

# Isoelectric Focusing of Proteins in the Native and Denatured States

## ANOMALOUS BEHAVIOUR OF PLASMA ALBUMIN

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1. An analytical technique of isoelectric focusing in thin layers of polyacrylamide gel has been used to determine the isoelectric point, pI, of several proteins in the presence and in the absence of concentrated urea. 2. The presence of urea did not greatly affect pI except for bovine plasma albumin, where an increase of approx. 1 pH unit was found. 3. Evidence is presented that this change in the pI of bovine plasma albumin is due to the normalization of certain ionizable groups on unfolding of the protein in urea. 4. Evidence is also presented that prolonged exposure of bovine plasma albumin to urea results in intramolecular disulphide interchange and that, on removal of urea, the new patterns of disulphide bonding stabilize abnormal conformations with pI values intermediate between those of the native and denatured states. 5. The studies demonstrate heterogeneity in bovine plasma albumin based on primary-sequence differences. 6. Isoelectric focusing of proteins in urea appears to be useful in the study of various aspects of protein structure.

The high-resolution technique of isoelectric focusing in the presence of carrier ampholytes is a powerful tool for studying protein heterogeneity. An analytical technique of isoelectric focusing in thin layers of polyacrylamide gel was developed to study immunoglobulin heterogeneity (Awdeh, Williamson & Askonas, 1968). In these early studies antibody-antigen precipitates were dissolved in urea and the antibodies focused in the presence of concentrated urea. The questions raised by this procedure led us to the present studies on the relationship between the conformation and isoelectric properties of proteins.

In this paper we describe the isoelectric focusing of several proteins in the presence and in the absence of urea. The results chiefly concern bovine plasma albumin, since the isoelectric properties of this protein were markedly influenced by the presence of urea. The potential of analytical isoelectric focusing for the study of various aspects of protein structure is illustrated by these studies.

## METHODS

**Chemicals.** Ampholine carrier ampholytes of various pH ranges were obtained from LKB-Producter, Stockholm, Sweden.

Acrylamide, *NN'*-methylenebisacrylamide and riboflavin were from BDH Chemicals Ltd., Poole, Dorset,

U.K.; *NNN'*-tetramethylethylenediamine was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Urea was from May and Baker Ltd., Dagenham, Essex, U.K. Aqueous solutions were deionized immediately before use by passage through a column of mixed-bed resin.

Bromophenol Blue was from BDH Chemicals Ltd. and Coomassie Blue (colour index 42660) from Raymond Lamb, Alport, Middx., U.K.

5,5'-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and *N*-ethylmaleimide were from Sigma Chemical Co., St Louis, Mo., U.S.A.; dithiothreitol was from P-L Biochemicals, Milwaukee, Wis., U.S.A.; iodoacetamide was from BDH Chemicals Ltd.

**Proteins.** Crystallized bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.;  $\alpha$ -chymotrypsinogen A (bovine) was from Sigma Chemical Co.; rabbit creatine phosphokinase (EC 2.7.3.2) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; pig insulin was from Novo Co., Copenhagen, Denmark; bovine  $\beta$ -lactoglobulin was from BDH Chemicals Ltd.; chicken ovalbumin was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. *Erwinia carotovora* L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) was a gift from Mr H. H. Robinson, Microbiological Research Establishment, Porton Down, Salisbury, Wilts., U.K. Horse myoglobin and sperm-whale myoglobin were a gift from Dr M. J. Crumpton of this Institute. G-myeloma protein 5563 was prepared from ascitic fluid of tumour-bearing mice by chromatography on DEAE-cellulose (Askonas, 1961).

**Isoelectric focusing.** Isoelectric focusing was carried out in thin-layer polyacrylamide gels by the method of Awdeh *et al.* (1968). Further details of the procedure together with various modifications are given below.

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The composition of the gel mixture was as follows: acrylamide (50 mg/ml); *NN'*-methylenebisacrylamide (1.35 mg/ml); *NNN'*-tetramethylethylenediamine (0.25  $\mu$ /ml); riboflavin (0.002 mg/ml); Ampholine (20 mg/ml); urea (6M) as required. The mixture was degassed with a water pump, and gels (20 cm  $\times$  15 cm  $\times$  0.13 cm) were cast between two plates (one being previously treated with silicone) separated by a frame of silicone rubber tubing. After photopolymerization, the silicone-treated plate was removed, leaving the gel firmly attached to the other plate.

Proteins were dissolved at a concentration of 2 mg/ml in 20 mM-sodium phosphate buffer, pH 7.0, or, for insulin, in 5 mM-HCl. Samples (75–100  $\mu$ g) were applied to the surface of the gel as a drop of liquid or on a small piece of filter paper. Loading was normally at the end of the gel furthest from the expected focusing position.

Isoelectric focusing was carried out by inverting the gel so that each end rested on one of two horizontally positioned carbon electrodes contained in a humidified chamber (33 cm  $\times$  23 cm  $\times$  9 cm). Before each new run the cathode was wetted with ethylenediamine and the anode with phosphoric acid. The run was carried out at approx. 4°C for 20–24 h. While the pH gradient was being established (2–3 h) the voltage was gradually increased without the current being permitted to exceed 6 mA. The potential difference between the electrodes was finally maintained at 500 V, the current falling to less than 2 mA. We have found that 20 h is sufficient time for the proteins used in this study to be focused at their isoelectric points. This was demonstrated for normal and urea gels by loading samples of the same protein near both positive and negative electrodes (see Plate 3).

After the run was terminated and before staining of the protein bands, for the purpose of determining the pH gradient, discs of gel (0.55 cm in diam.) were cut out with a cork-borer at intervals of 1 cm along a track parallel to the potential gradient. Each disc was suspended for 2 h in 0.5 ml of water and the pH was then measured at room temperature with a combined microelectrode M22DP (Activion Glass Ltd., Kinglassie, Fife, U.K.) attached to a model 46A pH-meter (E.I.L., Richmond, Surrey, U.K.). It was shown that the dilution of Ampholine buffer that occurs in this process, from 20 mg/ml in the gel to approx. 1.2 mg/ml, does not significantly affect its pH. Serial dilutions were made from 20 mg/ml solutions of stock Ampholine for pH ranges 4–6, 5–8 and 7–10. Some change in pH values was detectable for ranges 4–6 and 7–10, but this only amounted to an increase and decrease respectively of 0.1 pH unit at 0.06 mg/ml.

Isoelectric focusing was performed at 4°C, but local temperatures in the gel were not known. We therefore chose to make pH measurements at room temperature for convenience. It is probable that the pI values of proteins and Ampholine carrier ampholytes will have similar temperature coefficients; to the extent to which this relationship holds our pI values will relate to room temperature.

Discs of gels were eluted with water whether or not urea was present in the gel, and this point is dealt with in the Discussion section. We have investigated the effect of 6M-urea on the observed pH value of Ampholine buffer at 20 mg/ml. With each of the three pH ranges and at 23 or 0°C the effect was to raise the value by between 0.3 and 0.4 pH unit.

For staining of the protein bands plates were immersed in a 0.2% (w/v) solution of Bromophenol Blue in ethanol-acetic acid-water (10:1:9, by vol.) (Awdeh, 1969) or in a 0.1% (w/v) solution of Coomassie Blue in a similar solvent of composition 9:2:9 (by vol.). After 20–40 min the plates were destained in several changes of solvent (6:1:13, by vol.). For the detection of insulin, which was leached out of the gels in the above procedures, plates were immersed in 10% (w/v) trichloroacetic acid. The precipitated bands were clearly visible without staining.

*Blocking of thiol groups in bovine plasma albumin.* Our preparations of bovine plasma albumin were found by the procedure of Ellman (1959) to contain approx. 0.7 mol of free SH groups/mol of protein. Blocking of these groups was carried out by addition of 5 mol of *N*-ethylmaleimide/mol of protein in 20 mM-sodium phosphate buffer, pH 7. The reaction mixture was left at room temperature for 10 min and then dialysed as described below and in the Results section.

*Reduction and alkylation of bovine plasma albumin.* Reduction of the disulphide groups of bovine plasma albumin was carried out by treatment of the protein in 8M-urea with 0.1M-dithiothreitol for 2 h at room temperature at pH 8, and this was followed by alkylation of the thiol groups with a threefold molar excess of iodoacetamide at 4°C for 30 min. During alkylation the pH of the reaction mixture fell to approx. 7. The reaction mixture was finally dialysed against 8M-urea.

*Dialysis procedure.* Dialysis of albumin solutions against 20 mM-sodium phosphate buffer, pH 7, or 8M-urea was carried out at 4°C in Visking tubing (diameter 0.25 in) washed as described by Sogami, Petersen & Foster (1969).

## RESULTS

*Isoelectric point of proteins in the presence and in the absence of urea.* The technique of Awdeh *et al.* (1968) for isoelectric focusing in thin layers of polyacrylamide gel has been used to make comparative measurements of the isoelectric points of proteins in their native state and unfolded in the presence of high concentrations of urea.

Polyacrylamide gels, containing the appropriate range of Ampholine carrier ampholytes, were prepared in duplicate, one of them containing 6M-urea. Up to eight protein samples were loaded side by side on to the surface of each gel. After focusing had been carried out, the pH gradients were measured, the proteins were stained and their isoelectric points determined. Each protein was focused on at least two gels of each type. Mean values of isoelectric point and standard deviations were calculated (Table 1).

The focusing of protein components is highly reproducible, and the main source of error is in the procedure used for determination of pH gradients. It would be possible to improve the accuracy of this by decreasing the size and spacing of the gel samples cut for pH measurements, and by determining the pH gradient along each individual track. However, for the present study the simple methodology described gave adequate measurements. With the

Table 1. *Isoelectric point of various proteins in the presence and in the absence of 6M-urea*

Isoelectric points were determined as described in the Methods section. Where no standard deviation is given each value quoted is the mean of two observations differing by less than 0.3 pH unit. The number of observations was three for  $\beta$ -lactoglobulin and ovalbumin, and five for myoglobin (sperm whale), 5563 myeloma protein and bovine plasma albumin. Values of pI measured by isoelectric focusing should represent isoelectric points (Vesterberg, 1968). Values for isoelectric points at 20–25°C, where available, support this hypothesis. The low pI found for chymotrypsinogen is probably due to the technical difficulties in making measurements at the basic end of a pH gradient.

Protein	pI of major components		Notes	Isoelectric point
	Normal gel	Urea gel		
Chymotrypsinogen	8.5	8.5	In urea several minor bands appeared to the acidic side of the main band down to approx. pH 6.6; urea is probably causing release of fragments of the molecule produced by the action of chymotrypsin (Desnuelle, 1960)	9.4 (Tanford, 1962) 9.66 (Marini & Wunsch, 1963)
L-Asparaginase	8.0	7.8		
Myoglobin (sperm-whale)	8.01, 7.68 ( $\pm 0.18$ )	7.73, 7.47 ( $\pm 0.16$ )	Three minor bands at approx. pH 7.6, 7.4, 7.1 (normal) and pH 7.3, 7.2, 6.9 (urea)	7.86–7.80 (Breslow & Gurd, 1962)
Myoglobin (horse)	7.5, 7.1	7.4, 7.0	Minor bands at approx. pH 7.0, 6.8 (normal) and 6.9, 6.7 (urea)	7.40, 6.96 (Vesterberg & Svensson, 1966; Vesterberg, 1967)*
Creatine phosphokinase	6.9, 6.7, 6.6	7.0, 6.9	The change from three to two bands in urea suggests that the enzyme in muscle may contain two dissimilar subunits (cf. Dawson, Eppenberger & Eppenberger, 1968)	
Myeloma protein (5563)	5.94, 5.86, 5.79 ( $\pm 0.06$ )	6.12, 6.08, 6.04 ( $\pm 0.07$ )		5.6 (Tanford & Epstein, 1954)
Insulin	6.0	6.0		
$\beta$ -Lactoglobulin	5.34, 5.26 ( $\pm 0.07$ )	5.14, 5.07 ( $\pm 0.14$ )	Minor bands at approx. pH 5.1 (normal) and 5.05, 5.00 (urea)	5.39 (Nozaki, Bunville & Tanford, 1959)
Ovalbumin	4.98, 4.93 ( $\pm 0.21$ )	4.90, 4.83 ( $\pm 0.20$ )	In urea several new bands appeared in the range pH 5.2–5.9	4.9 (Cannan, Kibrick & Palmer, 1941)
Plasma albumin (bovine)	4.86, 4.82, 4.78 ( $\pm 0.10$ )	6.01, 5.93, 5.86 ( $\pm 0.10$ )	Minor bands are shown in Plate 3	5.3 (Foster, 1960)

\* Measured by isoelectric focusing in a sucrose density gradient.

single exception of bovine plasma albumin (a similar phenomenon has been observed with human albumin and rat albumin in work not reported here), the isoelectric point determined for each protein component was not greatly altered in the presence of 6M-urea. The implications of similar isoelectric properties in the presence and in the absence of urea are dealt with in the Discussion section.

*Effect of urea on the isoelectric properties of bovine plasma albumin.* The alteration of the isoelectric-focusing position of bovine plasma albumin caused by the presence of 6M-urea is depicted in Plate 1. Myeloma protein (5563), which exhibits bands at similar isoelectric points irrespective of the addition of urea, is shown for reference. In these experiments focusing was performed with wide-range (pH 3–10) Ampholine carrier ampholytes, and in the resultant steep pH gradient the multiple bands of the albumin are poorly resolved, particularly in the absence of urea, where an enclosing envelope of protein is stained (Plate 1 is better than usual resolution, cf. Plates 4 and 5). To assign isoelectric points to the various albumin bands in the native state it is necessary to carry out focusing in narrow-range pH gradients; broader bands are obtained in these shallow gradients, but resolution is higher (Plate 2). Three major bands isoelectric at pH 4.86, 4.82 and 4.78 ( $\pm 0.1$ ) respectively are clearly resolved, though the most basic component appears complex. The presence of 6M-urea in a shallow pH gradient permits the three major components to be more sharply resolved (Plate 3), with isoelectric points at pH 6.01, 5.93 and 5.86 ( $\pm 0.1$ ); the two more basic of these show a tendency towards splitting into two bands and a number of minor components are also seen.

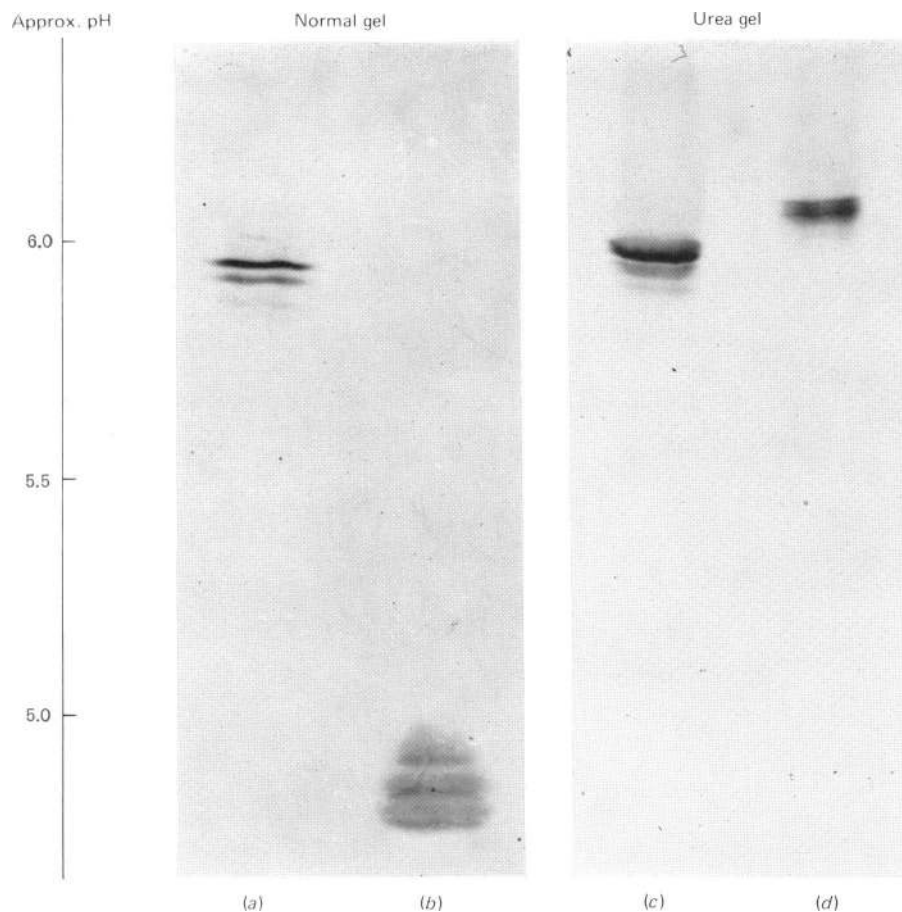
The persistence of the microheterogeneity of bovine plasma albumin in the presence of 6M-urea suggests that there must be a primary structural basis (either synthetic or post-synthetic) for the charge differences. This view was strengthened by investigating the focusing of bovine plasma albumin after complete reduction and alkylation (Plate 3). (In this case focusing was only possible in urea, since reduced and alkylated albumin is precipitated in its absence.) The banding pattern still shows microheterogeneity, with bands focusing at the same isoelectric points as those of unreduced albumin in 6M-urea. Calculations outlined in the Discussion section suggest that each component of bovine plasma albumin in urea differs from its neighbours by one unit charge. The reason for the changes in intensity of staining of the bands on reduction and alkylation is not known.

*Reversibility of the shift in the isoelectric point of bovine plasma albumin in urea.* A study of the reversibility of the large shift in isoelectric point

that bovine plasma albumin exhibits in urea was carried out in the hope that it might throw light on the cause of the shift. In an initial experiment the albumin was dissolved in solutions containing up to 10M-urea, and these samples were immediately loaded on to a gel not containing urea and focused in the normal way. No alteration in the banding pattern or in the isoelectric points of the components from those of the untreated albumin could be detected after the brief exposure to concentrated urea. Either the shift phenomenon had been completely reversed or it takes place slowly. During isoelectric focusing the albumin is exposed to urea for up to 24h, so reversibility was tested after treatments for a similar duration. A solution of bovine plasma albumin in 20mM-phosphate buffer, pH 7.0, was dialysed against 8M-urea for 24h followed by a further 24h dialysis against buffer. The sample was applied to a gel and focused in a pH 3–10 gradient in the absence of urea. The resultant heterogeneous pattern (Plate 4a) comprises between 10 and 20 bands focused at intervals over the pH range between the isoelectric points of albumin in the native state (Plate 4b) and in 6M-urea (close to the position of 5563 myeloma protein, Plate 4c). Prolonged exposure of albumin to concentrated urea followed by removal of the urea thus increased the number of components rather than demonstrating reversibility of the effect. To check that the increase in heterogeneity did not result from the slow removal of urea on dialysis, the albumin was dialysed against 8M-urea for 48h and then directly applied to a gel in the absence of urea; the pattern obtained showed no significant difference from the previous sample shown in Plate 4(a). Control samples of albumin dialysed for 48h against buffer showed the native focusing pattern.

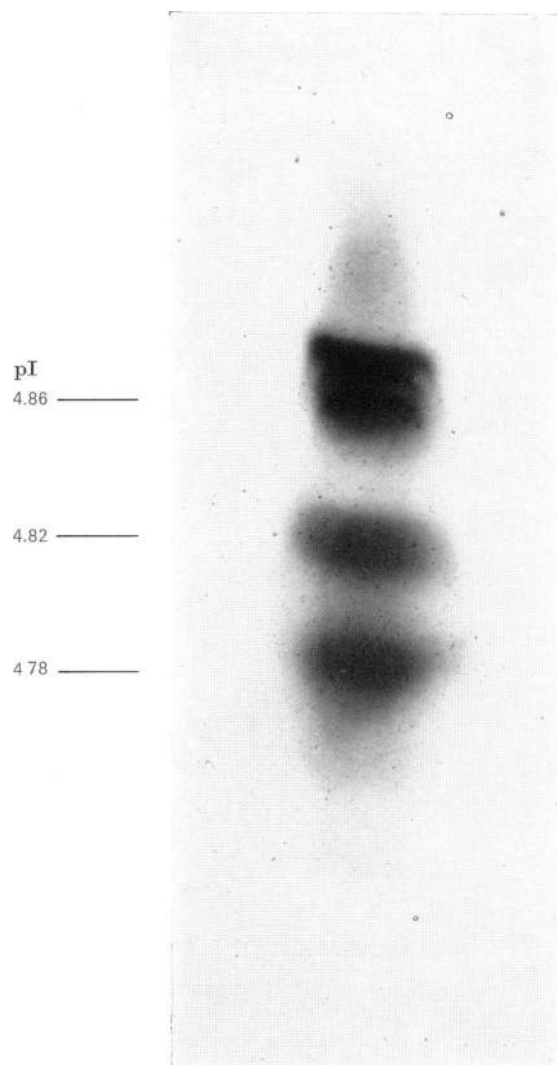
The samples exposed to urea for 24–48h and the controls were also examined by isoelectric focusing in the presence of 6M-urea. In both cases the isoelectric spectrum was similar and indistinguishable from the usual pattern in urea (Plates 1 and 3). In further control experiments 5563 myeloma protein was shown to retain its original isoelectric spectrum after being taken through any of the procedures applied to the albumin.

If the shift in the isoelectric point of bovine plasma albumin is due to unfolding of the molecule with normalization of certain ionizable groups, it would be expected to take place very rapidly and to be reversible. In the light of the experiment in which albumin was exposed to urea for a short period, the observations made when the protein was exposed to urea for prolonged periods remain compatible with this mechanism only if a secondary slow process also takes place in urea resulting, on removal of urea, in the formation of a series of stable conformational states intermediate between the



#### EXPLANATION OF PLATE I

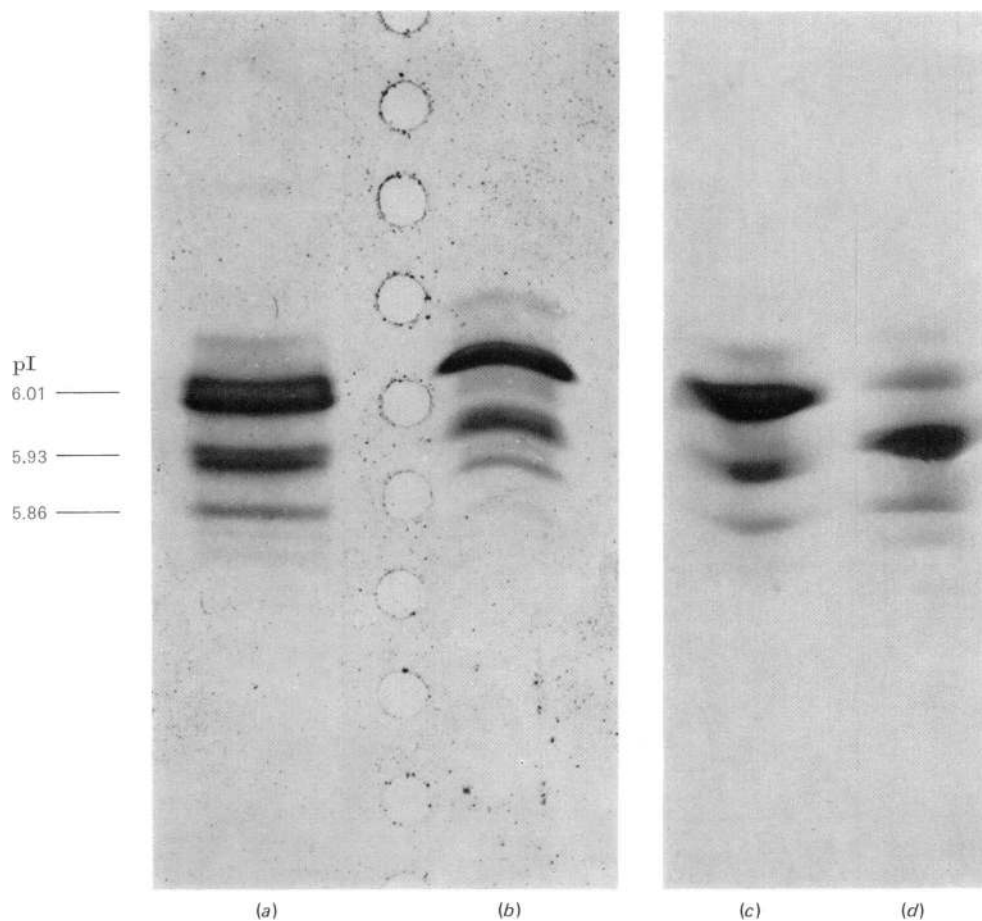
Isoelectric focusing of bovine plasma albumin and 5563 G-myeloma protein in the presence and in the absence of urea (polyacrylamide gels with and without 6M-urea, Ampholine range pH3-10). (a) Myeloma protein, no urea; (b) bovine plasma albumin, no urea; (c) bovine plasma albumin, 6M-urea; (d) myeloma protein, 6M-urea.



**EXPLANATION OF PLATE 2**

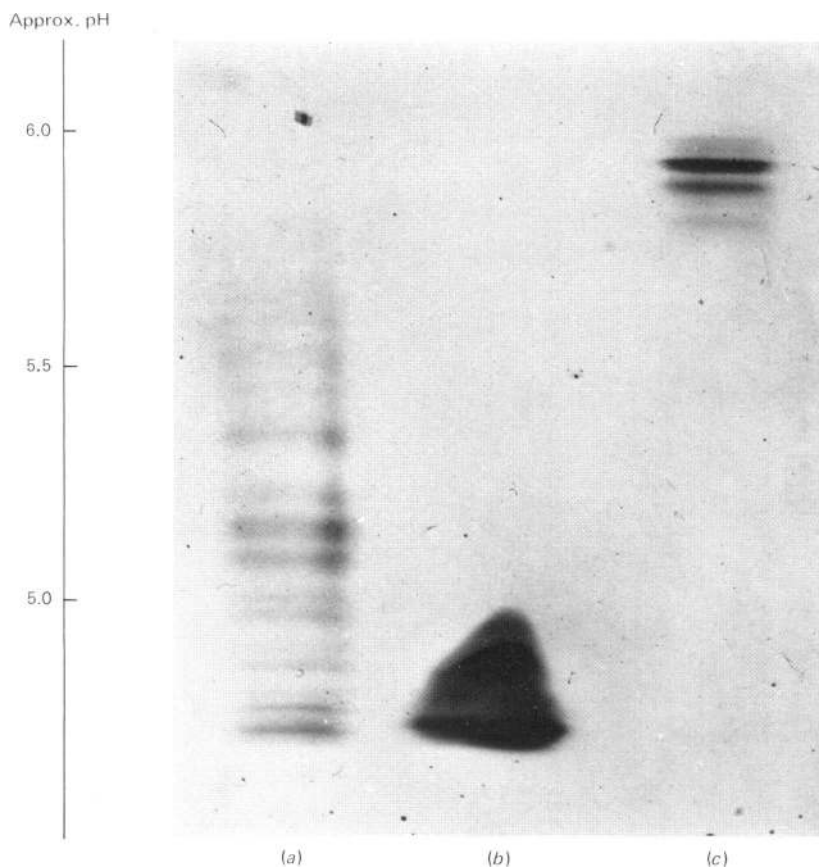
Isoelectric focusing of bovine plasma albumin in the absence of urea (polyacrylamide gel without urea, Ampholine range pH 4–6). Values of pI shown are mean values from Table 1.

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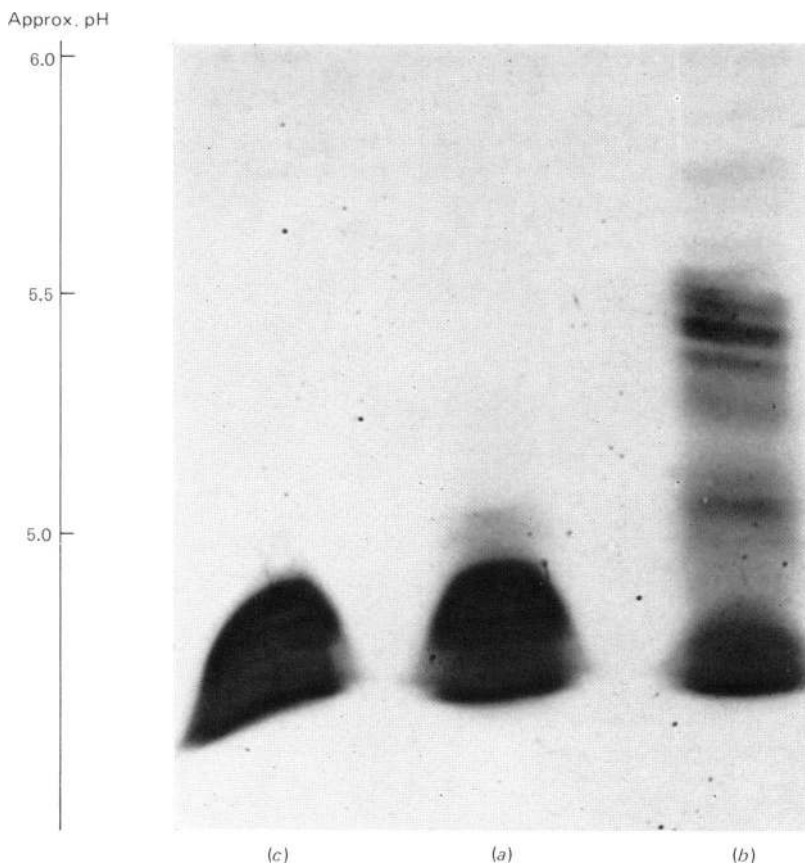
#### EXPLANATION OF PLATE 3

Isoelectric focusing of bovine plasma albumin and of reduced and alkylated bovine plasma albumin in the presence of urea (polyacrylamide gels containing 6M-urea, Ampholine range pH 5–8). Values of pI shown are mean values from Table 1. (a) and (c) Bovine plasma albumin (loaded at basic end); (b) bovine plasma albumin (loaded at acidic end); (d) reduced and alkylated bovine plasma albumin (loaded at basic end).



#### EXPLANATION OF PLATE 4

Isoelectric focusing in the absence of urea of bovine plasma albumin previously exposed to urea (polyacrylamide gel without urea, Ampholine range pH3-10). (a) Bovine plasma albumin that had been dialysed against 8M-urea for 24 h at 4°C and then against 20mM-phosphate buffer, pH7.0, for a further 24 h. For comparison are shown (b) undialysed bovine plasma albumin and (c) undialysed 5563 G-myeloma protein.



**EXPLANATION OF PLATE 5**

Isoelectric focusing in the absence of urea of 'blocked' bovine plasma albumin previously exposed to urea (polyacrylamide gel without urea, Ampholine range pH 3-10). (a) Bovine plasma albumin that had been treated with *N*-ethylmaleimide to block thiol groups before dialysis against 8M-urea for 24h at 4°C and then against 20mM-phosphate buffer, pH 7.0, for a further 24h. For comparison are shown (b) normal bovine plasma albumin that had been dialysed against 8M-urea for 24h at 4°C and then against 20mM-phosphate buffer, pH 7.0, for a further 24h, and (c) normal bovine plasma albumin that had been dialysed against 20mM-phosphate buffer, pH 7.0, for 48h at 4°C.

native and the unfolded states. A candidate for such a process would be the rearrangement of the intrachain disulphide bonds, and this possibility was examined.

The preparations of bovine plasma albumin used in these experiments contained approx. 0.7 mol of free thiol groups/mol, and to test whether this group could be involved in disulphide interchange a sample of the protein was treated with *N*-ethylmaleimide. This 'blocked' albumin lacking a free thiol group was taken through the various dialysis experiments described above. Prolonged exposure of 'blocked' albumin to concentrated urea did not significantly alter the isoelectric spectrum in the absence of urea (Plate 5a) relative to the native protein (Plate 5c). However, when focusing was carried out in the presence of 6M-urea, 'blocked' albumin showed the same increase in isoelectric point as did the untreated albumin.

### DISCUSSION

This study has shown that the isoelectric properties of bovine plasma albumin are markedly dependent on conformation; the native and denatured states of the protein have isoelectric points differing by approx. 1 pH unit. Moreover, bovine plasma albumin can apparently adopt other conformations intermediate in isoelectric properties between the native and the denatured states; these intermediate conformations are probably stabilized by abnormal patterns of disulphide bond formation. Andersson (1966) and Sogami *et al.* (1969) have shown that the free thiol group of bovine plasma albumin can initiate intramolecular disulphide interchange even in the native protein. In the presence of urea the possibilities for disulphide rearrangement are probably enhanced. During the removal of urea new patterns of intramolecular disulphide bonds could permit the albumin to refold in modified conformations. The number of conformations with different isoelectric points should be interpretable from the isoelectric spectrum of 'scrambled' albumin. We must, however, take into account the known microheterogeneity of bovine plasma albumin, which is shown in its isoelectric spectrum. The present study shows that the native, denatured and fully reduced and alkylated states all show similar microheterogeneity. We conclude that the components have different primary structures; such charge differences may not reflect genetically coded sequences, but could result from post-synthetic alteration of a single sequence. Any intermediate conformations presumably also reflect this charge heterogeneity, and consequently we can estimate from the number of intermediate isoelectric bands that there are probably no more than three or four such stable conformations. We refer to these

intermediate states as stable, because isoelectric focusing is an equilibrium technique and these components are focused as well-defined bands.

The above discussion of our results presupposes that the major effect of urea is on the conformation of bovine plasma albumin. We have excluded a number of other possibilities.

(1) Urea may dissociate ligands bound to albumin. Bovine plasma albumin has binding sites for many small molecules, which, if negatively charged, would lower the isoelectric point, and dissociation of such ligands in the presence of urea would therefore increase the value. This effect could, at most, account for only a small part of the observed pI shift. Fatty acids remain bound to the albumin as it is usually purified, but less than 1 mol/mol is present. Defatted albumin was examined and no difference in isoelectric spectrum was observed. Moreover, brief exposure of the albumin to 10M-urea or prolonged dialysis of 'blocked' albumin against 8M-urea (treatments that should dissociate bound ligands) do not irreversibly alter the isoelectric properties.

(2) Albumin may bind carrier ampholytes when focused in the absence of urea but not in its presence. The binding of Ampholine carrier ampholytes has been considered as a possible cause of the heterogeneity seen in most proteins studied by focusing techniques. However, Vesterberg (1969) was able to obtain complete separation of radioactively labelled Ampholine from human serum albumin on Sephadex G-50, suggesting that if any binding to the protein does occur it is fully reversible. In the particular case of bovine plasma albumin it should be noted that the isoelectric points determined in this study for the major components of the native protein are consistent with previous measurements (Alberty, 1949; Aoki & Foster, 1957a,b).

(3) Urea could directly affect the dissociation constants,  $pK$ , of groups contributing to the isoelectric point. Values of  $pK$  are indeed altered when measured in the presence of high concentrations of urea, and this can be explained in terms of decreased activity of water (Levy, 1958). This fundamental effect of urea on  $pK$  would of course apply to proteins and carrier ampholytes alike. We chose to measure pH after dilution of gel samples in water; pH gradients, thus measured, would be internally corrected for the effect of urea on  $pK$  values of ionizable groups in carrier ampholytes. Assuming that this shift in  $pK$  is quantitatively similar for both proteins and carrier ampholytes, then differences in the pI of proteins observed on focusing in the presence and in the absence of urea must be due to changes in the intramolecular environment rather than in the solvent. Isoelectric focusing of proteins in concentrated solutions of urea can thus be used to study the effect of conformation on their isoelectric properties.

The complete exposure of the charged groups of bovine plasma albumin in 6M-urea is suggested by the fact that the protein is focused at the same pI in urea irrespective of its former state: native, reduced and alkylated, 'scrambled' by dialysis against urea, or 'blocked' by treatment with *N*-ethylmaleimide. Further evidence for this point stems from the theoretical isoionic point of bovine plasma albumin.

An isoionic point was calculated for the albumin from the data of Spahr & Edsall (1964) for the amino acid composition; assuming  $pK_a$  values of 4.3 for carboxyl groups (Vijai & Foster, 1967) and 6.5 for imidazole groups (Dekker & Foster, 1967) generates an isoionic point of 6.03. This value agrees with that observed by us for bovine plasma albumin in urea and supports the idea that pI measurements in concentrated urea represent the isoionic values of unstructured protein. Error in the  $pK_a$  values that were chosen is not likely to affect this conclusion. A decrease of, for instance, 0.6 in the  $pK_a$  values chosen for the carboxyl or imidazole groups would decrease the calculated isoionic point only to respectively 5.86 or 5.69, and those used are already at the low end of the spectrum of normal values in native proteins (Foster, 1960). Further, they are in close agreement with values given by Tanford (1968) for randomly coiled polypeptide chains. Errors in amino acid composition would of course affect the result, but to lower the value to 5.3 (the observed isoionic point of native bovine plasma albumin; Foster, 1960) would require an underestimate of about 10 carboxyl groups or an overestimate of about 10 basic groups.

In view of the good agreement between the calculated pI of bovine plasma albumin and our measurements of the pI of the denatured albumin, the low pI of the native protein must be explained by peculiarities of tertiary structure. If we are to assume alteration of the  $pK$  values of charged groups due to protein conformation then *a priori* two possibilities could account for the low pI: (1) a number of carboxyl groups have abnormally low  $pK_a$  values (i.e. are unavailable for protonation); (2) a number of basic groups have abnormally low  $pK_a$  values (i.e. are held in the uncharged state at the pI of the protein).

Vijai & Foster (1967) have invoked the first possibility in relation to the N-F transition. This term refers to a conformational change that bovine plasma albumin undergoes on titration below pH 4.5 involving a partial expansion of the molecule and a change in its electrophoretic mobility consistent with an increase in positive charge on the protein (Foster, 1960). This increase in positivity is reflected in the acid branch of the titration curve of the protein. Vijai & Foster (1967) concluded from the abnormalities of the curve that in native bovine plasma albumin only about 60 of the

approximately 100 carboxyl groups are available for protonation. We therefore recalculated the isoionic point of bovine plasma albumin as above but assuming that 40 carboxyl groups remain unprotonated in the isoionic protein. The value obtained is only 0.07 pH unit less than the value of the unstructured protein. Clearly conformational effects on the  $pK$  of carboxyl groups can contribute very little to the low pI of native bovine plasma albumin.

The alternative proposal, that for conformational reasons several basic groups have drastically lowered  $pK_a$  values, could account for the pI of native bovine plasma albumin. Calculation shows that holding ten basic groups in the uncharged form (or seven groups if one allows for 40 unavailable carboxyl groups) generates a pI of approx. 5.3 for the albumin.

Green (1963) demonstrated heterogeneity of amino groups in bovine plasma albumin by studies on their reactivity with fluorodinitrobenzene, and he suggested that the more reactive groups were buried in hydrophobic regions. Recently N. M. Green & C. G. Knight (personal communication) have found that the first five or six dinitrophenyl groups substituting in bovine plasma albumin exhibit a low degree of availability to anti-dinitrophenyl antibody, thus providing strong confirmatory evidence that the most reactive amino groups of the protein are buried.

Evidence for the heterogeneity of amino groups in human serum albumin comes from the studies by Goldfarb (1966, 1970) on the reactivity of the groups with trinitrobenzenesulphonic acid. Under a variety of conditions between 6 and 10 amino groups/molecule were highly reactive. Goldfarb (1966) invoked conformation to explain the differences in reactivity, but placed the most reactive group at the 'outer surface' of the protein and less reactive groups were designated as buried. From a reinvestigation of the trinitrobenzenesulphonic acid reaction L.-O. Andersson (personal communication) has concluded that the reactive amino groups are situated in the high-affinity hydrophobic binding sites for organic molecules. To account for the low pI of bovine plasma albumin we postulate that about seven basic groups are buried in uncharged form. We equate these groups with the reactive amino groups demonstrated as described. The present findings thus support the evidence that reactive amino groups in plasma albumin are buried.

Why have we not observed with the other proteins studied similar dependence of pI on conformation? Either the  $pK$  values of ionizing groups are generally altered very little by changes in protein conformation, or there is usually a compensating change involving both acidic and basic groups. The burial of an amino group in the uncharged form is probably

a rare event, since proteins fold up at a pH distant from the  $pK$  of  $\epsilon$ -amino groups of lysine; only a small fraction of amino groups would be in the uncharged state, so there would be an energy debt to pay for their burial in that form.

This study shows the feasibility of performing analytical isoelectric focusing in concentrated urea solutions and points to the potential of this methodology for protein-conformational studies. With improvements in the technique of measuring pH much smaller differences in isoelectric point could be reproducibly measured and more subtle conformational influences might be detected.

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