Molecular-Sieve Chromatography and Electrophoresis in Polyacrylamide Gels

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1. The absolute electrophoretic mobilities of eight proteins have been measured at pH 8.76, I 0.05, in polyacrylamide gels of 20 different compositions at 10°C.
2. The partition coefficients of these proteins have been determined chromatographically under the same conditions by using columns of granulated polyacrylamide gel prepared simultaneously.
3. The electrophoretic mobilities are an exponential function of the gel concentrations when the latter are corrected for water uptake. The constants of this function have been determined by curve-fitting methods. They have been shown to be related to the free solution mobility and to the mean molecular radius respectively.
4. The reduced mobilities have been shown to be a linear function of the partition coefficients by statistical analyses.
5. The physical significance of the relation between electrophoretic mobility and chromatographic phase distribution in gel media is discussed in the context of these results.

The molecular retardation mechanisms in gel electrophoresis and molecular-sieve chromatography are clearly very similar in origin, and are essentially due to the obstruction to free molecular movement caused by the internal structure of the gel. A unified theory for the two processes would be of considerable value for the selection of the best method for a particular problem, especially for the estimation of molecular parameters. The derivation of a unified theory is, however, heavily dependent on the current theoretical treatment of the two methods.

The molecular exclusion model of Ogston (1958), as applied to molecular-sieve chromatography by Laurent & Killander (1964) and by Siegel & Monty (1966) for cross-linked dextran (Sephadex) gels, by Fawcett & Morris (1966) for polyacrylamide gels, and by Laurent (1967) for agarose gels. An alternative thermodynamic treatment (Hjertén, 1970), and a statistical mechanical treatment (Giddings, Kucera, Russell & Myers, 1968), lead to similar relations between solute dimensions and chromatographic behaviour. Ogston & Silpananta (1970) have also given a thermodynamic treatment of the partition process.

The theoretical treatment of gel electrophoresis is much less satisfactory, possibly because the quantity and quality of the experimental data are quite inadequate to test any hypothesis rigorously. Earlier suggestions by Smithies (1962) and by Ferguson (1964), based on the results of starch-gel electrophoresis, suffered from this defect, although Ferguson (1964) was the first to suggest the correct logarithmic relation between electrophoretic mobility and the concentration of starch in the gel.

Polyacrylamide gel is a particularly suitable medium for investigations of this nature, since the gel composition and consequently the retardation effect can be varied widely and continuously without any experimental complications. The current popularity of electrophoresis in polyacrylamide gels in protein and nucleic acid chemistry has led to several comparative investigations with this medium, mostly directed towards finding a relation between solute dimensions and electrophoretic mobility. Many of these studies have been invalidated by the inadequacies of their experimental technique or of the relevant theory. For example the models of Smithies (1962), of Raymond & Nakamichi (1964) and of Tombs (1965) are inconsistent, since they lead to the prediction of infinite electrophoretic mobility at zero gel concentration.

Morris (1967) confirmed the Ferguson (1964) logarithmic law for the electrophoresis of well-characterized model proteins in polyacrylamide gels of different compositions, and on the basis of a
comparison of these results with the earlier molecular-sieve chromatographic data of Fawcett & Morris (1966) with gels of the same compositions, proposed a unified theory for molecular-sieve chromatography and electrophoresis in gels. This was embodied in eqn. (1), where \( m' \) is the

\[
m' = aK_{av} + b
\]

(1)

reduced electrophoretic mobility of a solute molecule, i.e. its measured mobility at any gel concentration divided by its mobility \( m_0 \) at zero gel concentration, \( K_{av} \) is the partition coefficient of the solute in molecular-sieve chromatography with a granulated gel of the same composition, \( a \) is a numerical constant approximately equal to 1, and \( b \) is a small intercept constant. Morris (1967) showed that the mobility and partition data for nine test proteins at pH values of 4.3 and 8.8, by using polyacrylamide gels of 18 different compositions, were in fairly good agreement with eqn. (1).

The Ferguson (1964) law was also confirmed for polyacrylamide gels by Hedrick & Smith (1968) and Thorun (1971). Rodbard & Chrambach (1970) have subsequently given an alternative formulation of eqn. (1), which is also valid for non-spherical molecules, and have applied the theory (Rodbard & Chrambach, 1971) to derive values of the molecular radius, free mobility and valence of the solute molecules from measurements of their relative electrophoretic mobilities in polyacrylamide gels.

Morris' (1967) comparison was based on molecular-sieve data obtained by Fawcett & Morris (1966), with polyacrylamide gels prepared by a slightly different method, and using a different electrolyte medium as eluent. The purpose of the present investigation is to re-examine the validity of eqn. (1), by using gels prepared simultaneously from the same batch of chemicals for both chromatography and electrophoresis. Both series of measurements were carried out with the same electrolyte medium and at the same temperature under standardized conditions. The results have been analysed by computer-assisted statistical methods. They support conclusively both eqn. (1) and the Ferguson (1964) logarithmic law.

MATERIALS AND METHODS

Materials. Details of the proteins used together with their sources of supply and Stokes (\( R_\text{s} \)) and geometric mean (\( \bar{R} \)) radii are given in Table 1. The electrophoretic study provided some information on the homogeneity of these samples. Coeruloplasmin contained a small amount (<5%) of a fast-moving impurity. Bovine serum albumin contained a similar amount of the dimer. Ovalbumin and carbonic anhydrase were mixtures of the known genetic variants (ovalbumins A and B, carbonic anhydrases B and C) that could be resolved in some gel systems. The faster-moving ovalbumin component and the major carbonic anhydrase component (>80%) were used in these cases. All the other proteins were electrophoretically homogeneous under the conditions of this investigation. Only bovine serum albumin dimer in addition to the major components could be detected by molecular-sieve chromatography.

Blue Dextran 2000 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; acrylamide monomer and \( N'N' \)-methylene-bisacrylamide were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Two batches of these materials were used throughout the present investigation, and their equivalence was confirmed by comparative electrophoreses.

Tris free base, riboflavin, Naphthalene Black 12B and \( NNN'N' \)-tetramethyl-1,2-diaminoethane were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

Preparation of polyacrylamide gels. Unlike the pre-

<table>
<thead>
<tr>
<th>Table 1. Sources and molecular radii of model proteins</th>
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<tbody>
<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td>Coeruloplasmin, human</td>
</tr>
<tr>
<td>Transferrin, human</td>
</tr>
<tr>
<td>Bovine serum albumin, crystalline</td>
</tr>
<tr>
<td>Haemoglobin, human</td>
</tr>
<tr>
<td>Ovalbumin, 4 x recryst.</td>
</tr>
<tr>
<td>Carbonic anhydrase, human</td>
</tr>
<tr>
<td>Trypsin inhibitor, soya bean, cryst.</td>
</tr>
<tr>
<td>Lactalbumin 2 x recryst.</td>
</tr>
</tbody>
</table>
parations of Morris (1967), the gels were prepared by photochemical polymerization, by using riboflavin as initiator, the temperature being maintained at 30°C during the polymerization. The gels are described by the notation of Hjertén (1962), in which the first numeral \( T \) denotes the total weight of monomers (acrylamide plus \( NN' \)-methylene-bisacrylamide)/100 ml of solution, and the second numeral \( C \) denotes the amount of \( NN' \)-methylene-bisacrylamide expressed as a percentage (w/w) of the total amount of monomer. Thus a 5 x 5 gel contains 4.76 g of acrylamide and 0.25 g of \( NN' \)-methylene-bisacrylamide/100 ml of solution. Twenty different gel compositions, with \( C \) 1, 3, 5, 7 and 9\%, and \( T \) from 5 to 16\%, were used in the present investigation. They cover most of the practically useful range of polyacrylamide gels.

The calculated amounts of the monomers, together with \( NN'NN' \)-tetramethyl-1,2-diaminoethane (2.0 ml), were dissolved in 21 l of water at 30°C, and the solution was degassed in vacuo. A solution of riboflavin (10 mg) in water (50 ml) was mixed with the monomer solution, and 1550 ml of the mixture poured immediately into a Persepx multiple mould, which was provided with a Perspex outer jacket through which water at 30±1°C was circulated. The mould enclosed the solution completely so that there was no free air space. It provided five 43 cm x 12 cm x 0.5 cm slabs of gel after polymerization. The remaining 500 ml of monomer solution was poured into a screw-capped glass jar so as to fill it completely when the lid was in place. The inverted jar was then placed in a thermostat at 30±1°C and illuminated through the transparent bottom. Polymerization was initiated by illumination of the gels in both mould and jar with two 44 cm, 15 W fluorescent lighting tubes, and continued for 20 h at 30°C. The mould was then dismantled, and the gel slabs were removed and washed with water by continuous rocking in individual Perspex troughs for two 1 h periods. The slabs were then extracted with 0.5 m-NaCl for at least 24 h to remove soluble materials. They were washed three times with water to remove NaCl, and equilibrated with several changes of the tris-HCl buffer (pH 8.76, \( I = 0.05 \); see below) until no change in the conductance of the buffer could be detected. The washing-equilibration process occupied 6–7 days, and was essential for good reproducibility.

The polymerized gel in the glass jar was cut into suitably sized pieces and forced through a 120-mesh stainless-steel sieve by using a Perspex piston and cylinder apparatus similar to that described by Hjertén (1962). This granulation process was repeated once. The granulated gel was then graded in a Hamilton (1958) hydraulic particle classifier with de-ionized water at a flow rate up to 150 ml/min to remove fines. The gel was collected by filtration and extracted with 0.5 m-NaCl for 24 h. It was then filtered and washed with water to remove NaCl. It was then repeatedly equilibrated with changes of the tris-HCl buffer (pH 8.76, \( I = 0.05 \)) until no change in the conductance of the supernatant liquid could be detected. The gel suspension in tris-HCl buffer was then ready for packing into the chromatographic column.

Some variants of this procedure were investigated in the preliminary stages of this work. Recrystallization of either acrylamide or \( NN' \)-methylene-bisacrylamide (Loening, 1969) appeared to afford no advantages, and gave delayed polymerization at the lower \( T \) values. Polymerization with persulphate as initiator gave less-reproducible results. Polymerization at 10°C decreased the effective porosity of the gel and delayed polymerization. It appeared that the most reproducible gels were obtained when the polymerization proceeded relatively rapidly, so that carrying out the reaction at 30°C was beneficial in this respect.

Buffer. All chromatography and electrophoresis was carried out with a single buffer containing 302 g of tris free base and 250 ml of 2.00 m-HCl in 10 litres of water. This had pH 8.76, and 2.54 mmho at 20°C. It was nominally \( I = 0.05 \).

Water regains of polyacrylamide gels. These were carried out with portions of the gel slabs that had been extracted with 0.5 m-NaCl and equilibrated with tris-HCl buffer as described above. Pieces of the gel were carefully blotted with Whatman no. 50 filter paper to remove all surface moisture, weighed, and dried in vacuo over silica gel to constant weight. The water regains (g of water/g of dry polyacrylamide) were then calculated from the relation shown in eqn. (2).

\[
W_w = W/(X - 1.033 W)
\]  

(2)

\( W_w \) is the water regain from the tris-HCl buffer, \( W \) is the water content of the gel determined by the difference between the wet and dry weights, \( X \) is the total weight of the wet gel, and 1.033 is a factor which allows for the tris-HCl content of the dry residue.

Molecular-sieve chromatography. The chromatographic column was a 130 cm x 1.6 cm Persepx open-ended tube provided with a cooling jacket through which water at 10±0.2°C was circulated both during packing and chromatography. The gel bed was supported on a porous polythene plate incorporated into a plunger end-piece, which could be locked in position by expansion of a silicone rubber ring (Vere, 1966). A similar end-piece with a porous plate was used at the top of the column so that the gel bed was confined between the porous plates in an exactly defined volume with no free liquid spaces. The upper plunger was modified to incorporate a silicone rubber diaphragm, through which the protein sample could be injected directly into the buffer stream by means of a syringe and needle. Buffer was pumped through the column with an LKB Persepx pulsation-less peristaltic pump at a constant rate of 5.65 ml/h. The volume of the collected effluent was measured at intervals to check the constancy of flow rate. The effluent was monitored continuously at 254 or 280 nm by an LKB Uvicord absorbiositometer. All connexions between components were made with 1 mm internal diameter Teflon capillary tubing, the connexion between the column and the flow cell of the absorbiositometer being made as short as possible.

The column-packing procedure was similar to that recommended by Fischer (1969) for Sephadex gels. The column was extended by addition of a 70 cm-long tube of identical diameter terminating in a funnel at the upper end and provided with a cooling jacket. The column and extension were aligned in a vertical position, the lower plunger end-piece was locked in position and its porous plate covered with buffer. A thick suspension of the granulated polyacrylamide gel at 10°C was poured into the previously cooled column and extension so as to fill...
both. The effluent from the bottom of the column was led upwards through a Teflon capillary to terminate above the column. Packing was allowed to proceed with free liquid flow with an effective hydrostatic head of about 40 cm of water. Buffer flow through the column was continued for 18–20 h, until no further contraction of the gel bed could be detected. The extension and about 3 cm of the gel bed in the column were then removed and the upper end-piece was inserted and connected to the pump. The column was run for at least 24 h at 10°C before use. Any contraction of the gel bed could then be accommodated by adjustment of the upper plunger.

Most gel columns could be operated by downward flow as described. However, the $T = 5$ gel columns were best operated by upward flow because of the resistance caused by the compressibility of the soft gel particles. These columns were packed by downward flow with a somewhat thinner gel suspension, inverted, and upward buffer flow was resumed until the gel bed was uniformly distributed between the porous discs. Samples were injected at the bottom and the effluent was monitored at the top of columns operated in this way.

Retention volumes were measured from the point of injection marked on the recorder chart to the maximum of the solute elution zone. A 0.1 ml portion of a 3–5% (w/v) solution of the individual proteins was injected in most cases. The column void volume $V_0$ was determined from the retention volume of Blue Dextran 2000, and the total bed volume $V_t$ measured volumetrically with water as the volume between the two porous discs. Then if $V_A$ is the retention volume of solute A, and $K_{nA}$, is its partition coefficient:

$$K_{nA} = (V_A - V_0)/(V_t - V_0)$$

At least two determinations of $K_{nv}$ were carried out in all cases. The difference between replicates was always less than 3%. Because of the high density of the column packing, the $K_{nA}$ values tended to be somewhat lower than those obtained in dilute solution.

**Gel electrophoresis.** Electrophoresis was carried out on a horizontal apparatus with provision for cooling the 40 cm x 12 cm x 0.6 cm gel slab on both faces. Water at 10 ± 0.2°C was circulated through the two glass cooling plates during the experiment. The gel slab was separated from the upper cooling plate by a terylene (Melinex) film (0.15 mm thick) to permit easy removal of the plate. Portions (5 cm long) of the gel protruded from the cooling plates at either end to make contact with the electrolyte. These gel contacts were supported on inclined perforated Perspex plates and were immersed directly in the electrolyte. Their upper surfaces were covered with moist filter paper and polythene film to minimize evaporation. The anode compartment contained 1.75 litres and the cathode compartment 3.2 litres of the tris–HCl buffer. A multiple labyrinth system ensured that no pH or conductance change occurring at the electrodes could reach the vicinity of the gel. Platinum electrodes extending across the entire width of the electrode compartments supplied a uniform field. The potential gradient was provided by a Chadno 1200V, 0.3A power supply operated in the constant-current mode. The electric potential at two points 30 cm apart was measured at intervals by means of probes and a calibrated precision voltmeter. No variation in potential gradient across the gel was evident during 24 h operation at up to 50 W power dissipation.

The gel slab, after removal of excess of moisture by blotting, was transferred to the lower cooled plate of the electrophoresis apparatus and allowed to attain the working temperature. Ten 5 mm-wide slits were cut vertically in the gel 10 cm from the cathodic end of the cooled section by means of a multiple cutter made from razor blades. The test proteins (usually 15 µl of a 0.1% solution in a 1:3 dilution of the tris–HCl buffer) were applied on 5 mm x 4 mm pieces of Schleicher and Schull no. 2043b filter paper, and inserted into the slits in the gel. Duplicate applications of bovine serum albumin were made at both outer positions to provide a check on the long-term homogeneity of the electric field in the transverse direction. The gel cutter was then carefully removed, the surface of the gel covered with the terylene film, and the upper cooling plate placed in position. The gel ends were then covered and the potential was applied. Operating conditions were chosen to ensure that the slowest-moving protein (usually carbonic anhydrase) migrated at least 45 mm during 24 h. The potential gradient was 3, 4, 5 or 6 V/cm according to the conductance of the gel slab, and the current 0.08–0.12 A. Measurements of the operating temperature within the gel during electrophoresis showed that it never exceeded 11°C, and that it remained constant after the first 20 min of operation. Linearity of solute migration both with applied potential gradient and with time of migration was established in preliminary experiments.

Electrophoresis was continued for 24 h. The upper cooling plate and the terylene film were then removed, and the gel slab was trimmed and transferred to a plastic developing tray. The paper inserts were removed and the gel was stained with 0.2% (w/v) Naphthalene Black 12B inaq. 7% (v/v) acetic acid at an initial temperature of 100°C. Staining was continued for 18–24 h, and the slab then destained electrolytically in the apparatus of Ferris, Easterling & Budd (1963), withaq. 5% (v/v) acetic acid as electrolyte. Substitution of a nylon net for the filter paper recommended by these authors gave better support for the gel slab, and better access for the electrolyte. The distances from the starting points to the migrated zone maxima were measured directly on the gel with illumination by transmitted light. Replicate measurements could be made to better than 1 mm. Since the haemoglobin zone was visible on the unstained gel, the position of its zone maximum could be measured before and after staining, and provided a check on the dimensional stability of the gel slab through the staining and destaining processes. The gel was kept wet during handling and measurement to obviate shrinkage.

**Electrophoretic mobilities are expressed in 10⁻³ cm²/s per V. They are formally negative. At least three determinations were carried out with each protein at each gel composition and the recorded values are means.**

**RESULTS**

**Water regains.** Preliminary experiments had shown that the water regains of gels with low degrees of cross-linking (X 1 and X 3) were markedly dependent on the salt concentration of the solution.
It is clear that at the lower degrees of cross-linking, the variations of the measured water regains with the total polyelectrolyte concentration, $T$, and with the degree of cross-linking, $C$, is shown in Fig. 1.

The gel with the tris buffer, drying and correlation of the degree of cross-linking, $C$, is described in the Materials and Methods section and involves equilibration with the tris buffer. It was therefore necessary to work out the actual salt content of the water regain for the amount of tris hydrochloride present in the solution.

Table 2. Electrophoretic mobilities of eight model proteins in 20 different gel media

Electrophoretic mobilities were measured at pH 8.76, $T$ 0.05. Protein symbols are identified in Table 1.

<table>
<thead>
<tr>
<th>Nominal</th>
<th>$10^4 \times$ Mobilities (cm$^2$/s per V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Coe</td>
<td>5.16</td>
</tr>
<tr>
<td>Trans</td>
<td>3.00</td>
</tr>
<tr>
<td>BSA</td>
<td>6.69</td>
</tr>
<tr>
<td>Hb</td>
<td>2.89</td>
</tr>
<tr>
<td>Ova</td>
<td>6.36</td>
</tr>
<tr>
<td>CA</td>
<td>1.97</td>
</tr>
<tr>
<td>TI</td>
<td>6.75</td>
</tr>
<tr>
<td>Lac</td>
<td>4.25</td>
</tr>
</tbody>
</table>
error in any correlation with other physical properties.

**Mobilities.** The mobilities of the eight model proteins in the 20 different gel media are given in Table 2. The standard deviation \( \sigma \) of these values from their means for 428 values and 306 degrees of freedom is 0.05. Thus even for the lowest recorded mobility, 0.45, the confidence range of \( \pm 2 \sigma \) will correspond to an error of \( \pm 10\% \) in the absolute mobility, whereas in the majority of cases the error will be \( \pm 2-5\% \).

**Molecular-sieve chromatography.** The \( K_{av} \) values for the eight model proteins in the 20 gel media are given in Table 3. The reproducibility of the retention volumes suggests that, in most cases with \( K_{av} > 0.1 \), the errors will be less than in the mobility determinations. However, \( K_{av} \) values less than 0.05 are subject to considerable uncertainty as they are calculated with eqn. (3) from the difference of two large numbers of nearly equal magnitude. These \( K_{av} \) values, although included in Table 3 for comparison, have therefore not been used in the correlation with mobility data.

**DATA-PROCESSING**

Computations were carried out on an ICL series 1900 digital computer with programs compiled by Mr Ashley Hyams of this College.

The sets of mobility data for any one degree of cross-linking were fitted to a relation of the type (Ferguson, 1964; Morris, 1967) shown in eqn. (4)

\[
\ln m = \ln m_0 - K_T T
\]  

by a least-squares procedure. In eqn. (4) \( m \) is the mobility of the solute at the actual gel concentration \( T \), \( m_0 \) is the extrapolated mobility when \( T = 0 \), and \( K_T \) is a constant characteristic of the solute molecule. The fit of the experimental data to eqn. (4) was very good, the total sums of squares of residuals from the 40 individual equations being 0.874. The computed values of \( m_0 \) and \( K_T \) for the eight proteins at the five cross-linkings are given in Table 4 together with the mean \( m_0 \) values for each protein.

The computed best-fitting mobilities were re-converted into reduced mobilities \( (m') \) by division by the corresponding \( m_0 \) values from Table 4 \((m' = m/m_0)\). These reduced mobilities were then compared directly with the experimental \( K_{av} \) values of Table 3 by a computer program which calculated the regressions of \( m' \) on \( K_{av} \) (eqn. 1), of \( K_{av} \) on \( m' \) (eqn. 5), and the equation of the geometrically best-fitting line (eqn. 6) together with the sums of

\[
m' = aK_{av} + b \tag{1}
\]

\[
K_{av} = cm' + d \tag{5}
\]

\[
(K_{av} - K_{av})/(m' - \bar{m'}) = s \tag{6}
\]
Table 4. Computed values of free solution mobility, \( m_0 \), and retardation coefficient \( K_R \)

Protein symbols are identified in Table 1.

\[
\begin{array}{|c|c|c|c|c|c|c|c|}
\hline
(a) m_0 & \ldots & 1 & 3 & 5 & 7 & 9 & \text{Mean value} \\
\hline
\text{Coe} & 12.64 & 10.52 & 14.43 & 16.09 & 13.48 & 13.43 \pm 2.91 \\
\text{Trans} & 5.75 & 5.19 & 7.36 & 7.32 & 7.08 & 6.54 \pm 1.82 \\
\text{BSA} & 11.98 & 11.55 & 16.13 & 16.08 & 15.26 & 14.20 \pm 2.65 \\
\text{Hb} & 4.79 & 4.20 & 5.66 & 6.42 & 5.09 & 5.23 \pm 1.19 \\
\text{Ova} & 10.09 & 8.00 & 12.12 & 12.24 & 11.90 & 11.39 \pm 2.09 \\
\text{CA} & 3.11 & 2.37 & 3.45 & 3.11 & 2.80 & 2.97 \pm 0.60 \\
\text{TI} & 9.71 & 8.87 & 11.16 & 10.88 & 10.39 & 10.21 \pm 1.34 \\
\text{Lac} & 5.77 & 5.11 & 6.35 & 6.14 & 5.46 & 5.77 \pm 0.66 \\
\hline
\end{array}
\]

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
(b) K_R & \ldots & 1 & 3 & 5 & 7 & 9 \\
\hline
\text{Coe} & 0.273 & 0.295 & 0.340 & 0.318 & 0.283 \\
\text{Trans} & 0.195 & 0.221 & 0.269 & 0.242 & 0.235 \\
\text{BSA} & 0.169 & 0.205 & 0.256 & 0.231 & 0.219 \\
\text{Hb} & 0.158 & 0.179 & 0.224 & 0.213 & 0.182 \\
\text{Ova} & 0.137 & 0.153 & 0.195 & 0.176 & 0.175 \\
\text{CA} & 0.134 & 0.135 & 0.186 & 0.146 & 0.128 \\
\text{TI} & 0.108 & 0.123 & 0.154 & 0.132 & 0.131 \\
\text{Lac} & -0.095 & 0.108 & 0.134 & 0.112 & 0.103 \\
\hline
\end{array}
\]

Table 5. Computed parameters of the regression eqns. (1) and (5) and of the geometrically best-fitting line (6) for the correlation of the reduced mobility, \( m' \), and the partition coefficient, \( K_{av} \).

\( n \) is the number of comparisons, and \( \sum \) is the sum of squares of residuals from the best-fitting line.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
C & \ldots & 1 & 3 & 5 & 7 \\
\hline
a & 0.76 & 0.80 & 0.90 & 0.91 & 0.83 \\
b & 0.216 & 0.194 & 0.087 & 0.068 & 0.063 \\
c & 1.31 & 1.15 & 1.04 & 1.07 & 1.17 \\
d & -0.238 & -0.294 & -0.074 & -0.068 & -0.067 \\
\bar{m} & 0.45 & 0.37 & 0.29 & 0.27 & 0.29 \\
\bar{K}_{av} & 0.31 & 0.22 & 0.23 & 0.22 & 0.28 \\
s & 1.28 & 1.21 & 1.08 & 1.08 & 1.19 \\
\sum & 0.031 & 0.010 & 0.017 & 0.004 & 0.008 \\
n & 33 & 19 & 27 & 22 & 28 \\
\hline
\end{array}
\]

Rodbard & Chrambach (1970) have suggested that the coefficient \( K_R \) of eqn. (4) is related to the molecular radius \( R \) by an expression of the type shown in eqn. (7) for gel models of the type considered by Ogston (1958). In this equation \( p \) and \( q \) are constants. Two alternative values for \( R \) may be considered, the equivalent hydrodynamic or Stokes' radius \( R_s \), and the geometric mean radius \( R \), obtained from eqn. (8), where \( M \) is the molecular weight of the solute, \( \bar{v} \) is the partial specific volume and \( N \) is Avogadro's number. \( R \) is thus calculated on the assumptions that the molecule is not hydrated, and that \( \bar{v} \) is constant for all the proteins studied.

We have tested eqn. (7) by computation of the linear regressions and the geometrically best-fitting lines for \( K_{R}^{1/2} \) and both \( R_s \) and \( R \). The results do not discriminate clearly between the two alternatives, with sums of squares of residuals from the best line, \( K_{R}^{1/2} - R \), of 0.0037, and from the best line \( K_{R}^{1/2} - R_s \) of 0.0052. Plots of the former for the five series are shown in Fig. 3, whereas for the optimum X 5 series:

\[
R = 9.35 (K_R)^{1/2} - 1.87 \quad (9)
\]

\[
R_s = 13.52 (K_R)^{1/2} - 3.17 \quad (10)
\]
Fig. 2. Correlation of reduced mobilities $m'$ with partition coefficients, $K_{sv}$. ——— Geometrically best-fitting lines. Values of $T = 5$ (□), 7 (●), 9 (○), 11 (▲), 13 (△), 15 (■). (a) $C = 1$; (b) $C = 3$; (c) $C = 5$; (d) $C = 7$; (e) $C = 9$. 
Fig. 3. Correlation of the square root of the retardation coefficient ($K_r$) with the geometric mean radius ($R$). O, X1; •, X3; ▲, X5; △, X7.

**DISCUSSION**

*Measurement of $K_{xy}$.* This determination presents no special problems provided that the column dimensions are adequate, and measurement is restricted to the range $0.1 < K_{xy} < 0.8$. Extraction of the granulated gel with 0.5M-sodium chloride had been previously shown to be essential for good flow properties in long columns. It probably operates by removal of non-cross-linked material from the gel matrix. The use of a solvent with $I$ 0.05 minimized absorptive interactions of the protein solutes with the gel matrix and also prevented the excessive swelling of the softer gels, which is evident at very low ionic strengths.

*Measurement of electrophoretic mobilities in polyacrylamide gels.* The development of a theoretical background for gel electrophoresis has been greatly hindered by the virtual non-existence of experimental results of adequate quality. The majority of the published mobilities have been obtained by methods primarily developed for characterization purposes, and which are intrinsically unsuitable for physicochemical measurements. In particular there has usually been no control of the operating temperature, and the use of discontinuous electrolyte systems has resulted in uncertainty about the effective potential gradient. The migration distance has often been inadequate for precise measurement.

It was evident at the outset of this work that the maximum possible precision in mobility measurements would be essential for a rigorous test of eqn. (1), and an extended series of preliminary experiments was undertaken to investigate the main operational factors. The most important decision was to measure absolute rather than relative mobilities. This appeared to be necessary when results obtained over a considerable period of time are to be compared. However, it implies that for meaningful measurements the actual applied potential gradient must be measurable directly, preferably to ±1%, without the uncertainty of theoretical calculations. The solute migration must be linear both in time and in potential gradient. These conditions can be complied with only if a single, constant-composition electrolyte is employed throughout the gel and electrode compartments, so that no moving boundaries separating regions of different ionic composition (and different conductances) can migrate through the gel. Homogeneity of the electric field within the gel is also essential, and we found that this could be obtained only by direct immersion of the ends of the gel in the electrolyte, without the interposition of wicks of any kind. Extensive extraction and equilibration of the gel with the electrolyte also appeared to be essential for good reproducibility, and this is clearly impossible under the conditions of disc electrophoresis in tubes, at least with discontinuous buffer systems.

The relatively high ionic strength and electrolytic conductance of the buffers required for satisfactory molecular-sieve chromatography imposed special problems in this work which are absent in normal gel electrophoresis. In particular adequate temperature control was required at energy dissipations up to 50W. This was achieved by the use of thin (5mm) gel slabs and efficient glass cooling surfaces of large area. Preliminary experiments indicated that individual proteins have different temperature coefficients of mobility, in the range 3–5%/°C. Valid measurements of relative mobility can therefore only be made with strict temperature control to obviate any difference of this kind between solute and marker. Gel electrophoresis appears to be more exacting than electrophoresis in free solution in this respect.

Migration for distances adequate for precise measurement is obviously essential. Replicate measurements were reproducible to ±2%, even with the slowest-moving proteins used in this work.

The importance of a knowledge of the actual polyacrylamide content of the gels for comparative purposes is illustrated by the fact that the real $T$ values of 1% cross-linked gels approximate to half the nominal values. These corrections have been applied throughout the present computations.

*Relation between electrophoretic mobility and gel concentration.* The excellent fit of the experimental mobility results reported here to eqn. (4), together
with the earlier results of Morris (1967), Hedrick & Smith (1968) and Thorun (1971), leave no reasonable doubt that electrophoretic mobilities in polyacrylamide gels are correctly represented by an exponential function of the gel concentration. The limited data of Ferguson (1964) also support this relation for starch gels, and it is probably valid for other gels (such as agarose) with a fibrillar structure (Tsanev, Staynov, Kokileva & Mladenova, 1969).

Alternative relations expressing the mobility as a function of $1/T$ or $1/T^{1/2}$ are unattractive since they lead to the impossible result of infinite mobility at zero gel concentration, and in any case the very limited published experimental results are only an indifferent fit to these relations. An attempt to fit the present mobility results to a $m \propto 1/T^{1/2}$ relation gave sums of squares of residuals several orders of magnitude greater than with eqn. (4). On the other hand eqn. (4) makes the definite prediction that all the $m$ versus $T$ plots for the different cross-linkings should extrapolate to a single $m_0$ value at zero gel concentration, and that this should be equal to the free solution mobility of the solute. The 40 $m_0$ values of Table 4 provide a good test of this prediction. Each set of five values for a single protein is within $\pm 22\%$ of its mean value, and this agreement may be considered reasonably good in view of the length of the extrapolation of a log-log plot.

The second part of the prediction, that $m_0$ is equal to the free solution mobility of the protein under the same conditions, is examined in Table 6, which compares the published free solution mobility data with $m_0$ values corrected for differences in temperature and ionic strength. Correction for differences in operating temperature between the published values and $m_0$ values was made by multiplication of the latter by the ratio of the viscosities of water at the two temperatures (usually 0°C and 10°C), while correction for differences in ionic strength was carried out by multiplication by the square root of the ratio of ionic strengths (usually $1/2^{1/2}$). The agreement shown in Table 6 must be considered satisfactory, in view of the fact that the published values are 10–15 years old, and may in some cases have been obtained with protein preparations of dubious homogeneity (in only one case was a tris buffer used in the earlier determinations). The operating pH values are also not always identical. The present $m_0$ values for bovine serum albumin and ovalbumin are also in good agreement with those obtained earlier in this laboratory (Morris, 1967).

The evidence of Table 6 must be regarded as further support for eqn. (4) and also as independent confirmation of its physical interpretation.

Relation between electrophoretic mobility and molecular radius. Fig. 3 shows that the Rodbard & Chrambah (1970) eqn. (7) relating the coefficient $K_R$ to the mean molecular radius $\bar{R}$ is confirmed by our mobility data with a total sum of squares of residuals from the geometrically best lines of 0.003 for the five series. Nearly half of this total is contributed by the X5 series, whereas bovine serum albumin appears to have an anomalously high $K_R$ value in all the series.

The $K_R$ values appear to reach a maximum in the X5 series, and as the slopes of all the $K_R^{1/2} - \bar{R}$ plots are identical within experimental error, polyacrylamide gels with cross-linkings in the range 5–7% appear to be the most suitable for estimations of $\bar{R}$.

The precision of these determinations under the optimum conditions can be assessed from the regressions of $\bar{R}$ on $K_R^{1/2}$, and of $K_R^{1/2}$ on $\bar{R}$, which were obtained in the calculation of the best-fitting lines. The standard error of estimate, $S_x$, for the regression of $\bar{R}$ on $K_R^{1/2}$ for the X5 series was 0.061, so that the 95% confidence range of $\pm 2S_x$ corresponds to a range of $\pm 0.12$, or to an error of

<table>
<thead>
<tr>
<th>Protein</th>
<th>$10^5 m_0$ (corrected)</th>
<th>$10^5 m$ (published)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coe</td>
<td>6.85</td>
<td>5.26–6.14 (tris buffer)</td>
</tr>
<tr>
<td>Trans</td>
<td>2.93</td>
<td>3.1</td>
</tr>
<tr>
<td>BSA</td>
<td>7.24</td>
<td>6.6</td>
</tr>
<tr>
<td>Hb</td>
<td>2.67</td>
<td>2.5</td>
</tr>
<tr>
<td>Ova</td>
<td>5.6</td>
<td>5.2</td>
</tr>
<tr>
<td>CA</td>
<td>1.52</td>
<td>2.0</td>
</tr>
<tr>
<td>TI</td>
<td>5.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Lac</td>
<td>2.96</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 6. Comparison between published and calculated values for the free solution mobilities of the model proteins

Protein symbols are identified in Table 1.

Reference

Kasper & Deutsch (1963)
Schultze & Schwick (1959)
Longsworth & Jacobsen (1949)
Pauling, Itano, Singer & Wells (1949)
Longsworth (1941)
Peterman & Hakala (1942)
Kunitz (1947)
McMeekin (1952)
±8% for the lowest \( R \) (lactalbumin, 1.57\,nm) used in this work. The standard errors of estimate for the \( X_1, X_3 \) and \( X_7 \) series are very similar in magnitude, but as the numerical values of \( K_R \) are lower in these gels the overall errors may be greater.

This precision, although obtained at the cost of considerable experimental and computational effort, appears to be at least as good as that of other estimates of molecular parameters obtained by gel electrophoresis or chromatography. These methods, however, all share the common disadvantage that the measured variable is proportional to \( \exp(-R^2) \), and is thus only moderately sensitive to changes in \( R \). Gel electrophoresis may be superior to gel chromatography in this respect, owing to the intrinsic limitations on the resolving power of the latter (Giddings, 1967).

Relation between the electrophoretic mobility and the partition coefficient \( K_{av} \). The statistical analysis of the comparison of the 129 pairs of \( m' \) and \( K_{av} \), values given above shows that the relation between these parameters can be represented by a linear equation such as \( \text{eqn. (1)} \) with considerable precision (correlation coefficient 0.97). It is therefore highly probable that this reflects the presence of a common retardation mechanism in gel electrophoresis and gel chromatography which can be measured by either method as suggested by Morris (1967). On the other hand the greater number and precision of the present results allow some of the original conclusions of Morris (1967) to be modified and extended.

In the first place the slope constant \( a \) of eqn. (1) is not equal to 1, as surmised by Morris (1967), but is significantly less, varying from 0.76 for \( X_1 \) gels to a plateau about 0.9 for gels with cross-linking above 5. The possibility that \( a \) need not be equal to 1 was considered by Rodbard & Chrachmb (1970), who later (Rodbard & Chrachmb, 1971), found a value near 0.7 for this constant.

A theoretical treatment of electrophoretic migration in porous media has been given by Giddings & Boyack (1964), who have calculated the overall obstructive factor \( z \) (the fractional reduction in mobility in a tortuous structured medium) for various structural models of the medium. Polyacrylamide gels may be considered as being made up of interlacing fibres, themselves consisting of bundles of the linear polymer chains, held together by the cross-links (Fawcett & Morris, 1966). The spaces between the individual chains may or may not be permeable to water and small ions, depending on the extent of cross-linking, and evident in the swelling behaviour of the polymer. Thus with the weakly cross-linked polymers the electric field may actually pass through the fibres, which nevertheless present obstruction to movement of the macromolecular migrant species. Tight cross-linked polymers, on the other hand, may be impermeable both to macromolecules and small ions. The distinction between the 'tortuous path' and 'barrier' models of electrophoretic migration in porous media was early recognized by Synge (1957).

Giddings & Boyack (1964) concluded that \( z \) would vary from a value near 0.8 for non-swelling fibres to near 0.6 for fibres swelling twofold, a conclusion in agreement with the decrease in \( a \) with decreasing cross-linking and increasing water regain found in the present investigation. Giddings & Boyack (1964) also showed that if the barriers in the medium were permeable to small ions (and hence to the electric field) \( z \) would be increased for non-penetrant molecules such as proteins. Experimental values of \( a \) may thus reflect two opposing influences owing to the retarding and accelerative effects on \( z \). Our results are thus in general agreement with the predictions of Giddings & Boyack (1964) for media of this nature.

It is now evident that the intercept constant \( b \) of eqn. (1) is a function of the water regain of the gel, being largest with the \( X_1 \) and \( X_3 \) gels with high water regains, and tending to a low constant value for the \( X_7 \) and \( X_9 \) gels where the water regain has become constant. In this respect it reflects the marked sensitivity of \( m_0 \) to gel concentration. Weakly cross-linked gels with low real \( T \) values have high \( b \) values and therefore high reduced mobilities. The parameter \( K_R \), which effectively determines the fractionating power of the gel matrix, is not materially affected.

It is difficult to assign a physical interpretation to \( b \). The high values with the soft, weakly cross-linked gels suggest that these may be locally deformed under the influence of the electric field to allow increased rates of electrophoretic relative to diffusional migration, but the subject undoubtedly requires further investigation with gels of different structure.

The present investigations appear to have clarified certain aspects of the relation between electrical and purely diffusional migration in gels. It is to be hoped that they will also assist the application of both methods for the measurement of molecular parameters.

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REFERENCES
