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ISOELECTRIC POINTS OF PROTEINS, DETERMINED BY ISOELECTRIC FOCUSING IN THE PRESENCE OF UREA AND ETHANOL

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SUMMARY

Isoelectric points, pI_{app}, in sucrose–urea–water and glycerol–ethanol–water mixtures and isoelectric points, pI, in water have been determined at 25° for some carrier ampholytes. The differences, $pI_{app}-pI$, are shown to account for the primary medium effect and the pH measuring cell effect on the isoelectric point. The differences, $pI_{app}-pI$, for Ampholines are used to correct apparent isoelectric points of proteins. pI shifts resulting from the denaturing effect of urea and ethanol are discussed in terms of the conformation change.

INTRODUCTION

In a recent series of papers[1-3], we showed that the apparent isoelectric points, pI_{app}, of proteins, measured by isoelectric focusing in density gradients of sucrose, glycerol or ethylene glycol in the presence of carrier ampholytes, can be corrected for the primary medium effect and the pH measuring cell effect to give isoelectric points, pI, in water. The correction terms to be used, which were tabulated[3], depend on pI_{app}, the concentration of the non-electrolyte, the temperature and the chemical nature of the carrier ampholyte. In this approach, it was tacitly assumed that the conformation of proteins is not affected by the gradient-forming non-electrolyte. There is, indeed, enough evidence[4,5] in favour of this assumption with sucrose, glycerol and ethylene glycol at the concentrations used in isoelectric focusing.

In a paper dealing with the effect of urea on the behaviour of some proteins in isoelectric focusing, Ui[6] demonstrated the value of measurements of isoelectric points in the absence and presence of urea for conformational studies of proteins. However, in that paper, isoelectric points measured in the presence of urea were corrected for the primary medium effect and the pH measuring cell effect due to urea by an average correction term, independent of pI_{app}. In view of the evidence resulting from our work[1-3], this procedure appears to be incorrect[1]. Moreover, literature values of correction terms, $pI_{app}-pI$, associated with the effect of urea, have a considerable scatter, as is shown in Table I.

As we agree with Ui[6] on the value of isoelectric focusing for conformational

TABLE I

LITERATURE VALUES OF $pI_{app} - pI$ USED TO CORRECT FOR THE EFFECT OF UREA

Urea concentration (M)	Correction term, $pI_{app} - pI$	Range of pI_{app} for which correction term is stated to be valid	Reference
6	0.42	4–10	6
6	0.3; 0.4	4–6; 7–10	7
7	0.9	3–6	8
7	0.35	2.5–6	9

studies of proteins, we determined the appropriate correction terms accurately. For this purpose we measured values of $pI_{app} - pI$ for Ampholines in the presence of two reagents that have a conformation-changing effect on proteins, viz., urea and ethanol*, as a function of the concentration of these reagents and of the pI_{app} values of the Ampholines. As the effect of urea or ethanol on the conformation of proteins can be studied by density gradient isoelectric focusing, these $pI_{app} - pI$ values were determined also as a function of the concentration of two gradient-forming non-electrolytes, viz., sucrose and glycerol.

Supposing that urea and ethanol do not affect the conformation of Ampholines, these values of $pI_{app} - pI$ can be assumed to account correctly for the primary medium effect and the pH measuring cell effect of the reagents on the isoelectric point of a protein. Any difference between the isoelectric point of a protein in water and that obtained by correction of the pI_{app} value measured in the presence of the conformation-changing reagent can then be associated with the conformation change of the protein. It should be borne in mind, however, as was pointed out by Ui[6], that the absence of a pI shift due to a conformation-changing reagent cannot always be interpreted in terms of retention of the native configuration, as the pI value is informative only for the degree of dissociation of a few protolytic groups.

As a test of the validity of the correction procedure, we reappraised the literature values of isoelectric points of some proteins in the absence and presence of urea and measured the isoelectric points of two proteins, viz., ribonuclease and β-lactoglobulin, in the absence and presence of urea and ethanol.

In those instances where a significant shift in corrected isoelectric point was found, this shift was interpreted in terms of the conformation change by comparing the result with calculations on the basis of the equation of Linderstrøm-Lang and Nielsen[12], using the known chemical compositions of the proteins in question.

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* While urea and ethanol are both frequently used in conformational studies of proteins[10], only the former has been used as such in conjunction with isoelectric focusing. There is, however, no major objection to the use of ethanol in isoelectric focusing for conformational studies. Practical drawbacks are, of course, its low density, which induces a lower stability of glycerol density gradients, and its high volatility, which can be expected to render isoelectric focusing experiments on granulated gels more difficult. To our knowledge, such gel–isoelectric focusing experiments have not been carried out; experiments in a glycerol gradient, containing up to 60% of ethanol, have been performed by Lebedev et al.[11] for the separation of water-insoluble corn proteins.

EXPERIMENTAL

Measurements of isoelectric points of carrier ampholytes

The isoelectric points of 2% (w/v) solutions of Ampholines (LKB, Stockholm, Sweden) and Servalyte (Serva, Heidelberg, G.F.R.) in water (pI) and in the presence of urea, ethanol, urea + sucrose and ethanol + glycerol (pI_{app}) were determined at 25° as described earlier[3]. The chemicals used were urea (Merck, Darmstadt, G.F.R., Cat. No. 8488), ethanol (Baker, Phillipsburg, N.J., U.S.A., analysed grade), sucrose (Baker, analysed grade) and glycerol (Merck, p.a. grade). The specific conductivity of an aqueous 6 M solution of urea was 9.7 · 10^{-6} Ω^{-1}·cm^{-1} at 25°, indicating the absence of electrolytes. Urea was dissolved just before the measurements without raising the temperature above 25°, to prevent the formation of cyanate[13]. At relatively high concentrations of ethanol the reduced solubility of the carrier ampholytes resulted in turbid solutions.

Measurements of isoelectric points of proteins

Aliquots of 15 mg of bovine pancreatic ribonuclease (Sigma, St. Louis, Mo., U.S.A., Cat. No. R-4875) or bovine β-lactoglobulin (Serva, Cat. No. 27440) were focused in an electrofocusing column (LKB 8100-1) at a temperature of the cooling water of 4°. The concentration of Ampholines (pH range 9–11 for ribonuclease and 3.5–10 for β-lactoglobulin) was 2% (w/v). Density gradients were produced with a gradient mixer (LKB 8121) and ranged from 50 to 5% (w/w) of sucrose in the absence and from 42 to 4% (w/w) of sucrose in the presence of 6 M urea, and from 60 to 6% (w/w) of glycerol in the absence and presence of 30% (w/w) of ethanol. The catholyte was a 0.25 M sodium hydroxide solution; the anolyte was 0.16 M orthophosphoric acid or 0.01 M acetic acid.

After focusing at constant power (5 W) for 48 h (LKB 2103 power supply), the contents of the column were collected in fractions of 3 ml for ribonuclease and 1.5 ml for β-lactoglobulin. The extinction at 280 nm of these fractions was measured using a Vitatron Type MPS spectrophotometer. The pH at 25° of the fractions with maximum UV extinction was measured, and the sucrose or glycerol content was determined from the refractive index. We used calibration graphs established with solutions of 2% (w/v) Ampholines and 6 M urea in sucrose–water mixtures of varying sucrose content and with solutions of 2% (w/v) Ampholines and 30% (w/w) ethanol in glycerol–water mixtures with varying glycerol contents, respectively.

RESULTS

Isoelectric points of carrier ampholytes

The results are given in Table II. As an example, Figs. 1 and 2 give plots of pI_{app}—pI *versus* pI_{app} at a constant concentration (6 M) of urea in sucrose–water mixtures and at a constant concentration (30%, w/w) of ethanol in glycerol–water mixtures, respectively. Figs. 3 and 4 show values of Ip$_{app}$—pI *versus* pI_{app} in aqueous solutions of urea and ethanol, respectively.

Isoelectric points of proteins

The results for pI_{app} at 25° are given in Table III. Table III also includes pI

TABLE II

VALUES OF $pI_{app} - pI$ AT 25° FOR AMPHOLINES OF VARIOUS pH RANGES AND FOR SERVALYTE OF pH RANGE 2-4

$pI_{app} - pI$ values in parentheses denote that the corresponding measurement of pI_{app} was made in a turbid solution.

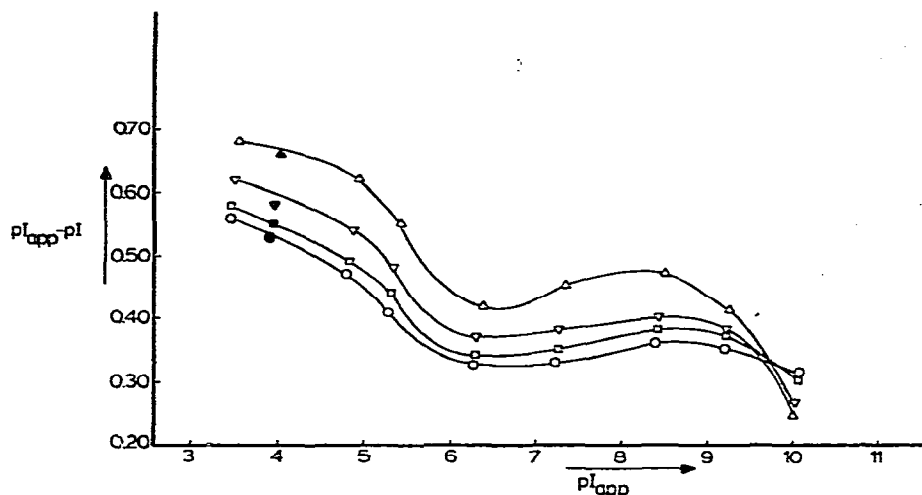| Denaturing agent and concentration	Gradient-forming agent (%, w/w)	Carrier ampholyte								Serva-lyte
		Ampholine								
		pH (2.5-4) pI 2.90	pH (3.5-5) pI 4.34	pH (4-6) pI 4.87	pH (5-7) pI 5.94	pH (6-8) pI 6.90	pH (7-9) pI 8.03	pH (8-9.5) pI 8.85	pH (9-11) pI 9.76	pH (2-4) pI 3.40
Urea (3 M)	Sucrose (0%)	0.27	0.21	0.20	0.16	0.16	0.17	0.17	0.15	0.27
	(15%)	0.27	0.21	0.19	0.15	0.16	0.17	0.16	0.11	0.26
	(30%)	0.27	0.23	0.21	0.16	0.17	0.17	0.15	0.07	0.26
	(45%)	0.30	0.26	0.24	0.19	0.21	0.20	0.15	0.02	0.28
	(55%)	0.33	0.32	0.29	0.23	0.26	0.24	0.17	0.01	0.33
Urea (6 M)	Sucrose (0%)	0.56	0.47	0.41	0.33	0.33	0.36	0.35	0.31	0.53
	(15%)	0.58	0.49	0.44	0.34	0.35	0.38	0.37	0.30	0.55
	(30%)	0.62	0.54	0.48	0.37	0.38	0.40	0.38	0.26	0.58
	(45%)	0.68	0.62	0.55	0.42	0.45	0.47	0.41	0.24	0.66
Urea (9 M)	Sucrose (0%)	0.85	0.74	0.66	0.52	0.52	0.57	0.58	0.50	0.80
	(15%)	0.89	0.80	0.71	0.55	0.56	0.61	0.60	0.49	0.84
Ethanol (30%, w/w)	Glycerol (0%)	0.38	0.36	0.28	0.09	0.11	0.11	0.08	-0.08	0.26
	(20%)	0.51	0.50	0.40	0.17	0.17	0.17	0.13	-0.07	0.38
	(40%)	0.71	0.70	0.59	0.29	0.25	0.24	0.21	-0.05	0.56
	(60%)	1.04	1.06	0.90	0.47	0.34	0.34	0.31	0.01	0.89
Ethanol (50%, w/w)	Glycerol (0%)	(0.75)	0.73	0.62	0.33	0.30	0.30	0.26	-0.02	(0.60)
	(20%)	(1.00)	0.95	0.81	0.45	0.38	0.37	0.33	0	(0.83)
	(40%)	(1.38)	1.34	1.13	0.65	0.48	0.45	0.43	0.06	(1.20)
Ethanol (72%, w/w)	Glycerol (0%)	(1.33)	(1.38)	(1.22)	(0.76)	(0.61)	(0.53)	0.47	0.08	(1.13)
	(20%)	(1.75)	(1.75)	(1.50)	0.87	0.60	0.54	0.50	0.07	(1.46)

Fig. 1. Values of $pI_{app} - pI$ versus pI_{app} for Ampholines (open symbols) and Servalyte (closed symbols) at 25° in 6 M solutions of urea in water (○) and sucrose–water mixtures containing 15 (□), 30 (▽) and 45% (w/w) (△) sucrose.

values at 25° calculated by applying correction terms, $pI_{app} - pI$, resulting from previous work[3] and Table II in this paper.

DISCUSSION

The results in Table II and Fig. 3 corroborate our prediction that the effect of urea on the apparent isoelectric point of an ampholyte depends on the acidity of the ampholyte. The same conclusion holds for ethanol (Fig. 4). Also in accordance with
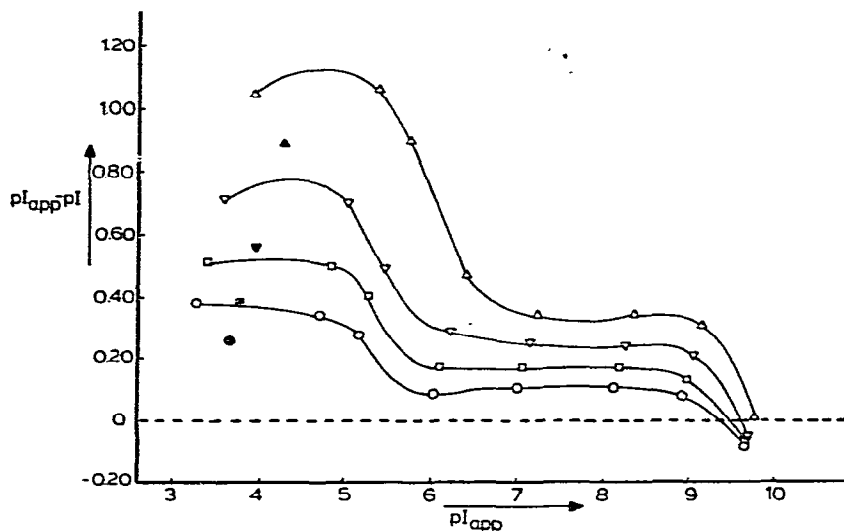

Fig. 2. Values of $pI_{app} - pI$ versus pI_{app} for Ampholines (open symbols) and Servalyte (closed symbols) at 25° in 30% (w/w) solutions of ethanol in water (○) and glycerol–water mixtures containing 20 (□), 40 (▽) and 60% (w/w) (△) glycerol.
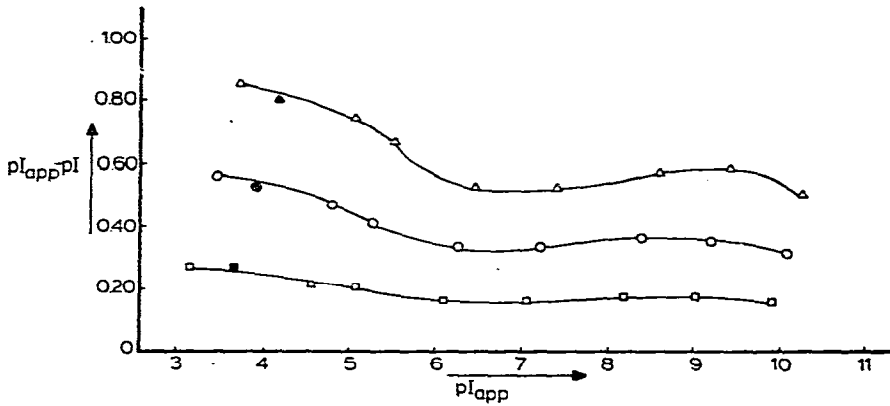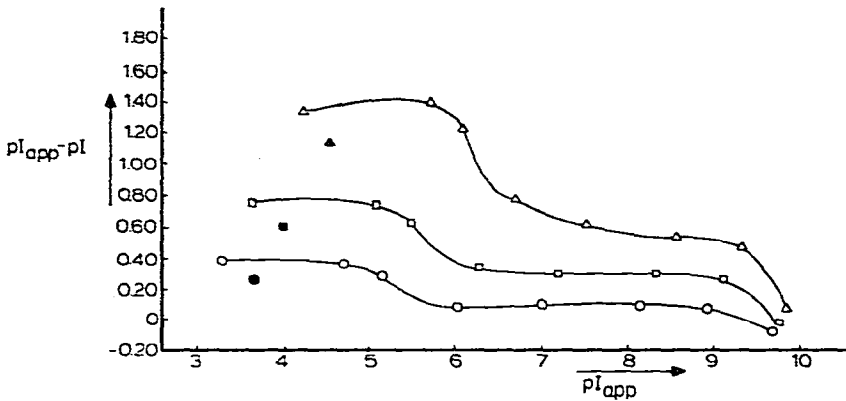
Fig. 3. Values. of $pI_{app} - pI$ versus pI_{app} for Ampholines (open symbols) and Servalyte (closed symbols) at 25° in aqueous solutions containing 3 (□), 6 (○) and 9 M (△) urea.

Fig. 4. Values of $pI_{app} - pI$ versus pI_{app} for Ampholines (open symbols) and Servalyte (closed symbols) at 25° in aqueous solutions containing 30 (○), 50 (□) and 72% (w/w) (△) ethanol.

TABLE III

ISOELECTRIC POINTS OF PROTEINS (25°)

Protein	Conformation-changing agent	pI_{app}	Gradient-forming agent (%, w/w)	$pI_{app} - pI$	pI
Ribonuclease	—	9.50	38.6% sucrose	−0.11	9.61
	—	9.53	40.7% sucrose	−0.12	9.65
	6 M urea	9.90	27.5% sucrose	0.28	9.62
	30% (w/w) ethanol	9.75	46.5% glycerol	−0.04	9.79
	30% (w/w) ethanol	9.81	48.6% glycerol	−0.06	9.87
	30% (w/w) ethanol	9.82	47.8% glycerol	−0.06	9.88
β-Lactoglobulin	—	4.98	34.2% sucrose	−0.04	5.02
	—	4.99	34.8% sucrose	−0.04	5.03
	6 M urea	5.40	28.0% sucrose	0.47	4.93
	6 M urea	5.38	28.2% sucrose	0.47	4.91
	30% (w/w) ethanol	5.76	49.3% glycerol	0.68	5.08
	30% (w/w) ethanol	5.74	47.3% glycerol	0.64	5.10

earlier findings[3], the effects of urea + sucrose and ethanol + glycerol (see Figs. 1 and 2) on the pI_{app} of Servalyte differ from those on the pI_{app} of Ampholines of comparable acidity.

A comparison of values of $pI_{app}-pI$ in sucrose–water and glycerol–water mixtures[3] with the results in Table II shows that the effects of sucrose and urea and of glycerol and ethanol, respectively, are not additive. As there are no literature data on the pK values of weak acids in ternary solvent systems, this finding cannot be commented upon.

The general appearance of the curves in Figs. 1 and 2, which show the effect of sucrose and glycerol, respectively, is similar to that in Figs. 3 and 4, which show the effect of urea and ethanol, respectively, and to that in Fig. 1 in ref. 3, demonstrating the effect of ethylene glycol. This suggests that the $pI_{app}-pI$ differences due to the five mentioned non-electrolytes are caused by the same effects, viz., the primary medium effect and the pH measuring cell effect.

As was pointed out in a preceding paper[3], by combining values of $pI_{app}-pI$ with values of δ, accounting for the pH measuring cell effect, values of pI^*-pI can be calculated:

$$pI^*-pI = pI_{app}-pI-\delta$$

Subsequently it was shown in that paper[3] that the pI^*-pI values of Ampholines calculated in this way are in between the pK^*-pK values of carboxylic acids and alkyl-substituted ammonium ions, giving additional support to the view that the $pI_{app}-pI$ differences of Ampholines due to sucrose, glycerol and ethylene glycol are indeed caused by the two effects mentioned above.

δ values due to ethanol are known[14]. Therefore, pI^*-pI values were calculated and are compared in Fig. 5 with literature values[15] of pK^*-pK. An analogous comparison for the effect of urea cannot be given, owing to the lack of corresponding δ values. One can compare, however, (see Fig. 6) the $pI_{app}-pI$ values of Ampholines with literature values of $pK_{app}-pK$ for acetic acid[16] and the n-butylammonium ion[17].

Figs. 5 and 6, which should be compared* with Fig. 3 in ref. 3, show that the effects of both urea and ethanol on the isoelectric points of Ampholines are indeed intermediate between the effects of these non-electrolytes on the dissociation constants of carboxylic acids and alkyl-substituted ammonium ions. We conclude, therefore, that the $pI_{app}-pI$ values in Table II can be used to correct for the primary medium effect and the pH measuring cell effect on the isoelectric points of proteins. Therefore, these values were used in a reappraisal of the literature values of the isoelectric points of some proteins, measured by isoelectric focusing in the presence of urea (see Table IV).

The data, confined to proteins that are not dissociated into subunits by the action of urea, clearly indicate the importance of the correction procedure.

For insulin and haemoglobin, there is no significant influence of urea on the pI value, which leads to the conclusion that these proteins contain no abnormally dissociating groups, as far as groups having an influence on the isoelectric point are concerned. For a comment on this conclusion, which was also arrived at by Ui[6], we

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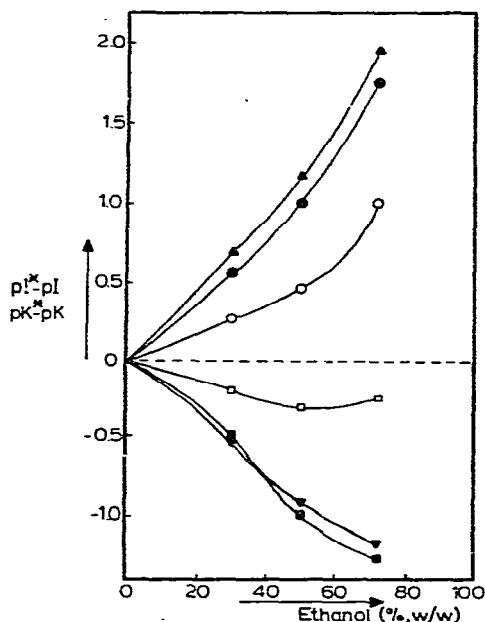* For that reason, data on propionic acid and n-propylamine are included in Fig. 5.

Fig. 5. Values at 25° of $pK^* - pK$ for acetic acid (●), propionic acid (▲), n-butylammónium ion (■) and n-propylammonium ion (▼) and $pI^* - pI$ for Ampholines, $pI = 2.90$ (○) and $pI = 9.76$ (□), in ethanol–water mixtures.

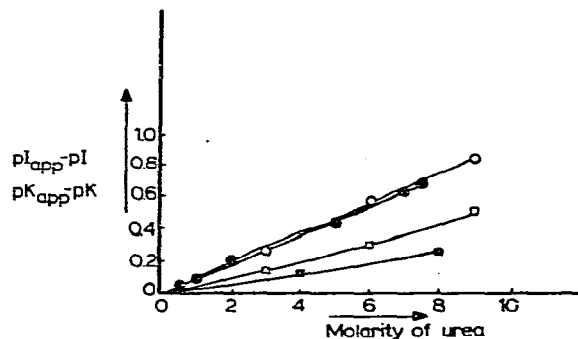Fig. 6. Values at 25° of $p\bar{K}_{app} - pK$ for acetic acid (●) and n-butylammonium ion (■) and $pI_{app} - pI$ for Ampholines, $pI = 2.90$ (○) and $pI = 9.76$ (□), in aqueous urea solutions.

TABLE IV

LITERATURE VALUES OF ISOELECTRIC POINTS IN THE PRESENCE AND ABSENCE OF UREA

Protein	Urea concentration (M)	pI_{app} (25°) *	Concentration of sucrose (%, w/w) **	$pI_{app} - pI$	pI (25°)	Reference
Insulin	0	5.69	20	−0.01	5.70	6
	6	6.15	20	0.36	5.79	6
Haemoglobin	0	7.07	38	0.01	7.06	6
	6	7.50	34	0.40	7.10	6
Ribonuclease	0	9.26	45	−0.08	9.34	6
	6	9.93	45	0.26	9.67	6
α-Casein	0	4.4***	—	—	4.4	
	7	4.66	~20	~0.8	3.9	8
β-Casein	0	4.5***	—	—	4.5	
	7	5.4	~20	~0.8	4.6	8
κ-Casein	0	3.7; 4.1***	—	—	3.7; 4.1	
	7	6.2	~20	~0.8	5.4	8

   * pI_{app} (25°) was obtained from the literature value $pI(t)$ using $\Delta pI/\Delta T$ values equal to the mean of the distribution of dpK/dT values given in Fig. 2 in ref. 3.
   ** Estimated from the descriptions of the focusing experiments and the graphs of the focusing patterns.
   *** Moving boundary values.

refer to his paper. For ribonuclease, however, the significant shift in pI due to urea would imply the presence of abnormally dissociating groups in the native state[6]. The data on caseins show, contrary to the conclusion that would be obtained from a comparison of pI_{app} values, that α-casein does and β-casein does not contain anomalously dissociating groups determining the isoelectric point in the native state. This is consistent with the evidence from viscosity measurements[18], indicating that β-casein is a random coil in the native state[5].

In Table III, our experimental results on ribonuclease are given. Whereas the value for pI estimated from the measurements in 6 M urea agrees with that found by Ui[6], the pI value found in the absence of urea differs considerably from his value (9.34). Our results are in good agreement with the pH of aqueous solutions of ribonuclease, found by Nozaki and Tanford[19] (9.60 at zero ionic strength and 9.71 in 0.15 M potassium chloride solution) and by Tanford and Hauenstein[20] (9.65 in 0.15 M potassium chloride solution). We conclude therefore, in contrast to Ui[6], that 6 M urea does not produce a significant pI shift. Further, there is no need for the assumption of an abnormally dissociating ε-amino group in the native protein. On the basis of the known[21] amino acid composition of ribonuclease and the equation of Linderstrøm-Lang and Nielsen[12], the pI value in water (9.63) can be explained by assuming that three out of six phenolic groups are dissociated (p$K = 9.95$), while the mean pK value of the ten ε-amino groups, considered to be identical, is 10.22. These figures agree with those required for the explanation of the entire titration curve[20].

The absence of a significant shift in pI under the action of 6 M urea should then be interpreted by assuming that the protein is barely denatured under the particular experimental conditions (at 4° and pH 9–11) and that an eventually occurring partial denaturation does not influence the degree of dissociation of the mentioned pI determining protolytic groups. The results of Nelson and Hummel[22] (denaturation by 8 M urea at 25° and pH 7.3 is incomplete) and viscosity and optical rotatory dispersion data for ribonuclease[5] in urea and guanidine hydrochloride solutions are consistent with this interpretation. Moreover, it is well known[23] that polyhydric alcohols (in this instance sucrose) protect the native conformation of ribonuclease against denaturation by urea.

In the presence of ethanol, which is known[23,24] to have a destabilizing effect on the native conformation of ribonuclease, the pI value differs significantly from that in water. We ascribe this pI shift to a general increase in the pK values of ε-amino groups upon denaturation. This increase, which is consistent with the fact that the pK values of side-chain amino groups required to explain the titration curves of native proteins are generally smaller than expected, was ascribed by Tanford[25] to the hydrophobicity of the major parts of lysine side-chains. These parts thereby tend to be buried in the interior of the native structure, resulting in stabilization of the uncharged form of the ε-amino groups relative to the charged form and hence in a decrease in their pK value. This view was confirmed by Nozaki and Tanford, who showed that the pK values of amino groups needed to explain the titration curves of amino acids[26] and proteins[19] in 6 M guanidine hydrochloride are close to the corresponding pK values of amino acids in aqueous dilute salt solution.

Consequently, we calculated the mean pK value of the ten ε-amino groups (considered to be identical) needed to explain the pI value (9.85) found in 30% ethanol assuming all other pK values to be unchanged relative to their effective values in the

native state. We obtained the result $pK = 10.55$, a value intermediate between the "expected" value according to Tanford[25] (10.4) and that (10.8) for lysine in aqueous solution[25].

The pI value (Table III) of β-lactoglobulin, *i.e.*, the approximately equimolar mixture of the genetic isomers A + B, calculated from measurements in a sucrose gradient, is slightly lower than expected from literature data. The latter are not consistent, however. By means of isoelectric focusing the following values are found: 5.26 (A)[7] and 5.34 (B)[7]; 5.21 (A)[27] and 5.34 (B)[27]; 5.13 (A)[28] and 5.23 (B)[28]; and 5.24 (A)[29] and 5.14 (A+B)[30]. The isoionic point of the protein (A+B) in pure water was found at pH 5.19 and to be invariable with ionic strength by Cannan *et al.*[31], but at pH 5.39 and to decrease with increasing ionic strength by Nozaki *et al.*[32]. pI values found by moving boundary electrophoresis are generally lower: 5.19 (ref. 33) and 5.10 (ref. 34). By means of the equation of Linderstrøm-Lang and Nielsen[12] and the pK values required for an adequate description[25] of the titration curve of β-lactoglobulin (A+B) in aqueous solution, a pI value of 5.36 can be calculated.

The $pI_{app} - pI$ correction terms are large in this instance, so the only comment that can be made on the pI shifts exerted by urea and ethanol is that they are small. A small effect is indeed expected. From the work of Tanford and co-workers[25,32], it is known that two out of fifty-three carboxyl groups are abnormally weak ($pK \approx 7.4$) in the native protein but behave as "expected" ($pK \approx 4.8$) in the denatured state. Calculation shows that this conformation change upon denaturation should result in a decrease in pI of 0.07 pH unit.

CONCLUSIONS

(1) $pI_{app} - pI$ values in sucrose–urea–water and glycerol–ethanol–water mixtures depend on the acidity/basicity and the chemical type of the ampholyte and on the solvent composition.

(2) $pI_{app} - pI$ values in these mixtures account for the primary medium effect and the pH measuring cell effect on the isoelectric point of Ampholines.

(3) $pI_{app} - pI$ values for Ampholines can be used to correct the apparent isoelectric points of proteins measured in these media, giving pI values which are useful for conformational studies.

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