NEW POSSIBILITIES IN THE DESIGN OF ULTRACENTRIFUGAL EQUILIBRIUM EXPERIMENTS

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For equilibrium experiments in the ultracentrifuge it is customary, except when the light absorption method is used, to make a separate determination of the initial solute concentration in terms of refractive index, because equilibrium patterns (both interference and schlieren) yield only differences between the concentrations at particular levels. The supplementary knowledge of the initial concentration makes it possible to convert this information into absolute terms. One method of obviating the additional step has been introduced by Yphantis (1964). The present communication deals with new modifications of technique which achieve the same object whilst being free from certain disadvantages of Yphantis' method.

Principles - When a layer of solvent is superimposed on solution in a synthetic boundary type cell, the solvent beyond a certain distance from the boundary is not detectably altered for a period of time which is dependent on several factors. If the Rayleigh interference fringes are photographed during this stage, and changes in them in

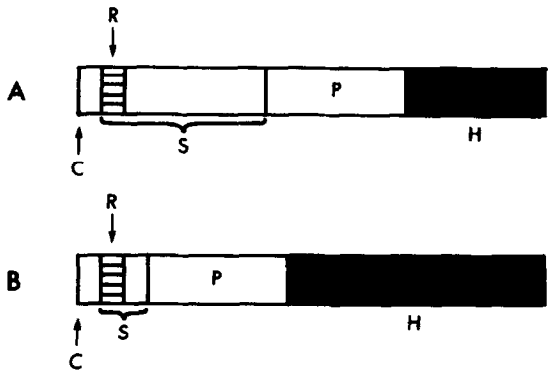

Fig. 1 - Disposition of sector contents after layering (A) a relatively large, (B) a relatively small volume of solvent (S) over protein solution (P). Capillary location is denoted by C, heavy fluid by H, and region for slit pictures by R.

this region (R in Fig. 1A) are followed by a sequence of slit pictures until equilibrium is established, absolute solute concentrations can be easily deduced.

This procedure (I) places limitations on the shortness of the column, but a reduction in column height is permissible (Fig. 1B) if the fractional part of the fringe shift at equilibrium is obtained by reference to base-line photographs (procedure II), provided that the initial photograph is recorded before there has been a shift of more than one fringe at the reference level, R.

In principle, the above sequences of fringe changes can be reversed if, after equilibrium has been attained, the rotor speed is increased sufficiently. These variations, which will be referred to as III and IV, correspond to procedures II and I respectively.

Experimental - The techniques illustrated in Fig. 1. require the use of a double synthetic boundary cell filled in such a way that, after the layering process is completed,

the meniscus in the solution sector is slightly below the capillary. The total height of the column is controlled by using appropriate volumes of heavy, inert fluid (FC43, perfluorobutylamine, as recommended by Yphantis (1960)).

A typical experiment of type II on bovine albumin (Table I) was done by putting 0.126 ml. of FC43 in each sector of a 12 mm. cell, 0.053 ml. of protein solution (thoroughly dialysed against the buffer) in one side, and 0.105 ml. of buffer in the other. Layering resulted in about 0.018 ml. of buffer flowing on to the solution so that the site of the boundary thus formed was about 0.5 mm. below the final meniscus level. Simple calculation indicated that it would be at least 14 minutes before diffusion could produce a shift as great as one fringe in the interference pattern at the meniscus. (Of course, with an unknown substance a conservative estimate of this time is made.) A full photograph (exposure 12 sec.) was recorded well before 14 minutes had elapsed. Immediately afterwards the Spinco swinging gate assembly, bearing the transparent plate with opaque horizontal scale markings, was installed in front of the camera. A second full photograph was taken with this arrangement, but at the end of 12 sec. the gate was closed so that light falling on the photographic plate was confined to a strip 1 mm. wide (Fig. 1). After the slit photograph (5 sec.) had been superimposed on the full photograph, slit photographs alone were recorded automatically at intervals of two minutes by means of the short shift mechanism. When about 60 slit photographs had been taken, the time interval was increased to 8 minutes.

Finally, when the fringe changes became very slow, slit and full photographs were superimposed, the gate was removed, and ordinary full photographs were recorded at hourly intervals.

From the slit pictures, the apparent alteration in the fringes is obtained relative to a superimposed scale line. However, as the whole image is liable to drift slightly relative to the scale over long periods of time, if identification of fringes is to be unequivocal the drift must be measured approximately. This is the purpose of incorporating the initial and late full pictures with superimposed slit pictures and scale lines. The

TABLE I

MOLECULAR WEIGHTS MEASURED BY THE NEW PROCEDURES

Solute	Sucrose	Bovine albumin	Bovine gamma globulin
Solvent	Water	Acetate buffer, pH 4.6, I = 0.1	Phosphate buffer, pH 7.0, I = 0.1
Speed (r.p.m.)	42,040	10,589	7,928
Total height of column (mm.)	5.5	2.0	1.0
Initial height of solution (mm.)	2.2	1.5	1.0
Initial solute concentration (mg./ml.)	20	3	1.6
Equilibrium concentration at meniscus (fringes)	21	5	4
M	343	66,700	149,000
Standard error (%)	0.23	0.54	0.76

accurate measurements are made on the final patterns taken in the absence of the scale, and on base-line photographs recorded at the same speed.

The first test material used was 'Analar' sucrose. Bovine albumin (Armour, Fraction V, batch FE 2170) was purified by passage through a column of Sephadex G-200 (Pharmacia, Uppsala, Sweden). Bovine gamma globulin was prepared from serum by a procedure which involved DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden). Both proteins were monodisperse when examined by velocity ultracentrifugation at a concentration of about 8 mg./ml.

Results - Molecular weights were calculated from the ideal form of the two-component equilibrium equation:

$$M = \frac{2RT}{\omega^2(1-\bar{v}\rho)} \frac{d(\ln c)}{d(r^2)}$$

in which the symbols have their usual significance. A plot of ln c against r^2 was always made, but the regression of ln c on r^2 was determined by the least squares method. Typical results, which were obtained by methods I, II, and III, respectively, are shown in Table I. The molecular weights are in satisfactory agreement with accepted values, and the standard errors of the regressions are of the same magnitudes as normally obtained in equilibrium experiments under comparable conditions.

Discussion - Lack of base-line stability or reproducibility is one of the most important factors which could affect the application of the new methods. The stringency of the requirements is least for method I, intermediate for II and III, and greatest for IV. In practice the only method which has given rise to this type of difficulty is IV.

The layering techniques (I and II) have the advantage that, with a rotor speed selected according to the specifications laid down by Pasternak et al. (1957), it is possible to reduce the time required to reach equilibrium. On the other hand, in experiments of the reverse type (III and IV), economy can be effected by a slight initial overspeeding, as advocated by Hexner et al. (1961). In all cases, equilibrium conditions can be chosen to avoid the steep gradients which occur in the method of Yphantis (1964).

The possible complications arising from charge effects, the slight modifications in technique which may be necessitated by the low speeds required for large molecules, and other relevant points will be dealt with in a future publication.

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