Biophysical Chemistry 9 (1979) 299–311

SCANNING MOLECULAR SIEVE CHROMATOGRAPHY OF INTERACTING PROTEIN SYSTEMS. II. DETERMINATION OF LARGE ZONE TRANSPORT PARAMETERS BY THE DIFFERENCE PROFILE METHOD AT LOW SOLUTE CONCENTRATION ☆

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Received 29 January 1978

The experimental determination of difference profiles for the study of large zone transport processes by scanning molecular sieve chromatography is described. Using the difference profile method, the progesterone-induced purple glycoprotein of the porcine uterus was found to exist as monomeric units in high ionic environment, with a partition coefficient of 0.269, partition cross-section of 0.488, partition radius of 25 Å and a molecular weight of 33 500 g/mole. The technique was further applied in examining the association–dissociation properties of oxyhemoglobin. In a high tonic environment, the partition coefficient was found to be 0.365 for dimer and the partition cross-section, 0.419; for the tetramer in low ionic strength solution, the partition coefficient was 0.275 and the partition cross-section 0.377, with a dissociation constant of 1.03×10^{-6} mole/l. This new technique should prove applicable in (1) readily locating the centroid positions of transport boundary profiles at the lowest practicable protein concentration limits, (2) demonstrating the characteristic boundary shape and concentration-dependent centroid position for an interacting solute, (3) determining the axial dispersion coefficient characteristic of solute turbulence within the gel matrix, and (4) distinguishing the boundary between low and high ionic strength solvent phases in the gel column.

1. Introduction

The recent introduction of direct ultraviolet scanning of a gel column [1,2] has greatly advanced previous elution techniques by permitting direct analysis of solute profiles at many stages during a single column experiment [3–5].

Continuous direct optical scanning of solute profiles in gel chromatography provides an accurate means of determining the shape of the boundary generated in a transport process by a chemically interacting solute. To date, this method has been applied in a variety of ways, among them (a) small or large zone experiments [6,7], (b) equilibrium saturation technique [2,3,5,6], (c) Brumbaugh-Ackers experiments [1,2,7], a modification of the Hümmel-Dreyer method [13], (d) stacked gel experiments or difference chromatography [3,8,25], (e) active enzyme gel chromatography [9,10], (f) fluorescence gel chromatography, and (g) single photon counting gel chromatography [12].

Large zone or saturation experiments [2,4] afford the most accurate determination of solute partition cross-section (ξ_i) and the partition coefficient (σ_i) [6,14], quantities from which molecular size and interaction parameters are readily derived. Large zone experiments offer the additional advantage of providing information on the time-dependent properties of the solute profiles in molecular sieve chromatography.

In this communication, we describe the experimental determination of difference profiles for the

☆ This work was supported by National Science Foundation Grant PCM 76-04367 and in part by General Research Support, College of Medicine, University of Florida. We are grateful for the assistance provided by the University of Florida computer center.

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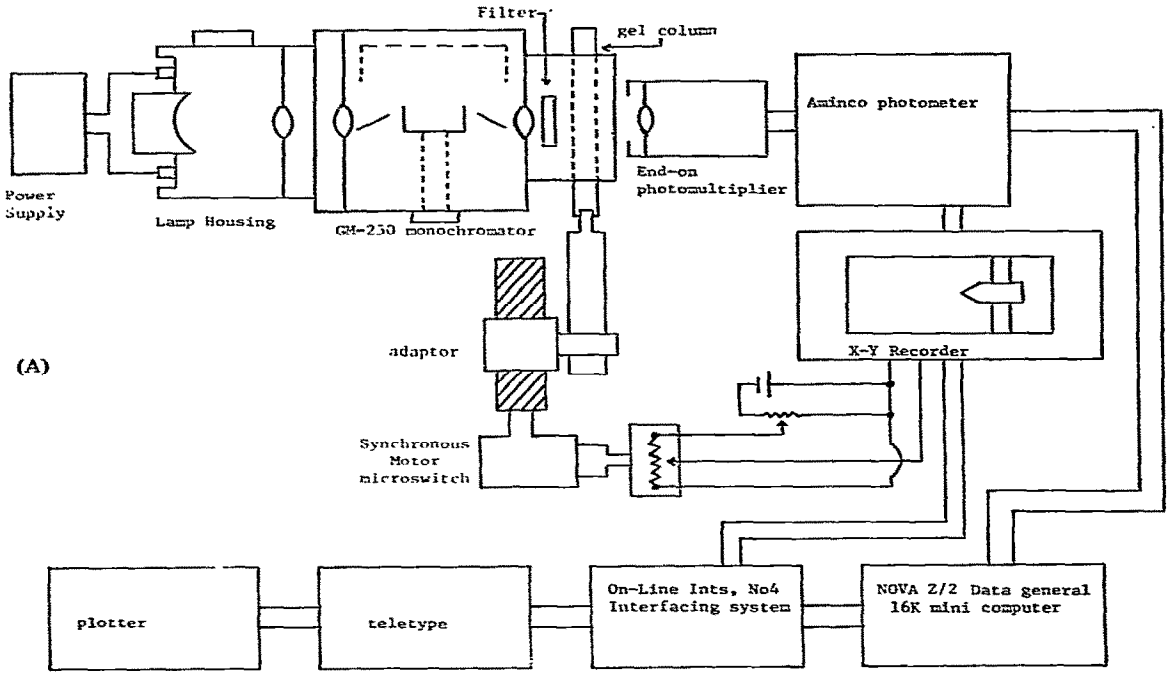
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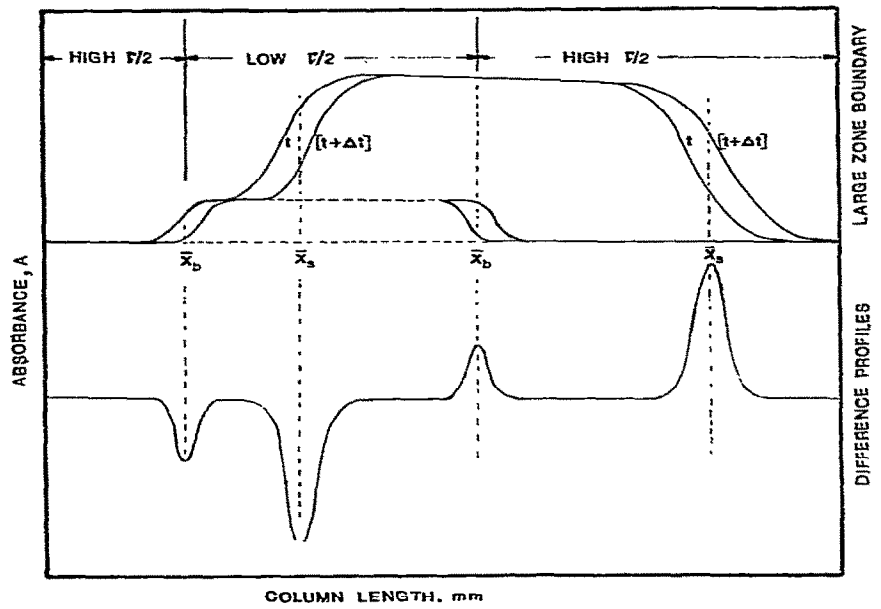
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ABSORBANCE, A
LARGE ZONE BOUNDARY
DIFFERENCE PROFILES
COLUMN LENGTH, mm

(B)

study of large zone transport processes by scanning molecular sieve chromatography, in an attempt to define the lowest concentration limits of this technique. In order to demonstrate the effectiveness of on-line data reduction in following the development of large zone boundary profiles, we examined samples of porcine purple glycoprotein and oxyhemoglobin in solution over a wide concentration range in 2 M NaCl or 0.05 M phosphate buffer, pH 7.4.

2. Experimental procedure

2.1. Materials and methods

All gel chromatography experiments were performed on Sephadex G-100 regular, obtained from Pharmacia. Marker proteins such as cytochrome C, chymotrypsin, sperm whale myoglobin and ovalbumin (5X) were purchased commercially from Schwarz-Mann (Orangeburg, N.Y.). Glycylglycine and progesterone (Lipo-lectin, aqueous suspension, 25 I.U./cc) were obtained from Sigma and Parke Davis and Co. (Detroit, MI.), respectively. Hemoglobin samples were prepared from freshly drawn blood by the method of Williams and Tsay [15]. Haptoglobin 1 : 1, isolated in quantity from plasma of normal H_p type 1 : 1 donors by the method of Cohen-Dix et al. [16], was provided for these experiments by Dr. Ackers' laboratory, Johns Hopkins University.

The progesterone-induced purple glycoprotein from the pig uterus [17,18] as purified by the method described by Chen et al., [19] was found to have extinction coefficients of $E^{1\%}_{280nm} = 10.12$ and $E^{1\%}_{545nm} = 0.75$. Concentration of the hemoglobin (HbA) in solution was determined by extinction coefficients of $E^{280nm}_{mM} = 34.0$ and $(E^{577nm}_{mM}/E^{542nm}_{mM}) = 1.066$.

2.2. Scanning chromatography with Nova Z/4 data acquisition system

The scanning system used in these experiments is essentially the same as that described elsewhere [1,2], with the exception of the monochromator and lamp housing, which are GM250 spectrophotometer model EU-700 (grating monochromator) and EU-701-50 (light source module) housing a Beckman deuterium lamp. All experimental data were obtained at 2200 Å with a single 2240 Å (±202 Å $\frac{1}{2}$-width) band pass optical filter (Baird-Atomic 35-02-0, 25 mm diameter) inserted between the monochromator and column compartment. (See fig. 1).

The data acquisition and processing system consists of a Nova Z/4 minicomputer (Data General Corp., 16 K, 16-bit words of core memory) and a multipurpose interface unit developed by On-line Instrument Systems (Olis Model No. 3600, 140 Featherwood Dr., Athens, Georgia). The data acquisition system is also connected to a Durrum stopped flow apparatus. The Olis multipurpose interface system offers two modes of operation: 1) the gel scanning mode and 2) stopped flow mode (which will not be considered here). Commands to the interface program are entered via an ASR33 teletype (Teletype Corp.). Data may be output to a storage oscilloscope (Tektronix D11), to $X-Y$ plotter (Hewlett-Packard 7005-B), and to punch tape and/or printed hard copy via the teletype.

2.3. System operation

The basic operating routine consists of moving a column through a horizontally-collimated beam of monochromatic light at a constant rate of approximately 1 mm per second for 100 s, during which time the computer acquires and stores a specified number of

Fig. 1A. Schematic diagram of scanning molecular sieve chromatography with Nova Z/4 date acquisition system. The optical scanning window is 1 mm in height. Scans were made at 220 nm. Scan rate is approximately 0.190 cm/sec, which is equivalent to 20 data points/cm.

Fig. 1B. Schematic diagram of a representative large zone boundary, above, and its corresponding difference profiles, below. $\bar{X}_s$ at high ionic strength indicates the centroid position of the difference profile of $F_{(t+\Delta t,x)} - F_{(t,x)}$ from eq. (7). $\bar{X}_b$ is the difference in the index of refraction between buffer systems of low and high ionic strength, from eq. (7). $\bar{X}_s$ at low ionic strength is the centroid position of the difference profile and $\bar{X}_b$ is the index of refraction between buffer systems of high and low ionic strength.

transmittance readings from the Amicon microphotometer. The block of data points taken during a scan (hereafter called a boundary record) may vary from 1 to 1400. Since the amount of core storage is limited, the number of records which may be stored at any given time is an inverse function of the size of a record. Most of the data presented here were obtained using a record length of 300 data points, allowing storage of 20 records (for comparison, a 400-point record length allows 18 record-storage slots) ‡. Upon completion of the data collection (in transmittance), the microprocessor converts each of the data points to absorbance values using a reference intensity obtained with the column out of the light beam just prior to the beginning of each scan. This absorbance record is then stored in any one of the available storage slots.

The Olis program allows storage records to be manipulated in five ways: 1) add one record to another, 2) subtract one record from another, 3) multiply each point in a record by a constant value entered via the teletype and, 4) calculate the natural logarithm, or 5) calculate the inverse of each point in a record.

2.4. Column packing

The quartz column (24 cm × 0.975 cm) was packed under gravity with Sephadex G-100 regular, previously swollen in 0.05 M Na-phosphate buffer, pH 7.4 (μ = 0.1). Glass wool served as the base on which the gel bed was packed. A porous polyethylene disc was used to stabilize the top of the gel column and an LKB varioperpex pump was used to maintain a constant flow rate of approximately 4.4 ml per hour. For these experiments, a single column was used throughout and the area was 0.712 cm^2 (radius, 0.476 cm).

2.5. Large zone profile transport experiments

Data on column partitioning were obtained exclusively by the use of large zone transport experiments. The gel column was equilibrated with 0.05 M Na-phosphate buffer, pH 7.4. In this type of experiment, a sample solution at the desired concentration was added continuously to the column, which is scanned repeatedly at regular intervals until a reproducible baseline is obtained. The criterion for reproducibility of such large zone scans was established to be that the mean difference in absorbance at each point in the column between two consecutive scans not exceed 0.001 absorbance units.

The column is scanned at regular time intervals as the solution/solvent boundary moves through the gel matrix at a constant flow rate of 4.4 ml/hour.

In the past, the rate of movement of the leading and trailing boundary has been determined by locating the centroid position for each scan, then plotting these distance values versus the time at which the scans were made [1–5].

We have found an alternative to this rather cumbersome procedure, which we have designated as the difference profile method of analyzing large zone experiments. In our simplified procedure, the baseline records of successive scans are subtracted from each other, yielding difference profiles. The problem of locating the centroid position of each boundary is then reduced to the simple problem of locating the peak position of these difference profiles.

Partitioning calibration parameters for a given column are obtained by determining the rate of movement of a series of sample macromolecules of known molecular radius [1–4,14]. The partition coefficient is calculated from

$$\sigma = \frac{(dt/d\bar{x})_p - (dt/d\bar{x})_o}{(dt/d\bar{x})_i - (dt/d\bar{x})_o} \qquad (1)$$

where $(dt/d\bar{x})$ is the slope of a plot of t versus $\bar{x}$ for a given sample marker, $(dt/d\bar{x})_o$ is the slope of the void volume marker and $(dt/d\bar{x})_i$, the slope of the internal volume marker. The values of $(dt/d\bar{x})_p$ for the protein sample correspond to $(1/F, dV/dx)$ where F is the flow rate and $(dV/d\bar{x}) = (\alpha + \beta\sigma)$, in which the slope of the plot of volume versus peak position, $\bar{x}$, is used to calculate the partition coefficient σ. α is the void volume per unit column length and cross-sectional area and β is the internal volume fraction of the gel phase per unit column length and cross-sectional area, i.e. $\xi = (\alpha + \beta\sigma)$. Note that the values of $\bar{x}$ were taken directly

‡ This limited storage availability, and the difficulties inherent in paper tape program input, could be alleviated considerably by the addition of a mass storage device such as a magnetic disc.

as the point numbers corresponding to difference profile peaks. Values of t, time, were calculated as the average between the times at which the two scans used to obtain the difference profile were taken, i.e. $t_{(x)} = t_{i-1} + [(t_i - t_{i-1})/2]$. The partition cross-section, ξ, is calculated from the following fundamental expression [4,6]

$$\xi A = F(\mathrm{d}t/\mathrm{d}\bar{x}). \tag{2}$$

The partition cross-section ξ represents the fraction of column cross-sectional area, A, which is accessible to the solute. This quantity ξ is dependent on the position of x within the gel bed. The expression for the weight average partition cross-section of interacting solute, which we denote as $\bar{\xi}_i$ is $\bar{\xi}_i = \Sigma_i(\xi_i C_i/C)$, where $C = \Sigma_i C_i$. For a given concentration C_i, the following expression if used, where erfc is the error function complement [14], in order to relate the partition coefficient σ_i to the molecular radius a_i.

$$a_i = a_0 + b_0 \ \mathrm{erfc}\ \sigma_i. \tag{3}$$

Here a_0 and b_0 are calibration constants of a particle of given radius in a given gel and are determined independently.

2.6. Evaluation of centroid positions ($\bar{x}$) of trailing and leading boundaries by the difference profile methods

In cases where determination of the difference profiles of leading and trailing boundaries requires numerical approximation of the delta function in terms of the time reference frame, i.e. $F_{(t,x)}$, this function may be expressed as [4,6,20]:

$$F_{(t,x)} = \frac{C_0}{\sqrt{4\pi L V t}} \exp\left\{-\frac{[x - (V/\xi)t]^2}{4LVt}\right\}. \tag{4}$$

Letting C_0 represent the plateau concentration, $a = 4LV$, where L is the axial dispersion coefficient and V is the solute distribution volume of the leading and trailing edges of the boundary. The centroid position, $\bar{x} = V/\xi$ and $C' = C_0/\sqrt{4\pi L V}$.

(i) The centroid position of the trailing boundary, $\bar{x}$, after difference profile determination, may be directly evaluated from ‡

$$F_{(t+\Delta t,x)} - F_{(t,x)} = C'\Delta t \int_{-\infty}^{x} \frac{\mathrm{d}}{\mathrm{d}t}\frac{1}{\sqrt{t}} \exp[-(x-\bar{x}t)^2/at]\,\mathrm{d}x \tag{5}$$

Assuming the time interval between scans, Δt, is limited to 5–10 min, then eq. (5) yields

$$F_{(t+\Delta t,x)} - F_{(t,x)} \approx \frac{C'\Delta t}{t^2}\{\bar{x}\exp[-(x-\bar{x}t)^2/at]\}, \tag{6}$$

providing that $\xi_{(x)}$ remains constant within this time interval and noting that $\xi_{(x)}$ varies with the distance coordinate in self-associating systems.

(ii) The centroid positions of leading boundary may be determined from the same equation (5), noting that the distribution curve is quite distinct from that of the trailing boundary. At the centroid position, $\bar{x}$, the distribution function is expressed as

$$F_{(t+\Delta t,x)} - F_{(t,x)} = \frac{C_0\bar{x}\Delta t}{\sqrt{4\pi L\bar{V}t^2}} \exp[-(x-\bar{x})^2/4LVt]. \tag{7}$$

In addition to the partition cross-section, which determines the average velocity coordinate ($\bar{x}/t$) of a solute within the column, it is also possible to evaluate the axial dispersion coefficient L from equation (7) utilizing second moment of distribution curve. Letting the second moment, $\bar{l}^2$, be expressed as

$$\bar{l}^2 = \int_{-\infty}^{\infty} \frac{C_0(x-\bar{x})\bar{x}\Delta t}{\sqrt{4\pi L\bar{V}t^2}} \exp[-(x-\bar{x})^2/4LVt]\,\mathrm{d}x. \tag{8}$$

Eq. (8) can be approximated by the difference profile method, i.e.

$$L = \bar{l}^2\sqrt{t}/2\Delta t V\bar{x}C_0. \tag{9}$$

Typical results for this type of calculation are consistent with our simulation parameters [21,22].

‡ Actual difference profile curve as diagrammed in fig. 1B is:

$$F_{(t+\Delta t,x)} - F_{(t,x)} = C'\Delta t\left[\int_{-\infty}^{x}\frac{1}{2\sqrt{t^3}}\exp[-(x-\bar{x}t)^2/at]\,\mathrm{d}x - \frac{\bar{x}}{\sqrt{t}}\exp[-(x-\bar{x}t)^2/at] + \frac{1}{at^2\sqrt{t}}\int_{-\infty}^{x}(x-\bar{x}t)^2 - \exp[-(x-\bar{x}t)^2/at]\,\mathrm{d}x\right].$$

First and last terms cancel when Δt is small. The middle term represents the distribution curve observed in an actual experiment.

3. Results and discussion

3.1. Calibration of sample protein markers by the difference profile method

The leading and trailing boundary profiles of a representative sample of porcine purple glycoprotein in 2M NaCl, 0.05 M Na-phosphate buffer, pH 7.4, are shown in fig. 2. The slant of the plateau region observed is indicative of some variation in the partition cross-section, ξ, with the x distance coordinate. The plateau concentrations of C_0 values are not always constant, indicating that ξ is dependent on the distance coordinate even though column packing is uniform. In general, both the leading and trailing boundaries should appear symmetrical; however, movement of the solute down the column causes an increased spreading of the boundary due to axial dispersion within the gel matrix [23].

A typical leading and trailing boundary profile from a large zone experiment after application of the difference profile method is shown in fig. 3. This profile is the result of subtracting leading and trailing boundary profiles of two successive scans of the purple glycoprotein sample made at five to ten minute intervals. The boundaries are well-defined, even though there is some boundary scatter at this low concentration of 20 μg/ml, and the centroid positions of the leading and trailing boundaries can be precisely determined by this technique. Note that application of our equation (5) will accurately predict both the boundary profiles and centroid position.

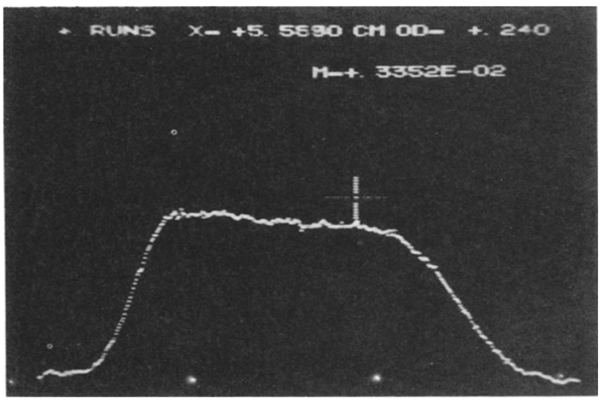


Fig. 2. Typical leading and trailing boundaries of a large zone experiment on Sephadex G-100 regular. A solution of porcine purple glycoprotein was added at a concentration of 50 μg/ml in 0.05 M Na-phosphate buffer, pH 7.4, containing 0.1 M NaCl. The scan was made 65 minutes after starting the leading edge. Y-axis (C'_T) scale is −0.10 A to 0.49 A. X-axis scale is 0 to 10 cm.

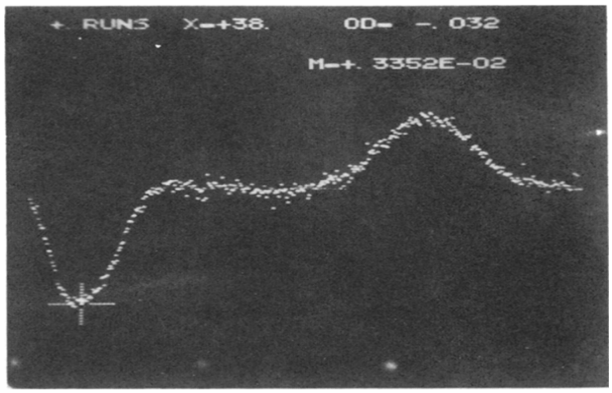


Fig. 3. Typical leading and trailing boundaries of a large zone by the difference profile method on Sephadex G-100 regular. Experimental conditions are identical to fig. 2, with the scan made 65 minutes after starting the leading edge. Porcine purple glycoprotein was added at a concentration of 20 μg/ml. Y-axis scale is −0.175 A to 0.175 A. X-axis scale is 0 to 300 data points. The difference profile was obtained at the cursor position, data point 38 of the trailing boundary.

Typical distance ($\bar{x}$) versus time data for the measurement of leading edges of a large zone by the difference profile method and correlation coefficients for a series of protein markers in solution are given in table 1. This table represents results of partition coefficient calculations and subsequent conversions to erfc $\bar{\sigma}$, which are plotted versus a, the molecular radius. As seen from this table, the partition coefficient and radius of known markers are in quite good agreement with earlier determinations [3,23].

It was noted during the passage of chymotrypsin through the column in a large zone experiment that both the leading and trailing edges were biphasic. The corresponding difference profiles exhibited two peaks, with the minor peak moving at a rate corresponding to that of the inclusion volume marker glycylglycine. We assume that this minor component is the dipeptide cleavage product resulting from an autocatalytically-

Table 1
Correlation coefficients and centroid position ($\bar{x}$) resulting from least square fits of t versus $\bar{x}$ data for a series of macromolecular species of known radii (difference profile from large zone leading boundary on Sephadex G-100 regular)

Species	Conc. (μg/ml)	$(dt/d\bar{x})$(min/pts)	corr. coeff.
haptoglobin 1 : 1 (Hp 1 : 1)	116	0.173	0.99993 [a]
dipeptide	–	0.488	0.99950
cytochrome C	100	0.364	0.99900 [b]
chymotrypsin	100	0.300	0.99991 [b]
ovalbumin	100	0.233	0.99840 [b]

Species	σ	erfc σ	a
cytochrome C	0.606	0.365	18.0
chymotrypsin	0.403	0.593	22.5
ovalbumin	0.187	0.935	27.6

[a] Time versus $\bar{x}$ data and corresponding slope for a solution of haptoglobin 1 : 1 (Hp 1 : 1), 116 μg/ml in 2 M NaCl, 0.05 M Na-phosphate buffer, pH 7.4. $\bar{x}$ (data point): 31, 60, 88, 117, 146, 176, for resp. time (min): 7.5, 12.5, 17.5, 22.5, 27.5, 32.5, gives $(dt/d\bar{x}) = 0.173$, corr. coeff. = 0.99993. $\alpha = 0.327$, $\beta = 0.596$ and $\xi = (0.327 + 0.596\,\sigma)$. $\xi = (F/A)(dt/d\bar{x}) = 1.893$ (pts min^{-1}) $(dt/d\bar{x})$(min pts^{-1}), where scanning rate = 0.204 cm s^{-1}, scanning distance = 16.32 cm. Number of points/unit distance in the scanner = 1.893 pts/cm.

[b] Molecular radius calculated from $(dt/d\bar{x})$ data in this table using $a_i = a_0 + b_0\,\mathrm{erfc}\,\sigma_i$ rather than $a_i = a_0 + b_0\,\mathrm{erfc}^{-1}\sigma_i$ [14].

produced component of chymotrypsinogen. In our subsequent calibrations this dipeptide component has been used as an inclusion volume marker.

3.2. Porcine purple glycoprotein

The application of the difference profile method in considering the leading boundary of typical large zone experiments on porcine purple glycoprotein is shown in figs. 4 and 5.

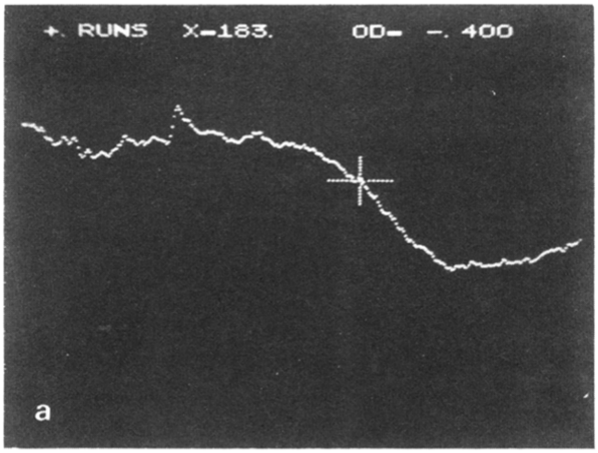
Figs. 4a and 4b show two successive scans made at 220 nm after a ten-minute interval, plotting the leading boundary of a sample in 2 M NaCl and Na-phosphate buffer, pH 7.4 at a concentration of 60 μg/ml. The cursor positions at the 183rd data point after 45 minutes and 55 minutes of flow time in successive scans are subtracted and, after base line correction, will yield the boundary shown in fig. 4c by the difference profile method.

The leading boundary profiles of two successive scans at 45 and 55 min of a large zone of purple glycoprotein at 12 μg/ml, after base line correction, are shown in figs. 5a and 5b. Note that at the lower concentration, more boundary scatter is evident in both plateau and baseline regions. Subtraction of the profile in 5b from that of 4a yields the difference boundary profile shown in fig. 6d.

In applying the difference profile method to porcine purple glycoprotein and oxyhemoglobin in solution, our purposes were three-fold: (1) to readily locate the centroid positions of the boundary profiles at the lowest practicable protein concentration limits, (2) to demonstrate the characteristic boundary shape and concentration-dependent centroid position for an interacting solute and (3) to determine the axial dispersion coefficient, characteristic of solute turbulence within the gel matrix.

The series of photographs in fig. 5 show difference profiles of scans taken at 45 min and 55 min after formation of the leading boundary, when the concentration is varied from 60 μg/ml to 2.4 μg/ml. The increasing degree of boundary scatter as the concentration was lowered is evident when comparing the entire series of scans. We found in our experiments however, that a recognizable centroid position could be determined at concentrations as low as 1.5 μg/ml by the difference profile technique.

The accurate determination of the centroid positions and solute turbulence of dynamic profiles such as these large zone experiments is essential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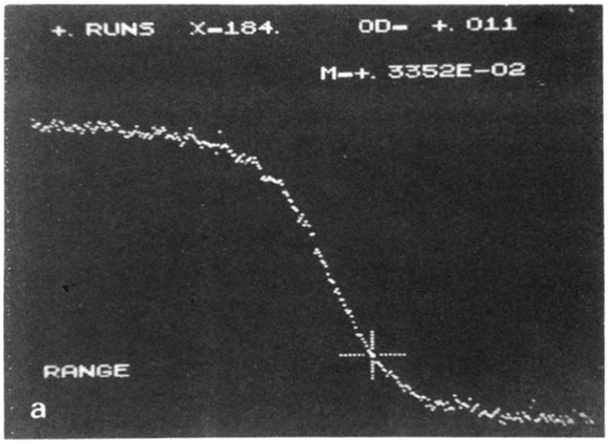
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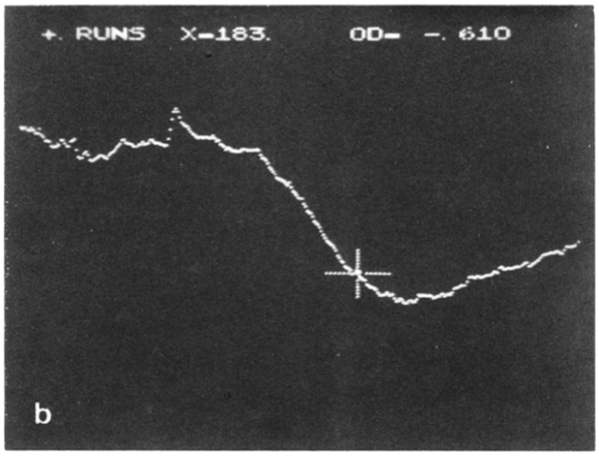
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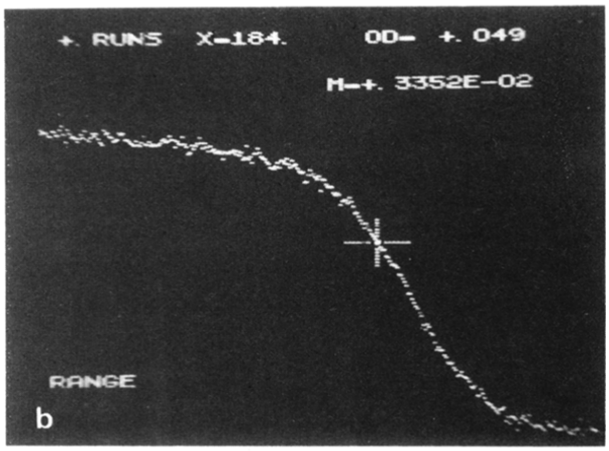
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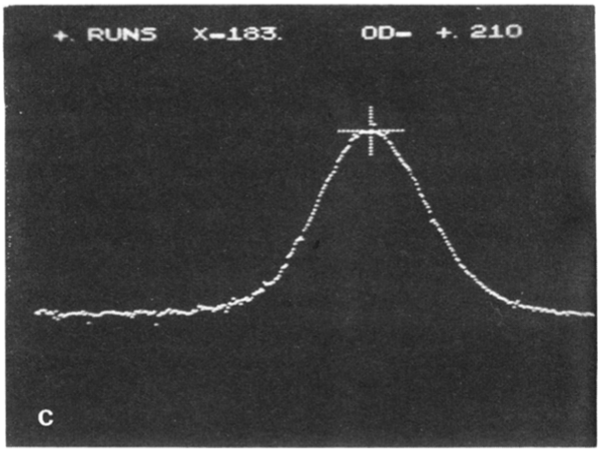
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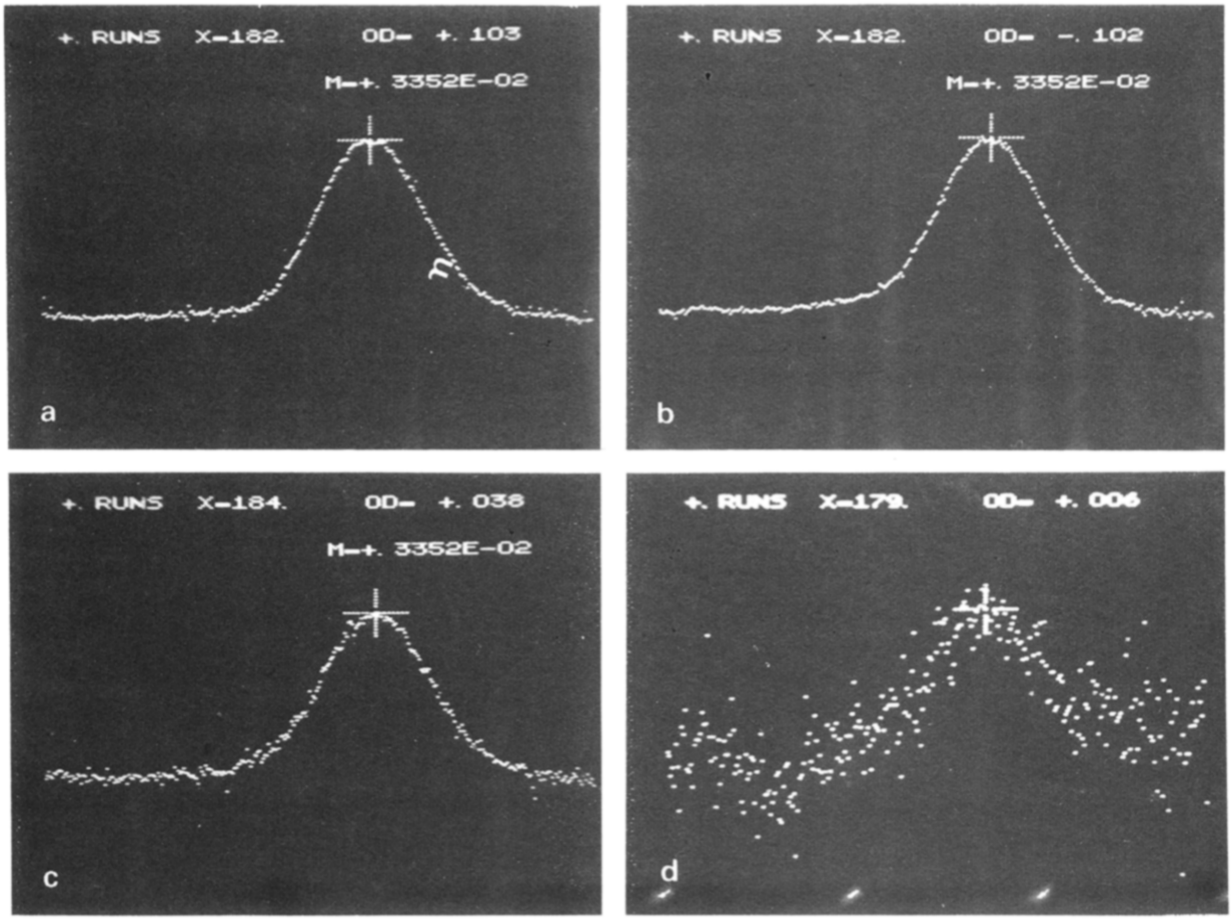
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Table 3
Slopes ($dt/d\bar{x}$) and partition coefficient at varying concentrations of porcine purple glycoprotein on Sephadex G-100 regular. Partition coefficients were calculated using table 1. Partition cross-sections were evaluated from eq. (2).

conc (μg/ml)	$dt/d\bar{x}$ (min/pts)	corr. coeff.	σ	ξ
2 M NaCl, 0.05 M Na-phosphate buffer, pH 7.4				
60	0.256	0.99996	0.263	0.485
30 (leading edge)	0.256	0.99990	0.263	0.485
30 (trailing edge)	0.259	0.99995	0.273	0.490
12	0.254	0.99993	0.257	0.487
2.4	0.259	0.99987	0.273	0.490
0.1 M NaCl, 0.05 M Na-phosphate buffer, pH 7.4				
50	0.259	0.99996	0.273	0.490
10	0.260	0.99991	0.276	0.492
0.1 M NaCl, 0.05 M Na-phosphate buffer, pH 7.4 *				
50	0.257	0.99991	0.276	0.487
10	0.259	0.99998	0.273	0.490

Average partition coefficient, $\sigma = 0.269 \pm 0.007$; average partition cross-section, $\xi = 0.488 \pm 0.004$; the partition radius, $a = 25$ Å.
* In the presence of 10^{-5} M Progesterone, Lipo-lectin, Parkee Davis & Company, Detroit, Michigan.

The axial dispersion coefficient, characteristic of solute turbulence within the gel matrix, was determined to be 3.94×10^{-4} cm^2/s for porcine purple glycoprotein. The molecular weight of this protein was found to be 33 500 ± 500 g/mole, with a partition radius of 25 Å. The conditions for these experiments, considering purple glycoprotein in a high ionic strength solution where the protein retains the monomeric form, were purposely selected so that we could consider a sample system under ideal or theta conditions in working out possible applications of the difference profile technique.

3.3. Dissociation–association properties of hemoglobin

The difference profile method was also applied to samples of human oxyhemoglobin and it was ascertained by the new technique that predominantly dimer($\alpha\beta$)–tetramer($\alpha_2\beta_2$) association was present over the concentration range studied, in both high and low ionic environments, and an accurate value was determined for the dimer–tetramer equilibrium constant, with a dissociation constant of 1.03×10^{-6} mole/l.

As shown in table 4, in 2 M NaCl, 0.05 M $NaPO_4$ buffer over the concentration range we examined, the partition coefficient of 0.365 and partition cross-section of 0.419 indicated the presence of 95% dimeric or dissociated oxyhemoglobin. At low ionic strength, the partition coefficient and partition cross-section of tetramer were found to be 0.275 and 0.377, respectively, which would indicate a theoretical partition coefficient of 0.340 for the dimeric species.

The large zone experimental difference profiles of oxyhemoglobin in solution between two solvent phases (as diagrammed in fig. 1B) are shown in figs. 7 and 8. Figs. 7a, b, c show the leading boundaries of the difference profiles in 2 M NaCl, 0.05 M Na-PO_4 buffer, pH 7.4, indicating with the cursor the movement of the centroid position as a function of column length as the hemoglobin sample moves down the column.

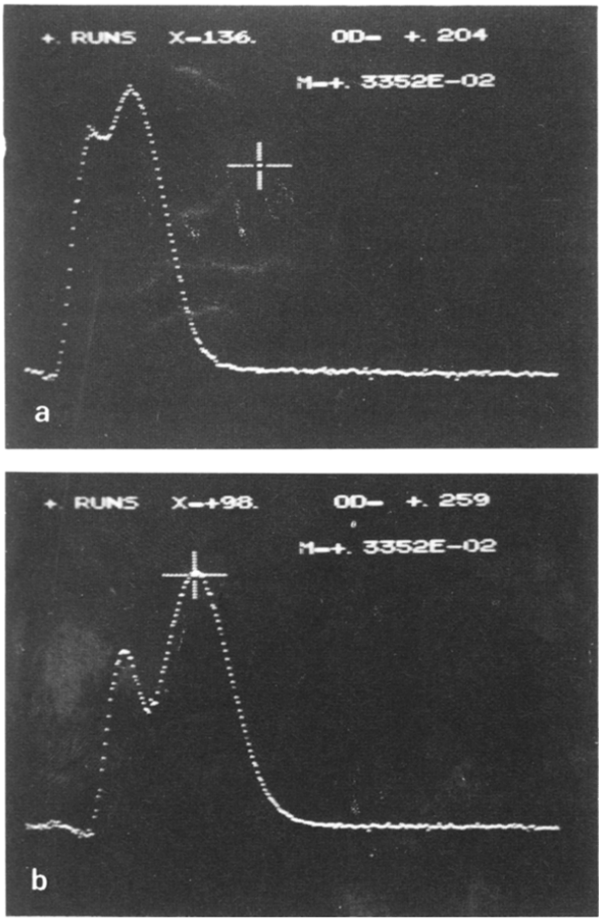
In figs. 8a, b, c, showing the trailing boundary of the difference profile, the location of the cursor again indicates the centroid position of the hemoglobin sample in low ionic strength buffer as it moves down the column.

In both figures, the slower moving components, which appear as either a peak or dip in the profile behind the centroid position, represent the difference in the index of refraction of the two solvent phases. Thus the slow-moving peak in fig. 7c represents the

Table 4
Slopes ($dt/d\bar{x}$) and partition coefficients at varying concentrations of human oxyhemoglobin on Sephadex G-100 regular. Partition coefficients were calculated using table 1 and the partition cross-sections evaluated from eq. (2)

conc (μg/ml)	$dt/d\bar{x}$(min/pts)	corr. coeff.	σ	ξ	erfc σ
Oxyhemoglobin, 2 M NaCl, 0.05 M Na-phosphate buffer, pH 7.4 *					
108	0.2660	0.99986	0.364	0.419	0.607
81	0.2670	0.99984	0.357	0.420	0.604
54	0.2674	0.99977	0.368	0.421	0.603
22	0.2651	0.99966	0.360	0.417	0.610
Oxyhemoglobin, 0.05 M Na-phosphate buffer, pH 7.4					
108	0.2393	0.99987	0.275	0.377	0.698
81	0.2455	0.99962	0.295	0.387	0.676
54	0.2458	0.99986	0.296	0.388	0.675
22	0.259	0.99990	0.340	0.408	0.630

* Hemoglobin solutions were prepared in the presence of 10^{-4} M Na_2EDTA, with no further addition of Na_2 EDTA after the sample was dialyzed in a high ionic strength environment.
$\sigma_{dimer} = 0.3750$ and $\sigma_{tetramer} = 0.2430$ in 0.1 M Tris/HCl, 0.1 M NaCl, 1 mM Na_2 EDTA, pH 7.4 at 21.5°C [24].



interface between high and low ionic strength buffer solutions. In contrast, the dip shown in fig. 8c represents the interface between low and high ionic strength buffer systems. Hence, these difference profiles show that the degree of solute-solvent interaction varies in the leading and trailing edge of the reaction boundary.

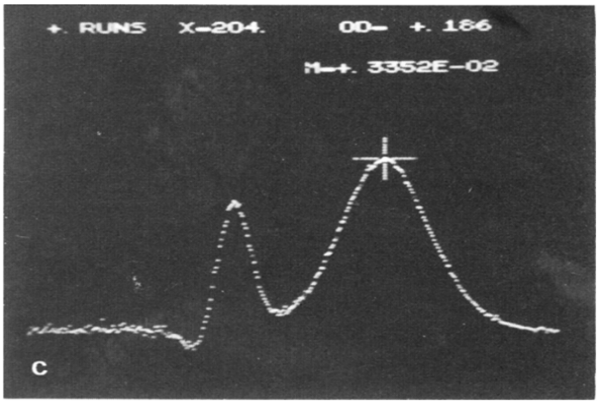


Fig. 7. Difference profiles of the leading boundaries for human oxyhemoglobin in high ionic strength buffer. All profiles represent the difference between scans taken at 108 μg/ml, 20, 30 and 60 minutes after formation of the large zone boundaries. Oxyhemoglobin in solution in 2 M NaCl, 0.05 M Na-PO_4 buffer, pH 7.4. a) $-0.05 \leqslant$ O.D. $\leqslant +0.35$; b) $-0.05 \leqslant$ O.D. $\leqslant +0.35$; c) $-0.05 \leqslant$ O.D. $\leqslant +0.25$.

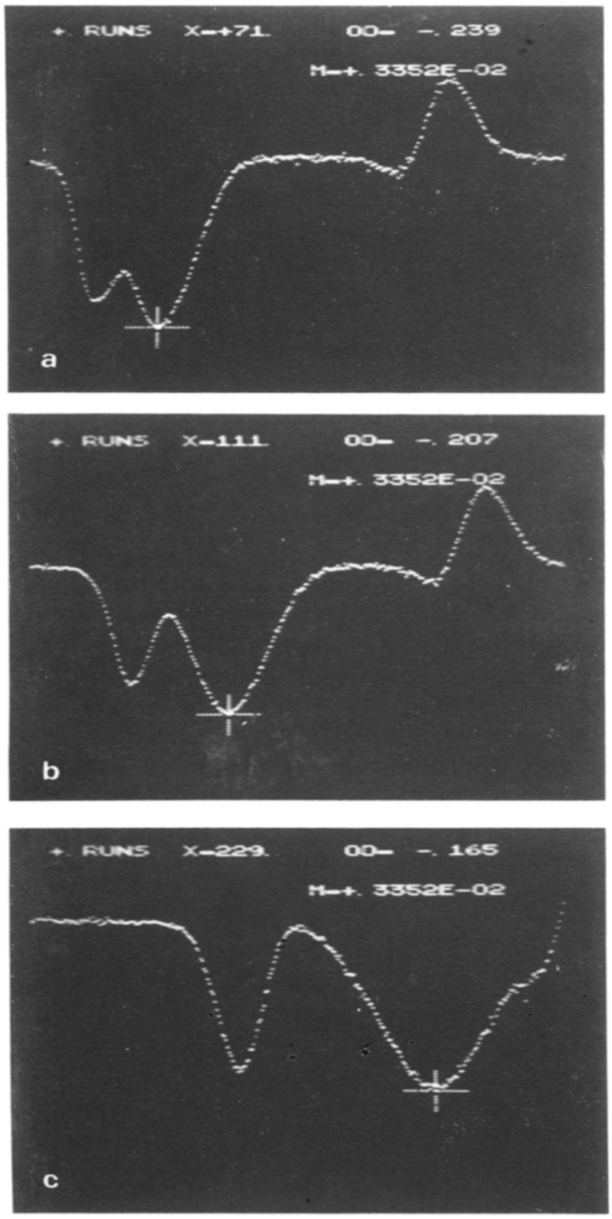


Fig. 8. Difference profiles of the trailing boundaries for human oxyhemoglobin in low ionic strength buffer. All profiles represent differences between scans taken at 108 μg/ml, 20, 30 and 60 minutes after formation of the large zone boundaries. Oxyhemoglobin samples are in 0.05 M $Na\text{-}PO_4$ buffer, pH 7.4. a) $-0.30 \leq$ O.D. $\leq +0.15$; b) $-0.30 \leq$ O.D. $\leq +0.15$; c) $-0.30 \leq$ O.D. $\leq +0.15$.

For oxyhemoglobin, fig. 7 and table 4 show that the partition cross-section determined from the leading boundary remains relatively constant in the 2 M NaCl ionic environment. At low ionic strength, the partition cross-section determined from the centroid position varies as a function of concentration, as seen in fig. 8 and table 4.

In these experiments, we have found the difference boundary profile technique was both effective and accurate in meeting the purposes we had set forth. It should be noted, however, that the accuracy of all subsequent calculations rests on the accurate determination of the centroid position. Care must be taken in making successive scans of the boundary profile of a sample over a time interval of five to ten minutes. Over a longer period, the determination of the true centroid position will be affected, as we have pointed out in our equation (7). With this word of caution, however, we feel the technique should prove extremely useful in its applicability to large zone transport experiments using scanning molecular sieve chromatography, and may be extended for use with the equilibrium saturation technique, Brumbaugh-Ackers experiments or difference chromatography [25].

Acknowledgement

We would like to thank Dr. R.M. Roberts and Dr. F.W. Bazer for supplying the purified uterine protein. This aspect of the work was supported by NIH grant HD-08560 and National Science Foundation grant PCM 74-18016 to Drs. Roberts and Bazer. Our thanks also to Charles Holtzer, Department of Environmental and Behavioral Sciences for his assistance in data computation.

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