Sluyterman, L. A. Ae (1968), *Biochim. Biophys. Acta 151*, 178.
Smith, E. L. (1958), *J. Biol. Chem. 233*, 1392.
Smith, E. L., Chavré, V. J., and Parker, M. J. (1958), *J. Biol. Chem. 230*, 283.
Smith, E. L., and Kimmel, J. R. (1960), *Enzymes 4*, 134.
Stockell, A., and Smith, E. L. (1957), *J. Biol. Chem. 227*, 1.
Webb, J. L. (1966), Enzyme and Metabolic Inhibitors, Vol. 3, New York, N. Y., Academic, p 11.
Whitaker, J. R. (1969), *Biochemistry 8*, 1896.
Whitaker, J. R., and Bender, M. L. (1965), *J. Am. Chem. Soc. 87*, 2728.
Whitaker, J. R., and Peréz-Villaseñor, J. (1968), *Arch. Biochem. Biophys. 124*, 70.
Williams, D. C., and Whitaker, J. R. (1969), *Plant Physiol.* (in press).
Wong, R. C., and Liener, I. E. (1964), *Biochem. Biophys. Res. Commun. 17*, 470.
Wright, C. J., Alden, R. A. and Kraut, J. (1969), *Nature 221*, 235.
Yankeelov, J. A., Jr., and Koshland, D. E., Jr. (1965), *J. Biol. Chem. 240*, 1593.

# The Estimation of Molecular Weights in Mixtures of Two Proteins by the Meniscus Depletion Method*

P. D. Jeffrey† and Margaret J. Pont

ABSTRACT: Sedimentation equilibrium experiments with mixtures of two proteins in different proportions have been performed at speeds high enough to deplete effectively the meniscus region of protein in an attempt to establish what conditions are necessary before the molecular weights and proportions of two proteins can be estimated with reasonable accuracy using this technique. The mixtures used were 20% ovalbumin plus plasma albumin, 5, 10, and 20% ribonuclease plus ovalbumin and 5, 10, and 20% ribonuclease plus plasma albumin. Molecular weights were evaluated from the slopes of $\ln j$ *vs.* $r^2$ plots, from extrapolation of reciprocal plots of $M_w$ and $M_n$ to infinite dilution and from "two species plots."

The possibility of estimating the proportions of the two proteins present in the original solution by extrapolating $M_n$ to the cell bottom was investigated. Experiments with the individual proteins are also reported. It is concluded that if $\sigma$ ($\sigma = \omega^2 M(1 - v\rho)/RT$) $\simeq 5$ for the species of lower molecular weight, the two species plot gives values for the molecular weight, $M_1$, of this species to better than 7% for the ribonuclease–plasma albumin mixtures ($M_2/M_1 = 5$) and within about 10–15% for the ribonuclease–ovalbumin mixtures ($M_2/M_1 = 3.4$) and is considerably better than the other methods tried for evaluating $M_1$. $M_2$ was obtained to better than 7% accuracy for all combinations above 5% ribonuclease–ovalbumin by using the two species plot. Attempts at estimating the proportions of the two species present were unsuccessful even in the mixture of 20% ribonuclease and plasma albumin because of the large errors in $M_n$ in solutions of low concentrations. It is thought that these errors were probably due to adsorption of protein on the walls of the centerpiece.

The method of measuring molecular weights at sedimentation equilibrium by running the ultracentrifuge at such high speeds that a region of the solution near the meniscus becomes effectively free of macromolecules was developed by Yphantis (1964) and has since been used widely. The main attractions of the method, referred to here as the meniscus depletion method, are the moderate times necessary to reach sedimentation equilibrium, the small amount of protein required, the fact that the initial concentration of the solution need not be measured and the increased sensitivity of detection of low molecular weight proteins in paucidiperse solutions which results from the centrifugal fractionation. An experimental investigation of the last point with mixtures of two proteins is the particular concern of the present paper.

In his paper Yphantis (1964) discusses thoroughly the pitfalls of the method and gives some general guide lines based on calculated distributions for the detection of the presence of smaller species for various combinations of molecular weight ratios and concentrations. We thought it would be useful to examine some actual mixtures of two proteins containing small amounts of the lower molecular weight protein to see under what conditions heterogeneity could be detected and how accurately the molecular weight of the smaller protein could be measured. Such information is of immediate interest in this laboratory in the investigation of wool and muscle proteins which are extremely difficult to purify completely and in which a small degree of molecular weight heterogeneity seems likely to persist even after repeated fractionation. After this work had been started, Roark and Yphantis

* From the Division of Protein Chemistry, CSIRO, Parkville (Melbourne), Victoria 3052, Australia. *Received May 9, 1969.*

† Correspondence should be addressed to this author at the above address.

presented a simple method for determining the molecular weights of both proteins in a mixture or associating system of two proteins (Roark and Yphantis, 1968) and we report here on the application of this method to our mixtures and an attempt to determine the proportions of the two proteins in the original solution.

Since the manuscript of this paper was submitted a paper by Hoagland and Teller (1969) has appeared which also makes use of the method of Roark and Yphantis, in this instance as a guide to which polymeric forms of a protein were taking part in a chemical equilibrium. This information was used as a starting point for a more detailed and critical analysis of the molecular weight *vs.* concentration data. In their paper Hoagland and Teller refer to two further papers which the present authors have not yet seen but which are likely to be significant in the analysis of sedimentation equilibrium experiments on mixtures or chemically reacting systems of macromolecules. The two papers are by Yphantis and Roark (1968) and Teller *et al.* (1968).

## Materials

*Buffer.* The buffer for all experiments was a solution of analytical reagent grade sodium dihydrogen phosphate (0.0016 M), disodium hydrogen phosphate (0.0028 M), and sodium chloride (0.1 M) in glass distilled water. The pH was adjusted to 7.00.

*Proteins.* The proteins used were bovine plasma albumin (Commonwealth Serum Laboratories, Australia, Cohn fraction V, batch no. 004), ovalbumin (Pentex five-times crystallized, lot 5), and ribonuclease (Sigma chromatographed grade type II salt and protease free, lot R100B-69). A sufficient amount of each of these was dissolved in 20 ml of the buffer solution to give a concentration slightly above 0.1 g/100 ml and dialyzed overnight in the cold against 250 ml of buffer. The optical density of each solution was then measured and its concentration adjusted to 0.10 g/100 ml by dilution with dialysate. The extinction coefficients were taken as 6.60 for bovine plasma albumin at 280 mμ (Tanford and Roberts, 1952), 6.63 for ovalbumin (Biochemists' Handbook, 1961) at 280 mμ and 7.15 for ribonuclease at 278 mμ (Wetlaufer, 1962). These solutions were used as stock solutions from which the mixtures were prepared.

## Methods

*Preparation of Mixtures.* The combinations of proteins which were used were ribonuclease and ovalbumin, ribonuclease, and plasma albumin and ovalbumin and plasma albumin. The first two combinations were prepared in the proportions 5, 10, and 20% ribonuclease plus the higher molecular weight species. 20% ovalbumin plus plasma albumin was the only mixture used for the other combination. The mixtures were prepared the day before the ultracentrifuge run from the 0.10 g/100-ml stock solutions by measuring out the appropriate volume of each solution with a micropipet calibrated in hundredths of a milliliter, to give 2 ml of solution of the required composition and total concentration 0.10 g/100 ml. This solution was then dialyzed overnight in the cold on a rocking dialyzer against 250 ml of the pH 7 buffer.

*Ultracentrifuge Experiments.* A Spinco Model E analytical ultracentrifuge equipped with the rayleigh interference optical system with the upper limiting aperture in the symmetrical position was used for all the sedimentation equilibrium experiments. A 12-mm Kel F coated Aluminium double-sector centerpiece and sapphire windows were used throughout the series. Volumes of 0.09 ml of the fluorochemical FC 43 were put into each sector of the cell. This large volume was used to move the protein solution away from the bottom of the centerpiece as some trouble with fringe distortion had been experienced even with sapphire windows. The volumes of protein solution and dialysate were 0.10 and 0.11 ml, respectively. The solution column was thus just under 3 mm long.

At the end of an experiment the assembled cell was emptied, rinsed three times with distilled water, filled with distilled water, run up to speed, left there for 5 min, and then decelerated. The cell was then emptied, rinsed once more with distilled water, and filled with the same volumes as for the initial experiment, with distilled water in place of solution and solvent. A blank run was then done under the same conditions as those used for the experiment with the protein solution. Photographs were taken after 5–10 min at the operating speed.

To fulfill the requirements for a meniscus depletion experiment in a 3-mm column the quantity $\sigma = \omega^2 M(1 - \bar{v}\rho)/RT$ must be $\geq 5$ cm$^{-2}$ to give sufficient length of flat baseline (Yphantis, 1964). In the experiments with the mixtures the speed was chosen to give $\sigma = 5$ for the low molecular weight species. The required speeds are 31,410 rpm for ovalbumin and 50,740 rpm for ribonuclease. The choice of a $\sigma$ of 5 cm$^{-2}$ for the low molecular weight species together with the relatively high total concentration (0.10 g/100 ml) for meniscus depletion experiments ensures that conditions are close to optimum for the detection of the low molecular weight material. Experiments were also done to characterize the individual proteins. The speeds were 25,980 rpm for plasma albumin, 31,410 rpm for ovalbumin, and 50,740 rpm for ribonuclease. The calculated times to reach sedimentation equilibrium for the conditions used were plasma albumin 9.4 hr, ovalbumin 7.2 hr, and ribonuclease 4.7 hr. Since all the experiments were run overnight it was considered that ample time for the attainment of equilibrium had been allowed. The experiments were done at 25° and the density of the solvent (dialysate) measured with a 50-ml pyncometer was 1.0032 g/100 ml. The values used for the partial specific volumes were plasma albumin, 0.734 ml/g (Dayhoff *et al.*, 1952); ovalbumin, 0.748 ml/g (Lamm and Polson, 1936); and ribonuclease, 0.695 ml/g (Van Holde and Baldwin, 1958).

*Measurements of Photographic Plates.* The photographs of the interference fringe patterns at sedimentation equilibrium were aligned with the radial direction parallel to the horizontal cross hair of a Nikon two-dimensional microcomparator by means of the reference fringes generated by the counter balance. The vertical position of the center of each of five fringes, three black, and two white was measured at increments of 0.5 mm on the plate until the displacement of the fringes relative to the meniscus region was about 100 μ. From then on measurements were taken at 0.1- or sometimes 0.05-mm intervals until the fringes became too crowded together to measure. The same procedure was followed on the blank photograph, the positions read being the same

distances from the center of rotation as those on the solution photograph. These readings on the blank were then used to correct those on the solution photograph for distortion as suggested by Yphantis (1964). The largest blank correction applied to any reading in the 0.1 g/100 ml solutions was only 10 $\mu$. The large volume of FC43 used as a false bottom was thus successful in minimizing optical distortion errors.

*Evaluation of Molecular Weights.* Measurement of the interference fringe patterns at sedimentation equilibrium after correction for optical distortion yields a set of readings of fringe displacement $j$ in microns on the photographic plate at equal radial increments $\Delta r$. These results were expressed as ln $j$ *vs.* $r^2$ and graphical plots made. A weight-average $\sigma$, $\sigma_w(r)$, can be evaluated from such plots by use of the relationship

$$\sigma_w(r) = \left(\frac{\mathrm{d} \ln j}{\mathrm{d}(r^2/2)}\right)_r \qquad (1)$$

If the solution is homogeneous and ideal ln $j$ *vs.* $r^2$ is a straight line whose slope gives the value of $\sigma$ corresponding to the molecular weight of the single species present. In the case of mixtures, if the fractionation is sufficiently great to give a region of solution where the lower molecular weight species is present virtually alone, the molecular weight of this species may be estimated from the initial slope of ln $j$ *vs.* $r^2$. We have calculated molecular weights for our mixtures by drawing the best straight line by eye through the initial points on the ln $j$ *vs.* $r^2$ plots to compare the molecular weights obtained by this method with those obtained in other ways. The criterion for a linear fit was that no point should deviate by more than about 5 $\mu$ from the line. In cases where there appeared to be a second linear region in the plots the same procedure was applied to evaluate a higher molecular weight.

Equation 1 was also used to evaluate point-average values of $\sigma$ from the relationship

$$\frac{\mathrm{d} \ln j}{\mathrm{d}(r^2/2)_{r_0}} = \frac{0.1}{r_0 \Delta r}(2 \ln j_{r_2} + \ln j_{r_1} - \ln j_{r_{-1}} - 2 \ln j_{r_{-2}}) \qquad (2)$$

where ln $j_{r_2} \ldots$ ln $j_{r_{-2}}$ are five data points spaced at equal increments $\Delta r$. The value of d ln $j$/d($r^2$/2) at $r_0$ the central point is equal to the slope of a least-squares straight line through the five points (Yphantis, 1964). No fringe displacements of less than 100 $\mu$ were used.

Number-average $\sigma$ values were evaluated from

$$\sigma n(r_k) \backsimeq \frac{j(r_k)}{I + \frac{\Delta r}{2m}\sum_{i=2}^{k} r_i j(r_i) + r_{i-1} j(r_{i-1})} \qquad (3)$$

where $I = j(r_1)/\sigma_w(r_1)$ and $m$ is the horizontal magnification factor of the ultracentrifuge optical system (Yphantis, 1964). $\sigma_w(r_1)$ was the first value of $\sigma_w$ evaluated by (2), *i.e.*, no fringe displacement of less than 100 $\mu$ was used and the displacement at $r_1$ was therefore about 110–150 $\mu$.

The quantity $\sigma$ has been operationally defined by eq 1. When measurements are carried out on mixtures it is assumed that the fringe displacement $j$ is composed of a contribution from each species in proportion to its concentration at the point $r$ in the cell. This will only be true if the specific refractive index increment of each species in the mixture is the same. The values for the proteins used here at 25° and 546 Å are plasma albumin, 185 × $10^{-5}$; ovalbumin, 182 × $10^{-5}$ (Halwer *et al.*, 1951); and ribonuclease 189 ± 2 × $10^{-5}$ (concentration in g/100 ml). The value for ribonuclease is the mean of two determinations at pH 7, 25° on solutions of concentration 0.4 and 0.8 g per 100 ml dialyzed against the phosphate–sodium chloride buffer. The concentrations of the solutions were obtained from the optical density at 278 m$\mu$ and an extinction coefficient of 7.15 (Wetlaufer, 1962). The refractive index increment of protein solution against dialysate was measured with a Brice-Phoenix differential refractometer calibrated with sodium chloride solutions and checked with potassium chloride solutions. The specific refractive index increments of the three proteins are probably sufficiently close that negligible errors are introduced by treating the results as if they were equal.

The weight-average molecular weight at a point $r$ is evaluated from $\sigma_w(r)$ by use of the relation

$$M_w(r) = \sigma_w(r) \frac{RT}{\omega^2(1 - \bar{v}\rho)} \qquad (4)$$

for an ideal system and where $(1 - \bar{v}\rho)$ is independent of pressure and concentration. If $\sigma_w(r)$ is measured on a mixture of proteins the true weight-average molecular weight is only obtained if all the species have identical refractive index increments and partial specific volumes. We have alluded to the first point above. With paucidisperse protein solutions the partial specific volumes will often be the same because the dispersity may be due to polymeric forms of the same protein. In the mixtures used here however there are large differences in the partial specific volumes of the individual proteins. Indeed the mixture of ribonuclease and ovalbumin probably presents the widest divergence of this quantity likely to be encountered with protein mixtures. There is no way of correcting the experimental observations for this without knowledge of what one is setting out to measure and a single value has to be used for $\bar{v}$. The experiment with ribonuclease alone showed that in the worst case, *i.e.*, the mixture containing 5% ribonuclease and 95% ovalbumin, the maximum error in the calculated weight-average molecular weight would be 10% if the value of $\bar{v}$ for ovalbumin were used. Similarly in the case of 5% ribonuclease–95% plasma albumin the maximum error in the measured molecular weight would be 5% if $\bar{v}$ for ribonuclease were used. Accordingly $\bar{v}$ for ovalbumin was used in evaluating molecular weights in mixtures containing ovalbumin and $\bar{v}$ for ribonuclease in mixtures containing plasma albumin. It should be emphasized that these are the maximum errors that occur in any value of the evaluated molecular weight for the mixtures where the effect is worst. The same considerations apply to the number-average molecular weight. It was assumed that the solutions were thermodynamically ideal because all the molecular weights were evaluated at concentrations below three interference fringes (about 0.08 g/100 ml). No evidence of nonideality was found experimentally. Reciprocal plots of the weight-average molecular weight and the number-average molecular weight *vs.* fringe displacement were made

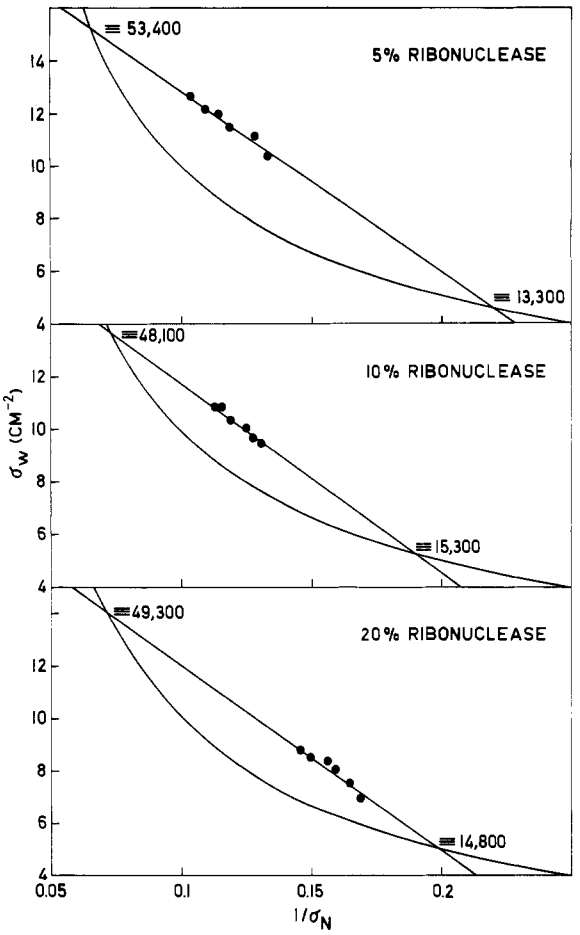


FIGURE 1: Two-species plots for ribonuclease–ovalbumin mixtures at 50,740 rpm. The numbers at the intersections of the line and the hyperbola give the molecular weights equivalent to the values of $\sigma$ at those points (eq 4).

and extrapolated to zero fringe displacement to see whether this procedure gave reasonable estimates of the lower molecular weight.

"*Two-Species Plots.*" A two-species plot is the term used by Roark and Yphantis (1968) to describe the graph obtained by plotting $M_k(r)$ against $1/M_{k-1}$ where $M_k = M_n$, $M_w$, $M_z$ or $M_{z+1}$ and $M_{k-1}$ is the next lower average.[1] $M_k(r)$ and $1/M_{k-1}(r)$ are related by the equation

$$M_k(r) = -M_1M_2[1/M_{k-1}(r)] + M_1 + M_2 \qquad (5)$$

where $M_1$ and $M_2$ are the molecular weights of the two species present. Equation 5 is valid only for systems containing two macromolecular species or components. From now on we will use $M_1$ and $M_2$ to refer to the species of lower and higher molecular weight, respectively. A plot of $M_k(r)$ *vs.* $1/M_{k-1}(r)$ is thus capable of yielding the molecular weights of two species. Equation 5 was derived for an ideal system and if nonideality is present a linear relationship is not obtained, the plot is concave to the abscissa. The presence of more than two species makes the plot convex to the abscissa. Rather than use eq 5 to obtain the two molecular weights one can use the intersections of the two species plot with the hyperbola obtained by plotting a suitable range of molecular weights *vs.* their reciprocals. Such a hyperbola is the locus, on the reciprocal plot, of all single species solutes and the two molecular weights we are interested in lie somewhere on it. Since they also lie on the two-species line they are uniquely defined by the intersections of this line with the hyperbola. This is the method used in the present paper where we have used the weight-average and number-average $\sigma$ values in the two species plots (Figure 1). The $\sigma$ values obey eq 5 and after obtaining $\sigma_1$ and $\sigma_2$ as outlined above, the corresponding molecular weights were evaluated from eq 4. In this case the appropriate values of the partial specific volumes were used.

*Proportions of Proteins in the Original Solution.* Having evaluated $M_1$ and $M_2$ it is desirable to have some estimate of the proportions of the two species in the initial mixture. Yphantis (1964) has shown that for ideal solutions the weight-average molecular weight over the whole solution is equal to the number-average molecular weight extrapolated to the cell bottom, *i.e.*

$$\overline{\sigma_w} = \sigma_n(b) \qquad (6)$$

when the concentration at the meniscus is negligible. If $M_1$ and $M_2$ are known it should be possible to calculate their proportions in the original solution from the expression for the weight-average molecular weight

$$\overline{M}_w = M_1f_1 + M_2f_2$$

where $f_1$ and $f_2$ are the fractions of the two species in the original solution. It is not possible to do this with the results of experiments in which $\sigma = 5$ cm$^{-2}$ for the lower species and the initial concentration is high (*e.g.*, 0.1 g/100 ml) because there is a large region near the cell bottom where the concentration gradient is so steep that measurements cannot be made, and consequently the extrapolation to get $\overline{M}_w$ is subject to unacceptably big errors. However it is possible to perform an experiment at low speed and initial concentration so that an accurate extrapolation of $\sigma_n$ to the cell bottom can be made. The results of such experiments are described later.

It should be pointed out that there is a possibility of complex formation in these mixtures as ribonuclease has a net positive charge at pH 7 and ovalbumin and plasma albumin have net negative charges. If there had been such interaction in significant amount it would have been expected to show up as the presence of a third molecular weight species in the two species plots. This behavior was not seen even in the experiments with the highest proportions of ribonuclease, it was therefore concluded that if any complexes were formed they were in too small amounts to be experimentally detectable. Erlander and Foster (1959) in Archibald approach to equilibrium experiments with mixtures found no evidence of interaction between ovalbumin and ribonuclease at 24° in 0.1 M sodium chloride (pH 5.7–6.6) when the two proteins were present in approximately equal amounts and the total concentration was 1.1 g/100 ml.

[1] Sophianopoulos and Van Holde (1964) utilized the relationship between the weight- and $z$-average molecular weights generalized in eq 5 in their study of lysozyme.

## Results

*Single Proteins.* A. RIBONUCLEASE. The Sigma ribonuclease which was used in the first meniscus depletion experiment was found to contain material of molecular weight less than 11,000 as well as material of molecular weight greater than 15,000 and was therefore not suitable for the experiments with mixtures. A sample of this ribonuclease was applied to a Sephadex G-200 column equilibrated with the buffer used in the ultracentrifuge experiments. The elution profile and experimental details are given in Figure 2. The area of the small peak which is assumed to correspond to aggregated ribonuclease is about 6% of the total area of the pattern. The presence of low molecular weight material is not detectable. A tube toward the leading edge of the main peak (tube 51) was chosen in order to eliminate low molecular weight material and after dilution to a suitable concentration the ribonuclease solution from this tube was used for a meniscus depletion experiment which gave the following results. The plot of ln $j$ *vs.* $r^2$ was apparently linear and gave a molecular weight of 14,500, however the values of the point-average molecular weights increased with concentration and the reciprocal plot gave an extrapolated value of 13,300. A two species plot was then made and gave values of 13,400 and 25,000 which correspond within experimental error to monomer and dimer.

Presumably the smaller peak in the Sephadex elution profile is due to the ribonuclease dimer. There was no evidence of any material of molecular weight less than 13,300 in the meniscus depletion experiment. Extrapolation of $M_n$ to the cell bottom gave $\bar{M}_w$ = 15,400 corresponding to 13% dimer in the original solution. This figure is certainly too high because the gel filtration indicated only 6% of aggregate in the whole sample before the cut was made. The inaccuracy of this figure is illustrative of the errors in the proportions of low molecular weight species calculated in this way which are discussed fully under Results. It was concluded that the solution of ribonuclease obtained after gel filtration contained no material of molecular weight lower than the ribonuclease monomer and less than 6% of ribonuclease dimer. The presence of some aggregated material in samples of ribonuclease which have been stored under refrigeration for a period of months as this sample had, has been reported before (Yphantis, 1960).

In order to see whether the presence of 6% of ribonuclease dimer was acceptable in the experiments with mixtures we calculated the concentration of each of the species ribonuclease, ribonuclease dimer and plasma albumin at $r$, the most centrifugal point in the cell at which the molecular weight was measured, from the expression

$$C_i(r) = A_i \exp(\sigma_i r^2/2) \quad (7)$$

where

$$A_i = 0.5\sigma_i C_0(b^2 - a^2)/[\exp(\sigma_i b^2/2) - \exp(\sigma_i a^2/2)] \quad (8)$$

$C_0$ is the initial concentration, $b$ is the position of the cell bottom, and $a$ is the position of the meniscus. The calculation was made for the mixture containing the highest proportion of ribonuclease, namely, 20% ribonuclease–80% plasma albumin. Assuming that the ribonuclease contained 6%

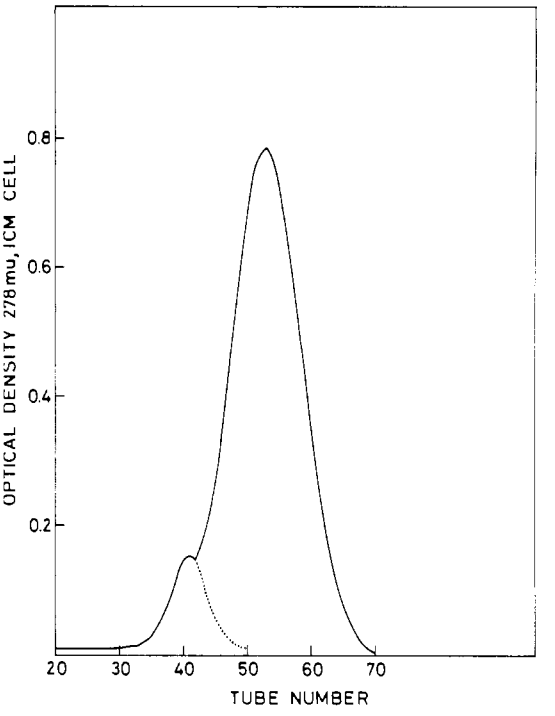


FIGURE 2: Elution profile of 158 mg of Sigma ribonuclease in 15 ml of sodium phosphate–sodium chloride buffer of pH 7.0 applied to 133 × 2.3 cm column of sieved Sephadex G-200, fraction size 7 ml.

dimer the composition of the original solution in weight/volume terms would be 18.8% ribonuclease monomer, 1.2% ribonuclease dimer, and 80% plasma albumin. The total initial concentration was taken as 0.10 g/100 ml, the speed of 50,740 rpm gives $\sigma$ (ribonuclease) = 4.76 $cm^{-2}$, $\sigma$ (ribonuclease dimer) = 9.52 $cm^{-2}$ and $\sigma$ (plasma albumin) = 20.92 $cm^{-2}$, $a$ = 6.6481 cm from the axis of rotation, $b$ = 6.9330 cm from the axis of rotation and the point $r$ at which the concentrations were calculated was 6.8877 cm from the axis of rotation. The latter three values were those obtained in an actual experiment with 20% ribonuclease–80% plasma albumin. The result of the calculation was that at the point $r$ 87.2% of the total concentration was due to ribonuclease monomer, 2.4% to ribonuclease dimer, and 10.4% to plasma albumin. The contribution of the dimer to the weight-average molecular weight at the point $r$ was 3.4%. This is of the same order as the precision of the molecular weight determinations and since the calculation was made for the experiment with most dimer present it was concluded that 6% or less dimer in the ribonuclease would not measurably affect the point-average molecular weights evaluated in mixtures of ribonuclease and the other proteins. Accordingly the ribonuclease solution obtained after gel filtration (tube 51) was used in the experiments with mixtures.

B. OVALBUMIN. The plot of ln $j$ *vs.* $r^2$ was linear and gave a molecular weight of 48,200. The point-average molecular weights increased with concentration and the reciprocal plot gave an extrapolated value of 45,800. The two species plot gave a value of 46,000 for $M_1$ but owing to the few points available and their scatter it was not possible to decide whether the aggregate was dimer or trimer. Values ranging from 90,000 to 136,000 could plausibly be obtained. In either event the

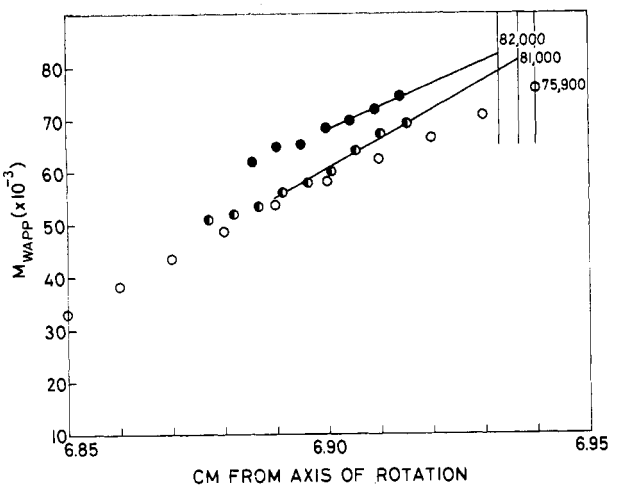


FIGURE 3: Theoretical and experimental weight average molecular weights in a mixture of 20% ribonuclease–plasma albumin. Initial concentration 0.02 g/100 ml, speed 29,500 rpm. meniscus 6.64 cm, bottom 6.94 cm, 25°. (● and ◐) Two separate experiments; (○) theoretical molecular weights calculated from eq 7 and 8. The experimental molecular weights were evaluated using the weight-average $\bar{v}$ at each point obtained from the theoretical distribution.

aggregates will not affect the calculated molecular weights in the region of the cell which is measured.

C. PLASMA ALBUMIN. The ln $j$ *vs.* $r^2$ plot was linear with a hint of upward curvature at the high end and gave a molecular weight of 69,000. The point-average molecular weights increased with increasing concentration and the reciprocal plot gave an extrapolated value of 69,000. The two species plot gave values of 67,500 and 136,000, *i.e.*, monomer and dimer. Extrapolation of $M_n$ to the cell bottom gave $\bar{M}_w$ = 73,000 corresponding to 8% of the dimer.

As we show later this method of estimating the proportion of low molecular weight material present in a mixture is subject to large errors, however it is thought that this figure of 8% for plasma albumin is probably quite close to the true value because it agrees with that measured by Yphantis (1964) for a sample of bovine plasma albumin. He obtained a value of 9.5–10.5% for the proportion of dimer on extrapolating $M_n$ and $M_w$ and this estimate was found to be in satisfactory agreement with a value of 8% obtained from sedimentation velocity experiments on the same preparation. The only place the proportion of plasma albumin dimer is made use of is in calculating theoretical distributions for a mixture of 20% ribonuclease and plasma albumin at low speed and low concentration (see under Results and Figure 5) and the contribution to the total fringe displacement of 8% of plasma albumin dimer is less than 5% to within 0.02 cm of the cell bottom. Thus only the order of magnitude of the amount of dimer is really required.

*Mixtures.* The experiments with mixtures of proteins had three objectives; the detection of heterogeneity, the evaluation of $M_1$ and $M_2$ and the determination of the proportions of the two proteins present in the original solution. The criteria for heterogeneity were the deviation of the ln $j$ *vs.* $r^2$ plot from linearity, the increase of $M_w$ or $M_n$ with concentration and the comparison of the variation of $M_w$ and $M_n$ with concentration. $M_1$ was evaluated from the initial slope of the ln $j$ *vs.* $r^2$ plot, from the extrapolation of reciprocal plots of $M_n$ and $M_w$ to zero concentration and by using the two species plot. $M_2$ was evaluated from the two species plot. We also wanted to see whether we could estimate the proportions of the two species in the original mixture by extrapolating $M_n$ to the cell bottom to give $\bar{M}_w$ and extrapolating $M_w$ to the cell bottom to give $\bar{M}_z$. To test this procedure we did an experiment with the 20% ribonuclease −80% plasma albumin mixture at a low total concentration (0.02 g/100 ml) to allow measurement of the interference fringe pattern right up to the cell bottom thus allowing precise extrapolation of $M_n$. The bovine ribonuclease used in this experiment was a sample from the Armour Pharmaceutical Co. which gel filtration on Sephadex G-200 and a meniscus depletion experiment showed had slightly less dimer present than the Sigma sample, and no low molecular weight material. Its molecular weight was measured to be 14,000 in a meniscus depletion experiment. The experiment with this mixture could not be done at $\sigma$ = 5 cm$^{-2}$ for ribonuclease because with this set of experimental conditions not enough measurable fringe pattern is observed at sedimentation equilibrium. Accordingly a compromise was made and the experiment was performed at $\sigma$ = 7 cm$^{-2}$ (29,500 rpm) for plasma albumin. The resulting weight-average molecular weights are shown in Figure 3 and the number-average molecular weights in Figure 4. The weight-average molecular weights extrapolated to give a value for $\bar{M}_z$ of 82,000 and the number averages to give a value for $\bar{M}_w$ of 74,500. Both of these figures correspond to the presence of less than 0% ribonuclease. The effect of the error involved in the method of evaluating $M_n$ by approximating I as indicated in eq 3 was assessed by using trapezoidal integration, *i.e.*, from

$$\sigma_n(r_k) = \frac{j(r_k)}{\frac{\Delta x}{2m}\sum_{i=2}^{k} r_i j(r_i) + r_{i-1} j(r_{i-1})} \qquad (9)$$

where the sum in the denominator is evaluated at equal increments all the way from the meniscus to the point $r_i$. As Figure 4 shows there is a difference of about 8% in the values of $M_n$ evaluated by the two methods at the low $r$ values but this rapidly falls off to a negligible difference near the cell bottom. This is in agreement with the comments of Yphantis (1964) on the effect of approximating part of the required integral by $I$. A second experiment with freshly prepared protein solutions and exactly the same experimental conditions was done to see what the experimental error in evaluating the weight- and number-average molecular weights was. The results of this experiment are also shown in Figures 3 and 4 and also give values of $\bar{M}_z$ and $\bar{M}_w$ which grossly underestimate the amount of ribonuclease present. The results of the two experiments however are in fairly good agreement, the error in $\bar{M}_z$ being about 1.2% and in $\bar{M}_w$ about 10%. As Figure 5 shows the error in the actual measured fringe displacement in the two experiments was only about ±7 $\mu$ which is close to that expected by Yphantis (1964) for this kind of experiment. Figure 5 also shows the theoretical fringe displacement as a function of distance for the two experiments. This was calculated from eq 7 and 8 and the experimental parameters and the presence of 8% of plasma albumin dimer was allowed for. The fringe displacement was

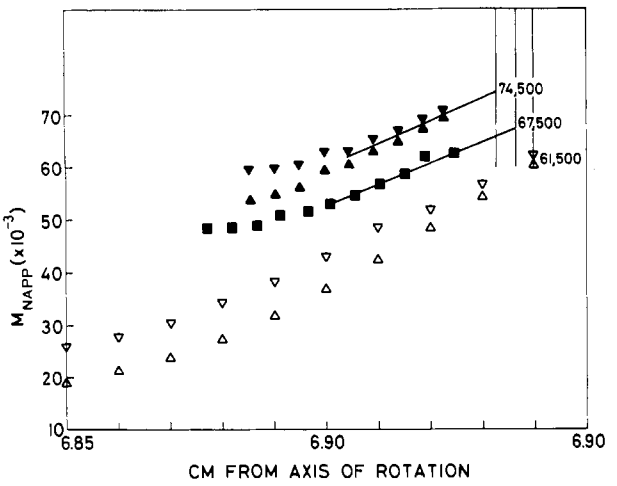


FIGURE 4: Theoretical and experimental number average molecular weights in a mixture of 20% ribonuclease–plasma albumin. Initial concentration 0.02 g/100 ml, speed 29,500 rpm, meniscus 6.64 cm, bottom 6.94 cm, 25°. (▼) Evaluated from eq 3, (▲) evaluated from eq 9, (■) separate experiment eq 3, (▽) theoretical molecular weights evaluated from eq 9 as described in the text, and (△) theoretical molecular weights evaluated from the calculated concentration distributions (eq 7 and 8). The experimental molecular weights were evaluated using the number-average $v$ at each point obtained from the theoretical distribution.

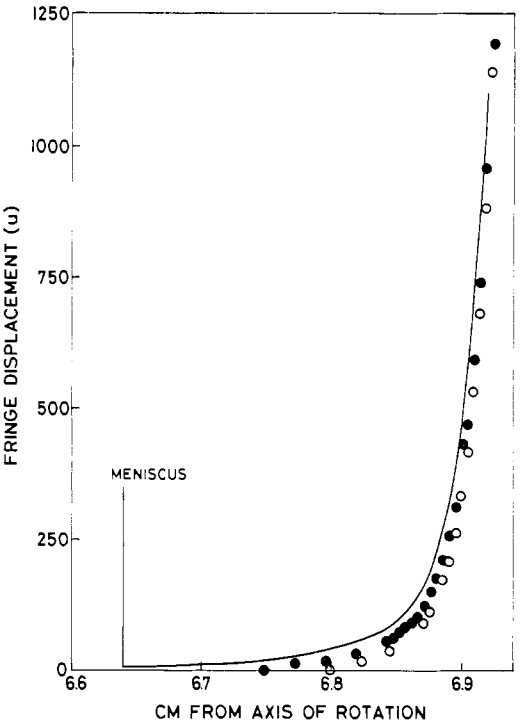


FIGURE 5: Theoretical and experimental fringe displacements in a mixture of 20% ribonuclease–plasma albumin. Initial concentration 0.02 g/100 ml, speed 29,500 rpm, meniscus 6.64 cm, bottom 6.94 cm, 25°. (● and ○) Two separate experiments, the solid line the theoretical distribution.

calculated from the concentration $\Delta C$ in g/100 ml from

$$j = \Delta n/\Delta c \cdot \Delta c \cdot 1/\lambda \qquad (10)$$

and a mean fringe displacement of 287 $\mu$/fringe where $j$ = the number of fringes generated by light of wavelength $\lambda$ = 5461 Å passing through a solution of concentration $\Delta c$ in g/100 ml of specific refractive index increment $\Delta n/\Delta c$ = 187 $\times$ $10^{-5}$ and of path length 1.2 cm. It can be seen in Figure 5 that there is a discrepancy of about 30 $\mu$ between the mean of the experimental fringe displacements and the theoretical at the meniscus end and of over 100 $\mu$ at the bottom and that this is too big to be experimental error. The result of this on the weight and number average molecular weights is shown in Figures 3 and 4 where the experimental molecular weights are compared with the theoretical ones. Obviously the error is much more serious in its effect on the number average molecular weights. The effect of using a base line evaluated on the same criterion as one would use experimentally (constant to within $\pm 5$ $\mu$), and evaluating number-average molecular weights by trapezoidal integration from the theoretical fringe displacements is shown in Figure 4. This is as well as one could possibly do experimentally and shows that the error in a number-average molecular weight at a given point could be as high as 25%. However the extrapolated $M_n$ should still be very close to its theoretical value.

We did an experiment with ribonuclease alone under exactly the same conditions as the two experiments discussed above to see whether this also showed a discrepancy between calculated and experimental fringe displacements. The concentration of ribonuclease was 0.004 g/100 ml, *i.e.*, the same as its concentration in the mixtures. The measured fringe displacements are compared with the theoretical ones in Figure 6 where it will be seen that the experimental displacements are low by about 15–45 $\mu$ between the meniscus and the cell bottom, respectively. It was thought that these low readings were probably due to adsorption of some of the protein from solution onto the walls of the centerpiece, an effect which has been stated to be potentially serious in solutions of low concentration (Yphantis, 1964). We attempted to test this by allowing a solution of ribonuclease of concentration 0.004 g/100 ml to stand in the solution sector of an assembled double-sector cell for 5 days, removing it by

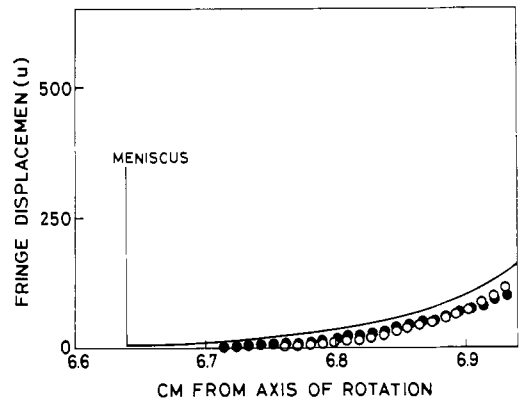


FIGURE 6: Theoretical and experimental fringe displacements in a ribonuclease solution of initial concentration 0.004 g/100 ml, speed 29,500 rpm, meniscus 6.64 cm, bottom 6.94 cm, 25°. (● and ○) Two separate experiments; (○) that after standing cell full of solution for 5 days before performing the run (see text). The solid line is the theoretical distribution.

TABLE I: Results of Meniscus Depletion Experiments with Mixtures of Two Proteins.[a]

| Protein Mixture | Ratio of $\sigma$ | Form of ln $j$ *vs.* $r^2$ Plot | Mol Wt from ln $j$ Plot | Mol Wt from Extrapolation of Reciprocal Plots | | Mol Wt from Two Species Plots | |
|---|---|---|---|---|---|---|---|
| | | | | $M_n$ | $M_w$ | $M_1$ | $M_2$ |
| 20% ovalbumin and plasma albumin | 1.5:1 | Linear | 65,000 | 64,000 | 65,000 | Unusable | |
| 5% ribonuclease and ovalbumin | 3.4:1 | Two linear regions | 41,600 and 24,400 | 20,700 | 24,700 | 13,300 | 53,400 |
| 10% ribonuclease and ovalbumin | 3.4:1 | Two linear regions | 34,600 and 22,500 | 18,300 | 28,900 | 15,300 | 48,100 |
| 20% ribonuclease and ovalbumin | 3.4:1 | Two linear regions | 19,000 and 16,200 | 18,200 | 22,600 | 14,800 | 49,300 |
| 5% ribonuclease and plasma albumin | 5.1:1 | Two linear regions | 39,500 and 16,400 | 15,000 | 25,000 | 13,000 | 71,400 |
| 10% ribonuclease and plasma albumin | 5.1:1 | Two linear regions | 24,400 and 13,400 | 13,300 | 13,200 | 12,700 | 68,900 |
| 20% ribonuclease and plasma albumin | 5.1:1 | Linear | 19,100 | 13,800 | 12,800 | 13,600 | 68,500 |

[a] Experiments in 12-mm cell, 3-mm column height, 25° at speeds to give $\sigma = 5$ cm$^{-2}$ for the lower species. Total concentration in each case 0.10 g/100 ml in phosphate buffer (pH 7.00)–0.1 M NaCl.

syringe as completely as possible then dismantling the cell and gently drying the centerpiece and windows with tissue. The reassembled cell was then loaded with ribonuclease solution of concentration 0.004 g/100 ml in exactly the same way as for the first experiment and the experiment repeated. It was hoped that prior equilibration of the interior of the cell with the solution would prevent further adsorption from a fresh solution. The results of this experiment (Figure 6) are in excellent agreement with those of the previous one with the low concentration ribonuclease solution so the long soaking apparently has accomplished nothing. A further experiment with a ribonuclease solution of the same concentration and under the same experimental conditions except that the FC 43 layering oil was omitted was done to see whether the FC 43 was removing any protein from solution. The discrepancy between the measured and calculated fringe displacements was even greater under these conditions and still in the same direction, that is the measured fringe displacements were too low. A duplicate experiment with a freshly prepared solution gave the same result. The mean difference between the calculated and experimental fringe displacements down the length of the solution column with FC 43 present was 21 $\mu$ and without FC 43, 36 $\mu$. The ratio of these two quantities is 1.7:1. Omission of the FC 43 allows the solution to come into contact with the bottom of the centerpiece and the ratio of the area of centerpiece available to the solution without FC 43 to that with it present (excluding the area of the sapphire windows) is 1.67:1. Thus apparently the difference in the calculated and experimental fringe displacements is directly proportional to the area of centerpiece available to the solution and it therefore seems very likely that this difference arises from adsorption of protein onto the walls of the centerpiece. The fact that prior equilibration of the centerpiece with protein solution did not prevent this is probably due to the difficulty of removing the solution used for equilibration without also removing the protein adsorbed to the centerpiece walls. The experiments with and without FC 43 indicate that FC 43 does not remove ribonuclease from solution as this would result in increased fringe displacements when the FC 43 was omitted whereas a decrease is observed. It is also unlikely that FC 43 causes aggregation of ribonuclease because one would then expect in the presence of FC 43 an upturn in the experimental fringe displacements near the cell bottom causing them to be higher than those calculated for the ribonuclease monomer. In fact the discrepancy between calculated and experimental fringe displacements tends to increase toward the cell bottom (Figure 6). It is felt therefore that the most likely explanation of the discrepancies shown in Figures 3–6 is adsorption of protein from solutions of low concentration. In the case of mixtures of species of low and high molecular weights this will be a serious problem for assessing the proportion of the lower species present because it will be the one which is present virtually alone at the meniscus end of the cell (*cf.* Figures 5 and 6) and therefore a disproportionate amount of it will be removed from solution in this important region.

## Discussion

The results of the experiments with mixtures are summarized in Table I. Heterogeneity was easily detectable in all of the mixtures except 20% ovalbumin and plasma albumin where only the systematic difference in $M_w$ and $M_n$ revealed the presence of more than one protein. For this reason it was thought not worthwhile to use mixtures of these proteins containing smaller amounts of ovalbumin. The results ob-

tained with this mixture suggest the possibility of missing heterogeneity in cases where the molecular weights of the proteins are not very different, *e.g.*, 2:1 or less and where there is less than about 20% of the smaller species. Results obtained for such mixtures are likely to be interpreted as showing the presence of a single protein with a molecular weight lower than that of the major component. This is even more likely to happen with nonideal solutions where variations in ln $j$ *vs.* $r^2$ and in $M_w$ and $M_n$ due to heterogeneity can be masked by those due to thermodynamic nonideality. This possibility has been shown before in experiments with SCM[2]-$\beta$-lactoglobulin and SCM-ovalbumin in 8 M urea. When 10% SCM-$\beta$-lactoglobulin was present ln $j$ *vs.* $r^2$ was linear, $M_{w,app}$ did not vary with concentration and a value of 37,000 was obtained for the molecular weight of the one protein apparently present (Jeffrey, 1968).

The molecular weights obtained for the lower molecular weight species from the ln $j$ *vs.* $r^2$ plot are in general not very satisfactory. Only in one case (10% ribonuclease plus plasma albumin) was the value correct. This method generally seems to overestimate the molecular weight even with two species whose molecular weights are in the ratio 5:1 and with $\sigma = 5$ for the lower species. With mixtures of species of molecular weights differing by a factor of 5 it seems that extrapolation of point-average molecular weights to infinite dilution gives reasonable estimates of $M_1$ providing there is 10% or more of this species present. Where there is a bigger difference in molecular weights presumably lower proportions would give equally good estimates. Again with the lower ratios and proportions the values obtained for $M_1$ tend to be too high although it is worth noting that extrapolation of $M_n$ is much more satisfactory than $M_w$ in these cases.

These results are consistent with those previously reported (Jeffrey, 1968) for a meniscus depletion experiment with 10% ribonuclease plus ovalbumin in 0.01 M sodium borate. In that case the speed was such as to give $\sigma = 7.5$ cm$^{-2}$ for ovalbumin as compared with $\sigma = 5$ cm$^{-2}$ for ribonuclease in the present series and $\bar{V}$ for ribonuclease was taken as 0.723 ml/g whereas it is now thought that the value used in the present paper 0.695 ml/g is more likely to be correct (Van Holde and Baldwin, 1958). Recalculation of the results obtained in 0.01 M sodium borate using the latter value gave values of 30,000 and 32,500 for $M_1$ from extrapolation of the reciprocal plots of $M_n$ and $M_w$, respectively. Heterogeneity was detectable from the ln $j$ *vs.* $r^2$ plot and the difference in $M_n$ and $M_w$, but there was insufficient fractionation at the speed used to allow the two species plot to be used in evaluating $M_1$ and $M_2$.

The two species plots in the present series give values for $M_2$ which are in fair agreement (7% or better) with those obtained for the single proteins for all combinations above 5% ribonuclease–ovalbumin. The agreement of $M_1$ with the known values is better than 7% for the ribonuclease–plasma albumin mixtures. This method is therefore rather better than that of extrapolation for obtaining $M_1$ and has the decided advantage of giving good values of $M_2$ at the same time.

It was not possible to use the two species plot to obtain values for $M_1$ and $M_2$ when a speed corresponding to a $\sigma$ of 5 cm$^{-2}$ for the higher species was used and probably the best approach when contamination of a major component with a small amount of a protein of lower molecular weight is suspected is to carry out a meniscus depletion experiment at a $\sigma$ of 5 cm$^{-2}$ or higher for the smaller protein (whose molecular weight may be approximately known from other experiments) at a high total concentration. The two species plot can then be used to obtain values of $M_1$ and $M_2$. Our attempts at estimating the proportions of the two species present even in the mixture with the highest ratio of molecular weights and 20% of the lower species were not successful. At least with these proteins it seemed that if the experiment was performed at a low enough concentration to allow precise extrapolation to the cell bottom, low values of the fringe displacement, probably caused by adsorption of protein, caused such large errors in the extrapolated values of $M_n$ and $M_w$ that they were virtually useless for the required purpose. It is felt therefore that proportions of low molecular weight species calculated from such extrapolations especially in solutions of low concentration should be very cautiously interpreted. It would be more satisfactory whenever it is possible to obtain the proportion and number of components by some other technique such as polyacrylamide gel electrophoresis where densitometry of the resulting protein bands allows their proportions to be determined with more certainty. This information can also be used in combination with the results of sedimentation equilibrium experiments to fix more precisely the values of the molecular weights of the components.

[2] SCM-*S*-carboxymethyl.

Acknowledgments

We wish to thank Professor D. A. Yphantis for sending us a prepublication copy of Studies of Self-Associating Systems by Equilibrium Ultracentrifugation by D. E. Roark and D. A. Yphantis and Mr. E. F. Woods for useful discussion and criticism of this work.

References

Biochemists' Handbook (1961), Long, C., Ed., London, E. and F. N. Spon, p 82.

Dayhoff, M. O., Perlmann, G. E., and MacInnes, D. A. (1952), *J. Am. Chem. Soc. 74*, 2515.

Erlander, S. R., and Foster, J. F. (1959), *J. Polymer Sci. 37*, 103.

Halwer, M., Nutting, G. C., and Brice, B. A. (1951), *J. Am. Chem. Soc. 73*, 2786.

Hoagland, V. D., Jr., and Teller, D. C. (1969), *Biochemistry 8*, 594.

Jeffrey, P. D. (1968), *Biochemistry 7*, 3345, 3352.

Lamm, O., and Polson, A. (1936), *Biochem. J. 30*, 528.

Roark, D. E., and Yphantis, D. A. (1968), *Biophys. J. 8*, A99.

Sophianopoulos, A. J., and Van Holde, K. E. (1964), *J. Biol. Chem. 239*, 2516.

Tanford, C., and Roberts, G. L., Jr. (1952), *J. Am. Chem. Soc. 74*, 2509.

Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1968), *Ann. N. Y. Acad. Sci.* (in press).

Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.

Wetlauffer, D. B. (1962), *Advan. Protein Chem. 17*, 376.

Yphantis, D. A. (1960), *Ann. N. Y. Acad. Sci. 88*, 586
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Yphantis, D. A., and Roark, D. E. (1968), *Ann. N .Y.Acad. Sci.* (in press).

# Lipid Composition of Synaptic Plasma Membranes Isolated from Rat Brain by Zonal Centrifugation*

Carl Cotman,† M. L. Blank, Anita Moehl, and Fred Snyder

ABSTRACT: A plasma membrane fraction derived from rat brain nerve endings or the synaptic plasma membrane fraction, isolated by zonal centrifugation, was analyzed for their lipid content and their composition of lipid classes and their aliphatic moieties. With the exception of glycolipids and sphingomyelin, the lipid classes of synaptic plasma membrane are very similar to whole brain. Phosphatidylcholine and phosphatidylethanolamine are the major lipids and make up 62% of the total membrane lipid. Cholesterol and ceramide make up the major neutral lipids and 21% of total brain lipid. The synaptic plasma membranes contain lower quantities of glyceryl ethers in phosphatidylethanolamine and phosphatidylcholine fractions than in whole brain. Certain distinctive features in fatty acid content were found; synaptic plasma membranes contained much less 18:1 acyl moieties in phosphatidylethanolamine and phosphatidylserine + phosphatidylinositol than found in whole brain, whereas most other acyl moieties with the exception of the longer chain unsaturated fatty acids are similar. The 22:6 acyl chains in synaptic plasma membrane account for approximately 32% of the total fatty acids in phosphatidylethanolamine and phosphatidylserine + phosphatidylinositol, but in whole brain the 22:6 fatty acids account for only about 26 and 20% of phosphatidylethanolamine and phosphatidylserine + phosphatidylinositol, respectively. The alk-1-enyl moieties in phosphatidylethanolamine are restricted to 16:0, 18:0, and 18:1 chain lengths in both synaptic plasma membrane and whole brain.

The components of neuronal membranes need to be known because elucidation of the structure and dynamic function of this membrane is dependent upon such knowledge. Specific lipids of membranes undoubtedly subserve specific functions; studies of model membranes and experiences with enzyme reconstitution illustrate the vital role and specificity of lipids in the functional properties of biomembranes. Tetrodotoxin, a molecule that specifically blocks changes in sodium conductance during nerve action potentials, interacts with and causes spreading of surface films made from nonpolar lipids extracted from squid axons, but polar lipids do not show such interactions (Villegas and Camejo, 1968; Camejo and Villegas, 1969). Phospholipids are essential for respiratory activity in mitochondria (Fleischer *et al.*, 1962), and the reconstitution of electron transport and oxidative phosphorylation from mitochondrial components has specific phospholipid requirements (Racker and Bruni, 1968). Structural protein preparations from mitochondria bind cardiolipin in larger quantities than other mitochondrial membrane lipids (Richardson *et al.*, 1964).

Certain phospholipids are effective in reactivating Na–K-ATPase activity (Tanaka and Strickland, 1965). Phosphatidylinositol has been found to have high affinity for calcium at physiological monovalent salt concentrations (Papahadjopoulos, 1968), and calcium is intimately involved in membrane bioelectric activity (Papahadjopoulos and Ohki, 1969). It is, therefore, appropriate to know the lipid composition of nerve membranes to evolve an understanding of their physiologic functioning.

The content, but no detailed class and fatty acid data, of various lipids in subcellular fractions of brain including SPM[1] has been reported by Lapetina and coworkers (1968). Whittaker (1966) has also reported previously unpublished data obtained by Sheltawg on certain lipid classes from synaptosome ghosts or SPM fractions. A detailed analysis of lipid classes from whole synaptosomes has been presented by Eichberg and coworkers (1964) and by Seminario and coworkers (1964). Kishimoto and coworkers (1969) have briefly

* From the Medical Division of the Oak Ridge Associated Universities, Oak Ridge, Tennessee, under contract with the U. S. Atomic Energy Commission, in collaboration with the Joint National Institutes of Health, Atomic Energy Commission Molecular Anatomy Program (the Molecular Anatomy Program is sponsored by the U. S. Atomic Energy Commission, the National Cancer Institute, the National Institute of General Medical Sciences, and the National Institute for Allergy and Infectious Diseases), Oak Ridge National Laboratory (operated for the U. S. Atomic Energy Commission by the Nuclear Division of Union Carbide Corp.), Oak Ridge, Tennessee 37830. *Received June 26, 1969.* This work has been supported in part by the American Cancer Society Grant No. P-470, and National Institute of Health Grant No. GM12562-06.

† Present address: Department of Psychobiology, University of California at Irvine, Irvine, Calif. 92664.

[1] Abbreviations used are: SPM, synaptic plasma membrane; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine.