DETERMINATION OF MOLECULAR WEIGHTS AND FRICTIONAL RATIOS
OF PROTEINS IN IMPURE SYSTEMS BY USE OF GEL FILTRATION
AND DENSITY GRADIENT CENTRIFUGATION. APPLICATION TO CRUDE
PREPARATIONS OF SULFITE AND HYDROXYLAMINE REDUCTASES

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(Received June 8th, 1965)

SUMMARY

The behavior of each of a series of proteins during chromatography on columns
of Sephadex G-200 may be correlated with the Stokes radius of the protein, but does
not correlate with molecular weight. Proteins with Stokes radii as high as 107 Å
or molecular weights as high as 1,000,000 may be characterized by the use of such
columns. The Sephadex data are used in a critique of earlier mathematical treatments
of the phenomenon known as "gel filtration".

With a Stokes radius measured by the chromatographic method and a sedimen-
tation coefficient determined by density gradient centrifugation, reasonable estimates
for both the molecular weight and the frictional ratio (f/fø) of a macromolecule are
available. Since both of these methods are applicable to proteins present in mixtures,
valuable information concerning the molecular weights and shapes of proteins may
be obtained in anticipation of the achievement of high degrees of purity. The determi-
nation of the molecular weight and the f/fø for each of several enzymes in unfraction-
ated extracts of Salmonella typhimurium and Neurospora crassa illustrates this
application.

INTRODUCTION

Gel filtration, the passage of solutes through chromatographic columns com-
posed of porous gel particles, provides a particularly attractive method for the sepa-
ration of substances on the basis of relative molecular size. Such chromatography
is based upon partition of solute materials between a phase interior to the gel grains
and a mobile exterior phase in the absence of adsorption phenomena.

Although there is widespread agreement that elution positions upon gel fil-
tration are correlated with general molecular "size", there is no consensus in the
literature as to which size parameter is fundamentally responsible for the observed
elution behavior of macromolecules. Thus, some workers have presented empirical
correlations of elution volume with molecular weight or sedimentation coefficient.
while others have found the data to correlate best with molecular (Stokes) radius or diffusion coefficient\(^{16-18}\). The underlying basis of this uncertainty appears to be that all of the proteins used as calibrating standards possess closely similar frictional ratios and partial specific volumes. For such proteins, a correlation of molecular weight with elution position is virtually indistinguishable from a correlation with molecular radius, as is evident from inspection of the classical Eqns. 1 and 2:

\[
M = \frac{6\pi \eta N s}{(1 - \phi)} \tag{1}
\]

\[
f/\phi = a \left( \frac{3M}{4\pi N} \right)^{1/2} \tag{2}
\]

where \(M\) = molecular weight, \(a\) = Stokes radius, \(s\) = sedimentation coefficient, \(\phi\) = partial specific volume, \(f/\phi\) = frictional ratio, \(\eta\) = viscosity of medium, \(\rho\) = density of medium, and \(N\) = Avogadro's number.

We shall provide evidence in the present communication which strongly indicates that the elution position of a protein upon Sephadex G-200 chromatography is not correlated with molecular weight but instead is a function of the Stokes radius. Whereas such a correlation would disappoint those desiring a rapid estimate of molecular weight, it actually makes gel filtration a considerably more valuable procedure when used in conjunction with a second physical method\(^{18}\). A calibrated gel filtration column may be used for estimation of the Stokes radius of a macromolecule present in impure form, even in unfraccionated cell extracts, provided a specific assay for the macromoleule is available. The sedimentation coefficient of such a macromolecule may be obtained by the method of sucrose density gradient centrifugation as described in greater detail by Martin and Ames\(^{20}\). If the partial specific volume can be reasonably estimated, as is the case with most proteins, one may obtain an accurate determination of both molecular weight and frictional ratio of macromolecules present in impure systems by means of Eqns. 1 and 2.

In a previous communication\(^{21}\), we determined the molecular weights and friction ratios for several aggregation states of urease by combining gel filtration measurements with sedimentation coefficients and partial specific volume data available in the literature. In the present manuscript, we have used combinations of the gel filtration and sucrose density gradient centrifugation techniques to define molecular parameters for the three species of TPN-tyrosylase reductase activity found in crude extracts of Neurospora crassa\(^{22,23}\) and for the sulfite reductase present in extracts of Salmonella typhimurium\(^{24-27}\).

**Experimental Procedures**

**Materials**

Bovine fibrinogen (type II), horse heart cytochrome c (type III), and crystalline yeast alcohol dehydrogenase, bovine serum albumin, bovine hemoglobin, and jackbean urease were obtained from Sigma Chemical Company. Crystalline ferritin (cadmium free) was purchased from Nutritional Biochemical Corporation. Worthington Biochemical Corporation provided a suspension of crystalline catalase. Salmonella bacteriophage P-22, mutant strain H-5, was prepared and stored according to the method of Hartman\(^{28}\). Sephadex G-200 was supplied by Pharmacia Fine Chemicals, Inc.
Unless otherwise stated, a 0.04 M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA was used.

**Gel filtration**

Dry Sephadex G-200 (bead form) was suspended in buffer and allowed to swell at 3° for three weeks, during which time fine particles were decanted. Glass columns of 30 mm diameter were fitted at the bottom with a porous disk, the latter being covered with a thin layer of a mixture of glass beads and sea sand. Gel was poured into these columns to a total length of 420 to 480 mm. Following the initial packing, a disk of Whatman No. 1 filter paper was applied to the top of the gel, and the column material was allowed to settle for four days under flow prior to application of any sample. By means of this procedure, the void volume for a given column, determined as the elution volume of P-22 phage, was found to vary by less than 1 ml over a period of several weeks.

Samples dissolved in buffer were applied in a total sample volume of 1.5 ml. The sample was allowed to settle into the gel, following which the gel surface was washed by addition of 5 ml of buffer. After the latter volume had settled into the gel, an overlying buffer layer was added, a reservoir attached, and elution allowed to proceed. Flow rates varied from 7 to 15 ml per h. No effect of variations in flow rate on the volume in which catalase or ferritin was eluted could be detected. Fractions of 1.0 or 1.6 ml were collected by means of an automatic fraction collector and were assayed for protein and/or enzymatic activity. The following quantities of the various proteins were used: urease, 0.1 mg; catalase, 1–10 mg; alcohol dehydrogenase, 5 mg; serum albumin, fibrinogen, hemoglobin, ferritin, or cytochrome c, 10 mg; *Salmonella typhimurium* 30–42 % ammonium sulfate fraction, 10 mg; *Neurospora crassa* extract, 20 mg; and P-22 phage with an infectivity titer of $3 \times 10^{11}$.

Gel filtration data are presented in terms of $K_d$, the parameter defined by Gelotte and used in the correlations of Ackers and of Porath. A similar parameter, $K_{nv}$, is utilized in the correlation proposed by Laurent and Killander. These parameters are defined as follows:

$$ K_d = \frac{V_e - V_0}{V_t - V_e - V_0} $$

$$ K_{nv} = \frac{V_e - V_0}{V_t - V_0} = \frac{V_t - V_e - V_0}{V_t - V_0} \cdot K_d $$

where $V_e =$ elution volume corresponding to the peak concentration of a solute; $V_0 =$ void volume of the column (elution volume of a substance which does not penetrate the solvent space interior to the gel grains); $V_e =$ volume not accessible to solvent (i.e., volume due to the component molecules of the gel); and $V_t =$ total volume of the gel bed. We have assumed that the void volume corresponds to the elution volume of phage P-22 (ref. 31). The volume occupied by the gel grains themselves, $V_g$, has been estimated from the following relation:

$$ V_g = \frac{V_t}{B \cdot d} $$

where $B =$ bed volume per gram of dry Sephadex G-200 (approx. 30 ml/g), and $d =$ density of dry Sephadex G-200 (1.65 g/ml [ref. 19]). For the columns used in

the present work, \( V_a = 7 \text{ ml} \) and \( k_v = 0.96 \ k_a \). The difference between the parameters \( k_d \) and \( k_v \) is obviously quite small.

**Density gradient centrifugation**

4.5-ml linear gradients of sucrose concentration were prepared in cellulose nitrate tubes from 15.5 and 33% (w/v) sucrose solutions in buffer. The devices employed for preparation of the gradients' and collection of fractions\(^9\) were kindly provided by Dr. T. P. Salo. Gradients were stored at \( 3^\circ \) for from 8 to 36 h prior to use. Linearity of selected sucrose gradients was determined by addition of phenol red to the sucrose solution of lower concentration. Following centrifugation and collection of fractions, the absorbance at 400 nm was found to increase in a linear fashion with fraction number.

In experiments involving centrifugation of *Neurospora crassa* extracts, 1 mg of catalase was added to 0.25 ml of extract, and 0.2 ml of this mixture was layered onto the gradient. Centrifugations were performed for 17.5 h (corrected for acceleration and deceleration time of the centrifuge by the method of Martin and Ames\(^6\)) at 39,000 rev./min at \( 3^\circ \) in the No. 39 SW rotor of the Spinco Model L ultracentrifuge. Fractions ranging from 54 to 76 in number were collected and assayed for catalase, hydroxylamine reductase, and/or sulfite reductase activities.

In experiments with *Salmonella typhimurium* extracts, 1 mg of catalase was added to 0.15 ml of a 30-42% ammonium sulfate fraction of *Salmonella* protein, and 0.1 ml of this mixture was layered onto the gradient. Centrifugation was performed at 39,000 rev./min for 4.2 h (corrected as above\(^8\)). 40 or 47 fractions were collected and assayed for catalase and TPNH-cytochrome c reductase activities.

**Preparation of extracts**

*Neurospora crassa* wild-type strain 5297a and strain 80702 Ra were used in these experiments. Growth of Neurospora mycelia and preparation of extracts were as described previously\(^19,20\).

*Salmonella typhimurium* strain LT-2 was grown with shaking at 37\(^\circ\) on the medium "E" of Vogel and Bonner\(^28\) with MgCl\(_2\) added in place of MgSO\(_4\). L-Djenkolic acid (1.5 \times 10^{-4} \text{ M}) was used as the sole source of sulfur in order to maintain a maximal degree of derepression of sulfite reductase\(^24,25\). Cultures were harvested during the logarithmic phase of growth, and extracts were prepared by sonic disruption of the cell suspensions in 0.5 M potassium phosphate buffer (pH 7.7), followed by a centrifugation at 30,000 \times \text{g} \text{ for 60 min}. The supernatant fluid was subjected to a second centrifugation at 120,000 \times \text{g} \text{ for 90 min}. The resulting supernatant was subjected to fractionation by addition of a saturated ammonium sulfate solution at pH 5.6. The protein precipitating between 30 and 42% saturation with ammonium sulfate was dissolved in 0.04 M sodium phosphate buffer (pH 8.0). The sulfite reductase activity was concentrated in this fashion and purified four-fold as compared to the 30,000 \times \text{g} \text{ supernatant}.

**Assays**

Neurospora sulfite reductase was assayed by measurement of H\(_2\)S production from sulfite using the method of Siegel\(^8\). TPNH- and DPNH-hydroxylamine

\(^{*}\text{T. P. Salo and D. M. Kouns, unpublished data.}\)

RESULTS AND DISCUSSION

Gel filtration of standard proteins

The results of chromatography of P-22 phage and the proteins listed in Table I are presented in Table I. It is seen from a comparison of the data presented in these tables that the order of elution of proteins from the Sephadex G-200 column cor-

<table>
<thead>
<tr>
<th>Species</th>
<th>Stokes radius (Å)</th>
<th>Sedimentation coefficient (× 10^23 sec)</th>
<th>Molecular weight</th>
<th>f/f₀</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>107</td>
<td>7.9</td>
<td>330,000</td>
<td>3.34</td>
<td>44</td>
</tr>
<tr>
<td>Ferritin</td>
<td>79</td>
<td>6.5</td>
<td>1,300,000</td>
<td>1.14</td>
<td>45</td>
</tr>
<tr>
<td>Urease**</td>
<td>61</td>
<td>18.6</td>
<td>483,000</td>
<td>1.19</td>
<td>50, 47</td>
</tr>
<tr>
<td>Catalase</td>
<td>52</td>
<td>11.3</td>
<td>230,000</td>
<td>1.25</td>
<td>48</td>
</tr>
<tr>
<td>Yeast alcohol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>45</td>
<td>7.4</td>
<td>150,000</td>
<td>1.28</td>
<td>49</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>35</td>
<td>4.3</td>
<td>65,000</td>
<td>1.30</td>
<td>50</td>
</tr>
<tr>
<td>Hemoglobin***</td>
<td>24</td>
<td>2.8</td>
<td>32,000</td>
<td>1.14</td>
<td>7, 18, 54</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>17</td>
<td>1.9</td>
<td>12,400</td>
<td>1.09</td>
<td>51</td>
</tr>
</tbody>
</table>

* Ferritin was determined spectrophotometrically as apoferritin–iron complex. Rothem¹⁴ has shown that the average complex contains two molecules of apoferritin and, because of the iron present, possesses a molecular weight 2.8 times that of apoferritin. The Stokes radius of the average ferritin molecule has been calculated from the sedimentation coefficient, partial specific volume, and molecular weight determined for the mean apoferritin–iron complex by Kortman⁴⁶.

** The urease preparation used contained a mixture of monomer, dimer, trimer, and higher polymers, the species being readily distinguishable by gel filtration⁴⁶. Values for the monomer are given.

*** Andrews⁵¹ has shown that, at the concentrations used in the present experiments, hemoglobin is highly dissociated upon gel filtration. The molecular parameters reported here refer to the halfomer of hemoglobin.

I With the exceptions of ferritin and hemoglobin, Stokes radii were calculated from diffusion coefficients reported in the literature by means of the equation

\[ a = kT/6\pi\eta D \]

where \( k \) is the Boltzmann constant; \( T \), absolute temperature; \( \eta \), viscosity of the medium; and \( D \), the diffusion coefficient.
Molecular Parameters of Impure Proteins

Table II

Gel Filtration of Standard Proteins

1.5-ml samples containing each of the substances listed below (in quantities stated in the text) were applied to a 4.50 mm x 30 mm column of Sephadex G-200 equilibrated with 0.04 M sodium phosphate buffer (pH 8.0), containing 0.005 M EDTA. Elution was performed with the same buffer and fractions of 1.5 ml were collected.

<table>
<thead>
<tr>
<th>Species</th>
<th>Elution volume (ml)</th>
<th>$K_4$</th>
<th>Calculated $r^*$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-22 phage</td>
<td>81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>88</td>
<td>0.03</td>
<td>193</td>
</tr>
<tr>
<td>Ferritin</td>
<td>109</td>
<td>0.11</td>
<td>204</td>
</tr>
<tr>
<td>Urease (monomer)</td>
<td>132</td>
<td>0.20</td>
<td>201</td>
</tr>
<tr>
<td>Catalase</td>
<td>148</td>
<td>0.26</td>
<td>199</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>152</td>
<td>0.32</td>
<td>197</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>191</td>
<td>0.43</td>
<td>198</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>224</td>
<td>0.58</td>
<td>198</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>284</td>
<td>0.80</td>
<td>318</td>
</tr>
</tbody>
</table>

$^*$ Values for the parameter $r$ were calculated by the method of Ackers.

relates well with Stokes radius. That elution volume does not depend fundamentally upon molecular weight is apparent from the behavior of ferritin and fibrinogen. With this pair of proteins, the member with the larger molecular weight possesses the smaller molecular radius. As may be seen from the data of Table II, fibrinogen, which has the larger Stokes radius, was eluted first from the column, while ferritin, which has the larger molecular weight, was eluted later. A similar relationship exists between the urease monomer and fibrinogen. Thus, the effective molecular radius, not the molecular weight, is the parameter which determines the behavior of a macromolecule upon gel filtration on Sephadex G-200.

Several mathematical correlations of elution volume with Stokes radius have appeared in the literature. The relations proposed by Porath, Laurent and Killander, and Squire are based upon theories which assume equilibrium partitioning of solute molecules between mobile exterior liquid and microregions of volume within the gel particles, these "penetrable" volumes being greater for smaller molecules than for larger ones. The correlations differ only in their assumptions as to the microscopic structure of penetrable space within the gel particles. The equations proposed by these authors are as follows ($\alpha$ = Stokes radius):

Porath:

$$K_{av}^\alpha = \alpha - \beta \alpha$$  \hspace{1cm} (6)

Laurent and Killander:

$$(-\log K_{av})^{1/2} = \alpha/\beta + a$$  \hspace{1cm} (7)

Squire:

$$(V_a/V_0)^{1/2} = \alpha - \beta \alpha$$  \hspace{1cm} (8)

The significance of the constants $\alpha$ and $\beta$ differs for each equation.

ACKERS\textsuperscript{18} has proposed a theory based upon a mechanism of both steric and frictional hindrance to molecular diffusion within the gel matrix. His relation, based on the RENKIN equation\textsuperscript{40} is:

$$K_d = \left[1 - \left(\frac{a}{r}\right)^2 \right] \left[1 - 2.104 \left(\frac{a}{r}\right) + 2.09 \left(\frac{a}{r}\right)^3 - 0.05 \left(\frac{a}{r}\right)^5 \right]$$

(9)

Unlike the previous equations which possess two arbitrary constants, that of ACKERS possesses a single constant, \(r\), the value of which is dependent upon the type of bed material used. ACKERS\textsuperscript{18} has published an extensive table of theoretical values of \(a/r\) corresponding to observed values of \(K_d\). Thus, if Eqn. 9 were valid, it should be possible to calibrate a gel filtration column by means of two substances, an excluded macromolecule and a single protein of known Stokes radius. A minimum of three substances would be required to evaluate the constants in any of the other correlations.

We have used the data of Table I to test the validity of the various equations. As may be seen from Figs. 1 and 2, satisfactory linear relationships are obtained by means of Eqns. 6 and 7. That data fit the equation of ACKERS is shown by the excellent agreement obtained for the value of the constant \(r\), using different standard proteins as demonstrated in Table II. The data are not accommodated by the Eqn. 8 of SQUIRE\textsuperscript{13}, since the plot of \((V_0/V_d)^{1/2}\) versus Stokes radius is decidedly non-linear. The behavior of cytochrome \(c\) is anomalous in all of the correlations.

When ferritin, alcohol dehydrogenase, catalase, and serum albumin were chromatographed in a mixture, the elution volumes obtained for each protein differed by less than 1 ml from the values found when the proteins were subjected to gel

![Graph](image_url)

**Fig. 1.** Correlation of \(K_d\) with Stokes radius. The gel filtration data of Table II were plotted according to the correlation of PORATH\textsuperscript{10}, Eqn. 6 in the text.

**Fig. 2.** Correlation of \(K_d\) with Stokes radius. The gel filtration data of Table II were plotted according to the correlation of LAURENT and KILLANDER\textsuperscript{18}, Eqn. 7 in the text.

filtration individually. Identical results were obtained when ferritin, catalase, alcohol dehydrogenase, and hemoglobin were chromatographed individually or together with Neurospora extract. Thus it is possible to determine the Stokes radius of a protein even in the presence of contaminating macromolecules.

### Molecular weights and frictional ratios of standard proteins

A molecular weight and $f/f_0$ value for each of the proteins of Table I was calculated by means of Eqns. 1 and 2 using values of the Stokes radii obtained by means of the Ackers correlation with catalase serving as reference molecule ($r = 199$ Å). In making this calculation, a partial specific volume of 0.725 cm$^3$/g was used, as this value was selected by Martin and Ames$^{28}$ as representative of most proteins for their sucrose density centrifugation studies. Sedimentation coefficients were obtained from the literature (Table I); the values used for catalase and alcohol dehydrogenase were determined by density gradient centrifugation$^{28}$.

### Table III

**Calculated Molecular Parameters of Standard Proteins**

Table II shows the radii for the proteins listed below were calculated from the gel filtration data of Table I by the method of Ackers$^{28}$ with catalase ($r = 199$ Å) as reference compound. Molecular weights and frictional ratios were determined by means of Eqns. 1 and 2, using the sedimentation coefficients of Table I, the calculated Stokes radii, and an assumed partial specific volume of 0.725 cm$^3$/g.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stokes radius (Å)</th>
<th>Molecular weight</th>
<th>$f/f_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>110</td>
<td>360 000</td>
<td>2.35</td>
</tr>
<tr>
<td>Urease (monomer)</td>
<td>61</td>
<td>480 000</td>
<td>1.18</td>
</tr>
<tr>
<td>Catalase</td>
<td>52</td>
<td>240 000</td>
<td>1.28</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>46</td>
<td>140 000</td>
<td>1.34</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>35</td>
<td>62 000</td>
<td>1.34</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>24</td>
<td>28 000</td>
<td>1.20</td>
</tr>
<tr>
<td>Ferritin</td>
<td>78</td>
<td>2 100 000</td>
<td>0.92</td>
</tr>
<tr>
<td>Cytochrome $c$</td>
<td>10</td>
<td>8 900</td>
<td>0.75</td>
</tr>
</tbody>
</table>

It may be seen by comparison of the data of Tables I and III that the agreement between the calculated molecular parameters for fibrinogen, urease monomer, catalase, alcohol dehydrogenase, serum albumin, and the hemoglobin half-mer, and the literature values for these parameters is quite good. Such a result is to be expected if the gel filtration data do indeed provide an accurate estimate of Stokes radius, since the partial specific volume of each of these molecules is quite close to the assumed value.

Poor agreement for molecular weight and frictional ratio is obtained for ferritin as a result of the large discrepancy between the assumed $v$ and the actual value of 0.59 cm$^3$/g (ref. 45). The agreement with the actual value for Stokes radius, however, is seen to be quite satisfactory. Cytochrome $c$, which may have been adsorbed to the gel matrix in these experiments due to the basic nature of this protein$^{57}$, behaves anomalously, yielding very low values for Stokes radius, molecular weight and

It should be noted that if the molecular parameters of these two proteins had been unknown, the impossible values of $ff_0$ obtained would have caused the experimenter to suspect anomalous behavior of these molecules.

**Molecular parameters of sulfate and hydroxylamine reductases**

The techniques of gel filtration and density gradient centrifugation were applied to the determination of the molecular weight and frictional ratio for each of the following enzymes: sulfate reductase ($H_2S$:TPNH oxidoreductase, EC 1.8.1.2) of *Neurospora crassa* and *Salmonella typhimurium* and the Peak-B and -C hydroxylamine reductases of *Neurospora*. Properties of these enzymes have been described in other communications from this laboratory. Briefly, *Neurospora* possesses three species of TPNH-hydroxylamine reductase activity readily separable by density gradient centrifugation or gel filtration. The most rapidly sedimenting species, termed Peak A, is identical with sulfate reductase and possesses no DPNH-hydroxylamine reductase activity. The species of intermediate and lowest sedimentation rate, termed Peaks B and C, respectively, will accept either DPNH or TPNH as electron donors. Peak-A and -B hydroxylamine reductases overlap to some extent upon gel filtration, but it is a simple matter to distinguish between the two enzymes by assaying for sulfate reductase and DPNH-hydroxylamine reductase activities. The sulfate reductase of *Salmonella typhimurium* possesses a TPNH-cytochrome c reductase activity, and it was this latter activity which was used in these experiments because of its high $v_{\text{max}}$ value as compared to sulfate reduction.

![Fig. 3](image)

Fig. 3. Gel filtration of *Salmonella* sulfite reductase. A 1.5-ml sample containing the 30–42% ammonium sulfate fraction from *Salmonella typhimurium*, ferritin, and catalase was applied to a 4.7-cm × 30-cm column of Sephadex G-200 equilibrated with 0.04 M sodium phosphate buffer (pH 8.0), containing 0.004 M EDTA. Elution was performed with the same buffer and fractions of 1.0 ml each were collected. The column had previously been calibrated using P-22 phage, ferritin, catalase, alcohol dehydrogenase, and serum albumin. Quantities of the various substances used for chromatography were as described in the text. The $r$ value of ACKERS calculated for the column was 167 A with respect to catalase and to the average value for all of the markers used. Fractions were assayed by methods described in the text for the following activities (units in parentheses): Peak 2, sulfite reductase assayed by means of its TPNH-cytochrome c reductase activity (Δ_A475/min per 0.5 ml); Peak 3, ferritin ($A_{415} / 0.5$); Peak 4, catalase (Δ_A580/min per 50 μl). The elution profile of P-22 phage (Peak 1), determined by A_560 measurements was obtained in a separate experiment.

Gel filtration

The elution patterns resulting from chromatography of a Neurospora or Salmonella extract together with ferritin and catalase as markers are presented in Figs. 3 and 4. The Sephadex G-200 column used had previously been calibrated with P-22 phage, ferritin, catalase, alcohol dehydrogenase, and serum albumin. The r value for this column was 197 Å with respect to both catalase and the average value derived for all of the markers used.

![Graph of elution patterns](image)

Fig. 4. Gel filtration of Neurospora hydroxylamine reductases. A 1.5-ml sample containing Neurospora crassa 3357a extract (10,000 x g supernatant), ferritin, and catalase was applied to a previously calibrated 30 mm x 30 mm Sephadex G-200 column described in Fig. 3. Quantities of the proteins used were as described in the text. Elution was performed with 0.04 M sodium phosphate buffer (pH 8.0), containing 0.005 M EDTA, and fractions of 1.6 ml each were collected. Fractions were assayed for the following activities (units in parentheses): Peak 2, sulfite reductase (μmoles H₂S per 10 min per 0.4 ml x 10); Peak 3, ferritin (A₄₁₅); Peak 4, DPNH-hydroxylamine reductase (Peak A₄₄₀) (ΔA₄₄₀ per 10 min per 0.4 ml); Peak 5, catalase (ΔA₄₄₀ per min per 50 μl); Peak 6, DPNH-hydroxylamine reductase (Peak C₄₈₅) (ΔA₄₄₀ per 10 min per 0.4 ml x 5). The elution profile of P-22 phage (Peak 1), determined by A₂₆₀ (multiplied by 2 in the above figure) measurements in a previous calibration experiment with the column, indicates the magnitude of the void volume. Assays were performed as described in the text.

The sulfite reductase activity from both organisms was eluted earlier than ferritin but later than P-22 phage. The Peak-B hydroxylamine reductase of Neurospora was eluted at a slightly larger volume than was ferritin. The Peak-C activity appeared considerably after catalase. Values for the Stokes radii of the several species, computed using the correlations of ACKERS, LAURENT AND KILLANDER, and PORATE, are tabulated in Table IV. The estimates of Stokes radius obtained by the several methods differ in the most extreme case by only 7%.

Density gradient centrifugation

Sedimentation patterns, obtained by sucrose density gradient centrifugation, showing the movement relative to catalase for each of the three hydroxylamine reductases of Neurospora and for the sulfite reductase of Salmonella are presented elsewhere. Using the values of DÉ DUVE et al., for the density and viscosity of sucrose solutions as functions of concentration and temperature, the numerical integration of Eqn. 10 was performed for a 15.5–33 % gradient of sucrose, in a volume of 4.5 ml in tubes fitting the Spinco 39 SW rotor, at a centrifugation temperature of

### TABLE IV

**Molecular Parameters of Sulfite and Hydroxylamine Reductases**

Sucrose density gradient centrifugation of the 30-45% ammonium sulfate fraction from *Salmonella typhimurium* and of the *Neurospora crassa* extract was performed as described in the text with catalase present as reference compound. Movement relative to catalase is equal to: (total number of fractions minus fraction number of peak activity) divided by (total number of fractions minus fraction number of catalase activity maximum). Sedimentation coefficients (s<sub>20,w</sub>) were determined from this parameter by means of Fig. 5, with partial specific volume assumed to be 0.725 cm<sup>3</sup>/g. Stokes radii were determined from the data of Figs. 3 and 4 using the correlations of Ackers<sup>18</sup>, Laurent and Killander<sup>19</sup>, and Porath<sup>20</sup>, respectively. Molecular weights and frictional ratios were calculated by means of Eqsns. 1 and 2 using the s<sub>20,w</sub> values obtained by density gradient centrifugation, the Stokes radii obtained by gel filtration, and an assumed partial specific volume of 0.725 cm<sup>3</sup>/g. Values obtained using the various correlations of elution volume with Stokes radius upon gel filtration are shown for comparative purposes (Ack = correlation of Ackers<sup>18</sup>, L&K = correlation of Laurent and Killander<sup>19</sup>, and Por = correlation of Porath<sup>20</sup>).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Movement relative to catalase</th>
<th>s&lt;sub&gt;20,w&lt;/sub&gt; (X 10&lt;sup&gt;12&lt;/sup&gt; sec)</th>
<th>Stokes radius (Å)</th>
<th>Mol. weight × 10&lt;sup&gt;-3&lt;/sup&gt;</th>
<th>f/f&lt;sub&gt;0&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ack</td>
<td>L&amp;K</td>
<td>Por</td>
</tr>
<tr>
<td>Salmonella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfite reductase</td>
<td>1.53 (2)</td>
<td>17.7</td>
<td>116</td>
<td>114</td>
<td>111</td>
</tr>
<tr>
<td>Neurospora</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfite reductase</td>
<td>1.09 (7)</td>
<td>13.5</td>
<td>98</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td>NH&lt;sub&gt;2&lt;/sub&gt;OH reductase (Peak B)</td>
<td>0.81 (5)</td>
<td>9.4</td>
<td>74</td>
<td>74</td>
<td>75</td>
</tr>
<tr>
<td>NH&lt;sub&gt;3&lt;/sub&gt;OH reductase (Peak C)</td>
<td>0.37 (5)</td>
<td>3.9</td>
<td>28</td>
<td>27</td>
<td>29</td>
</tr>
</tbody>
</table>

* Number of determinations in parentheses.
The results of this calculation for proteins of $\theta = 0.725$ cm$^3$/g are illustrated in Fig. 5.

$$s_{20, w} = \int_{z_0}^{z} \frac{n_x(\rho_p - \rho_{20, w})}{n_{z_0}(\rho_p - \theta)} \frac{dx}{x}$$  \hspace{1cm} (10)$$

where $s_{20, w}$ = sedimentation coefficient corrected to water at 20$^\circ$; $\omega$ = angular velocity of the rotor in radians per second; $t$ = time of centrifugation in seconds; $x$ = distance from the center of rotation to the center of the solute peak; $z_0$ = distance from the center of rotation to the meniscus (6.1 cm in our experiments); $n_x = \text{viscosity of the medium at the point } x \text{ at the temperature of centrifugation}$; $\rho_x = \text{density of the medium at the point } x \text{ at the temperature of centrifugation}$; $n_{20, w} = \text{viscosity of water at 20$^\circ$}$; $\rho_{20, w} = \text{density of water at 20$^\circ$}$; and $\rho_p = \text{density of solute (assumed to be equal to $\theta^{-1}$ in these experiments)}$.

Fig. 5. Motion of solutes upon centrifugation in a 15.5-33% sucrose gradient. The bold line indicates the distance travelled by a solute molecule of sedimentation coefficient $s_{20, w}$ and partial specific volume 0.725 cm$^3$/g in a 4.5 ml linear gradient of 15.5-33% (w/v) sucrose as a function of $\omega^2 t$, as calculated by numerical integration of Eqn. 11. Temperature of centrifugation is assumed to be 3$^\circ$. Dimensions of the tube in which the gradient resides are those described by Martin and Ames. The volume of sample applied is 0.1 ml. The light line is present merely to indicate the deviation of the predicted motion from linearity. Catalase was centrifuged under the conditions described in the text at 30,000 rev./min for 4.2 and 17.5 h (time corrected for acceleration and deceleration by the method of Martin and Ames). The open circles indicate that the motion of catalase ($s^2_{20, w} = 11.3$ S, see ref. 20) closely follows that predicted by Eqn. 11.

The gradients containing catalase and Neurospora extract were centrifuged for 17.5 h and those containing catalase and Salmonella sulfite reductase for 4.2 h. As is seen from Fig. 5, the observed distance travelled by catalase for each of these time periods is in good agreement with that predicted from the theoretical curve. From the observed distance of travel, the time and speed of centrifugation, and the assumed $\rho_p^{-1}$ of 0.725 cm$^3$/g, one may determine by means of Fig. 5 the value of the

sedimentation coefficient (defined as \( s_{0,725}^{0.725} \) by Martin and Ames\(^{50} \)) for each of the activities examined. These values are presented in Table IV.

**Calculation of molecular parameters**

Using the Stokes radii and sedimentation coefficients obtained by gel filtration and density gradient centrifugation, respectively, together with the assumed \( \bar{v} \) of 0.725, we have calculated molecular weights and frictional ratios for the four activities assayed. It is evident from the results presented in Table IV that the three methods of calculating the Stokes radius from the elution volume yield virtually identical results, and therefore are equally suitable for the calculation of molecular weight and \( f/f_0 \).

The sulfate reductases of Salmonella and Neurospora possess molecular weights of approx. 800000 and 540000, respectively. These results are consistent with the known multi-component nature of these enzymes\(^{52-53} \). The Salmonella sulfate reductase is dependent upon the integrity of six cistrons\(^{54,55} \) and is known to pass electrons from TPNH to sulfate in a minimum of two enzymatic steps\(^{56,57} \). The Neurospora enzyme is dependent upon at least three genetically separable loci\(^{58,59} \), and experiments concerned with cofactor requirements for TPNH oxidation and the production of \( H_2S \) (ref. 23) have suggested a complexity of the electron flow in this protein.

The Neurospora Peak-B hydroxylamine reductase, identical to the nitrate-adaptive enzyme described by Zucker and Nason\(^{40} \), is also quite large, possessing a molecular weight of 290000. The Peak-C hydroxylamine reductase, about which little is known other than its pyridine nucleotide specificity, is quite small relative to the other enzymes, possessing a molecular weight of approx. 45000.

The calculated frictional ratios indicate that the sulfate reductases are quite asymmetric, possessing frictional ratios of approx. 1.8 each, as is the hydroxylamine reductase Peak-B activity, with \( f/f_0 \) equal to 1.7. The Peak-C activity possesses a frictional ratio of approx. 1.2.

It must be emphasized that a large error in the assumed value for \( \bar{v} \) may lead to quite a significant error in the calculated values for sedimentation coefficient, molecular weight, and frictional ratio. Adsorption onto the Sephadex G-200 gel will lead to low values of Stokes radius, molecular weight and \( f/f_0 \). Since the \( f/f_0 \) values calculated for the sulfate reductases and Peak-B hydroxylamine reductases are quite high, the occurrence of adsorption artifacts with these proteins is quite unlikely.

In support of this conclusion, the behavior of the Salmonella and Neurospora enzymes upon chromatography on a Sephadex G-200 column equilibrated with buffer of high ionic strength (0.1 M sodium pyrophosphate, pH 8.3) was identical to that on the standard column. The possibility of error due to the assumption of too low a partial specific volume, as would be the case if one or more of the enzymes contained significant lipid material, has been diminished by the observation that the sulfate reductase from Salmonella will sediment into sucrose solutions having densities at 3° of 1.17 and 1.25 g/cm³ upon centrifugation at a maximal speed for 48 h in the 39 SW rotor. This behavior of the enzyme indicates that its density in hydrated form is at least 1.25 g/cm³, corresponding to a maximal hydrated specific volume of 0.80 cm³/g. The behavior of catalase in these experiments was identical with that of the sulfate reductase, which finding was consistent with the value for catalase of \( \bar{v} = 0.73 \) cm³/g reported earlier\(^{48} \). It is interesting to note that a buoyant density

was assigned to the enzyme β-galactosidase by use of density gradient centrifugation in cesium chloride solutions. There is no obvious reason why similar approaches should not be applicable to the determination of $\bar{v}$ for many enzymes present in polydisperse systems.

**General remarks**

Using an extensive series of globular proteins, Whitaker, Andrews, and Andrews et al. have demonstrated an excellent correlation between elution volume upon chromatography on columns of Sephadex G-75, G-100, and G-200 and the logarithm of molecular weight. The data of Andrews et al. are also readily accommodated by Eqn. 7, the linear transformation of a relationship proposed by Laurent and Killander between elution volume and Stokes radius. Of particular significance is the fact that, as shown in Fig. 6, the plots of $(-\log K_v)^{1/2}$ versus

![Graph showing correlation between Stokes radius and $(-\log K_v)^{1/2}$](image)

Fig. 6. Correlation of the data of Andrews with Stokes radius. $K_v$ values were determined from elution positions for various proteins of known Stokes radius found by Andrews and Andrews et al. upon chromatography on columns of Sephadex G-75, G-100, and G-200. The data are plotted according to the correlation of Laurent and Killander.

Stokes radius obtained with these data intersect at a common point on the abscissa. The value of the abscissa at this point, $\beta$ in Eqn. 7, is approx. 2 Å. According to the model proposed by Laurent and Killander, the constant $\beta$ in Eqn. 7 represents the axial radius of a straight dextran chain and thus should remain constant for all of the Sephadex grades used, since these grades are composed of a common dextran chain and vary only in degree of cross-linkage. The latter authors have indicated that the radius of such a straight polysaccharide chain is approx. 2–3 Å. The constancy of $\beta$ in the correlation of Eqn. 7 and the excellent agreement of the experimentally

*Biophys. Acta, 112 (1966) 346-362*
determined value with that predicted by the model of Laurent and Killander provide strong support for the validity of that model.

Independent of the precise microscopic mechanism of gel filtration, however, is the fact that all of the data in the literature concerning such chromatography may be reconciled by an empirical correlation of elution volume with Stokes radius. The data concerning relative elution positions of fibrinogen, ferritin, and urease monomer presented in this manuscript cannot be reconciled, on the other hand, by a correlation with molecular weight. It is perhaps unfortunate for the present endeavor that there is no unique molecular weight for ferritin complexes. Rothern's states that the average iron-containing complex contains two molecules of apo-ferritin (see legend for Table I). Similarly, the urease preparation which we used in these experiments was polydisperse, composed of monomer and a series of polymers. It must be borne in mind, however, that the molecular weights of even the monomer species of ferritin (apo-ferritin, molecular weight 457,000) and urease (molecular weight 483,000) are considerably greater than that of fibrinogen (see Table I). Thus, the presence of high-molecular-weight components in the ferritin and urease preparations only strengthens the significance of the observation that these proteins are eluted later than the less heavy fibrinogen.

One may note the discrepancy between the data of Fig. 2 and those of Andrews et al. presented in Fig. 6 for Sephadex G-200. The data for elution volumes of proteins presented in the present communication are in rather close agreement with measurements presented by Laurent and Killander, Ackers, Roskes and Thompson, and Wieland et al. K_d values calculated from the data of Andrews et al., in contrast, are considerably higher than the values presented by other authors. A possible explanation for this discrepancy is the extensive time (three months) which Andrews et al. allow for complete hydration of Sephadex G-200. It is possible that the properties of the gel have been changed during this period so as to allow greatly improved equilibration between the solute and the solvent interior to the gel phase, thus increasing the effective "pore size" of the gel. In this context, Ackers has reported that the K_d values obtained following chromatography of proteins on columns of Sephadex G-200 allowed to swell for less than one week are considerably lower than the values obtained in static batch equilibrium experiments with the gel.

From inspection of Eqns. 1 and 2, it is seen that the value of molecular weight is dependent upon the values of four parameters—Stokes radius, sedimentation coefficient, frictional ratio, and partial specific volume—only three of which are truly independent variables. The estimation of molecular weight by either the density gradient centrifugation technique of Martin and Ames or the gel filtration method of Whitaker and Andrews involves experimental determination of but a single parameter and assumptions as to the values of the two other independent parameters. Combination of the two experimental approaches allows a much more reliable estimation of molecular weight since a third parameter, the partial specific volume, varies over quite a narrow range for most proteins. Of particular importance is the fact that when values for the Stokes radius, sedimentation coefficient, and partial specific volume are established, then the fractional ratio may be readily calculated. It must be emphasized, however, as is indicated by the case of ferritin in the present manuscript, that, so long as the partial specific volume for a protein is not established with any degree of certainty, the results obtained by combination

of the gel filtration and centrifugation techniques must be regarded as tentative. A correct estimate of $\bar{v}$ is particularly important since the determination of the sedimentation coefficient by the technique of sucrose density gradient centrifugation relies upon an assumed value for the partial specific volume of the protein under investigation (see Eqn. 10; Fig. 5; and refs. 20, 41). The limited experiments described here and also with the enzyme $\beta$-galactosidase\(^{32}\) suggest that an estimate of $\bar{v}$ is also obtainable, however, by centrifugal studies, even with polydisperse systems.

The utility of a method for the determination of molecular weight and frictional ratio for macromolecules in heterogeneous solutions seems evident. The techniques described in the present work have been applied to determination of the specific degree of aggregation of various species present in a solution of crystalline urease\(^{34}\). The data indicated that the change in frictional ratio of urease upon polymerization into dimer and trimer was quite slight, a result which places serious restrictions on possible models for the mechanism of the aggregation. Similarly, Rogers et al.\(^{39}\) have determined the molecular weight of glutamate dehydrogenase at the low protein concentrations normally used for enzymatic assay by combining gel filtration data with measurements performed on the analytical ultracentrifuge. The method described in the present manuscript might readily be applied also to studies of the structure of enzymes too labile to be purified without structural alteration, of gross conformational changes of proteins, and of the effects of genetic damage upon the structure of specific gene products.

ACKNOWLEDGEMENTS

Publication No. 21 from the Department of Biochemistry, University of Tennessee. These endeavors were supported in part by Contract AEC AT (40-1)3082 from the United States Atomic Energy Commission and by Grant No. GM-17,008 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES


54 A. G. Kirscher and C. Tanford, Biochemistry, 3 (1964) 201.