total concentration, determined by suitable analytical methods, the absolute amount of each component may be determined. If the specific refractive indices differ markedly, appropriate corrections for this difference must be made. The concentration of each component may be found by combining Eqns. 2 and 3 and rearranging:

$$C_o = \frac{N}{a}\left[\frac{X_n}{X_o}\right]^2 = \frac{A_n}{aM_E^2 M_S M_C ab \tan \Psi}\left[\frac{X_n}{X_o}\right]^2$$

(Eqn. 12)

If resolution of one or more of the components is incomplete but all components are resolved from the meniscus, the above methods may still be used. It will be necessary, though, to reconstruct the incompletely resolved curves into their component parts. Methods for performing this reconstruction are given by Svedberg (1940).

Incomplete resolution from the meniscus is a much more serious problem and until recently the concentration of the unresolved component could be obtained only by determining the concentration of all the other components and subtracting them from the initial total concentration. In the case of a complex mixture

such as the cytoplasmic proteins, this procedure proved exceedingly difficult. As an alternative, during the early phases of this work, when the whole cytoplasms of a number of species of plants were examined and the proportion of Fraction I protein estimated, an approximate method was used. The areas under the Fraction I protein peak and under the peak representing the incompletely resolved proteins were measured in several photographs where the 19S component was completely resolved from the more slowly moving components. No dilution corrections were applied for distance down the cell. The specific refractive increment was assumed to be the same for all components and the percent of the total refractive increment contributed by each component was computed. These values were averaged for the available exposures. By this method, the percent of Fraction I protein showed a steady decrease with distance through the cell, as would be expected from both dilution effects and from increased resolution of low molecular weight material from the meniscus. Since at that time there was no way of knowing what the refractive area of the incompletely resolved component should be, the accuracy of the method was unknown. By measurement of the area under the 19S peak in a series of samples run at identical concentrations in the ultracentrifuge, it was possible, though, to get good comparisons of the relative amounts of Fraction I protein in a series of samples even if the absolute amount of it could not be determined.

An accurate method of determining the absolute amount of Fraction I proteins was necessary, however, for studies of the physiology and biochemistry of Fraction I. Accordingly, considerable time was spent in attempting to develop an exact assay procedure for this protein.

The particular centrifuge employed for these studies presented two problems which had to be overcome. The first and most serious of these problems was the "fuzzy" knife edge shadow and attendent difficulties in properly locating the base line so that corrected area measurements would be constant to within a few per cent. A partial solution to this problem has been discussed above, but it is at best only an expedient, and not a substitute for optical perfection. It still leaves an element of doubt as to the exact location of the base line.

The second problem was to determine the amount of protein the area under a dn/dx, x curve corresponds to, since the constants of the optical components in the centrifuge are not known. The constants involved are M_S, M_C, a, b, and Ψ in Eqn. 12. If these factors are held constant and α is also assumed to be constant for all proteins, then

$$C_o = \frac{N}{\alpha} \left[\frac{X_n}{X_o}\right]^2 = \frac{A_n}{M_E^2 K} \left[\frac{X_n}{X_o}\right]^2 \qquad \text{(Eqn. 13)}$$

where $K = (\alpha)(M_S)(M_C)$ (a) (b)(tan Ψ). Since C_o may be determined independently, the value of K may be experimently determined by

calibration with a homogenous protein, such as crystalline bovine serum albumin (BSA), in the cell. The procedure employed will be described in detail.

Two calibration runs were made in a 12.00 mm cell, using bovine serum albumin at a concentration of 3.80 and 7.55 mg./ml. in 0.1 ionic strength potassium maleate buffer of pH 7.0. The slit angle was 1°. An appropriate stop was employed during the calibration runs and the reference run with buffer alone. The runs were continued until the albumin peak was about 60°/o of the way through the cell. Exposures intervals were so timed that 3 - 5 photographs in which there was considerable base line on either side of the peak were available for measurements. Tracings of the dn/dx, x patterns were made at an enlarger magnification of 13.05 diameters. The base line was arbitrarily located from the reference run as described in Section C above. The areas under the dn/dx, x peaks were measured with a planimeter.* Measurements were repeated until the range of three values was within 1°/o of the total area measured. The average areas measured, in planimeter units, and the appropriate correction factors for dilution are given in Table XXI. The average of the corrected areas corresponds to 178 and 174 planimeter units per mg. protein for protein concentrations of 7.55 and 3.80 mg./ml., respectively. The average, 176, may be used to convert area measurements to protein concentration as long as the planimeter constant and

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* Lasico Serial No. 1613 - Chem. Dept. No. 2803.

enlarger magnification remain the same. For use with other planimeters or with a different enlarger magnification, the value of K in Eqn. 13 may be computed.

The planimeter constant was found to be 99.94 units per cm^2. Employing the average value of 176 planimeter units/mg. protein/ml., the value of K is found from:

$$K = \frac{A_o/\text{planimeter constant}}{M_E^2 \; C_o} = \frac{176/99.94}{(13.05)^2 \; (1.0)}$$

$$= 1.034 \times 10^{-2} \; cm^2/mg. \text{ protein.}$$

This value is applicable under any conditions in this particular ultracentrifgue provided only:

1. That the material be examined in a 12.00 mm sector.
2. That the material have the same specific refractive increment as BSA.
3. That the slit angle be set at 1^o.

By using the "area constant" and the arbitrary method of positioning the base line, outlined above, it has been possible to determine the amount of Fraction I protein in whole cytoplasm to about $\pm 5^o/o$ of the amount present. The estimated amounts measured in this way are probably low because it has been found that as sedimentation proceeds the corrected area decreases. This anomalous decrease in area is probably due to incorrect positioning of the base line. The whole cytoplasm system is convenient to work with for there is very little elevation of the base line in

front of the 19S or 26S peaks, leaving little ambiguity in interpretation of the areas. In twice-sedimented Fraction I protein preparations, however, the base line in front of the 19S and 26S peaks is elevated considerably, apparently due to the presence of heterodisperse material that does not form discrete peaks. This makes area measurement and correction difficult. This in turn leads to errors which, percentage wise, are negligible for Fraction I protein assay but which have a very large effect on the small amount of slow component computed by difference between the total protein content and the Fraction I protein content. When it is necessary to know the concentration of low molecular weight material accurately, a new type of cell recently developed by Schachman (1952) has proven useful.

Low molecular weight material is not resolved from the meniscus in the normal cell because the rate of diffusion back to the meniscus is as great as the rate of sedimentation in the gravitational fields employed. The "synthetic boundary" cell developed by Schachman obviates this difficulty by forming a sharp, stable protein solution-buffer boundary after the rotor is running at 60 to 80 r.p.s. This creates a sharp gradient between the solution and solvent, just as is formed during sedimentation. The fast moving components sediment as usual and the low molecular weight material remains more or less stationary at the solution-solvent interface, where its concentration may be

measured by the change in refractive increment across the interface. Since the low molecular weight material is heterogeneous, the heaviest portion of it is sedimenting and the lighter portion is diffusing backward through the buffer layer. The buffer layer, however, is of sufficient depth that material does not diffuse completely across it before the fast component has sedimented clear, affording a measurement of the true concentration gradient across the solution-solvent interface. This technique sensitively detects concentration changes across an interface, hence it is necessary to eliminate all gradients except the gradient due to protein alone. This is accomplished by throughly dialyzing the protein solution against the same buffer solution used to overlay it in the cell. This eliminates all salt concentration effects except that due to the Donnan equilibrium across the dialysis membrane. By means of this technique it is possible to estimate accurately the concentration of only a few percent of a low molecular weight component in a mixture of predominantly high molecular weight material. Simultaneous estimation of large percentages of the high molecular weight material present in high concentration is difficult because there is very limited space available between the solution-solvent boundary and the region at the end of the cell where anomalies occur, and because concentration of the high molecular weight components in a mixture where only a few percent of low molecular weight material is being assayed is

so great that all of the corresponding dn/dx peak cannot be photographed (cf. Figure 17).

The analyses of a sample of spinach whole cytoplasm by the different methods used during these investigations will be presented as a summary. Photographic records of the analyses in the normal cell and in the synthetic boundary cell are shown in Figures 23A and 23B, respectively. Estimations of the amount of the 19S component in this sample, computed in three different ways, are tabulated in Table XXII.

The relative amount of the total refractive area in the 2100 and 2400 second exposures in Figure 23A that is due to the 19S component is shown in Section A, Table XXII. Because the low molecular weight, heterogeneous component is not completely resolved, its area continues to increase as the heavier portion of it slowly sediments. Simultaneously, the area under the 19S peak decreases because of the dilution effect as this component moves through the cell. The net effect, on the basis of relative, uncorrected areas, is to decrease the apparent amount of the 19S component as sedimentation proceeds. Thus, choice of the particular exposures measured will have a significant effect upon the amount of Fraction I protein determined by this method. This is illustrated by the observed difference between the two exposures measured.

It should be emphasized that the relative area method used here is basically unsound unless the area of all components

TABLE XXII

COMPARISON OF THE AMOUNT OF FRACTION I PROTEIN IN A SPINACH WHOLE CYTOPLASM PREPARATION AS ASSAYED IN THE NORMAL CELL AND IN THE SYNTHETIC BOUNDARY CELL

Initial Protein Concentration 7.20 mg./ml.

A. Normal cell--Relative area method.

Exposure**	Area* under 19S peak	Area* under slow peak	Total Area*	Percent of total area under 19S peak	Percent of total area under slow peak
2100 sec.	395	642	1037	38.2	61.8
2400 sec.	382	650	1032	36.9	63.1

B. Normal cell--Corrected area-protein calibration method.

Average corrected area under 19S peak	453*
Protein equivalent of corrected area	2.58 mg.
% Fraction I protein (2.58/7.20)	35.8

C. Synthetic boundary cell--both methods of computation.

Average corrected area under 19S peak	485*
Average corrected area under slow peak	944*
Total area	1429*
% Fraction I protein based on total refractive increment (485/1429).	34.0
Protein equivalent of average area under 19S peak.	2.76 mg.
% Fraction I protein based on amount of 19S component in mixture (2.76/7.20).	38.3

* Arbitrary units. 1.0 mg. protein equivalent to 176 units.

** Refers to Figure 23A.

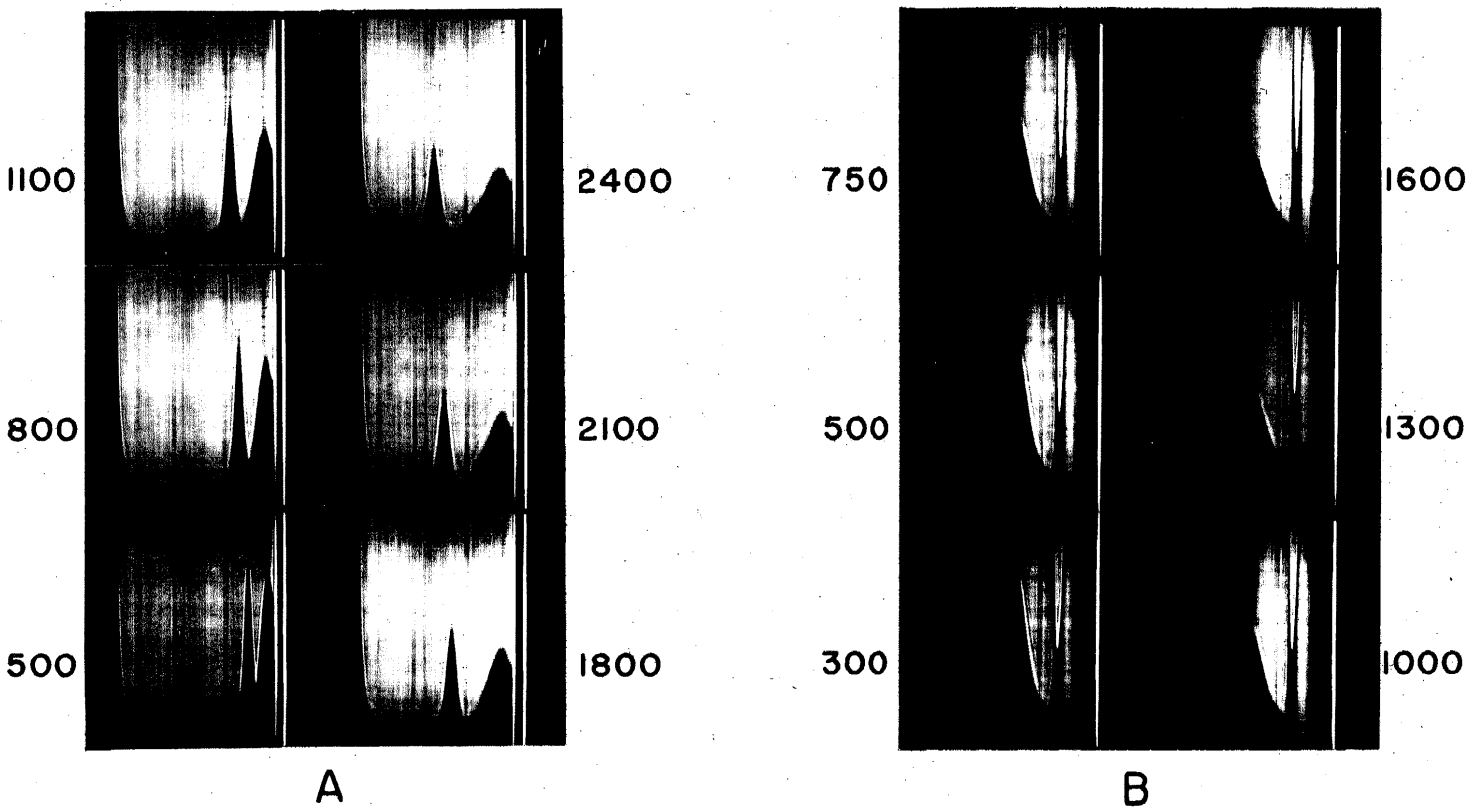

Figure 23. Ultracontrifugal examination of a spinach whole cytoplasm in the normal cell (A) and the synthetic boundary cell (B). Sedimentation proceeds from right to left. Solvent, 0.1 μ k-maleate buffer, pH 7.0. Protein concentration, 7.2 mg./ml. Rotor speed: A, 751, r.p.s.; B, 852 r.p.s. Note complete absence of any component sedimenting faster that 19S.

can be measured and corrected for all factors which affect the area. The agreement between the results in Section A and those in Sections B or C of Table XXII is entirely fortuitous. If the molecular weight distribution of the low molecular weight fraction had been such that the weight average molecular weight of the fraction was much lower, then the proportion of the protein in this fraction that was resolved from the meniscus would have been lower, the measured area less and the apparent relative amount of Fraction I protein much higher.

As indicated earlier in this discussion, the inadequacies of the relative area method where one component is not completely resolved from the meniscus may be overcome by conversion of the observed area of the resolved component(s), after suitable correction for dilution effects, into absolute amounts of protein. The results of calculations made from measurements on the 1100, 1800, 2100 and 2400 second exposures in Figure 23A are presented in Section B, Table XXII. The results obtained by this method are basically sound and may, therefore, be compared with those obtained by use of the synthetic boundary cell.

The results of similar measurements and computations for the synthetic boundary cell run (Figure 23B) are given in Section C, Table XXII. In this case, since all components are resolved, a direct comparison of the relative area method and the "absolute amount" method is possible. The area under the 19S component peak was taken as the average corrected area measured in the first

four exposures; that of the low molecular weight component was taken as the average corrected area under the slow peak in exposures 2 through 5. In exposure 6, a marked decrease in the corrected area of the slow component, indicating that some of the very low molecular weight portion of this fraction had diffused backward to the meniscus, was observed.

The average value for the amount of Fraction I protein determined in the synthetic boundary cell by the two different methods, 36%, is the same as that determined in the normal cell on the basis of the protein area calibration factor. The value of 38.3% of Fraction I protein as determined from the corrected area under this peak in the synthetic boundary cell is probably high due to improper location of the base line. This is believed to be so because the average total refractive area measured was ca. 12% greater than it should have been on the basis of the measured protein concentration of the mixture and the protein area calibration factor. All methods of computation, except for the method in Section A, Table XXII, produced values within ± 5% of the average value.

A further use of the synthetic boundary cell to determine the amount of low molecular weight protein in Fraction I protein solutions is shown in Figure 17 (presented in Section III, Part II).

On the basis of the considerations discussed above, the following procedures are recommended for studying the cytoplasmic proteins in the ultracentrifuge:

1. For determination of the sedimentation constants of components which are resolved from the meniscus, use the normal cell because of the extra sedimentation distance available. The low molecular weight fraction is so heterogeneous that a "sedimentation rate" for it, which may be obtained from the early stages of a run in the synthetic boundary cell, is meaningless.

2. For assay of Fraction I protein in whole cytoplasm, use the normal cell and determine the absolute amount of this component by means of the area calibration method described above. At low total protein concentrations, the synthetic boundary cell may also be used advantageously. Where this is possible, the relative area method will give satisfactory results.

3. For assay of low molecular weight material in either whole cytoplasm or in Fraction I protein preparations, the synthetic boundary cell must be used. In general, simultaneous assay of the low molecular weight fraction and the amount of the 19S component in Fraction I protein preparations will not be possible. The amount of the 19S component may, however, be assayed in the same solutions in the normal cell.

On the basis of these procedures, it is possible to estimate the amount of Fraction I protein in any mixture to a probable accuracy of ca. 5°/o of the amount present.

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