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DETERMINATION OF DISSOCIATION CONSTANTS OF COMPLEXES OF TRYPSIN AND ITS LOW MOLECULAR WEIGHT INHIBITORS BY AFFINITY CHROMATOGRAPHY IN ZONAL AND FRONTAL ANALYSIS ARRANGEMENT

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The inhibitory constants of complexes of trypsin and its soluble or immobilized inhibitors were determined from volumes in which trypsin emerged from the column of its immobilized inhibitor (*p*-aminobenzamidine coupled through hexamethylenediamine to hydroxyalkyl methacrylate gel, Spheron) eluted by solutions of soluble trypsin inhibitors (benzylamine, benzoyl-L-arginine, *N*-butylamine, benzamidine, and *p*-aminobenzamidine). The values of constants obtained by affinity chromatography in the zonal and frontal analysis arrangement were in good agreement and in accordance with data obtained kinetically. The plot of $1/(V_i - V_0)$ versus $1/K_I$ (determined by zonal analysis) or of V_i versus $K_I(V - V_i)$ (determined by frontal analysis) for identical concentrations of various inhibitors was linear. The fact that the dissociation constant of the complex of trypsin and immobilized *p*-aminobenzamidine ($1.6\text{–}3.7 \times 10^{-6}$ M) is lower than the dissociation constant of the complex of trypsin and free *p*-aminobenzamidine (1.9×10^{-5} M) seems to indicate possibilities of nonspecific adsorption in the binding of trypsin to *p*-aminobenzamidine–NH_2–Spheron.

INTRODUCTION

Affinity chromatography, a method based on the unique property of biologically active compounds to form rigid, specific, and reversible complexes, is used not only for the isolation of these compounds but also in studies on their specific interactions. Affinity chromatography as a tool for the determination of dissociation constants of complexes of enzymes and their inhibitors has been developed mainly by Dunn and Chaiken (1–3) and by Kasai and Ishii (4). Both methods are based on the determination of the volume, in which the enzyme emerges from a column of its immobilized

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inhibitor, eluted by solutions of free inhibitors at various concentrations. Dunn and Chaiken (2) use the zonal analysis type of experiment, whereas Kasai and Ishii (4) use frontal analysis. Both methods permit the determination of dissociation constants of complexes formed by the enzyme and either its free inhibitor, used for its displacement, or the inhibitor immobilized to a solid matrix. There are two advantages to both methods: First, they permit the determination of the dissociation constants of the complex formed by the enzyme and its immobilized inhibitor, which is one of the most important factors in affinity chromatography; and second, if the same inhibitor is used for the preparation of a specific adsorbent and also for specific elution, it is possible to estimate the effect of both the matrix and the attachment from the agreement or disagreement of the determined values.

This study has been designed to compare the dissociation constants of complexes of trypsin and its low molecular weight inhibitors determined by zonal analysis according to Dunn and Chaiken (2), by frontal analysis according to Kasai and Ishii (4), and by kinetic measurements according to Dixon (5). Another aim of this study was to estimate the effect of immobilization of the inhibitor on this interaction with trypsin from the knowledge of the dissociation constants of the complex formed by trypsin and either free or immobilized *p*-aminobenzamidine.

MATERIALS AND METHODS

The hydroxyalkyl methacrylate gels (Spheron 300), particle size 100–200 μm, were prepared as described earlier (6) as well as Spheron with covalently attached hexamethylenediamine (NH_2–Spheron) (7, 8). *p*-Aminobenzamidine was coupled to NH_2–Spheron by soluble carbodiimide according to Hixson and Nishikawa (9). Lyophilized trypsin was supplied by Léčiva (Dolní Měcholupy, Czechoslovakia) and its activity was determined with benzoyl-L-arginine *p*-nitroanilide (BAPA) as substrate according to Erlanger et al. (10) (1.55 μmol/min · mg at pH 8). BAPA, benzylamine, and benzoyl-L-arginine were products of Fluka A. G., Buchs, Switzerland. *p*-Aminobenzamidine was from Serva, FRG, benzamidine-HCl from Aldrich, United States, *N*-butylamine from Merck, FRG.

Adsorption of Trypsin to NH_2-Benzamidine–NH_2–Spheron as Function of pH and Ionic Strength

Trypsin (2.1 mg) was dissolved in 30 ml of individual buffers whose composition is shown in the legend to Fig. 1. The solution was

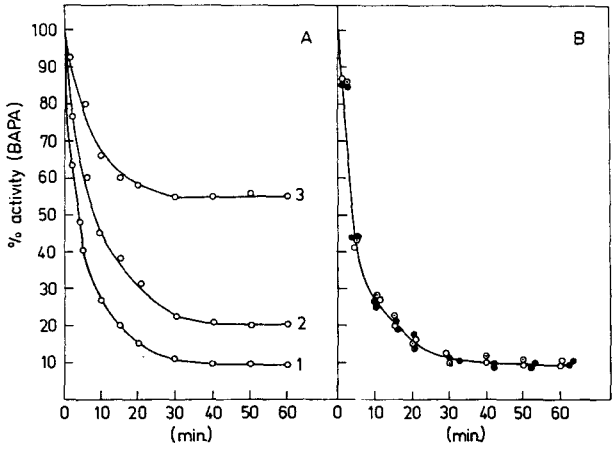

FIG. 1. Adsorption of trypsin to NH_2–benzamidine–NH_2–Spheron: (A) as function of pH: (1) 0.05 M Tris-HCl buffer containing 0.02 M $CaCl_2$, pH 8.0; (2) 0.05 M Tris-maleate buffer containing 0.02 M $CaCl_2$, pH 7.0; (3) 0.05 M Tris-maleate buffer containing 0.02 M $CaCl_2$, pH 6.0 (B) As function of ionic strength: Tris-HCl buffer containing 0.02 M $CaCl_2$, pH 8.0: ●, 0.05 M; ○, 0.1 M; ⊗, 0.2 M; ⊙, 0.5 M.

subsequently mixed with 9 ml of gel slurry (equilibrated in the same buffer). A trypsin solution in the same buffer yet without the addition of the gel served as a control in all experiments. The activity of the suspensions and solutions was determined with BAPA (10) as substrate at fixed time intervals and the quantity of trypsin adsorbed was calculated from the activity value (see Fig. 1A and 1B).

Determination of Working Capacity of NH_2–Benzamidine–NH_2–Spheron Column

Trypsin (5 mg) was dissolved in 1 ml of 0.05 M Tris–HCl buffer, pH 8, containing 0.02 M $CaCl_2$ and applied onto a column of NH_2–benzamidine–NH_2–Spheron (0.8 × 19 cm). The column was eluted by the same buffer. The enzyme was displaced by 0.1 M acetic acid, pH 3.0 (adjusted by ammonia). The weight of desorbed trypsin (working capacity of column) was calculated from the quantity of amino acids (μmol) determined in acid hydrolysate of an aliquot of the desalted, lyohilized fraction by the method of Spackman and co-workers (11).

Determination of Dissociation Constants of Trypsin–Inhibitor Complex According to Dunn and Chaiken (2)

Columns (0.8×19 cm) of NH_2–benzamidine–NH_2–Spheron were equilibrated on 0.05 M Tris-HCl buffer–0.02 M $CaCl_2$, pH 8, of varying inhibitor concentration (for benzylamine, 1.5×10^{-1} to $1.0\times10-3$ M; for benzoyl-L-arginine: 1.5×10^{-2} to 1.0×10^{-3} M; for *N*-butylamine, 1.5×10^{-2} to 1.0×10^{-3} M; for benzamidine and *p*-aminobenzamidine-HCl, 1.0×10^{-3} to 5.0×10^{-5} M). The columns were always charged with the same quantity of trypsin (1 mg dissolved in 0.1 ml of equilibrating buffer) and eluted with the corresponding equilibrating buffer. The activity of the effluent was determined with BAPA (10) as substrate and the absorbance of the effluent measured at 410 nm. Figures 2 and 3 show the determination of the dissociation constants of trypsin with the aid of benzylamine. Between each determination of dissociation constant of individual inhibitors the gel was suspended in 6 M guanidine-HCl and subsequently washed with water until the conductivity of the effluent was the same as that of water. The chromatography was carried out at room temperature.

Determination of Dissociation Constants of Trypsin–Inhibitor Complex According to Kasai and Ishii (4)

Columns (0.8×19 cm) of NH_2–benzamidine–NH_2–Spheron were equilibrated either in 0.05 M Tris–HCl buffer containing 0.02 M $CaCl_2$, pH 8.0, or in the same buffer containing varying inhibitor concentrations (for benzylamine, 1.0×10^{-2} to 1.0×10^{-3} M; for benzoyl-L-arginine, 5.0×10^{-1} to 1.0×10^{-3} M; for *N*-butylamine, 6.2×10^{-2} to 1.0×10^{-3} M; for benzamidine, 1.0×10^{-3} to 1.0×10^{-5} M; for *p*-aminobenzamidineHCl. 1.0×10^{-3} to 1.8×10^{-5} M). The columns were subsequently charged with a trypsin solution (15 mg in 100 ml of equilibrating buffer) until the activity of the effluent was the same as the activity of the trypsin solution (100%) applied to the column. The activity of the effluent was determined again with BAPA as substrate. The effluent volume in which the activity was 50% of original activity was taken for V_i. A example of the determination of the dissociation constants of the complex of trypsin with free benzoyl-L-arginine and immobilized *p*-aminobenzamidine is shown in Figs. 4 and 5. After each determination of dissociation constant with one soluble inhibitor the gel was washed as described in the preceding section. The chromatography was carried out at room temperature.

Determination of Void Volume (V_m) in Column of NH_2–Benzamidine–NH_2–Spheron

A column (0.8 × 19 cm) of NH_2–benzamidine–NH_2–Spheron prewashed with water was charged with a solution of 20 mg of dextran in 0.5 ml of water. The elution volume of dextran was measured in a Knauer 2050 differential refractometer.

Kinetic Determination of Dissociation Constants of Trypsin–Inhibitor Complex According to Dixon (5)

A solution (2.5 ml) of BAPA (final concentration 0.68×10^{-3}, 0.51×10^{-3}, $0.34 - 10^{-3}$, and 0.17×10^{-3} M) in 0.05 M Tris-HCl buffer containing 0.02 M $CaCl_2$, pH 8.0, was mixed with 200 μl of trypsin solution (final concentration 1.62×10^{-7} M) inhibited by a defined inhibitor concentration (for benzylamine 3.4×10^{-4} to 0.85×10^{-4} M; for benzoyl-L-arginine, 4.6×10^{-3} to 1.2×10^{-3} M; for *N*-butylamine, 1.34×10^{-3} to 0.84×10^{-3} M; for benzamidine-HCl, 2.7×10^{-5} to 0.67×10^{-5} M; for *p*-aminobenzamidine-HCl, 7.88×10^{-6} to 1.97×10^{-6} M). The cleavage of BAPA was discontinued after 10 min of incubation at 23°C by the addition of 1 ml of 30% acetic acid in dioxane.

RESULTS AND DISCUSSION

Making use of our previous experience with the isolation of chymotrypsin and trypsin on specific adsorbents prepared by coupling of low molecular weight synthetic inhibitors to Spheron (12, 13) we concentrated in this study on the examination of the effect of support and binding on specific interaction of immobilized *p*-aminobenzamidine with trypsin. The dependence of the quantity of trypsin adsorbed to *p*-aminobenzamidine–NH_2–Spheron on pH (shown in Fig. 1A) and the dependence of the quantity of trypsin adsorbed on ionic strength (shown in Fig. 1B) demonstrate that an equilibrium is achieved in 40 min in all cases. The quantity of trypsin adsorbed to the adsorbent decreases with the decreasing pH and remains practically constant when the molarity of the Tris-HCl buffer lies between 0.05 and 0.5 M.

There are two methods of investigating the specific interaction of the enzyme with its immobilized low molecular weight inhibitor by affinity chromatography. Dunn and Chaiken (1, 2) derived equations for the calculation of dissociation constants of enzyme complexes with their free (K_I) or immobilized (K_L) inhibitors based on the zonal arrangement of

affinity chromatrography. Similar equations based on the frontal analysis arrangement of affinity chromatography were derived by Kasai and Ishii (4). We compared both methods.

Figure 2 shows elution curve profiles obtained by affinity chromatography of trypsin on columns of *p*-aminobenzamidine–NH_2–Spheron according to Dunn and Chaiken (2). The column of the specific adsorbent was equilibrated with alkaline buffer of varying inhibitor concentrations, in this case of benzylamine. The columns equilibrated in this manner were always charged with the same quantity of trypsin. Trypsin was eluted by the equilibrating buffer. As can be seen in Fig. 2, the lower the inhibitor concentration of the buffer, the higher was the volume in which the enzyme emerged; the protein peak broadened simultaneously. By contrast, increasing inhibitor concentrations led to a decrease of the elution volume down to a value at which the volume did not decrease any further with the increasing inhibitor concentration. This volume is designated V_0 and represents the effluent volume of the enzyme when there is no interaction between trypsin and immobilized *p*-aminobenzamidine. The volume of V_i for individual inhibitor concentrations was measured when the effluent activity was maximum. Figure 3 shows the plot of $1/(V_i - V_0)$ versus

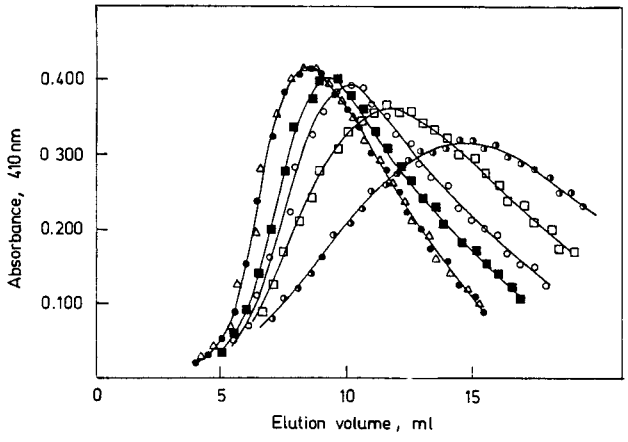

FIG. 2. Chromatography of trypsin on NH_2-benzamidine–NH_2–Spheron. Different benzylamine concentrations in eluting buffer were used in the experiment carried out according to Dunn and Chaiken (2). To the column (0.8 × 19 cm) equilibrated with 0.05 M Tris–HCl buffer containing 0.02 M $CaCl_2$, pH 8.0, and benzylamine in the following concentrations: ● 1.5×10^{-1} M; △, 9.0×10^{-2} M; ■, 1.0×10^{-2} M; ○, 5.0×10^{-3} M; □, 2.5×10^{-3} M; ◑, 1.0×10^{-3} M, was always applied the same quantity of trypsin (1 mg); the latter was eluted from the column by the corresponding equilibrating buffer. Fractions of 0.5 ml were collected at 5-min intervals. The elution profile obtained with 7.5×10^{-3} M benzylamine is not shown in the figure for the sake of clarity.

inhibitor concentration used for elution $[I_0]$ which is linear as expected according to the equations described by Dunn and Chaiken (2). The slope of the line is given by the term $1/K_I A$ where $A = (V_0 - V_m) \times (L/K_L)$. The intercept on the $1/(V_i - V_0)$ coordinate is $1/A$. In this case we obtained the value of the dissociation constant of the trypsin–benzylamine complex ($K_I = 3.1 \times 10^{-4}$ M) from the ratio of intercept to slope. When 9.0×10^{-2} and 1.5×10^{-1} M solutions of benzylamine were used, V_0 for trypsin was 8.5 ml. The void volume ($V_m = 4.0$ ml) was determined by chromatography of dextran on *p*-aminobenzamidine–NH_2–Spheron. The concentration of immobilized *p*-aminobenzamidine $[L] = 1.2 \times 10^{-5}$ M was calculated from the working capacity of the *p*-aminobenzamidine–NH_2–Spheron column estimated in earlier experiments. The value of $1/A$ was read on the $1/(V_i - V_0)$ coordinate and inserted into $K_L = (V_0 - V_m)[L]/A$; thus the dissociation constant of the complex of trypsin and immobilized *p*-aminobenzamidine, $K_L = 1.9 \times 10^{-6}$ M, was obtained. In an analogous manner trypsin was eluted from the *p*-aminobenzamidine–NH_2–Spheron column by solutions of benzoyl-L-arginine, *N*-butgylamine, benzamidine, and *p*-aminobenzamidine of different concentrations. The plot of $1/(V_i - V_0)$ versus inhibitor concentration $[I_0]$ was linear in all cases. The values of the K_I and K_L-constants are given in Table 1.

The elution profiles obtained by affinity chromatography of trypsin on the *p*-aminobenzamidine–NH_2–Spheron column by the method of Kasai and Ishii (4) are shown in Fig. 4. The column of the specific adsorbent was equilibrated either with the alkaline buffer only or with the buffer containing different concentrations of the inhibitor (benzoyl-L-arginine in the example given). A trypsin solution in the corresponding equilibrating

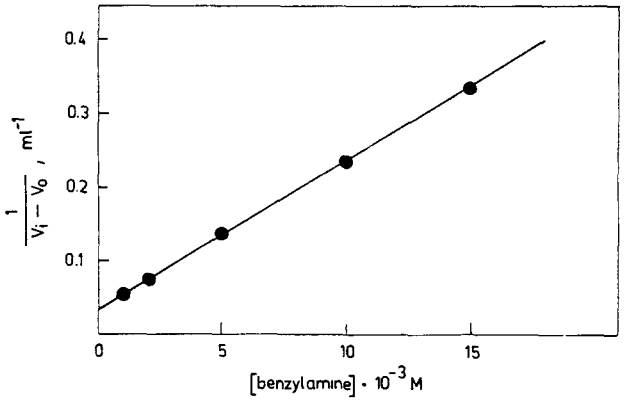

FIG. 3. Plot of $1/(V_i - V_0)$ (ml^{-1}) versus molar concentration of benzylamine in eluent. The data given in Fig. 2 were used.

TABLE 1. **Dissociation Constants of Trypsin Complexes K_I and K_L Determined by Affinity Chromatography on *p*-Aminobenzamidine–NH_2–Spheron**

Free inhibitor	Dunn and Chaiken (2)		Kasai and Ishii (4)		Dixon (5)	Published values
	K_I (M)	K_L (M)	K_I (M)	K_L (M)	K_I (M)	K_I (M)
Benzoyl-L-arginine	5.3×10^{-3}	1.7×10^{-6}	5.5×10^{-3}	3.7×10^{-6}	5.6×10^{-3}	$5.8+10^{-3}$ (14)
Benzylamine	3.1×10^{-4}	3.2×10^{-6}	5.0×10^{-4}	3.7×10^{-6}	4.5×10^{-4}	6.0×10^{-4} (15)
N-Butylamine	1.7×10^{-3}	1.9×10^{-6}	3.7×10^{-3}	3.7×10^{-6}	1.8×10^{-3}	1.7×10^{-3} (15)
Benzamidine	6.7×10^{-5}	1.9×10^{-6}	5.2×10^{-5}	3.7×10^{-6}	1.8×10^{-5}	1.8×10^{-5} (16)
p-Aminobenzamidine	1.9×10^{-5}	1.6×10^{-6}	1.9×10^{-5}	3.7×10^{-6}	8.0×10^{-6}	8.3×10^{-6} (17)

buffer was continuously applied to the equilibrated column of *p*-amino-benzamidine–NH_2–Spheron until the activity of the enzyme solution emerging from the column equaled the activity of the solution applied. When affinity chromatography is carried out in the frontal analysis arrangement V_i equals the elution volume at which the enzyme concentration is half that of the plateau. With the increasing concentration of the inhibitor in the elution buffer, V_i decreased down to V_0, i.e., to the volume which did not change with the increasing concentration $[I_0]$. When trypsin was eluted by 3.5×10^{-1} M benzoyl-L-arginine the V_i value was the same as that obtained by the elution with 5×10^{-1} M benzoyl-L-arginine; the V_0 value was 8.5 ml. There was no interaction between trypsin and its immobilized inhibitor at this concentration. The V value was obtained by chromatography of trypsin on the same column yet in the absence of the inhibitor. As shown in Fig. 4, $V = 41$ ml. Using the values shown in Fig. 4, V_i was plotted versus $(V - V_i)/[I_0]$ (see Fig. 5). The plot was linear, as expected from the equations derived by Kasai and Ishii (4). The value of the dissociation complex of trypsin and benzoyl-L-arginine, $K_I = 5.5\times10^{-3}$ M, was calculated from the formula $K_I = (V_i - V_0)/(V - V_i)\times[I_0]$. The advantage of frontal analysis is that once we know the V and V_0 values for the given column of immobilized inhibitor, we can determine the K_I values of the remaining free inhibitors from one single chromatography run using the equation just given. If $[E_0] \ll K_L$, the

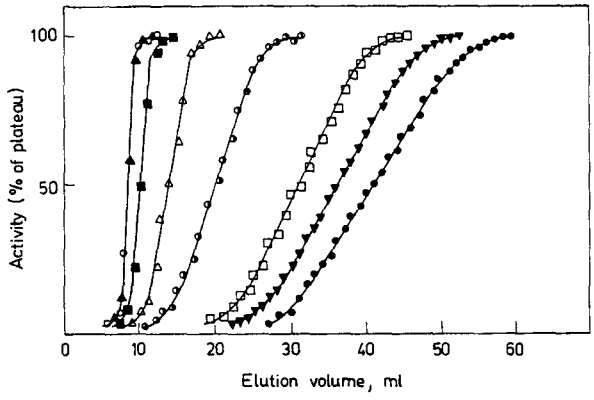

FIG 4. Chromatography of trypsin on NH_2–benzamidine–NH_2–Spheron. Different benzoyl-L-arginine concentrations were used in the experiment carried out according to Kasai and Ishii (4). The column was equilibrated with 0.05 M Tris-HCl buffer + 0.02 M $CaCl_2$, pH 8.0, containing benzoyl-L-arginine in the following concentrations: ○, 5.0×10^{-1} M; ▲, 3.5×10^{-1} M; ■, 12.4×10^{-2} M; △, 2.7×10^{-2} M; ◑, 10.5×10^{-3} M; □, 2.5×10^{-3} M; ▼, 1.0×10^{-3} M. The trypsin solution (15 mg/100 ml) in the equilibrating buffer was then continually absorbed to the column. The fractions were collected at 5 min intervals.

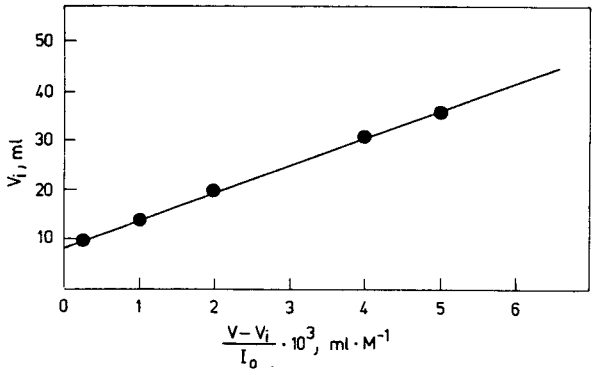

FIG. 5. Plot of elution volume V_i (ml) versus $(V - V_i)/[I_0]$. The data given in Fig. 4 were used.

dissociation constant of the complex of trypsin with immobilized *p*-aminobenzamidine can be calculated from the equation $K_L = L_t/(V - V_0)$, where L_t is the total quantity of efficient immobilized inhibitor determined from the working capacity of the adsorbent (in our case $L_t = 0.12$ μmol). When we arrived at $V = 41$ ml and $V_0 = 8.5$ ml, we calculated the dissociation constant of the complex of trypsin and immobilized *p*-aminobenzamidine, $K_L = 3.7 \times 10^{-6}$ M. As follows from the formula used for its calculation, the dissociation constant does not depend on the free inhibitor used. Since in our case it did not hold fully that $[E_0] \ll K_L$, the K_L value obtained by frontal analysis is in good agreement with the data obtained according to Dunn and Chaiken (2). Trypsin was chromatographed in an analogous manner on the *p*-aminobenzamidine–NH_2–Spheron column in the presence of different concentrations of benzylamine, *N*-butylamine, benzamidine, and *p*-aminobenzamidine. When V_i was plotted versus $(V - V_i)/[I_0]$, a straight line was obtained in all cases. The values of the K_I and K_L determined are shown in Table 1.

As follows from Table 1 the K_I and K_L values determined by both methods are in good agreement. The table also shows the dissociation constants of complexes of trypsin with free inhibitors determined kinetically according to Dixon (5). The last column shows the corresponding K_I values recorded in the literature (14–17). The K_L value of the complex of trypsin and immobilized *p*-aminobenzamidine ($K_L = 1.6 \times 10^{-6}$ and 3.7×10^{-6} M, respectively) is lower than the K_I value of the complex of trypsin and free *p*-aminobenzamidine ($K_I = 1.9 \times 10^{-5}$ M); this shows the positive effect of adsorbent during adsorption. Similar results were also obtained by Kasai and Ishii (4) who examined the adsorption of trypsin to glycyl-glycyl-arginine-Sepharose. By contrast, Dunn and Chaiken (2) studying affinity

chromatography of nuclease thymidine 3′-(*p*-Sepharose-aminophenyl phosphate)-5′-phosphate obtained practically the same K_I and K_L values, showing that the interaction of the enzyme with the inhibitor was not affected either by the support or by the coupling method. The K_L values determined by zonal analysis vary over the range 1.6×10^{-6} to 3.2×10^{-6} M, which demonstrates that the value of the dissociation constant of trypsin is independent of the nature of the inhibitor used for elution. The equation used for the calculation of the K_L value from frontal analysis contains terms characterizing only the total quantity of efficient immobilized inhibitor and the V and V_0 values which are necessarily identical for any soluble inhibitor; therefore, only one value, K_L, has to be calculated in such a case.

As shown by the data in Fig. 1B, the behavior of trypsin was different from that of ribonuclease on the uridine-5′-(Sepharose-4-azminophenyl-phosphoryl)-2′-(3′-phosphate) (3) and its elution volume did not change in the molarity range of Tris-HCl buffer 0.05–0.5 M.

Figures 6 and 7 show the plot of dissociation constants K_I of complexes of trypsin and the inhibitors studied versus elution volumes obtained with identical (1 mM) concentrations of the corresponding inhibitors. As can be seen in Fig. 6 when the values obtained by zonal analysis are used in the $1/(V_i - V_0)$ versus $1/K_I$ plot a straight line is obtained, as

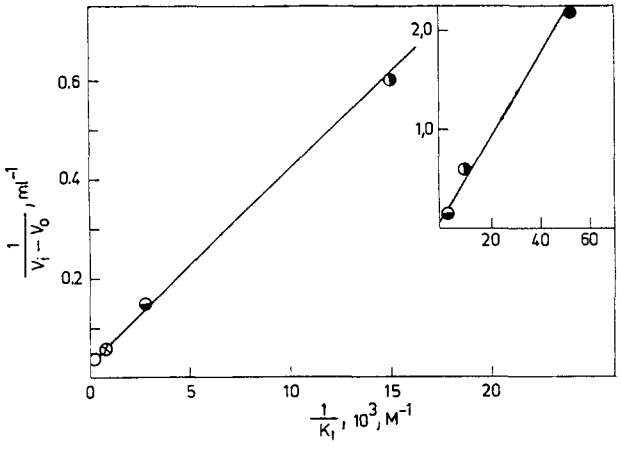

FIG. 6. Plot of $1/(V_i - V_0)$ versus $1/K$, where K_I and V_i were determined for the individual inhibitors according to Dunn and Chaiken (2). V_i stands for elution volume of trypsin displaced by 1×10^{-3} M solutions of the followsing inhibitors: ●, *p*-amino-benzamidine; ◑, benzamidine; ◒, benzylamine; ⊗, butylamine; ○, benzoyl-L-arginine.

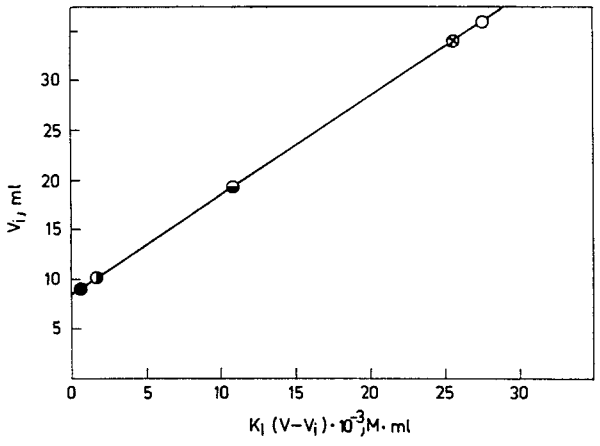

FIG. 7. Plot of V_i versus $K_I(V - V_i)$ where V_i and K_I were determined for the individual inhibitors according to Kasai and Ishii (4). V_i stands for elution volume of trypsin displaced by 1×10^{-3} M solutions of the following inhibitors: ●, *p*-amino-benzamidine; ◑, benzamidine; ◒, benzylamine; ⊗, butylamine; ○, benzoyl-L-arginine.

when the V_i values are plotted versus $K_I \times (V - V_i)$ obtained by frontal analysis (Fig. 7). If, therefore, we have a column of immobilized inhibitor for which the corresponding curve has been constructed, we can read from the graph the dissociation constants of the complex formed by the enzyme and any inhibitor provided the proper inhibitor concentration has been used for the elution. The linear course of the experimental plots also shows that we are justified to use the equations derived over the entire range of dissociation constants.

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