halides.[30] The fact that the magnitudes of the stability constants correlate with the crystallographic radii of the metal ions rather than their hydrated radii may not mean that the unhydrated ions are actually bound in the complexes. The experimental results are explicable in terms of the concept of localized hydrolysis discussed by Robinson and Harned.[31] If a cation M^+ polarizes the solvent to a sufficient extent a kind of ion pair $M^+ \ldots OH^- - H^+ \ldots A^-$ may be formed with an anion A^- which is a proton acceptor. According to this concept the stability of the complex would be greater for smaller M^+ and stronger bases A^-. This latter point is borne out by the fact that the binding of the various alkali metal cations[9] and of the various alkaline earth cations by P^{-2} and AMP^{-2} correlates with the acid strengths of the corresponding acids. Similarly the weak binding by CrP^{-3} correlates with the low pK_a' of the phosphate group.

The relative values of the stability constants for the more basic forms of the anions are in the order to be expected from the magnitude of the charge: $ATP^{-4} > ADP^{-3} > P^{-2}, AMP^{-2}$. It is of interest to note that the complexes of ATP^{-3} are much less stable than the complexes of ADP^{-3}. This probably results from the greater localization of charge in ADP^{-3}.

The values of the apparent stability constants for the orthophosphate complexes at 0° are also given in Table III. If it is assumed that ΔH for the formation of these complexes is constant from 0 to 25°, the values of ΔH and ΔS may be calculated. The values of ΔH are each about +5 kcal. $mole^{-1}$, and the values of ΔS are each about 25 kcal. $deg.^{-1}$ $mole^{-1}$. Thus these complexes are formed because of the favorable entropy change which reflects the relatively greater freedom of the solvent molecules in the presence of the products than in the presence of the reactants.[32]

The fact that the stability constants for the complexes of the divalent cations with ADP^{-2} and ATP^{-3} and their conjugate basic forms have been determined makes it possible to calculate the pK_a' values for the acidic forms of the various complex ions. The values for the apparent dissociation constants of the complexes are given by the expression $K_a'K_1'/K_2'$. The calculated pK_a' values are summarized in Table IV.

TABLE IV

pK_a' VALUES AT 0.2 IONIC STRENGTH AND 25°

ADP^{-2}	6.68	ATP^{-3}	6.95
ADPSr	5.5	$ATPSr^{-1}$	5.4
ADPCa	5.4	$ATPCa^{-1}$	5.3
ADPMg	5.1	$ATPMg^{-1}$	5.0
ADPMn	4.6	$ATPMn^{-1}$	4.5

Whereas the uncertainties in the pK_a' values for ADP^{-2} and ATP^{-3} are ±0.02, the uncertainties in the pK_a' values for the complexes are ±0.1 for the ADP complexes and ±0.2 for the ATP complexes. It is of interest to note that for both ADP and ATP the acid strengths of the complexes increase in the order of increasing affinity of the more basic form of the anion for the metal ion, that is, $Sr^{++} < Ca^{++} < Mg^{++} < Mn^{++}$.

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MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

A Centrifugal Light Scattering Cell for the Ultraclarification of Liquids

BY W. B. DANDLIKER AND J. KRAUT[1]

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A glass light scattering cell capable of being centrifuged at high speeds (centrifugal cell) is described. The use of this cell makes it possible to clarify liquids to a very high degree and then, without transfer, to make scattering measurements upon them. In addition, the cell has a small volume (6 ml.) and exhibits extremely low stray light down to 26° from the incident beam. It is found that this method of clarifying liquids is superior to all other methods tried, such as high speed centrifugation followed by transfer to a light scattering cell, filtration through plastic filters or filtration through ultrafine sintered glass.

Introduction

Although light scattering is widely used for the determination of size, shape and activity coefficient of macromolecules in solution,[2] the problem of clarification of liquids has continued to present serious obstacles to reliable and accurate measurements. The variety of methods which have been applied to clarifying liquids utilize separations based upon distillation, filtration or centrifugation. Filtration procedures include the use of bacterial filters such as the Seitz asbestos type after impregnation with bakelite,[3] "ultrafine" sintered glass filters,[2] plastic filters of the Millipore type[4] and graded cellulose nitrate membranes.[5] Centrifuga-

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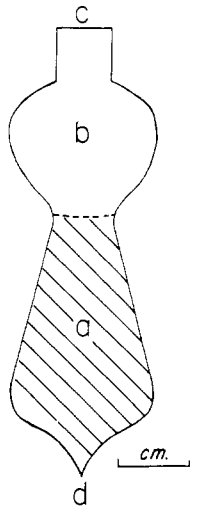

Fig. 1.—Centrifugal light scattering cell.

tion of solutions at high speeds followed by transfer to the scattering cell has been employed in many laboratories.[2,3]

The basic weakness in all these methods lies in the fact that the solution must be transferred from the container where it is cleaned to the cell in which measurements are made. Schulz and co-workers[6] have eliminated this difficulty by using a stainless steel and glass scattering cell which could be subjected to centrifugal fields of about 10,000 times gravity after the solution was introduced. However, the glass wall of Schulz' cell is in the form of a right circular cylinder which in this case was made quite heavy to withstand the centrifugal field. Cylindrical cells may give rise to multiple reflections and high stray light as was pointed out by Zimm[3]; Schulz' finding that the scattering from toluene showed an apparent dissymmetry of 1.03 to 1.04 probably indicates the presence of stray light at low angles. Under these circumstances measurements at angles close to the incident beam are subject to large corrections and the results are thus somewhat uncertain. In addition the use of the Schulz cell is limited to non-corrosive liquids because of contact with metal parts.

The ideal light-scattering cell should embody several essential features. First of all, the stray light should be negligibly small over a wide angular range; for most studies on macromolecules the range from $\theta = 25$ to 135° would be adequate. Secondly, the process of clarification of the solution before observation should be carried out entirely within the light scattering cell so that no transfer of the clean solution is necessary. Other somewhat less important qualities of the ideal cell are small volume, chemical inertness, cheapness and ease of construction. It is the purpose of this paper to discuss a centrifugal light scattering cell meeting these requirements.

Experimental

Apparatus.—The instrument described here is an adaptation of that used by Zimm.[3] It is comprised of two parts: a thin-walled conical inner cell containing the scattering liquid and an outer cell filled with a liquid having approximately the same refractive index as that contained in the inner cell. A cross section of our inner cell is shown in Fig. 1; it was constructed from selected 8 mm. Pyrex tubing. The inner cell is filled so that the meniscus is at the constriction between the two bulbs (~6 ml. is required). The incident beam passes through the lower conical bulb (a) in a direction perpendicular to the axis of symmetry. The upper bulb (b), containing air, furnishes the necessary buoyancy and hydrostatic stability so that the filled cell floats upright in a liquid of suitable density. This feature makes it possible to centrifuge the inner cell at very high speeds without direct support from the wall of the centrifuge tube. The cell aligns its axis parallel to the resultant of the gravitational and centrifugal fields. The principle involved here is well known and has been applied recently to sedimentation analysis in floating glass or quartz capillaries.[7]

In this work it has been found convenient to use 75% glycerol in water as a flotation medium, allowing bulb (b)

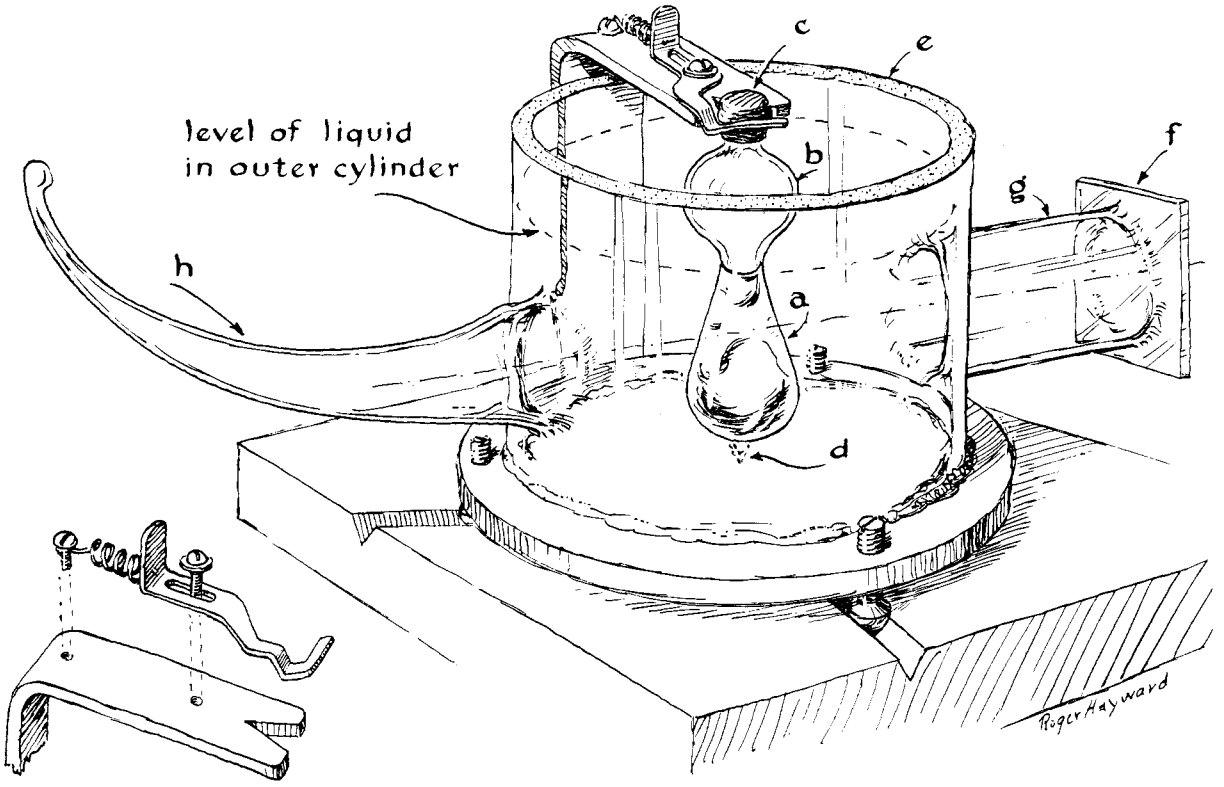

Fig. 2.—Complete light scattering cell assembly. Details in text.

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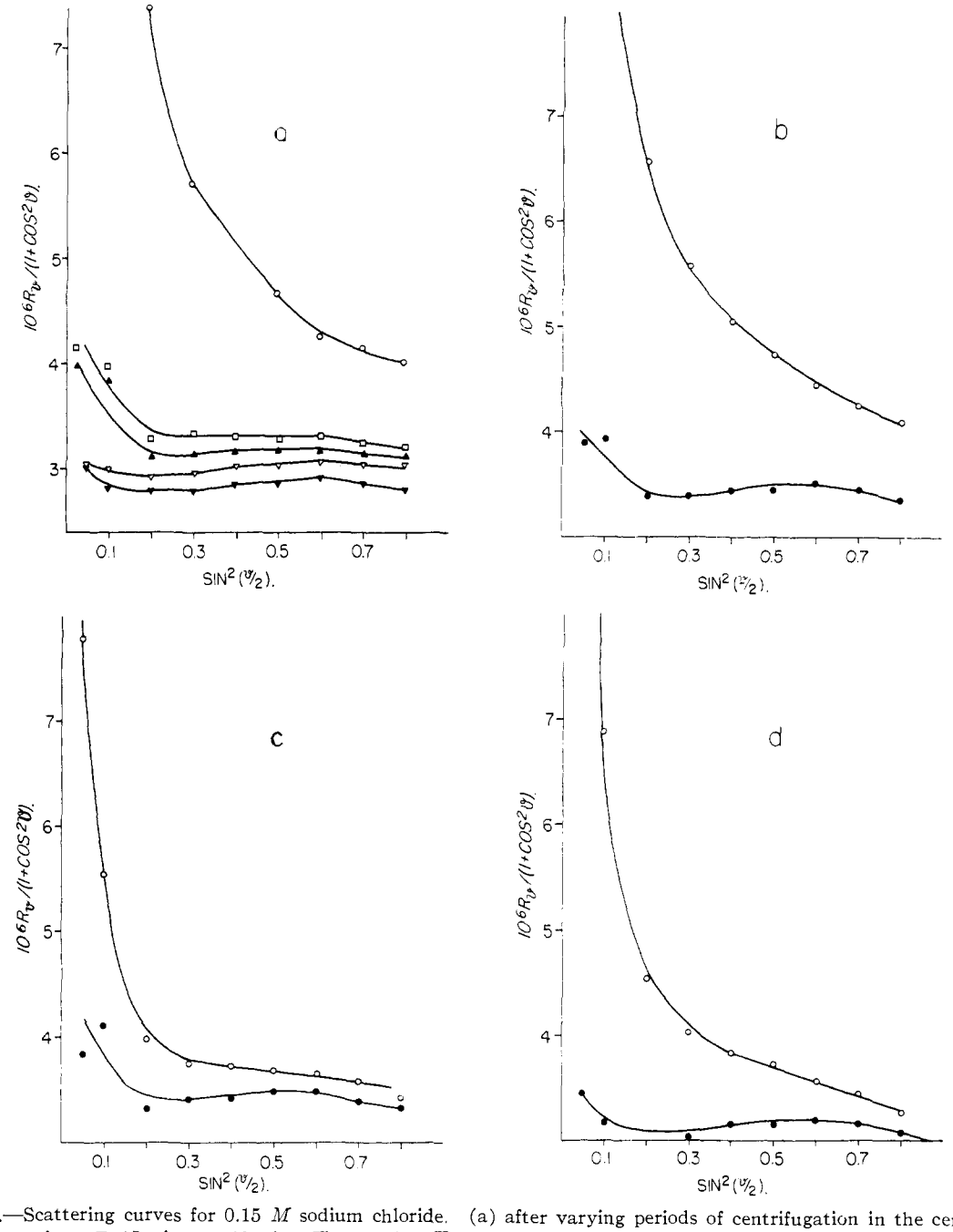

Fig. 3.—Scattering curves for 0.15 *M* sodium chloride. (a) after varying periods of centrifugation in the centrifugal cell: O, zero time; □, 15 min.; ▲, 60 min.; ▽, 120 min.; ▼, water for 120 min. (b) O, centrifuged for 2 hr. at 90,000 times gravity and transferred to the centrifugal cell; ●, after an additional 30 min. of centrifugation in the centrifugal cell. (c) O, filtered through an ultrafine sintered glass filter; ●, followed by 30 min. of centrifugation in the centrifugal cell. (d) O, filtered through a Millipore filter; ●, followed by 30 min. of centrifugation in the centrifugal cell.

to be about half immersed. The filling hole (c) at the top of the cell is closed during centrifugation and subsequent operations by a piece of Parafilm or by a hooded serological rubber stopper. The drawn-out tip (d) at the bottom serves both to center the cell in the outer cell assembly by fitting into a small conical hole and to entrap material sedimented out of the solution. Centrifugation of these cells is carried out in a Spinco Model L ultracentrifuge equipped with a swinging bucket rotor[8] (#SW 25.1) capable of withstanding a maximum speed of 25,000 r.p.m. and provided with three buckets for 1 in. × 3 in. plastic cups.

(8) We are indebted to the Spinco Division, Beckman Instruments, Inc., Belmont, California, for putting a rotor at our disposal.

The cells have routinely been exposed to fields of more than 50,000 and occasionally to fields of about 80,000 times gravity (25,000 r.p.m.). Newly constructed cells are tested at maximum speed before putting them into routine use. Occasional breakage occurs at this stage but we have found that once tested in this manner cells may be expected to last indefinitely. All four of our original cells have been in use almost daily at 55,000 times gravity for several months.

Figure 2 shows the appearance of the entire cell assembly. The large center cylinder (e) (58 mm. high) was constructed from a piece of 70 mm. (o.d.) Pyrex tubing. Stray light from the entrance window (f), consisting of a piece of microscope slide, was made negligible by the long entrance tube (g), 25 mm. o.d. by 46 mm. long. The troublesome back

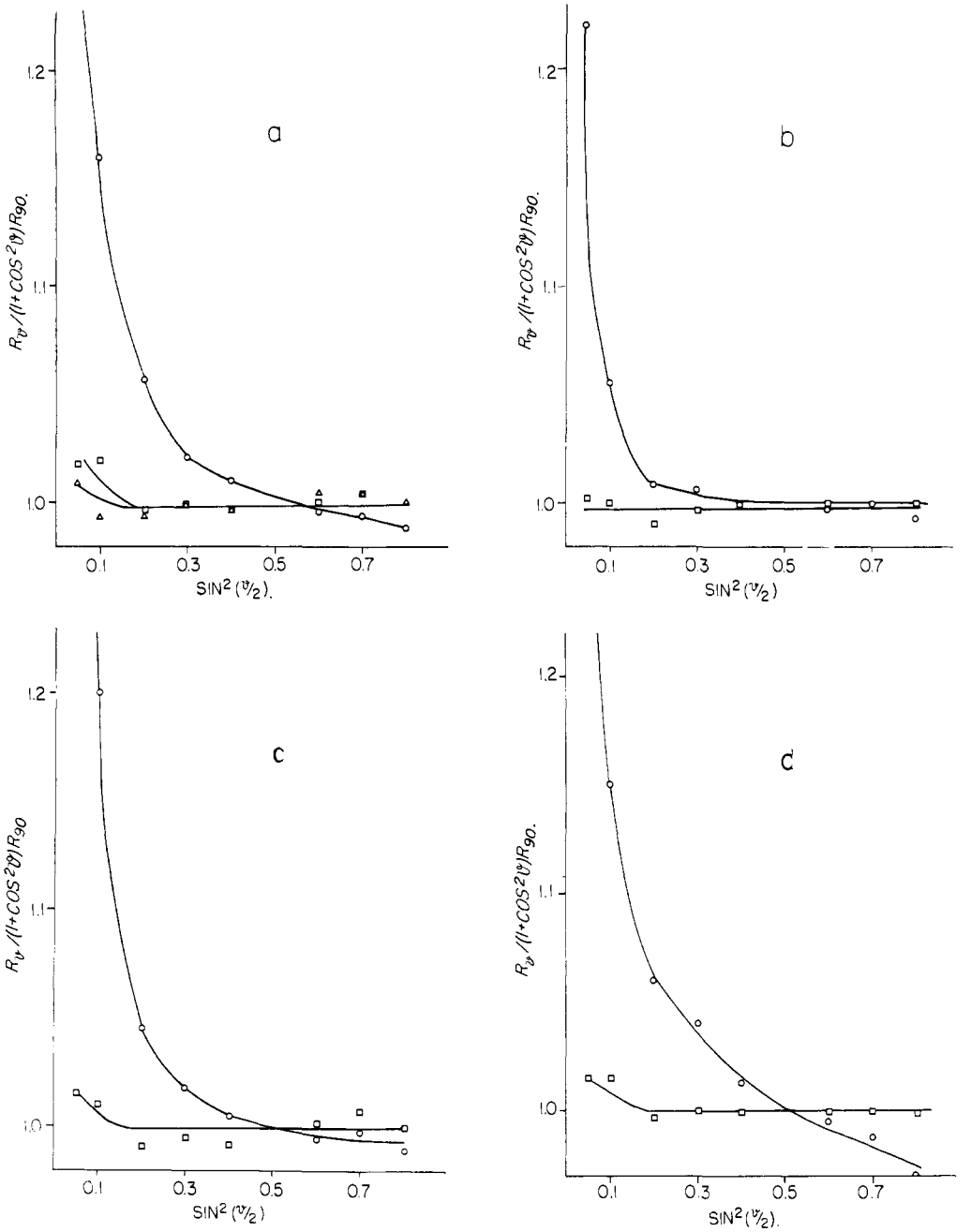

Fig. 4.—Scattering curves for 0.1% albumin. (a) after varying periods of centrifugation in the centrifugal cell: O, zero time; □, 30 min.; △, 180 min. (b) O, centrifuged for 2 hr. at 90,000 times gravity and transferred to the centrifugal cell; □, after an additional 30 min. of centrifugation in the centrifugal cell. (c) O, filtered through an ultrafine sintered glass filter; □, followed by 30 min. of centrifugation in the centrifugal cell. (d) O, filtered through a Millipore filter; □, followed by 30 min. of centrifugation in the centrifugal cell.

reflection from the usual exit window has been eliminated by the use of a Wood's horn (h) to absorb the transmitted beam. A thermoplastic cement (Cenco-Sealstix) was used for the glass-to-glass and glass-to-aluminum joints.

The inner cell is centered on the bottom by resting in a small conical cavity and on top by a V-slot and spring arrangement; orientation is fixed by means of a permanent index mark on the inner cell. The entire outer cell assembly is positioned in the instrument[9] by three spherical feet which fit into V-grooves oriented 120° apart. All surfaces not required to transmit light were painted black.

In order to minimize scattering in the outer liquid, the outer cell was filled with clean water from an all-glass still before each series of measurements and fitted with a cover.

Materials and Procedure.—The centrifugal cells were

(9) Obtained from the Phoenix Precision Instrument Co., Philadelphia, Pennsylvania. The instrument was modified so as to focus an image of an illuminated slit into the center of the scattering cell. This was accomplished by increasing the distance between the source and cell to about 50 cm. and removing the cylindrical lens. A rectangular slit about 2 × 10 mm. consisting of 4 razor blades was placed as close to the source as possible and focussed by the remaining lens into the center of the cell. The diaphragm was used to limit the convergence of the incident beam to a maximum of 5°. Scattered light originating in the lens was largely removed by a diaphragm just *outside* the incident beam near the entrance window of the outer cell.

calibrated by the transmission method using Ludox silica sols.[10,11] The intensity of the incident beam was measured with the photometer set at 0° and with the entire cell assembly removed. Corrections for the change in illuminated volume viewed by the photometer at different angles were obtained by measuring the fluorescent light from fluorescein solutions.[3] The correction factor was found in all cases to be nearly identical with sin θ.

The effectiveness of the centrifugal cell was investigated by comparative experiments with several commonly used methods for clarifying liquids. The solutions used were 0.15 M sodium chloride and an isoionic solution of Pentex bovine plasma albumin in 0.15 M sodium chloride (10 mg. protein/ml.).

Results of these experiments are shown in Figs. 3 and 4 in which R_θ denotes the Rayleigh ratio of angle θ for unpolarized incident light. In these figures, ordinates have been so chosen that a Rayleigh scatterer would result in a horizontal straight line. All measurements were made at $\lambda_0 = 4358$ Å. In Fig. 3 for 0.15 M sodium chloride we have plotted total observed intensities which in general include contributions from stray light, solvent and solute scattering, *i.e.*, no deductions have been made for stray light. Figure 4, however, shows excess scattering due to solute (albumin) only. For this latter figure the necessary stray light and solvent corrections were obtained from the next to the lowermost curve in Fig. 3a representing the scattering from clean sodium chloride. In all cases the centrifugal cell was run at 20,000 r.p.m. (~55,000 times gravity).

Figure 3a shows the gradual disappearance of large-particle scattering from 0.15 M sodium chloride in the centrifugal cell as the time of centrifugation was varied.

After 15 min. no particles could be seen by the naked eye on viewing the solution at low angles. After 2 hr. the solution was clean and the 90° scattering was only 6% greater than that for water alone. The water curve (lowermost in Fig. 3a) obtained after 2 hr. of centrifugation in the centrifugal cell indicates a Rayleigh ratio at 90° of 2.86×10^{-6} cm.$^{-1}$ which is the same as that reported[12] for water purified by multiple distillation. The upward convexity of the plots for clean water and sodium chloride observed in Fig. 3 is real and due to the molecular anisotropy[12] of water. The water curve of Fig. 3 shows that the stray light contribution for the cell assembly of Fig. 2 is only of the order of 10% of the scattering of water (λ_0 4358) at angles as low as 26°. At higher angles the stray light rapidly becomes negligibly small compared to the water scattering and for most common organic solvents could be neglected at all angles between 26 and 135°. Figure 4a shows the results of the corresponding experiment with an albumin solution.

In Figs. 3b and 4b the performance of the centrifugal cell is compared with the results of high speed centrifugation followed by transfer of the liquid. The solutions were first centrifuged for 2 hr. at 90,000 times gravity and transferred to the centrifugal cell with dust-free pipets. Scattering measurements then gave the upper curves; 0.5 hr. of additional centrifugation in the centrifugal cell resulted in the lower curves.

Figures 3c and d and Figs. 4c and d show the results of experiments using preliminary clarification with a Corning ultrafine sintered glass filter and with a Millipore filter.

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It should be pointed out that with proteins even as small as serum albumin it is possible to form appreciable concentration gradients during the clarifying centrifugation. For example, on centrifuging a 0.1% albumin solution for 1.5 hr. at 55,000 times gravity, the protein concentration at the top of the cell was 79% of that at the bottom. Near the center of the cell, however, the concentration was within 2% of the initial value. Use of the centrifuge brake during deceleration (after a 1.5-hr. period) brings the extremes of concentration to within 10% of one another without stirring up the sedimented dust. A period of 0.5 hr. centrifugation followed by deceleration with the brake results in barely detectable gradients which rapidly disappear by diffusion, convection and stirring upon handling.

Even very large gradients probably could be readily destroyed by gentle rotation of the cell about its axis to produce a slight stirring of the contents. Precautions were taken in this work to insure that gradients were not present during light scattering measurements.

Discussion

In every case investigated it was found that solutions clarified by usual methods still contain large particles which raise the low angle scattering. Centrifugation of these solutions in the centrifugal cell removes these particles rapidly and completely; it may be appropriate to term this latter process *ultraclarification*.

It appears that the dust remaining after ordinary clarification has little effect on the scattering at high angles. At low angles, however, the decrease in scattering after ultraclarification is important to the problem of the accurate determination of molecular sizes and shapes by light scattering.

For large particles a study of the angular dependence curves as a function of time may be useful as a means of detecting the presence of contaminating materials having appreciably different sedimentation constants than that of the molecule under investigation.

In practice it may be advantageous to precede the use of the centrifugal cell by other methods of clarification. For example, it is evident that the centrifugal cell can be effective only for the removal of particles denser than the surrounding medium. It may be quite common to find small amounts of low density fatty materials in protein preparations. If these are present a residue may tend to collect on the inside walls of the cell and to increase the stray light. Usually, impurities of this sort may be removed by a preliminary centrifugation followed by discarding any material rising to the surface. This procedure was found to be desirable for our preparation of bovine albumin.

SEATTLE, WASHINGTON