An Ultracentrifugal Field Relaxation Method for Molecular Weights*

GERSON KEGELES AND CHO LU SIA†

From the Department of Chemistry, Clark University, Worcester, Mass.

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The field relaxation procedure involves the layering of solvent over solution in the ultracentrifuge cell, and acceleration to a speed sufficient to cause appreciable sedimentation, followed by careful deceleration to a sufficiently low speed to permit horizontal extrapolation of the schlieren pattern to the image of the bottom of the fluid column. The concentration function is obtained by summing the areas under the free peak and near the bottom of the schlieren pattern, leading to enhanced rather than reduced accuracy when these areas become comparable in magnitude. Simple projection and tracing of patterns, followed by polar planimeter measurements, provide sufficient accuracy. Under conditions of low speed operation, solvent reference experiments may be dispensed with, so that the entire measurement requires only one ultracentrifuge cell filling. It has been found that this technique offers quick sensitive means for the detection of high molecular weight contaminants as well as for the detection of reversible aggregation and gel or precipitate formation at high concentration, and it is expected that this method will offer the protein chemist an easily applicable new criterion for protein homogeneity.

Eigen (1954, 1957) and others have employed relaxation methods for the study of rapid chemical reactions. Our attempt to apply this concept to study interacting protein systems by an ultracentrifugal field relaxation technique has been only partially successful. Since the relaxation, in a dissociating system, of a buildup of concentration produced by sedimentation is controlled in a complicated way by diffusion and residual sedimentation, as well as by dissociation, the information which has been obtained has served to verify quickly the existence of a chemical reaction, but not to measure its velocity. In the case of nonreacting systems, however, the technique has found ready applicability for assessing the purity of protein preparations and the effectiveness of purification techniques.

Experimental Procedure

It was found (Kegeles, 1949, unpublished observations) that 16% ammonium sulfate solutions which had been submitted to ultracentrifugation at 60,000 rpm gave rise to two peaks in the schlieren diagram, one from either end of the liquid column, when the instrument was decelerated. The interpretation was made that when the angular velocity approaches zero the concentration gradient must also approach zero at the impervious cell boundaries, since this gradient is proportional to the diffusional flow at zero angular velocity. Hence the schlieren pattern, which shows a strong upsweep at high angular velocity, must show a maximum at some point in the cell as the angular velocity is decreased. Advantage has now been taken of this phenomenon by selecting as a lower speed that value which allows the schlieren pattern to be extrapolated approximately horizontally to the image of the cell bottom. In this respect the technique resembles that described by Ehrenberg (1957), who, however, obtained his data from the upper meniscus just prior to free boundary formation at relatively high ultracentrifuge velocities. In order to prevent too large an accumulation of protein at the cell bottom, we have found it desirable to form a free boundary by layering solvent (dialysate) over solution (Kegeles, 1952; Pickels *et al.*, 1952), rather than wait for a free boundary to be formed by sedimentation. Acceleration is performed as usual, but deceleration is performed by coasting. Whereas the procedure of Archibald (1947) as used in this laboratory (Klainer and Kegeles, 1955; 1956) requires two cell fillings and two-coordinate microcomparator readings of the photographic plates, and Ehrenberg's technique (1957) although permitting enlarger and polar planimeter measurements still requires two ultracentrifuge experiments, only one cell filling for one ultracentrifuge experiment is required in the field relaxation method (Kegeles and Sia, 1963). In order to observe the lower meniscus, a thin transparent layer of a heavier liquid, fluorocarbon FC-43, is placed below the aqueous column (Ginsburg *et al.*, 1956).

Typical ultracentrifugal field relaxation photographs are shown in Figure 1, illustrating two separate experiments on 1.2% crystalline (Worthington) α-chymotrypsin in 0.05 ionic strength, *p*H 8.45 Veronal buffer. In each experiment, the sample was accelerated to 20,410 rpm and decelerated at approximately 1,000 rpm min^{-1}. In the photograph at the left, the sample was allowed to remain at this higher speed for 6 minutes before being decelerated to 3,500 rpm, and it was photographed after 12 minutes at the lower speed. In the photograph at the right, the sample was allowed to remain at the higher speed for only 3 minutes prior to deceleration to 4,000 rpm, and was photographed after 20 minutes at the lower speed. The pronounced elevation in the height of the schlieren pattern at the bottom of the column in the first experiment is caused by the effect on the association reaction of the larger accumulation of protein during the longer period at 20,410 rpm.

To illustrate that this effect for chymotrypsin is due to aggregation, diagrams obtained from a similar pair of experiments performed on crystalline Armour bovine plasma albumin in *p*H 4.41 sodium chloride–sodium acetate–acetic acid buffer at 0.17 ionic strength are shown in Figure 2. In both photographs of Figure 2, the sample was accelerated to 20,410 rpm and decelerated at the rate of 1000 rpm min^{-1} to 6,000 rpm. In the photograph at the left the sample was allowed to remain at the higher speed for 6 minutes prior to deceleration, while in the photograph at the right only 3 minutes were allowed at 20,410 rpm before deceleration. A comparison of the regions near the cell bottom in Figures 1 and 2 serves to differentiate between chymotrypsin, which aggregates to hexamers under these conditions (Sia, 1963) and bovine plasma albumin, which does not appear to undergo rapid aggregation. This contrast and the data obtained from these patterns

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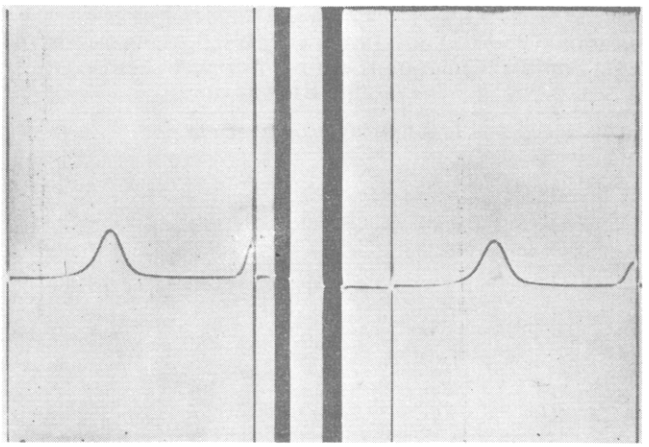

FIG. 1.—Ultracentrifugal field relaxation schlieren patterns for α-chymotrypsin; 1.23% solution in 0.05 ionic strength, *p*H 8.45 Veronal buffer. Sedimentation proceeds from left to right. Left-hand pattern, 6-minute ultracentrifugation at 20,410 rpm before relaxation to 3,500 rpm; right-hand pattern, 3-minute ultracentrifugation at 20,410 rpm before relaxation to 4,000 rpm.

described under Results serve in part to answer a question raised repeatedly, whether the field relaxation method makes all proteins behave as aggregating systems.

A further possible conjecture, that all proteins necessarily precipitate at the lower interface between the aqueous and fluorocarbon phases, and that this precipitate redissolves on field relaxation, can be tested by relaxing the centrifugal field still further than in the experiments shown in Figures 1 and 2. As indicated in the first paragraph of this section, the condition requiring no flow at this interface also requires that the limit of the flow, which approaches the diffusional flow, must be zero as the centrifugal field approaches zero. In mathematical terms:

$$\lim_{\omega \to 0} J = \lim_{\omega \to 0} \left(-D \frac{\partial c}{\partial x} \right) = 0$$

Hence as the velocity approaches zero the concentration gradient and the refractive index gradient must approach zero at the cell bottom, and under practical conditions of large field relaxation the refractive index gradient curve goes through a maximum, as shown in two experiments, Figure 3. Here the samples of protein were accelerated to 20,410 rpm in 5 minutes, permitted to remain at this speed for 4 minutes, decelerated to 3,000 rpm at a rate of 1,000 rpm min^{-1}, and photographed after remaining at the lower speed for 8 minutes. In the diagram at the left, the sample is 1% chymotrypsin in 0.05 ionic strength, *p*H 8.4 Veronal buffer. In the diagram at the right, the sample is 1% Armour crystalline bovine plasma albumin in 0.17 ionic strength, *p*H 4.41 sodium chloride–sodium acetate–acetic acid buffer.

On the other hand, should a precipitate or gel form at the lower interface, which then redissolves upon relaxation of the field, the condition that there is no flow at the bottom of the aqueous column no longer holds. In the language of diffusion, there is now a source at the cell bottom in the limit when the centrifugal field goes to zero. Hence:

$$\lim_{\omega \to 0} J = \lim_{\omega \to 0} \left(-D \frac{\partial c}{\partial x} \right) > 0$$

It is physically obvious that the concentration function has its maximum in this case at the surface of the precipitate or gel. Although the problem has not been

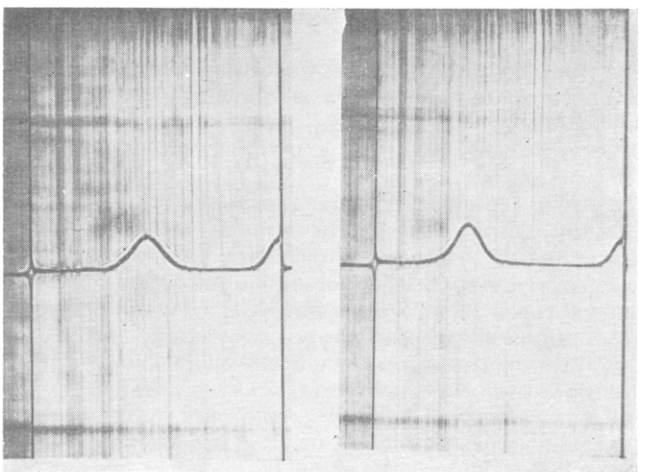

FIG. 2.—Ultracentrifugal field relaxation schlieren patterns for Armour crystalline bovine plasma albumin; 1% solution in 0.17 ionic strength, *p*H 4.41 sodium chloride–sodium acetate–acetic acid buffer. Sedimentation proceeds from left to right. Left-hand pattern, 6-minute ultracentrifugation at 20,410 rpm before relaxation to 6,000 rpm; right-hand pattern, 3-minute ultracentrifugation at 20,410 rpm before relaxation to 6,000 rpm.

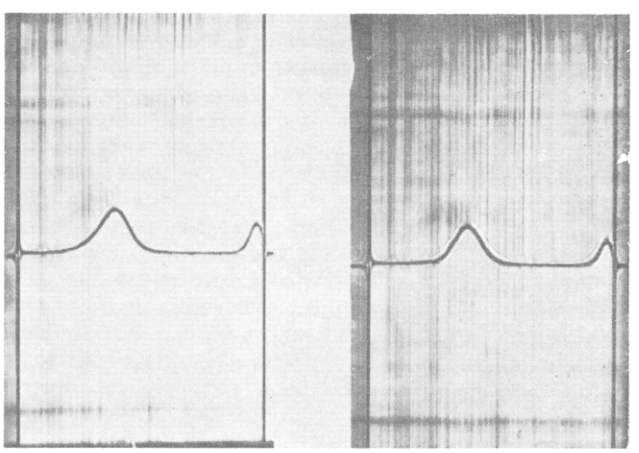

FIG. 3.—Ultracentrifugal field relaxation schlieren patterns for 1% chymotrypsin (left) and for 1% Armour crystalline bovine plasma albumin (right). Sedimentation proceeds from left to right. Both patterns, 4-minute ultracentrifugation at 20,410 rpm before relaxation to 3,000 rpm; photographs taken after 8 minutes at 3,000 rpm.

analyzed, it is also probable that the concentration gradient will increase continuously as the cell bottom is approached. Hence, at any practically attainable low velocity it should be impossible to make the field relaxation refractive index gradient curve bend over significantly toward the baseline if reversible precipitation or gel formation has occurred.

Our experience has indicated that, on the basis of this criterion, chymotrypsin, bovine plasma albumin, and human hemoglobin preparations (Green, 1963, unpublished observations) show no evidence of reversible precipitation or gel formation at the aqueous-fluorocarbon interface. However, a sample of an abnormal globulin (Kochwa, 1963, unpublished observations), which produced some visible gel formation on centrifugation in an angle-head rotor, also could not be made to provide diagrams which bent toward the baseline at low fields. Thus the field relaxation procedure clearly appears not to involve reversible precipitation of proteins as a *general* phenomenon, and the procedure also appears capable of distinguishing between systems which remain in the aqueous phase

and those which form a second phase at the lower interface.

Actual computations of molecular weights at the cell bottom are made from the following equation:

$$M_w = \frac{EF\,RTZ_b}{(1 - V\rho)\omega^2 x_b(A_1 + A_2)} \qquad (1)$$

Here E is the enlarger projection magnification factor, F is the enlargement by the camera lens in the ultracentrifuge of distances along the axis of rotation, Z_b is the extrapolated deflection above the estimated baseline of the schlieren diagram at the lower meniscus, as obtained from the *enlarger* projection, and $A_1 + A_2$ represent the sum of the areas below the free peak and near the cell bottom image, as measured from the enlarger projection. Three points may be noted here. The value of M_w must represent the true weight-average molecular weight of the material which is seen at the cell bottom at any instant, provided only that no continuing convection is occurring. Although convection does occur occasionally, the effect of convection at an impervious interface must disappear at the instant when convection stops. This is apparent from the requirement that all the forces causing transport of the solute through the impervious aqueous-oil meniscus must instantaneously balance to zero, independent of the past history of the experiment (Kegeles *et al.*, 1957). Moreover, the cell bottom is a region of high density gradient and consequent stability against convection. Second, M_w does not represent the weight-average molecular weight of the original sample, but depends in a complicated way on what materials are present at the lower meniscus at a given instant. Third, it is not necessary that there be a "plateau" region between the free peak and the region where protein piles up at the cell bottom in order to use equation (1). However, there must be no elevation of the schlieren curve above the baseline in the region between the air-aqueous phase meniscus and the free peak, a condition which offers no difficulty at all when using layering techniques with solutions of macromolecules.

Results

In Table I are shown the results for the experiments on α-chymotrypsin illustrated in Figure 1. The pronounced effect on the observed molecular weight of the longer period at 20,410 rpm is apparent. We wished to investigate whether this is caused by rapid reversible aggregation to polymers (Schwert, 1949; Schwert and Kaufman, 1951; Steiner, 1954; Tinoco, 1957; Neurath and Dreyer, 1955; Massey *et al.*, 1955; Gilbert, 1955; Rao and Kegeles, 1958; Bethune and Kegeles, 1961). Therefore crystalline bovine plasma albumin (Armour), a presumably chemically inert system, at least for short periods, was subjected to ultracentrifugal field relaxation measurements, Figure 2, under similar schedules of acceleration and deceleration.

Table I

1.23% α-Chymotrypsin in *p*H 8.45 Veronal Buffer, 0.05 Ionic Strength

Field Relaxation Method			
Min at 20,410 rpm	Reduced Speed	Time[a] (min)	M_w Bottom
3	4,000 rpm	37	133,600
6	3,500 rpm	38	223,600

[a] From commencement of acceleration.

Table II

Armour Crystalline Bovine Plasma Albumin in *p*H 4.41 Sodium Chloride–Sodium Acetate–Acetic Acid, 0.17 Ionic Strength

Field Relaxation Method				
Maximum Speed (rpm)	Time at Max. Speed (min)	Reduced Speed (rpm)	Total Time[a] (min)	M_w Bottom
20,410	3	6,000	33	97,000
20,410	6	6,000	40	98,500
20,410	4	6,000	38	97,000
20,410	4	6,000	43	98,000
20,410	4	5,000	59	95,000
20,410	4	5,000	75	98,000

[a] From commencement of acceleration.

The results obtained for bovine plasma albumin are shown in the first two rows of Table II. In contrast with Table I, there is within experimental error no observed dependence of the observed molecular weight on the time of sedimentation at the high speed. Such behavior would not be expected for a grossly impure mixture, however.

The reproducibility of the results for this albumin sample in three separate experiments is also shown in Table II. The variations in the results obtained lie well within expected experimental errors of measurement. Although the sample showed a visible high molecular weight shoulder in sedimentation velocity experiments, field relaxation molecular weights proved to be relatively insensitive to the choice of high speed or the time during which the sample was held at such speeds prior to deceleration. However, the field relaxation results indicated a molecular weight of 97,000 for 1.3% crystalline bovine plasma albumin in *p*H 4.41 sodium chloride–sodium acetate–acetic acid buffer at 0.1 ionic strength. A similar solution of the protein was therefore centrifuged in the angle-head rotor for 4.5 hours at 40,000 rpm, and the upper third of the sample was collected for quantitative re-examination. Sedimentation velocity still indicated a small skewing of the pattern toward the cell bottom. Identical redialyzed samples were subjected to molecular weight measurements by the Archibald method, the Ehrenberg method, and the field relaxation method, and the results are shown in Table III, which also serves to indicate reproducibility in two field relaxation experiments.

In order to obtain some idea of the weighting of the observed molecular weight toward heavier species present, a mixture of 0.5% raffinose and 0.5% glycine

Table III

1.2% Crystalline Bovine Plasma Albumin, Upper Third of Sample Centrifuged 4.5 hr at 40,000 rpm; Measured in *p*H 4.41 NaCl-CH_3COONa-CH_3COOH Buffer, 0.17 Ionic Strength

Method	Speed (rpm)	Time (min)	M_w Top	M_w Bottom
Archibald	7,928	15	66,800	72,300
Ehrenberg	17,500	38	67,600	
Field relaxation	6,300	45		84,700
	6,300	50		85,200
	5,000	73		83,000
	5,000	83		84,700
	5,000	50		84,000
	5,000	60		85,300

was subjected to field relaxation measurement. The sample was accelerated to 60,000 rpm, left at this speed for 10 minutes, and then decelerated to 43,000 over a period of 10 minutes. The photograph was taken after 4 minutes at the lower speed. In this case, an identical water reference run was made for comparison, to eliminate the local effects of window distortion. The weight average molecular weight M_w was computed from equations (2), (3), and (4):

$$C = C_1 + C_2 \quad (2)$$

$$C_1M_1(1 - V_1\rho) + C_2M_2(1 - V_2\rho) = RT(dC/dx)_b/\omega^2x_b \quad (3)$$

$$M_w = (C_1M_1 + C_2M_2)/(C_1 + C_2) \quad (4)$$

Here C_1 and C_2 represent the unknown concentrations of raffinose and glycine at the cell bottom where the observed total concentration is C. M_1 is taken as 504.5 and M_2 as 75.1. The $(1 - V_1\rho)$ factor for raffinose is taken as 0.3919, based on the measurements of Longsworth (1953) and of McDonald and Gross (1954). The factor $(1 - V_2\rho)$ for glycine is taken as 0.4154, based on the work of Dalton and Schmidt (1933).

This measurement resulted in a weight-average molecular weight of 424. The weight average value for the original sample is only 289.6. This measurement is subject to two errors. First, the raffinose alone was found to give a value of 535 with the same technique indicating the presence of some impurity. Second, no account was taken of the difference in specific refractive index increments of raffinose and glycine in aqueous solution.

Discussion

We have noted the marked dependence of the field relaxation molecular weight results for α-chymotrypsin, Figure 1 and Table I, on the time of ultracentrifugation at the higher speed, and the relative insensitivity of such results, Figure 2 and Table II, to the program of the experiment for bovine plasma albumin. From this comparison, it has been deduced that we have a quick and extremely sensitive method for detecting the existence of rapid concentration-dependent chemical interactions of proteins, such as polymerizations and the formation of complexes.

From Figure 3 and its description in the section on experimental procedure we have demonstrated that it is possible to use the field relaxation method to distinguish between systems which precipitate or gel at the cell bottom and those which do not.

From the results described above for the raffinose-glycine mixture, it is clear that the field-relaxation molecular weights are weighted heavily toward the high molecular weight components in a mixture.

From Table III, it is seen that a relatively pure protein gives the expected molecular weight with the Ehrenberg technique, and only a small indication of impurity when the results from the cell top and the cell bottom are compared in the Archibald method. Yet the same sample has shown a decrease of molecular weight only from 97,000 to 85,000 with the field relaxation method, which clearly indicates the need for still further purification. Thus it is felt that our ultracentrifugal field relaxation procedure constitutes a very sensitive tool for following the removal of high molecular weight contaminants from purified proteins, and an additional, easily applicable criterion for protein homogeneity.

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