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SOME THEORETICAL ASPECTS OF AFFINITY ELECTROPHORESIS

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SUMMARY

The applicability of affinity electrophoresis to the determination of dissociation constants of protein–ligand complexes is demonstrated for conditions more general than those originally adopted (Hořejší *et al.*, *Biochim. Biophys. Acta*, 499 (1977) 290–300). In particular, the effects of incomplete immobilization of the "immobilized" ligand, the mobility of the protein-free ligand complex, the effect of protein concentration and the kinetics of the protein–ligand complex formation are treated in detail. It is shown that dissociation constants can be determined even when these complicating factors are not negligible; in fact, in some cases these factors may be useful for obtaining additional information about the complex. The applicability of affinity electrophoresis to the study of the kinetics of slow protein–ligand reactions is demonstrated. The interaction of lectins with sugars in this system can be considered very fast, and its kinetics has practically no effect on the dissociation constant determination.

INTRODUCTION

The principle of affinity electrophoresis in polyacrylamide gel was used first by Takeo and Nakamura for the quantitative study of phosphorylase interaction with glycogen[1]. Later we used affinity electrophoresis on O-glycosyl polyacrylamide gels as a means of identification of lectins in a complex mixture[2]. After refinement and simplification of this technique, we developed it into a versatile method applicable to the quantitative study of lectin–sugar[3–5] and enzyme–inhibitor[6] interactions.

A variant of this method using agarose gel instead of polyacrylamide gel is being currently developed by Bøg-Hansen and his co-workers, and seems to be very promising for the study of macromolecule–macromolecule interactions[7–9].

Important features of our improved method[3,4] are:

(a) Immobilization of the ligand in the gel is achieved by preparation of a suitable macromolecular derivative (*e.g.*, soluble O-glycosyl polyacrylamide copolymers[3–5,10] or coupling to a dextran of high molecular weight[6]). This macromolecular derivative is then added in the desired amount to the polymerization mixture used for the preparation of polyacrylamide gels. After completion of polymerization the macro-

molecular derivative which carries the ligand is presumably "immobilized" by physical entrapment into the gel network.

(b) In addition to the immobilized ligand, a free ligand (in general different from the immobilized one) can also be incorporated into the gel. The interaction with free ligand counteracts the retardation caused by immobilized ligand, so that not only the dissociation constant of the protein-immobilized ligand complex (K_i) but also that of the protein-free ligand complex (K) can be easily estimated from the dependence of mobility on the concentrations of both the immobilized and the free ligand[3].

(c) The applicability of this method and especially the evaluation of the results (dissociation constant estimation) are based on several assumptions:

(i) The concentration of protein in the migrating zone is much lower than concentration of immobilized or free ligand.

(ii) The mobility of the protein-free ligand complex is identical with that of the free protein.

(iii) The immobilization of the ligand by means of its macromolecular derivative is complete, so that the protein-immobilized ligand complex has zero mobility.

(iv) The complex formation is very fast in comparison with the movement of the protein band in the gel.

(v) The basic eqn. 1 used for the evaluation of K_i and K in our simple system[3] was derived from an analogous equation used for the description of a similar affinity chromatographic system[11], not directly from the notion of the mechanism of the electrophoretic system.

$$\frac{d}{d_0 - d} = \frac{K_i}{c_i}\left(1 + \frac{c}{K}\right) \tag{1}$$

The present communication is an attempt to investigate the applicability of affinity electrophoresis to cases where some of the above assumptions are not valid and to evaluate the effect of violation of these assumptions on the values of the dissociation constants obtained.

SYMBOLS

a	total protein concentration in the moving zone (M).
$(a)_{ac}$	actual concentration of free protein in the moving zone (M).
c	total concentration of "free" ligand (low molecular-weight) in the affinity gel (M).
$(c)_{ac}$	actual concentration of free ligand within the moving zone (M).
c_i	total concentration of "immobilized" ligand (in the form of macromolecular derivative) in the affinity gel (M).
$(c_i)_{ac}$	actual concentration of "immobilized" ligand (uncomplexed) within the moving zone (M).
d	distance travelled by the protein band from the start during the whole electrophoretic experiment (time t_0) in the affinity gel; *i.e.*, mobility in the affinity gel (mm).
d_0	distance travelled by the protein band from start during time t_0 in the control (non-interacting) gel; *i.e.*, mobility in the control gel (mm).

d_{eq} mobility of the protein band in affinity gel under equilibrium conditions (very fast reaction or preincubation of the protein with ligand before electrophoresis) (mm).
Δd difference of mobilities of the protein band under non-equilibrium and equilibrium conditions (mm).
D mobility of the protein-free ligand complex (*i.e.* the distance reached by this complex during time t_0) (mm).
D_i mobility of the protein–"immobilized" ligand complex (mm).
k_1 rate constant of the protein–ligand complex formation (M^{-1} sec^{-1}).
K dissociation constant of the protein-free ligand complex (M).
K_i dissociation constant of the protein–immobilized ligand complex (M).
K_i^{-1} equilibrium (association) constant of the protein–immobilized ligand complex (M^{-1}).
t time (t_1, t_2, t_3 specified in the text) (sec).
t_0 duration of the electrophoretic experiment (sec).
v_0 rate of electrophoretic movement of the protein zone in the control (non-interacting) gel (mm sec^{-1}).
x abscissa.
y ordinate.
$[X]$ concentration of the protein-free ligand complex (M); also used for the complex alone.
$[X_i]$ concentration of the protein–immobilized ligand complex (M); also used for the complex alone.

Dissociation constants were used rather than equilibrium (association) ones because most of the equations are then obtained in a more compact form.

RESULTS AND DISCUSSION

Independent derivation of eqn. 1

During the entire affinity electrophoretic experiment (time t_0) the protein zone moves a distance d_0 in the control gel and a distance d ($d < d_0$) in the affinity gel containing immobilized (c_i) and free ligand (c). When the above-mentioned simplifying assumptions hold, the time t_1 spent by each protein molecule either in the free state or in the complex with free ligand is $t_1 = d/d_0 \cdot t_0$ and the time spent in the complex with immobilized ligand is $t_2 = t_0(d_0 - d)/d_0$. Thus, at any moment of the separation, eqns. 2 and 3 are valid:

$$\frac{(a)_{ac} + [X]}{[X_i]} = \frac{t_1}{t_2} = \frac{d}{d_0 - d} \qquad t_1 + t_2 = t_0 \tag{2}$$

$$K_i = \frac{(a)_{ac} \cdot (c_i)_{ac}}{[X_i]} \qquad K = \frac{(a)_{ac} \cdot (c)_{ac}}{[X]} \tag{3}$$

If $c_i \gg a \ll c$, then $(c_i)_{ac} \doteq c_i$ and $(c)_{ac} \doteq c$, and eqns. 3 can be written as

$$K_i = \frac{(a)_{ac} \cdot c_i}{[X_i]} \qquad K = \frac{(a)_{ac} \cdot c}{[X]} \tag{3a}$$

Putting $[X_i]$ and $[X]$ from eqns. 3a into eqn. 2 yields directly eqn. 1:

$$\frac{d}{d_0 - d} = \frac{K_i}{c_i}\left(1 + \frac{c}{K}\right) \tag{1}$$

Generalization of eqn. 1 for the case when $D \neq d_0$ and $D_i \neq 0$

The equation describing the generalized case can be derived in a way essentially identical with that in the above paragraph. The following equation can be written:

$$d = d_1 + d_2 + d_3 \tag{4}$$

where d_1, d_2 and d_3 are distances travelled in the form of free protein, complex $[X]$ and complex $[X_i]$, respectively. Clearly,

$$d = t_1 \cdot \frac{d_0}{t_0} + t_2 \cdot \frac{D}{t_0} + t_3 \cdot \frac{D_i}{t_0} \tag{4a}$$

$$t_0 = t_1 + t_2 + t_3 \tag{4b}$$

where t_1, t_2 and t_3 are the times spent in the form of free protein, complex $[X]$ and complex $[X_i]$, respectively, and d_0, D and D_i are the mobilities of these species. Again, the ratios of actual concentrations of these forms are proportional to the total times of their existence:

$$\frac{[X_i]}{[X]} = \frac{t_3}{t_2} \qquad \frac{[X_i]}{(a)_{ac}} = \frac{t_3}{t_1} \qquad \frac{[X]}{(a)_{ac}} = \frac{t_2}{t_1} \tag{5}$$

Combination of eqns. 3, 4a, 4b and 5, together with the assumption that $c_i \gg a \ll c$ yields, after several simple algebraic operations, the general eqn. 6:

$$\frac{d}{D - d} = \frac{K_i \cdot D}{K \cdot (DK_i + Dc_i - K_i d_0 - c_i D_i)} \cdot c + \frac{K_i d_0 + c_i D_i}{DK_i + Dc_i - K_i d_0 - c_i D_i} \tag{6}$$

This is again a straight-line equation and, as in the case of eqn. 1 (which is a special case of eqn. 6 for $D = d_0$ and $D_i = 0$) it allows us to estimate both K_i and K (Fig. 1a). The intercept of the straight line with the y-axis yields K_i because d_0 and c_i are known parameters and D_i and D can be determined separately. Similarly, K is given by the intercept of the straight line with the x-axis, which is

$$-X = K\left(\frac{d_0}{D} + \frac{c_i D_i}{K_i D}\right) \tag{6a}$$

It is important that eqn. 6 is valid irrespective of the intrinsic mobility of the immobilized or free ligand. Obviously, in the case of neutral ligands (*e.g.* O-glycosyl polyacrylamide copolymers and sugars) this intrinsic mobility is zero but in the case of charged ligands the intrinsic mobilities will be generally non-zero (positive or negative, depending on pH).

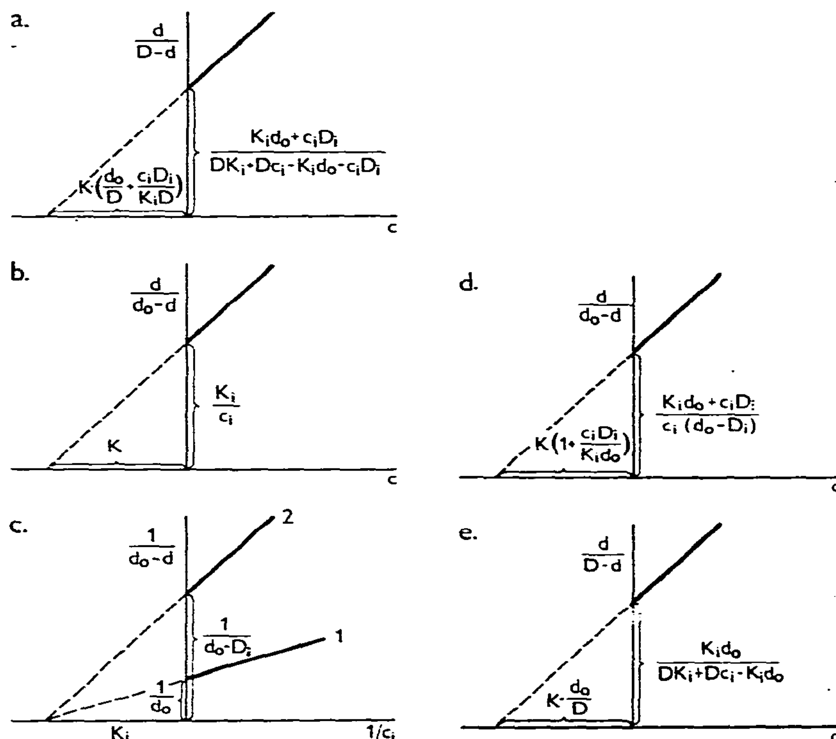

Fig. 1. Graphical estimation of K_i and K. (a) General case (eqn. 6). (b) $D = d_0$, $D_i = 0$ (eqn. 1). (c) $c = 0$ (eqn. 7); line 1; $D_i = 0$; line 2: $D_i > 0$. (d) $D = d_0$, $D_i > 0$ (eqn. 8). (e) $D \neq d_0$, $D_i = 0$ (eqn. 9).

Experimental consequences of eqn. 6. The prerequisite for K_i and K determination in the general case is the knowledge of D_i and D. According to their definitions, D_i can be determined as the limit of d for $c_i \to \infty$ and D as the limit of d for fixed c_i (*e.g.*, zero) and $c \to \infty$. For this purpose eqn. 7 can be used, which allows for an easy estimation of D; similarly, the D value can be obtained by plotting $1/(d_0 - d)$ *vs.* $1/c$ in the gels devoid of immobilized ligand (the controls should contain identical concentrations of substance similar to the free ligand but non-interacting with the protein). Clearly, the differences $(d_0 - d)$ will be usually much lower when measured as a function of c than in the case of dependence on c_i.

Typical experimental results expectable during D_i and D estimation and the relationship between d_0, D, D_i and d are schematically shown in Fig. 2.

The need for preliminary determination of D_i and D might be avoided by using an alternative approach in which d is expressed directly as a non-linear function of c, with known parameters c_i and d_0 and unknown parameters K, K_i, D and D_i. All the unknown parameters can be estimated by some approximating computerized method of fitting several experimental points with this curve. Application of this approach is now under study in this laboratory.

Eqn. 6 can be partially simplified in special cases.

When $D = d_0$ and $D_i = 0$, eqn. 6 becomes eqn. 1 (Fig. 1b).

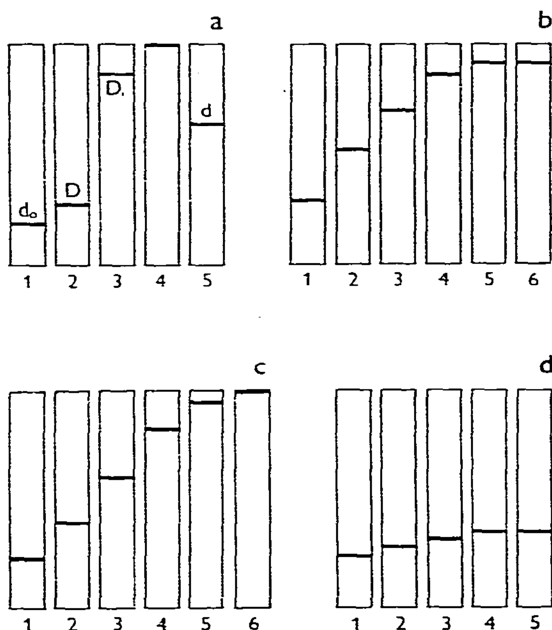

Fig. 2. Schematic representation of the patterns of affinity gels expected under various conditions. All samples were run from the top of gels for identical times (t_0). (a) Comparison of typical magnitude of the mobilities d, d_0, D, and D_i. 1 = Control gel; 2 = gel containing high concentration of the free ligand ($D < d_0$); 3 = affinity gel containing high concentration of immobilized ligand ($D_i > 0$); 4 = the same as under 3, except that $D_i = 0$; 5 = affinity gel containing optimal concentrations of immobilized and free ligand. (b) Estimation of D_i ($D_i > 0$). 1 = Control gel; 2–6 = affinity gels containing increasing c_i. (c) The same as under (b) except that $D_i = 0$. (d) Estimation of D ($D < d_0$). 1 = Control gel; 2–5 = gels containing increasing concentrations of free ligand.

When $c = 0$, an experimentally important case, only the dependence of mobility on c_i is measured and K_i is determined. Eqn. 6 transforms into 6b:

$$\frac{d}{d_0 - d} = \frac{K_i d_0 + c_i D_i}{d_0 c_i - c_i D_i} \tag{6b}$$

which is equivalent to

$$\frac{1}{d_0 - d} = \frac{K_i}{d_0 - D_i} \cdot \frac{1}{c_i} + \frac{1}{d_0 - D_i} \tag{7}$$

This equation is further simplified when $D_i = 0$, *i.e.* in the case of complete immobilization of the ligand:

$$\frac{1}{d_0 - d} = \frac{K_i}{d_0} \cdot \frac{1}{c_i} + \frac{1}{d_0} \tag{7a}$$

Eqn. 7a is a more convenient form of the equations used in our previous studies[3,5,6] for K_i determination.

Experimental consequences of eqn. 7. Eqn. 7 describes the situation when the macromolecular derivative used for immobilization of the ligand is too small to be completely entrapped in the gel network. It is important that regardless of the D_i value, K_i can be determined directly from the intercept of the straight line ($1/[d_0 - d]$ *vs.* $1/c_i$) (Fig. 1c) with the x-axis. The value of D_i is obtained from the intercept of this straight line with y-axis ($1/[d_0 - D_i]$).

Bøg-Hansen and co-workers[7–9] studied the interaction of concanavalin A (Con A) with some glycoproteins, employing the methodology of affinity electrophoresis in agarose gel in a series of agarose gel slabs containing increasing concentrations of Con A. Electrophoresis was done under conditions when the intrinsic mobility of Con A was approximately zero. The retardation of glycoproteins observed on the affinity gels containing Con A was dependent on the Con A concentration. Obviously, this is exactly the case described by eqn. 7. In fact, the possibility of K_i determination regardless of the D_i value was recently mentioned by Bøg-Hansen and Takeo (T. C. Bøg-Hansen, personal communication).

The applicability of eqn. 7 for the quantitative study of protein A–IgG interactions was also confirmed by Zikán[12].

It seems likely that eqn. 7 may be generally useful for the study of macromolecule–macromolecule interactions. In conjunction with the molecular weights of both reacting components D_i should provide information on the stoicheiometry of the complex.

In the case when $D = d_0$ and $D_i \neq 0$, the interaction of the protein with free ligand has only a negligible effect on its mobility and the "immobilized ligand" is not fully immobile.

Eqn. 6 can be written in this case as

$$\frac{d}{d_0 - d} = \frac{K_i \cdot d_0}{Kc_i(d_0 - D_i)}\, c + \frac{K_i d_0 + c_i D_i}{c_i(d_0 - D_i)} \qquad (8)$$

Thus, K_i is determined from the intercept of the straight line with the y-axis and K from the intercept with the x-axis ($-x = K(1 + [c_i\, D_i/K_i\, d_0])$) (Fig. 1d). Unfortunately, also in this case a separate determination of D_i is necessary, and the conclusions reached in the discussion of eqn. 6 hold also for eqn. 8.

In the experimentally important case when $D \neq d_0$ and $D_i = 0$, the immobilization of the ligand is complete but the complex of protein with the free ligand has a mobility different from that of free protein. Eqn. 6 simplifies to:

$$\frac{d}{D - d} = \frac{K_i D}{K(DK_i + Dc_i - K_i d_0)}\, c + \frac{K_i d_0}{DK_i + Dc_i - K_i d_0} \qquad (9)$$

In this case the K value can be determined without knowledge of K_i, but a separate determination of D is again necessary because it appears in the dependent variable and because the intercept of the straight line with the x-axis is equal to $-K \cdot (d_0/D)$ (Fig. 1e).

The effect of protein concentration

The effect of protein concentration in the migrating zone on the value of dis-

sociation constants determined by affinity electrophoresis will be described here only for the simplest case covered by eqn. 7a.

Using the same type of reasoning as above, the following set of equations can be written:

$$\frac{d}{d_0 - d} = \frac{(a)_{ac}}{[X_i]} \qquad K_i = \frac{(a)_{ac}(c_i - [X]_i)}{[X_i]} \qquad (a)_{ac} = a - [X_i]$$

After elimination of $(a)_{ac}$ and $[X_i]$ from these equations we obtain

$$K_i = \frac{d}{d_0 - d} c_i - a \cdot \frac{d}{d_0} \tag{10}$$

which can be rewritten as

$$\frac{1}{d_0 - d} = \frac{K_i}{d_0} \frac{1}{c_i} + \frac{1}{d_0} + \frac{ad}{c_i d_0^2} \tag{10a}$$

Analogously, eqn. 10b can be derived to describe the more general case ($D_i \neq 0$):

$$\frac{1}{d_0 - d} = \frac{K_i}{(d_0 - D_i)} \cdot \frac{1}{c_i} + \frac{1}{d_0 - D_i} + \frac{a(d - D_i)}{c_i(d_0 - D_i)^2} \tag{10b}$$

Eqn. 10a is formally analogous to eqn. 7a, which holds in the cases when the protein concentration can be considered negligible. When a is not negligible its effect will be seen as a non-linearity of the plot $1/(d_0 - d)$ *vs.* $1/c_i$ (Fig. 3a).

However, this non-linearity can be eliminated by rewriting eqn. 10 in the form:

$$\frac{d}{d_0} = \frac{1}{a} \cdot \frac{dc_i}{d_0 - d} + \frac{K_i}{a} \tag{10c}$$

and plotting d/d_0 *vs.* $dc_i/(d_0 - d)$. This straight line yields K_i directly from its intercept with the x-axis (Fig. 3b).

Experimental consequences of eqns. 10a and 10c. Although in most cases the value of a is known approximately, it may be difficult to determine its exact value experimentally owing to stacking and diffusion phenomena during the electrophoresis. Thus, it is advantageous that the effective a value can be obtained directly from eqn. 10b as the tangent of the curve. However, during affinity electrophoresis the phenomenon of "sharpening" of interacting zones in affinity gels is observed. The sharpening increases with c_i and it is a direct consequence of decrease of the time spent by the protein molecules in a free (diffusible) state. Thus, the effective protein concentration will differ to some extent for gels with different c_i. This effect should be corrected by measurement of the zone width and recalculation of protein concentrations. All these complicating factors prompt us to use $a \ll c_i$ whenever possible. This condition is difficult to meet in cases of strong interactions when the lowest applicable protein concentration is limited by the sensitivity of common staining techniques. Sufficient in-

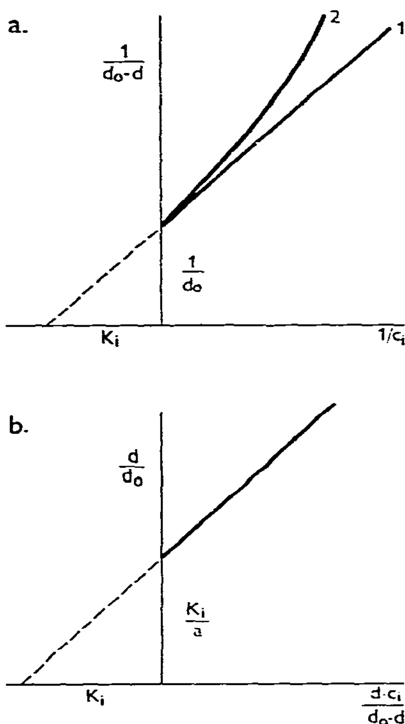

Fig. 3. The effect of protein concentration on K_i determination. (a) Graphical representation of eqns. 7a (line 1) and 10a (curve 2). (b) Estimation of K_i using eqn. 10c.

crease of the detection sensitivity may be achieved, *e.g.* by application of specific enzyme-staining techniques[6] or radioactively labelled proteins[2].

To illustrate the effect of protein concentration, an example from our previous work may be taken[3,4]. In the case of a lectin of mol.wt. 50,000, its concentration during affinity electrophoresis is typically $a = 2.5 \cdot 10^{-5}$ M, whereas c_i are 10–100 times higher. The error caused by neglecting protein concentration is *ca.* 1–10%. This value lies within the limits of experimental error. However, in the case of strong interactions (*e.g.*, *Helix pomatia* lectin with D-GalNAc[3] or enzyme–blue dextran[6]) neglecting the protein concentration may lead to serious errors. Reinvestigation of some of our previous results with respect to the effect of protein concentration on K_i values is now under study in this laboratory.

The effect of the kinetics of complex formation

The effect of the kinetics will be described here again only for the simple case covered by eqn. 7a ($c = 0$, $D_i = 0$, $D = d_0$, protein with single binding site), assuming that $c_i \gg a$, that diffusion and stacking can be neglected and that the zone width is very small. Under these conditions (which can be reasonably met in practice), the kinetics of the protein–immobilized ligand complex formation during the movement of protein zone through the affinity gel is approximately the same as in the case of complex formation within the small vessel with a volume equal to that of the protein zone containing the ligand (c_i). After reaching the equilibrium concentration of the

complex, the ratio $(a)_{ac}/[X_i]$ will be conserved throughout the rest of experiment (movement in the "ocean" of the ligand). Thus, during the later phases of the experiment the separation will proceed under equilibrium conditions so that only a "transition phenomenon" at the beginning of the electrophoretic run may be expected.

Under the above conditions the complex formation is a pseudo-monomolecular reaction described by the differential kinetic eqn. 11:

$$\frac{d[X_i]}{dt} = k_i c_i (a - [X_i]) - k_1 K_i [X_i] \tag{11}$$

which can be integrated to

$$[X_i] = \frac{c_i a}{c_i + K_i} (1 - e^{-(c_i + K_i)k_1 t}) \tag{11a}$$

describing $[X_i]$ as a function of time.

As described above, in our simple case the ratio of the mobilities of a protein zone in the affinity gel (containing only immobilized ligand —c_i) to that in a control gel is generally

$$\frac{d}{d_0} = \frac{t_1}{t_0} = \frac{(a)_{ac}}{a} = \frac{a - [X_i]}{a} \tag{11b}$$

where t_1 is the time spent by a protein molecule in the uncomplexed (free) state and t_0 is the time of duration of the entire electrophoretic experiment.

However, when $[X_i]$ and thus a are functions of time, eqn. 11b holds in its differential form:

$$\frac{dd}{dt} = v_0 \cdot \frac{a - [X_i]}{a}; \qquad v_0 = \frac{d_0}{t_0} \tag{11c}$$

After introduction of the expression for $[X_i]$ from eqn. 11a, this gives the differential eqn. 11d:

$$\frac{dd}{dt} = v_0 \left[1 - \frac{c_i}{c_i + K_i} (1 - e^{-(c_i + K_i)k_1 t})\right] \tag{11d}$$

which can be integrated to

$$d = v_0 t \left(1 - \frac{c_i}{c_i + K_i}\right) + v_0 \cdot \frac{c_i}{(c_i + K_i)^2 k_1} (1 - e^{-(c_i + K_i)k_1 t}) \tag{12}$$

Eqn. 12 describes the distance travelled by the protein zone in an affinity gel as a function of time. When the complex formation is very fast ($k_1 \to \infty$), the second term of this equation becomes negligible and eqn. 12 is transformed into a simple form equivalent to eqn. 7a. As expected, the effect of complex formation kinetics on the experimentally measured d value is such that it causes an increase in comparison with

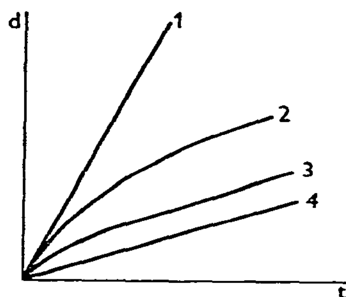

Fig. 4. The effect of kinetics of protein–immobilized ligand complex formation on the electrophoretic mobility in affinity gel (eqn. 12). 1 = Control gel ($c_i = 0$); 2–4 = affinity gels $(k_1)_2 < (k_1)_3 < (k_1)_4 = \infty$).

the experiment performed under equilibrium conditions (Fig. 4). This increase is described by the second term of eqn. 12; the maximum absolute value of this increment is

$$\Delta d_{\max} = v_0 \cdot \frac{c_i}{(c_i + K_i)^2 \, k_1} \qquad (t \to \infty) \tag{12a}$$

However, the relative effect of the kinetics will be at a maximum at the very beginning of the experiment when $\Delta d/d_{eq}$ or $\Delta d/d_0$ is at a maximum. The effect of the kinetics depends primarily on the k_1 value in relation to the K_i value.

Experimental consequences of eqn. 12. The predictible effect of the kinetics of the complex formation on the experimentally measurable d values can be shown by introduction of values into eqn. 12 for a particular case. Thus, in the case of Con A interaction with immobilized α-D-mannosyl residues under the conditions of affinity electrophoresis in an acidic buffer system[3], the following approximate values can be used: $v_0 = 10^{-2}$ mm sec^{-1}, $c_i = 10^{-4}\,M$, $K_i = 4 \cdot 10^{-5}\,M$; the value of k_1 is *ca.* $10^5\,M^{-1}$ sec^{-1} (ref. 13).

These values yield the following approximate corrections of measured d values as compared with the experiment performed under equilibrium conditions: for $t = 1$ sec, 20%; $t = 10$ sec, 2%; $t = 100$ sec, 0.2%; $t = 1$ h, 0.006%.

The reaction can be considered very fast and the effects of the kinetics are not observed. Moreover, the lectin–sugar complex formation is a relatively slow reaction[3] so that in most other cases its effect on the results of affinity electrophoresis need not be considered.

In cases when all effects of the kinetics must be eliminated, it is advisable to prepare the electrophoretic sample of the protein in the medium containing the same concentration of the ligand (in the form of its soluble macromolecular derivative) as that present in the gel. After sufficient incubation time an equilibrium is reached, and the sample can be subjected to electrophoresis under equilibrium conditions.

In the cases of sufficiently slow reactions, eqn. 12 could be used even for the determination of the rate constants of the reaction. For this purpose the K_i value should be determined under equilibrium conditions (preincubation with the ligand), and then the difference Δd between the distances travelled by the protein zone under

the non-equilibrium and equilibrium conditions (at identical t) could be measured as a function of time:

$$\Delta d = \frac{A}{k_1}(1 - e^{-Bk_1t}); \qquad A = \frac{v_0 c_i}{(K_i + c_i)^2}; \qquad B = c_i + K_i$$

which is equivalent to

$$e^{-Bk_1t} = 1 - \frac{\Delta d}{A} k_1 \qquad (12b)$$

This equation can be solved graphically and the root k_1 is obtained as the intercept of the left-hand side exponential function and the right-hand side straight line. For this purpose a single Δd value is sufficient in principle.

Thus, at least in principle, affinity electrophoresis might serve also for the study of the kinetics of molecular interactions. For example, rate constants can easily be measured in the case of a hypothetical complex formation characterized by the same parameters (k_1, d_0) as in the above example (Con A–α-D-mannoside complex) but the K_i being four orders of magnitude lower ($K_i = 4 \cdot 10^{-9}$ M) and consequently also c_i four orders of magnitude lower —10^{-8} M). In such a case, $\Delta d/d_{eq}$ at $t = 1$ h is *ca.* 0.5, *i.e.* a 50% deviation from the equilibrium experiment.

Thus it appears that affinity electrophoresis is applicable under conditions more general than those originally adopted (eqns. 6, 6b, 7, 8, 9, 10a, 10c). The effect of the kinetics of complex formation on the results of affinity electrophoresis is shown at least in a simple case (eqn. 12), and the applicability of affinity electrophoresis to the study of kinetics of complex formation is demonstrated (eqn. 12b).

However, several other problems remain to be solved. First, most of the equations presented here hold exactly only for proteins with a single ligand-binding site. Multiple interactions and their effects, especially on the K_i value, are still to be examined. Also the effect of microscopic non-homogeneity of the immobilized ligand concentration (islets of higher local concentration on the macromolecules of the carrier) has not been treated theoretically or experimentally so far. It would be desirable to develop simple practical methods for solving the general equations and possibly to examine the applicability of other equations (10a, 10c, 12) under more general conditions ($D_i \neq 0$, $D \neq d_0$, $c \neq 0$).

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REFERENCES

1 K. Takeo and S. Nakamura, *Arch. Biochem. Biophys.*, 153 (1972) 1–7.
2 V. Hořejší and J. Kocourek, *Methods Enzymol.*, 34 (1974) 178–181.
3 V. Hořejší, M. Tichá and J. Kocourek, *Biochim. Biophys. Acta*, 499 (1977) 290–300.

4 V. Hořejší, M. Tichá and J. Kocourek, *Biochim. Biophys. Acta*, 499 (1977) 301–308.
5 K. Hauzer, M. Tichá, V. Hořejši and J. Kocourek, *Biochim. Biophys. Acta*, in press.
6 M. Tichá, V. Hořejši and J. Barthová, *Biochim. Biophys. Acta*, 534 (1978) 58–63.
7 T. C. Bøg-Hansen, *Anal. Biochem.*, 56 (1973) 480–488.
8 T. C. Bøg-Hansen, *Abstr. Vol. 10th Intern. Congress Biochem., Hamburg, 1976*, p. 188.
9 T. C. Bøg-Hansen, O. J. Bjerrum and C. H. Brogren, *Anal. Biochem.*, 81 (1977) 78–87.
10 V. Hořejši and J. Kocourek, *Experientia*, 34 (1978) 307.
11 B. M. Dunn and I. M. Chaiken, *Biochemistry*, 14 (1975) 2343–2349.
12 J. Zikán, *Folia Microbiol. (Prague)*, in press.
13 R. M. Clegg, F. G. Loontiens and F. M. Jovin, *Biochemistry*, 16 (1977) 167–174.