Journal of Chromatography, 324 (1985) 395–406
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 570

ELECTROPHORETIC STUDIES OF BLOOD GLOBIN PREPARATIONS

S. KANKO* and K. AUTIO
Technical Research Centre of Finland, Food Research Laboratory, Biologinkuja 1, SF-02150 Espoo (Finland)
(Received January 17th, 1985)

SUMMARY

Isoelectric focusing (IEF) across an urea gradient, and titration curves obtained by IEF–electrophoresis with and without urea, were used to characterize porcine and bovine haemoglobin and globins prepared either by the cold-acetone method (native globin) or by a new method based on haem precipitation with a dilute carboxymethylcellulose (CMC) solution at acidic pH. CMC-treated bovine globins dissociated at moderately low urea concentration into α and β subunits. In native bovine globin and in CMC-treated porcine globin, one intense band consisting of both α and β subunits was stable to urea at the isoelectric point (p*I*), but was dissociated into subunits below the p*I*. Common to all titration curves of the globins was a marked reduction in mobility at pH below 5.0 in the case of bovine globin and below 6.0 in the case of porcine globin because of the formation of an aggregate. In all globin samples except spray-dried bovine globin the main band remained stable between the p*I* and the pH of aggregation.

INTRODUCTION

Animal blood is a valuable source of edible protein. It contains about 18% crude protein, of which 80% is found in the blood corpuscles and 20% in the plasma. If blood corpuscle concentrate is to find wide utilization in the food industry, however, the strong flavour and dark colour must be eliminated. This can be achieved through the separation of haemoglobin into haem and globin fractions.

Conventionally, globin has been prepared from haemoglobin by use of different solvents,[1–3] or by a method based on carboxymethylcellulose column chromatography in the acidic pH range[4]. A new, simple separation method, recently described by Autio *et al.*[5], has been developed in this laboratory. The method is based on precipitation of haem with a dilute carboxymethylcellulose (CMC)–water solution in the acidic pH range, where haem reacts with CMC and can be separated from the globin solution by centrifugation.

Haemoglobin is a haemoprotein composed of four separate polypeptide chains ($\alpha_2\beta_2$) which are arranged in the form of a spherical structure. Each of the four chains contains a haem group embedded in the interior of the molecule rich in non-polar

0021-9673/85/$03.30

residues. The iron of the haem is linked at two sites to a histidine imidazole nitrogen atom of the polypeptide chain. When the haem is removed from the haemoglobin the stability of the molecule is decreased[6]. Moreover, both the processing method and the origin of the globin seem to have a great influence on its functional properties, which in turn depend on the structure[7,8]. Electrophoretic methods are effective for fractionation and characterization of proteins[9,10]. Isoelectric focusing (IEF) is a simple and powerful method for the detection of changes in the charge of protein molecules. Vertebrate globin has a high histidine content (*ca.* 8%)[11]. When the high-order structure of globin is destroyed these basic amino acids will move from the interior to the surface of the molecule and the isoelectric point (p*I*) will increase. However, the IEF technique does not give any information about the subunit structure or molecular weights of the IEF bands.

An interesting approach to the characterization of the subunit structure of IEF bands is the use of a urea concentration gradient in gels. Urea is a denaturant which dissociates non-covalently associated subunits: in the case of globin the native protein is expected to give rise to two subunit bands at high urea concentration. The first report of this method was presented by Hobart[12].

Hydrogen-ion titration by isoelectric focusing–electrophoresis is another new method which, among other things, gives information about the subunit interactions at different pH values. This method was developed by Rosengren *et al.*[13].

The above methods are here applied to characterize porcine and bovine globins processed by different methods.

MATERIALS AND METHODS

Preparation of globins

Globin was prepared from bovine and porcine blood cells by the CMC-precipitation method, described in detail earlier by Autio[5,14]. The principle of the method is as follows. Blood cells are haemolyzed, the pH is adjusted to below 2 with HCl, dilute CMC–water solution is added and the haem–CMC precipitate is centrifuged off. The protein solution obtained was either frozen or concentrated by ultrafiltration and spray-dried.

Native globin was prepared from bovine blood cells by the cold acetone–hydrochloric acid method described by Clegg *et al.*[15].

Isoelectric focusing

Isoelectric focusing was performed horizontally in ultrathin (0.5 mm) polyacrylamide gel[16,17] {T[=(g acrylamide + cross-linker)/100 ml solution] = 7.5%, C (= g cross-linker/%T) = 3%} on an LKB 2217 Ultrophor apparatus. Carrier ampholytes of pH 7.0–9.0 and 8.0–9.5 (LKB) were added to a final concentration of 1.6 and 0.8%, respectively. The concentrations of ammonium persulphate and N,N,N′,N′-tetramethylethylenediamine (TEMED) in the gel were 0.07 and 0.1%, respectively.

Isoelectric focusing was also performed in acrylamide gel containing a urea gradient (0–8 *M*) and a small acrylamide gradient (T = 5.4–4.2%, C = 5.9%) perpendicular to the direction of electrofocusing[18]. The final concentrations of carrier ampholytes were 1.6% (pH 7.0–9.0) and 0.8% (pH 8.0–9.5), and those of ammonium persulphate and TEMED were 0.04 and 0.03%, respectively.

Samples were applied to sample application papers which were laid on the surface of the gel. Electrofocusing was carried out at +15°C, with a voltage of 500 V applied for 10 min without samples, then 500 V for 30 min and 1200 V for about 4 h with samples. The pH gradient at +15°C was determined by measuring the pH in the gel layer with a microglass electrode (Ingold Type 10455 3006). In the case of urea the pH readings were reduced by 0.42[9]. Gels were fixed, stained and destained[17] and the intensity of the stained bands was measured by densitometry (Quick Scan R & D densitometry, Helena Laboratories).

Titration curves

Titration by combined isoelectric focusing–electrophoresis[19,20] was carried out in ultrathin polycrylamide gels (T = 5%, C = 3%). The concentrations of ammonium persulphate and TEMED were 0.04 and 0.02%, respectively. The carrier ampholyte was Ampholine pH 3.5–10 (LKB), used at a concentration of 2%. The pH gradient was first established without samples (1200 V, 50 min) and then the electrophoresis (600 V, 10 min) was carried out perpendicular to the pH gradient. Titration curves were also obtained in 8 *M* urea.

RESULTS AND DISCUSSION

Bovine globin

When haem is removed from globin, an unfolding reaction of globin occurs and as a result the isoelectric point of globin is higher than that of haemoglobin[21] (Fig. 1). In the case of the spray-dried CMC-treated globins, the further processing,

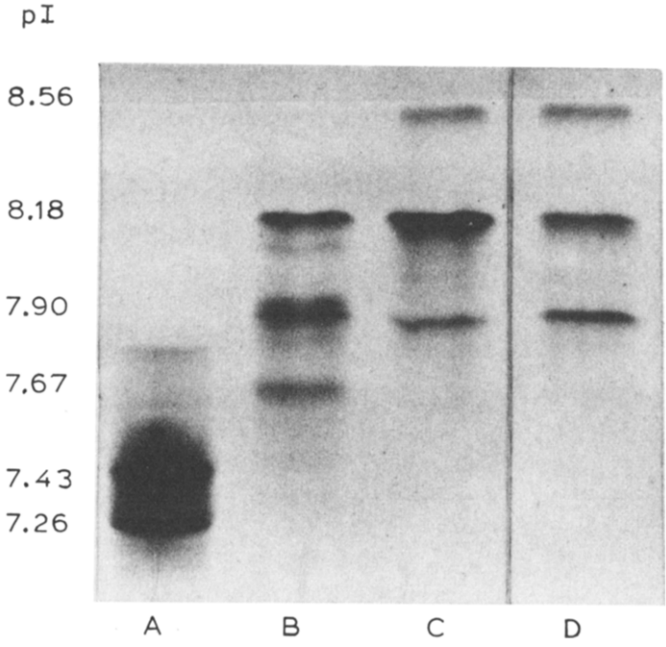

Fig. 1. Isoelectric focusing of bovine haemoglobin and globins. A = Haemoglobin; B = native globin; C = frozen CMC-treated globin; D = spray-dried CMC-treated globin.

concentration and drying, decreased the intensity of the band with p*I* 8.18, and there are strong new bands with p*I* 7.90 and 8.56 (Fig. 2).

When the IEF bands were further characterized across a urea gradient different patterns were obtained for haemoglobin and the variously processed globins (Fig. 3). Haemoglobin seems to be quite resistant to urea. The main component with p*I* 7.26 ($\alpha_2\beta_2$) did not dissociate in 8 *M* urea. However, a small amount of dimer (p*I* 7.43) contained in the bovine haemoglobin was dissociated into subunits at around 4 *M* urea. Earlier it was reported that 6 *M* urea has no influence on the p*I* of human haemoglobin[9].

The patterns for the globins were more complicated. In native globin (Fig. 3B) the band with p*I* 8.18 was found to dissociate at moderately low urea concentration

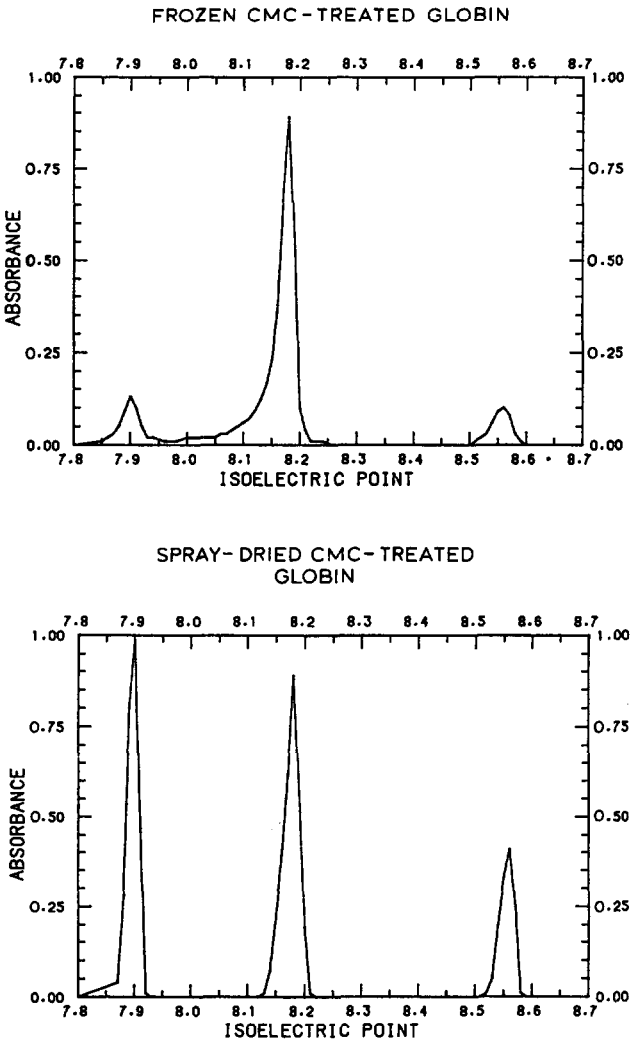

Fig. 2. Intensity of bovine globin bands in isoelectric focusing gels.

to an α subunit (p*I* 8.26) and probably to a stable aggregate with p*I* 7.75. The fact that in the IEF pattern of the subunits only β chain was present suggests that unsymmetric aggregates may be present. It may be that a β chain is dissociated from the band with p*I* 7.90 and as a result a stable symmetric aggregate is formed. Perutz *et al.*[22] have reported that, in addition to the splitting of haemoglobin into symmetrical species, there is also a splitting into unsymmetrical species. Quantitative investigation of the stained bands cannot resolve the nature of the species since the staining is based on ammonium groups which are strongly represented in the α chain.

In contrast to native globin, in frozen globin (Fig. 3C) the band with p*I* 7.90 was weak and the main band with p*I* 8.18 was dissociated into α and β subunits. Another important difference was the existence of the α subunit in the frozen globin, whereas only the β subunit or its polymer was present in the native globin. Likewise in the spray-dried globin (Fig. 3D), the main band was dissociated into α and β subunits, but instead of the one intense β chain of the frozen globin, many faint bands could be seen.

The titration curves were obtained with and without 8 *M* urea. The results are presented in Figs. 4 and 5. In the presence of urea, bovine haemoglobin did not dissociate into subunits at the isoelectric point, whereas on the acidic and alkaline sides at least two curves could be observed (Fig. 4A). The CMC-treated frozen and spray-dried globins, by contrast, existed as subunits in the presence of denaturant also at the p*I* (Fig. 4C and D). The patterns indicate the presence of several different α and β chains, especially in the spray-dried globin. In native globin the band with p*I* 7.75 really contains an aggregate in which both subunits are dissociated below the p*I* (Fig. 4B).

Common to all the titration curves of globins in the absence of urea was a marked reduction in mobility at around pH 5. The molecular weight and the shape of a protein are known to affect the rate at which it migrates through polyacrylamide gels; but since the mobility is not reduced in the presence of urea, unfolding cannot be the reason. Rather it seems that the α chain has reacted with the main band to form an aggregate.

The pattern obtained from the spray-dried globin (Fig. 5D) is more complicated. The main bands with p*I* 8.18 and 7.90 are of aggregates which are supposed to dissociate at around pH 6 to several α and β subunits (Fig. 6). Probably the spray-dried globin contains unsymmetric aggregates derived from altered α and β chains and hence the aggregate is less resistant to dissociation by H^+. In the case of frozen and native globin a more stable symmetric structure was formed after the removal of α chain (Fig. 5B and C). It is interesting that, in contrast to native globin, the main component of frozen globin dissociated into subunits under the influence of urea but did not dissociate under the influence of H^+.

Porcine globin

Porcine globin has been reported to possess poorer functional properties than bovine globin[8]. The reason for this must be the different amino acid sequences which determine the high-order structure of the globins. The further characterization of the bands obtained by IEF (Fig. 7) revealed that, in contrast to CMC-treated bovine globins, only the bands with p*I* 8.01 and 8.11 were dissociated into subunits in the urea gradient; the band with p*I* 8.26 was due to an aggregate, which is resistant to

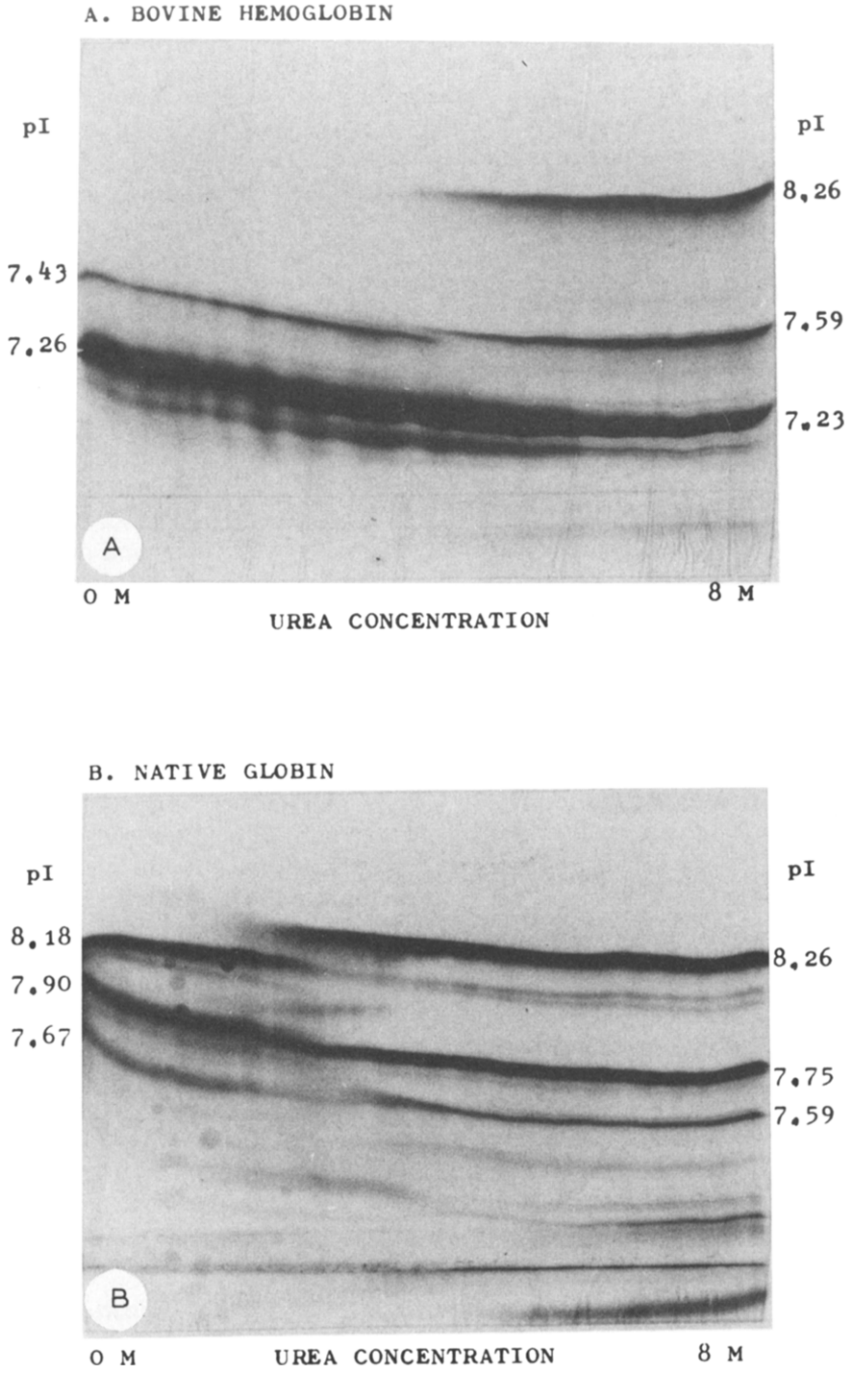
A. BOVINE HEMOGLOBIN
pI
pI
8.26
7.43
7.59
7.26
7.23
A
0 M
8 M
UREA CONCENTRATION
B. NATIVE GLOBIN
pI
pI
8.18
8.26
7.90
7.67
7.75
7.59
B
0 M
UREA CONCENTRATION
8 M

Fig. 3.

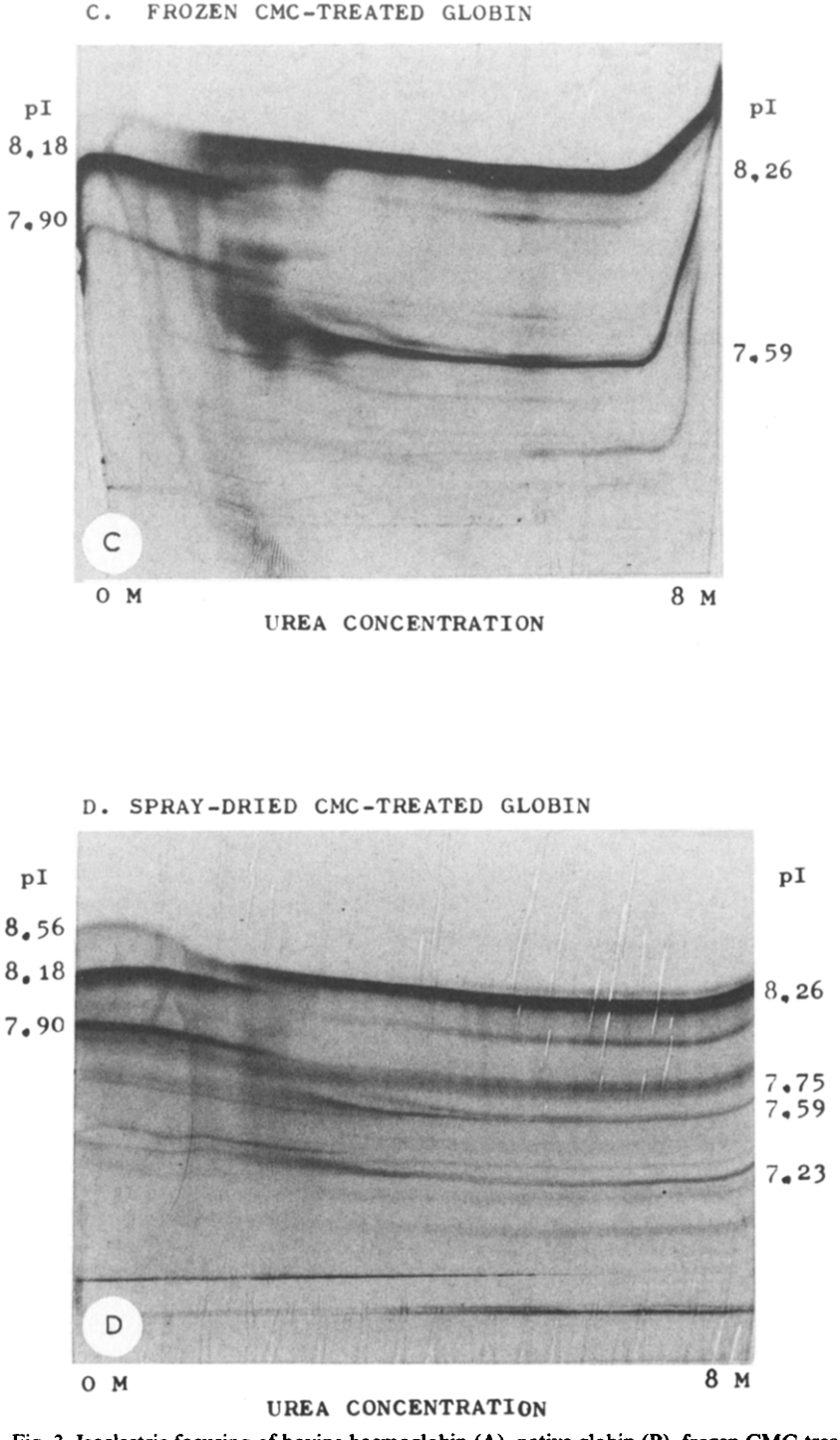

Fig. 3. Isoelectric focusing of bovine haemoglobin (A), native globin (B), frozen CMC-treated globin (C) and spray-dried CMC-treated globin (D) in an urea gradient.

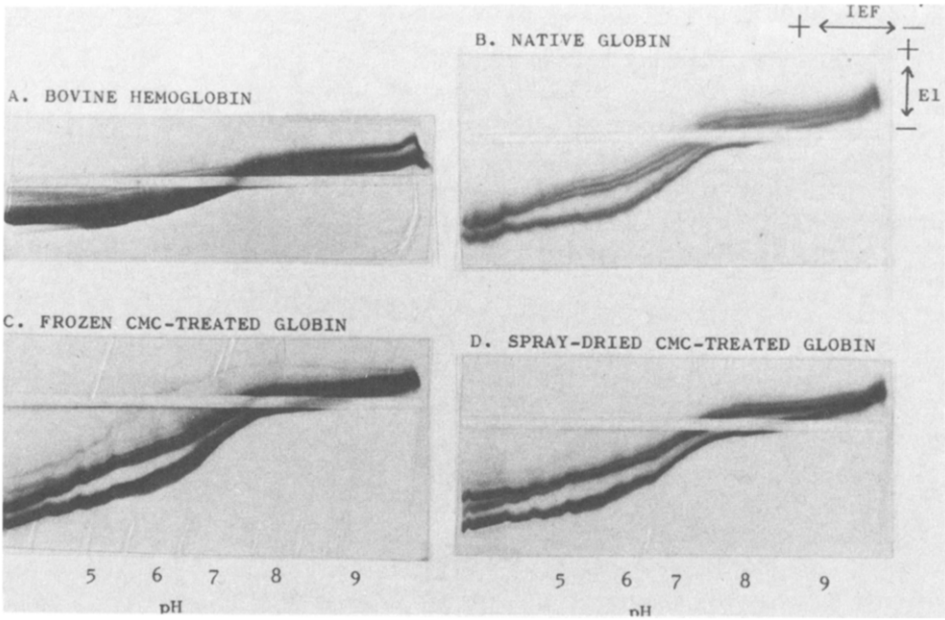

Fig. 4. Titration curves of bovine haemoglobin and globins in 8 *M* urea. Details as in Fig. 3.

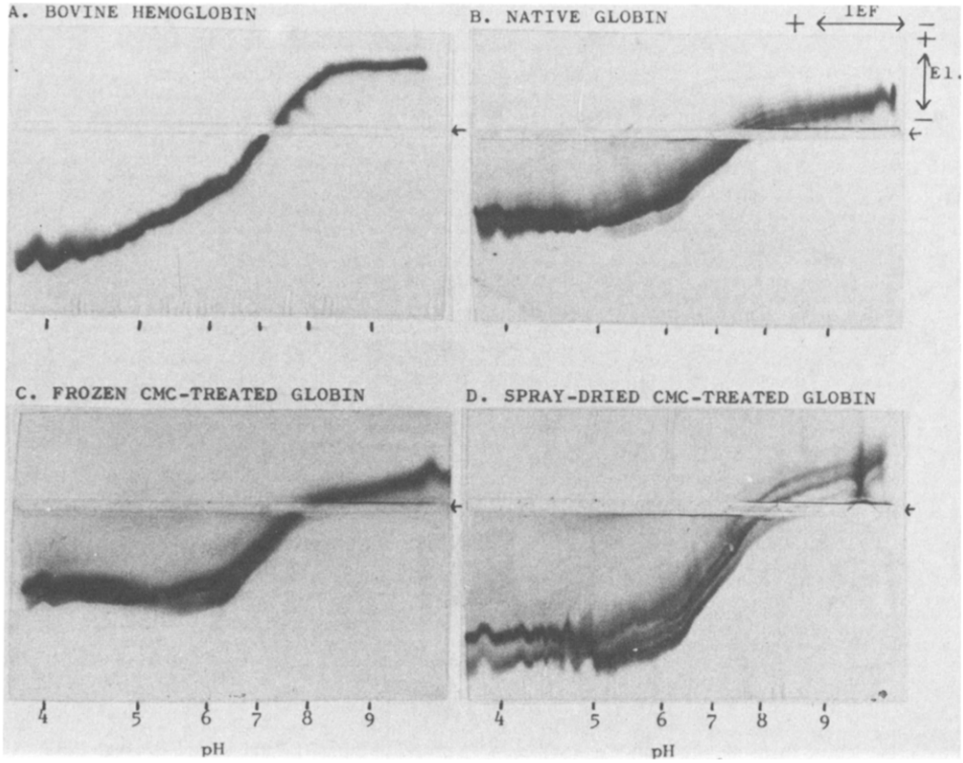

Fig. 5. Titration curves of bovine haemoglobin and globins in the absence of denaturants. Details as in Fig. 3.

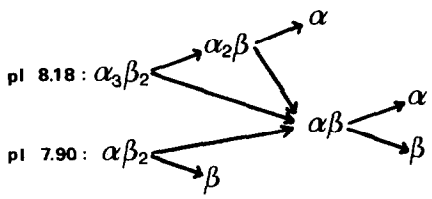

Fig. 6. Hypothesis of the dissociation of the globin aggregates.

urea at the p*I* (Fig. 8B, C). In the presence of denaturant the band with p*I* 8.26 is dissociated into α and β chains below the p*I* (Fig. 9). The titration curve of spray-dried CMC-treated porcine globin in the absence of urea (Fig. 9) shows the main band to be more stable to dissociation caused by H^+ than that of bovine globin, but the aggregate was formed at higher pH for porcine than for bovine globin.

The pattern of porcine haemoglobin in the urea gradient is very similar to that of bovine haemoglobin (Figs. 3A and 8A). The results demonstrate the importance of both haem and globin contacts and the α–β interaction for the stability of the structure of haemoglobin.

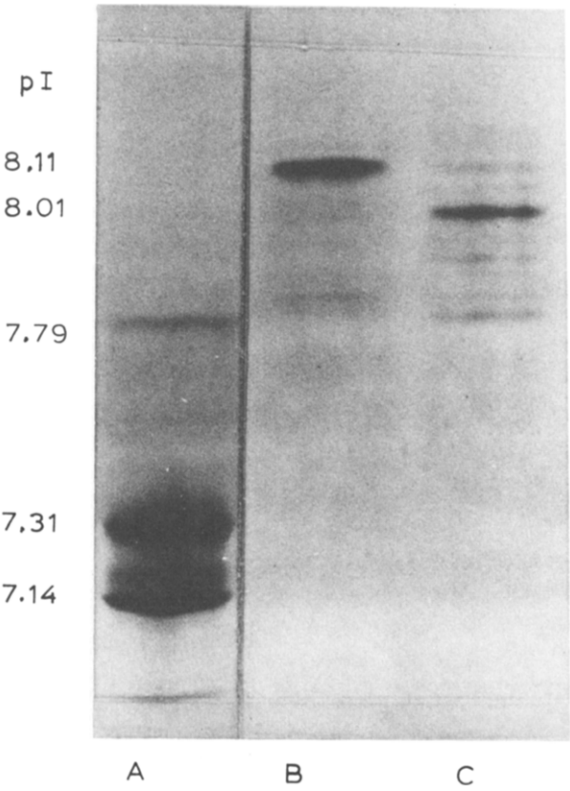

Fig. 7. Isoelectric focusing of porcine haemoglobin and globins. A = Haemoglobin; B = frozen CMC-treated globin; C = spray-dried CMC-treated globin.

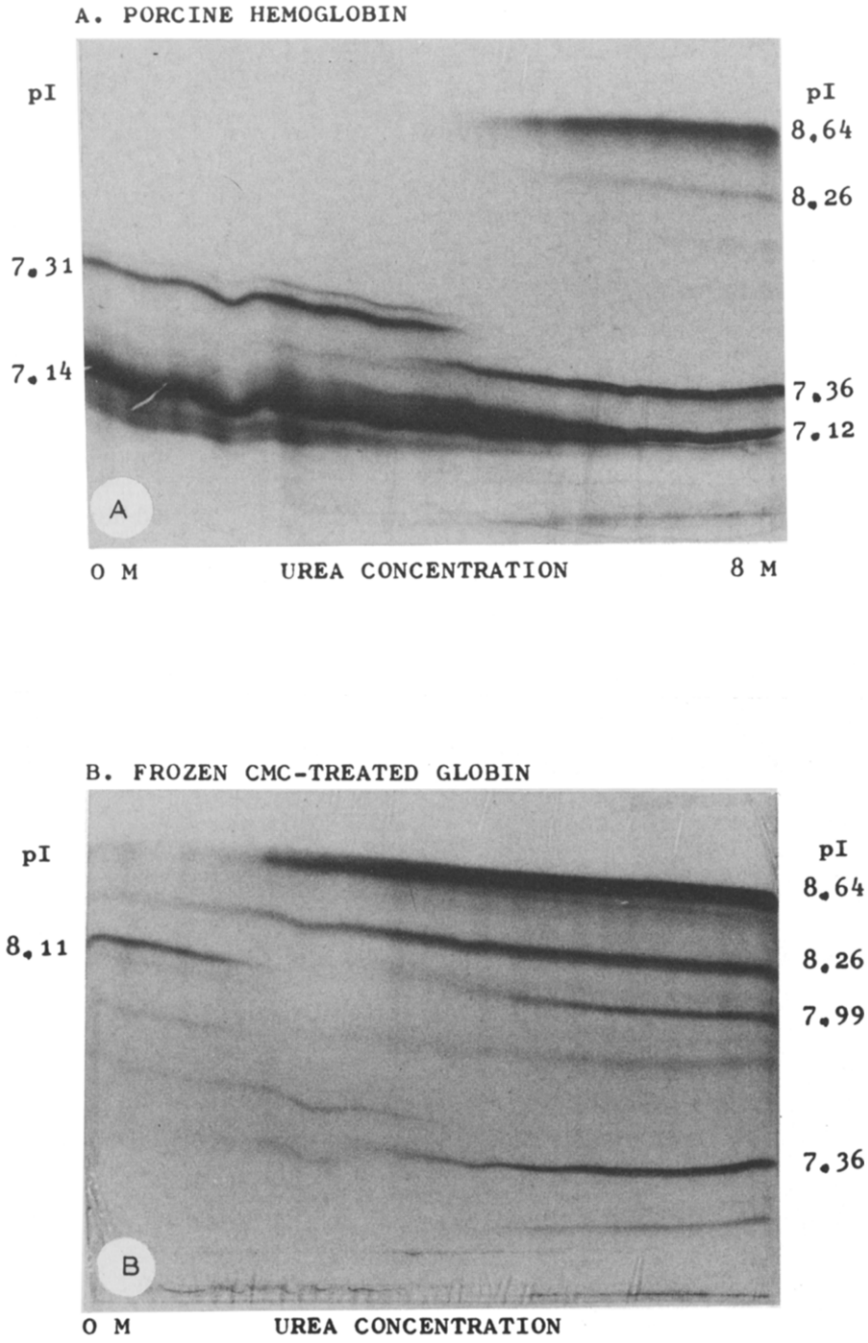
A. PORCINE HEMOGLOBIN
pI
7.31
7.14
pI
8.64
8.26
7.36
7.12
A
0 M
UREA CONCENTRATION
8 M
B. FROZEN CMC-TREATED GLOBIN
pI
8.11
pI
8.64
8.26
7.99
7.36
B
0 M
UREA CONCENTRATION

Fig. 8.

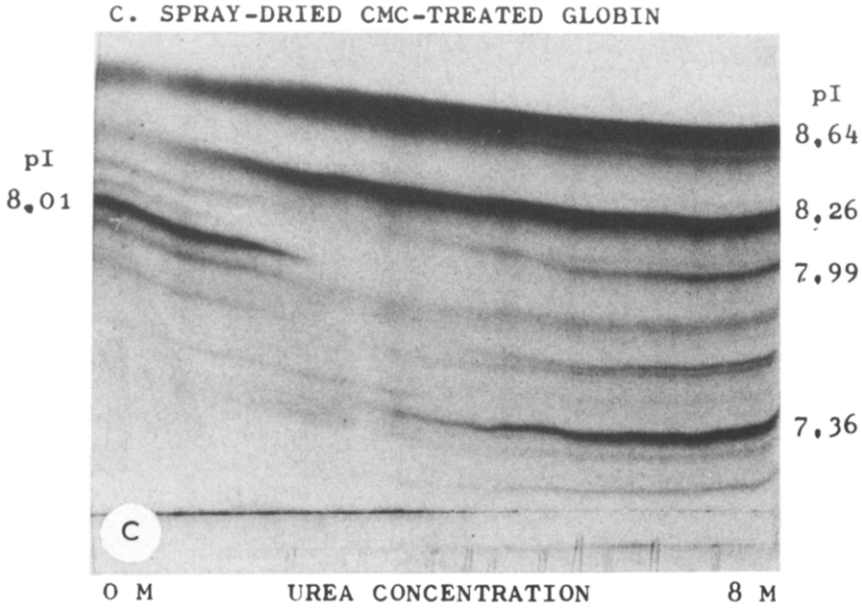

Fig. 8. Isoelectric focusing of porcine haemoglobin and globins in an urea gradient. Details as in Fig. 7.

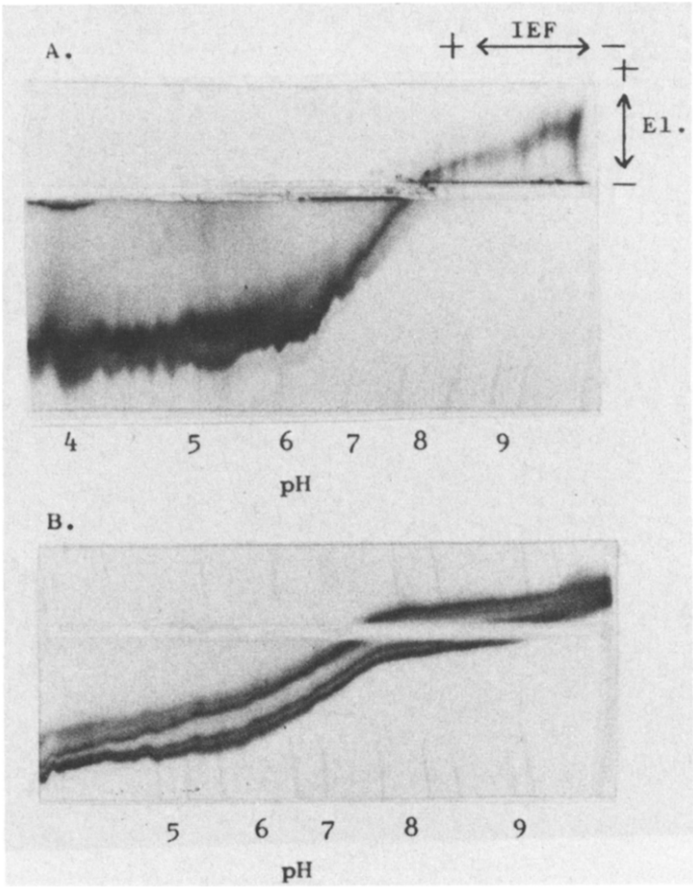

Fig. 9. Titration curves of porcine CMC-treated spray-dried globins: A, in the absence of denaturants; B, in 8 *M* urea.

REFERENCES

1 E. M. Jope, H. M. Jope and J. R. P. O'Brien, *Nature (London)*, 164 (1949) 622.
2 P. T. Tybor, C. W. Dill and W. A. Landmann, *J. Food Sci.*, 38 (1973) 4.
3 P. Lindroos, *Swedish Pat.*, 389596 (1977).
4 Y. Sato, S. Hayakawa and M. Hayakawa, *J. Food Technol.*, 16 (1981) 81.
5 K. Autio, M. Kiesvaara and Y. Mälkki, *Finnish Pat.*, 63243 (Appl. 1982).
6 H. Polet and J. Steinhardt, *Biochemistry*, 8 (1969) 857.
7 K. Autio, S. Kanko and M. Kiesvaara, *Proceedings, 30th European Meeting of Meat Research Workers, Bristol, September 9–14, 1984*, p. 369.
8 K. Autio, H. Lyytikäinen, Y. Mälkki and S. Kanko, *J. Food Sci.*, in press.
9 N. Ui, *Biochim. Biophys. Acta*, 229 (1971) 567.
10 N. Ui, *Ann. N.Y. Acad. Sci.*, 209 (1973) 198.
11 F. Haurowitz and R. L. Hardin, in H. Neurath and K. Bailey (Editors), *The Proteins*, Vol. II, Part A, Academic Press, New York, 1954, p. 288.
12 M. J. Hobart, in J. P. Arbuthnott and J. A. Beeley (Editors), *Isoelectric Focusing*, Butterworths, London, 1975, p. 275.
13 Å. Rosengren, B. Bjellqvist and V. Gasparic, in B.J. Radola and D. Graessling (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, 1977, p. 165.
14 K. Autio, *Proceedings II, 29th European Congress of Meat Research Workers, Salsomaggiore, August 29–September 2, 1983*, p. 765.
15 J. B. Clegg, M. A. Naughton and D. J. Weatherall, *J. Mol. Biol.*, 19 (1966) 91.
16 A. Görg, W. Postel and R. Westermeier, *Anal. Biochem.*, 89 (1978) 60.
17 A. Görg, W. Postel, R. Westermeier, P. G. Righetti and K. Ek, *LKB Application Note 320*, 1981.
18 T. E. Creighton, *J. Mol. Biol.*, 129 (1979) 235.
19 K. Ek, *LKB Application Note 319*, 1981.
20 P. G. Righetti, R. Krishnamoorthy, C. Lapoumeroulie and D. Labie, *J. Chromatogr.*, 177 (1979) 219.
21 K. Autio, M. Kiesvaara, Y. Mälkki and S. Kanko, *J. Food Sci.*, 49 (1984) 859.
22 M. F. Perutz, H. Muirhead, J. M. Cox and L. C. G. Goaman, *Nature (London)*, 219 (1968) 131.