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ACTIVE ENZYME GEL CHROMATOGRAPHY. I. Experimental aspects ‡

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Transport properties of active enzyme species can be studied effectively by layering a small band of enzyme-containing sample on a gel chromatographic column previously saturated with substrate. The column is optically scanned at successive time intervals to yield profiles representing the appearance of chromophoric product or disappearance of chromophoric substrate. These profiles permit determination of the specific activity and rate of transport of the active species. Initial studies on mechanics of the technique establish the feasibility of accurately determining transport properties of active enzyme species chromatographed on gel columns. Illustrative results are presented for L-glutamate dehydrogenase and for homoserine dehydrogenase studied in both forward and reverse reactions. It is shown that the partition cross sections derived from the rates of motion of catalytic activity are the same as those determined by equilibrium saturation experiments which directly measure the degree of partitioning by the protein. These results establish the validity of the technique for a variety of future studies. Active enzyme gel chromatography appears comparable in precision to the active enzyme sedimentation technique, at current stages of development.

1. Introduction

Characterization of proteins in terms of their molecular size, weight, and interaction properties has most commonly been carried out using techniques (e.g., ultracentrifugation) requiring sample concentrations on the order of milligrams per ml. The range of concentration frequently accessible in such studies far exceeds the catalytic concentrations of enzymes used in steady-state kinetic investigations. Consequently, desired correlations between molecular size and functional properties must be made with great caution and can sometimes lead to false conclusions regarding the active units of enzymic species.

In order to circumvent these difficulties the technique of "active enzyme sedimentation" has been pioneered and developed by Cohen and his colleagues in a series of elegant studies [1–6]. In this technique a small band of enzyme is layered over a solution of substrate in a centrifuge cell. It is possible to study the transport properties of active enzyme–substrate complexes containing microgram (or even nanogram) quantities of enzyme, by monitoring the enzyme reaction spectrophotometrically through the appearance of a chromophoric product or the disappearance of a chromophoric substrate. Since only the active portion of any protein present is observed, impure enzyme samples can be studied by this technique.

The principle of active enzyme transport can of course be extended to other transport methods besides centrifugation. In this paper we describe initial studies aimed at developing the corresponding technique of active enzyme gel chromatography. Using the approach of direct optical scanning we have carried out studies to explore the feasibility of accurately determining transport properties of active enzyme species chromatographed on gel columns. We will present some studies on the mechanics of the technique as well as the types of data analysis required, and will show illustrative results with two enzyme systems: bovine liver L-glutamate dehydrogenase and aspartokinase I-homoserine dehydrogenase I from *E. Coli*. Methods for analysis of boundaries from scanning gel chromatography experiments are described in an accompanying paper from this laboratory [7]. A

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second paper on the active enzyme transport method by Brown and Zimmerman [8] describes a wider range of predicted effects and analyses, based upon numerical simulation.

From the theory of gel chromatography the molecular size-dependent parameter most directly related to the transport of both single component and multicomponent solutes is the partition cross section, ξ. This quantity represents the average fraction of the column's cross section which is available to the solute [9]. The rate of motion of the centroid position, $\bar{X}$, of the dynamic profiles is inversely proportional to ξ, according to the relationship

$$d\bar{X}/dt = F/\xi A, \quad (1)$$

where F is the solvent flow rate and A is the cross-sectional area of the column.

To confirm that partition cross sections calculated from profiles observed within the gel column by direct optical scanning agree with those determined from equilibrium saturation experiments, a study of several noninteracting solute species was carried out and is described in the accompanying paper [7]. Methods for location of the centroid position of such profiles, corrected to a constant time frame, were also developed in that study. Here we have carried out studies to determine the feasibility of applying the same relationship, eq. (1), to the motion of an enzyme–substrate complex, as observed through the catalytic activity of the enzyme, rather than that of the protein alone.

The only assumption necessary to the validity of experimental results is that of steady-state reaction, i.e., the ratio of enzyme to substrate must not exceed the conditions for linearity of assay. This condition may be readily verified by independent activity measurement. In order to interpret results of product profile motion straightforwardly, it is generally necessary to make the additional assumption that all active species have constant specific activity throughout a given experiment [1–6]. It should be noted, however, that the method is potentially useful for testing just such assumptions, and could be used to determine which, among several interconverting species, has the highest specific activity.

The types of profiles that must be determined from experimental measurement are shown schematically in fig. 1. Fig. 1(a) corresponds to a reaction in which substrate is chromophoric. Curve A represents the initial baseline which is measured when the column is saturated with (transparent) buffer. The spike on the left represents the porous disc which defines the top of the gel bed. The baseline A within the column is attributable to light scattered by the gel column bed. Curve B is a baseline measured after saturation of the column with chromophoric substrate. Curve C is the enzyme activity profile measured after the enzyme band has reached point $\bar{X}$, corresponding to the centroid of the "trailing boundary" profile indicated by the dashed line. In order to calculate this centroid the reagent-saturated baseline B must serve as the profile plateau and a new "baseline" whose absorbance is determined by the peak absorbance (X_1) of the activity profile must be determined. This is accomplished by using the partition cross section, $\xi(X)$, determined at each point in the gel from saturation of the column by the low molecular weight chromophore (e.g., NADPH), and the absorbance $A(X_1)$ at the point X_1, to create baseline $D(X)$ according to the equation:

$$\{[D(X_1) - A(X_1)]/\xi(X_1)\}\,\xi(X) = D(X) - A(X). \quad (2)$$

A new "apparent partition cross section" $P(X)$ for the chromophoric reagents is determined as $B - D$ from the absorbance of the new baseline and the reagent saturated scan at each point in the gel. Then the centroid of the profile is determined by solving the equation:

$$\int_{X_1}^{X_2} (C - D)\,dX = \int_{\bar{X}}^{X_2} P\,dX. \quad (3)$$

This type of experiment corresponds to the experimental study to be described of the homoserine dehydrogenase reaction in the "forward" direction, as written below from left to right:

Aspartic semialdehyde + NADPH + H^+ $\rightleftarrows$ Homoserine + $NADP^+$. In contrast, an experiment in which a product of the enzymatic reaction is chromophoric is illustrated in fig. 1(b). This situation corresponds for example, to the "reverse" reaction of homoserine dehydrogenase and to the "forward" reaction of L-glutamate dehydrogenase:

L-Glutamate + NAD^+ $\rightleftarrows$ α-ketoglutarate + NADH + NH_4^+

Scan A in fig. 1(b) represents the buffer baseline and scan B is again the reagent saturated baseline. Since $NADP^+$ does not absorb appreciably at the wavelength of interest (e.g., 340 nm), scan B does not rise much above the buffer baseline. The partition cross sections at every

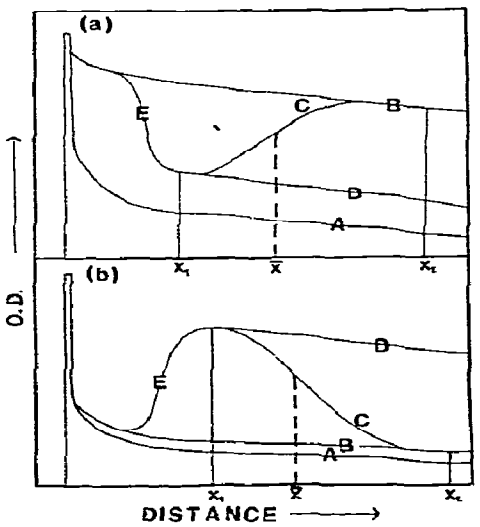

Fig. 1. Schematic diagram of active enzyme scans in gel chromatography. The profiles in (a) correspond to the case where chromophoric substrate is depleted giving rise to profile C. A and B are buffer baseline and chromophoric product traces, respectively. Curve D is an apparent baseline representing the extent of steady-state substrate depletion as the enzyme band moves down the column (left to right). The centroid of boundary C denoted by $\bar{X}$ corresponds to the effective position of enzyme band. X_1 and X_2 are reference positions used in calculating the centroid. Curve E denotes the boundary created by entering buffer. The profiles shown in (b) represent the case where the product of the reaction is chromophoric. Curves A, B, C, D, have the same significance as in diagram (a).

point in the scan needed to create the artificial reference plateau, represented by scan D, are calculated from independently determined partition cross sections of the chromophore NADPH. By inverting the scheme in fig. 1(b) it will be seen to be nearly the mirror image of the scheme in fig. 1(a). Eq. (2) can also be used to create $D(X)$ in this case from which $P(X)$ is calculated. Then the centroid position (X) of the profile can be located by eq. (3) for this type of experiment as well.

2. Materials and methods

2.1. Materials

All gel chromatography experiments were carried out using Sephadex G-200 (Pharmacia) in a precision quartz column (0.945 × 17 cm). Glycylglycine was from Worthington. Thyroglobulin was a gift from Dr. Richard Easterday (Pharmacia Fine Chemicals). Glutamate dehydrogenase was purchased from Sigma.

Homoserine dehydrogenase (aspartokinase I-homoserine dehydrogenase I) was isolated and purified from *E. Coli.* cells by a method described by Clark [10], with the minor modification of including an additional chromatographic step at the end using Sepharose 4B. The homogeneity of the protein preparations were determined by the existence of a single band on polyacrylamide gel electrophoresis. The protein concentration of the purified enzyme was determined spectrophotometrically, using an extinction coefficient of 0.67 absorbance units-cm^2mg^{-1} at 280 nm [10].

The amino acids DL-homoserine and L-threonine, were purchased from Calbiochem. All other chemicals used in the active enzyme experiments were from Sigma. They included nicotinamide-adenine dinucleotide phosphate in oxidized and reduced forms, nicotinamide-adenine dinucleotide in oxidized form, and L-glutamic acid.

Aspartic semialdehyde was prepared from allylglycine by the method of Black and Wright [11].

2.2. Instrumentation

All active enzyme transport experiments were performed using the scanning gel chromatograph initially described by Brumbaugh and Ackers [12], equipped with a digital data acquisition system. Flow was controlled by an LKB varioperpex pump. The rates were 3.1 ml/hr for glutamate dehydrogenase and 1.4 ml/hr for the homoserine dehydrogenase experiments. The monochromator slit width was 0.75 mm for measurements at 220 nm and 0.125 for measurements at 340 nm. The general operating procedures, data acquisition, and solute profile determinations were the same as those described in the accompanying paper [7]. Experiments were carred out at 23°C.

2.3. Enzymatic activities

Enzymatic activities were assayed by following the change in NADPH concentration spectrophotometrically at 340 nm.

2.3.1. Homoserine dehydrogenase

The initial saturating substrate levels chosen for the forward reaction assay were 0.25 M potassium phosphate (pH 7.6), 1.4 nM β-aspartic semialdehyde and 184 μM NADPH. A 10 mM concentration of L-threonine was used for studies in the presence of this feedback inhibitor. The gel column was saturated with 0.25 M potassium phosphate (pH 7.6) in order to determine the reference baseline and dilutions of stock enzyme were made in 0.25 M phosphate or 0.25 M phosphate plus 10 mM threonine.

For the reverse reaction, studies at pH 8.9 were performed in the presence of threonine, and in the presence of the activating $MgATP^{2-}$ complex. Initial reagent concentrations were 0.1M Tris buffer pH 8.9, 0.4 M KCl, 25 mM DL-homoserine and 320 μM $NADP^+$. Threonine, when used, was at 10 mM, while the levels of $MgCl_2$ and the dipotassium salt of ATP were 1.0 mM each. Reverse reaction active enzyme assays were also performed in 0.1 M Tris buffer pH 8.0 in the presence and absence of threonine. All reagents were at the same concentration levels as in the pH 8.9 assays. Reference baselines were made with Tris-HCl at the appropriate pH.

2.3.2. Glutamate dehydrogenase

Glutamate dehydrogenase was assayed in the "forward" direction described above. In this reaction, at pH 7.6, the enzyme is one-fifteenth as active as it is in catalyzing the reverse reaction [13].

The gel column was saturated with a reaction mixture of 0.25 M potassium phosphate buffer (pH 7.6), 50 mM sodium glutamate (made to pH 7.6), and 2 mM NAD^+. The stock glutamate dehydrogenase was 10 mg/ml. It was diluted to appropriate concentration (10 μg/ml) with the same 0.25 M phosphate buffer.

2.3.3. Linearity of assays

To determine the linear part of the rate curve under various conditions of initial substrate concentrations, assays were run in cuvettes with a 1.0 cm path length employing 3.0 ml reaction mixtures. The reactions were followed spectrophotometrically at 340 nm with a Gilford spectrophotometer equipped with a Sargent SRL recorder, and cell compartment thermostated at 25°C.

For homoserine dehydrogenase the reaction mixtures studied were those described above (section 2.3.1.). A 10 μl sample of stock enzyme preparation (stored in 10 mM phosphate buffer, pH 7.6, 5 mM threonine, 0.5 mM dithiothreitol) whose concentration was 1.32 mg/ml protein, was used to initiate the reaction. The maximum absorbance change obtained before deviation of 1–2% from the linear activity course was noted. These limits were then compared with plateau levels of absorbance change in the active enzyme experiments. Similar determinations were made using increased levels of the substrates. In the forward reaction, levels of aspartic β-semialdehyde and NADPH two, three, and five times as great as those defined above were tested. In the reverse direction two times the homoserine and $NADPH^+$ levels were tested for the conditions defined at pH 8.9. Such increased levels which yielded higher linearity limits were used to saturate the gel column when greater enzyme activities were anticipated from concentrated enzyme samples.

For the glutamate dehydrogenase assays similar studies were performed to ensure that linearity was not exceeded in any of the active enzyme chromatography experiments.

2.3.4. Determination of initial sample dilution upon entering the gel column

In order to determine whether small zone samples of varying volume are diluted to the same extent upon entering the gel column, samples, ranging in volume from 10 μl to 125 μl, of protein solution (glyceraldehyde-3-phosphate dehydrogenase) of known absorbance were applied to the gel column saturated with the same 0.01 M phosphate buffer used to make up the protein solution. Once the sample passed through the porous disc, the column was rinsed with the buffer and more phosphate buffer was applied to develop the column.

Stock glyceraldehyde-3-phosphate dehydrogenase was diluted 1:9 with 0.01 M potassium phosphate buffer (pH 7.6) for an absorbance determination at 220 nm. The absorbance of the stock solution was found to be 19.1. Samples of the stock were diluted in the 0.01 M phosphate buffer to give a concentration whose peak absorbance at 220 nm would range between 0.15 and 0.60 in the gel when aliquots, ranging in volume between 10 μl and 125 μl, were applied to the column saturated with the same buffer.

The column was repeatedly scanned at 220 nm as the small zone of protein moved down the column, spreading out and being diluted as it moved. The peak

absorbance of each scanned profile was then compared with the initial absorbance of the applied sample. The ratio of these absorbances was used as an estimate of the degree of dilution the protein sample had undergone.

2.3.5. Procedure for active enzyme gel chromatography experiments

The column was first saturated with either potassium phosphate or Tris buffer of appropriate pH, and a reproducible reference baseline was obtained by repeatedly scanning the column. Sixty milliliters of appropriate reaction mixture containing each component at the same concentration as would be used in a standard spectrophotometric assay were prepared and filtered twice through No. 2 filter paper. A Pasteur pipette was used to twice rinse the inside of the column above the disc with the reaction mixture before the actual addition of the reagents to the column and the initiation of saturation was begun. The column was carefully rinsed with reagent mixture before each subsequent addition of reagents.

The process of column saturation was monitored by driving the column to its uppermost position and observing the absorbance at that point in the column as recorded on the digital voltmeter of the data acquisition system. When the absorbance reached a maximum, the column was scanned to obtain a reagent saturated reference baseline.

A 500 μl sample of enzyme of appropriate concentration was then prepared by dilution of stock enzyme with the column buffer, either phosphate or Tris. A 100 μl aliquot of this diluted enzyme preparation was applied to the column as a small zone. The timer was started as the aliquot was placed on the polyethylene disc and the time at which the zone passed completely into the disc was noted. A 25 μl aliquot of the diluting buffer was applied as a "chaser". Once the chaser passed into the disc, the column above the disc was twice rinsed with reagent mixture and then filled with more reagent. The column was driven to its lowest position, the dark current and internal zero of the photometer were set, the teletype turned on and the Sargent recorder set to begin the scan. As each scan was begun, the time since t_0 was noted and the recorder turned on.

2.3.6. Specific activity determination

The activity of each small zone of enzyme applied to the gel column was determined by the amount of product produced between successive scans. The determination of this amount of product is made by integrating the area of each baseline subtracted scan. Each scan area is corrected for any deviation in the absorbance of the baseline as represented by that portion of the column into which the enzyme zone has not progressed. The successive scan areas are then subtracted from each other to determine the total product produced in the time between each pair of scans. Since the total amount of enzyme applied to the column is known, a specific activity, in terms of product per mg protein, can be calculated for each scan interval.

3. Results

3.1. Sample size studies

The decrease in peak absorbance corresponding to each sample volume was determined as the ratio of absorbances of the applied sample and that of the solute profile peak within the column. Results are shown in table 1. It can be seen that the change in absorbency of the sample upon entering the gel bed is highly dependent on sample size. The magnitude of change is

Table 1
Change in peak absorbance relative to applied sample absorbency [a] according to sample volume

Sample volume	Ratio of peak absorbency [b]	
	Scan 1	Scan 2
10 μl (undiluted stock GAPDH)	175	400
25 μl (undiluted stock)	100	147
50 μl (1:4 dilution of stock)	37.3	75
75 μl (1:9 dilution of stock)	18.2	33.5
100 μl (1:9 dilution of stock)	8.5	10
125 μl (1:9 dilution of stock)	6.5	9.7

a) Glyceraldehyde-3-phosphate dehydrogenase was prepared in 0.1 M Potassium phosphate buffer to a concentration which had an absorbance of 19.1. The G-200 gel column was saturated with 0.01 M phosphate buffer and a sample of protein of the size and composition noted was applied as a small zone to the column.

b) The small zone motion was observed by direct scanning and the peak absorbance was compared to that of the sample applied.

minimized by increasing the size and a nearly constant dilution factor is obtained for sample volumes of 100 μl or greater. For this reason, all the active enzyme chromatography experiments reported here were performed with 100 μl enzyme samples.

The physical bases of the large apparent dilution effects shown for small samples in table 1 are not entirely understood. Dilution within the gel bed is clearly not the only contributing process. It is clear, for example, that if the change in absorbance observed were due exclusively to dilution of the sample, a 10 μl zone would have been distributed into 1.75 ml at its peak during the sample's first seven minutes on the column. For a column of these dimensions, that would necessitate that the profile move almost two centimeters into the gel bed in this time. This simply does not occur. Therefore, other effects must contribute to the decrease in protein absorbance observed. Most likely, some protein adheres to the porous disc before entering the gel.

3.2. Glutamate dehydrogenase

The activity of glutamate dehydrogenase, which undergoes indefinite self-association of 313000 MW "monomers", has been shown to be independent of state of aggregation [14]. At the concentration of GDH used (10 μg/ml) the enzyme will exist almost entirely in the form of monomers [15].

The active enzyme scans observed for a 100 μl sample of glutamate dehydrogenase at an initial sample concentration of 10 μg/ml appear in fig. 2. Computation of the area under each profile was performed and the successive changes in area between consecutive scans yielded specific activities on a per milligram protein basis. These results are shown in table 2. Except for the first pair of scans, the change in area between consecutive scans was found to be constant throughout the column, implying constant specific activity.

The centroid position of each active enzyme boundary

Table 2
Change in area and specific activity between consecutive scans for glutamate dehydrogenase activity [a]

Scan interval	Change in area [b] (absorbance units × number of points)	Specific activity [c] (change in absorbane per mg protein)
1–2	3.537	3537
2–3	4.071	4071
3–4	3.791	3791
5–6	3.887	3887
6–7	3.824	3824

a) Stock glutamate dehydrogenase (10 mg/ml) was diluted to 10 μg/ml with 0.25 M potassium phosphate buffer (pH 7.6). The activity was assayed in the direction where L-glutamate is converted to α-ketoglutarate. A 100 μl sample of enzyme was applied to a G-200 column (0.934 × 17 cm) saturated with 0.25 M potassium phosphate, 50 mM glutamate, and 2 mM NAD^+. The production of NADH was followed at 340 nm.

b) Area of first scan profile is subtracted from area of second scan profile. Difference represents amount of product in time period between two scans.

c) Specific activity is reported as total change in absorbance per mg protein for the same amount of time (7.36 minutes) per scan interval.

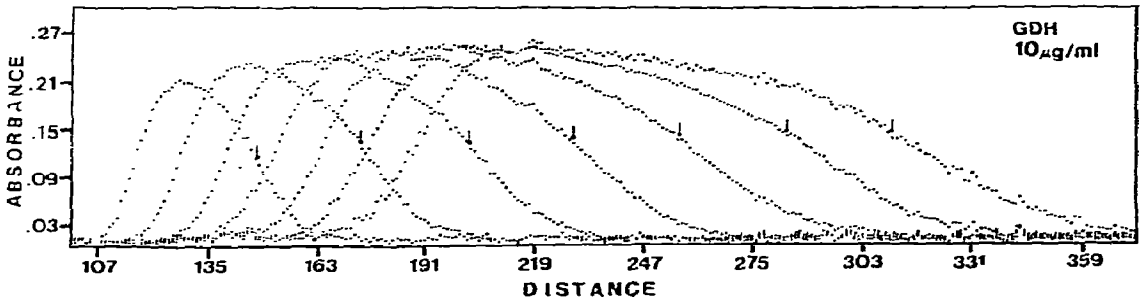

Fig. 2. Active enzyme gel chromatography profiles of glutamate dehydrogenase. Stock glutamate dehydrogenase (10 mg/ml) was diluted to 10 μg/ml with 0.25 M potassium phosphate buffer (pH 7.6). A 100 μl sample was applied to a G-200 column saturated with 0.25 M potassium phosphate buffer, 50 mM sodium glutamate, and 2 mM NAD^+. A 25 μl plug of phosphate buffer followed the enzyme zone. The column was scanned at 340 nm to observe the production of NADH. The column flow rate was 3.1 ml/hr.

Table 3
Profile motion parameters for glutamate dehydrogenase activity

Data point of centroid	Centroid distance		Rate of centroid motion, V_c (mm/s)	Partition cross section $\xi_{GDH}^{V_c}$
	Initial $\bar{X}$ (mm into gel bed)	Corrected $\bar{X}$ (mm into gel bed)		
120	9.15	8.86	0.0261	0.4058
147.33	21.66	20.96	0.0268	0.3954
174.45	34.07	32.96	0.0270	0.3915
202.39	46.85	45.29	0.0278	0.3810
228.74	58.91	57.05	0.0262	0.4039
255.35	71.09	68.82	0.0265	0.3992
283.06	83.77	81.01	0.0274	0.3864
311.47	96.77	93.52	0.0279	0.3789

produced by glutamate dehydrogenase was determined by methods described in the accompanying paper [7]. Corrections to a constant time frame were made on each centroid position and the corrected rate of motion of the centroids was calculated. From these rates the partition cross section ξ was determined at each centroid position according to eq. (1). The results appear in table 3.

The partition cross section for glutamate dehydrogenase (ξ_{GDH}) by direct scanning at 220 nm of the column saturated with the protein was compared to that calculated from enzyme activity profiles at each centroid point ($\xi_{GDH}^{V_c}$). The corresponding values are presented in the first two columns of table 4. Also included in the table are the ξ's obtained from saturation data for glycylglycine (ξ_{GLY}) and thyroglobulin (ξ_{THY}), used as markers for internal and void volumes, respectively. The partition coefficient for glutamate dehydrogenase (σ_{GDH}) at each centroid point was calculated from the formula:

$$\sigma_{GDH} = (\xi_{GDH}^{V_c} - \xi_{THY})/\xi_{GLY}.$$

The values of the partition cross section which were derived from the motion of the activity profiles appear to be more constant than those from saturation data and obviously do not reflect local fluctuations in packing as vividly. However, as had been observed for the motion of large zones of noninteracting solutes, the ξ's calculated from activity profile centroids agree with saturation data to within 4% for all scans except for scans 2 and 3. Excluding scans 2, 3, and 4, the average of the partition coefficients which were calculated by use of ξ from the centroid rate ($\xi_{GDH}^{V_c}$) is 0.49. This value is consistent with the expected motion of a 313 000 molecular weight molecule which is nearly excluded from the G-200 gel.

3.3. Homoserine dehydrogenase forward reaction

Active enzyme gel chromatography was carried out with the homoserine dehydrogenase forward reaction in the presence and absence of threonine over a twenty-fold range of initial enzyme concentration (1.32 μg/ml to 26.4 μg/ml). This concentration range is below the levels studied by stopped flow and temperature jump methods in which no concentration dependence of enzyme activity was observed [16,17]. Typical baseline subtracted active enzyme scans appear in fig. 3. The profiles appear as negative changes in absorbance since the chromophore NADPH is oxidized during the reaction and the product $NADP^+$ does not absorb appreciably at 340 nm.

The first scans in each experiment appear as sharp, relatively symmetrical, peaks with long leading boundary plateaus. As the enzyme zone move farther into the gel bed the second plateau "grows" to eventually merge with the sharp trailing boundary.

The "biphasic" feature of the activity profiles is primarily attributable to an initial dilution of the chromophoric substrate in the small volume of gel directly below the porous disc as the enzyme sample enters the column. The sample is introduced into the gel in buffer alone; no chromophoric substrate is included in this volume or in the buffer "chase" which follows the enzyme zone. Consequently, as long as it alone occupies

Table 4
Partition cross sections for glutamate dehydrogenase determined from rate of centroid motion and from equilibrium saturation and calculated partition coefficient

Centroid data point number	Partition cross sections				
	$\xi^{V_c}_{GDH}$	ξ_{GDH} [a]	ξ_{GLY}	ξ_{THY}	σ_{GDH}
120.0	0.406	0.404	0.920	0.362	0.048
147.3	0.395	0.436	0.949	0.385	0.011
174.4	0.392	0.423	0.947	0.380	0.012
202.4	0.381	0.397	0.964	0.364	0.017
228.7	0.404	0.403	0.956	0.354	0.052
255.4	0.399	0.406	0.962	0.351	0.050
283.1	0.386	0.396	0.955	0.342	0.047
311.5	0.379	0.384	0.957	0.333	0.048

[a] Saturation ξ's were calculated by equilibrating the same column used for the active enzyme study with 0.25 M phosphate buffer to determine a baseline and then saturating with a protein solution to determine the equilibrium saturation function. The glutamate dehydrogenase solution used had a concentration of 0.048 mg/ml and the glycylglycine and thyroglobulin solutions were approximately 0.1 mg/ml. The column was scanned at 220 nm.

this gel region, the concentration of chromophore is reduced, thereby lowering the absorbance in that region. Since new substrate must move into the gel regions already partially depleted of NADPH before the profile returns to its baseline, intermediate scans appear "biphasic". However, the profile corresponding to the actual position of the active enzyme is the broader second plateau and not the sharp initial peak.

Specific activities calculated from successive areas of these scans were found to decrease slightly. This decrease was found to be consistent with the observed deterioration of the aspartic semialdehyde substrate during the four hours duration of the experiment.

3.4. Homoserine dehydrogenase reverse reaction

Active enzyme experiments with homoserine dehydrogenase were also performed with the reverse reaction assay at pH 8.9 in the presence of threonine and in the presence of the $MgATP^{2-}$ complex, and at pH 8.0 in the presence and absence of threonine. In each case the en-

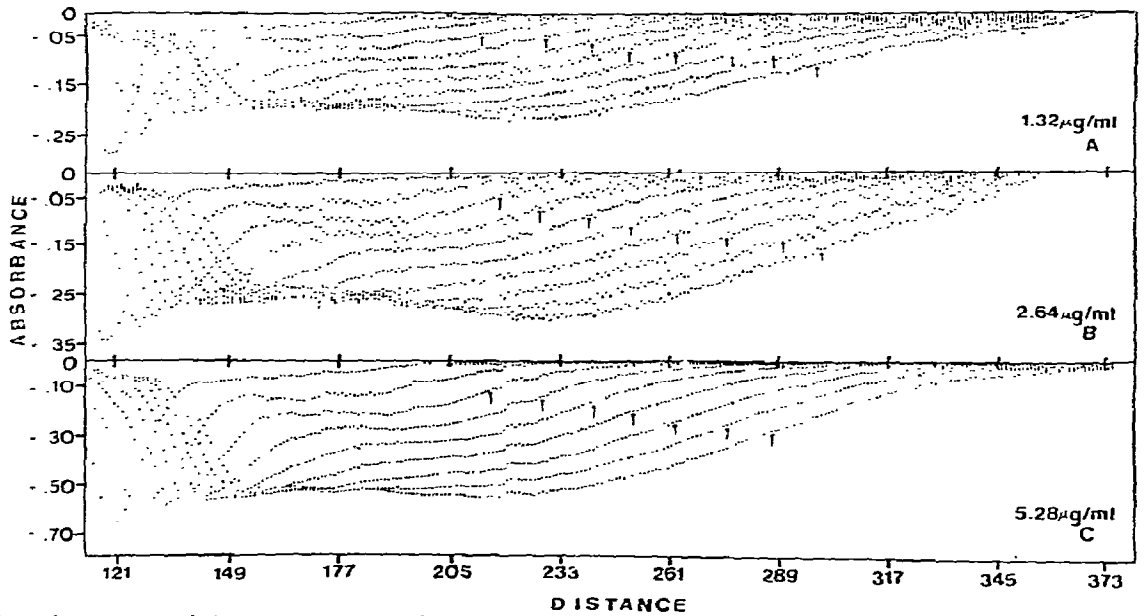

Fig. 3. Active enzyme gel chromatography profiles of homoserine dehydrogenase assayed in the forward direction in the absence of threonine. Stock enzyme was diluted to the concentrations noted with 0.25 M potassium phosphate buffer (pH 7.6). A 100 µl sample of each diluted enzyme was applied to a G-200 column saturated with the following reagents: for enzyme samples 1.32 µg/ml and 2.64 µg/ml, 0.25 M phosphate buffer, 1.41 mM aspartic semialdehyde, and 184 µM NADPH; for enzyme sample 5.28 µg/ml, 0.25 M phosphate buffer, 2.82 mM aspartic semialdehyde and 378 µM NADPH. Each enzyme sample was followed by a 25 µl sample of phosphate buffer. "Absence of threonine" results from the dilution of the threonine (5 mM) in which the enzyme was stored. The column flow rate was approximately 1.4 ml/hr. The reaction was followed at 340 nm.

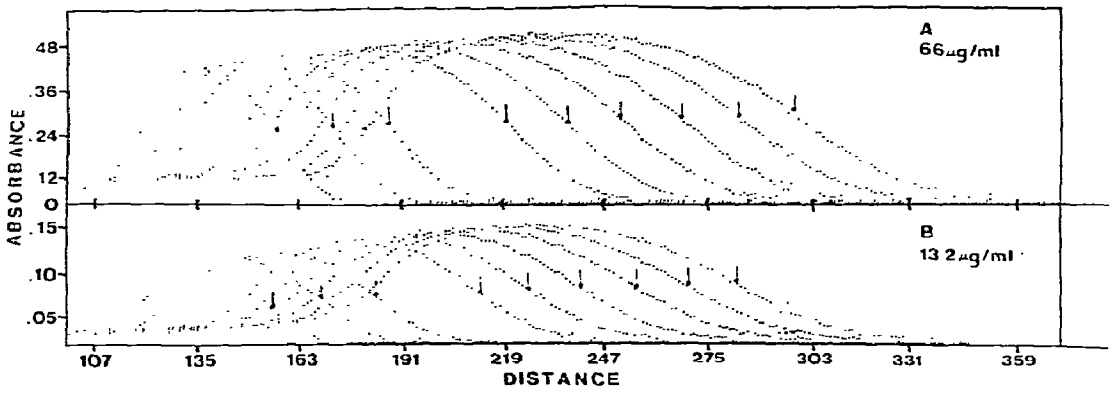

Fig. 4. Active enzyme gel chromatography profiles of homoserine dehydrogenase assayed in the reverse direction in the presence of threonine at pH 8.9. Stock enzyme (1.32 mg/ml) was diluted to the concentration noted with 0.1 M Tris buffer (pH 8.9) plus 0.01 M threonine. A 100 μl sample of diluted enzyme was applied to a G-200 column saturated with the following reagents: for enzyme sample 13.2 μg/ml, 0.1 M Tris buffer, 0.4 M KCl, 25 mM L-homoserine, 320 μM $NADP^+$ and 0.01 M threonine; for the enzyme sample 66 μg/ml, homoserine and $NADP^+$ concentrations were at 50 mM and 640 μM, respectively. A 25 μl sample of Tris buffer plus threonine followed the enzyme zone. The reaction was followed at 340 nm. The column flow rate was approximately 1.4 ml/hr.

zyme concentration was varied over a 12.5 fold range (5.28 μg/ml to 66 μg/ml).

The particular effector conditions chosen for this series of experiments were dictated by the observation that the enzyme at pH 8.9 will rapidly lose activity in the absence of threonine. Recent studies have shown that in the presence of the $MgATP^{2-}$ complex and the substrate homoserine, enzyme activity is protected [18]. The protection is essentially as effective as that of threonine.

Scans from a pair of active enzyme experiments for the reverse reaction appear in fig. 4. The profiles appear as increases in absorbance at each point in the scan, since the chromophore NADPH is produced during the reaction. The reverse reaction is much slower than the forward reaction thereby yielding smaller changes in absorbance. The scans do not appear biphasic because the entering buffer containing the enzyme does not lead to dilution of a chromophoric substrate. A notable feature for the reverse reaction experiments is the inability to bring the reaction profile back down to the pre-experiment baseline by introducing fresh reactants to the gel. The most likely explanation for this observation is the partial adsorption of the NADPH product to the Sephadex gel. This effect could be eliminated in desired cases by use of a polyacrylamide gel for the column. In the present experiments the adsorption is of no consequence to the analysis of the enzyme profiles.

Similar profiles for the activity of homoserine dehydrogenase at the defined conditions over the entire concentration range were integrated for total product, and specific activities were calculated. No discernible concentration dependence of the specific activity was found.

3.5. Rate of active enzyme transport

Centroid positions and their rates of motion for the forward reaction activity are shown in table 5.

Table 5
Profile motion parameters for homoserine dehydrogenase assayed in forward direction in absence of threonine

Enzyme concen. (μg/ml)	Average rate of centroid motion (mm/s)		Average $\xi^{V_c}_{HDH}$	
	mean	st. dev.	mean	st. dev.
26.4	0.0152	0.001	0.359	0.026
13.2	0.0134	0.003	0.418	0.128
5.28	0.0123	0.002	0.451	0.074
2.64	0.0123	0.001	0.447	0.044
1.32	0.0119	0.002	0.467	0.088

Table 6
Profile motion parameters for homoserine dehydrogenase assayed in forward direction in presence of threonine

Enzyme concen. (μg/ml)	Average rate of centroid motion (mm/s)		Average $\xi_{HDH}^{V_c}$	
	mean	st.dev.	mean	st.dev.
26.4	0.0168	0.001	0.3251	0.023
2.64	0.0153	0.001	0.3580	0.038

There is considerable scatter in the data as evidenced by the large standard deviation. A slight trend toward slower rate of profile motion and a larger partition cross section with decrease in enzyme concentration would suggest that enzyme dissociation is occurring, and species which are dissociated from active tetramer are contributing to the forward reaction activity. The enzyme is known to dissociate from the tetrameric state to dimers under conditions similar to those of these experiments [18].

No change in specific activity occurs with a change in enzyme concentration in the presence of threonine, which is known to promote association of homoserine dehydrogenase at pH 7.6 conditions [18,19]. It seems likely that the dimeric form of the enzyme makes a smaller contribution to total enzyme activity observed in the presence of threonine than in the absence of threonine at similar enzyme concentrations. A comparison of the rates of motion of activity profile centroids and the resultant partition cross sections for two sample concentrations which were shown to be of uniquely different size when run without the threonine were made in the presence of threonine. The results appear in table 6.

In each case the two parameters indicate active species which are larger than those observed without threonine at the same enzyme concentration. The ξ determined for the more dilute sample with threonine is smaller (indicating larger species) than that observed for the most concentrated sample without threonine.

Similar motion parameters were determined for experiments run in the reverse reaction at pH 8.9 in the presence of threonine and at pH 8.0 in the absence of threonine. The information is tabulated in table 7.

In each case the more dilute enzyme samples moved with an apparent molecular size smaller than that of the concentrated enzyme samples. The magnitude of the ξ's obtained at the two enzyme concentrations for the two sets of conditions are much more similar than those determined for homoserine dehydrogenase run in the presence and absence of threonine at forward reaction assay conditions on a different gel column.

In light of the fact that no appreciable concentration dependence on specific activity was observed either at pH 8.9 in the presence of threonine or the $MgATP^{2-}$ complex or at pH 8.0 in the presence and absence of threonine, there is serious doubt that such a phenomenon exists with the reverse reaction conditions.

4. Discussion

The active enzyme transport technique employes the motion of an enzyme–substrate complex, rather than that of the protein alone, to define the molecular size properties of the enzyme. The possibility of applying the basic concept to gel chromatography depends critically on the accurate correspondence of the fundamental partitioning parameter ξ with that calculated from the motion of the dynamic profile of the protein or protein–substrate complex. The theory of operation

Table 7
Profile motion parameters for homoserine dehydrogenase assayed in reverse direction

Conditions	Enzyme concen. (μg/ml)	Average rate of centroid motion (mm/s)		Average $\xi_{HDH}^{V_c}$	
		mean	st.dev.	mean	st.dev.
pH 8.9 with THR	66	0.0157	0.001	0.340	0.012
	13.2	0.0145	0.001	0.375	0.016
pH 8.0 without THR	66	0.0169	0.001	0.325	0.025
	13.2	0.0152	0.002	0.361	0.040

of gel chromatography predicts that the rate of motion of the centroid of such profiles is inversely related to ξ, the partition cross section of the protein. Using the technique of direct optical scanning of gel columns, dynamic profiles of large zones of simple noninteracting species or the activity associated with a small quantity of enzyme moving down the column could be observed. The partition cross sections determined from independent saturation experiments could then be compared with those determined from the profile motion.

The agreement between the column parameter ξ derived from saturation data and that from the rate of motion of the activity profile centroid of glutamate dehydrogenase, is similar to that observed for the motion of large protein zones of noninteracting solute species [7]. The results indicate that the active enzyme–substrate complex behaves in an essentially identical manner to the protein alone and that basic gel chromatography theory derived for the observation of proteins alone can be adapted for the study of protein–substrate complexes. Values of the partition cross section determined in this case are in good agreement with values expected for a 313 000 molecular weight species. These results and the constancy of specific activities determined from the successive area calculations imply that the active enzyme data accurately reflect both the size of the active species, and the level of activity associated with the active form.

Analysis of the dynamic profiles of large zones of noninteracting solute species and active enzyme studies of glutamate dehydrogenase lead to the conclusion that partitioning parameters can be accurately determined to within at least 4% of "true" saturation values by the direct optical scanning of gel columns. Precise knowledge of the reference baseline and the protein absorbency can improve this value substantially. Cohen and Mire have defined the precision in molecular size parameters determined from active enzyme sedimentation when calculated by both rigorous and approximation methods to be about 1% for sedimentation coefficients and 10% for diffusion coefficients. However, the errors on sedimentation coefficients they reported for test studies of the technique averaged 5% of their reported $s_{20,w}$. The sedimentation and chromatographic techniques thus appear to be roughly comparable at present stages of development.

The stability provided by the stationary phase of active enzyme gel chromatography is substantial. It eliminates the need to create a density gradient throughout the reaction mixture in order to prevent convection currents and a density difference between reactants and enzyme, both of which are important in sedimentation. The ability to carry out experiments under one atmosphere of pressure and the high photometric accuracy obtainable in scanning gel columns make it a particularly appealing method for many applications.

In general the same concentrations of enzyme can be analyzed by the two techniques, but, as was shown earlier, the volume in which the sample is added in active enzyme gel chromatography is critical and is greater than that used in sedimentation. Consequently, higher levels of substrates may be required to study upper limits of concentration dependence with the gel system.

Results obtained with the homoserine dehydrogenase enzyme serve to illustrate the characteristics of active enzyme transport for forward and reverse reactions in a single system. The present results do not permit any detailed analyses of subunit interactions nor relative specific activities of interconverting species in that system. However they provide the background for future extensions to such analyses.

This study has not been an exhaustive investigation of the possibilities of the active enzyme gel chromatography technique. Rather, it was intended to define the experimental conditions which would be most promising in assuring precision in calculation of column parameters and maximum utility in further studies. It is expected that future applications will refine these conditions to eliminate some of the possible sources of error already noted, and to include additional ways of analyzing the data. Careful calibration of the columns for molecular size is an obvious extension of present methods. Additional information can be obtained by fitting the detailed shape of an active enzyme profile to mechanistic models. It has been established that changes in porosity and flow rate can alter the shape of such curves dramatically. Extensive information might ultimately be obtained from such numerical fitting, even though the equations which describe the profiles cannot be solved analytically. The accompanying paper by Brown and Zimmerman [8] represents highly valuable initial explorations in this direction.

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