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Electrophoresis in Highly Cross-linked Polyacrylamide Gels

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Summary

The properties of polyacrylamide gels with 1 to 50% cross-linking (%C) were studied using electrophoresis in multiphasic buffer systems of five proteins with known molecular weight. The extrapolated free mobility (Y_0 or M_0) varies in a biphasic manner with %C, reaching a maximum when %C is 5 to 7%, and falling to a constant plateau value specific for each protein when %C is greater than 20%.

The retardation coefficient (K_R) shows a different type of biphasic relationship with %C: it increases with %C over the range 1–5%C and then falls asymptotically to zero, irrespective of molecular size.

High %C provides a substantial increase in the effective pore size of the gel and enables one to fractionate macromolecules in the multimillion molecular weight range. These gels should be useful when it is desired to minimize molecular sieving, e.g., for isotachophoresis and isoelectric focusing.

INTRODUCTION

In polyacrylamide gel electrophoresis (PAGE) generally 1–5% cross-linked gels have been used for fractionation of proteins. However, 20% cross-linked gels have been used since Ornstein and Davis (*1*) introduced PAGE in "discontinuous" (or "multiphasic") buffer systems. The

"stacking" gel ("concentration" or "upper" gel) of these systems is 20% cross-linked in order to minimize the "molecular sieving" of macromolecules in these gels. The finding by Ornstein and Davis that such highly cross-linked gels exhibited a larger pore size than obtainable by gels of low percent cross-linking was implicit in their application to stacking gels, but was not explicitly stated in the literature. Fawcett and Morris (*2*) were the first to investigate quantitatively large pore gels of high degree of cross-linking using gel filtration. By using Ogston's model (*3*), they estimated the median pore radii ($R_{0.5}$) for 15 and 25% cross-linked (%C) polyacrylamide gels of total gel concentrations (%T) from 6.5 to 10%T [nomenclature of Hjerten (*4*)]. For the 25%C gels, $R_{0.5}$ ranged from 3 to about 3.7 nm; for 15%C gels, $R_{0.5}$ ranged from 2 to 3 nm. These pore sizes were larger than obtainable from 1, 2, or 5%C gels for similar values of %T. Gel electrophoresis can be used in a similar manner to estimate median pore size (*5*, *6*).

Further impetus for the study of highly cross-linked gels came from the theoretical predictions of corollaries to Ogston's model (*3*), which state that in an ideal gel composed of a random suspension of points or spheres (0-D gel) the behavior of a molecule would be primarily determined by its volume, whereas in a gel composed of a random suspension of linear fibers (1-D gels), the behavior of a molecule is governed by its surface area (*3*, *5*, *7*, *8*). Thus, if one could develop "ideal" 0-D and 1-D gels, one might be able to obtain an estimate of the surface/volume ratio of macromolecules and, thus, derive the axial ratios for particular geometric forms. Also, if the density or partial specific volume of a protein or series of proteins were known (or at least uniform), then behavior in gels sensitive to volume would also be expected to provide a better correlation with mass (molecular weight) than behavior in a gel sensitive to surface area. It seemed plausible that, by changing the ratio of cross-linking agent to total monomer (%C), one might effect a change from a (predominantly) 1-D gel to a 0-D gel. Every cross-linkage could be considered to represent the "ends" of at least two linear fibers, and thus, increasing %C would decrease the mean length of the linear fibers between branch points. Also, if "bundling" occurs, then the total length of the linear fiber would decrease (*2*). Richards and Temple (*9*) have proposed a very similar model, "to equate the crosslinks or clusters with the point elements, and the rods with the acrylamide chains," and demonstrated that this theory is compatible with gel swelling and absorbance properties of gels with 1 to 10%C, 2 to 20%T.

Thus, it seemed possible, though by no means certain, that increasing %C would result in formation of a 0-D gel. The experimental test of this hypothesis is complicated by the fact that the dimensions of either the "gel fiber" or the "gel point" cannot be neglected; rather, they are of the same order of magnitude as many of the molecules under study. Also, the effects of the hydration shells of both the macromolecule and the gel, the role of electrostatic interactions, distortion of the electrical field by the elements of the gel, and "ballistic" distortion of the gel by the macromolecule (*6*, *10*) are too complex to be predicted. Finally, the postulates that (*5*, *11*, *12*)

$$K_{av} = f \quad \text{and} \quad M/M_0 = f \tag{1}$$

must be regarded as working hypotheses, since these are based on static rather than on kinetic considerations.

In order to test the hypothesis that highly cross-linked gels are sensitive to "volume," whereas slightly cross-linked gels are sensitive to "surface area," it would be necessary to perform studies on a wide variety of globular and highly asymmetrical, well-characterized molecules. Fibrinogen, nucleic acids (*10*), sodium dodecyl sulfonate (SDS) coated proteins (*13*), and polysaccharides are available for this purpose. However, these molecules have usually been characterized by classical methods at high ionic strengths. Since the conformations of these molecules are highly dependent on ionic strength and display strong electrostatic interactions, characterization by classical methods does not provide standards applicable to electrophoretic studies.

The test of the volume–surface area hypothesis in electrophoresis with highly cross-linked polyacrylamide gels was begun by the present preliminary study using five proteins. It showed that polyacrylamide gels of up to 50%C could be made and that the gels provide very large pore sizes. This extends the use of PAGE to the multimillion molecular weight range (*14*). Electrophoresis in these gels gives rise to linear Ferguson plots (*15*): both the slope (retardation coefficient, K_R) and y-intercept of the Ferguson plot (Y_0) vary systematically with %C in a fashion that requires a reinterpretation of the physical meaning of these two parameters as measures of molecular size and net charge (*15*).

MATERIALS AND METHODS

Proteins

Low-density lipoprotein (LDL) (see Table 1) of human serum (obtained from H. Pollard, NIH) was characterized as a globular lipo-

TABLE 1

List of Proteins Used

Protein	Mol wt $\times 10^3$	Shape	Ref.	Source
Low-density lipoprotein (LDL)	3200	Globular	*27*	H. Pollard, NIH
R-Phycoerythrin	290	Globular	*8*	H. W. Siegelman, Brookhaven Natl. Lab.
Human fibrinogen	340	Rod-shaped	*28*	J. Finlayson, NIH
Human hemoglobins A and S (cyanmet)	64	Globular	*28*	M. Feld, NIH
BSA	65	Globular	*28*	Armour

protein with mol wt = 3.2×10^6. R-Phycoerythrin extract of *Ceramium rubrum*, designated "phycoerythrin" (obtained from Dr. H. W. Siegelman, Brookhaven National Laboratory, Long Island, N.Y) was assumed to be globular and to have the same molecular weight as R-phycoerythrin from another source (*4*), i.e., mol wt = 290,000. Human fibrinogen (a gift of Dr. John Finlayson, NIH) had been purified and characterized by high-speed sedimentation equilibrium. Human hemoglobins A and S (obtained as a hemolysate from M. Feld, NIH) were converted to cyanmethemoglobins (*16*) and stored at 4°C. Bovine serum albumin (BSA) (Armour) was used. Solutions were made in upper buffer (see below), 25% in sucrose, and stored at 4°C.

Polymerization

The procedure of polymerization was similar to that previously described (*15*). However, due to the low solubility of monomer solution with high %C, several modifications were required.

Acrylamide–*N*,*N*′-methylenebisacrylamide (Bis) stock solutions (*15*) were prepared from the purified reagents as a 30%T solution for 1 to 7.5%C gels and as a 20%T solution for 10 to 20%C gels. Volume ratios and initiator concentrations are indicated in Table 2.

For the preparation of 50% formamide gels, acrylamide–Bis stock solutions were prepared in 100% formamide (BDH No. 28422, Gallard Schlesinger). Aqueous buffers, initiator solutions, and TEMED concentrations were identical to other gels.

Gels of 30 to 50%C were prepared from 10%T stock solutions in lower gel buffer (final concentration). Stock solutions were prepared by heating to 40°C with stirring and filtration through Whatman No. 1 paper. Before storing the stock solutions, dilutions were made to the degree permitted by the final gel concentration required. Immediately prior to deaeration, 1% *K*-persulfate (KP) in lower gel buffer (final concentration; designated LGB × 1) was added to give a final concentra-

TABLE 2

Polyacrylamide Gel Recipes for Multiphasic Buffer System A

Temperature 25°C, pH(ZETA) = 8.93; pH(PI) = 9.45
Stacking limits (upper gel): RM(1, 4) = 0.098; RM(2, 2) = 0.947
Unstacking limit (lower gel): RM(1, 9) = 0.244

Buffer	Components/100 ml[a]		pH	KAPPA (μmhos/cm)
Upper	Glycine, 0.385 g	Tris, 0.663 g	8.92	338
Upper gel	1 *N* H_3PO_4,[b] 6.40 ml	Tris, 0.71 g	7.18	3020
Lower gel	1 *N* HCl, 6.0 ml	Tris, 4.54 g	8.79	5241
Lower	1 *N* HCl, 5.0 ml	Tris, 0.757 g	7.41	4471

	Monomer solution		Catalyst		
	%T (g/100 ml)	%C	KP (mg 100 ml)	RN (mg/100 ml)	TEMED[c] (μl/100 ml)
Upper Gel	3.125	20	5.	0.5	25.
Lower Gel	2.0	1–50	50.	0	100
	↓	↓			↓
	15.0	1–20	50.	0	25

TEMED[b] Levels (μl/100 ml Gel) for Lower Gel

%T	2	3	4	4.5	5	7	8.5	10	12.5	15
TEMED	100	90	75	60	50	45	40	35	30	25

[a] All concentrations refer to final polymerization mixture.
[b] 1 *N* = 0.5 *M*.
[c] TEMED = *N*,*N*,*N*′,*N*′-tetramethylethylenediamnine

tion of 0.2%. An example follows:

6.86 %T: 7 ml 10%T, 50%C in LGB × 1
+ 3 ml LGB × 1
+ 0.2 ml 1% KP in LGB × 1

Deaeration was conducted at about 40°C for gels with %T values exceeding 7%. Gel tubes were fire-polished and sealed with Parafilm. In order to provide a mechanical support, a plug of 0.2 ml of a 7–10%T, 2%C gel was polymerized at the bottom of the tubes. An alternative but less effective way to stabilize gels mechanically was to stretch a piece of nylon mesh (ASTM 400-37) across the bottom of the gel by use of a Tygon sleeve. Polymerization was carried out at 25°C in the thermostated lower buffer reservoir of an electrophoresis apparatus which provides hydrostatic equilibration of gel tubes and improved heat exchange through continuous stirring (*17*).

Buffer System

The multiphasic buffer system of Davis (*1*), modified in the form designated system A (*15*) and analyzed by the program of Jovin (*18*) was used at 25°C. Table 2 defines the system and lists some of its computed operative parameters.

Electrophoresis

PAGE was carried out at a current density of 2 mA/0.28 cm^2 as previously described (*15*) in the same apparatus used for polymerization (*17*).

Fixation and Staining

Gels were removed from the tube, after rimming of the 7%T gel plug, by application of a few drops of water on top of the gels and application of slight air pressure. In the case of 30 to 50%C gels, staining with Coomassie Blue in 12.5%TCA (*19*) was reduced to 10 min, since these gels exhibit an appreciable background of irreversibly adsorbed stain. If mechanical fragility of the gels prohibited manipulation, gels were stained overnight in the same 40-ml vials used for fixation by addition of 1 ml 0.05% Coomassie Blue in 12.5% trichloroacetic acid (TCA) to 40 ml 12.5%TCA.

Alternatively, 30–50%C gels were stained with 0.1% Amidoblack in 7% acetic acid for 2 hr. Gels were gently poured onto cheesecloth supports lining rectangular chambers of a destaining rack; destaining was effected by continuous pumping of 7% acetic acid through the vessel surrounding the destaining rack and through a charcoal bed.

Other Procedures

Photography of unstained and stained gels, measurement of relative electrophoretic mobilities (R_f) for each band, computation of retardation coefficients (K_R), and y-intercepts (Y_0) were carried out as described previously (*15*).

RESULTS

Figure 1 depicts the change of retardation coefficient, K_R, as a function of %C for the five proteins studied (mol wt from 6×10^4 to 3×10^6). As %C increases, the K_R first increases to a maximum in the region

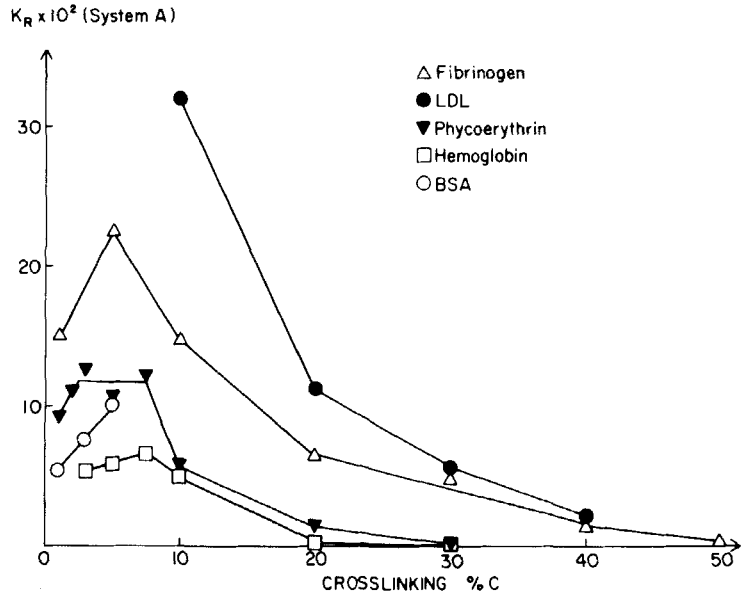

FIG. 1. Retardation coefficients (K_R) of fibrinogen, LDL, phycoerythrin, human hemoglobins A and S, and BSA vs degree of cross-linking (%C) of polyacrylamide gels.

of 5 to 8%C and then decreases. This confirms previous findings of Morris and Morris (*6*). Figure 1, furthermore, shows that, as %C increases from 10 to 50%, the K_R values of molecules with widely differing molecular weight progressively approach each other and approach zero asymptotically. At all %C values, fibrinogen exhibits K_R values intermediate between LDL and hemoglobin or phycoerythrin.

Figure 2 shows the change of y-intercept of the Ferguson plots, Y_0, as a function of %C. As for the K_R vs %C curves (Fig. 1), the Y_0 vs %C curves exhibit a maximum when %C is approximately 10%C. However,

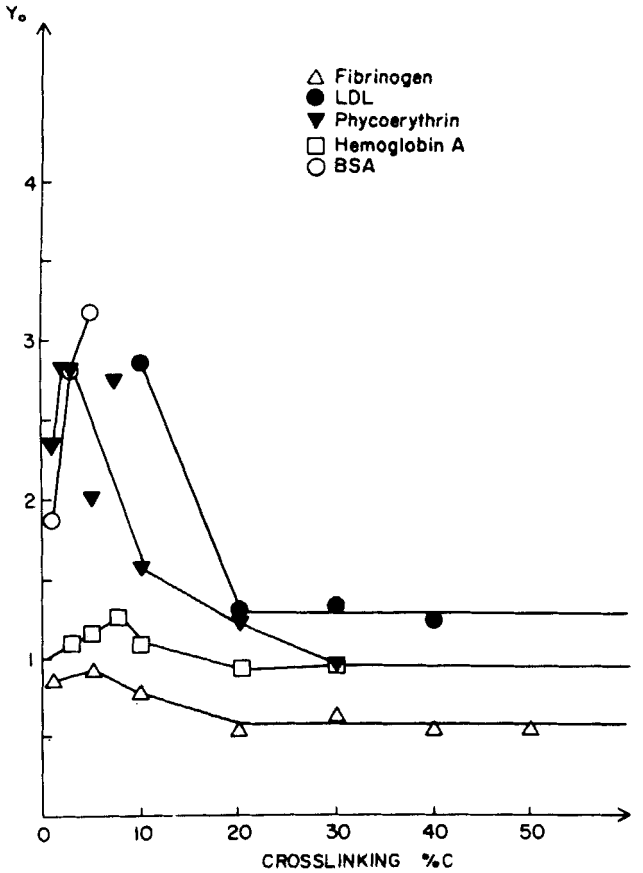

FIG. 2. The y-intercepts (Y_0) of the Ferguson plots for five proteins vs degree of cross-linking (%C) of polyacrylamide gels.

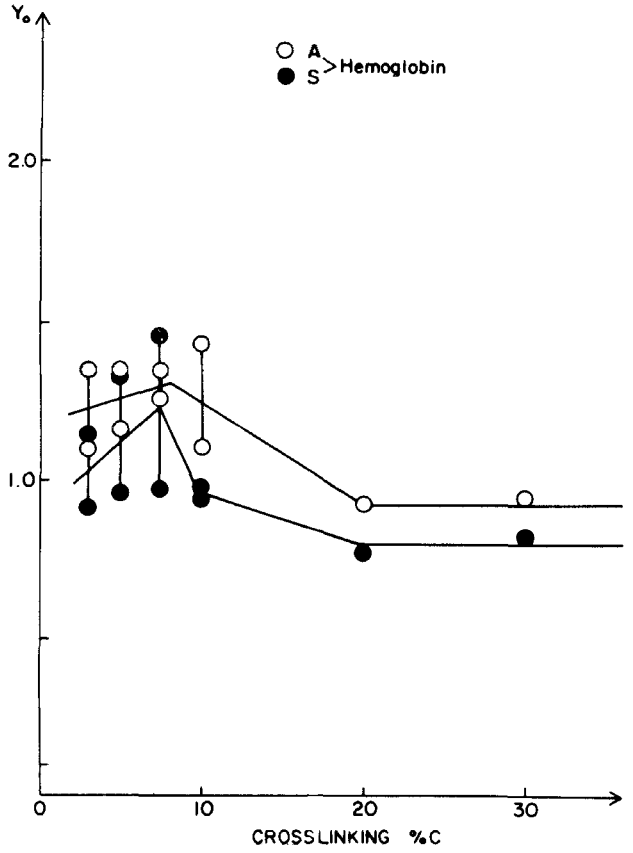

FIG. 3. Plot of Y_0 vs %C for human hemoglobins A and S.

as %C increases above 10%, Y_0 assumes plateau values, characteristic for each of the proteins, which appear to be consistently lower than the Y_0 value extrapolated to 0%C.

Figure 3 shows the same relation between Y_0 and %C for hemoglobins A and S. The constant Y_0 values at high %C were 0.93 and 0.80 for hemoglobin A and S, respectively. These values were used to compute apparent molecular net charge (valence, V) (*15*) for the two hemoglobin species. Results are shown in Table 3. A difference of 0.84 in valence between hemoglobins S and A was found in the buffer system used (pH 9.5, $I = 0.0158$).

TABLE 3

Summary of Computer Output (15) Giving the Calculated Free Electrophoretic Mobility (M_0) and the Molecular Net Charge (Valence V) of Hemoglobins A and S Derived from Y_0 Values at 20 to 30%C Seen in Fig. 3

```
DATE :   10    27    71

SYSTEM 400      CROSSLINKING  30      PROTEIN  91      Y0 = .931828
RM[1,9]         IONIC STR.    TEMP.           MU[SODIUM+]     MU[FRONT]
-.244           .0158          25              4.72500E-04    -1.1290E-04


FREE MOBILITY [CM/SEC]/[VOLTS/CM] =       -1.07430E-04
PROTEIN RADIUS =                           2.66459E-07
COUNTERION RADIUS =                        2.50000E-08
DEBYE-HUCKEL RECIPROCAL THICKNESS =        4.13779E+06
HENRYS FUNCTION OF  1.10255  = X1 =        1.03204
CHARGE [COULOMBS/MOLECULE] = Q =          -9.34128E-12
VALENCE[ NET PROTONS/MOLECULE] = V1 =     -5.83468

MOBILITY AT I =  .1   IS =                -7.06605E-05
HENRY'S FUNCTION OF 2.77377  = X1          1.08687

-------------------------------------------------------------------


DATE :   10    27    71

SYSTEM 400      CROSSLINKING  30      PROTEIN  92      Y0 = .798103
RM[1,9]         IONIC STR.    TEMP.           MU[SODIUM+]     MU[FRONT]
-.244           .0158          25              4.72500E-04    -1.1290E-04


FREE MOBILITY [CM/SEC]/[VOLTS/CM] =       -9.20133E-05
PROTEIN RADIUS =                           2.66459E-07
COUNTERION RADIUS =                        2.50000E-08
DEBYE-HUCKEL RECIPROCAL THICKNESS =        4.13779E+06
HENRYS FUNCTION OF  1.10255  = X1 =        1.03204
CHARGE [COULOMBS/MOLECULE] = Q =          -8.00073E-12
VALENCE[ NET PROTONS/MOLECULE] = V1 =     -4.99735

MOBILITY AT I =  .1   IS =                -6.05202E-05
HENRY'S FUNCTION OF 2.77377  = X1          1.08687
```

Figure 4 shows the K_R vs %C curve of fibrinogen in water and 50% formamide. Use of 50% formamide as gel solvent produces an increase in K_R. This may be due to a loosening of protein conformation in the mixed solvent or to a change in the pore size of the gel.

DISCUSSION

The present study indicates that polyacrylamide gels with up to 50% cross-linking can be made and used as a medium for zone electrophoresis.

The problem of the low solubility of monomer solutions with high Bis content has been at least partially solved by (a) adjustment of volume ratios of the acrylamide–Bis solution, buffer and catalysts in the polymerization mixture, (b) increased temperature of the polymerization mixture prior to polymerization, and (c) use of formamide solutions as solvent for the polymerization mixture.

However, due to solubility problems, at the present time, the total gel concentration is restricted to <10%T for a 50%C gel. This does not appear to be a severe problem, since these gels appear to be most applicable to high-molecular-weight macromolecules, where a low %T appears desirable. Also, since K_R approaches zero with increasing %C, mobility becomes (almost) independent of gel concentration (%T).

Gels with 10%C have previously been utilized by Elson and Jovin (*20*) to minimize the degree of swelling, which was otherwise problematic [swelling of the gel may lead to obstruction and occlusion of the elution chamber in preparative (elution) PAGE apparatus]. White and Dorion (*21*), Richards and Temple (*9*), and Morris and Morris (*6*) studied the water regain properties of gels in detail: as %C increased from 1 to 10%, the water regain decreased markedly.

Gels with 15% or higher cross-linking appear opaque. This may inter-

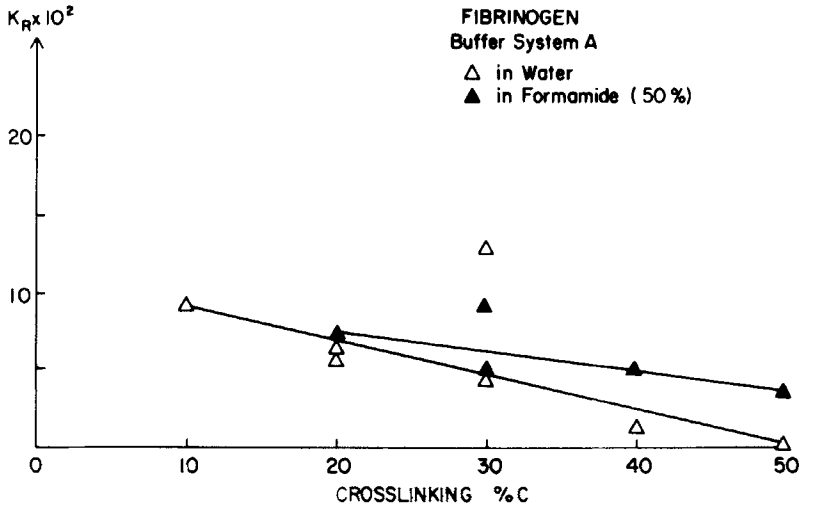

FIG. 4. Plot of K_R vs %C for fibrinogen in polyacrylamide gels made in aqueous buffer or in 50% formamide.

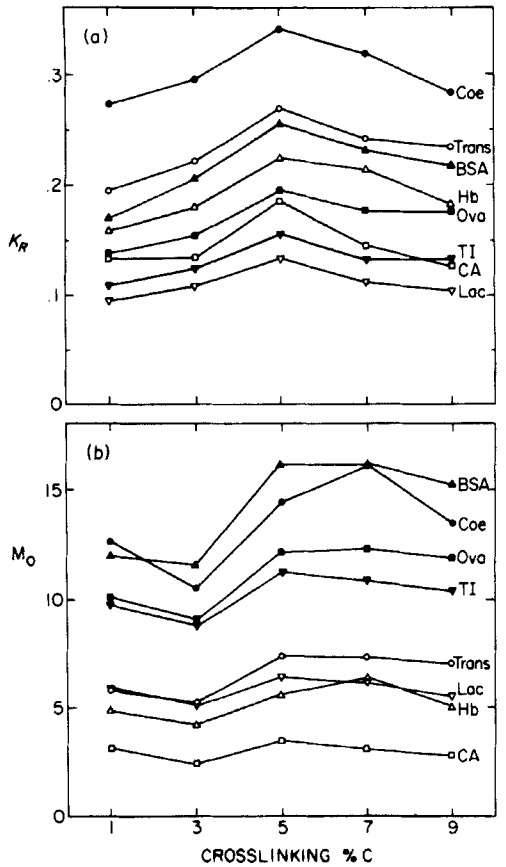

FIG. 5. (A) Plot of K_R vs %C for eight proteins (data from Table 4 of Ref. 6). (B) Plot of M_0 vs %C for eight proteins (data from Table 4 of Ref. 6).

fere with the detection of protein bands in stained gels, and provides a high background for densitometry. However, minor modifications of previous staining techniques gave satisfactory results. Highly cross-linked gels also showed mechanical lability and relatively poor adherence to glass. However, these problems were overcome by (a) use of hydrostatic equilibration of the gels, (b) use of a gel plug of high %T, low %C, as a mechanical support, (c) use of a nylon-mesh support, and (d) minor modification of techniques of handling the gels, e.g., use of

diffusion rather than electrophoretic destaining of gels stained with Amidoblack and removal of gels from glass tubes by cracking the glass tube with a hammer rather than by rimming.

The present results both confirm and extend the recent findings of Morris and co-workers (*2*, *6*). They showed that K_R values vary systematically as a function of %C, with a maximum when %C is approximately 5 (Fig. 5A depicts the data from Table 4 of Ref. *6*). The electrophoretic data of Morris et al. were restricted to the range $1 <$ %C < 9, whereas our findings cover the range $1 <$ %C < 50. Thus, Morris et al. did not observe that K_R approaches zero, irrespective of molecular size, as %C increases from 10 to 50%.

Morris and Morris (*6*) also measured the extrapolated free mobility as a function of %C (Fig. 5B). They interpreted their results as showing a random, rather than systematic, variation of M_0 with %C, and calculated the standard deviation of M_0 values obtained at five different values of %C. However, in retrospect, after viewing the systematic relationship between Y_0 and %C shown in Fig. 2 and 3 of the present report, it appears that the data of Morris et al. also show a similar relationship (Fig. 5), i.e., M_0 increases as %C increases from 1 to 5%, reaches a maximum, and then falls as %C increases further to 7 or 9%. Certain methodological differences between the study of Morris and co-workers and ours should be pointed out. These are summarized in Table 4.

Despite these differences, the overall results obtained are remarkably similar (compare Fig. 5A with Fig. 1; compare Fig. 5B with Fig. 2 and

TABLE 4

Comparison between Conditions of PAGE Used by Morris and Morris (*6*) and in This Report

Parameters	Morris and Morris (*6*)	Present authors
Buffer	Continuous	Multiphasic (discontinuous)
	Tris–HCl	Tris–glycinate
pH	8.76	9.45
Ionic strength	0.05	0.015
Temperature	10°C	25°C
Gel purification	Diffusion	None

3). However, due to the differences in methodology, the values of K_R and Y_0 (or M_0) cannot be compared quantitatively for the two proteins (BSA and hemoglobin) which appear in both series.

Use of multiphasic buffer systems yields a value of Y_0 which can be converted to free mobility, M_0, as follows (*15*):

$$M_0 = Y_0 \times \mathrm{RM}(1, 9) \times u_{\mathrm{Na}^+} \tag{2}$$

where u_{Na^+} represents the mobility of the sodium ion at the same ionic strength and temperature as used in electrophoresis, and RM(1, 9) is the mobility, relative to Na^+, of glycinate in the operative separation phase in the buffer system used here. Thus, the Y_0 values of Fig. 2 and 3 can be converted, by a direct proportionality, to values of free mobility which should have the same physical meaning as the M_0 values obtained (relatively) directly by Morris. Conversion of Y_0 values into free mobilities makes the implicit assumption that u_{Na^+} and RM(1, 9) (or at least the product of these two parameters) are unaffected by either %T or %C. Alternatively, if the product, RM(1, 9) $\times$ u_{Na^+}, varies systematically with %T in a fashion analogous to the Ferguson relationship (*22*), then the values of Y_0 obtained by the procedure employed are still valid estimates of Y_0, and values of M_0 calculated from Eq. (2) will also be correct.

The present findings, although regarded as preliminary, have important and unexpected implications for the measurement of free mobility by PAGE, using either continuous (*6*) or multiphasic (*14*, *18*) buffer systems. If Y_0 (and M_0) vary systematically with %C (as appears to be the case from Fig. 2, 3, and 6), then it is unclear as to which, if any, of the Y_0 values is the "correct" one. One approach would be to extrapolate Y_0 to 0%T and 0%C (analogous to the double extrapolation utilized in the Zimm plot for light scattering). Thus, one could obtain Y_0 for several values of %C in the range $1 < \%C < 5$ and extrapolate to 0%C. However, the slope of this relationship can be rather high (at least in some cases). Also, the validity of linear extrapolation in the region below 1%C remains to be investigated or established, and this will be quite difficult, due to the extreme mechanical lability of gels with 1% or less %C. Indeed, a curvilinear relationship is suggested by Figs. 2 and 3. Of course, any extrapolation procedure increases the uncertainty of the results with a loss of precision of the estimate.

An alternative approach is suggested by the finding (Figs. 2 and 3) that Y_0 approaches a constant value when $10 < \%C < 50$. This plateau value is remarkably close to the values obtained by extrapolation of Y_0

to 0%C, using the approach described above. Calculation of the valence (net charge per molecule) for hemoglobins A and S by use of these plateau values for Y_0 reveals net charges of 5.0 and 5.8, respectively. This appears compatible with the known substitution of a single amino acid in 2 of the 4 chains of the hemoglobin molecule. One might expect less than a full two-charge difference if one of the two glutamic acid residues is not exposed at the surface of the molecule. However, the calculation of net charge involves many assumptions (*15*); therefore, this analysis does not have sufficient precision to enable us to discriminate between alternative approaches to obtaining a "true" M_0. However, the constancy of Y_0 (and M_0) in highly cross-linked gels may be used empirically. Also, Morris has shown an excellent correlation between the mean M_0 (of values obtained at 1, 3, 5, 7, and 9%C) and the free mobility estimated by classical moving boundary electrophoresis. This would appear to validate use of M_0 values obtained by PAGE (also cf. Fig. 5 of Ref. *15*), at least for certain purposes.

Unfortunately, the number of M_0 values reported in the literature is limited. Also, before making comparisons between M_0's from PAGE and classical methods, it is necessary to introduce correction factors for temperature, viscosity, and ionic strength. The validity of these correction factors is subject to considerable uncertainty. Finally, the same protein preparations have not been studied by both the classical and the PAGE methods, and the buffer constituents used were usually different in the two cases.

What is the explanation for the dependence of Y_0 and M_0 on %C? It may be due to a subtle change in the properties of the gel (e.g., distortion of the electrical field by the gel matrix). However, it is also possible that it is due to a conformational change in the protein, secondary to a change in its ionic milieu. There is also a possibility (though small) that it is a statistical artifact. Since the %C affects K_R and since errors in Y_0 and K_R are (positively) correlated, part of the relationship between Y_0 and %C may be a statistical artifact. However, the most likely explanation is that the Ogston model for a gel does not apply exactly to polyacrylamide gels and, therefore, application of the Ogston model to electrophoresis via the assumption that $M/M_0 = f(6, 5)$ may break down. There is some evidence for this:

1. Neville (*23*) has shown nonlinearity of Ferguson plots for SDS derivatives of proteins at high gel concentrations (although the mobilities are so low that this may have been due to measurement error).

2. Morris and Morris (*6*) have shown a small but definite, systematic departure from the theoretical ideal that $K_{av} = M/M_0$.

3. The linearity of the Ferguson plot may break down, especially in the very low %T region not readily available for experimental study. Indeed, the linearity of the analogous relationship between log K_{av} and %T must also break down for 5%C gels, since linear extrapolation of the data of Fawcett and Morris (*2*) would lead to the physically untenable or impossible value of $K_{av} = 1.2$ for several proteins (*5*).

4. Fisher and Dingman (*10*) have shown that mobility is affected by voltage gradient and suggested that this may be due to orientation of the asymmetrical molecule in the electrical field and/or a ballistic effect with the macromolecule distorting the gel structure. The latter possibility has also been entertained by Morris and Morris (*6*) and by the present authors.

This departure from ideal behavior would be expected on the basis of the Ogston model, if the gel consists of a combination of both 0-D (point) and 1-D (linear) elements (*3*, *9*) since the probabilities of "hits" and "cuts" (or "tangent" and "end" contacts) are not entirely independent of each other (*5*).

The observation that K_R approaches zero as %C increases, irrespective of molecular size, may indicate that the effective length or density of the gel is approaching zero. Another possibility is that the radius of the gel fiber or point (r) becomes so large relative to the size of the buffer or macroions (R), that the term ($R + r$) becomes constant, irrespective of R (i.e., size discrimination is lost). Accordingly, the true K_R for the trailing ion across the moving boundary in the separation phase becomes equal to the K_R of macromolecules, so that the observed K_R for macromolecules is zero. Thus, the "front" and the protein are retarded to the same extent. This possibility is open to experimental test (*23*).

The present results do not support the hope that PAGE will provide a simple method for evaluation of the configurations (axial ratio or surface/volume ratio) of macromolecules. Thus, Fig. 1 shows that the retardation coefficient of fibrinogen (presumably a rod-shaped molecule, with axial ratio 30:1, at least under some ionic conditions) behaves in very much the same manner as K_R for several (presumably) globular proteins.

If low %C gels were 1-D and high %C gels were 0-D, then one would expect fibrinogen to display a very high K_R in the low %C series (com-

pared with a K_R for a globular protein of similar molecular weight) but a "normal" K_R in the high %C gels. Instead, fibrinogen behaves very similar to the other standard proteins. It is possible that the expected effect does exist but is not detectable due to relative imprecision of the method. Again, the finite thickness of the gel, r, will tend to obscure the expected effect of molecular asymmetry. However, further studies with asymmetrical molecules will be needed to rule out the possibility that the observed behavior of fibrinogen was peculiar to this molecule or to the particular preparation studied. For such future studies it is encouraging to note that at a single %C, discrimination between single-stranded nucleic acids is possible on the basis of K_R alone (*10*).

The present results emphasize the need to utilize a constant %C when obtaining "standard curves" or "calibration curves" for the empirically observed relationship between square root of K_R and R or between K_R and molecular weight. We have previously indicated the need for separate calibration curves for each %C (*14*, *15*); the present data confirm this conclusion. Thus, one cannot utilize a standard curve, obtained at 2%C, to interpret the K_R in terms of molecular radius for an unknown preparation studied at 5%C. Also, for purposes of estimation of the molecular weight (or radius) of an unknown protein in the mol wt range of 60,000 to 300,000, it appears that a 5%C gel is best (cf. Fig. 1). However, for larger proteins (LDL), it may be desirable to operate at a higher %C (e.g., 10%), in order to obtain lower K_R's and, thus, higher mobilities that can be measured more accurately.

The present studies indicate that gels with high %C (10–50%) may be useful for fractionation of macromolecules in the multimillion molecular weight range. Indeed, use of high %C may be viewed as an alternative to use of very low %T, in order to achieve a sufficiently large median pore size to permit these large macromolecules to enter the gel. Both of these approaches have their practical difficulties; e.g., use of very low %T (1–3 %T) leads to loss of mechanical stability, so that polymerization combined with gelation of agarose is necessary (*24*, *25*). Use of very high %C also leads to mechanical lability and possible changes in gel structure, but at least it is not necessary to superimpose another type of gel matrix on polyacrylamide, and the chemical nature of the gel remains unchanged. Also, at least over the observable range of total gel concentration, linear Ferguson plots are still obtained (cf. Fig. 1). Thus, high %C should enable us to extend the working range (in terms of molecular weight) of PAGE to higher limits than heretofore available. Also, since K_R appears to approach zero, irrespective

of molecular size, as %C increases progressively, the mobility in the gel may provide a measure of free mobility.

The present studies are regarded as preliminary; it will be desirable to extend these observations to several additional well-characterized macromolecules in each of the globular, rod-shaped, and random-coiled classes.

It will also be desirable to extend the present observations to other cross-linking agents, providing different lengths of the cross-linking bridges. Also, studies in linear polyacrylamide solutions (0%) may help to elucidate the problem of determining the correct values for Y_0 or M_0.

Several of the findings of this study [and the related study of Morris and Morris (*6*)] may be explained by "failure" of the basic Ogston model; i.e., either the Ogston model for a gel does not apply to polyacrylamide, or, the assumptions made to apply the Ogston model to electrophoresis break down. Nevertheless, the Ogston model remains the best available model for prediction of the properties of polyacrylamide gels as applied to both gel filtration and gel electrophoresis. The formulation of a more realistic model for the polyacrylamide gel "pore" will depend on studies aimed at a direct evaluation of the physical states of polyacrylamide. Related studies on the diffusion coefficient in polyacrylamide gels have been initiated (*26*) that may also shed light on this problem. Also, studies of the macroscopic properties of gels (*6*, *9*) have contributed to understanding of the role of cross-linking in determining the nature of the polyacrylamide gel.

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