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The Ultracentrifuge: Problems and Prospects*

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Progress in ultracentrifugation in recent years has stemmed from both theoretical and experimental advances. Among the latter has been the adaptation to the ultracentrifuge of the methods of interferometry which have led to tremendous gains in the accuracy of sedimentation analysis. Despite the power of the interference techniques there are limitations in their optical sensitivity; and, moreover, different chemical species cannot be distinguished from one another optically by these or the older refractometric methods. As a consequence there has been renewed interest in the application of the much more discriminating absorption optical system. This review deals with a variety of the problems inherent in the implementation of interference and absorption optics for ultracentrifugal analysis, and emphasis is directed toward the prospects of further developments in the application of these techniques. In consideration of the difficulties encountered in the adaptation of the Rayleigh interference system to the ultracentrifuge, particular attention is given to the problem of locating the white light (or zero-order) fringe and using it for the evaluation of possible preferential interactions in multicomponent systems. Illustrations are given of the use of the Rayleigh optical system for the determination of concentrations of both large and small molecules and the binding of low molecular weight solutes to proteins. Also a discussion is included dealing with the application of interference optics for the determination of molecular weights by sedimentation equilibrium. Studies with both homogeneous and heterogeneous protein preparations are illustrated.

The absorption optical system, which has remained virtually unchanged since its introduction by Svedberg and his co-workers over twenty years ago, suffers from several deficiencies. Despite this, the versatility and sensitivity of the method have led to its utilization for a host of special problems. Some of these are illustrated with examples from zone centrifugation experiments. In order to eliminate some of the imperfections in the absorption optical system as well as to provide direct viewing of the ultracentrifuge patterns, the photography and densitometry employed heretofore have been replaced by a newly developed automatic split-beam scanning absorption system. The unit consists of a drive assembly which controls the movement of a photomultiplier across the magnified image of the ultracentrifuge cell. This operates in conjunction with the appropriate electronic circuitry necessary to convert the light pulses into curves of optical density versus distance. High resolution is achieved by mounting a slit only 35 μ in width at the entrance to the photomultiplier housing. Double-sector cells are used in the rotor with the result that two light bursts strike the photomultiplier in quick succession followed by a long dark period while the rotor completes one revolution. An electronic switching arrangement is incorporated to effect the separation of the light pulses and the routing of the two signals into two separate holding circuits, one representing the logarithm of the transmittance of the solvent and the other the solution. The outputs of these holding circuits are then fed to a difference amplifier where subtraction is achieved to give a direct plot of optical density versus position in the cell. In effect the single light beam is converted by the chopping action of the rotor into two beams thereby permitting automatic compensation for optical defects. Various applications of the optical system are given including sedimentation coefficient determinations, the evaluation of concentrations, and the determination of molecular weights by the sedimentation equilibrium technique. Also the use of this system in conjunction with a monochromator is illustrated with spectral studies of different sedimenting materials. Finally, applications are given of the analysis of interacting systems and the measurement of small differences in sedimentation coefficients.

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The past ten years have witnessed revolutionary advances in the development and application of the ultracentrifuge as a research tool. As a result, precision and accuracy not previously contemplated are now

commonplace and measurements hardly visualized a few years ago are becoming routine.

Many new trends are apparent. Thus sedimentation velocity experiments, long the favorite of the research worker, are now often inadequate and sedimentation equilibrium experiments frequently take their place. Studies of the homogeneity of macromolecules with respect to sedimentation rate are becoming rare; instead interest is focused on the distribution of molecular weights and buoyant densities. The analysis of interacting systems, formerly the province of other physical chemical techniques, is now a principal target of research with the ultracentrifuge. Uncertainties and anomalies of a few years ago are now well resolved and accounted for in terms of rigorous, powerful, theoretical treatments.

Accompanying the obsolescence of older techniques and experiments are other changes. Simplicity and repetition in experimentation have given way to complexity and diversity. Microgram quantities frequently suffice now where previously even milligram amounts were inadequate. Convenience in the optical registration of the movement and distribution of molecules in a centrifugal field is sacrificed in part because of the more pressing demands for enhanced accuracy. Accordingly the recently adapted interference optical methods are gaining wide popularity for certain types of experiments; and the older, highly perfected cylinder lens schlieren optical system is no longer employed indiscriminately but is relegated to those specific investigations for which it is best suited. Meanwhile the requirements for greater sensitivity and the need to distinguish among the various chemical species present in solutions led to the rebirth of the light absorption optical system which had been prematurely discarded and too-long ignored. In addition to various optical systems the armament of the research worker now includes synthetic-boundary cells, double-sector cells, partition cells, short column cells, wedge-window cells, and compressibility cells. On the shelf are found multicell rotors, high speed rotors of titanium, and special heavy rotors for low speed experiments.

Despite the diversity of existing techniques and their implementation for attacks on the fascinating problems of biochemistry and biophysics, progress should continue unabated as the demands of the research worker become more exacting and advances in technology open new avenues for further exploration. Today's preliminary results herald even more radical developments and progress for tomorrow. It is not feasible to present here a comprehensive treatment of all forefronts of research with the ultracentrifuge. Therefore only a fraction of the recent developments is summarized in this communication. The selection of material for consideration is based on the research activities of the author's laboratory and on the conviction that we have succumbed too long to the attractiveness of the schlieren optical system. Thus the discussion which follows is devoted largely to developments in and applications of the interference and absorption optical systems. This occasion also furnishes the opportunity to illustrate and consider various types of zone (or density gradient) centrifugation which now are so widely used in biochemical investigations.

Rayleigh Interference Optical System

General Considerations.—Despite the apparent success of the Rayleigh optical system in producing ultracentrifuge patterns of high quality this interference system[1] as yet has not been used widely in sedimentation experiments (Mommaerts and Aldrich, 1958; Johnson *et al.*, 1959; Richards and Schachman, 1959; Schachman, 1960; La Bar and Baldwin, 1962; Creeth, 1962). It is not surprising therefore that there is insufficient understanding of the function of some of the components in the optical system. Judging from the history of the development of the schlieren optical system to its present high level of perfection we can anticipate that concerted studies of the Rayleigh system will lead to a powerful and versatile tool for the analysis of ultracentrifuge experiments. Hence no apologies need be offered for outlining here some of the problems inherent in the adaptation of the Rayleigh interferometer.

In the initial adaptation of the Rayleigh interferometer for the ultracentrifuge (Spinco, 1958; Johnson *et al.*, 1959; Richards and Schachman, 1959) the so-called Rayleigh mask which functioned as a beam splitter was incorporated directly in the cell housing. This was accomplished by including a pair of parallel slits (0.510 mm wide) in the lower window holder. Thus light traversed only a small segment of the cross section of each half of the double-sector cells, and the conditions for interference were satisfied only when the rotor was so located during each revolution that the light rays passed through both sectors simultaneously. Because of the relatively large size of the schlieren lenses (compared to the cross section of the cell) the conditions for interference of the light beams were satisfied while the rotor was turning through a considerable angle. To prevent the optical system from "seeing the cell" while the rotor turned through this large angle, an additional aperture containing two parallel slits (0.75 mm wide) was fastened on the upper schlieren lens holder (Spinco, 1958; Richards and Schachman, 1959). The two slits in this upper aperture mask were arranged symmetrically about and parallel to a radius from the center of rotation, and the axis of the cylinder lens was oriented relative to that radius.[2]

Although the Rayleigh interference patterns are of high quality (Richards and Schachman, 1959; Schachman, 1960; La Bar and Baldwin, 1962), they are not completely satisfactory when there are large refractive index gradients in the cells (Clark and Schachman, unpublished data). In a centrifugal field the boundaries (or surfaces corresponding to a fixed value of the refractive index) are not planar but rather in the form of cylindrical surfaces. As a consequence the cylinder lens cannot be oriented correctly in relation to the entire cross section of the cells. Assume for the moment that the cell is stopped in the center of the optical path and the schlieren optical system is in use.

[1] The Jamin interferometer also has been employed for ultracentrifuge experiments (Beams *et al.*, 1954, 1962; Hexner *et al.*, 1962a, b; Beams, 1963). No discussion of this technique is given here but it should be noted that the Jamin interferometer as used by Beams and coworkers possesses certain features which make it especially attractive for sedimentation equilibrium experiments. Apparently the Jamin system has not been employed for sedimentation velocity experiments; and although no equilibrium patterns have been published in recent years, presumably these are now of much higher quality than those shown some years ago (Beams *et al.*, 1955).

[2] An alternative arrangement also has been employed for the fixed upper aperture. This consists of a pair of slits one of which is directly above a radius from the center of rotation with the second displaced from but still parallel to it. With this offset-aperture mask conjugate levels are not compared, and laborious and even uncertain corrections must be applied if there are large refractive index gradients in both compartments (see Richards and Schachman, 1959, for a discussion of the relative merits of these different arrangements).

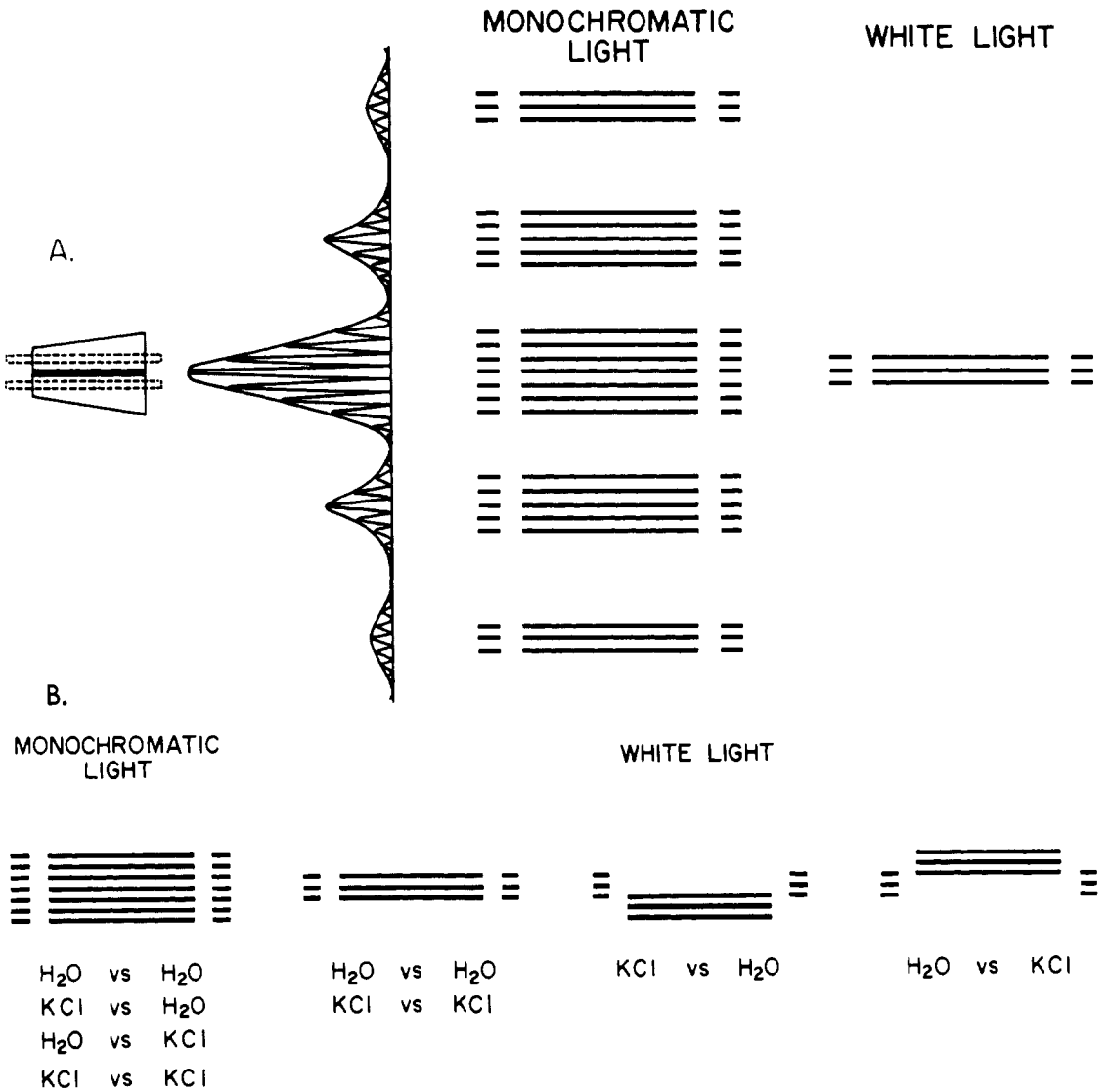

FIG. 1.—(A) Schematic diagram of the diffraction pattern and interference fringes produced with monochromatic and white light when the cell compartments are filled with liquids having the same index of refraction. On the left is shown the double-sector cell along with the pair of slits which acts as the beam splitter (or Rayleigh mask). Next to it is a diagram outlining the light distribution which results from diffraction of light by a pair of slits. The diffraction envelopes are not continuous bands of light but consist, instead, of a series of alternate bright and dark interference fringes. Only the central diffraction envelope is generally visible because of the large decrease in light intensity in the subsidiary envelopes. Though interference fringes are present in the latter they can be detected only by overexposure of the photographic plates. With nonmonochromatic (white) light the fringes, except for the central (achromatic) or zero-order fringe corresponding to equal geometric paths for the light rays through the two cell compartments, are almost obliterated. Generally one less distinct fringe can be detected on either side of the achromatic fringe, and thus three fringes are observed. The short fringe segments on either side of the longer interference fringes represent the pattern from the double slits in the counterbalance cell. (B) Schematic diagram of the interference patterns caused by different combinations of liquids. With monochromatic light (as seen on the left) the central diffraction envelope shows a series of fringes which appear qualitatively the same even when liquids of different refractive index are compared. With white light, however, the location of the visible fringes depends on the relative refractive indices of the two liquids. If they are the same (either water or KCl in both compartments) the achromatic fringes will be centrally located. When one refractive index is higher (or lower) than the other the zero-order fringe is shifted to compensate for the difference in optical paths of the light rays passing through the two liquids. In this way the total optical paths (including the distances through air as well as the optical paths through the two different solutions) are identical. The two diagrams on the right show a fringe shift (down or up) of only 2 fringes. For large differences in refractive index (corresponding, for example, to a concentration of 1 g/100 ml) the shift of the achromatic fringes may amount to 40 fringes.

Only that section of the cell midway between the radial walls of the cell has a refractive index gradient in the proper orientation relative to the axis of the cylinder lens, since the latter is aligned with its axis parallel to the radius through the center of the optical path. All other segments of the cross section of the cell have refractive index gradients at varying angles relative to the axis of the cylinder lens. Since the optical system records refractive index gradients in terms of a series of planes (and not cylindrical surfaces), the gradient recorded for each plane represents some average of the refractive index gradients which are "seen" by the

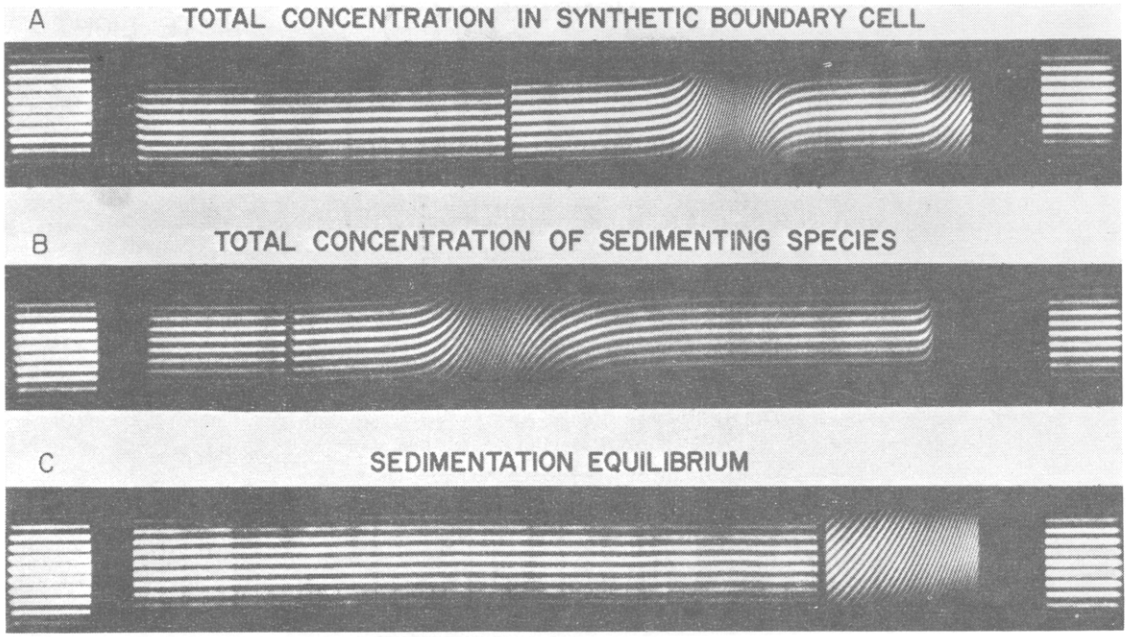

FIG. 2.—Interference patterns representing concentration determinations, sedimentation velocity, and sedimentation equilibrium experiments. The upper pattern was obtained from an experiment in a synthetic-boundary cell; it provides a measure of the total concentration (in fringes) of protein (bovine serum albumin) and sodium dodecyl sulfate in the solution. To obtain this pattern the solvent was layered over the solution containing the protein and detergent. After the boundary spread for some time due to diffusion, the photograph was taken while the rotor was operating at about 10,000 rpm. The second pattern was obtained from an independent sedimentation velocity experiment on the same mixture. This pattern was photographed after the ultracentrifuge was operating for about 1 hour at 59,780 rpm. The number of fringes in this pattern compared to the upper pattern provides a measure of the amount of detergent bound to the protein and sedimenting with it as protein-detergent complexes. The lower interference pattern was obtained at sedimentation equilibrium after the ultracentrifuge operated at 7928 rpm for 18.5 hours (Ramel *et al.*, 1961).

cylinder lens when the cell is in the optical path. Narrowing the aperture above the cell so that only a small region of the cell is observed at a time tends to minimize this complication. Similar considerations apply to the implementation of an interference optical system which employs a cylinder lens. The interferometer ideally should compare only conjugate levels in the two compartments of the double sector cell. If the slits in the upper aperture are too wide, the effect due to the curvature of the surfaces of constant refractive index is serious and the resulting interference patterns are blurred or even annihilated in that region of the image conjugate to the large gradients. Moreover the deviation of the light rays in the direction of increasing refractive index gradient leads to a distortion of the interference fringes. To obviate these difficulties the slits in the upper aperture have been narrowed even further (0.24–0.36 mm) and the slit separation has been reduced to about 3.6 mm (Clark and Schachman, unpublished data). Although this tends to lead to longer exposure times some compensation is achieved by redesign of the window holder in the cell itself. The sapphire windows now available suffer much less distortion than the quartz windows formerly used and the incorporation of a narrow mask on the cell is no longer necessary. Many factors remain to be examined more thoroughly but this tentative arrangement seems superior to that employed heretofore.

The use of very narrow slits in the upper aperture (which functions as the optically active component) leads to wider diffraction envelopes and an increased number of fringes in the central envelope. Although measurements on the plate are slightly more tedious, the resulting patterns have proven especially useful in experiments aimed at determining directly the fringe number by location of the white light (or zero-order) fringe. Preliminary results (Clark and Schachman, unpublished data) indicate that concentrations can be determined directly, without recourse to synthetic-boundary cell experiments, by comparing the white light patterns with those produced by monochromatic light. A schematic diagram illustrating the technique is shown in Figure 1. With monochromatic light no obvious difference in the fringe pattern can be detected even with various combinations of solutions in the two compartments of the double-sector cell. Depending on the refractive indices of the two solutions, the fringes are shifted up or down, but higher order fringes replace those shifted out of view. Therefore a cursory examination of the interference pattern in the central diffraction envelope reveals no differences. With white light, however, only the achromatic (white light) fringes are well defined and their location, relative to the fringes corresponding to the counterbalance cell, depends on the refractive indices of the two liquids. Changing the refractive index of the liquid in one of the compartments causes the achromatic fringes to be shifted up or down. If the refractive index difference between the two liquids is very large, this shift may be many millimeters (equivalent to many fringes, where the number of fringes is the distance divided by the fringe spacing with monochromatic light). By suitable selection of photographic emulsions and with the wide diffraction envelopes produced by the narrow slits, a displacement of the achromatic fringes equivalent to about 40 fringes can be measured directly. Thus protein concentrations of about 1 g/100 ml or even greater can be determined directly from the fringe patterns even in the absence of boundaries. In order to obtain results that are not subject to experimental errors from

TABLE I
CONCENTRATION DETERMINATIONS FROM INTERFERENCE PATTERNS

Radial Dilution During Sedimentation Velocity Experiments[a]

1 t (min)	2 x_H (cm)	3 c_t (fringes)	4 Δc_t (fringes)	5 $c_t + \Delta c_t$ (fringes)	6 c_0 (fringes)	7 $c_t(\bar{x}_t/x_m)^2$ (fringes)
4	5.924	15.51	0.11	15.62	15.72	15.71
24	6.118	14.58	1.04	15.62	15.72	15.76
48	6.360	13.46	2.17	15.63	15.73	15.72
74	6.633	12.36	3.27	15.63	15.73	15.70

Absolute Concentrations from Synthetic-Boundary Cell Experiments[b]

	Concentration in Fringes		Per Cent Error
	Calculated	Measured	
Sucrose			
1	16.10	16.06	−0.25
2	32.26	32.30	+0.12
3	48.52	48.48	−0.08
Bovine serum albumin			
1	40.23	40.13	−0.25
2	40.23	40.21	+0.05

[a] The sedimenting material was bushy stunt virus at a concentration of 0.4 g/100 ml in 0.1 M potassium phosphate buffer at pH 6.8. The speed was 14,290 rpm. The reference liquid contained 1,3-butanediol (Richards and Schachman, 1959).
[b] All experiments were performed in a capillary-type, double-sector synthetic-boundary cell. The calculated fringe numbers were based on dry weight concentrations and independent measurements in a diffusion apparatus (Richards and Schachman, in preparation).

uncontrollable variations in the assembly and distortion of the ultracentrifuge cell, a special cell has been designed and is now being tested (Clark and Schachman, unpublished data). This cell, containing a separate compartment for a known reference liquid, automatically provides a reference for the shift of the zero-order fringe. At the same time it produces the pattern for the solution relative to the solvent. In this way the effect of cell or window distortion is obviated. Some problems still remain but the preliminary results are sufficiently promising to warrant further efforts in this direction.

Determination of Concentrations.—Figure 2a is a typical interference pattern from an experiment employing a double-sector synthetic-boundary cell. Mere counting of the fringes across the boundary immediately provides a value for the difference in refractive index between the solution and the solvent. In conjunction with the measured value of the specific refractive increment of the solute and knowledge of the cell thickness and the wavelength of the light, this fringe count gives a direct measurement of the solute concentration. For proteins (in a 12-mm cell and a wavelength of 546 mμ) a solution at a concentration of 1 g/100 ml produces about 40 fringes. Since measurements can be made to an accuracy of about ± 0.02 fringes, the concentration of such a solution can be evaluated with an accuracy of about ± 0.05%. No other optical system can rival the interference systems for such determinations. Often it is highly desirable to determine concentrations not from layering experiments performed with a synthetic-boundary cell but from actual sedimentation velocity experiments in which the fringe count is made from patterns of the migrating boundary. Such a pattern is shown in Figure 2b, and Table I illustrates the precision with which concentration measurements can be made from a sedimentation velocity experiment. These data (Richards and Schachman, 1959) provide also a critical test of the radial dilution equation of the ultracentrifuge (Svedberg and Rinde, 1924; Trautman and Schumaker, 1954). Columns 1 and 2 record the times after attaining the desired speed and the positions of the boundary. The number of fringes, c_t, across the boundary is listed in column 3, and column 4 gives the change in concentration (Δc_t) in the plateau region as measured there by the lateral shift of the fringes across the pattern. As shown in column 5, the sum of the values in columns 3 and 4 is remarkably constant, indicating that the progressive decrease in the number of fringes across the boundary is fully accounted for by the dilution measured in the plateau region.[3] Since a slight change in concentration had occurred before the first pattern was photographed, the values in column 5 were corrected to give the initial concentration (c_0) shown in column 6. This correction accounts as well for the slight dilution of the cell contents during acceleration of the rotor. Finally, c_0 is calculated (column 7) by means of the well-known radial dilution equation from the c_t values in column 3 and the squares of the positions of the boundaries at specific times relative to the square of the distance from the meniscus to the axis of rotation. The agreement is excellent.

Absolute concentrations as well can be determined with considerable accuracy as shown in experiments with sucrose and bovine serum albumin (Richards and Schachman, in preparation). In these experiments, some of which are illustrated in Table I, solutions of known dry weight concentration were prepared and the fringe count was measured in a synthetic-boundary cell after layering the solvent over the solution. Uncertainties in the value of the optical thickness of the liquid column constitute the largest source of error, since the plastic centerpiece of the ultracentrifuge cell is compressed during tightening of the cell and there may be some bulging of the windows under the influence of the centrifugal field. Measurement of the value of optical thickness is necessary and feasible, but correction for window distortion remains a problem which is as yet unsolved.

[3] Such measurements with schlieren optics are not possible, since only the difference in refractive index (concentration) across a boundary is measurable and not the change with time in a region of uniform concentration.

The convenience and accuracy of the Rayleigh system for concentration determinations make it especially valuable for the analysis of mixtures of sedimenting components in terms of the relative amounts of the different materials. No tedious integration procedures are required, as with the schlieren patterns, and corrections for the redistribution of buffer salts are unnecessary.

Evaluation of Preferential Interactions.—Structural investigations on proteins and other macromolecules frequently involve physical chemical measurements on solutions containing appreciable amounts of denaturing and dissociating agents such as urea, guanidine, or sodium dodecyl sulfate. Unless special precautions are taken in the design and execution of these experiments (Casassa and Eisenberg, 1960, 1961; Eisenberg, 1961; Eisenberg and Woodside, 1962) ambiguities exist because of possible preferential interactions between the macromolecules and either the low molecular weight solute (urea, guanidine, sodium dodecyl sulfate) or the solvent (water). For some systems the dialysis procedure (Casassa and Eisenberg, 1961; Eisenberg, 1962) may not be feasible and it is mandatory, therefore, that the preferential interactions, if any, be evaluated. Then the necessary correction term can be included in the multicomponent theory for interpreting the physical chemical data (Stockmayer, 1950; Kirkwood and Goldberg, 1950; Schachman and Lauffer, 1950; Wales and Williams, 1952; Ogston, 1954; Katz and Schachman, 1955; Williams *et al.*, 1958; Baldwin, 1958; Peller, 1958; Schachman, 1959, 1960; Fujita, 1962).

It should be emphasized that for many systems there may be no preferential interaction between the macromolecules and either of the other two components. In such cases the multicomponent theory reduces to the simple two-component equations in which the mixed solvent is treated as a one-component solvent. Before final interpretations can be drawn from data for complicated systems the interactions must be examined. Different methods are available and have been employed for evaluating the extent of these interactions (see, e.g., Lauffer and Bendet, 1954; Katz and Schachman, 1955; Kielley and Harrington, 1960; Cox and Schumaker, 1961; Vinograd and Hearst, 1962). No single technique is completely satisfactory, however, and the development of additional methods is desirable.

For studies of preferential binding of sodium dodecyl sulfate, which is particularly effective in causing the dissociation of macromolecules into subunits, preliminary results indicate that the interference optical system can be of value. The patterns shown in Figure 2 were obtained from a solution containing bovine serum albumin and sodium dodecyl sulfate. The upper pattern, from an experiment with a synthetic-boundary cell, provides a measure of the total concentration (in fringes) of the protein and detergent. Below this is the pattern from a sedimentation velocity experiment using the same mixture. Fewer fringes can be seen across this boundary than in the upper pattern, thereby demonstrating that not all the detergent is bound as part of the sedimenting species. Data from such experiments are presented in Table II. With the protein alone virtually all the fringes measured in the synthetic-boundary cell were accounted for by the moving boundary. A solution made by mixing the detergent and the protein gave 24.17 fringes in the synthetic-boundary cell whereas the sum of the contributions from the two components examined separately was 24.03.[4] As seen in the bottom row of the table, not all the fringes could be accounted for in the sedimentation velocity experiment. Comparison of this value, 23.45, with that of 24.17 showed that 90% of the sodium dodecyl sulfate was bound. Many aspects of this problem as yet have not been investigated. There are complications in the interpretation of data from chemically reacting systems (Gilbert and Jenkins, 1956, 1959) and corrections must be applied for the different refractive increments. Thus using these data for calculation of equilibrium constants would be hazardous. The data are sufficiently free of ambiguity, however, to warrant their use in the evaluation of the correction term (which itself is not very great) for studies of the size of subunits in proteins.

TABLE II

EVALUATION OF PREFERENTIAL INTERACTIONS IN MULTICOMPONENT SYSTEMS BY ANALYSIS OF INTERFERENCE PATTERNS[a]

Material	Method	Concentration in Fringes
BSA	SBC	16.76
BSA	Sed. vel.	16.75
SDS	SBC	7.28
BSA + SDS	Calculated	24.03
BSA + SDS	SBC	24.17
BSA + SDS	Sed. vel.	23.45

[a] These data, representing the binding of sodium dodecyl sulfate (SDS) to bovine serum albumin (BSA), were obtained from patterns such as those in Figure 2. Fringe counts were made from patterns of synthetic-boundary cell experiments (SBC) and conventional sedimentation velocity experiments. Baselines from the fringe patterns were determined before and after each experiment (Ramel *et al.*, 1961).

It seems likely that some macromolecules in multicomponent systems may show preferential binding of water, with ions or other small molecules (like urea or sucrose) being excluded from the molecular domain of the macromolecule. The degree of exclusion may be considered as a measure of the lack of permeability of the macromolecules. Hydrodynamic data, though providing an indication of the molecular domain of the kinetic unit, cannot reveal whether, within this domain, there is a substantial amount of water which is incapable of acting as a solvent for urea, sucrose, or buffer ions. If such preferential interaction with water did exist, its magnitude could be determined, in principle, by equilibrium dialysis. This would be seen as negative binding of the low molecular weight solute molecules (Katz and Schachman, 1955; Katz, 1956). Generally equilibrium dialysis studies have been aimed at evaluating positive binding of the low molecular weight solutes (Klotz, 1953), and the technique apparently has not been used extensively to search for exclusion of small solute molecules from the domain of large molecules. Even if negative binding did exist, the degree of enhancement of the small solute molecules on the outside of the dialysis bag would be small and might escape detection. Accordingly experiments were done (Richards and Schachman, 1961) to determine the feasibility of a centrifugal technique for investigating this problem. Although the method still does not possess the desired precision a few comments regarding it seem in order.

The solution containing the macromolecules, small solute molecules (indicator), and solvent may be con-

[4] A slight deviation from additivity may be expected as a result of the interaction between the protein and the detergent (see Rosenberg and Klotz, 1955, for measurements of the volume change resulting from the addition of sodium dodecyl sulfate to serum albumin).

sidered as filling the ultracentrifuge cell. After centrifugation the macromolecules are packed on the bottom of the cell as a separate phase, with the supernatant liquid remaining above. The volume of this liquid phase is less than that of the original solution because of the removal of the macromolecules. Hence the indicator concentration is increased, and the enhancement gives a measure of the preferential interaction and the volume from which the indicator molecules are excluded.[5] Since the measurement is, in effect, one involving the small difference between two large numbers, the development of a direct, differential method is required. With the Rayleigh interferometer adapted for the location of the white light fringe such a measurement of the concentration difference should be not only precise but simple. In this application the interferometer measures the concentration difference between the supernatant liquid (just behind the moving boundary) and a reference solution of the indicator at a known concentration. The results indicate that some of the liquid associated with the macromolecules as part of the hydrodynamic units is incapable of acting as a solvent for the indicators. The availability of an internal reference cell with provisions for the unambiguous location of the white light fringe (Clark and Schachman, unpublished data) should provide the means for obtaining quantitative data regarding these preferential interactions. Thereby implications can be drawn about the permeation of macromolecules by different reagents (Schachman, 1960).

Molecular Weight Determination.—The Rayleigh optical system is now finding its widest application in the determination of molecular weights by sedimentation equilibrium. Counting of the fringes across the cell in a sedimentation equilibrium pattern like that in Figure 2c, coupled with the value of the initial concentration (also in fringes) from a synthetic-boundary cell experiment, gives the data required for the evaluation of weight average molecular weights (Lansing and Kraemer, 1935). The simplicity of the computations, the directness and rigorous theoretical basis of the procedure, and the reduction in time required to attain equilibrium by the use of short liquid columns (Svedberg and Pedersen, 1940; Van Holde and Baldwin, 1958; Yphantis, 1960)[6] all have contributed toward making the sedimentation equilibrium method popular once again. But the attractiveness of this computational method is not without its pitfalls; and it would be most unfortunate if in this type of sedimentation experiment (as previously with the sedimentation velocity method) much of the valuable information from the experiment remained unextracted from the photographic records. Not only is important information regarding the heterogeneity or homogeneity of the sample lost if this simple calculation alone is used, but even the result itself may be seriously in error.

It is not unusual for solutions of macromolecules to contain a substantial amount of aggregated material which is packed slowly on the bottom of the cell (or at the interface between the aqueous solution and the dense, inert liquid placed in the cell to provide a transparent cell bottom of the proper shape [Ginsburg *et al.*, 1956]). If, in the equilibrium pattern, this aggregated material does not contribute to the number of fringes across the cell but is included in the determination of the initial concentration, then the computed molecular weight will be too low. Similarly, the presence of low molecular weight material at different concentrations in the sample and in the reference liquid can lead to values of the molecular weight that are too low.

With suitable precautions in handling the data neither of these special (but not unusual) cases need cause concern. Moreover the "contamination" of a protein preparation by either a large or small molecular weight impurity can be detected. As shown in the early work with the sedimentation equilibrium method (Svedberg and Pedersen, 1940), plots of the logarithm of concentration versus the square of the distance from the axis of rotation are straight for homogeneous materials and concave upward for polydisperse substances (assuming ideal behavior for the solutions). To make such plots, as shown on Figure 3 for two proteins, the concentration at each level in the cell must be evaluated. Different methods are available for doing this and all should be used if at all possible. First and foremost, the concentration at the meniscus (and hence at every other level) can be calculated from an equation expressing the conservation of mass (Richards and Schachman, 1959). Second, the one level at equilibrium corresponding to the initial concentration can be determined by location of the white light fringe. This position, known as the hinge point (Archibald, 1947), can be found by a variety of methods. If dilute solutions are employed it can be found directly from patterns obtained with the filter removed from the optical system. Alternatively, an inert liquid such as 1,3-butanediol (Richards and Schachman, 1959) can be added to the solvent to give a refractive index equal to that of the solution of the macromolecules. Discretion must be exercised, of course, in the choice of this liquid for the reference cell. Its molecular weight and partial specific volume must be such that it does not redistribute under the influence of the centrifugal field (Richards and Schachman, 1959; La Bar and Baldwin, 1962). In the absence of such a substance still another procedure can be used. Since the concentration in the vicinity of the hinge point changes only slightly and very slowly (Archibald, 1947; Waugh and Yphantis, 1953), continuous photography of the pattern in the vicinity of the hinge point permits its location and, by this means, the fringes are readily identified in terms of concentration (Spinco, 1958; Richards and Schachman, 1959; Richards, 1960). Yphantis (1962) has suggested and tested still another method for identifying the fringes in terms of known concentrations. This involves the use of dilute solutions and operation of the rotor at speeds sufficiently high that the concentration of macromolecules at the meniscus is reduced to zero. Thus the fringes are easily numbered unambiguously. At the bottom of the column the fringes may be packed too closely to be resolved on the photographic plate and hence information about the weight average molecular weight and heterogeneity is sacrificed.

It is clear that tedious calculations are required for a complete evaluation of the sedimentation equilibrium patterns. The temptation to avoid them is great, but the return for the time and effort invested can be considerable as seen in the plots (Fig. 3) for both a

[5] This simple experiment can be visualized in another manner. Consider a solution of a low molecular weight solute at a known concentration. Dry protein molecules are introduced into the solution and sedimented through the liquid to the bottom of the ultracentrifuge cell. An analysis of the concentration of the supernatant liquid relative to that of the original solution provides the information required for an evaluation of the preferential interaction (see Sihtola and Svedberg, 1948, for related studies).

[6] An additional reduction in the time required to attain equilibrium is achieved readily by using a preformed concentration gradient in the ultracentrifuge cell (Pasternak *et al.*, 1959) or by overspeeding the rotor for a calculated period of time followed by reduction to the desired speed (Richards and Schachman, 1959; Richards, 1960; Hexner *et al.*, 1961).

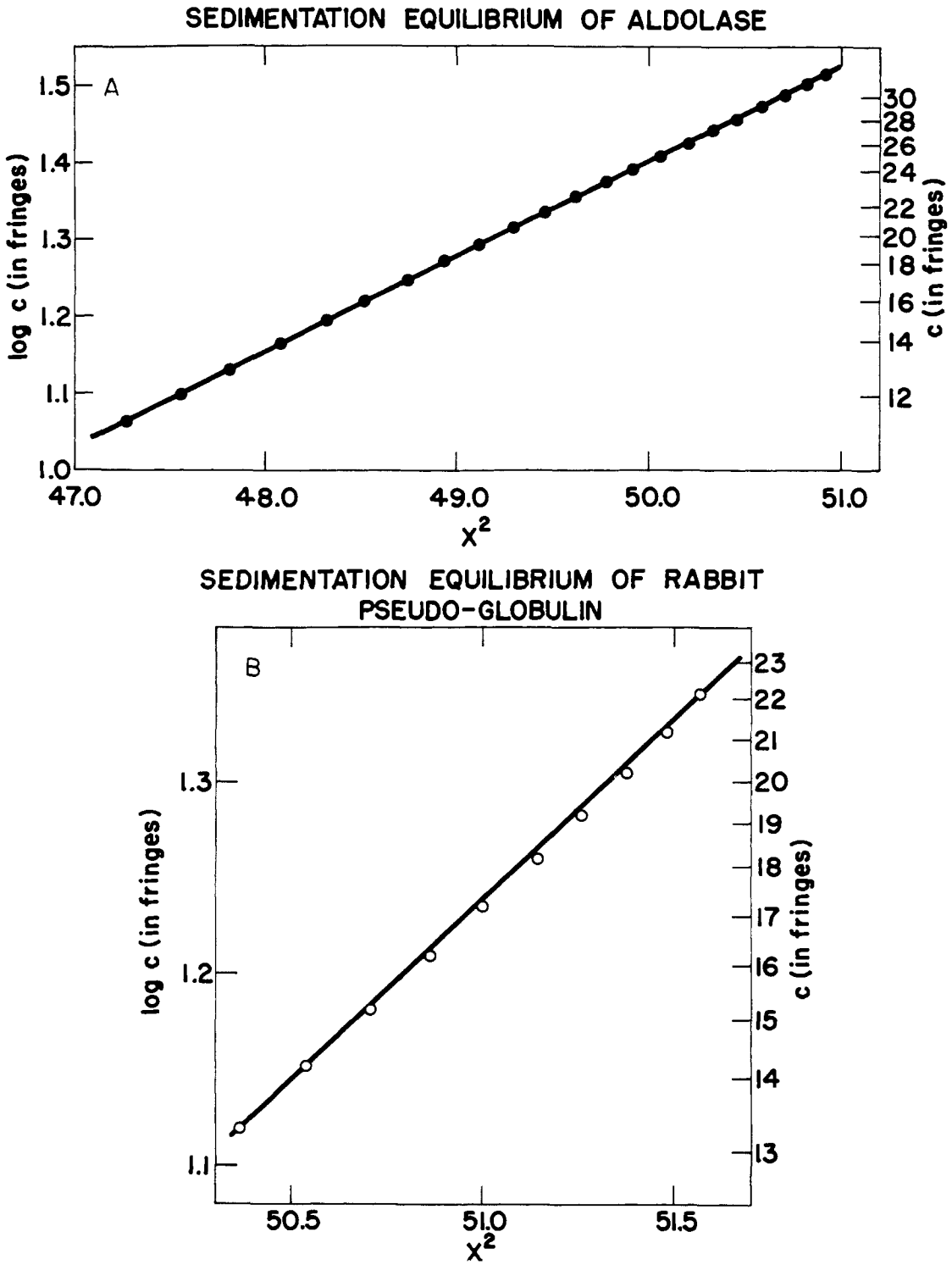

FIG. 3.—Molecular weight determination of proteins by sedimentation equilibrium. The ordinate gives the logarithm of the concentration (in fringes) and the abscissa the square of the distance from the axis of rotation (in cm^2). (A) Data for a homogeneous protein, aldolase (Stellwagen and Schachman, 1962). (B) Data for an inhomogeneous protein, rabbit pseudoglobulin (Richards and Schachman, unpublished data).

homogeneous and polydisperse protein preparation.[7] These computations are now programmed for a computer (Teller and Schachman, unpublished data) and the tentative programs include the calculation of the concentration at the meniscus (based on the conservation of mass) as well as a detailed presentation of the logarithm of concentration versus the square of the distance from the axis of rotation. Included also are the weight average and z-average molecular weights. The availability of such computational techniques now permits the analysis of the patterns for mixtures of macromolecules of different sizes (Rinde, 1928; Svedberg and Pedersen, 1940). In this way it may be possible to describe the heterogeneity of the pseudo-globulin preparation in terms of a mixture of a few species of markedly different molecular weights or as a mixture of

[7] To illustrate the heterogeneity of the preparation of the pseudoglobulin a straight line was drawn through the first and last experimental points. The differences in the slopes of the best fitting lines at the meniscus and cell bottom provide a measure of the heterogeneity of the preparation.

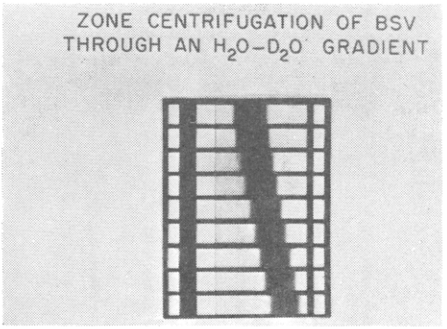

FIG. 4.—Zone centrifugation followed by absorption optics. The moving zone represents a band of bushy stunt virus (A_{260} = 1.0) migrating through a density gradient created by layering a buffered solution of the virus in H_2O over a slightly more concentrated buffer in D_2O. Sedimentation is from right to left and the pictures reading downward are at 2-minute intervals. The patterns are prints of the original photographic plate and the black band represents the ultraviolet-light-absorbing virus particles (Hersh and Schachman, 1958).

many different species of relatively similar molecular weights. Such information obviously would be very valuable.

ZONE CENTRIFUGATION

Biochemical investigations in recent years have been facilitated greatly by the development and application of the technique of zone (or density-gradient) centrifugation (Brakke, 1953; Anderson, 1956; Meselson *et al.*, 1957; de Duve *et al.*, 1959). Yet very few quantitative studies have been conducted to explore the limitations imposed by possible convective disturbances in these experiments. Convection occurs whenever a dense liquid (or volume element within a liquid) is closer to the axis of revolution than any other less dense liquid; i.e., unless the density increases monotonically with increasing distance from the axis of rotation there will be mass movement of liquid from one region of the cell (or tube) to other regions. As a consequence sedimentation experiments in the ultracentrifuge have involved traditionally a homogeneous phase (the solution) and the movement of individual solute molecules through the liquid in a centrifugal direction. The movement of these molecules is observed in the region of the trailing molecules where there is a "boundary" between the less dense solvent (supernatant) and the solution. In flotation experiments (Gofman *et al.*, 1949) the solute molecules migrate in a centripetal direction leaving behind a more dense solvent. A trailing boundary is observed in both cases and quantitative measurements are made from the shape and movement of this boundary.

Leading boundaries in the ultracentrifuge are intrinsically unstable and are annihilated by the convective flow of liquid which occurs whenever there is a density inversion in the liquid. To prevent this bulk flow of liquid from encompassing the whole liquid column in special experiments with leading boundaries, stabilization is effected by the addition of another solute and the formation of a density gradient. This is illustrated in Figure 4, which shows a zone of bushy stunt virus moving through an ultracentrifuge cell containing a density gradient due largely to D_2O. This experiment (Hersh and Schachman, 1958) was performed to assess the stabilizing effect of a density gradient in complicated systems containing more than two components. The absence of ultraviolet light–absorbing material to the right of the band due to the virus shows that bulk flow of liquid to the bottom of the cell did not occur in this experiment. But it should be noted that minute eddies of circulation of liquid at the leading edge of the zone may have occurred. The movement of the virus particles in the centrifugal direction produces an increase in the density of the liquid which the particles enter. In the absence of a gradient of density ahead, streaming of liquid would

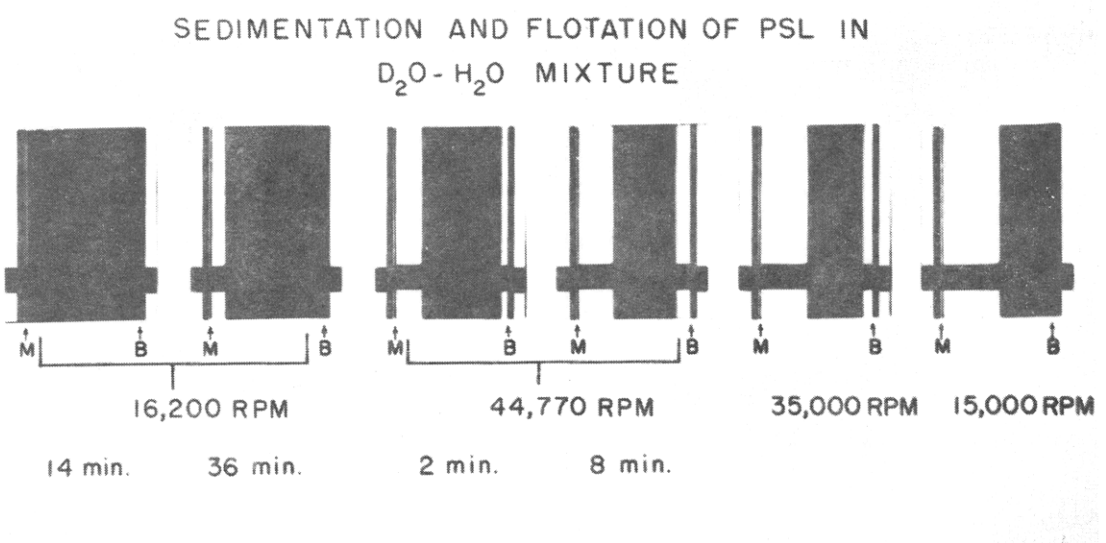

FIG. 5.—Ultracentrifuge patterns from a single experiment illustrating the sedimentation and flotation of polystyrene latex particles in a D_2O·H_2O solution. Sedimentation is to the right and flotation to the left. The arrows marked M and B refer, respectively, to the liquid meniscus and bottom of the ultracentrifuge cell. Pictures are photographic positives from the schlieren optical system and the dark regions in the cell correspond to the solution of the latex particles. The scattering of light by particles was so great that light corresponding to those regions of the cell in which the particles were present did not reach the photographic plate, as if the particles actually absorbed the incident light. From left to right the pictures correspond to 14 and 36 minutes at 16,200 rpm, 2 and 8 minutes at 44,770, and at 35,000 and 15,000 rpm (Cheng and Schachman, 1955).

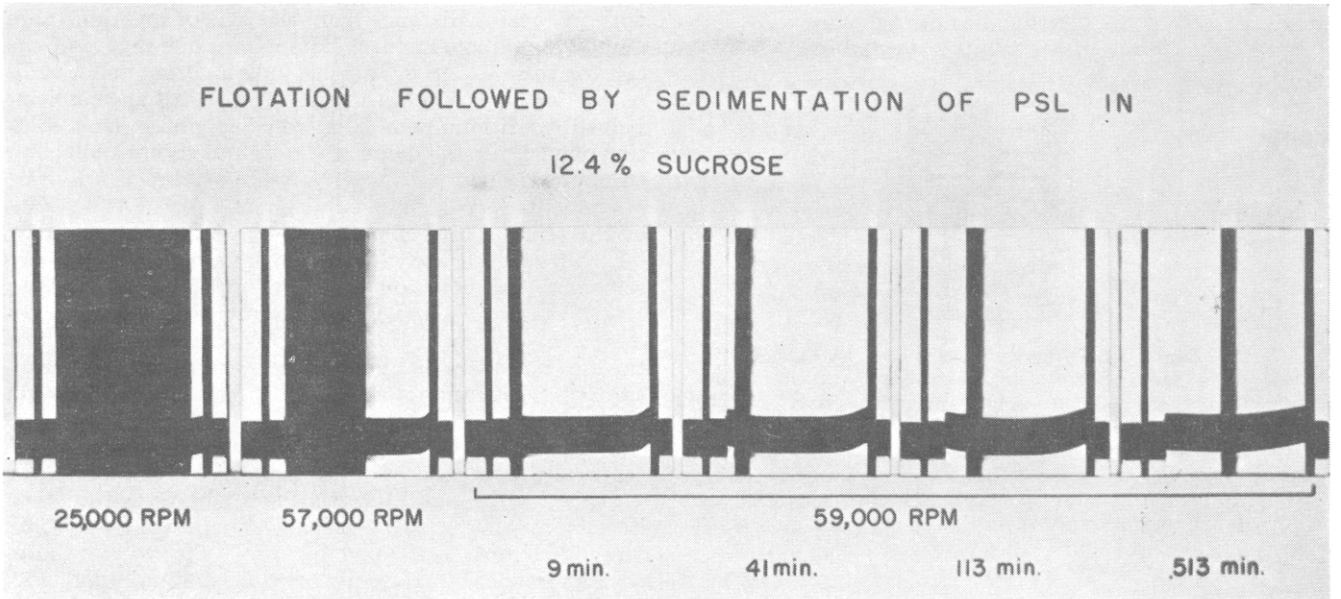

FIG. 6.—Zone centrifugation in density-gradient sedimentation equilibrium experiment. These are schlieren patterns from a study using polystyrene latex particles (PSL) as a probe for following the redistribution of sucrose during the approach to sedimentation equilibrium. Centrifugal direction is to the right. The sucrose concentration initially was 12.4 g/100 ml. Pictures are photographic positives and the dark regions in these prints correspond to regions in the cell where the scattering of light by the particles prevented the light from reaching the photographic plate. From left to right the patterns correspond to 25,000 rpm and 57,000 rpm, and for 9 minutes, 41 minutes, 113 minutes, and 513 minutes at 59,780 rpm. During the acceleration of the rotor the PSL migrated centripetally to the top of the solution column and then migrated as a zone in the centrifugal direction as the sucrose redistributed during the approach to sedimentation equilibrium (Cheng and Schachman cited by Ginsburg *et al.*, 1956).

occur with the consequent deterioration of the leading boundary. If the moving zone is to be stable, the density of the liquid as a whole must increase monotonically through the cell; whether stability exists or convection occurs depends on *both* the density gradient due to the small molecules and the negative gradient contributed by the macromolecules at the front of the zone. The latter density gradient depends on the shape of the leading boundary as well as the concentration and density of the macromolecules. Sharp leading boundaries and high concentrations of sedimenting molecules tend to cause instability, but their deleterious effects can be offset somewhat by steeper density gradients due to the small molecules. Despite studies by Brakke (1953), Anderson (1956), and Svensson *et al.* (1957), no satisfactory quantitative treatment of this problem is yet available. Often the convective flow is limited to a small region in the vicinity of the leading boundary and there is no general circulation of flow throughout the cell.

Experiments such as that illustrated in Figure 4, in which an optical system is used in conjunction with sector-shaped cells,[8] may provide the means for an understanding of this complicated problem. A detailed analysis of the shape of both the leading and trailing boundaries as well as the thickness of the band as a function of the density gradient would furnish valuable data.

It should be noted, however, that not all zone centrifugation experiments involve convective flow. Figure 5 (from Cheng and Schachman, 1955) shows the formation of a zone due to the simultaneous sedimentation and flotation of polystyrene latex particles (PSL)[9] in a D_2O–H_2O solution. At low speed the presence of the latex particles throughout the cell caused the photograph to appear white (note that Fig. 5 is a positive print of the original plate) because the scattering of light by the large particles prevented the light from striking the photographic plate. Sedimentation (in the second frame) created a transparent supernatant liquid and the trailing boundary resulting from the sedimenting particles is seen in the picture. Upon acceleration of the rotor the density of the liquid at the bottom of the cell became larger than that of the PSL because of the greater compressibility of the liquid, and the particles there began to migrate in a centripetal direction. Gradually the latex particles collected as a band near the center of the cell. Actually the sedimenting boundary migrated more slowly near the center of the cell as the centrifugal field was increased because the particles approached an *iso*-dense region of the liquid. Upon deceleration of the rotor this almost stationary boundary began to move again, and the particles (at the right-hand end of the band) which had been migrating in a centripetal direction reversed their direction and began sedimenting. In this experiment the band formation in the density gradient (due to the imposed pressure gradient) occurred with no density inversions. Trailing boundaries were involved throughout (until the rotor was decelerated at which point the right-hand edge became a leading boundary). This type of experiment and variations of it (see Cheng and Schachman, 1955, for a discussion) provide useful information about the compressibility of particles or macromolecules and also afford the opportunity for investigations of the heterogeneity of the particles with respect to density. Whereas variations

[8] Zone centrifuge experiments generally employ swinging bucket rotors or other preparative rotors. The analysis of the transport of macromolecules in these rotors is even more difficult because of the additional convective flow of liquid caused by the particles striking the nonradial walls of the tubes.

[9] Abbreviations used in this paper: PSL, polystyrene latex; RHP, *Rhodospirillum* heme protein; LDH, lactic dehydrogenase.

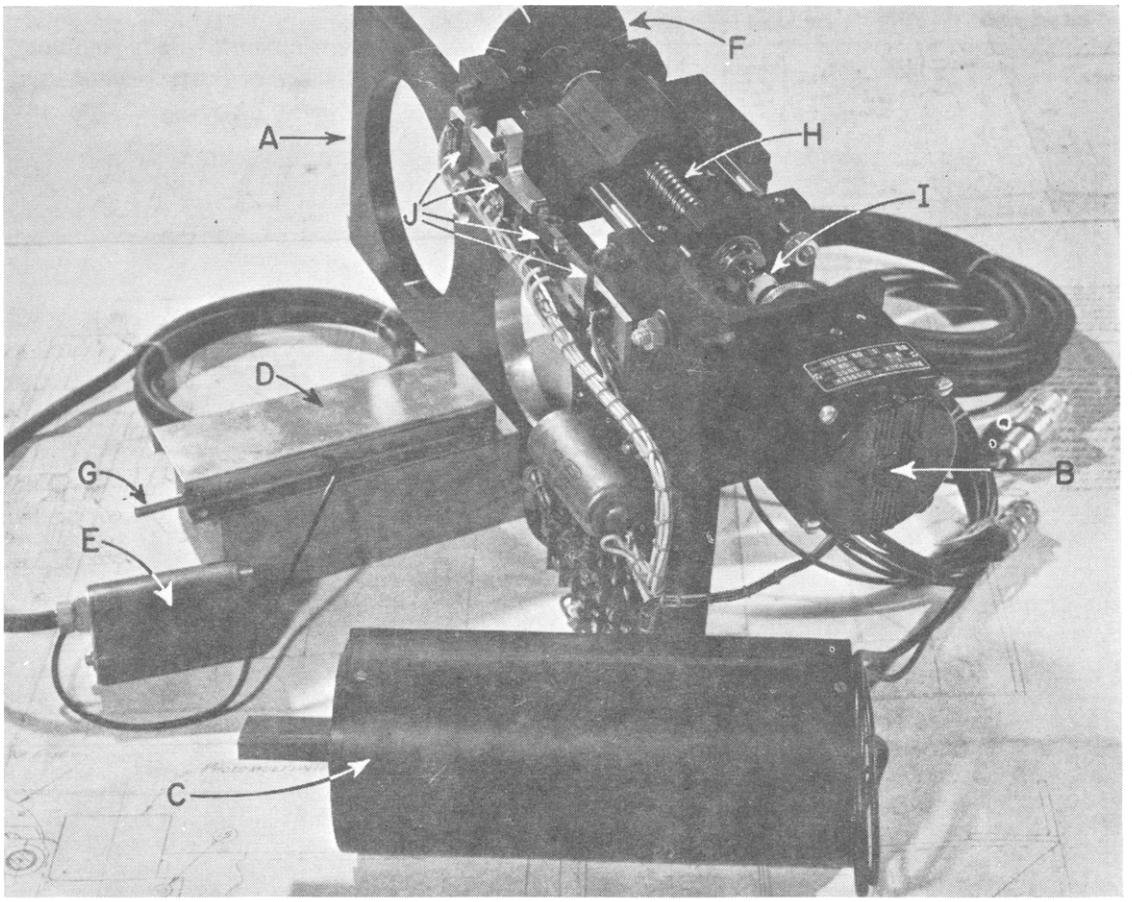

FIG. 7.—A photograph of the partially disassembled scanning unit. Shown in the photograph are: (A) the support bracket with the large hole on the left for mounting the whole unit on the dust tube of the schlieren optical system. Seen in the center of the bracket is another large opening. When the unit is mounted on the ultracentrifuge, it is at this opening that the image of the cell in the absorption system appears; this is the region scanned by the moving photomultiplier tube; (B) the drive motor; (C) the photomultiplier housing with the light shield projecting from the left-hand end; (D) the sheet metal dust cover which is removed in this photograph from its normal position above the lead screw; (E) the light for the timing generator responsible for ruling the recorder paper transversely in terms of the revolution of the lead screw; this light housing is normally mounted just beyond the slotted metal disk seen on the end of the shaft of the lead screw; (F) the slotted metal disk; (G) the photosensitive element which is part of the timing generator; (H) the lead screw; (I) the flexible self-centering coupling between the drive motor and the lead screw; (J) the limit and safety switches.

in density of the particles of 0.01% would lead to differences in sedimentation rate (in H_2O) of only 0.2%, under the conditions of this particular experiment the same distribution of particle densities would cause a 10% variation in sedimentation coefficient. The absence of such a spread in sedimentation coefficients in H_2O–D_2O mixtures indicated that the polystyrene latex particles were exceedingly homogeneous in terms of density.

Another illustration of zone centrifugation is given in Figure 6. Here the movement of the latex particles is governed by the redistribution of the small solute molecules (sucrose) and the consequent density change throughout the cell. Since the density of the original sucrose solution was slightly larger than that of the latex particles the latter rapidly floated to the top of the column. However, the gradual decrease in the concentration of sucrose at the top of the cell caused a decrease in density and the consequent movement of the band in a centrifugal direction. We see here a leading boundary, i.e., migrating particles entering a region in which there were no particles a short time earlier. This represents an example of zone centrifugation in which the leading boundary is stabilized by the density gradient created by the redistribution of dissolved solute. In this type of experiment (Cheng and Schachman [1955], cited by Ginsburg *et al.*, 1956) the latex particles were used as an indicator to examine the redistribution of the smaller solute molecules and to provide a means for the location of the hinge point (Archibald, 1947) and for an approximate evaluation of the molecular weight of the redistributing solute molecules.

The more recent density gradient sedimentation equilibrium experiments of Meselson *et al.* (1957) can be considered as the converse of that shown in Figure 6. In these elegant experiments the redistribution of the small solute molecules of known molecular weight caused, in turn, the sedimentation and flotation of the much larger deoxyribonucleic acid (DNA) molecules which were the subject of interest. Here the spreading of the zone of DNA and also the location of the band provide an indication of the molecular weight and density of the macromolecules. In the one case the large particles were used as an indicator of the redistribution of the small molecules, whereas in the other the redistribution of the small molecules was used as a probe for investigations of the macromolecules. The important role of the latter type of experiment for the investigation of many biological problems is so well known as not to require documentation here (for a review see Vinograd and Hearst, 1962).

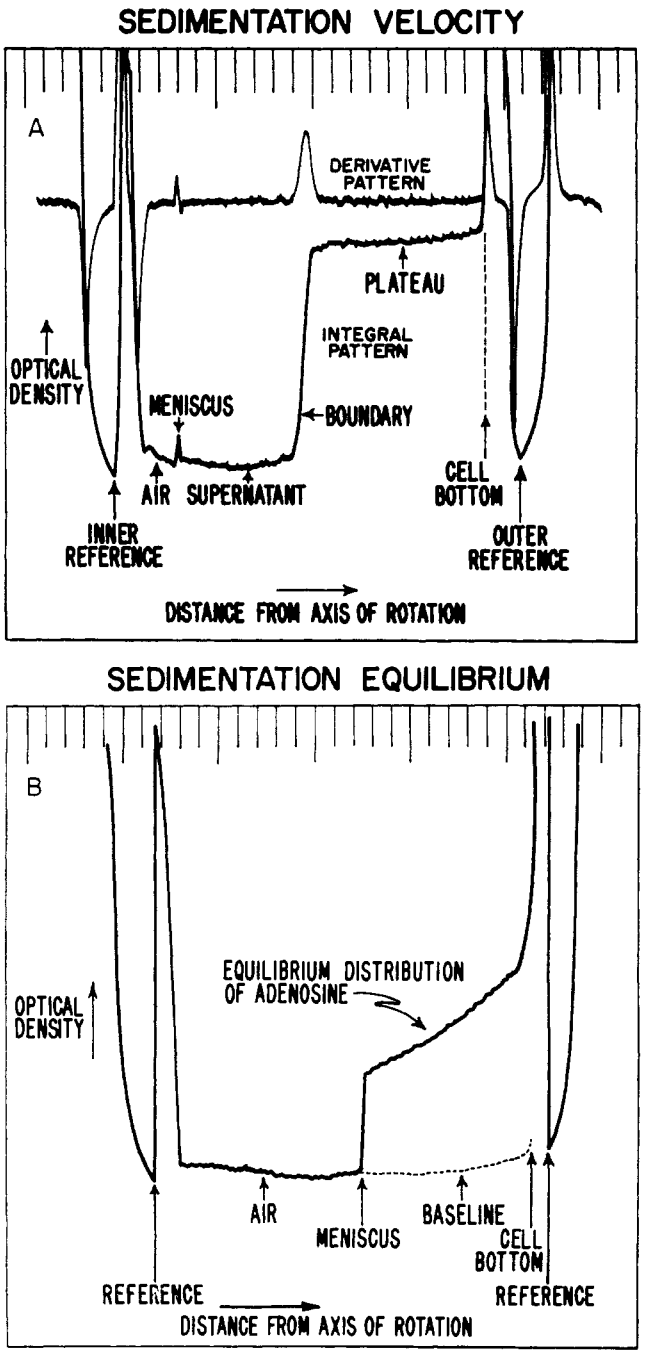

FIG. 8.—Tracings of scanner diagrams. (A) Sedimentation velocity pattern of bushy stunt virus (A_{260} = 0.9). (B) Sedimentation equilibrium pattern of adenosine (A_{260} = 0.5).

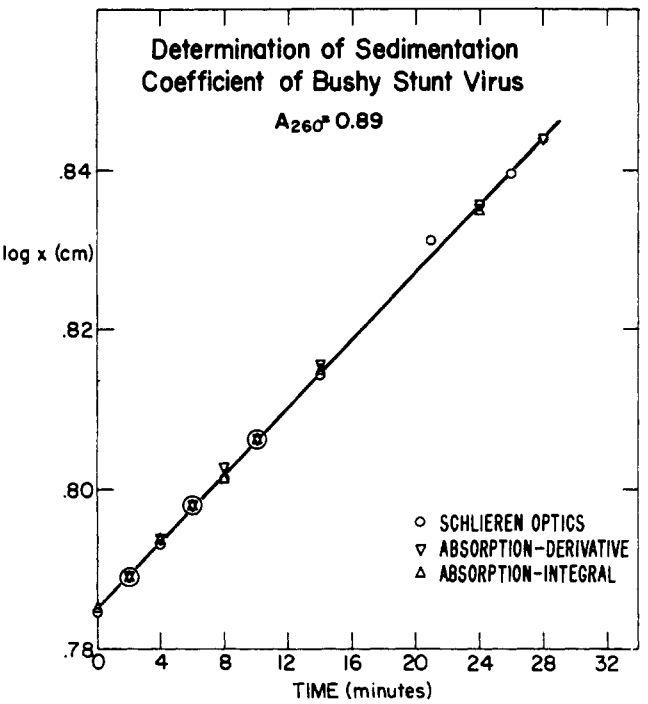

FIG. 9.—Determination of the sedimentation coefficient of bushy stunt virus. The ordinate gives the logarithm of the boundary position in cm from the axis of rotation and the abscissa the time in minutes after reaching speed (23,150 rpm). Data indicated by O were obtained from the schlieren patterns. The points represented by ▽ were obtained from the maximum ordinate of the derivative traces of the absorption scanning system, and the data shown as △ were from the integral curves and correspond to the half-concentration level (Hanlon *et al.*, 1962).

ABSORPTION OPTICAL SYSTEM

General Remarks.—"Inflexible," "inaccurate," "inconvenient," "laborious," "time-consuming," (and even "impossible") have been some of the invectives characterizing the absorption optical system and presaging its early demise. It should come as no surprise that these features, plus the psychological drawback resulting from experimenting without observing, "flying blind" so to speak, led to the virtual abandonment of this technique. Historically, of course, the absorption method was the first optical system to be applied to the ultracentrifuge (Svedberg and Nichols, 1923), but the methods developed subsequently seemed so much more satisfactory that they took precedence. Despite the versatility, convenience, and accuracy of the schlieren method and the adverse qualities of the absorption method, the latter, once again, is being used extensively even in the primitive form originally devised (Svedberg and Pedersen, 1940).

The recent sudden surge in popularity of absorption optics can be attributed to two factors, sensitivity and selectivity. Owing to the inherent limitations in the sensitivity of refractometric and interference optical techniques, solutions of relatively high concentration are required; and frequently the ultracentrifuge patterns for concentrated solutions are so ambiguous and anomalous as to be meaningless.[10] Also, since these methods are responsive to changes in refractive index only and since most solutes cause approximately equal increments in refractive index, no possibility is afforded for distinguishing or identifying different chemical species in a solution. With the schlieren and interference optical systems boundaries due to serum albumin, ribonucleic acid, and ribosomes "look alike." In contrast, the absorption system, for many important biological macromolecules, works efficiently with solutions having concentrations even less than one-tenth those used with the other optical methods. Moreover, the absorption system has the great advantage of discrimination since different components can be distinguished one from another by way of variations in their absorption properties. Clearly the removal of some of the disadvantages of the absorption system, and particularly that stemming from the inability to view directly the sedimentation process during an experiment, would contribute substantially to the power

[10] This applies both to the determination of the size and shape of macromolecules like deoxyribonucleic acid as well as to studies of the homogeneity of the sedimenting material (Peacocke and Schachman, 1954; Shooter and Butler, 1956; Schumaker and Schachman, 1957).

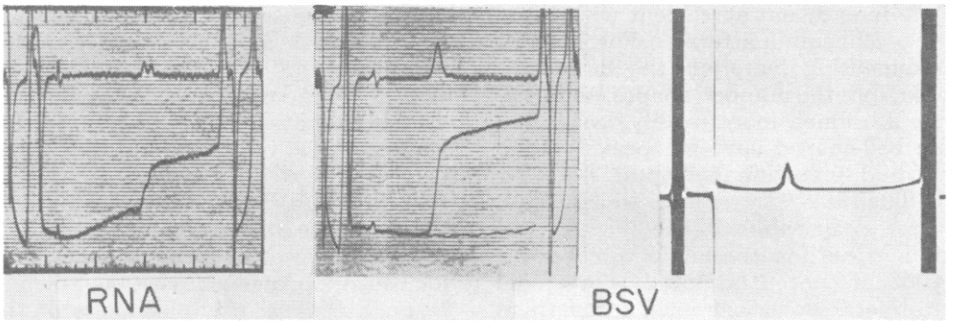

FIG. 10.—Patterns illustrating the advantages of both the integral and the derivative curves. The pattern on the left is from a sedimentation velocity experiment on a preparation of partially degraded ribonucleic acid isolated from tobacco mosaic virus. The derivative pattern shows clearly the presence of two components while the integral curve shows that there is also polydisperse slowly sedimenting and rapidly migrating material. On the right are two patterns from a sedimentation velocity experiment with bushy stunt virus. The absorption pattern obtained with the single-beam scanning system shows clearly (in the integral curve) the presence of a considerable amount of aggregated material in the preparation. Neither the schlieren pattern on the right nor the derivative pattern from the scanning system shows the presence of this material so vividly.

of the method. Accordingly considerable effort has been devoted in this laboratory to provide an automatic "direct viewing" absorption system, incorporating the facilities resulting from the technological advances since the time Svedberg and his co-workers first employed this method.

Single-Beam Photoelectric Scanning System.—The development in recent years of photomultipliers of extreme sensitivity has stimulated successful efforts to replace tedious photographic methods with automatic photoelectric techniques. Thus it is no surprise that a photoelectric scanning absorption system for the ultracentrifuge has been shown to be practical (Goring and Bryson, 1959; Schachman, 1960; Hanlon and Schachman, 1961; Aten and Schouten, 1961). A detailed account of the design, performance, and application of a single-beam scanning system coupled with a monochromator has been presented elsewhere (Hanlon *et al.*, 1962; Schachman *et al.*, 1962). Only the general features of the system are described here along with a few illustrations of its application to biochemical problems.

Figure 7 shows the scanning unit (without the electronic components) in a partially disassembled form. The entire unit is compact and light in weight so that it can be fastened onto and supported by the optical dust tube of the schlieren system. Very little modification of the ultracentrifuge is required and the operation of the scanning absorption system is compatible with simultaneous use of either Rayleigh or schlieren optical systems. For the purpose of illustrating the components, the housing containing the photomultiplier was removed from the bracket which supports it. An end-window EMI 6256 S photomultiplier (E.M.I. Electronics, Ltd.) is used and the slit in front of the photocathode is sufficiently narrow (25–100 μ depending on the experiment) that high resolution is achieved in traversing the image of the cell. This image, magnified about 1.5-fold, is about 3.0 cm in length. The recorder and the synchronous motor, which drives the lead screw by means of a flexible, self-centering coupling, are actuated by external timing impulses from the automatic photographic unit of the ultracentrifuge. Limit switches mounted on the support bracket are engaged by a ramp-shaped cam which moves with the photomultiplier. One of these disengages the chart drive of the recorder and reverses the scanner motor at the end of the scan, thereby causing the photomultiplier housing to return to its starting position until the next signal from the automatic photographic timer. At the end of the lead screw which imparts linear motion to the photomultiplier housing is a slotted metal disk which, in conjunction with a small light and a photosensitive element, comprises the timing generator responsible for the transverse ruling of the recorder traces. This timing generator, by ruling the recorder traces unambiguously in terms of the linear motion of the photomultiplier, furnishes a check on the correlation and performance of the mechanically independent scanner and recorder drives. A single scan of the image requires about 6 revolutions of the screw in a period of 5 seconds.

The train of light pulses received by the photomultiplier during its movement across the image is converted by means of a data sampling filter and other electronic circuitry into a profile of relative light transmission versus distance. Even though the photomultiplier senses only light intensity the voltage output of the data sampling filter is proportional to the product of the light intensity and the duration of the pulses. In this respect the data sampling technique gives results analogous to a photographic plate which responds to both the intensity of light and the exposure time. After suitable amplification the signals from the filter are fed to one of the galvanometers of the 14-channel recorder or, alternatively, to a "log" circuit first and then to the recorder after additional amplification. Thus, by means of a simple switching arrangement, plots are produced of either transmittance or optical density as a function of position in the cell. A differentiating circuit is also included so as to produce curves analogous to the derivative curves portrayed by the schlieren optical system. Sample tracings of typical recorder curves are given in Figure 8. In Figure 8A are the integral and derivative curves from a sedimentation velocity experiment with bushy stunt virus, and the curve in Figure 8B is the integral pattern, corresponding to concentration versus distance, from a sedimentation equilibrium experiment with adenosine. The base line, shown as a dotted line, was obtained at the conclusion of the sedimentation equilibrium experiment after rinsing the cell and filling it with solvent followed by acceleration of the rotor to the same speed as that used in the actual equilibrium experiment.

The measurement of boundary positions, and hence the determination of sedimentation coefficients, was made readily from the derivative patterns although the integral curves can be used as well. As seen in Figure 9, representing data from a single experiment, the boundary positions determined from the integral and

derivative traces are in excellent agreement with those evaluated from the schlieren patterns. For cursory examination of sedimenting materials the derivative curves are preferable since the number, shapes, and positions of boundaries are much more readily recognized and analyzed from bell-shaped curves ("peaks") than from the contours and inflection points of S-shaped curves. This is illustrated by the sedimentation velocity pattern of a preparation of ribonucleic acid from tobacco mosaic virus (on the left of Figure 10). In the determination of concentrations, however, the integral curves are preferable; measurements from them are simple, rapid and accurate. Table III shows that

TABLE III

CONCENTRATION DETERMINATIONS WITH THE SPLIT-BEAM SCANNING ABSORPTION SYSTEM[a]

Boundary Position Distance from Axis of Rotation (cm)	Recorder Deflection Observed (cm)	Recorder Deflection Corrected (cm)	Absorbance (at 280 mμ)
6.21	5.6	5.9	0.98
6.37	5.5	6.1	1.01
6.42	5.4	6.1	1.01
6.50	5.2	6.0	0.99
6.63	5.0	6.0	0.99
6.88	4.7	6.1	1.01

[a] The sedimenting material was chicken heart lactic dehydrogenase in a 12-mm ultracentrifuge cell at an optical density (1.0-cm cell) of 1.00. Boundary positions (in cm from the axis of rotation) were measured from the integral patterns using the half concentration level. The recorder deflections were measured in the plateau region. The third column gives the recorder deflections corrected for radial dilution according to the equation, $c_t = c_0(\bar{x}/x_m)^2$, where c_t and c_0 are the concentrations at time t and zero time, respectively, and $\bar{x}$ and x_m represent the distances from the axis of rotation to the boundary and to the meniscus. The latter position was 6.05 cm. The fourth column gives the calculated absorbances with the calibration factor 6.0_3 taken as equivalent to an absorbance of 1.00.

accuracy with which concentrations can be measured from sedimentation velocity patterns. The dilution in the plateau region is accounted for accurately in terms of the radial dilution equation (Svedberg and Rinde, 1924; Trautman and Schumaker, 1954). It should be noted that concentrations in the plateau region are measured directly and unambiguously (in terms of optical density). Another advantage of the integral curve is illustrated by the sedimentation velocity patterns of bushy stunt virus in the right of Figure 10. Neither of the derivative patterns (from the scanning absorption system or the schlieren optical system) reveals the relatively large amount of rapidly sedimenting material in the preparation. However the aggregated material is detected readily in the integral curve as a sloping line in the so-called plateau region. Obviously the availability of both the integral and derivative patterns simultaneously is a special virtue of the scanning absorption system.

Split-Beam Scanning Absorption System.—Despite the reliability of the single-beam system described above, imperfections in the optical system are sufficiently serious that the experimental accuracy achieved is not yet comparable to that provided by the schlieren or Rayleigh optical systems. The limitations stem from nonuniform illumination, fluctuations in light intensity, reflections from lenses, and scattering from the oil and dust accumulating on the optical surfaces during operation of the ultracentrifuge. In order to minimize the difficulties caused by these factors the scanning system now has been modified to convert it, in effect, into a split-beam optical system (Lamers *et al.*, in preparation). To accomplish this, a double-sector cell was used in the rotor, the slit in front of the photomultiplier was shortened in length so that it was smaller than the image of the center rib in the double sector ultracentrifuge cell, and, finally, the electronic recording system was modified by the incorporation of holding circuits and a system of "logic" which permits the separation, identification, and comparison of the light pulses passing through the two compartments of the cell. Imagine the photomultiplier at some fixed position in the image of the ultracentrifuge cell. As the cell in the rotor sweeps by the optical system there are two quick bursts of light separated by 6 μsec (when the rotor is operating at 60,000 rpm) followed by a long "dark" period while the rotor completes one revolution. By appropriate filling of the cell the first of the two light pulses can be made to represent the transmittance (or the logarithm of the transmittance) of the solvent and the second pulse is a corresponding measure for the solution. Since the two light pulses (corresponding to the transmittance at conjugate levels in the cell) do not occur simultaneously the circuitry must permit storage of the first pulse (in the form of the logarithm) for subsequent comparison with its "mate." This requires the incorporation of two independent holding circuits as well as a sensing system which routes the individual light pulses to the correct holding circuits, one for the reference and the second for the sample. Instead of incorporating an external sensing system which can phase the switching circuit in terms of a signal from the rotor, the "logic" was dictated by the light pulses themselves. The decrease in the light intensity at the end of the reference (solvent) pulse activates an electronic switch, automatically closing the gate to the reference holding circuit and simultaneously opening the gate to the sample holding circuit, so that the second (solution) pulse is directed to the correct place. Since the sample gate remains open for a period of only 400 μsec, the first pulse detected after one complete rotation of the rotor is once again shunted into the reference holding circuit. In this manner the pulses, in the form of the logarithms of the intensities, are routed into separate holding circuits and the outputs from them are fed into a difference amplifier and finally to the recorder. Details of the construction and performance of this split-beam scanning system are given elsewhere (Lamers *et al.*, in preparation); but a few applications are given here to illustrate the potential of the optical system.

Figure 11 shows typical unretouched recorder traces from a sedimentation velocity experiment with chicken heart lactic dehydrogenase. Only the integral curves are shown since, as yet, the derivative curves produced by the split-beam system with the low light levels from the monochromator are not as satisfactory as desired. It should be recognized that the conversion to the split-beam system necessitates a sacrifice in light intensity because the slit in the scanning unit had to be shortened so that the photomultiplier could not "see" both compartments of the cell simultaneously while the cell was moving by the optical system. The concentration for this experiment was only 0.07 g/100 ml, corresponding to an optical density (at 280 mμ in a 1.0-cm cell) of 0.9. With the present sensitivity of the scanning system such solutions may be diluted almost 10-fold before quantitative studies become difficult. Obviously the sensitivity of absorption optics exceeds that of refractometric or interference methods. Moreover, preliminary experiments show that with further tech-

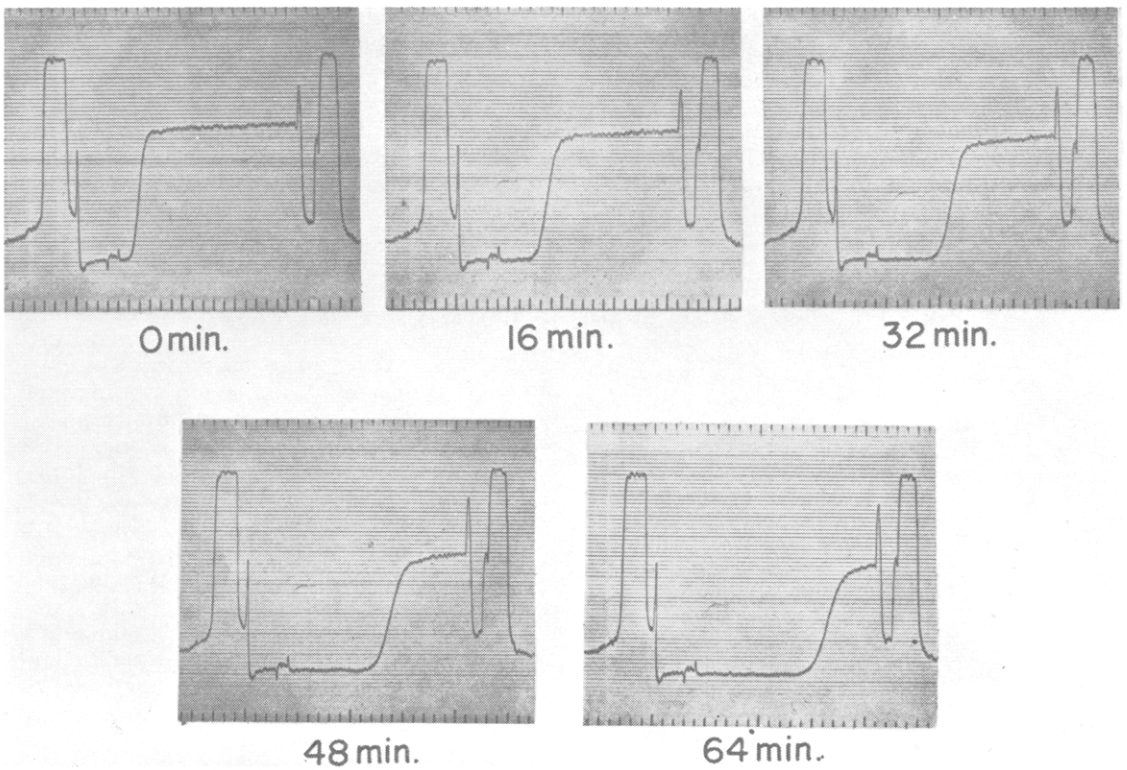

FIG. 11.—Sedimentation velocity patterns of chicken heart lactic dehydrogenase. All patterns were obtained with the split-beam scanning system and a monochromator in the optical system. The wavelength was 280 mμ (monochromator slit 2.0 mm) and the concentration was 0.075 g/100 ml. The times after reaching a speed of 59,780 rpm are indicated under each pattern.

nical improvements it should be possible to use light with a wavelength of 230 mμ, thereby enhancing the sensitivity of the system and permitting quantitative sedimentation studies with only a few micrograms of protein.

Spectral Analysis of Sedimenting Materials.—To test the reliability of the split-beam scanning system in conjunction with the monochromator, the absorption spectrum of myoglobin was measured during an actual sedimentation velocity experiment. The data shown in Figure 12 were obtained by measuring the recorder deflection (in the plateau region) as a function of wavelength. As seen in Figure 12, reliable absorption spectra can be obtained with the ultracentrifuge equipped with the split-beam scanning system; the agreement with the spectrum measured in a conventional spectrophotometer is excellent. It is worth noting also that the concentration employed in this experiment was only 0.005 g/100 ml, and excellent sedimentation velocity patterns were obtained even at these great dilutions (see Schumaker and Schachman 1957; and Schachman, 1959, for a discussion of possible convective disturbances in studies of dilute solutions).

As indicated in a previous section, one of the principal virtues of the absorption optical system is the discrimination it provides in distinguishing different chemical components on the basis of their absorption spectra. This unique advantage of the absorption system is illustrated by the data in Table IV from a sedimentation velocity experiment on a mixture of an iron-containing protein and *Rhodospirillum* heme protein (RHP) from the bacterium *Chromatium* (Bartsch and Kamen, 1960). Both the spectra and the sedimentation coefficients of the proteins were markedly different. It was to be expected, therefore, that spectral ratios in different regions of the boundary would reveal the composition of the mixture at each level. That this was achieved is seen readily in the data summarized in Table IV. At the trailing end of the boundary, where there was very little light-absorbing material (at any wavelength), the spectral ratios corresponded closely with those for the iron-containing protein which has a sedimentation coefficient of 1.5 S. When the spectrum was measured at a level corresponding to more rapidly sedimenting material the contribution of the RHP (of sedimentation coefficient 3 S) was apparent. This is clear from the changing spectral ratios which gradually

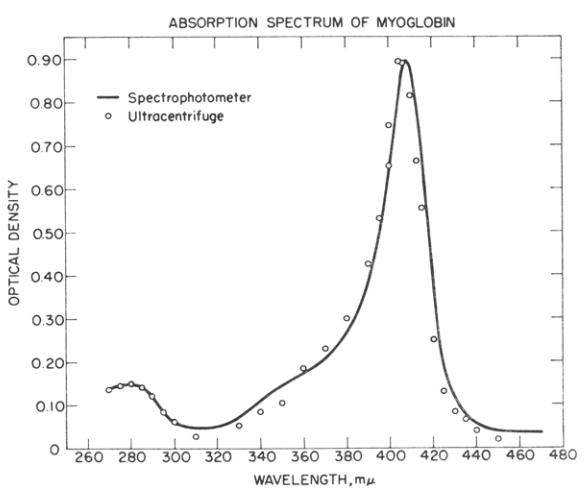

FIG. 12.—Absorption spectrum of myoglobin during a sedimentation experiment. The ordinate gives the optical density (normalized from the recorder deflections at the various wavelengths to the value at 406 mμ) and the abscissa represents the wavelength. For readings below 300 mμ the slit on the monochromator was 2.0 mm and at higher wavelengths it was 0.5 or 0.2 mm. The speed was 20,000 rpm and the scanner was positioned at a level corresponding to the plateau region.

TABLE IV
ANALYSIS OF MIXTURES FROM SPECTRAL ANALYSIS OF A SEDIMENTING BOUNDARY

Sample	Position in Boundary[a]	A_{400}	A_{335}	A_{280}
Iron-containing protein	—	1.00	0.93	2.6
RHP	—	1.00	0.26	0.26
Mixture	—	1.00	0.51	0.81
Mixture	Trailing end	1.00	0.80	2.2
Mixture	One-third level	1.00	0.81	1.7
Mixture	Three-fourths level	1.00	0.53	0.97

[a] The mixture was centrifuged for 90 minutes at 59,780 rpm, the scanner was moved to different regions of the image of the cell, and the recorder deflections were measured for light of the wavelengths indicated in the table. All absorbance values were normalized relative to the deflection at 400 mμ. The optical density of the mixture was 1.1 at 400 mμ which corresponds to about 6 cm deflection of the recorder when used at low sensitivity. For the measurements of the spectral ratios at the trailing end of the boundary where the optical density was less than 0.1 the recorder trace was expanded electronically to give deflections about 2 cm.

approached the values for the mixture of the two proteins. From data such as those in Table IV the presence of more than one chemical component can be demonstrated unequivocally.

Analysis of Interacting Systems.—In view of the direct relationship between sedimentation coefficients and molecular weights the velocity method should be uniquely suited for the detection and quantitative measurement of the binding of small molecules to macromolecules. Experiments of this type are not free of ambiguity, however, because of possible complications from coupled flows (Gosting, 1956) and the re-equilibration of the mixture if the equilibrium is disturbed due to differential sedimentation of the various molecular species (Gilbert and Jenkins, 1956, 1959). Despite these uncertainties and the consequent reservations attending a quantitative interpretation of binding data, such experiments frequently are profitable. This has been shown elsewhere (Schachman, 1960; Schachman *et al.*, 1962) in measurements of the binding of methyl orange to bovine serum albumin and in studies of the interaction between antidinitrophenyl antibody and the hapten, dinitrophenyllysine. The latter interaction is very strong (binding constant about 10^5) even in the presence of 8 M urea (Velick *et al.*, 1960; Bratley and Schachman, unpublished data). As an additional illustration of the utility of the transport method, data are given here from a study of an enzyme-coenzyme interaction.

Figure 13 shows representative sedimentation velocity patterns from a series of experiments with different mixtures of reduced diphosphopyridine nucleotide (DPNH) and chicken heart lactic dehydrogenase (LDH). The pattern of DPNH alone (on the left) shows that all the light-absorbing material (at 340 mμ) migrated slowly with a sedimentation coefficient about 0.2 S. Upon the addition of enzyme (1 mole LDH per 12 moles DPNH) almost one-third (28%) of the

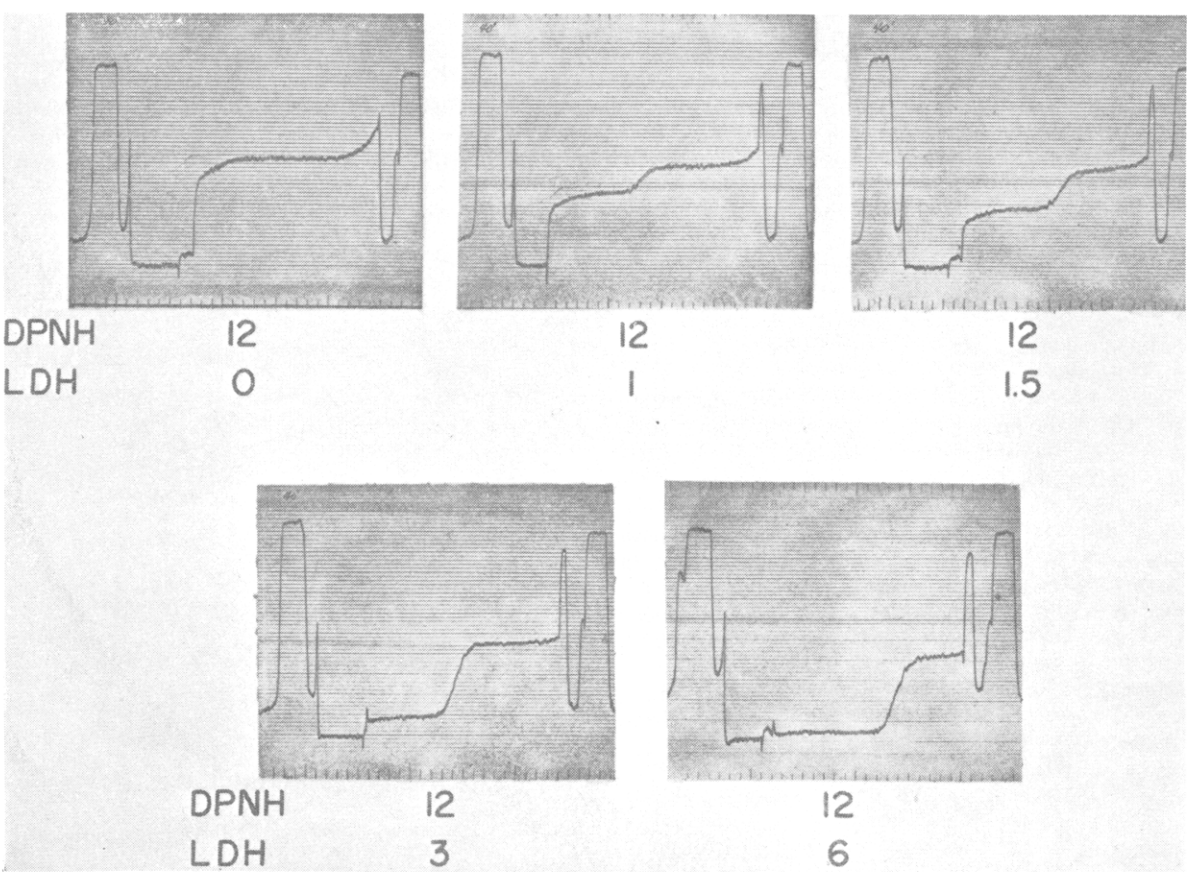

FIG. 13.—Sedimentation velocity patterns from an interacting system involving an enzyme [lactic dehydrogenase (LDH)] and a coenzyme [reduced diphosphopyridine nucleotide (DPNH)]. The wavelength of the light was 334 mμ and the slit width on the monochromator was 1.0 mm. The concentration of DPNH in all experiments was 1.3×10^{-4} M in 0.1 M phosphate buffer at *p*H 7. The concentration of LDH was varied in the different experiments to give the molar ratios indicated below each pattern. Sedimentation is from left to right. All experiments were at 59,780 rpm and the patterns were taken 40 minutes after reaching speed.

coenzyme sedimented rapidly with a coefficient of 7 S, the value for the pure enzyme. When the enzyme concentration was increased so that the molar ratio of LDH to DPNH was 1–8, about one-half (43%) of the DPNH appeared in the bound form. Increasing the enzyme concentration still more (as seen in the 4th pattern where the ratio was 1–4) caused the bulk (80%) of the DPNH to sediment at the same rate as the pure enzyme with the remainder migrating at the rate characteristic of free DPNH. Finally, as seen in the fifth pattern, only a slight change in the pattern resulted when the enzyme concentration was raised again (1 mole enzyme per 2 moles coenzyme); 93% of the DPNH appeared as high molecular weight complexes. When the data from these experiments are plotted in terms of multiple equilibria (Klotz, 1953) the number of binding sites on the enzyme is found to be about four, in agreement with the value deduced from other measurements (Cahn *et al.*, 1962).

The binding constants calculated from these patterns are not given here because the magnitude of the potential errors outlined above has not as yet been evaluated. But it should be noted that the pitfalls of the transport method can be avoided by the use of the sedimentation equilibrium technique described earlier (Schachman *et al.*, 1962). Each of these experiments is, in effect, equivalent to many equilibrium dialysis experiments (Klotz *et al.*, 1946) at different protein concentrations and identical concentrations of the free low molecular weight component. Coupling the data from the Rayleigh interference optical system with that from the absorption system provides the information required for a plot of the concentration of the small molecule as a function of the concentration of the macromolecules. Suitable treatment of the data (Steinberg and Schachman, in preparation) gives the amounts of the small component both free in solution and bound in the form of high molecular weight complexes. The limited results obtained thus far by both the transport and the equilibrium techniques suggest that, in accord with theoretical considerations (Gilbert and Jenkins, 1956, 1959; Richards and Schachman, 1957; Schachman, 1959; Fujita, 1962), only very small errors result in measurement by the transport method if the interactions involve large molecules with much smaller ions or molecules.

Both the transport and equilibrium techniques show considerable promise as tools for the analysis of interacting systems. Despite the complexity of the apparatus, the measurements themselves are rapid, simple, and accurate. Moreover, only very small amounts of material are required and the hazards of the equilibrium dialysis method (slow diffusion through and adsorption on the membrane) are avoided.

Direct Measurement of Small Differences in Sedimentation Coefficients.—Frequently the sedimentation coefficient of a substance may be altered so little as a result of a specific chemical or enzymic treatment that the effect cannot be described quantitatively because of limitations in experimental precision. With the absorption optical system, in particular, where the precision is less than that with schlieren or interference optics, a small alteration in the conformation or molecular weight of a protein cannot be detected because the

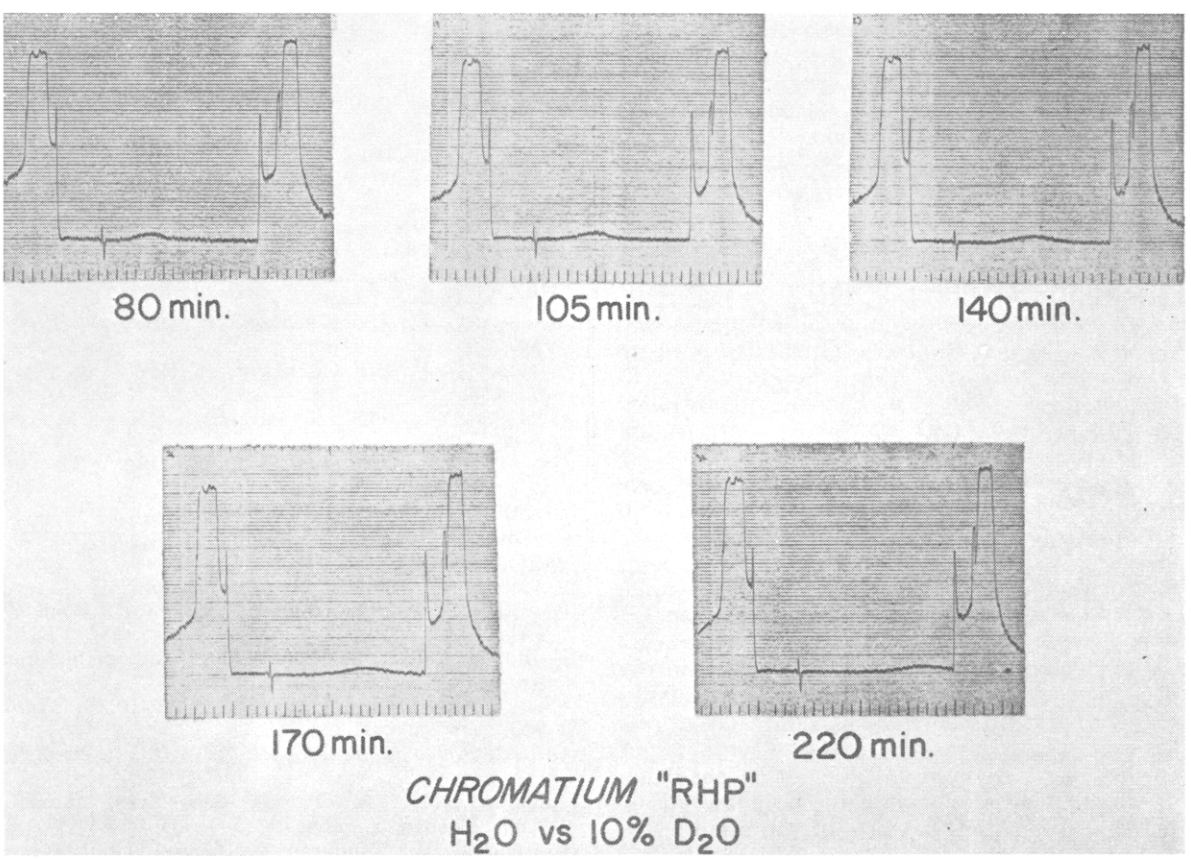

FIG. 14.—The measurement of small differences in sedimentation coefficients. Both compartments of the cell contained *Chromatium Rhodospirillum* heme protein (RHP) at identical concentrations. The solvent was 0.2 M NaCl–0.01 M phosphate buffer at *p*H 7. One solution contained 10% D_2O and the other was 100% H_2O. The volumes of solutions placed in the two compartments of the cell were measured carefully so that the menisci almost coincided. The wavelength was 405 mμ and the monochromator slit width was 0.2 mm. The optical density of the solution was 0.3 at 405 mμ. The figures below the patterns represent the time in minutes after the rotor attained a speed of 59,780 rpm.

change in the sedimentation coefficient may be smaller than the experimental errors in each measurement. For such situations there is a need, obviously, for a differential technique which permits the direct measurement (in one experiment) of the small difference between the two sedimentation coefficients. In this way errors due to variations in speed or temperature as well as in reading two sets of ultracentrifuge patterns are cancelled. It has been shown previously (Richards and Schachman, 1957, 1959) that the Rayleigh interference system can be used to subtract one sedimentation velocity pattern from the other to give curves related directly to the difference between the sedimentation coefficients of the substances in the two halves of the double-sector cell. Although the results produced by this technique were encouraging and the precision in measuring a 1% change in sedimentation coefficients was excellent, the accuracy is still not as great as that expected on the basis of the experimental parameters. With the availability of the split-beam scanning system, such experiments can now be followed by means of absorption optics.

Figure 14 shows a series of difference patterns from an experiment aimed at detecting a small change in the sedimentation coefficient. The sedimenting material in both cells was *Chromatium* RHP (s = 3 S) at the same concentration. The solution in one of the compartments contained sufficient D_2O to cause a decrease in the sedimentation rate of 5%. As seen in the difference patterns the curved region moved progressively down the cell and the area (more properly the first moment of the area) increased continuously as the boundaries became more separated due to the difference in the two sedimentation coefficients. When the solutions were identical and the menisci were accurately superimposed the difference pattern showed a straight horizontal line as if there were no sedimenting material in the cells. A quantitative test of this method in terms of the theoretical equations (Richards and Schachman, 1959) has not yet been made but it is clear that this difference technique may prove useful for biochemical investigations.

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Added in Proof

The zone-centrifugation technique used by Hersh and Schachman (1958) has now been employed in a slightly modified form for sedimentation velocity experiments (Vinograd *et al.*, 1963). Narrower bands than those shown in Figure 4 were obtained by layering smaller amounts of the upper solution in a synthetic-boundary cell. Stabilization of the leading boundary was achieved, as in Figure 4, by means of additional salt or D_2O in the lower liquid.

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