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TEMPERATURE DEPENDENCE OF AMPHOLINE pH GRADIENTS USED IN ISOELECTRIC FOCUSING

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SUMMARY

The pH shifts accompanying the increase of temperature from 4 to 25° have been accurately measured for some pH gradients created by isoelectric focusing of Ampholine carrier ampholytes in sucrose density gradient at 4°. By means of these shifts, the apparent pI value evaluated for a protein, for instance, from an isoelectric focusing run at 4° followed by pH measurements at 25° may be converted into a true value at 4°.

The influence of the sucrose concentration on the pH shifts was also studied. Except a very small increase of $|\,\mathrm{dpH/d}T\,|$ in the range 7–9, no sucrose dependence was observed.

INTRODUCTION

The development of the method of isoelectric focusing (IEF) has involved a rapid accumulation of isoelectric points (pI) of proteins in the literature. In a recent compilation[1], Righetti and Caravaggio listed *ca.* 800 pI values. However, many of these isoelectric points can never serve as the important tools for protein identification and characterization which might be desired. In fact, as correctly pointed out by the authors, the majority of the more than 300 workers quoted in ref. 1 have neglected to evaluate the pI values under controlled conditions or to report these conditions. Especially, there is quite a degree of uncertainty as to the temperature of the pH measurement. Most workers either do not report the temperature or simply refer to measurements made at room temperature.

According to a recent study of model proteins[2], the pI value should be expected to decrease with increasing temperature. The magnitude of the temperature coefficient $\mathrm{dpI/d}T$ depends on the protolytic composition of the protein and, to a lesser extent, on the temperature. For a strongly acidic protein, $\mathrm{dpI/d}T$ should be *ca.* −0.005 pH unit per degree around 4°, whereas for a strongly basic protein, it should be *ca.* −0.03 pH unit per degree. Consequently, when given a pI value for a protein at a certain temperature, it is impossible, in most cases, to transform this value to another

temperature. It is thus of utmost importance to the utility of the pI data henceforth reported that they refer to a settled standard temperature.

For several reasons[2], the IEF of proteins should preferably be performed at 4°. Ideally, the resulting pH gradient should also be measured at this temperature, although pH measurements at 25°, for instance, would be much easier. If the temperatures of the focusing and the pH measurement do not coincide, it may well be that the pH value assigned to the concentration maximum of the focused protein will not represent the true isoelectric point. Model calculations have indicated (see ref. 2, Fig. 1) that for a weakly acidic protein, for instance, the (apparent) pI evaluated from an IEF run at 4° followed by pH measurements at 25° may differ by as much as 0.2 pH unit from the true value at 25°.

There is a theoretical possibility, however, of circumventing the disadvantage of making pH measurements at 4°. Provided that the buffer capacity of the focused protein is negligible in comparison to that of the carrier ampholytes and that the shifts of the pH values of the latter with temperature are known, it would be possible to transform the apparent pI value of the protein at 25° into a true value at 4°.

Suppose that a protein has been focused at 4° and that the pH values of the fractions containing the protein have been measured at 25°. Further suppose that the pH value corresponding to the concentration maximum of the focused protein has been evaluated by interpolation. As pointed out above, this pH value will not necessarily be the true pI of the protein at 25°. Therefore it is properly denoted by pI^a_{25}, where *a* stands for apparent.

Although pI^a_{25} is *per se* dubious as an isoelectric point, it will still have a definite and useful physico-chemical meaning if, in the actual run, the total protein concentration is kept low enough to permit the carrier ampholyte molecules to dictate the pH in the focused zone, and thereby in the corresponding fractions. Then pI^a_{25} means the pH at 25° of that carrier ampholyte fraction being isoelectric at the same pH as the protein at 4°. Consequently, if the pH shift (ΔpH) which accompanies the warming of this carrier ampholyte fraction from 4 to 25° were known from separate measurements, it would be possible to calculate the pI of the fraction at 4°, and thus the true pI (pI_4) of the protein at this temperature, from the equation:

$$pI_4 + \Delta pH = pI^a_{25} \qquad (1)$$

In this paper, accurate values of ΔpH as a function of pI^a_{25} are presented for pH gradients formed by the Ampholine carrier ampholyte system (Aminkemi, Stockholm, Sweden) in sucrose density gradients. The effect of sucrose on dpH/dT is also considered.

EXPERIMENTAL

Isoelectric focusing

Ampholine pH gradients covering the pH ranges 3.5–10 (six runs), 5–7 (one run), and 7–9 (one run) were created in an LKB column (Type 8100-1) by density-gradient IEF for 72 h at a cooling-water temperature of 4.0 ± 0.1°. The final voltage was 600 V; the final power *ca.* 0.5 W.

In five of the runs, the column was filled with the following system of solutions

for an upward current direction: 11 ml of anode solution (containing 0.085 mole of sulphuric acid and 530 g of sucrose per litre), 98 ml of density-gradient solution (1%, w/v, of Ampholine, 50–500 g/l of sucrose), and 6 ml of cathode solution (0.025 *M* sodium hydroxide). In order to detect a possible influence of the sucrose concentration on dpH/dT, three of the pH gradients 3.5–10 were made with the top electrode as anode. Then the concentrations of sulphuric acid and sodium hydroxide were changed to 0.025 and 0.1 *M*, respectively.

The filling procedure was somewhat different from that recommended by the column manufacturer. The bottom electrode solution was slowly pumped into the column through the bottom plug *after* the density gradient had been introduced through the top nipple in the usual way. According to a recent investigation by Jonsson[3], this modification minimizes the risk of a skew initial distribution of the bottom electrolyte, which would give rise to slanting carrier ampholyte zones at focusing.

After the runs were finished, 1.5-ml fractions of the column contents were collected under a stream of moistened nitrogen in 2.5-ml polyethylene vials with captive snap closures. The vial were stored in a freezer until required for pH measurement.

Measurement of pH

The pH values of the fractions were measured at 25.0° and at 4.0° by means of a digital high-precision pH meter (Radiometer Type PHM64) and a combined electrode (Radiometer Type 2123C). Except for the lower 20 mm, the electrode was surrounded by a plastic cooling-mantle through which water of 25.0 or 4.0 $\pm$ 0.1° was circulated from a thermostat (Hetofrig Type CB4, Heto, Birkerød, Denmark). The cooling-mantle, in turn, and the water tube from the thermostat were isolated by a 1-cm layer of foam plastic. The bottom of the mantle fitted into the vials and contained holes for inlet and outlet of moistened nitrogen.

At delivery, a Radiometer combined electrode is filled with a saturated solution of potassium chloride. Consequently, cooling to 4° results in considerable precipitation of salt. After some days, this precipitate tends to block the porous pin of the above-mentioned electrode (probably owing to recrystallization) and thereby makes the pH response slow and non-repeatable. The original solution of KCl was therefore exchanged for the supernatant obtained by cooling the Radiometer refill solution (Type S-4004) to 0°.

The thermostat was also used for maintaining the fractions at the temperature of measurement. The original lid of the thermostat tank was replaced by a plastic tray, the bottom of which just reached down to the water surface. In the bottom, 100 holes were drilled with such a good fit as to allow the vials to hang down from them into the water. With this device, the *ca.* 75 fractions from one run, as well as a number of vials with buffer samples for standardization of the electrode, could be thermostated simultaneously.

The thermostated fractions of a run were measured in the order of increasing pH. Before being immersed in the fraction to be measured, the tip of the standardized electrode was rinsed with distilled water and blotted gently with paper tissue. When measuring at 4°, it proved necessary to pre-cool the rinsing water in an ice–water bath to obtain a sufficiently stable pH meter reading (see below) within a reasonable time.

About 1 min after the electrode had been immersed in the fraction, the latter was stirred for a few seconds by means of the electrode tip in order to accelerate the establishment of the pH and temperature equilibria within the glass membrane.

The time of measurement at each temperature (4 min at 25°, 5 min at 4°) was chosen on the basis of plots of pH meter reading *vs.* time of immersion of the electrode in a buffer sample and in a fraction. During the fourth minute at 25°, or the fifth minute at 4°, the reading usually changed only +0.001 or 0.002 pH unit. Being about the same at either temperature and for the Ampholine fractions as well as the buffers, this small drift should be insignificant.

The electrode was standardized by means of the following Radiometer buffers: S-1510 (pH 7.410 ± 0.005 at 25°; pH 7.505 ± 0.005 at 4°), S-1316 (4.01 ± 0.01; 4.00 ± 0.01), S-1326 (7.00 ± 0.01; 7.095 ± 0.01), and S-1336 (9.18 ± 0.01; 9.405 ± 0.01). The pH values referring to 4° were obtained by interpolation of data at 0, 5, 10, and 15° given by the Radiometer. The electrical zero of the electrode was adjusted by means of buffer S-1510 and the sensitivity by means of buffer S-1316 (acid region) or buffer S-1336 (alkaline region). Normally it was not necessary to re-adjust the sensitivity when going from the acid to the alkaline region. An additional check of the correct positioning of the pH scale could be made with the buffer S-1326.

A small lag of pH was observed when the electrode was transferred between solutions of widely different pH values (*e.g.*, the buffers S-1510 and S-1316) even though the electrode had been rinsed with water in between. Therefore the electrode was always pre-buffered in an "old" portion of the buffer to be measured before the final reading was made with a fresh portion. On the other hand, it would have been too time-consuming to pre-buffer the electrode before measuring the pH value of each fraction; nor should this be necessary, since the pH difference between two successive fractions was generally <0.1 pH unit.

In measuring the fractions, the electrode was checked against the Radiometer buffers each time an integer pH value of the gradient was about to be passed. In some cases, a small drift (<0.01 pH unit) of the electrical zero was disclosed. Then a linear correction was applied to the pH values of those fractions measured after the preceding control.

To minimize the lag effect, the electrode was pre-buffered in a buffer with the actual integer pH value before the measurements on the fractions were continued.

Treatment of pH data

The difference (ΔpH) between the pH values measured at 25 and 4° for each fraction of a given pH gradient was calculated and plotted *vs.* the pH value at 25° (pI^a_{25}). Then the values of ΔpH corresponding to every 0.1 pH unit of the gradient at 25° (*i.e.*, pI^a_{25} = ..., 6.6, 6.7, ...) were estimated by interpolation in the plot. The six series of ΔpH values for pH gradients 3.5–10 thus obtained were divided into two groups on the basis of the electrode polarity used at IEF. Within each group, the arithmetic mean and the standard deviation of the values of ΔpH pertinent to each value of pI^a_{25} were calculated.

RESULTS AND DISCUSSION

The arithmetic means and the standard deviations of the ΔpH values measured

TABLE I

SHIFTS OF pH ACCOMPANYING THE WARMING TO 25° OF AMPHOLINE pH GRADIENTS GENERATED BY ISOELECTRIC FOCUSING IN SUCROSE DENSITY GRADIENT AT 4°

pI^{a}_{15}*	*pH 3.5–10*				*pH 5–7, anode,* $-\Delta pH^{\S}$	*pH 7–9, anode,* $-\Delta pH^{\S}$
	Anode at bottom		*Cathode at bottom*			
	$-\Delta pH$**	σ***	$-\Delta pH$**	σ***		
3.5	0.054	0.002	0.069	0.004		
3.6	0.066	0.008	0.069	0.004		
3.7	0.073	0.011	0.068	0.005		
3.8	0.074	0.014	0.068	0.005		
3.9	0.078	0.015	0.073	0.006		
4.0	0.080	0.014	0.081	0.006		
4.1	0.085	0.013	0.083	0.005		
4.2	0.092	0.010	0.089	0.005		
4.3	0.104	0.008	0.092	0.001		
4.4	0.120	0.009	0.116	0.004		
4.5	0.131	0.008	0.128	0.005	0.13	
4.6	0.144	0.007	0.141	0.005	0.14	
4.7	0.157	0.007	0.154	0.009	0.15	
4.8	0.167	0.004	0.167	0.006	0.16	
4.9	0.180	0.006	0.177	0.005	0.18	
5.0	0.195	0.005	0.196	0.004	0.19	
5.1	0.203	0.005	0.211	0.004	0.21	
5.2	0.210	0.006	0.220	0.002	0.22	
5.3	0.227	0.004	0.231	0.003	0.23	
5.4	0.240	0.007	0.247	0.002	0.24	
5.5	0.259	0.004	0.263	0.005	0.25	
5.6	0.274	0.002	0.278	0.003	0.27	
5.7	0.287	0.002	0.292	0.006	0.29	
5.8	0.296	0.003	0.302	0.011	0.29	
5.9	0.307	0.012	0.311	0.007	0.30	
6.0	0.313	0.005	0.318	0.001	0.30	
6.1	0.318	0.005	0.333	0.000	0.31	
6.2	0.324	0.004	0.339	0.005	0.31	
6.3	0.333	0.005	0.341	0.008	0.32	
6.4	0.346	0.006	0.345	0.010	0.33	
6.5	0.363	0.004	0.358	0.006	0.34	0.38
6.6	0.374	0.004	0.379	0.005	0.36	0.38
6.7	0.381	0.006	0.383	0.012	0.37	0.39
6.8	0.390	0.005	0.390	0.006	0.38	0.40
6.9	0.394	0.007	0.404	0.005	0.38	0.40
7.0	0.396	0.007	0.411	0.006	0.39	0.41
7.1	0.401	0.003	0.417	0.006	0.39	0.41
7.2	0.400	0.002	0.421	0.007	0.40	0.41
7.3	0.402	0.006	0.425	0.009	0.40	0.42
7.4	0.407	0.008	0.426	0.006	0.40	0.42
7.5	0.411	0.006	0.423	0.004	0.41	0.43
7.6	0.414	0.008	0.432	0.004		0.44
7.7	0.420	0.009	0.443	0.005		0.44
7.8	0.426	0.007	0.446	0.002		0.44
7.9	0.434	0.005	0.450	0.005		0.45

(Continued on p. 352)

TABLE I (*continued*)

pI^a_{25}*	*pH 3.5–10*				*pH 5–7, anode, $-\Delta pH$§*	*pH 7–9, anode, $-\Delta pH$§*
	Anode at bottom		*Cathode at bottom*			
	*$-\Delta pH$***	*σ****	*$-\Delta pH$***	*σ****		
8.0	0.435	0.006	0.454	0.007		0.45
8.1	0.434	0.008	0.456	0.007		0.46
8.2	0.441	0.009	0.462	0.005		0.46
8.3	0.449	0.014	0.470	0.002		0.47
8.4	0.463	0.008	0.480	0.006		0.47
8.5	0.471	0.011	0.487	0.005		0.48
8.6	0.475	0.002	0.496	0.006		0.49
8.7	0.480	0.004	0.501	0.005		0.49
8.8	0.488	0.009	0.506	0.007		0.50
8.9	0.494	0.012	0.515	0.007		0.51
9.0	0.512	0.003	0.526	0.010		0.53
9.1	0.530	0 009	0.535	0.006		0.53
9.2	0.535	0.007	0.540	0.008		0.54
9.3	0.536	0.010	0.545	0.007		0.54
9.4	0.551	0.010	0.551	0.007		0.55
9.5	0.562	0.010	0.558	0.003		0.55
9.6	0.574	0.010	0.558	0.004		
9.7	0.580	0.015	0.558	0.008		
9.8	0.575	0.004	0.558	0.008		
9.9	0.578	0.008	0.558	0.009		
10.0	0.578	0.008	0.561	0.005		

* Apparent isoelectric point at 25°.

** ΔpH is defined as apparent isoelectric point at 25° minus true isoelectric point at 4°; the figures represent arithmetic mean values of data from three pH gradients.

*** Standard deviation of ΔpH.

§ Data from single pH gradient.

for the Ampholine pH gradients 3.5–10 are presented in Table I as a function of pI^a_{25}. The table also contains the ΔpH values obtained for single pH gradients 5–7 and 7–9. The latter values have been rounded to two decimal places.

As indicated by the standard deviations, the ΔpH values corresponding to a given value of pI^a_{25} were highly reproducible from one pH gradient to another as long as the latter covered the same pH range and referred to the same electrode polarities. In itself, a standard deviation based on only three observations should certainly be viewed with great caution. However, as 95% of the σ values listed are $\leqslant$0.012 pH unit, it is evident that the reproducibility of the ΔpH data reported is fully adequate.

It is also worth noting that only two of the six pH gradients 3.5–10 were generated with Ampholine from the same batch. This had no significant influence on the reproducibility of ΔpH.

As already mentioned, half of the pH gradients 3.5–10 were made with reversed electrode polarity in order to estimate the influence of the sucrose concentration on the ΔpH values. The results are shown in Fig. 1, from which can be drawn the qualitative conclusion that for Ampholine species isoelectric in the pH range 7–9, $|\Delta pH|$ is increased to some extent by the presence of sucrose. For $5 < pH < 7$, the sucrose concentration is about the same, irrespective of the electrode polarity, and

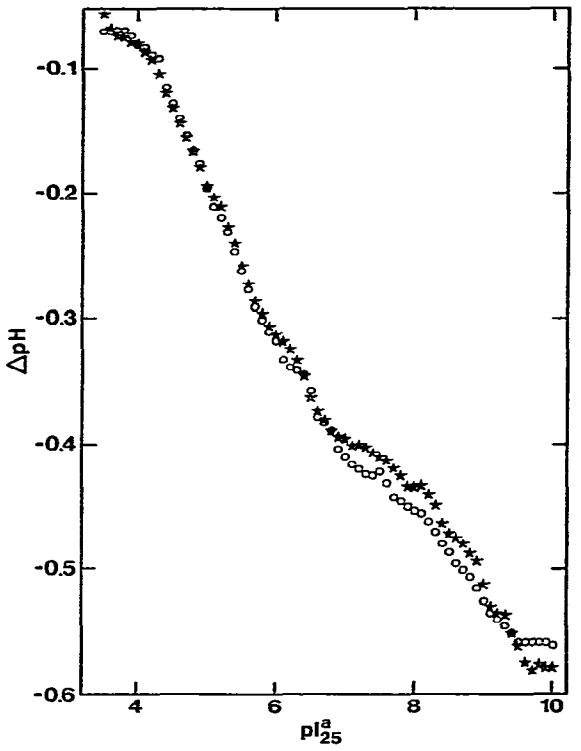

Fig. 1. Influence of sucrose concentration on the pH shifts accompanying the warming to 25° of Ampholine pH gradients 3.5–10 generated by isoelectric focusing in sucrose density gradient at 4°. Data from Table I, column 2 (asterisks) and column 4 (open circles).

therefore a small effect of sucrose in this region cannot be excluded. On the other hand, the sucrose dependence seems to be negligible below pH 5.

Unfortunately it is not feasible, on the basis of the present IEF runs, to make a quantitative evaluation of ΔpH as a function of the sucrose concentration, primarily because it is difficult to determine this concentration accurately in presence of Ampholine. However, if the density gradient is assumed to be linear and a correction is applied for its drift during a run, the sucrose concentration of, for instance, every tenth fraction can be estimated. By comparing these concentrations with the pertinent values of ΔpH in Table I, one comes to the conclusion that the increase of ΔpH is probably less than 0.01 pH unit per 100 g/l of sucrose.

The values of ΔpH given in Table I are thus strictly valid only for the Ampholine pH gradients and electrode polarities specified. If the effect of sucrose is of the size estimated above, it would be possible, however, to make a fairly accurate transformation of the data of Table I to runs with other Ampholine pH ranges and/or sucrose density gradients, as well as to runs in sucrose-free media such as Sephadex or polyacrylamide gels.

A careful determination of pI data by means of IEF also requires a knowledge of the temperature distribution in the density-gradient column or gel plate at the end of the run. A difference of only one degree between the zone focusing temperature

and the cooling-water temperature will induce, on the average, an uncertainty of 0.015 pH unit in the isoelectric point evaluated (*cf.* Fig. 1 of ref. 2 and Table I above).

According to temperature measurements made by Lundin *et al.*[4], the cooling is very efficient in the type of column (LKB 8100-1) used in this work. At a final power of 3 W, the temperature in the column, as measured at different levels with a pin-point thermistor, was only 0.4–0.9° higher than that of the cooling water. Since the final power was 0.5 W or less in the runs reported here, the difference between the column temperature and the cooling-water temperature should be negligible.

In order to test the accuracy of the ΔpH data, and also the validity of eqn. 1, the latter was applied to some proteins for which both apparent pI's at 25° and true pI's at 4° are known from reported runs in sucrose density gradient. The results are in Table II. The pI values at 4° calculated from eqn. 1 agree very well with those measured; the small deviations (0.01–0.03 unit) are all within the total experimental limits of error.

TABLE II

TEST OF VALIDITY OF EQN. 1 AND ΔpH DATA OF TABLE I

Protein	pI_{25}^{a}*	ΔpH**	pI_4*** *(calc.)*	pI_4§ *(ref.)*
Bovine β-lactoglobulin A	5.13 (5)	−0.21	5.34	5.35 (6) 5.35 (6)§§
Bovine β-lactoglobulin B	5.23 (5)	−0.22	5.45	5.46 (6) 5.48 (6)§§
Bovine carbonic anhydrase B	5.89 (7)	−0.30	6.19	6.18 (6) 6.17 (6)§§
Horse myoglobin $MbII_1$	6.96 (8)	−0.39	7.35	7.32 (8)
Horse myoglobin $MbII_2$	6.91 (8)	−0.39	7.30	7.27 (8)

* Apparent pI at 25° as given in the reference cited (in parentheses); measured in sucrose density gradient

** Estimated from Table I.

*** True pI at 4° as calculated by means of eqn. 1.

§ True pI at 4° as given in the reference cited (in parentheses).

§§ Measured in polyacrylamide gel.

For comparison, some pI values measured in gels at 4° are also in Table II. It is seen that these values too deviate less than 0.03 pH unit from those calculated, thus confirming, at least for the pH range 5–7, that the effect of sucrose on ΔpH of Ampholine is small.

The excellent conformity of the gel and density-gradient data also indicates that the apprehensions recently expressed by Gelsema and De Ligny[9] concerning the evaluation of pI values from runs in sucrose density gradient are considerably exaggerated. According to these authors, the sucrose solvent effect upon the isoelectric points of carrier ampholytes and proteins should give rise to errors of several tenths of a pH unit.

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