DIFFUSION COEFFICIENTS OF PROTEINS IN CARRIER AMPHOLYTE *VERSUS* IMMOBILINE GELS

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SUMMARY

The apparent diffusion coefficients of proteins in carrier ampholyte isoelectric focusing (CA-IEF) and in immobilized pH gradients (IPGs) are strongly dependent on the amount of buffering ions present in the system. However, whereas in CA-IEF increased levels of ampholytes facilitate diffusion, in IPGs they strongly quench it. It is concluded that a protein in an IPG matrix is isoelectric but not isoionic, in the sense that it forms a salt with the surrounding ions bound to the polyacrylamide matrix. This salt formation is beneficial as it greatly increases protein solubility at the pI. It is suggested that, when performing zymograms *in situ*, the IPG gel should contain at least twice the standard amount of Immobiline, so as to keep sharp enzyme bands even with prolonged incubation periods.

INTRODUCTION

In his pioneering work, Catsimpoolas[1,2] proposed the interesting concept of measuring protein diffusion coefficients in polyacrylamide gel isoelectric focusing (IEF) by following kinetically zone spreading during the defocusing stage. Measurements of the variance (σ^2) of a diffusing zone as a function of time yields a linear relationship, the slope of which should correspond to the apparent diffusion coefficient (D) of a given protein. This approach appeared to have several advantages: (a) the concentration distribution of the IEF zone should be Gaussian at zero time, (b) diffusion would start from the smallest possible zone due to focusing and (c) the diffusing species are at their isoelectric point where electrostatic effects are minimal. Notwithstanding the theoretically sound approach, the apparent D values measured in this way were so much larger (*e.g.*, for ovalbumin 100% greater, *i.e.* $14 \cdot 10^{-7}$ cm^2/s *vs.* $7 \cdot 10^{-7}$ cm^2/s in an ultracentrifuge) than the true diffusion coefficients measured in a free-liquid phase that the method was abandoned.

For conventional IEF in presence of carrier ampholytes (CA), Rilbe[3] proposed a plausible model in which, at the steady state, a protein in IEF is both isoelectric and isoionic, the latter term meaning that at the pI the protein is stripped free from any potential ligand, such as the buffer components. In simple terms, this definition

excludes artefactual protein zones due to partial saturation of the macromolecule with the different amphoteric buffers surrounding it. It should be noted that, in conventional zone electrophoresis, proteins are in general assumed to be extensively bound to the buffering groups (phosphate,citrate, borate, etc.), resulting in marked discrepancies between the pIs determined with the two techniques and, in some instances, in spurious bands[4]. Rilbe's model, except for the very few instances in which proteins with peculiar amino acid compositions have been demonstrated to be extensively coated with CAs[5], has been assumed to be generally valid.

With the advent of immobilized pH gradients (IPG)[6], the situation seems to have completely changed. In order to explain some peculiar phenomena of focusing in IPG matrices, such as the very sharp bands in diluted matrices and the remarkable loading ability in preparative runs, we have repeatedly proposed that, in an Immobiline environment, the protein is isoelectric but not isoionic[7-9]. Proteins seem to form a salt with the surrounding polymer coils, a sort of "protein–Immobilinate" or "Immobiline–proteinate", depending on which of the species acts as a carboxyl donor. However strongly we felt that this proposal was valid, we were never able to supply direct proof. Perhaps the original idea of Catsimpoolas of measuring diffusion coefficients could now provide the missing link.

EXPERIMENTAL

Materials

Immobilines, Ampholine (pH 6–8), acrylamide, N,N'-methylenebisacrylamide (Bis), persulphate, N,N,N',N'-tetramethylethylenediamine (TEMED), Gel Bond PAG, a Multiphor 2 chamber, a Multitemp thermostat and a Macrodrive 5 power supply were obtained from LKB (Bromma, Sweden).

Horse heart myoglobin (H.H.Myo) was purchased from Sigma (St. Louis, MO, U.S.A.). Human adult haemoglobin (Hb) was obtained from normal individuals and was purified from minor components (especially glycosylated forms) by preparative IPG[8].

Methods

Conventional IEF. This was performed in 0.5 mm thin gels[10] at 5%T* or 3%T and in 1–4% CA buffers (pH 6–8).

IEF in IPGs. This was run in 0.5 mm thin gels[11] in the pH range 6.5–8.5 with Immobiline concentrations ranging from standard (10 mM buffering ion, 1 ×) to 2 × up to 4 × concentrations in either 3%T or 5%T polyacrylamide gels.

Measurement of apparent diffusion coefficients (D). In all instances IEF and IPG gels were run at the same %T, same electrode distance, same pH range (2 pH units), same focusing temperature and same protein loads. Only the final focusing voltage was different (1500 V in CA-IEF, 2000–2500 V in IPGs) so that our D measurements were biased in favour of CA-IEF (by driving the proteins into sharper bands in IPGs, D should be higher in IPGs than in IEF). As soon as the current was switched off, the gel was placed in an LKB laser scanner (coupled to an Apple II computer) and the main myoglobin [or the haeme-oxidized haemoglobin (Met-Hb)]

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* %T = (grams of acrylamide + grams of Bis)/100 ml.

zones were scanned at regular time intervals at 25°C. For measurements of D, we used the classical equation derived for the ultracentrifuge (Fick's second law), linking the ratio of peak area (A) to peak height (h) to the time and to the diffusion coefficient (D):

$$A^2/h^2 = 4 \pi D t \tag{1}$$

By plotting A^2/h^2 vs. time (t), a line of positive slope is obtained; this slope, divided by 4π, will be the apparent D value. According to Catsimpoolas[2], D is defined as the peak variance (σ^2) as a function of time:

$$D = 1/2 \, (d\sigma^2/dt) \tag{2}$$

On the other hand, as given elsewhere[1], the standard deviation is defined as

$$\sigma = (\text{peak area/peak height})/\sqrt{2} \pi \tag{3}$$

Combination of eqns. 2 and 3 gives

$$D = (A^2/h^2)/(4 \pi t) \tag{4}$$

which is our eqn. 1.

RESULTS

Fig. 1 shows a series of representative scans of diffusing peaks during the defocusing stage in a CA-IEF vs. an Immobiline gel. Both gels contained 5%T, the former 2% Ampholine and the latter the standard 1 × Immobiline concentration (10 mM buffering ion). The latter two concentrations are assumed to be virtually equivalent, as 2% Ampholine has been equated to a 10 mM buffer solution[12]. It can be seen that the myoglobin peak diffuses faster in CA-IEF than IPGs. In fact, by plotting the data according to eqn. 1, $D = 4.2 \cdot 10^{-6}$ cm^2/s is obtained in CA-IEF vs. $D = 2.9 \cdot 10^{-6}$ cm^2/s in the equivalent IPG gel. If our starting hypothesis is correct, progressively increasing Immobiline concentrations should result in parallel decrease in D in IPG gels. As shown in Fig. 2, these expectations were fully confirmed experimentally: when myoglobin was run in a 3%T gel containing Immobiline levels ranging from 1/2 × to 3 ×, D decreased from $3.8 \cdot 10^{-6}$ to $2.2 \cdot 10^{-6}$ cm^2/s. The decreases in D, however, although substantial, levelled off at the 2 × Immobiline concentration. We suggest that perhaps myoglobin, being a rather small protein, would not be a representative model and the binding effect could be minimized by rapid saturation of the protein surface charge by the Immobiline counter ions present in the matrix at the low levels. Therefore the experiments were continued with haemoglobin (in the oxidized form, Met-Hb) at Immobiline concentrations ranging from 1/2 × to 3 × and at two different matrix concentrations, 5%T and 3%T.

As shown in Fig. 3A, the apparent D value of Met-Hb decreases by a much greater factor (three-fold) on going form 1/2 × to 3 × Immobiline, compared with the myoglobin experiment in Fig. 2 (from $D = 2.78 \cdot 10^{-6}$ to $D = 0.88 \cdot 10^{-6}$ cm^2/s in a 3 × gel, 5%T). When the experiments were repeated in 3%T gel, similar data were obtained (Fig. 3B); in fact, except for a small difference in the D value obtained
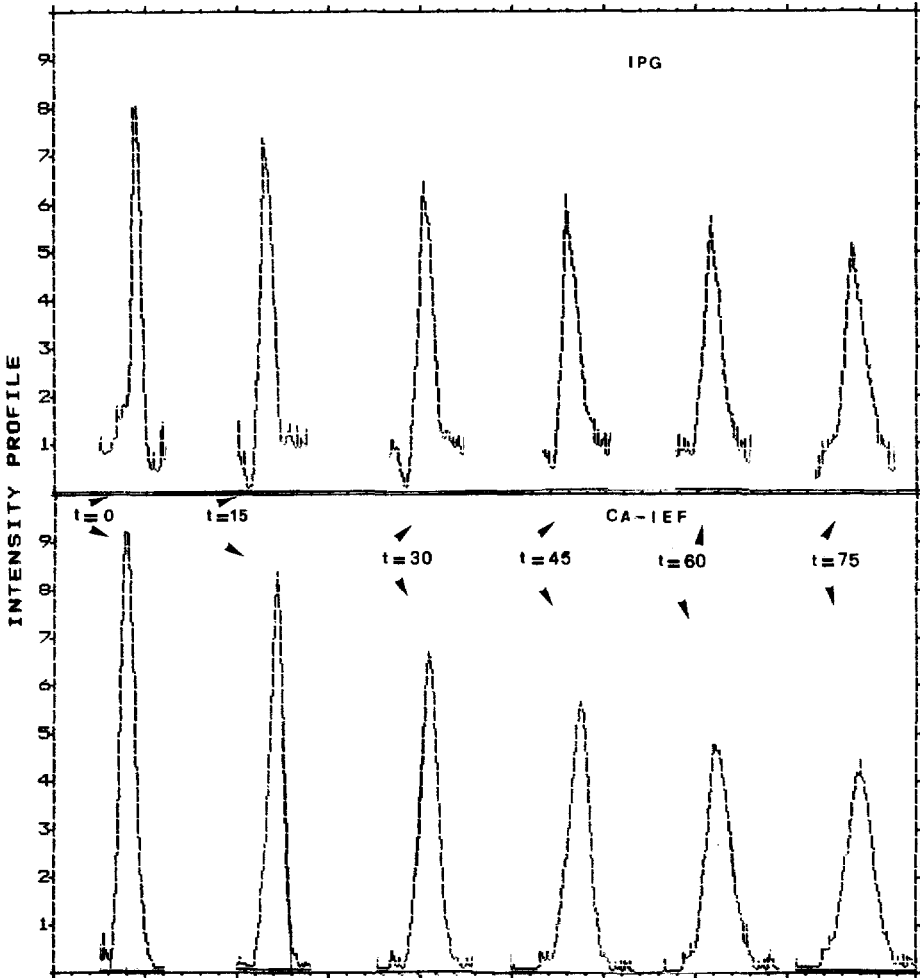
Fig. 1. Representative series of scans of diffusion in the absence of an electric field; 50 μg of myoglobin were loaded in each sample track. upper row: 5%T polyacrylamide gel with 2% Ampholine (pH 6–8), focused at 10°C, 1500 V. Lower row: 5%T polyacrylamide gel with standard Immobiline concentration (1 ×) in the pH range 6.5–8.5. The time of diffusion is marked on each peak. Densitometry with an LKB laser scanner coupled to an Apple II computer.

with 1/2 × Immobiline, the two sets of D values obtained at the two different gel porosities are essentially identical. This further strengthens the hypothesis that diffusion in IPG gels is regulated more by the level of Immobilines than by the relative matrix porosity (see also Discussion). However, when comparing proteins of different size, the apparent D values are clearly dependent on the molecular mass (compare Fig. 2 with Fig. 3B).

The above data are summarized in Fig. 4, which reports the dependence of the apparent D value on the Immobiline concentration in the gel, for two different proteins (myoglobin and Met-Hb) and at two different gel concentrations (5%T and
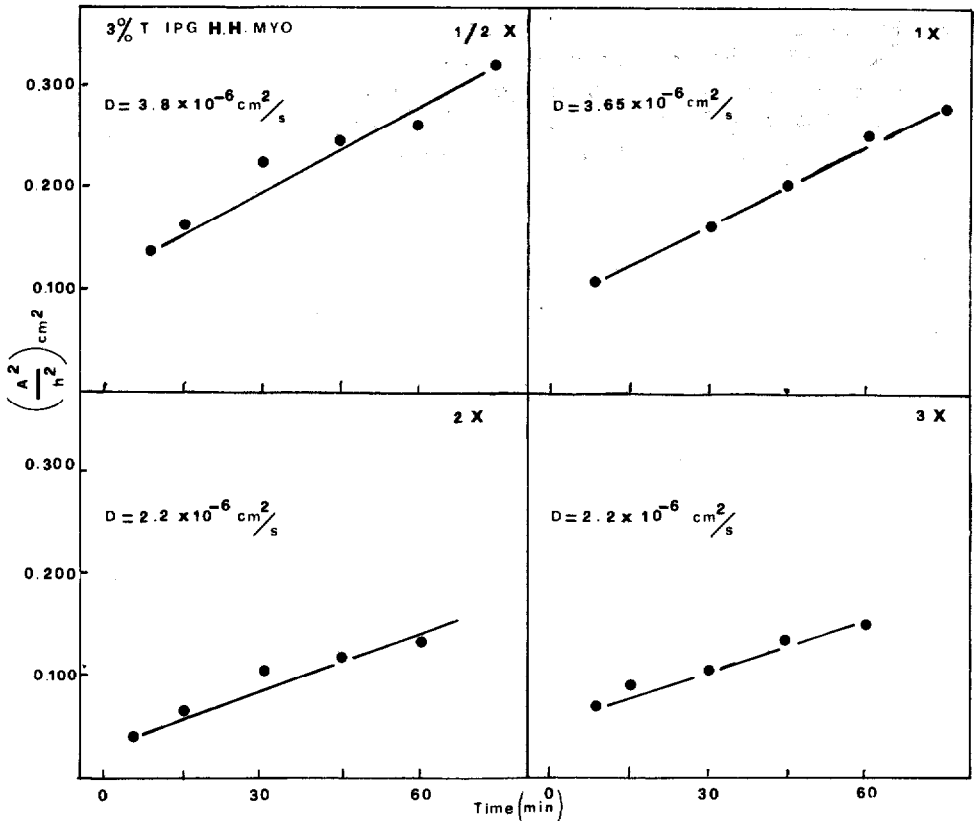
Fig. 2. Measurement of apparent diffusion coefficents (*D*) of horse heart myoglobin (H.H.Myo). A 3%T polyacrylamide matrix was gelled to contain ½ ×, 1 ×, 2 × or 3 × Immobiline concentration in the pH range 6.5–8.5 (for the recipe, see LKB Application Note No. 324; the Immobiline values given in this formulation represent the standard, 1 × molarity). After focusing, diffusion was followed at the times marked on the abscissa (in minutes) with an LKB laser scanner at 25°C. The ordinate represents the ratio of peak area to peak height (A^2/h^2). The slope of the curve, divided by 4 π, gives the apparent diffusion coefficient (*D*). For calculation of *D*, the time scale in the abscissa is converted from minutes to seconds.

3%T). The line of negative slope, in all instances, is again an indication of progressive binding of the different proteins to the IPG matrix.

At this point it was of interest to see how the same proteins would behave in a carrier ampholyte-impregnated gel, *i.e.*, in conventional isoelectric focusing in the presence of amphoteric, non-covalently affixed buffers. In Fig. 1 it was seen that myoglobin diffuses faster in CA-IEF than in IPGs, but that was not quantified. Fig. 5 shows the results of direct measurements of *D* for Met-Hb in the presence of different levels of carrier ampholytes (1, 2, 3 and 4%, but only the 1% and 4% CA data are given for brevity) and at two different gel concentrations (5%T and 3%T). Surprisingly, in all instances the *D* values substantially increase at higher CA levels as though, when switching off the current, the concomitant diffusion of the amphoteric buffers would facilitate rather than hinder the protein diffusional process. This seems to be a general phenomenon; when myoglobin was replaced with Met-Hb, it also

exhibited higher apparent D values at higher CA concentrations (Fig. 6). These data are summarized in Fig. 7; it can be seen that the apparent D measured in carrier ampholyte gels is a line of positive slope as a function of %CA, just the opposite behaviour of D in IPG gels (*cf.*, Fig. 4). Only at low CA levels (1–2%) does there seem to be an apparent levelling off of the diffusion coefficient (lower graphs in Fig. 7).

DISCUSSION

Some interesting practical aspects can be discussed on the basis of the above results.

Apparent diffusion coefficients

We agree with Catsimpoolas[1] that the apparent D measured in polyacrylamide gels does not represent a true diffusion coefficient (*e.g.*, as measured by ultracentrifugation) but at best a "dispersion coefficient". First, to be meaningful our data should be extrapolated to zero gel and zero protein concentrations, whereas we have only extrapolated to vanishing Immobiline and CA molarities. In addition, it is as-
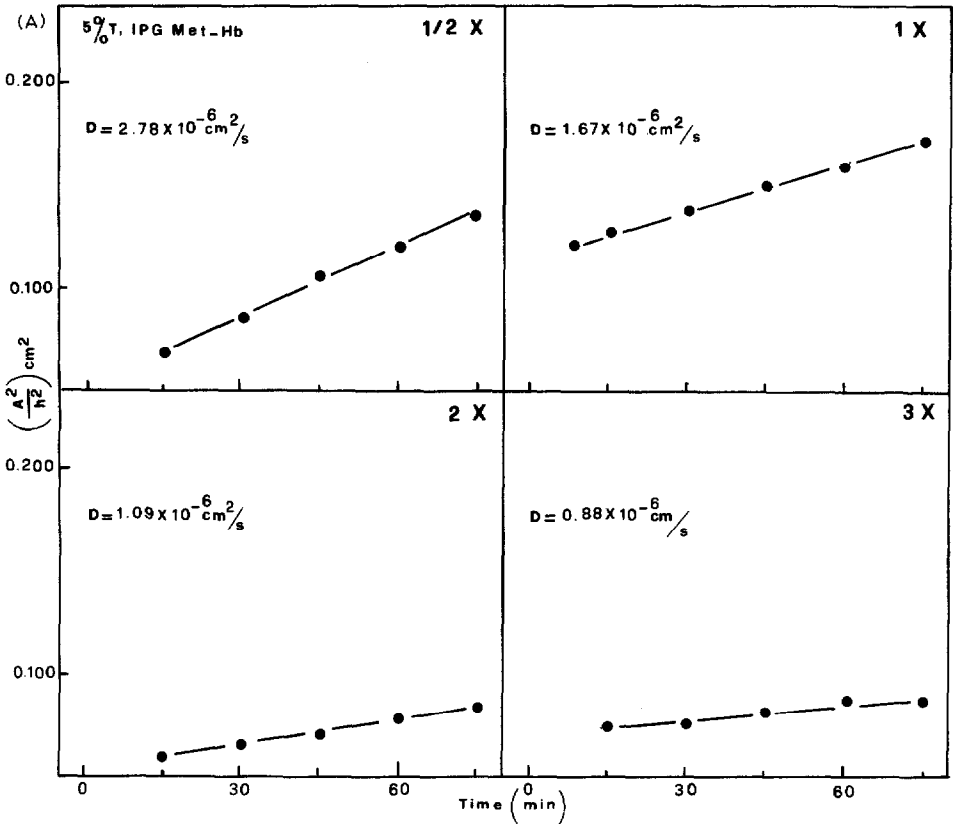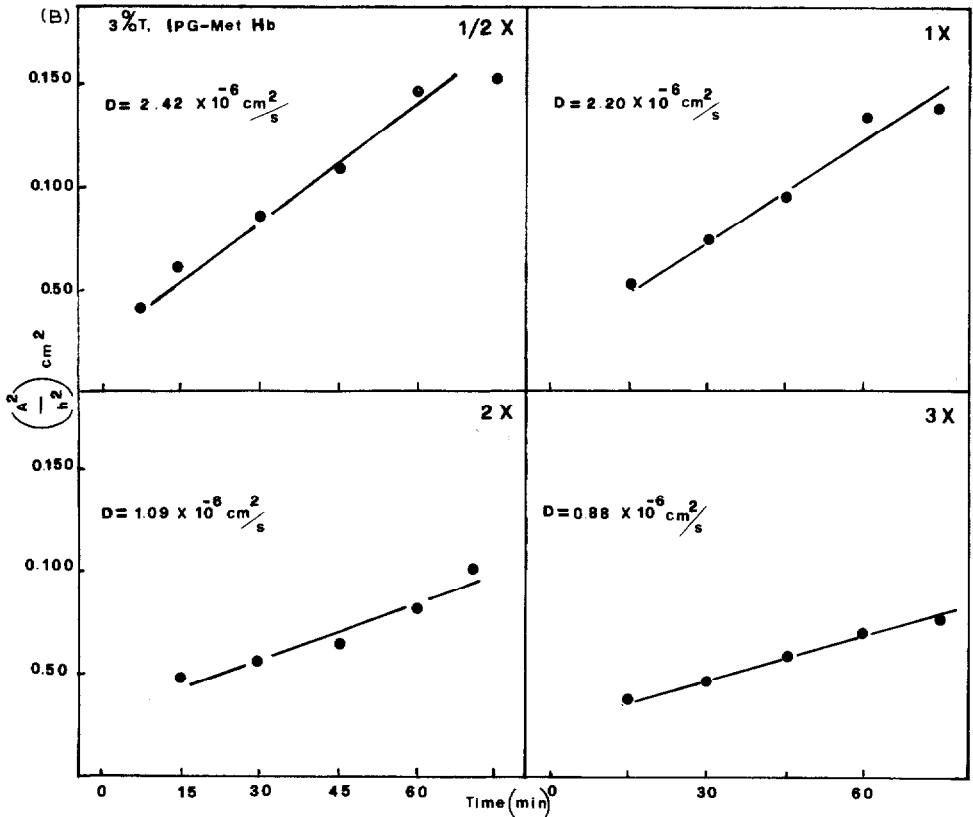

Fig. 3.

Fig. 3. Measurements of apparent diffusion coefficients of oxidized haemoglobin (Met-Hb). (A) The D values are calculated as a function of Immobiline molarity (from $\frac{1}{2}$ × to 3 ×) in a 5%T gel. (B) The D values are derived as a function of Immobiline concentration (from $\frac{1}{2}$ × to 3 ×) in a 3%T matrix. All other experimental conditions as in Fig. 2.

sumed that the viscosity is constant along the diffusion path; although this might be true in IPGs, it can hardly be so in CA-IEF, where the amphoteric buffers are condensed in a linear array of sharp peaks and valleys. In fact, it might be this very uneven CA distribution that is responsible for the unexpected finding of increased D values at higher Ampholine concentrations; once the current is switched off, the highly condensed material packed in the peak could quickly roll down the valley and transport away the protein zone in this process. The transport will be symmetric in both directions, as the chances are that proteins will focus in a valley rather than in a peak. The reasons are as follows. According to Almgren[13], minima of conductivity are found at Ampholine peak maxima, whereas the highest conductivity is at the intersection between adjacent peaks. It follows that, once a protein is in a valley, the low voltage gradient in this region will not facilitate the climb to the top of the peak. In addition, in CA-IEF there will also be substantial differences in osmotic pressure along the diffusion path, as water is drawn in the regions of higher conductivity, which will also be regions of higher ionic strength during the focusing process (or,
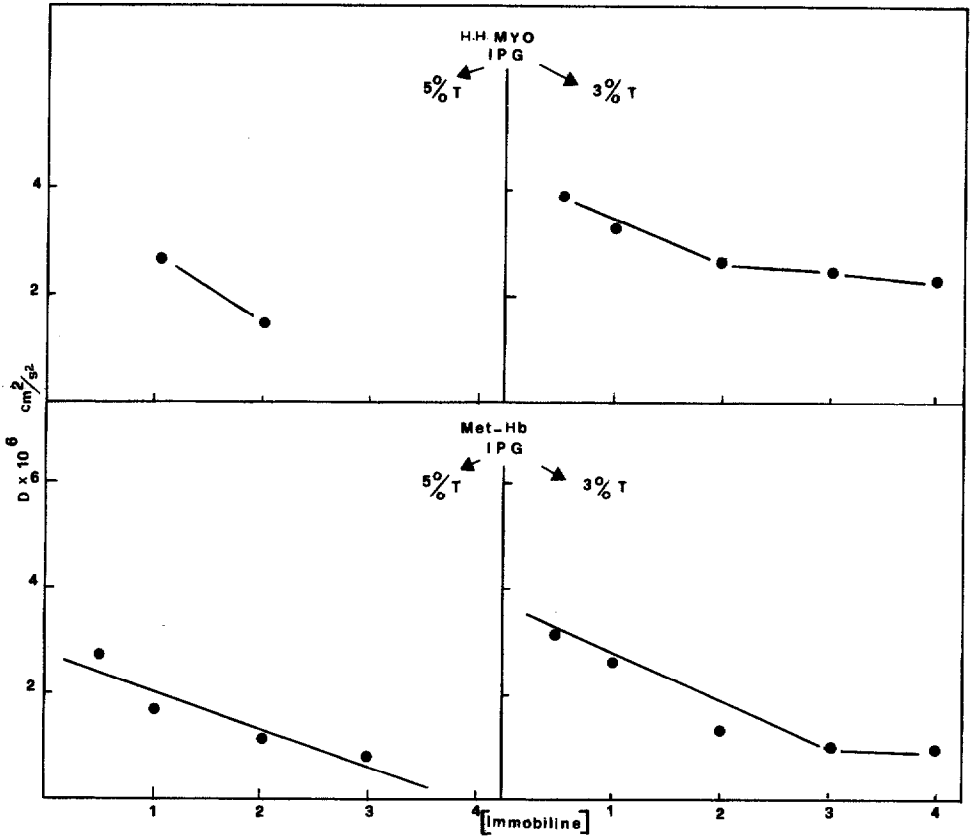
Fig. 4. Dependence of the diffusion coefficients on the Immobiline molarity in an IPG gel. Upper graphs: *D* values of horse heart myoglobin in a 5%T (left) and a 3%T (right) gel. Lower graphs: *D* values of oxidized haemoglobin in a 5%T (left) and a 3%T (right) gel. The *D* values have been derived from Figs. 2, 3A and 3B.

according to another model, water could be accumulated on an Ampholine peak, due to the shuttling back and forth of charged anions and cations in equilibrium with the isoelectric species; they would pick up water away from the p*I* and discharge it in the p*I* region, where they become isoelectric again). Independently of the exact location of the osmotic ridges, there will surely be discontinuities of osmotic pressure along the focusing path.

Concept of isoelectric and isoionic

Perhaps the time has come to untie this intricate knot. We strongly believe that Rilbe[3] was right in his original definition and that the instances in which a protein in CA-IEF has been found coated with the amphoteric buffers, in the p*I* region (away form the p*I* there can be many interactions), are very few.[5] On the other hand, the present experiments clearly demonstrate that in IPGs the protein is isoelectric but not isoionic, in the sense that it forms a salt with the surrounding Immobiline buffers and titrants bound to the polyacrylamide coils. This result in strong
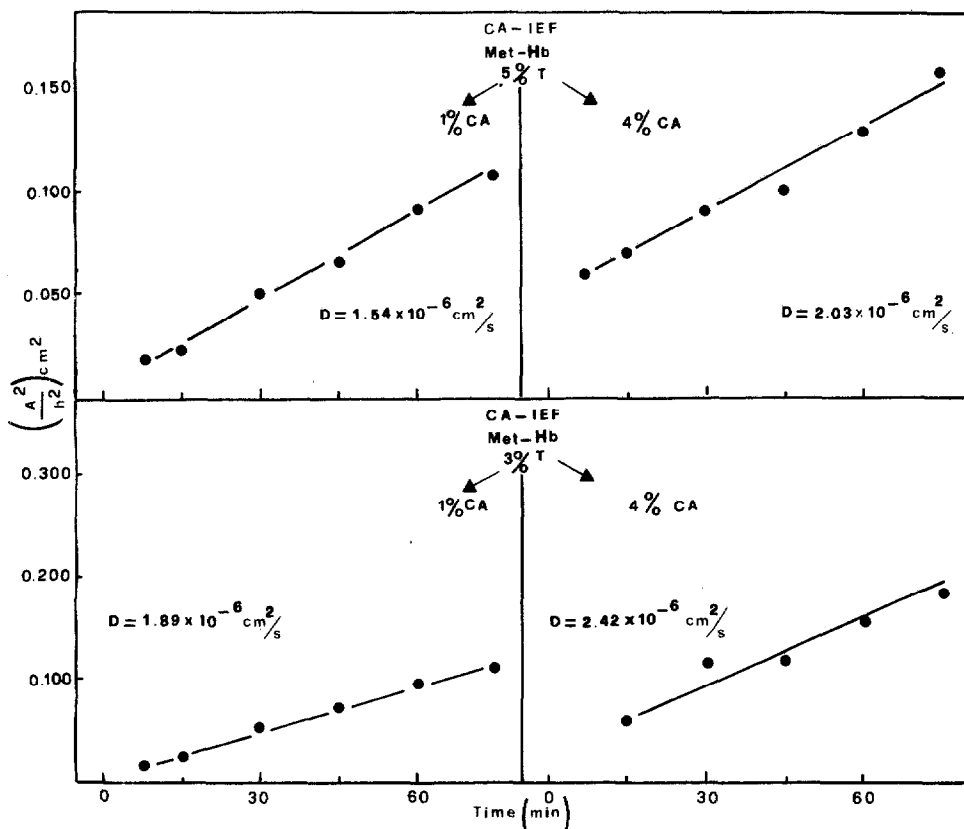
Fig. 5. Measurements of apparent diffusion coefficients of haemoglobin by conventional isoelectric focusing in amphoteric buffers (CA). Upper graphs, in a 5%T gel; lower graphs in a 3%T matrix. Experiments were run in 1%, 2%, 3% and 4% CAs but, for brevity, only the extreme data (1% and 4% CA) are given. All other experimental conditions as in Fig. 2.

quenching of diffusion in the absence of the electric field. Is this good or bad? From an experimental point of view, this is a most positive aspect. We have demonstrated that, under equivalent conditons, IPG matrices can afford a protein load up to 10 times higher than CA-IEF media[7,8], so much so that IPGs were equated to "salting-in" media whereas CA-IEF gels were related more to "salting-out" environments. The reason is now clear: by providing counter ions (different from protons) to an isoelectric protein and allowing for salt formation (concomitant with supplying higher ionic strengths) the protein solubility at the pI is greatly increased. However, from a theoretical point of view this could be a disaster, as in principle an IPG protein should have a different pI to a CA-IEF protein, the former being related to the Immobiline-complexed species and the latter to a truly isoionic form. This aspect has been debated for a long time among the members of the three groups who originally developed IPGs (B. Bjellqvist in Bromma and A. Görg in Munich, in addition to our own group); unusual as it sounds, we are unable to see this effect. In principle, when seeding in the same gel widely varying protein concentrations, if at the higher con-
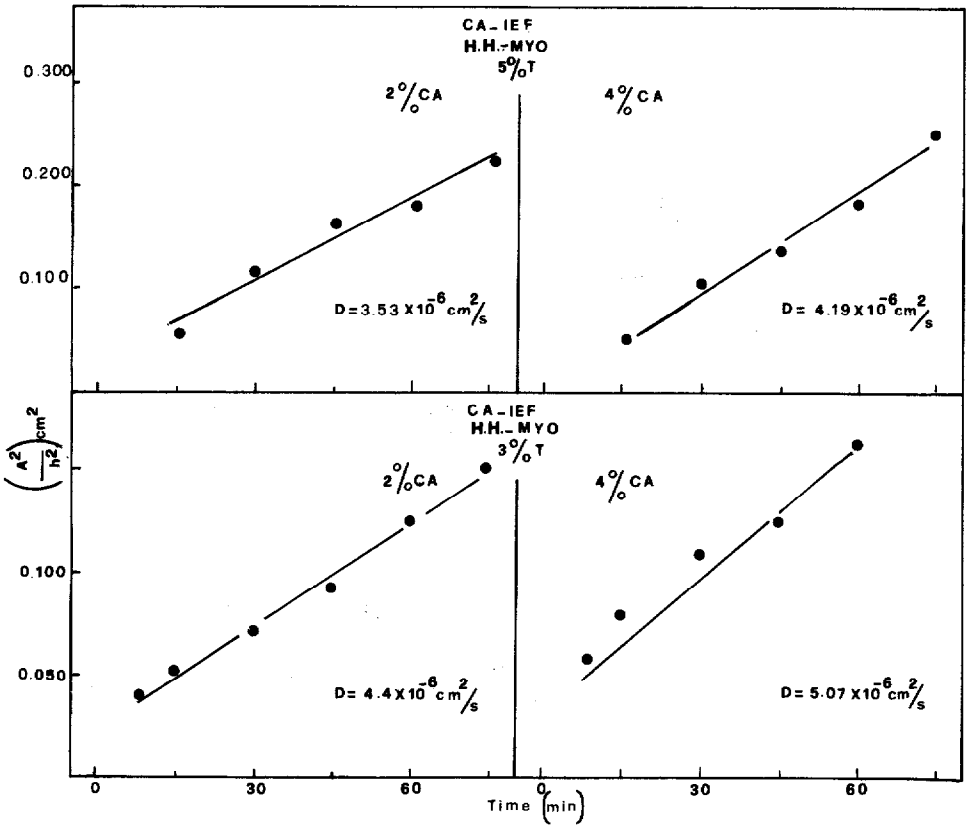
Fig. 6. Measurements of apparent diffusion coefficients of myoglobin by conventional isoelectric focusing in amphoteric buffers (CA). All experimental details as in Fig. 5. For calculation of D values, see Fig. 2.

centrations the equilibrium is driven towards salt formation the complexed species should have different pIs; hence the bands should not be aligned in a straight line (parallel to the Pt electrode wires) but be bow-shaped, with a positive or negative slope at the higher protein loads. In a number of diagrams we have published[6-9] we have never seen this effect. How we could reconcile these two opposite extremes (knowing that we have salt formation in IPGs and yet that the protein does not change the pI) is not clear at present. We shall try the following explanation. When the protein forms a salt in an IPG matrix it does not do it with "unlike" ions (phosphate, borate, oligoanions such as citrate, etc., as typical of zone electrophoresis) but with "like" ions (carboxyls and amino groups). In addition, the infinitesimal gel layer surrounding the isoelectric protein band will also be isoelectric; adding isoelectric species among themselves might not change the pI.

Practical hints

There are some important experimental applications of our findings. Doubling (sometimes tripling) the Immobiline concentration in a gel has two beneficial effects: the bands at the pI become sharper and tighter and, in the absence of the electric
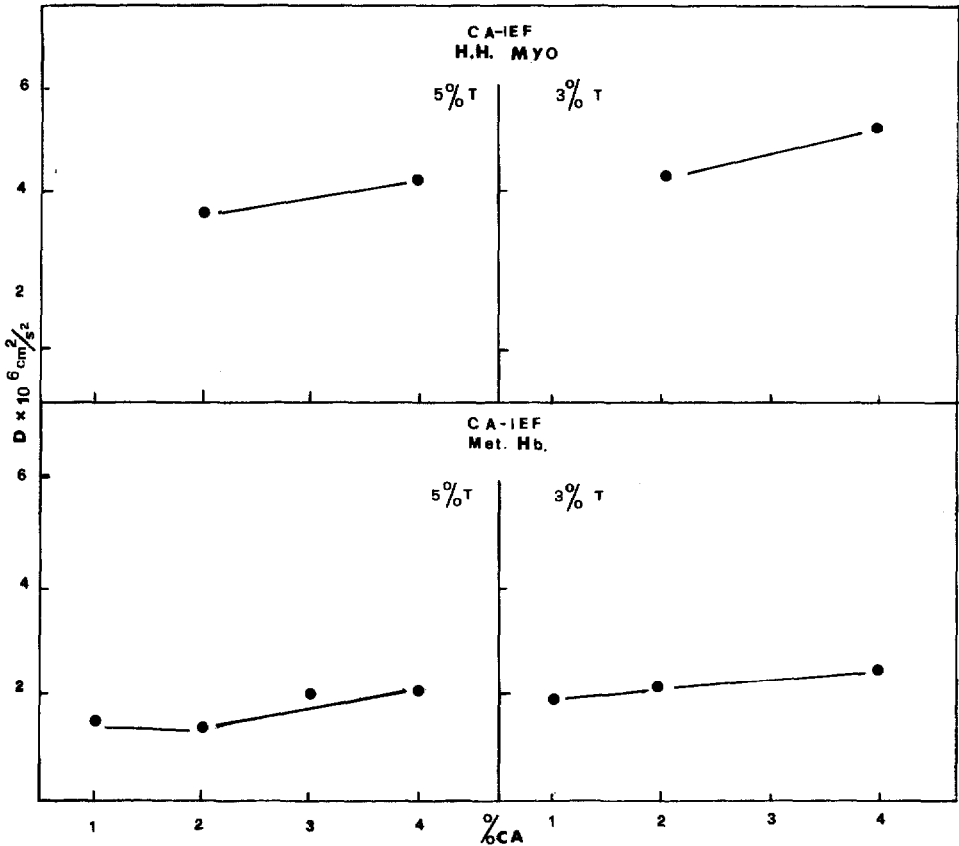
Fig. 7. Dependence of the diffusion coefficients on the carrier ampholyte concentration in a conventional IEF gel. Upper graphs, D values of myoglobin in a 5%T (left) and 3%T (right) gel; lower graphs, D values of oxidized haemoglobin in a 5%T (left) and 3%T (right) gel. The D values have been derived from Figs. 5 and 6. Note the positive slope of these graphs, in contrast to the negative slopes in IPG gels (Fig. 4).

field, they diffuse considerably less. Thus, when developing zymograms *in situ*, high Immobiline concentrations in the gel (2 × or 3 ×) will allow long incubation times, when needed, with a substantially reduced diffusion of the focused bands. We had noticed this recently, when performing several different enzyme stainings in Immobiline gels[14,15]: in the case of alkaline phosphatase in human sera we had to incubate the gel for as long as 2 h, yet the resulting zymogram was extremely sharp. It turned out that we were using a 2 × Immobiline gel.

CONCLUSION

The results can be summarized as follows.

(1) In CA-IEF, higher levels of buffering ions facilitate difusion of the focused zone in the absence of an electric field.

(2) In contrast, in IPG gels, higher levels of Immobilines strongly quench dif-

fusion of the isoelectric bands, this being consistent with a model of protein–Immobiline salt formation in the pI zone.

(3) This effect, in IPG gels, is dependent on molecular mass, being smaller for myoglobin and much greater for Met-Hb, suggesting that average-sized or large proteins are ideally suited for *in situ* zymogramming or time-dependent reactions, while retaining much of the resolution and sharpness of the focusing process.

(4) The diffusion-quenching process in IPG gel plateaus at 3 × Immobiline levels, suggesting that, for zymogramming or *in situ* manipulations after the IEF process, 2 × or 3 × Immobiline gels will be ideally suited.

ACKNOWLEDGEMENTS

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