9. *An Ultrafiltration Method for the Optical Clarification of Protein Solutions in Light-scattering Investigations.*

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In the application of the light-scattering method to molecular-weight and -size determinations of dissolved macromolecules, removal of aggregated material and all impurity is essential. The more usual methods of filtration are inadequate and a simple ultrafiltration technique based on Elford's Gradacol membranes has been evolved. By its use molecular-weight values in fair agreement with accepted values have been obtained as well as low and reproducible dissymmetry values.

Since the light-scattering method yields a weight-average molecular weight (M) for dissolved macromolecules, erroneously large values are caused by the presence of relatively small amounts of heavy aggregates or impurity. In a current light-scattering investigation considerable difficulty was found in removing such aggregates from solutions of certain globulin-type seed proteins. For aqueous systems the ordinary filtration methods employing fine sintered discs, Seitz or cellulose-pulp pads were ineffective; high-speed centrifugation (12 000 r.p.m.) only partially cleared the solutions, for it was found impossible not to stir up sediment when removing tubes from the rotor. An ultrafiltration technique based on the Gradacol membranes developed by Elford (*Trans. Faraday Soc.*, 1937, **33**, 1094) was finally adopted, with which clear solutions were obtained, giving molecular weights near the accepted values. The light-scattering apparatus used is described elsewhere (Goring and Johnson, publication pending).

Experimental

Membranes were made in the same way as the thimble-type osmometer membranes described by Adair (*Proc. Roy. Soc.*, 1925, *A*, **108**, 627) and Alexander and Johnson ("Colloid Science," Oxford Univ. Press, 1949). A suitable nitrocellulose solution was poured evenly over a test tube-shaped mould perforated by a small hole at the rounded end; the mould was rotated at about 15 r.p.m. Each coat was allowed to dry for a given time before the next was applied. No heat was used for drying, and the temperature and humidity were not controlled. With Elford's Gradacol membranes, intended for gradation of particle size, reproducible and uniform pore diameters are ensured by strict control of temperature and humidity in the drying process. Since the purpose here was merely removal of aggregates, such care in preparation was considered unnecessary. After soaking in water overnight the thimble was removed by forcing water under pressure between the film and the glass through the perforation. High quality rubber pressure tubing was used for mounting and the final size of the membrane was *ca.* 40 mm. long by 10 mm. diameter, giving a filter area of 12 sq. cm. Membranes were stored in 40% saturated aqueous ammonium sulphate and were cleaned for re-use with N/10-hydrochloric acid or 0·1% sodium hydroxide solution. More concentrated solutions of alkali caused the nitrocellulose to become brittle and crack.

Absolute ethanol, sodium-dried ether, and AnalaR acetone, amyl alcohol, and glacial acetic acid were used as solvents; the nitrocellulose was a sample (grade HL 120/170) recommended by Alexander and Johnson for membrane formation. The most useful membranes for filtration of seed-globulin solutions ($M \approx 300{,}000$) were made by applying three coats of a solution of the following composition: nitrocellulose (2 g.), ether (18 g.), alcohol (7 g.), acetone (9 g.), amyl alcohol (4 g.), and acetic acid (0·13 g.).

As reported by Elford the pore size was found to be sensitive to the concentration of acetic acid, and membranes with greater or smaller permeabilities were made by using, respectively, less or more acetic acid in the nitrocellulose solution. Times of drying before the second and the third coat were applied were 2 and 3 minutes, respectively, and, for the final drying, moulds were suspended in a vertical position for 1 hour before being soaked. The permeability of these membranes to water was 0·7 c.c./minute under a pressure of 5 cm. of mercury and was fairly reproducible ($\pm 10\%$). With protein solutions, flow rates were slower because of the increased viscosity of the fluid and probably the blockage of the pores by large aggregates.

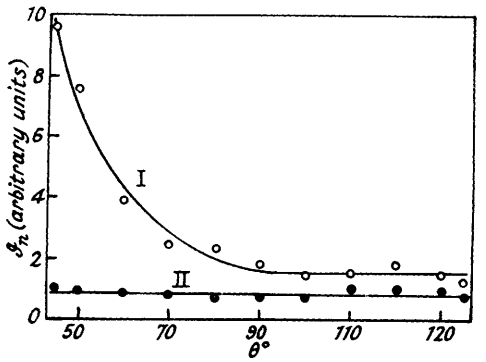
By means of a mercury manometer, protein solutions were forced through the membranes

under pressures of 5—15 cm. Hg. If the solution was so cloudy that filtration proved very slow, a preliminary filtration through a sintered glass disc was found preferable to the use of greater pressures which tended to pollute the solution. The proportion of material held up by the filter varied with the protein preparation, the state of the solution, and the ionic strength, I. With serum albumin ($I = 0{\cdot}1$) and edestin solutions (from hemp seed) ($I = 1$), respectively, the hold-up of material was 15% and 25%, while for strongly aggregated solutions of arachin (from ground-nuts) ($I = 0{\cdot}1$) less than half of the protein was found in the filtrate.

Easy filtration with small hold-up was favoured by solutions of high ionic strength. For example, with a solution of legumin (from peas) in phosphate buffer of pH 7·7 and $I = 0{\cdot}1$, the hold-up was 75%, falling to 20% when the ionic strength was increased to 0·5. This effect may have been due to either the increasing tendency to aggregation at low values of I (due to the lowered solubility of the globulin), or an increase in particle–membrane interaction at low ionic strengths.

An example of the effectiveness of the filters is given in the figure which shows the normalised intensity distributions of scattered light for buffers cleaned, respectively, by filtration through

Plot of $\mathscr{I}_n$ against θ for buffer solutions filtered through a No. 4 " Pyrex " sintered-glass disc (I) *and an ultrafiltration membrane* (II).



a No. 4. Pyrex sintered-glass disc, and by the present ultrafiltration technique. The normalised intensity $\mathscr{I}_n$ is given by :

$$\mathscr{I}_n = \mathscr{I} \sin\theta/(1 + \cos^2\theta)$$

where $\mathscr{I}$ is the observed intensity and θ is the angle the scattered light makes with the transmitted beam. For the ultra-filtered buffer, $\mathscr{I}_n$ is constant and the dissymmetry, $\mathscr{I}_{60°}/\mathscr{I}_{120°}$, is virtually unity, indicating that heavy material had been completely removed by the filter. The irregularities in the graph are due to small imperfections in the light-scattering apparatus which become apparent at such low values of $\mathscr{I}_n$; here $\mathscr{I}_n$ for the ultra-filtered buffer is less than 1% of the scatter shown by a 0·3% solution of arachin. With the buffer filtered by the sintered-glass disc, $\mathscr{I}_n$ increased markedly for smaller values of θ, and $\mathscr{I}_{60°}/\mathscr{I}_{120°}$ was 2·7, showing the presence of large scattering particles in the buffer.

As a further test, solutions of serum albumin and arachin, known to contain a considerable amount of aggregated material, were filtered first through a No. 4. " Pyrex " sintered-glass disc and then through a membrane. Light-scattering determinations of molecular weight were made after each filtration. From the results of these measurements, shown in the Table, it is

Decrease in Molecular Weight and Dissymmetry after Ultrafiltration.

	M			$\mathscr{I}_{60}/\mathscr{I}_{120}$	
Protein	Accepted value	found, before ultrafiltration	found, after ultrafiltration	before ultrafiltration	after ultrafiltration
Arachin	330,000	3,260,000	542,000	1·95	1·26
Serum albumin	70,000	140,000	85,000	1·69	1·04

clear that much of the aggregated material passed through the sintered-glass disc to give high values of M and $\mathscr{I}_{60}/\mathscr{I}_{120}$ which were considerably reduced after ultrafiltration. The M values were still somewhat higher than the accepted molecular weights because strongly aggregated

solutions were especially chosen to test the membranes. Freshly prepared solutions gave accurate values for the molecular weight of serum albumin; for the seed globulins, arachin, legumin, and edestin, molecular weights only 10% higher than the accepted values were noted. This discrepancy is not necessarily attributed entirely to the light-scattering determination.

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