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SCANNING ISOELECTRIC FOCUSING IN SMALL DENSITY-GRADIENT COLUMNS

IV. THE USE OF DEUTERIUM OXIDE FOR PREPARING THE DENSITY GRADIENT AND ITS EFFECTS ON ISOELECTRIC POINTS OF PROTEINS*

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SUMMARY

The use of deuterium oxide as a substitute for sucrose in preparing density gradients for small isoelectrofocusing columns has been investigated. A density gradient was created directly in a 1.5-ml column by free inter-diffusion of three deuterium oxide solutions for 3 min. The resulting deuterium oxide concentration course (as shown by measurement of the refractive-index gradient) had a very high degree of linearity. Test runs with β-lactoglobulin and sperm-whale myoglobin showed that the strength and stability of the deuterium oxide density gradient should normally be sufficient for stabilization of protein zones against convection during isoelectric focusing. The isoelectric points of β-lactoglobulins A and B were found to increase as the concentration ratio of deuterium oxide to water at focusing level increased. Within the limits of experimental error, the p*I* shift corresponding to a given change in solvent composition was equal for both components. These findings are accounted for in terms of the deuterium isotope effect on the dissociation constants of protolytic groups and the shift in the asymmetry potential of the glass electrode in deuterium oxide as compared with water. The spontaneous reduction of sperm-whale met-myoglobin to ferrous myoglobin, which occurs on prolonged isoelectric focusing, is discussed, as are the benefits and drawbacks of deuterium oxide as a density-gradient solute.

INTRODUCTION

For several reasons, sucrose has been the solute of choice for density-gradient stabilization of isoelectrofocusing columns. It has a relatively large density increment, it is harmless or even protective to proteins, and it is obtainable in grades of high purity at a reasonable price. Sucrose density gradients, however, have some disad-

* A preliminary report on this work has been published[1].

vantages. Thus, they cannot be used for focusing enzymes that utilize sucrose as a substrate. Moreover, sucrose (and its usual substitutes glycerol and 1,2-ethanediol) considerably increases the viscosity in the lower part of the column, and thereby lengthens the focusing time at a given voltage. There is also the risk of forming interfering degradation products of sucrose near the electrodes because of the extreme pH values prevailing there.

An interesting substitute for sucrose, namely deuterium oxide (D_2O), has not so far been used. One reason for this is that it would be too costly to produce such a gradient for commercial 110-ml and 440-ml columns, and another is that the density increment obtainable (0.1 g/ml) would be too small for these columns. In the 1.5-ml column described by Jonsson *et al.*[2], however, D_2O should be a possible alternative to sucrose.

Preliminary runs with bovine β-lactoglobulin as sample confirmed that D_2O could be used to establish a density gradient in such a column, but the isoelectric points were shifted in the presence of D_2O. Subsequent experiments, therefore, were directed towards further elucidating the correlation between the isoelectric point of a protein component and the concentration of D_2O at the focusing level.

EXPERIMENTAL

Isoelectrofocusing column

The isoelectrofocusing column utilized in this work is a modification of one described earlier[2,3]. The lower electrode (of platinum wire) has been exchanged for a sheet of palladium (0.1 mm thick). Part of this sheet (4×10.5 mm^2) serves as cathode and covers the bottom of the focusing chamber (A in Fig. 1), whereas part of it (1×55 mm^2) serves as electrical lead and passes through the capillary tube (B in Fig. 1).

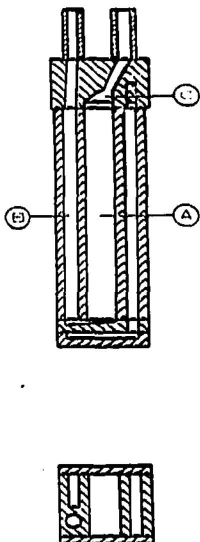

Fig. 1. Schematic drawing of spectrophotometer cell used as column. A, focusing chamber; B, capillary tube for cathode electrical lead; C, anode compartment. (For further explanation of column, see, for example, Fig. 1 of ref. 2.)

The cell (manufactured by Hellma, Müllheim, G.F.R.), was delivered with a separate bottom section; this enabled the electrode part of the palladium sheet to be positioned between the bottom section and the rest of the cell. The two cell pieces were then joined by means of silicone rubber (Dewcon Silite 100, from Dewcon, Danvers, Mass., U.S.A.).

After an experiment, absorbed hydrogen was removed from the palladium electrode by running the sheet as anode until it gassed. As electrolyte, an old aqueous solution of 1% Ampholine pH 3–6 was used; the current was kept at about 4 mA.

To prevent distortion of protein bands due to electro-osmosis, the inner walls of the cell were silanized with dimethyldichlorosilane[4].

Solutions

The cell was normally filled with the following system of aqueous solutions for a downward current direction: 0.10 ml of anode solution (containing $1 \cdot 10^{-4}$ ml of concentrated H_2SO_4 per ml of H_2O), 0.40 ml of top solution [1% (w/v) Ampholine], 0.70 ml of intermediate solution [1% of Ampholine, 50.0% (v/v) of D_2O and a suitable amount of sample], 0.35 ml of bottom solution (1% of Ampholine and 97.5% of D_2O), and 0.18 ml of cathode solution (1% of Ampholine of a less acidic or more alkaline pH range than above and 97.5% of D_2O).

In some experiments, the sample was introduced in a special sample solution (0.20 ml) below the top solution or above the bottom solution; in these experiments, the volume of intermediate solution was reduced to 0.50 ml. The D_2O contents of the sample solutions were 35 and 65%, respectively. No isoelectric points were evaluated from these experiments.

In addition, four runs were made in sucrose density gradients having a constant concentration of D_2O, the sucrose concentrations of the initial solutions being identical with those used by Jonsson *et al.*[2]. As well as pure D_2O, three H_2O–D_2O mixtures containing 10.0, 25.0 and 80.0% of D_2O, respectively, were used as solvents.

Chemicals

Deuterium oxide (99.8%, w/w) was obtained from Norsk Hydro (Oslo, Norway), and sucrose of analytical-reagent grade from Mallinckrodt (St. Louis, Mo., U.S.A.). Ampholine carrier ampholytes were supplied by LKB Produkter (Stockholm, Sweden). Bovine β-lactoglobulin (BDH, Poole, Great Britain) and sperm-whale myoglobin (Koch-Light Lab., Colnbrook, Great Britain) were used as test substances.

Preparation of density gradients

The D_2O and sucrose density gradients were prepared by free diffusion[5] using the equipment described by Jonsson *et al.*[2]. The cell was held in the horizontal position for 3 and 15 min, respectively, and the D_2O concentration course in the focusing chamber was analyzed by measurement of the refractive-index gradient as described by Rilbe and Pettersson[5].

Scanning isoelectric focusing

The equipment and technique described earlier[2,3] were used, with the following modifications. The resistor[2] was omitted, as the new positioning of the cathode allowed a higher electrical load at the beginning of a run. The capillary tube (B in Fig.

1) was kept closed during the run to avoid displacement of the column contents due to evaporation of H_2O from the anode compartment.

The scanning device[2] was extended to contain two optical systems, perpendicular to each other and including the same cell elevator. One optical system comprised a mercury lamp and a grating monochromator type 33-86-01 for UV radiation (Bausch & Lomb, Rochester, N.Y., U.S.A.), the other a tungsten lamp and a grating monochromator for visible light (Bausch & Lomb, type 33-86-02). The cell elevator could be turned round its vertical axis, so permitting the cell to be scanned with either UV or visible light. By means of a switch, the signal from the appropriate photomultiplier could be fed into the amplifier. To allow compensation for possible variations in the baseline position, a short air-filled cuvette (1 × 1 × 1 cm) was fixed in the cell holder just below the cell. The temperature of the cell was kept at 5°.

Determination of pH course

Fractionation of the column contents after completion of a run and subsequent evaluation of the pH course were carried out as before[3].

RESULTS AND DISCUSSION

Linearity and stability of the D_2O density gradient

The D_2O concentration course in the focusing chamber, as obtained by measurement of the refractive-index gradient[5], is shown in Fig. 2. It can be seen that the concentration course obtained by keeping the cell in the horizontal position for 3 min at 5°, and then raising it, had a high degree of linearity. The bottom and top concentrations of D_2O were 83 and 13% by volume, respectively; the remaining con-

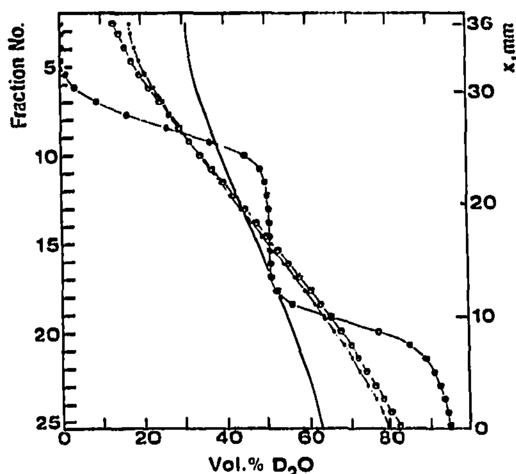

Fig. 2. Concentration courses for D_2O in focusing chamber of cell as obtained after introduction of three aqueous solutions containing 0.0, 50.0 and 97.5% of D_2O by volume (●—●); after 3-min diffusion with cell in horizontal position (○—○); and after diffusion for 2 h (•—•) and 24 h (——) with cell in upright position. The volumes of the initial solutions were 0.50, 0.70 and 0.53 ml, respectively. The concentration courses were determined by analysis of measured refractive-index gradients[5]. The vertical distance from the bottom of the focusing chamber is shown by x.

centration difference was thus 70%, or almost three-quarters of the initial concentration difference.

Fig. 2 also shows the stability of the D_2O density gradient while the cell was maintained upright. After 2 h, the total concentration difference had decreased to 63%; 22 h later, the concentration course was still very linear, the concentration interval being 32%.

Thus it is possible, in only 5 min, to create a linear density gradient in the 1.5-ml cell by applying the free-diffusion method[5] to, in principle, three aqueous solutions having different contents of D_2O (0.0, 50.0, and 97.5% by volume, respectively).

If the regression line is calculated from the resulting concentration course as given in Fig. 2, a concentration gradient of 20.39% of D_2O cm^{-1} is obtained. Since the densities of D_2O and H_2O at 5° are 1.106 and 1.000 g/ml, respectively[6], and since D_2O and H_2O form ideal solutions, this means a density gradient of 0.0216 g/ml·cm*. An analogous calculation of the sucrose density gradient used by Jonsson *et al.*[2] gives the value 0.0184 g/ml·cm. Thus, the initial D_2O density gradient is almost 20% larger than the sucrose density gradient obtained in the same cell with 200 g of sucrose per litre as the initial bottom concentration.

The stability of the density gradient with time is less with D_2O than with sucrose, as would be anticipated from the diausion coefficients of the two solutes. The D_2O density gradient decreased to 0.0202 g/ml·cm (*i.e.*, by about 6%) when the cell was kept upright for 2 h*; the corresponding change in the sucrose density gradient was too small to be measured[2].

The D_2O density gradient remaining after diffusion for 2 h, however, is still more than twice as large as the sucrose density gradient obtainable in an LKB 110-ml column with 500 g of sucrose per litre as the initial bottom concentration. Consequently, the D_2O density gradient described should normally be sufficient for stabilizing protein zones. This conclusion is supported by the results of test runs with bovine β-lactoglobulin and sperm-whale myoglobin.

Isoelectric focusing of bovine β-lactoglobulin

In the first series of experiments, the initial solutions (except anolyte and catholyte) contained Ampholine pH 4–6. Since the bottom electrode of the present cell is positioned inside the focusing chamber, there was no need for a highly conductive catholyte such as the 0.02 *M* sodium hydroxide solution used earlier[2,3]; this could be replaced by 1% Ampholine of pH range 5–7 without discernible convection at the cathode.

Isoelectric focusing was performed at constant voltages of 105 and 175 V, *i.e.*, at average field strengths of 30 and 50 V/cm, respectively. At the lower field strength, β-lactoglobulin focused well, yielding two symmetrical, well-resolved peaks representing the genetic variants A and B; this is shown by Fig. 3 (and also by Fig. 1 of ref. 1). Repeated scanning of the cell showed that an isoelectric spectrum of maximum sharpness was obtained after about 75 min, irrespective of whether the protein was loaded in all the intermediate solution, or in special sample solutions below the top or above the bottom solutions. By this time, the initial current of about 0.75 mA had decreased

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* In the preliminary report, the D_2O density gradients obtained after diffusion for 0 and 2 h with cell upright were erroneously stated to be 0.018 and 0.016 g/ml·cm, respectively.

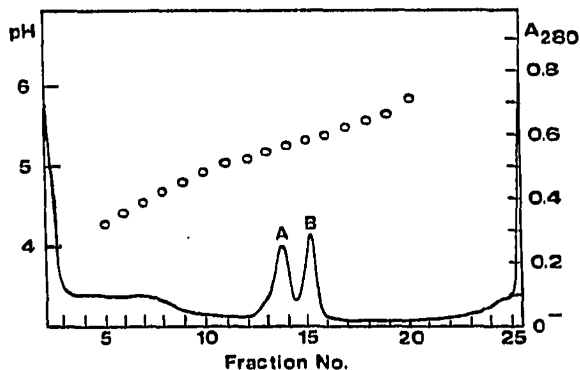

Fig. 3. Final scan of cell at 280 nm (solid curve) obtained after D_2O density gradient isoelectric focusing of 80 μg of β-lactoglobulin in 1% Ampholine for 75 min (Experiment 4 in Table I). Average field strength 30 V/cm. The curve shown by the circles represents the pH values of 60-mm^3 fractions of the column contents (ref. 3). Peak A represents β-lactoglobulin A, and peak B β-lactoglobulin B.

to 0.26 mA. The final peaks were less sharp than those obtained in a sucrose density gradient (*cf.*, Fig. 2c of ref. 3), but this is to be expected, owing to the higher diffusion velocities of the protein components in the less viscous D_2O gradient.

When isoelectric focusing was performed at an average field strength of 50 V/cm, the bands were distorted and less well-resolved.

The isoelectric points of β-lactoglobulins A and B as evaluated from the pH course at 25° were about 0.1 pH unit higher than those obtained in a sucrose density gradient (see Table I, Experiments 1–3). Examination of the literature revealed that this could be explained by taking into account the effects of D_2O on the acid-dissociation constants of weak acids, and on the asymmetry potential of the glass electrode.

In order to elucidate further the correlation between the isoelectric point of a protein component and the concentration of D_2O at the focusing level, experiment 4 in Table I was made. By exchanging 25% of the Ampholine pH 4–6 for Ampholine pH 5–7 in the top, intermediate and bottom solutions, β-lactoglobulins A and B were forced to focus nearer the anode, *i.e.*, at lower concentrations of D_2O. In a subsequent run, the proportion of Ampholine pH 5–7 was increased to 75% of the total (Experiment 5).

The concentration interval that can be studied by this technique is limited to about 30–65% of D_2O, as focusing near the electrodes may give rise to erroneous p*I* values due to diffusion of anolyte and catholyte during fractionation of the column contents (*cf.* ref. 3, p. 582). Additional runs were therefore made in sucrose density gradients having fixed concentrations of D_2O (Experiments 6–9), the Ampholine composition being the same as in Experiments 2 and 3.

Experiments 1–9 show that p*I* values as evaluated from pH courses measured in isoelectrofocusing systems containing D_2O are consistently higher than those obtained from measurements in D_2O-free systems. Moreover, they increase as the concentration ratio of D_2O to H_2O increases at the focusing level. Qualitatively, these results are plausible in view of the effect of D_2O on the acid-dissociation constants of weak acids[7]. Several studies have shown that p*K* values increase when D is substituted for H in the functional groups of the acid molecule through addition of D_2O

TABLE I

APPARENT AND ABSOLUTE ISOELECTRIC POINTS AT 25.0° OF β-LACTOGLOBULINS A AND B

Values obtained under experimental conditions described in text.

β-Lactoglobulin	*Experiment No.*	pI_{app} *	*x (mm)* **	*n* ***	$\Delta p(DH)$ §	pI_n §§
A	1	5.13	—	0	0	5.13[3]
	2	5.23	13.9	0.56	0.20	5.43
	3	5.24	14.3	0.55	0.20	5.44
	4	5.23	18.2	0.47	0.16	5.39
	5	5.16	26.4	0.30	0.10	5.26
	6	5.16	18.7	0.10	0.03	5.19
	7	5.18	18.2	0.24	0.08	5.26
	8	5.25	17.8	0.77	0.29	5.54
	9	5.25	18.0	0.96	0.38	5.63
B	1	5.23	—	0	0	5.23[3]
	2	5.34	12.1	0.60	0.21	5.55
	3	5.34	12.6	0.59	0.21	5.55
	4	5.33	16.2	0.52	0.18	5.51
	5	5.28	24.1	0.34	0.12	5.40
	6	5.26	16.6	0.10	0.03	5.29
	7	5.29	15.9	0.24	0.08	5.37
	8	5.34	15.9	0.77	0.29	5.63
	9	5.35	16.1	0.96	0.38	5.73

* Isoelectric point evaluated from pH measurements of $6 \cdot 10^{-2}$-ml fractions.
** Vertical distance from bottom of focusing chamber to peak level.
*** Atom fraction of D at peak level.
§ Value of p(DH) minus pH-meter reading at peak level[10].
§§ Sum of pI_{app} and $\Delta p(DH)$.

to the solvent H_2O. According to Halevi *et al.*[8], the increase is almost proportional to the atom fraction (n) of D in the H_2O–D_2O mixture.

Quantitative evaluation of the observed shifts in pI is hampered by the lack of isoelectric points in the literature referring to D_2O as solvent. Nevertheless, it has been possible in this instance to relate these shifts to the corresponding changes in the intrinsic dissociation constant (pK_{int}) of normal side-chain carboxyl-groups of the protein. The latter changes can then be compared with data published for small organic molecules. However, before discussing quantitatively isoelectric points obtained from runs in the two types of density gradient, the change in asymmetry potential of the glass electrode in D_2O compared with H_2O must be considered.

Various workers have reported that the pH meter reading in D_2O solution at 25° is about 0.4 units lower than that in H_2O solution of equal acidity[9]. As shown by Glasoe and Long[10], and by Salomaa *et al.*[11], the deviation $\Delta p(DH)$* for mixtures of H_2O and D_2O is almost proportional to n. Thus, the pI values of β-

* In conformity with the acidity scales for pure H_2O and pure D_2O, an acidity scale may be defined for H_2O–D_2O mixtures. The latter "pH" scale is preferably denoted by p(DH) to point out that p(DH) depends on both the activities of H and D. The deviation $\Delta p(DH)$ is defined as p(DH) minus pH-meter reading.

lactoglobulin as evaluated from measured pH courses and given in column 2 of Table I are only apparent ones; to obtain absolute values, the pertinent values of Δp(DH) must be added.

In order to ascertain whether the literature values of Δp(DH) were valid for the glass capillary electrode (Radiometer G297/G2; see ref. 3) used here, pH-meter readings were made on 10 hydrochloric acid solutions that contained the same concentration of acid (0.01 *M*) but different amounts of D_2O (0–90%). It was found that the pH-meter reading for a given solution (taken 1 min after filling the glass capillary) tended to rise somewhat after repeated fillings. When the capillary was filled 10 times with each of the 10 solutions in order of increasing concentration of D_2O, the values of Δp(DH) calculated from the final reading for each solution deviated more and more from those of refs. 10 and 11. The final Δp(DH) value (corresponding to 90% of D_2O) was 0.04 pH units lower than the value evaluated from Fig. 1 of ref. 10. Moreover, if, in this position, the glass capillary was filled with the D_2O-free 0.01 *M* hydrochloric acid, the pH-meter reading was about 0.04 pH units higher than the constant reading originally obtained with this solution. After subsequent fillings with the D_2O-free hydrochloric acid, the readings slowly decreased to the original value.

On the other hand, when the glass capillary was filled only twice with each H_2O–D_2O mixture and, in addition, with several portions of D_2O-free 0.01 *M* hydrochloric acid before changing from one mixture to the next, the resulting values of Δp(DH) agreed within 0.01 pH unit with those evaluated from ref. 10. The agreement with the equation proposed by Salomaa *et al.*[11] was somewhat less good.

According to Covington *et al.*[12], Δp(DH) at a given concentration of D_2O should be slightly higher for the moderately acid fractions of Experiments 1–9 than for 0.01 *M* hydrochloric acid. As the effect is about 0.01 pH unit for pure D_2O, it is neglected here, however.

Small positive shifts (0.01–0.03 pH units) in pH-meter readings were also observed for the calibration buffers[3] after measurements had been made on the 25 fractions of an isoelectrofocusing run. As the fractions were measured in order of increasing D_2O concentration, with no rinse in between, a negative correction proportional to the fraction number was applied to the pH-meter reading for each fraction. The p*I* values in column 2 of Table I were evaluated from such corrected pH courses.

Estimation of Δp(DH) from the data of ref. 10 requires *n* to be known at the focusing level and at the moment of fractionation. In Experiments 2–5, the latter occurred about 1.5 h after the cell was brought back into the vertical position. Interpolation in Fig. 2 between the curves of diffusion for 3 min and for 2 h adequately gives the percentage of D_2O by volume at the particular level in the cell. Since the molar volumes of pure H_2O and pure D_2O are almost equal (18.02 and 18.10 ml/mole, respectively), and since H_2O and D_2O form ideal mixtures, *n* can be derived with sufficient accuracy by dividing the obtained percentage of D_2O by 100. The effect on *n* of added Ampholine (due to its share of the volume and to partial exchange of H for D in its protolytic groups) is negligible.

In Experiment 6, the value of *n* is given with sufficient accuracy by the composition of the H_2O–D_2O mixture used for preparing all the initial solutions used in the experiment. In Experiments 7–9, however, small corrections were made to *n* to take account of partial exchange of H for D in the hydroxy-groups of the sucrose (the pertinent sucrose concentrations were estimated from Fig. 2 of ref. 2).

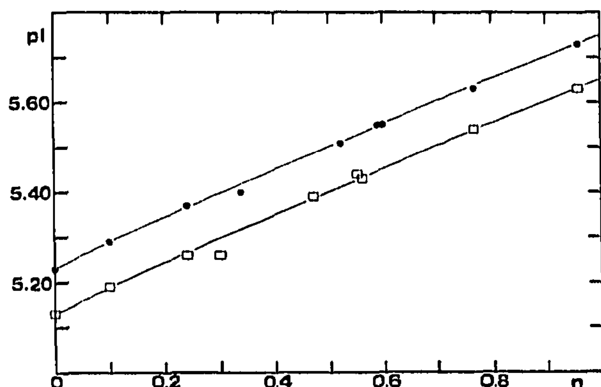

Fig. 4. Dependence of pI values of β-lactoglobulins A (□) and B (●) on the D atom fraction of the aqueous solvent. The curves correspond to the regression line in Fig. 5.

The resulting values of n, Δp(DH) and the absolute isoelectric points (pI_n*) are listed in Table I, columns 4–6. The pI_n values are plotted against n in Fig. 4, from which it can be seen that the isoelectric point of each β-lactoglobulin component varies almost linearly with the atom fraction of D in the solvent at the focusing level. Within the limits of experimental error, the shift in pI corresponding to a given value of n seems to be equal for both components. An attempt has been made to account for these findings in terms of the well-known Linderstrøm–Lang equation and the protolytic composition of the protein on one hand, and a general equation for the D isotope effect in aqueous solutions on the other.

The equation of Linderstrøm–Lang

$$\mathrm{pH} = \mathrm{p}K^{i}_{\mathrm{int.}} + \log\left[x_i/(1 - x_i)\right] - 0.868wZ \qquad (1)$$

correlates the dissociation of ionizable groups on proteins with the prevailing pH value [x_i refers to the average degree of dissociation at any pH of groups of type i having an intrinsic dissociation constant ($K^{i}_{\mathrm{int.}}$), Z to the net charge of the protein, and w to a parameter accounting for the electrostatic interaction between charged groups[13]]. At the isoelectric pH, Z is zero, and eqn. 1 is simplified to

$$\mathrm{p}I = \mathrm{p}K^{i}_{\mathrm{int.}} + \log\left[x_i/(1 - x_i)\right] \qquad (2)$$

If n_i is the total number of ionizable groups of type i, the isoelectric condition of $Z = 0$ is expressed by the equation

$$\Sigma n_i z_i = 0 \qquad (3)$$

where z_i is the average charge on each group of type i and is equal to $(1 - x_i)$ for cationic groups and to $-x_i$ for anionic groups.

* Henceforth, parameters pertinent to a mixed solvent will be indicated by the subscript n. Subscripts H and D will refer to pure H_2O and pure D_2O as solvents.

According to Basch and Timasheff[14], and to Tanford[13], β-lactoglobulin A contains the following numbers of ionizable groups per dimer ($pK_{int,H}$ at 25° in parentheses): 2 α-carboxyls (3.75), 50 normal side-chain carboxyls (4.66), 2 anomalous side-chain carboxyls (7.25), 4 imidazoles (7.25), 2 α-amino groups (7.80), and 44 other groups (>9.5). The total number of cationic groups is 40. In the β-lactoglobulin B dimer, the number of normal side-chain carboxyls is 48.

Since the isoelectric point of β-lactoglobulin A in H_2O is 5.13, eqn. 2 indicates that the 40 cationic groups, as well as the 2 α-carboxyls, are almost fully ionized at this pH. At the same pH, x should be about 0.76 for the 50 normal side-chain carboxyls. The remaining groups should be practically uncharged.

In D_2O, the pK value of each protolytic group is increased. As shown by Li *et al.*[15] (and by others) for a number of organic acids, the magnitude of the increase depends on the type of group affected (–COOH, $-NH_3^+$, $-ImH^+$, etc.) and on the pK_H of this group. This means that the values of $pK^i_{int.}$ for β-lactoglobulin will all increase in D_2O relative to H_2O, but to a different extent. In consequence, small shifts can be expected in the x_i values at the isoelectric pH of, *e.g.*, β-lactoglobulin A. As regards the normal side-chain carboxyls, however, application of eqns. 2 and 3 indicates that the resulting effect on the term $\log [x_i/(1 - x_i)]$ is less than 0.01 pH unit, provided that the shifts in $pK_{int.}$ for the other ionizable groups are of about the magnitude suggested by the data of Li *et al.* It is then evident from eqn. 2 that measured shifts in pI for β-lactoglobulin (A or B) reflect equal shifts in $pK_{int.}$ of its normal side-chain carboxyls*. It is also evident that the increase in pI accompanying any increase in n should be equal for both genetic variants.

Although eqn. 2 is certainly an approximation based on several simplifying assumptions (*cf.* Tanford's review[13]), it has, in combination with eqn. 3, been very successful in calculating theoretical pI values for small globular proteins and for disclosing shifts in $pK_{int.}$ accompanying conformational changes in such proteins[16,17]. Thus, it could *a priori* be supposed to give reliable results for the small and compact β-lactoglobulin dimers. That this is so is confirmed, since the pI values of the A and B genetic variants in H_2O are calculated to be 5.16 and 5.24, whereas the experimental values are 5.13 and 5.23. The theoretical difference in pI values for β-lactoglobulins A and B is calculated to be 0.08 pH units, which is in good agreement with the experimental value of 0.10.

In further discussion, it thus seems fully justifiable to replace observed shifts in pI with equal shifts in $pK_{int.}$ for the normal side-chain carboxyls. On the other hand, it is doubtful whether it is also justifiable to apply to the intrinsic dissociation constant of a polyacid equations derived for the dissociation constant of a monobasic weak acid in H_2O–D_2O mixtures. Nevertheless, experimental data fit very well an equation of the type given by Halevi *et al.*[8]:

$$K_H/K_n = (K_H/K_D)^{n\gamma} \tag{4}$$

* Though an increase in pI due to the presence of sucrose could be well expected, as the latter lowers the dielectric constant of the solvent, earlier runs involving focusing at various sucrose concentrations have failed to exhibit such an effect. Moreover, in the runs in sucrose density gradients reported in Table I, the focusing level (and thereby the sucrose concentration) was kept almost constant. Thus, the effect of sucrose on the pI shifts studied should be negligible.

This equation, in which γ is an empirical parameter "presumably not very different from unity", is an approximation of a general equation for the D isotope effect for aqueous solutions and is based on the assumption that variations in K are due almost solely to changes in the standard state for the ionization process.

Substituting $K_{\mathrm{int.}}$ for K in eqn. 4 and entering the operator p = —log, one obtains

$$\mathrm{p}K_{\mathrm{int.},n} - \mathrm{p}K_{\mathrm{int.},H} = (\mathrm{p}K_{\mathrm{int.},D} - \mathrm{p}K_{\mathrm{int.},H})\, n^{\gamma}$$

or, simplified,

$$\Delta \mathrm{p}K_{Hn} = \Delta \mathrm{p}K_{HD}\, n^{\gamma} \quad (5)$$

where $\Delta \mathrm{p}K_{Hn}$ means the shift in $\mathrm{p}K_{\mathrm{int.}}$ for a given value of n, and $\Delta \mathrm{p}K_{HD}$ is the corresponding shift for $n = 1$.

Eqn. 5 may be written in the linear form:

$$\log \Delta \mathrm{p}K_{Hn} = \log \Delta \mathrm{p}K_{HD} + \gamma \log n \quad (6)$$

If $\log \Delta \mathrm{p}K_{Hn}$ is calculated from the data in column 6 of Table I, and plotted *vs.* $\log n$, the result is the graph in Fig. 5. Thus it can be seen that eqn. 6 agrees excellently with experimental values for both β-lactoglobulins (deviation $\leqslant 0.01$ pK unit), apart from the points coming from experiment 5 (deviation 0.04 and 0.02). If these latter points are excluded, the regression line obtained has the slope $\gamma = 0.94$ and the constant term $\log \Delta \mathrm{p}K_{HD} = -0.283$; curves corresponding to this regression line have also been introduced in Fig. 3.

The D isotope effect on the intrinsic dissociation constant of normal side-chain carboxyls in β-lactoglobulin (as obtained from the constant term of the regression line) is $\Delta \mathrm{p}K_{HD}$, which is equal to 0.52. This value is difficult to compare with literature data, primarily because data pertinent to intrinsic dissociation constants of polyacids

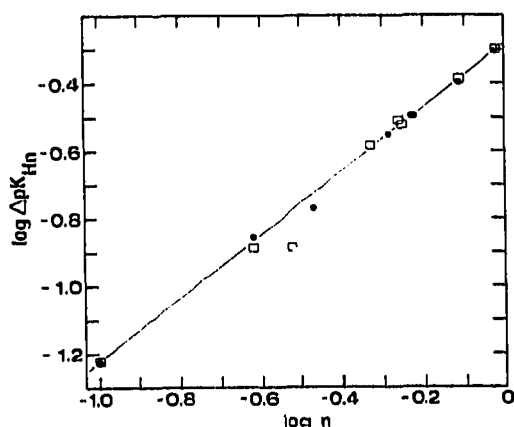

Fig. 5. Dependence of $\log \Delta \mathrm{p}K_{Hn}$ on $\log n$. For explanation of parameters, see text accompanying eqns. 4–6; the solid line is the regression line according to eqn. 6. β-Lactoglobulin A, □; β-lactoglobulin B, ●.

are lacking. The pK shifts hitherto published for carboxyls (and other ionizable groups) all refer to individual groups on small molecules. Moreover, those small molecules being of most interest to the present discussion, *viz.*, ROOC–$(CH_2)_x$–COOH and CH_3–CO–$(CH_2)_x$–COOH, x having the value 2 or 3 (see ref. 13), have not been studied in D_2O.

Among the acids examined in D_2O, succinic acid is the one most closely related to aspartic and glutamic acid residues. If it is remembered that a molecule with two identical dissociable groups will have a dissociation constant that is twice the value for either group alone, the data of Robinson *et al.*[18] give the following intrinsic values for the succinic acid carboxyls: $pK_H = 4.51$ and $pK_D = 5.05$. This means that ΔpK is 0.54, which agrees well with the shift observed here for the side-chain carboxyls (0.52). It may also be mentioned that, for acetic acid ($pK_H = 4.76$), $\Delta pK = 0.56$ (see ref. 18). On the other hand, application of equations of the form $\Delta pK = a + b\ pK_H$ (as proposed in refs. 15, 19 and 20), in which a and b are constants, to the side-chain carboxyls, is of limited value. Such equations yield ΔpK_{int} values between 0.50 and 0.66.

Isoelectric focusing of sperm-whale myoglobin

In order to examine whether or not the spontaneous reduction of ferric Mb I observed by Jonsson *et al.*[2] on prolonged focusing of sperm-whale myoglobin was caused by sucrose degradation products, a run of this protein in a D_2O density gradient was made.

The top solution contained Ampholine pH 5–7 (0.48%) and pH 7–9 (0.52%), whereas the rest of the initial solutions contained Ampholine pH 7–9 only. The sample (35 μg) was dissolved in the intermediate solution, and the voltage used was 105 V.

Repeated scanning of the cell disclosed that the absorbancy at 410 nm of zone 1 (containing ferric Mb I, *cf.* ref. 2, p. 12) passes through a maximum after about 150 min. Simultaneously, the absorbance of zone 2 tended to rise. Subsequent scans at 435 nm confirmed that ferric myoglobin was reduced even in the present system.

Although it is still plausible to assume, as did Jonsson *et al.*[2] (and earlier Satterlee and Snyder[21]), that the observed reduction of the heme iron is due to some product formed by electrolysis at the adjacent cathode, the above observations exclude sucrose as the source of such a product. The alternative (that reduction is caused by molecular hydrogen diffusing up from the cathode) seems most unlikely, since the cathode in the present cell is non-gassing. Further, attempts to reduce the protein with hydrogen in a test-tube were unsuccessful.

Quinn[22] recently reported that ferric bovine myoglobin is converted into a mixture of oxygenated and deoxygenated forms of the ferrous protein by the combined effects of Ampholine and either of the radical generators persulfate or riboflavin; the reduction was observed in isoelectrofocusing gels as well as in acrylamide-free solution. In the present work, therefore, it cannot be excluded that atomic hydrogen close to the cathode surface started some radical reaction by which reducing and more acid fragments of the carrier ampholytes were formed. However, this can hardly be the explanation in the case of Jonsson *et al.*, notwithstanding experimental indications in this laboratory of a chemical modification of the Ampholine on prolonged focusing[23]. Jonsson *et al.* used sodium hydroxide as catholyte, and this should have prevented the carrier ampholytes from approaching the cathode surface.

It should also be noted that another heme protein, ferricytochrome *c*, shows a tendency to spontaneous reduction to the ferrous form at pH 9–10. This reduction can be observed in a test-tube[24,25], but proceeds at a higher rate during such procedures as paper electrophoresis[24] and isoelectric focusing[26]. According to Boeri[25], this so-called "auto-reduction" is a complex chain process, which is accompanied by liberation of hydrogen ions, and is facilitated by low oxygen pressures. Oxidizable residues of the peptide chain, along with OH^- (and HO_2^-) from the solvent, are possible reducing agents.

A similar chain process is conceivable for ferric sperm-whale myoglobin in the isoelectrofocusing column, although it does not proceed to a measurable extent at the isoelectric pH in a test-tube and the ferrous form of this protein tends to autoxidize. At the isoelectric level of Mb I, the conversion from the ferric into the ferrous form should be greatly enhanced, as the product (because of its negative charge) is forced to move away from the reaction zone and the oxygen pressure should be low in this part of the column.

Accuracy of pI_n values

The effects of D_2O on pK values and pH-meter readings of course complicate evaluation and interpretation of isoelectric points from runs in D_2O density gradients. To obtain absolute pI values in such a gradient, one must compensate for the change of asymmetry potential of the glass electrode, and this requires that n be known at the level of the focused protein component. In addition, an absolute pI referring to a protein component focused in a D_2O gradient is of little use unless accompanied by the pertinent value of n.

Evaluation of the change in n with height in the cell is time-consuming, and, because of disturbing refractive-index increments from the carrier ampholytes, has to be performed on a separate density gradient in which Ampholine has been replaced by H_2O. Therefore, the course of the D_2O density gradient cannot be determined in conjunction with each run. Instead, one is forced to rely on the conformity of the gradient from one run to another. An additional source of error inherent in evaluation of the D_2O concentration course from refractive-index measurements is the small difference in refractive index between D_2O and H_2O.

It is thus important to know to what extent pI_n is influenced by an incorrect estimation of n at the level of a peak. Inspection of Fig. 1 of ref. 10 indicates that, if the error in n is assumed to be ± 0.05 (which corresponds to $\pm 5\%$ of D_2O), the error thereby induced in pI_n is only ± 0.02 for those n values comprised by the density gradient (for $n > 0.9$, the error in pI_n is ± 0.03). Since the real uncertainty in n is probably less than ± 0.05, highly accurate pI values should still be obtainable when D_2O is used instead of sucrose in the 1.5-ml column.

CONCLUSIONS

The results reported in this paper have shown that a D_2O density gradient created in a 1.5-ml column by diffusion is sufficiently strong and sufficiently stable with time to be able to stabilize protein zones against convection during isoelectric focusing.

Owing to the smaller viscosity increment of D_2O as compared with sucrose, the

focusing time is shorter with a D_2O density gradient than with a sucrose gradient. However, the focusing time of 75 min obtained here should not be compared directly with the focusing time of about 120 min reported by Jonsson *et al.*[2]. The introduction in the present cell of a palladium cathode covering the bottom of the focusing chamber considerably facilitates passage of current and permits the use of higher electrical loads. Thus, the focusing time of a run in a sucrose density gradient corresponding to experiments 2 and 3 was about 90 min. On the other hand, almost 15 min is saved when preparing the density gradient as the initial D_2O solutions interdiffuse more rapidly than do sucrose solutions.

As already pointed out, the effects of D_2O on p*I* values and on the asymmetry potential of the glass electrode complicate evaluation and interpretation of isoelectric spectra from runs in D_2O density gradients. In routine work, where the species are well-known and absolute p*I* values are of minor interest, this should generally be unimportant, although it is conceivable that closely adjacent peaks may change positions in D_2O and sucrose density gradients, respectively.

Isoelectric focusing in a D_2O density gradient, or in a sucrose density gradient having a fixed concentration of D_2O, may in favourable circumstances (as for β-lactoglobulin) give information about the magnitude of the D isotope effect on a single intrinsic dissociation constant. It is also conceivable that this technique could be used to study conformational changes induced by D_2O (*cf.*, Ui's work with urea[16,27]).

Another related potential use of isoelectric focusing in the presence of D_2O is as a tool to study the role of water in the association–dissociation equilibrium of sub-unit proteins. Among such proteins already studied in D_2O (by cellulose acetate electrophoresis, for example) are lactate and bovine-liver glutamate dehydrogenases. According to Henderson and Henderson[28], D_2O stabilized lactate dehydrogenase at low ionic strength and temperature (8°) and inhibited sub-unit interchange between the heart- and muscle-type enzymes. With glutamate dehydrogenase, D_2O appeared to facilitate the association of monomers. These results were interpreted as being due to isotope effects on equilibrium processes involving exchangeable protons in the proteins.

When studying effects of D_2O on isoelectrically focused proteins, the "mixed" gradients utilized in experiments 6–9 may be preferable. By superimposing a fixed D_2O concentration on a sucrose density gradient, it is possible to cover a larger concentration interval of D_2O. It is also an advantage that the particular value of n is calculable from solvent composition and sucrose concentration only, although a correction for D–H exchange in the sucrose may be necessary.

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