Electrophoresis as a Criterion of Purity in Proteins[1]

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IT IS CUSTOMARY to adopt a standard of purity commensurate with the operations applied to a particular system. For example, a good grade of conductivity water still contains many diverse molecules if one considers the isotopes of hydrogen and oxygen. Yet for most chemical uses conductivity water is a purer grade of water than is necessary. Few chemical uses require that optical isomers be resolved. In contrast, however, optical isomers of natural products become immediately important in the chemical reactions occurring in the living organism, because such operational distinctions are made by the organism itself. The concept of purity as applied to proteins must likewise be defined in an operational sense. Thus a protein may be considered pure as judged by sedimentation velocity, and impure as judged by electrophoretic analysis or immunochemical tests. It seems quite probable that a protein judged pure on the basis of phase rule solubility studies may prove impure when studied by the reversible electrophoretic spreading (*1, 2*) or the Gouy interference diffusion techniques (*3–5*). Crystalline β-lactoglobulin has been shown by Ogston (*6*) and ovalbumin by Kegeles (*7*) to be heterogeneous as judged by the Gouy method.

A system that cannot be fractionated is in a thermodynamic sense a single component (*8*). One meets with difficulty when applying this definition to proteins, however, because adequate chemical and physical fractionation techniques for protein systems are still in the incipient phases of development. The limitations of Kirkwood's electrophoresis-convection method for protein fractionation are still unexplored. So far, the method has been applied to the γ-globulins of serum with considerable success (*9, 10*). Chromatographic methods (*11–13*) are as yet not sufficiently developed to be critically evaluated either for fractionation or for analysis. This also applies to a system devised by Svensson and Brattsten (*14*), which has the merits of both simplicity and mildness of operation. Another variation in electrophoretic technique utilizes electrophoresis on paper (*15–17*). Although it is too early

to discern the ultimate value of this method, it promises to be useful for both fractionation and analysis.

The term "electrophoretically homogeneous," as it has been commonly employed in the older literature and is frequently employed in the present biological literature, is misleading. What is usually meant is that a single symmetrical peak is obtained at one pH and at one ionic strength. A number of proteins earlier considered homogeneous have been found to resolve into several peaks when examined over a wider pH range (*18, 19*). Other proteins, although showing a single symmetrical boundary over a wide range in pH at an ionic strength (μ) of 0.10, give markedly unsymmetrical boundaries at $\mu = 0.01$ (*1, 20*). Almost without exception, when the potential field is reversed in an electrophoresis experiment, the boundary, which has been growing broader with time, will begin to sharpen. This reversible spreading is enhanced at lower ionic strengths. In an operational sense the term "electrophoretic homogeneity" should refer to the most critical technique employed. Thus used, the term would imply that, over the pH range within which the protein is not denatured and at low ionic strength values, a single boundary is obtained. Furthermore, the term implies that under conditions of low ionic strength at the isoelectric point reversible spreading does not occur. Several factors are involved in applying this definition.

Specific ion effects. Interaction between proteins and buffer ions must be considered in evaluating electrophoretic patterns. These specific ion effects may adversely affect the resolution of components, particularly where the mobilities are close to one another (*21*). Chloride ion, for example, is bound to the extent of more than eleven ions per molecule of albumin (*22*). That these binding effects influence the isoelectric point when it is measured electrophoretically has been shown by Longsworth and Jacobsen (*23*).

Hydrogen ion concentration. As mentioned above, components that exhibit the same mobility at one pH move with widely different mobilities at other hydrogen ion concentrations. An example is the case of the two mucoproteins in serum, which both have mobilities of approximately -6.4×10^{-5} cm^2 $volt^{-1}$ sec^{-1} at pH 8.6 and an ionic strength of 0.10 in veronal buffer, and mobilities of approximately -3.8×10^{-5} and

-0.3×10^{-5} cm²/volt⁻¹/sec⁻¹, respectively, when examined at pH 4.5, $\mu = 0.10$ in acetate buffer (24).

Ionic strength effect. Electrophoretic mobility is a function of ionic strength (25). This is attributed in part to the ion atmosphere surrounding the moving protein ion (26), and in part to specific ion effects mentioned above, since the isoelectric points are known to shift with ionic strength (25), as is shown in Fig. 1. Mobilities become more sensitive to pH
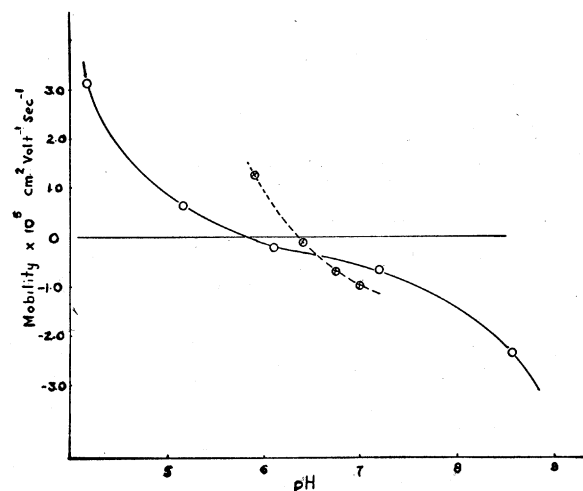

FIG. 1. pH Mobility curves for bovine serum γ 1 globulins (20) ; ○, μ = 0.10 ; ⊕, μ = 0.01.

changes as the ionic strength is lowered, as can also be seen in Fig. 1. Consequently, resolution of components having nearly identical mobilities is increased at lower ionic strengths. Conversely, more symmetrical and sharper boundaries are obtained at higher ionic strengths. Any adequate evaluation of homogeneity requires that electrophoretic studies be performed at several ionic strengths. The factor of electroosmosis along the cell wall must be considered in low ionic strength experiments. Until this factor has been studied more adequately it seems inadvisable to work at an ionic strength less than 0.02.

The potential gradient. Since resolution in electrophoresis is a function of the field strength, it is desirable to use as large a potential gradient as possible without causing convection. In this way maximum resolution with respect to the time the current passes through the cell may be achieved. Thermal convection gradients are more likely when higher field strengths are employed, because of the larger amount of heat developed in the system. The maximum feasible field strength (E) that does not produce thermal convection is a function of the specific conductivity (K) of the

solution. $E = \sqrt{\dfrac{H}{K}}$ volt cm⁻¹, where H is the power in watts cm⁻³ that can be dissipated in the cell. As can be seen from this equation, greater potential gradients can be employed with buffers of lower ionic strength.

The factors of resolution and sensitivity. In using electrophoresis as a criterion of purity it is necessary to consider the resolving power of the optical system. This raises the question, what is the least amount of a component that one can detect with the optical method employed? With the use of the 25-mm cell, the better instruments detect a gradient corresponding to refractive index change in the cell of 2×10^{-5} for the green line of mercury (27–29). The least refractive index difference that can be resolved varies inversely with the cell thickness. Consequently, with smaller cells such as the 15-mm cell, it is not possible to detect a refractive index difference less than approximately 3.5×10^{-5} at a wavelength of 5,461 A. The specific

refractive increment $\dfrac{\Delta n}{\rho} = k$ for bovine serum albumin

is equal to 0.0019 (30). It is possible, therefore, when using a 25-mm cell, to detect an amount of serum albumin corresponding to 0.01 g/100 ml of solution. In the analysis of a protein system where the concentration of the solution in the cell is 1 per cent, it is possible to detect bovine serum albumin present as a contaminant to the extent of 1 per cent of the total protein, provided the gradient due to albumin is completely resolved from the gradients due to other components in the system. If the concentration in the cell is increased to 2 per cent it is possible to detect a component in the system present to the extent of 0.5 per cent of the total protein in solution. Such resolution is seldom achieved, however. Optical imperfections in the lenses and cells, as well as the use of widened horizontal slits to overcome light absorption, all serve to decrease the resolving power of the system.

Even when the instrument is capable of such resolution, it is necessary to operate under the maximum conditions of sensitivity to observe this small gradient. The sensitivity, corresponding to the least detectable height of the curve in the pattern, depends upon the magnifying power of the camera and the cylinder lenses, the cell thickness, the distance of the cell from the diagonal slit, and the slit angle. The sensitivity of the apparatus can be increased threefold by increasing the diagonal slit angle from 30° to 60°. Few existing optical systems are sufficiently perfect to allow the use of the latter angle.

Where the mobilities of the contaminants are close to that of the main component ($\Delta u < 1$ cm² sec⁻¹ volt⁻¹) the contaminants may be present to the extent of several per cent and remain undetected, and there is good reason to suspect that this occurs frequently. The specific refractive increment for most proteins that have been measured is approximately 0.0019. Accordingly, in the best electrophoretic work impurities present to the extent of 0.5 per cent remain undetected, and more commonly several per cent of substances other than the main component remain imperceptible.

It is common practice, also, to centrifuge the solution prior to introducing it into the cell in order to remove small amounts of insolubles. The latter may indicate that the solubility limits of one or more components in the system have been exceeded, or they may

indicate insoluble contaminants in the preparation not involved in the electrophoretic analysis itself. It is incorrect to call such a preparation homogeneous unless the insoluble material is investigated and shown to result from exceeding the solubility limits of the solvent system.

Reversible electrical spreading. The high refractive index gradient existing between the buffer and the protein solution at the time of the formation of the boundary gradually approaches zero as diffusion occurs. This spreading of the boundary takes place even without the application of an electric field, and diffusion coefficients are frequently determined in this fashion. With the application of an electric field, additional changes in the sharpness of the boundary generally occur. Superimposed upon the diffusion process, which causes both ascending and descending patterns to broaden, are the electrical spreading factors.
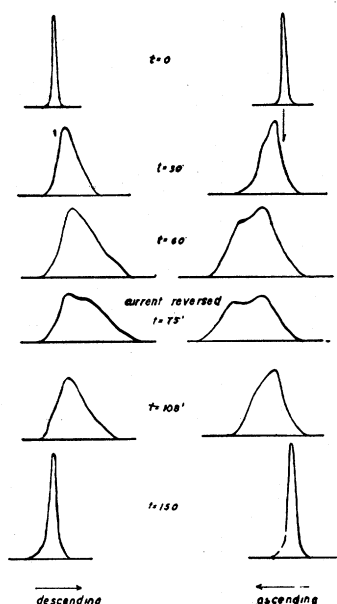
FIG. 2. Reversible electrophoretic spreading of bovine γ 1 globulins at pH = 5.95, μ = 0.01 (20).

It is commonly observed that the ascending boundary is sharper than the descending boundary in the electrophoresis of protein systems. Longsworth has shown that the reverse may occur (31). This phenomenon is due to the salt gradients that exist through the boundaries, as shown by Longworth and MacInnes (21, 32). As a result of these salt gradients, conductivity and ionic strength variations occur throughout the cell. Both contribute to the anomalous behavior, although the conductivity effect is of larger magnitude. Likewise, there are differences in pH on opposite sides of a boundary that may enhance or counteract the described phenomenon. These effects, which are predicted by theory (33) and have been quantitatively treated (34, 35), are functions of the net change on the protein molecule and diminish as the molecule approaches an isoelectric condition.

Both the pH and conductivity effects are minimized when the experiment is conducted at the isoelectric point. In this instance, if thermal and mechanical convection are not involved, and if the spreading is greater than can be attributed to diffusion, electrical inhomogeneity of the protein is indicated (36). This type of spreading reverses when the polarity of the field is reversed, in contrast to spreading caused by thermal and mechanical convection. This phenomenon is illustrated in Fig. 2.

Reversible electrical spreading has been dealt with by Sharp and co-workers for the case where diffusion is negligible compared with electrical spreading (37). The case in which diffusion is not negligible, but in which the mobility distribution may be represented by a Gaussian distribution function, has been treated by Alberty (1, 38). More recently, Brown and Cann (2) have generalized the treatment to include non-Gaussian distributions of mobilities and have shown that the equation derived by Alberty (1) is applicable in the generalized case, provided the apparent diffusion coefficient is calculated from the second moments of the gradient curve. The equation is:

$$D' = \frac{\sigma^2 - \sigma_0^2}{2t_E} = D + \frac{E^2\beta^2}{2}\, t_E,$$

where D' is the "apparent diffusion coefficient," and D is the true diffusion coefficient. σ_0 is the second moment of the gradient curve at the instant the electric field is applied, and σ the second moment at a time t_E. A plot of the measured apparent diffusion coefficient against the time of electrophoresis gives a linear relation, the slope of which is $\dfrac{E^2\beta^2}{2}$. The heterogeneity constant β is equal to $\dfrac{\sqrt{2S}}{E}$, where E is the potential gradient and S the experimentally determined slope.

A zero slope is proof of homogeneity. Yet even this criterion is invalid if, by chance, a mixture of two homogeneous proteins have coincident isoelectric points. In the latter instance, however, it is unlikely that the mobilities would coincide over an extended pH range. The heterogeneity constant has the same dimensions as mobility (cm^2 $volt^{-1}$ sec^{-1}) and measures the standard deviation of the mobility distribution.

Another test for electrophoretic homogeneity was recently reported by Hoch in England (39). Hoch has shown that, for a homogenous protein under a constant potential gradient, a steady state develops in the ascending limb of the cell. The "steady state boundary criterion" had already been described and used by Alberty and Anderson (40) in 1948, although Hoch was unaware of this at the time he published. This steady state results when the spreading caused by thermal diffusion and pH variation through the boundary is exactly counterbalanced by the sharpening that is due to the conductivity effect. When the steady state is reached, the boundary no longer changes with respect to time. Inability to obtain a steady state is indicative of electrical heterogeneity.

Hoch demonstrated that oxyhemoglobin fulfilled the requirements of homogeneity, whereas crystalline bovine serum albumin did not. Hoch's method has the disadvantage that no quantitative measure of the extent of heterogeneity is obtained. Other limitations of the "steady state boundary criterion" are mentioned by Alberty and Anderson (40). In the reversible spreading technique the extent of heterogeneity is measured in terms of the standard deviation of the mobility distribution. Hoch's method can be applied, nevertheless, where the reversible spreading technique is not possible, as in the case of those proteins almost completely insoluble at their isoelectric point.

Two aspects of the electrophoresis-convection method developed by Kirkwood and associates (9) should be emphasized. These workers fractionated proteins, which moved as a single peak in the electrophoresis cell. Hess and Deutsch (20) had previously accomplished such fractionation by chemical methods. Kirkwood et al. also showed that the mobility distributions of the fractions, when added, give the mobility distribution of the original starting material. Such evidence leaves little doubt that a single symmetrical peak in an electrophoretic pattern is insufficient evidence of homogeneity.

Conclusions. Two things become apparent: First, the concept of protein purity will be revised as more sensitive tests become available and are applied. Second, and less obvious, a protein may be judged pure for one operation and impure for another. For example, a protein obeying the phase rule solubility test for purity may not obey a biological test of purity, yet it may be a sufficiently "pure" protein for purposes not involving its biological activity.

The concept of purity is a relative term, and a particular protein should not be termed "homogeneous" when judged by a single operation. Only if it has withstood all the known tests should it be considered as meeting the present standards of homogeneity. Electrophoresis is not a particularly sensitive criterion of purity as the operation is customarily performed.

The reversible electrical spreading technique is an exceedingly sensitive method for detecting heterogeneity. The term "electrophoretically homogeneous" should be reserved for those proteins that have a

heterogeneity constant $\beta = 0.0$. It appears doubtful whether any protein has yet been obtained sufficiently "pure" to satisfy this criterion.

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