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MICROBORE COLUMN EXCLUSION CHROMATOGRAPHIC METHOD FOR STUDYING PROTEIN ASSOCIATION AND ITS RELATION WITH ENZYMATIC ACTIVITY

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SUMMARY

The principles of treating chromatographic data are considered to allow the determination of number of components, equilibrium and kinetic association constants of proteins by means of micro-column exclusion chromatography. The method is developed to study protein association in cases complicated by adsorption. The potentialities of the chromatographic method have been examined for the study of the mechanism of enzyme catalytic action.

INTRODUCTION

Association and biological function of proteins are closely interrelated. The interest in studying equilibrium and kinetics of protein association has increased particularly during the last years, since these reactions have been recognized to be fundamental to many metabolic control mechanisms in vivo. Of particular importance are those systems that contain two kinetically different forms of an enzyme which are capable of reversible interconversion. For example, such is the case when only one of the components has affinity to a ligand, taking the part of activator or of inhibitor in enzymatic catalysis. Biochemical aspects of correlations between enzymatic activity and protein self-association have been discussed in the review of Frieden [1], who cited as an example a number of self-interacting enzymes demonstrating the flexibility in the activity control which may be attained by means of reversible association.

The present work deals with methodological problems of studying protein association and its correlation with enzymatic activity by means of exclusion liquid chromatography. Apart from using microbore columns, the novelty in this field consists in expanding the potentialities of the chromatographic method for determination of the rate constants of these reactions and for the case of

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protein adsorption on the column. The methodological principles that are developed in this paper for protein association should also be useful for studying other reactions of biopolymers such as complex formation, ligand binding, etc.

EXPERIMENTAL

A microbore-column liquid chromatograph Kh-Zh-1305 (Special Design Office of Analytical Instrument-making, Academy of Science, U.S.S.R.) was used for the experimental study of protein association. It was equipped with a multi-wavelength spectrophotometric detector (cell volume 1 μl) and precision syringe pump (syringe volume 2 ml, flow-rate 8—4000 μl/h). Glass columns (250 mm × 1 mm I.D.) were packed with Sephadex G-75 superfine (Pharmacia, Uppsala, Sweden), or Bio-Gel P-60, <400 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.). The procedure of microbore-column filling is described in ref. 2.

The absorbance of samples was monitored in the range 220—300 nm. Wide-zone technique was used with a sample volume of 200 μl and flow-rate of 500 μl/h.

Purified samples of phospholipase A_2 (isozymes E_2 and E_3) and dihexanoyl-lecithin were obtained from the Institute of Bioorganic Chemistry (Academy of Science of the U.S.S.R.). Lysozyme activity A (Sojuzreaktiv, U.S.S.R.) was purified on a Sephadex G-75 preparative column with UV detection. Standard proteins for calibration were obtained from Serva (Heidelberg, G.F.R.). All other reagents (Sojuzreaktiv, U.S.S.R.) were of analytical grade, with the exception of potassium chloride, which was of reagent grade.

RESULTS AND DISCUSSION

Steric exclusion chromatography (SEC) which is the traditional method of biopolymer separation according to molecular size, appears also to be a convenient means of studying physicochemical properties of reacting systems. The reversible association does not allow the monomer and polymer zones of the protein to be resolved completely since polymer would always be formed in the monomer chromatographic zone, whereas in its own zone polymer would dissociate. Depending on the ratio of the reaction rate to the rate of chromatographic separation, different shapes of the concentration profile would be observed on the chromatogram. When the ratio is small (complete separation would be attained if it were high) no separation would be observed. Comparable rates of separation and interconversion of the components would result in partially resolved zones. On the other hand, association reaction causes the additional spreading of chromatographic zones due to the different chromatographic mobilities of the components and random distribution of the times which the macromolecule spends in its different states of aggregation. Thus the location of peaks on the chromatogram and the degree of its spreading reflect the properties of associating protein migrating along the column. A quantitative theory has been developed [3—9] which provides the possibility of determining equilibrium and kinetic constants for association reactions by studying concentration dependences of the first and the second moments of the chromatogram.

Carrying out SEC experiments on capillary microbore columns with an inner diameter of 0.5—1 mm, results in a significant reduction in the amount of protein required (100-fold when the column diameter is reduced from 5 mm to 0.5 mm). This clears the way to studying reactions of deficient proteins. Micro-column SEC also provides certain advantages when compared with the widespread sedimentation method of association studies. Among these the possibility of studying an extremely wide concentration range (down to 10^{-4} M with detection at 220 nm) is an important advantage.

Protein association should be studied in the following stages: (1) diagnostics of association; (2) determination of the number of components and the degree of association; (3) determination of equilibrium and kinetic constants; (4) studying the correlation between association parameters and functional activity of the protein. Micro-column SEC (MCSEC) appears to be useful at every level of study, though sometimes other methods should also be used to obtain additional information (sedimentation, diffusion).

The most complex problem in the analysis of associating protein is to determine the number of protein oligomers. The following types of association are especially frequent: (A) one-stage equilibrium monomer—n-mer (1); (B) multistage equilibrium — indefinite association with subsequent addition of monomer units and equal change in the free energy at every stage (2, 3).

$$n\mathrm{M}_1 \underset{}{\overset{K_{1,n}}{\rightleftharpoons}} \mathrm{M}_n;\ K_{1,n} = [\mathrm{M}_n]/[\mathrm{M}_1]^n,\ n \geqslant 1 \tag{1}$$

$$\begin{cases} \mathrm{M}_1 + \mathrm{M}_1 \overset{K_{1,2}}{\rightleftharpoons} \mathrm{M}_2 & K_{i,i+1} = [\mathrm{M}_{i+1}]/[\mathrm{M}_i]\,[\mathrm{M}_1] \quad (2) \\ \mathrm{M}_2 + \mathrm{M}_1 \overset{K_{2,3}}{\rightleftharpoons} \mathrm{M}_3 & K_{1,2} = K_{2,3} = \ldots = K_{i,i+1} \equiv K = \quad (3) \\ \ldots\ldots & \\ \mathrm{M}_i + \mathrm{M}_1 \overset{K_{i,i+1}}{\rightleftharpoons} \mathrm{M}_{i+1} & = (1/C_1)(1 - \sqrt{C_i/C}) \end{cases}$$

where C is the total concentration of protein. Between these limiting cases the intermediate mechanisms are possible, among which model 4 is realized frequently.

$$q\mathrm{M}_1 \underset{}{\overset{K_{m,n}}{\rightleftharpoons}} s\mathrm{M}_m + r\mathrm{M}_n \qquad n > m > 1 \tag{4}$$

$$K_{m,n} = \frac{[\mathrm{M}_m]^s\,[\mathrm{M}_n]^r}{[\mathrm{M}_1]^q} = K_{1,m}^s \cdot K_{1,n}^r \tag{5}$$

Two-component reaction of monomer—n-mer type 1 may be distinguished with comparative ease. It is usually carried out on the basis of the Sophianopolous and Van Holde criterion [10], which predicts for two-component systems the inverse linear relationship between z-average ($\bar{V}_z$) and weight-average ($\bar{V}_w$) elution volumes of polydisperse protein.

$$\bar{V}_z = (V_1 + V_n) - V_1 V_n \bar{V}_w^{-1} \tag{6}$$

According to definition of averages

$$\bar{V}_w = \sum_1^n V_i C_i / \sum_1^n C_i \tag{7}$$

$$\bar{V}_z = \sum_1^n V_i^2 C_i^2 / \sum_1^n V_i C_i \tag{8}$$

$\bar{V}_w$ and $\bar{V}_z$ are calculated from wide-zone frontal chromatograms by means of the following formulae

$$\bar{V}_w = \int_0^{C_0} V(C)\mathrm{d}C / C_0 \tag{9}$$

$$\bar{V}_z = \int_0^{C_0} V^2(C)\mathrm{d}C / \int_0^{C_0} V(C)\mathrm{d}C \tag{10}$$

where C_0 is the total protein concentration. If criterion 6 shows the number of components $n > 2$, there is no other way than sorting out the models of association. This means varying the equilibrium constants for the reactions 2 and 3 and to draw a conclusion about the reaction mechanism on the basis of the best fit between experimental and computed dependences $V_w(C)$. The uncertainty of such an analysis may be reduced by making use of additional sedimentation data on the concentration dependence of $\bar{M}_w$ (weight-average mol. wt.) or by means of an independent method of evaluating the number of components. Unfortunately, the only method to be used for this purpose is the measurement of instantaneous electrophoretic mobility [11] based on the Doppler effect in light scattering.

When the number of components is known, it is possible to determine equilibrium association constants from the concentration dependence of the weight-average elution volume [5]. The frontal mode of chromatographic analysis should be used, since it provides a constant composition in the plateau region. A typical chromatogram, obtained under these conditions, is depicted in Fig. 1, and the position of centroid, which corresponds to the weight-average elution volume ($\bar{V}_w$), is defined by eqn. 9.

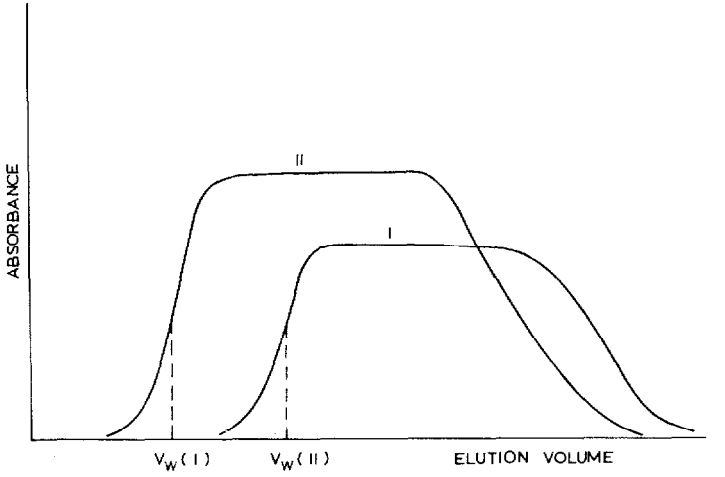

Fig. 1. Wide-zone chromatograms obtained by MCSEC. I = lysozyme (n = 2), pH = 7.0, μ = 0.2; II = α-chymotrypsin (n = 3), pH = 6.2, μ = 0.2, n = number of components.

For the reaction 1, eqns. 7 and 1 lead to the following relationship

$$\bar{V}_w(C_0) = \frac{V_1 + K_{1,n} C_1 V_n}{1 + K_{1,n} C_1} \tag{11}$$

where monomer concentration (C_1) in the plateau region is related to C_0 and $K_{1,n}$ as follows:

$$K_{1,n} C_1^n = C_n; \qquad C_1 + C_n = C_0 \tag{12}$$

The monomer elution volume (V_1) may be determined from $V_w(C)$ by extrapolation to infinite dilution, and the polymer elution volume (V_n) from the calibration curve. It should be kept in mind that individual proteins are extremely different in terms of solvation, asymmetry of the native conformation, and ability to interact with the sorbent. That is why molecular mass calibration is unreliable, whereas hydrodynamic radius calibration is not possible in some cases (for instance, in the presence of adsorption).

For swelling gels Stokes' radii (R) calibration of the distribution coefficient (K_d) is based on Ackers' equation [4]

$$R = a + b \ \mathrm{erfc}^{-1} \ K_d \tag{13}$$

where a and b are constants, $\mathrm{erfc}\ K_d = \frac{\sqrt{\pi}}{2} - \int_0^{K_d} e^{-t^2} dt$, $\mathrm{erfc}^{-1} K_d$ the inverse function.

For rigid gels the best calibration results are obtained with the so-called random-sphere model [12] which presents an adequate geometrical image of the structure of porous silica-based materials. According to the random-sphere model,

$$K_d = \psi^{(1+R/R_s)^3 - 1} \tag{14}$$

where ψ is the inner porosity of the sorbent, R_s the radius of elementary SiO_2 microsphere in the matrix of the silica gel. Figs. 2 and 3 show calibration relationships for globular proteins obtained on the columns with Sephadex G-75 (Fig. 2) and TSK-gel SW 3000 (Fig. 3).

When the protein under investigation is being adsorbed on the column, the problem of association study becomes rather difficult, since V_i values of oligomer elution volumes are in this case unknown. Just this very case is realized with lysozyme, the protein which is known to interact even with inert carbohydrate and polyacrylamide gels. However, with this protein it is possible to overcome these adsorption-based complications and to determine the dimerization constant by means of MCSEC. For this purpose special methods for treating the experimental data have been developed [13]. Assuming Langmuir-type protein adsorption, the equilibrium dimerization constant and two parameters of the adsorption isotherm may be found from the experimental $V_w(C)$ dependence and eqn. 6 on the basis of the best-fit values for these parameters, providing the closest correspondence of calculated and experimental $\bar{V}_w$ and $\bar{V}_z$. The result is shown in Figs. 4 and 5. It is worth mentioning that an identical value for the dimerization constant ($K_{1,2}$ = 0.9 ± 0.2 dl/g) has been obtained for both dextran and polyacrylamide gels despite the difference in adsorption isotherms for these gels. This fact, together with the good fit of our result with sedimentation equilibrium data, demonstrates the reliability of the meth-

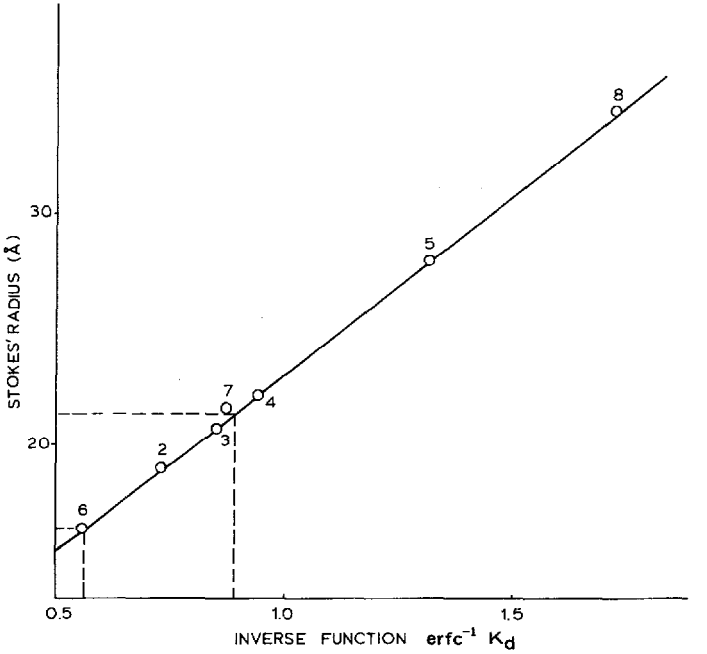

Fig. 2. Calibration dependence of distribution coefficient K_d on the Stokes' radii of proteins for microbore column with Sephadex G-75. V_i = protein elution volume; V_0 = void volume; V_p = pore volume. 1 = cytochrome *c*, 2 = ribonuclease, 3 = myoglobin, 4 = chymotrypsinogen A, 5 = ovalbumin, 6 = phospholipase A_2 (monomer), 7 = phospholipase A_2 (dimer), 8 = bovine serum albumin (BSA).

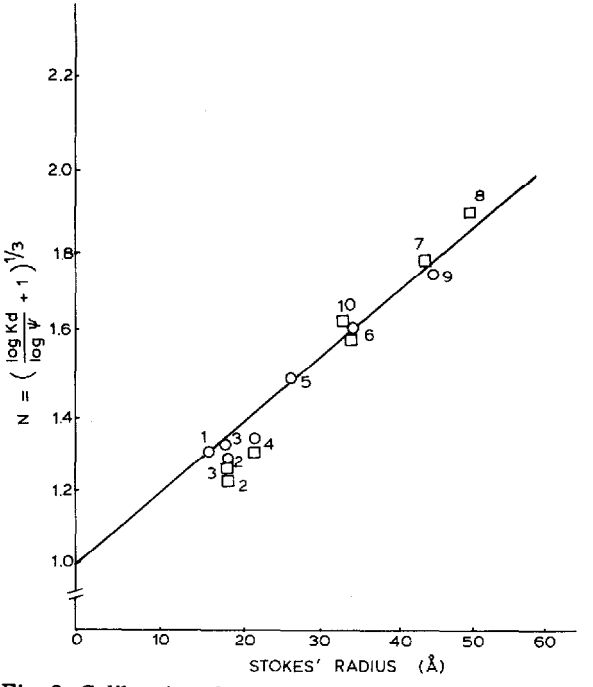

Fig. 3. Calibration dependence for TSK-gel SW 3000, based on the random-sphere model of porous structure. Experimental data are from refs. 15 and 16. R_s = 59.5 Å; ψ = 0.708; 1—5 as in Fig. 2, 6 = BSA, 7 = BSA—dimer, 8 = BSA—trimer, 9 = aldolase.

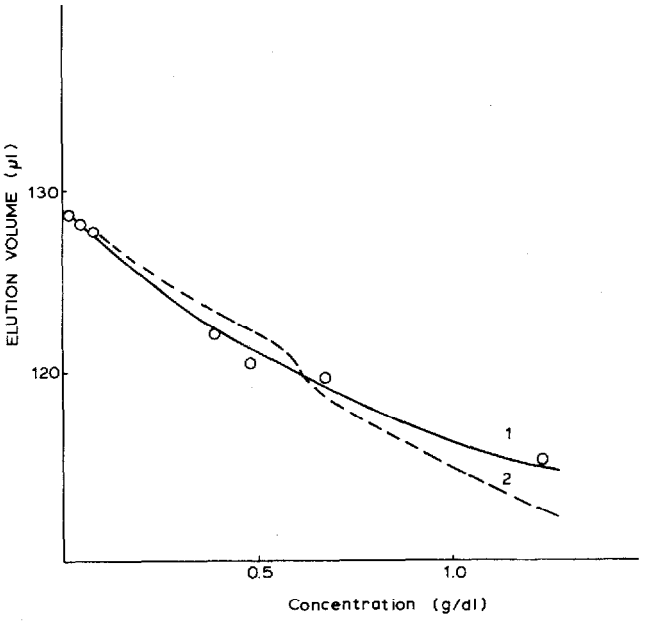

Fig. 4. Concentration dependence of V_w for lysozyme (Bio-Gel P-60 column). 1 = curve, calculated for $K_{1,2}$ = 0,9 dl/g; 2 = curve, calculated for the doubled dimerization constant. (o) Experimental data.

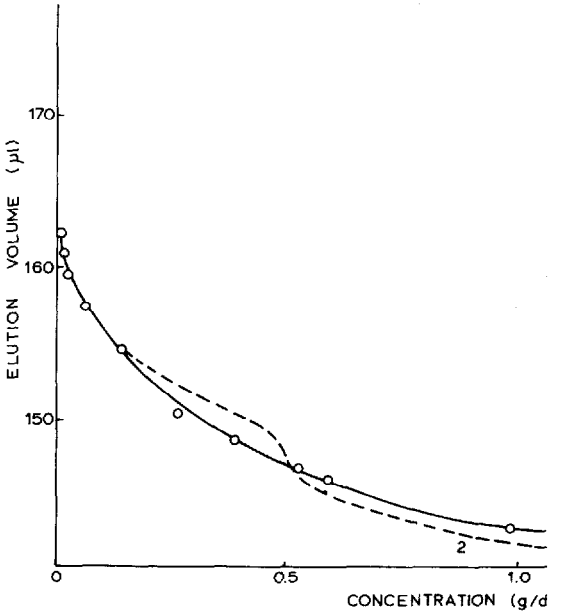

Fig. 5. Concentration dependence of V_w for lysozyme (Sephadex G-75 column). Designations correspond to those in Fig. 4.

od developed by us for chromatographic study of protein association complicated by protein adsorption.

Now consider the principles of chromatographic estimation of the rate constants for protein self-association reactions. The equilibrium mixture of monomer with the associated form of protein tends to separate when it is moving in the column, but the equilibrium composition would be re-established in partially resolved zones with a certain lag time. Therefore, the spreading of chromatographic boundary of reversibly associated protein is increased in comparison with a non-interacting mixture of the same composition. Besides longitudinal and eddy diffusion, which are usual factors of spreading, for reacting systems there is another type of spreading caused by different times for protein to move as monomer or as associate. Thus the measured dispersion coefficient $L_{V(M)}$ may be represented as the sum of the weight-average dispersion coefficient and a certain kinetic term [2].

In the simplest case of dimerization

$$L_{V(M)} = \frac{1}{N} + L_K \tag{15}$$

$$L_K = 2F(V_1 - V_2)^2 K_{1,2} C_1 / (1 + K_{1,2} C_1)^3 V_w^3 k_2 \tag{16}$$

In these equations $L_V = \frac{\sigma_V^2}{V^2}$, σ_V^2 is the volume dispersion of boundary, F is volume flow-rate, N is the number of theoretical plates, $k_2 = \frac{k_1 C_1^2}{C_2}$ is rate constant

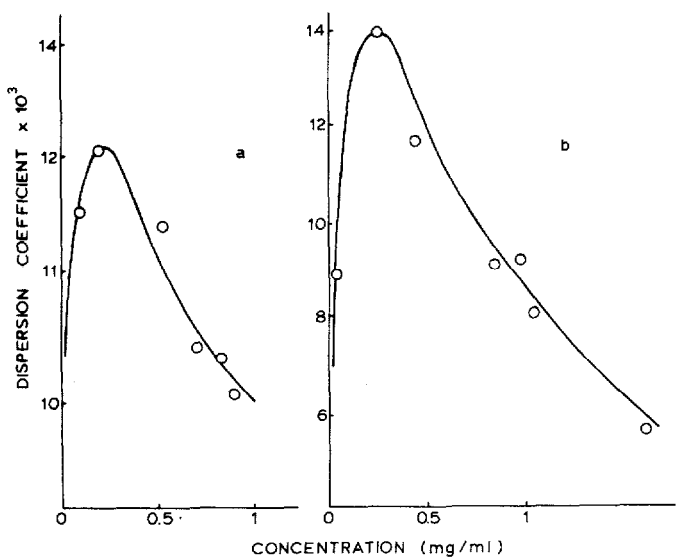

Fig. 6. Concentration dependence of the dispersion coefficient $L_{V(M)} = \sigma_V^2/\bar{V}_w^2$ for phospholipase A_2 leading boundary of chromatogram. (a) $C_{Ca^{2+}} = 0$; (b) $C_{Ca^{2+}} = 0.02$ M. Curves are the theoretical results based on the data of Table I for $N = 200$ theoretical plates. (○) Experimental points.

for the reverse and k_1 for the direct reaction. Eqn. 16 may be used for the estimation of rate constants. The results obtained for phospholipase A_2 are presented in Fig. 6 and Table I. The rate constants for dimerization of this enzyme appeared to be sensitive to the presence of activator (Ca^{2+}).

TABLE I

RATE CONSTANTS FOR PHOSPHOLIPASE A_2

Ca^{2+} concentration	$K_{1,2} \cdot 10^{-4}$ (M^{-1})	$k_1 \cdot 10^{-4}$ (M^{-1} sec^{-1})	k_2 (sec^{-1})	$\tau = k_2^{-1}$ (sec)
0	6.0 ± 0.5	4.2 ± 0.5	0.7 ± 0.1	1.5
0.02	6.3 ± 0.5	1.27 ± 0.32	0.2 ± 0.05	5

It can be seen from Fig. 6 that there is a maximum in $L_{V(M)}$ versus C_0 plot. It is explained as follows. When $C_0 \to 0$ (complete dissociation) and $C_0 \to \infty$ (complete association), $L_{V(M)} \to 1/N$, whereas the intermediate range of concentration is characterized by mixed kinetics, when association and mass transfer are equally rate-limiting.

We have considered physicochemical aspects of chromatographic study of associating proteins. To illustrate the biochemical potentialities of this method let us cite as an example the use of MCSEC to ascertain the mechanism of catalytic action of phospholipase A_2 [14]. This enzyme hydrolyzes the fatty ester bound at position 2 of 1,2-diacyl-*sn*-phosphoglycerides.

The rate of lipolysis is effectively increased when the substrate is in micellar form. By means of MCSEC, dimerization of this enzyme was investigated, whereas the activity data have shown the dimer to be the only active form of phospholipase A_2. We examined the influence of substrate (dihexanoyl-lecithin) in molecular form and in micelles on the dimerization equilibrium of phospholipase A_2, and the influence of activator (Ca^{2+}) and inhibitor (Ba^{2+}) on complex formation of the enzyme with the micelle. Fig. 7 shows the dependence of reduced elution volume on the total concentration of phospholipase A_2 isozyme E_3. The ordinate in Fig. 7 corresponds to the centroid elution volume of the leading boundary which is normalized by $V_t = V_p + V_0$, since several columns had been used. V_p is the pore volume of the gel, V_0 the void volume. From curves 1 and 2 the dimerization constants $K_{1,2}$ (4.5 ± 0.2 ml/g) and $K_{1,2}$ (1.6 ± 0.1 ml/g) have been calculated for the systems Tris–HCl–KCl and Tris–HCl–KCl–$BaCl_2$; Tris–HCl–KCl–$BaCl_2$–monomer substrate, respectively. This result has been confirmed by sedimentation equilibrium data on the weight-average molecular mass. Thus it was found that inhibitor (Ba^{2+}) shifted slightly the equilibrium of dimerization to monomer, whereas the substrate in its molecular form had no influence on dimerization. The effect of micellar substrate is quite different (curve 3 in Fig. 7). In this case there is no $V_w(C)$ dependence, which is typical for reversible association, and the weight-average elution volume corresponds to dimer (E_2) elution volume, which is significantly higher than the elution volume of the dimer–micelle complex E_2S_n. Two important conclusions may be drawn from this: (1) the dimer–micelle complex is not formed in the presence of Ba^{2+}; (2) in the presence of mi-

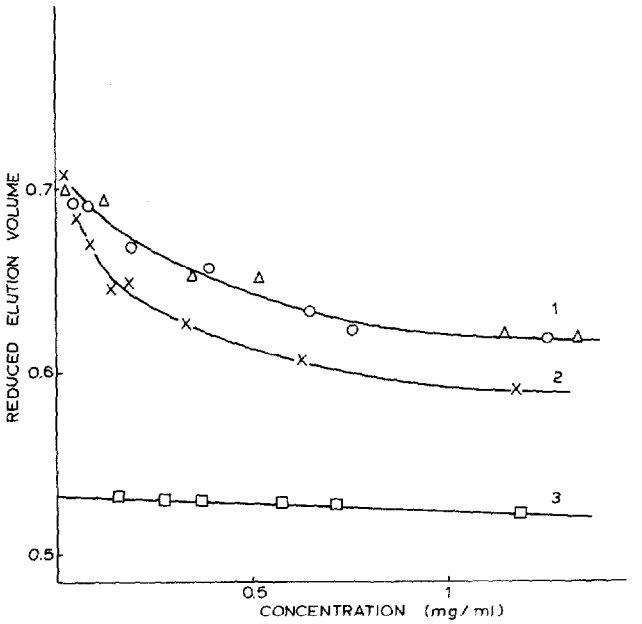

Fig. 7. Concentration dependence of the reduced weight-average elution volume V_w/V_t for phospholipase A_2 (Sephadex G-75 column) obtained in the systems: Tris—HCl—KCl (2, ×); Tris—HCl—KCl—$BaCl_2$ (1, ○); Tris—HCl—KCl—$BaCl_2$—S ([S] = 1 mg/ml < CMC) (1, △); and Tris—HCl—KCl—$BaCl_2$—S ([S] = 20 mg/ml ≥ CMC) (3, □), S = substrate (dihexanoyl-lecithin); CMC = critical micelle concentration.

cellar substrate, the association equilibrium of phospholipase A_2 is entirely shifted towards active dimer.

The constancy of the dispersion coefficient in the examined range of concentration is also indicative of one-component behaviour of dimerized phospholipase A_2 in the system containing substrate micelles.

Thus we arrive at the conclusion that Ca^{2+} and Ba^{2+} promote, or respectively suppress, enzymatic activity at the stage of complex formation, but not at the stage of association. These results, obtained by means of MCSEC, have clarified to some extent the mechanism of catalytic action of phospholipase A_2. In conclusion, the specific character of the MCSEC experiment is worth noting. In our experiments as little as 1 mg of protein was sufficient for a complete study of association equilibrium and kinetics. For detection we used a spectrophotometer with a cell volume of ca. 1 μl. Due to low dead volumes of the cell and connection tubes the contribution of extra-column spreading to total dispersion did not exceed 5%.

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