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CARRIER AMPHOLYTE-MEDIATED OXIDATION OF PROTEINS IN ISOELECTRIC FOCUSING

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SUMMARY

It has been found that all commercially available carrier ampholytes (the amphoteric buffers used to create and stabilize the pH gradient in isoelectric focusing, IEF) (*e.g.*, Ampholine, Pharmalyte) are extensively oxidized by persulphate during gel polymerization. These oxidized species are formed for all pH ranges, from acidic to basic, and exhibit chromophoric peaks in the 300–360 nm range. It is believed that these oxidized products are N-oxides (possibly even nitrosamines). Owing to their high redox potentials during IEF, especially in alkaline pH ranges, they are able to capture two H^+ and two electrons and be reduced, while oxidizing in proteins SH group of Cys to –S–S– (possibly even to cysteic acid). This generates artefactual band heterogeneity, not present in the starting sample, which could never be eliminated as, on rerunning the zone, additional oxidation will take place. In such cases, the presentation of proof of sample homogeneity to agencies such as the FDA, for recombinant DNA products for human consumption, might be impossible. Such a cause of artefactual sample heterogeneity can be virtually eliminated by simply not exposing the carrier ampholytes to persulphate. In the proposed IEF protocol, "empty" gels are polymerized, washed, dried and reswollen in the desired pH interval.

INTRODUCTION

For at least a decade users of the isoelectric focusing (IEF) technique have debated extensively on potential artefacts arising from its very principle, *i.e.*, isoelectric condensation of amphoteric macroions. It was believed that if proteins, during migration to the p*I* position, were to bind to the carrier buffers (CA) [a multitude of oligoamino, oligocarboxylic amphoteric species, having M_r from 600 (the most basic) to 900 Da (the most acidic)][1], multiple equilibria would then ensue, a pattern of $n + 1$ zones being generated, representing n complexed states with n different CA species plus one uncomplexed protein zone originally in equilibrium with all the others[2].

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Our group has extensively analysed these phenomena over the years. Indeed, Galante *et al.*[3], on the basis of variations of melting points of tRNA in presence of Ampholine, have demonstrated that the mechanism of IEF fractionation of nucleic acids is strong complex formation between them and the CA species used to generate and stabilize the pH gradient in IEF. The complex is strongly pH dependent: it is almost non-existent at neutral pH (7.4), weak at pH 5.4 and very strong at pH 4.2 and lower, *i.e.*, in the typical pH range of the apparent isoelectric fractionation of tRNAs and mRNAs.

Subsequently, in the analysis of heparin (a carboxylated and sulphated polysaccharide), Righetti and co-workers[4,5], on the basis of spectra taken in solution and in focused gels, of repeated runs of isolated, focused bands, of IEF fractionations performed in the presence and absence of urea, at variable Ampholine-to-heparin ratios and with heparins with varying degrees of substitution, demonstrated the IEF heparin profile to be artefactually elicited by interaction with carrier ampholytes. It was thus found that the 21 IEF heparin fractions were indeed 21 different complexes of the same macromolecule with 21 specific Ampholine molecules determining the apparent p*I* of the focused zone. It was then understood[6] that the anomalous fractionations of nucleic acids and heparin were just particular cases of a more general phenomenon, by which all polyanions (*e.g.*, polyphosphates, polysulphates, polycarboxylates and even acidic dyes[7]) would elicit complex formation with carrier ampholytes in the range 3–5, with a strongly pH-dependent affinity, being *ca.* 10 000 fold greater at pH 3 than at pH 7.

Correspondingly, at the opposite extreme of the pH scale, it was found that polycations were able to complex CAs too, although only poly-Arg could form stable complexes, poly-Lys and poly-His exhibiting no appreciable binding[8].

The situation described above would appear to be hopeless; on the contrary, the artefacts here reported are unique examples and refer to limiting structures, *i.e.*, mostly to polyanions and polycations, which, lacking counter ions in their chemical framework, have to bind any other counter ion available in solution. There is no evidence that CAs will elicit the same multimodal distribution by interacting with proteins. In fact, according to Rilbe[9], in IEF a protein should be both isoelectric and isoionic, the latter term indicating that the isoelectric protein is stripped free of any other counter ions available in the surrounding buffer, except from protons deriving from the protolytic equilibria due to its amino acid composition. This is made possible by the fact that both proteins and carrier ampholytes are amphoteric species, so that at their respective isoelectric points they can form an "inner" salt, with no tendency to bind to foreign ions. This is certainly not the case in zone electrophoresis with conventional buffers. For example, it is known that, by electrophoresis in borate buffers, bovine serum albumin (BSA) is always split into two bands, representing the equilibrium[10] free BSA $\rightleftharpoons$ borate–BSA.

If IEF is then a safe fractionation procedure, are there any instances in which artefactual bands can be generated? We report here an undesirable phenomenon that must have plagued the technique since at least 1971, when gel IEF was first described[11]: when focusing proteins, free SH groups can be oxidized during the run, producing zones with higher p*I*. The oxidation power comes from the carrier buffers themselves, oxidized by the persulphate during the gel polymerization step. The phenomenon is described here and remedies are proposed.

EXPERIMENTAL

Materials

Repel- and Bind-Silane, Gel Bond PAG, the Multiphor 2 chamber, Multitemp thermostat and Macrodrive power supply were from LKB (Bromma, Sweden) and Pharmalyte and Ampholyte buffers and a protein p*I* marker kit were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, N,N′-methylenebisacrylamide (Bis), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulphate and Coomassie Brilliant Blue R-250 were from Bio-Rad Labs. (Richmond, CA, U.S.A.) and dithiothreitol (DTT) and urea from Merck (Darmstadt, F.R.G.). Human globin chains were prepared by the acetone–acid method of Clegg *et al.*[12].

Spectrophotometric analyses

UV–VIS spectrophotometry was performed with a Cary 219 instrument (Varian, Palo Alto, CA, U.S.A.). Ampholine and Pharmalyte pH ranges were analysed for potential oxidation by persulphate during the gel polymerization step. Solutions of 1.5% of the pH intervals used for haemoglobulin focusing (pH 6–9) were prepared in 100 m*M* buffer (phosphate, titrated to neutral pH) and standard amounts of catalysts were added (1 μl of TEMED and 1 μl of 40% ammonium persulphate per 1 ml of gelling solution) in the absence of gelling monomers (acrylamide and Bis). As a blank, the same amounts of TEMED and persulphate, diluted in 100 m*M* phosphate buffer (pH 7.0) were used. All cuvettes were subjected to the standard treatment utilized for gel polymerization (1 h, 50°C) and then spectra were taken at room temperature in the range 280–450 nm at 2 A full-scale, with automatic baseline correction.

Conventional IEF in carrier ampholytes

Human globin chains were taken as a model for the potential oxidation of SH groups duing focusing. Three types of gels were prepared. In one, the gel was polymerized directly in the presence of 2.5% carrier ampholytes (CA; a 1:1 mixture of pH 6–8 and pH 7–9 ranges plus a small amount of pH 3–10) and 8 *M* urea and used as such with a 30-min prerun.

A second gel type was polymerized in the absence of any additive ("empty" gel), washed first in 100 m*M* ascorbic acid for 30 min, then twice in distilled water, dried and reswollen in the presence of 2.5% CA and 8 *M* urea. This gel was run using also 100 m*M* ascorbic acid as anolyte.

A third gel type was treated exactly as the second gel, except that it was run using 50 m*M* phosphoric acid as anolyte. All gels were made to contain a 5%T, 4%C matrix and were polymerized with the same amounts of catalysts (1 μl of TEMED and 1 μl of 40% ammonium persulphate per 1 ml of gelling solution) at 50°C for 1 h. Human globin chains, freshly dissolved in 8 *M* urea, 2% CA in the pH range 6–8 and 5% 2-mercaptoethanol, were applied in pockets precast at the anode in amounts ranging from 15 to 20 μg. Focusing was performed at 10 W (maximum) and 1500 V (at equilibrium) for 2–4 h. Staining was made in Coomassie Blue in the presence of copper(II) sulphate[13]. The globin chain focusing technique is essentially as described by Righetti *et al.*[14], except that addition of detergent to the sample and gel layer was omitted, as it was not the aim of this work to separate the A_γ and G_γ chains.

RESULTS

Action of persulphate on carrier ampholytes

Fig. 1 shows the results of incubating carrier ampholytes with persulphate, under conditions mimicking those used for preparing standard polyacrylamide gels. The spectra of the *ca.* neutral pH ranges (pH 6–8 Ampholine and pH 5–8 Pharmalyte) show that, in the two controls, essentially no chromophores are apparent, whereas on contact with AP, strong chromophores appear. Ampholine exhibits a bimodal distribution, with peaks centred on 300 and 345 nm, whereas Pharmalyte produces a single strong chromophore centred on 316 nm.

Action of oxidized carrier ampholytes on proteins

The presence of these oxidation products in Ampholine and Pharmalyte could be deleterious to protein fractionation because, even in the absence of persulphate (*e.g.*,

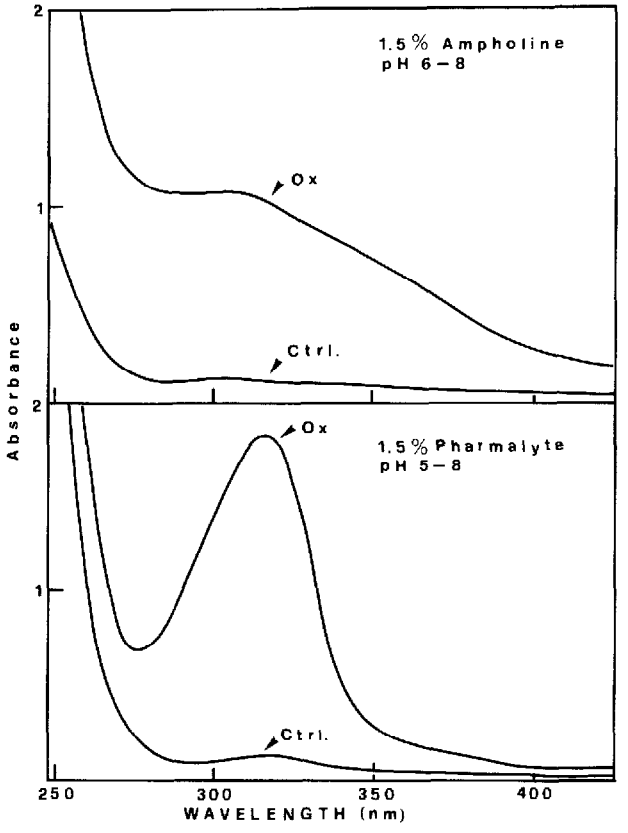


Fig. 1. Oxidation of carrier ampholytes by persulphate; 1.5% solutions of either Ampholine (pH 6–8) or Pharmalyte (pH 5–8) in 100 m*M* phosphate buffer (pH 7.0) were incubated for 1 h at 50°C either as such (control, Ctrl.) or in presence of 0.04% ammonium persulphate and TEMED (oxidized, Ox). Note, in this last instance, the formation of strong chromophores in the 300–360 nm region, which we attribute to the formation of N-oxides.

even if the gel had been extensively pre-run to discharge persulphate and its degradation products to the anode), the focusing gel could bear an intrinsic oxidation power present all along the pH gradient as oxidized buffering species. When focusing in alkaline pH ranges, proteins could be directly oxidized, while reducing the oxidized buffers present along the migration path. That this could be the case is shown in Fig. 2A: when globin chains are focused in an Ampholine gel (containing oxidized Ampholine species) the α-globin chains are clearly split into two bands, the upper zone (representing oxidized α-chains, as discussed below) constituting *ca.* 40% of the two components. Even β-globin chains are split into two bands, whereas foetal chains are resolved into a single component. If the run is performed in presence of Pharmalyte,

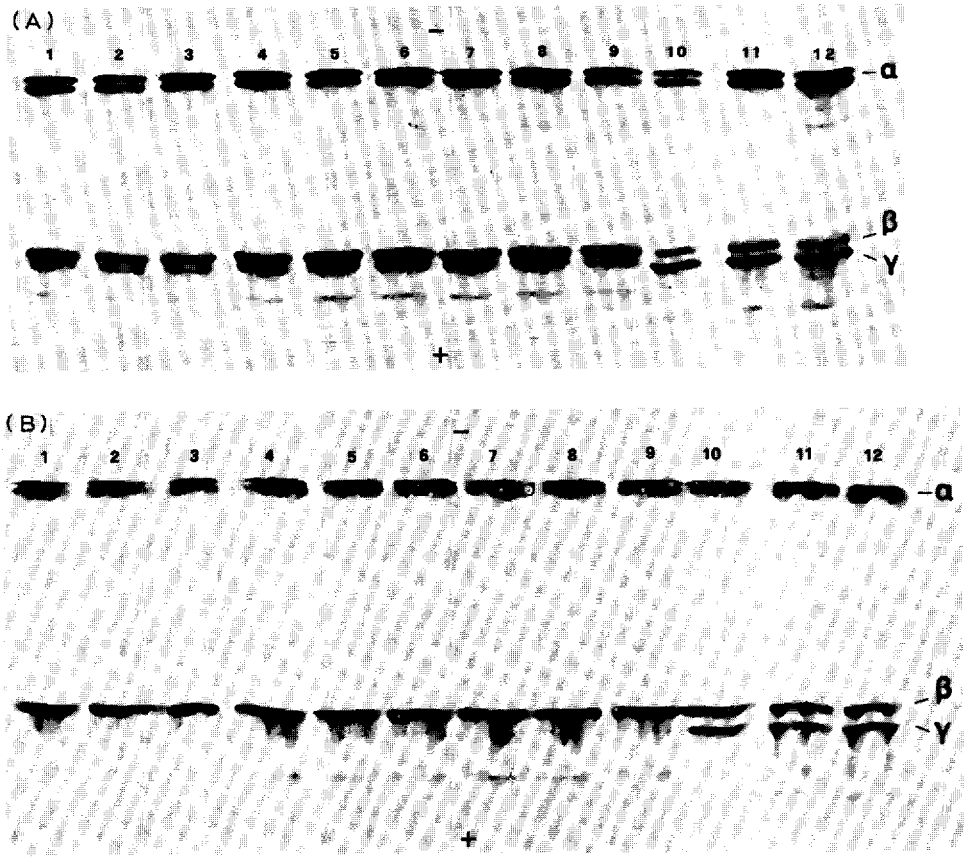


Fig. 2. Focusing of human globin chains. Gels: 5%T, 4%C polyacrylamide, in the presence of 8 *M* urea and 2.5% carrier ampholytes. (A) Gel containing a total of 2.5% Ampholines in the pH ranges 6–8, 7–9 and 3.5–10, in the ratio 45:45:10. (B) Gel containing a total of 2.5% Pharmalytes in the pH ranges 6–8, 6.5–9 and 3–10 in the ratio 45:45:10. Both gels were polymerized directly in the presence of urea and carrier ampholytes with 0.04% persulphate and TEMED. Samples: 1–9, globin chains from normal human adults; 10–12, globin chains from umbilical cord blood. The samples were applied anodically at a concentration of *ca.* 20 μg total protein. The gels were prefocused for 30 min at 400 V and the run was continued for 2 h at 1100 V and an additional 30 min at 1800 V. Staining with Coomassie Brilliant Blue in Cu^{2+}. Note the splitting of α-chains into two bands, more pronounced in Ampholine IEF.

the α-chains are still split into two components, whereas the β-globins appear as a single zone (Fig. 2B). In both instances, the foetal chains are unaffected by the presence of oxidized carrier ampholyte species.

In order to check the above hypothesis (oxidation of globin chains by oxidized carrier ampholyte species), a control experiment was run as follows: an empty gel was prepared (in the absence of ampholytes and urea), washed first in ascorbic acid and then in distilled water, and dried. The dried gel was then reswollen in the presence of urea and either Ampholine or Pharmalyte. As shown in Fig. 3A, now α-globins focus as a single zone, indicating the absence of oxidation products, whereas β-chains are still resolved into two fractions. However, if the experiment is performed with Pharmalytes, both α- and β-globins focus as single zones (Fig. 3B). In both instances, the foetal chains are unaffected and always focus as a single component.

The ability of persulphate to oxidize globin chains (mediated by the present of oxidized carrier ampholyte species during the IEF run) was investigated by running a series of gels polymerized with different levels of persulphate. Three types of gels were

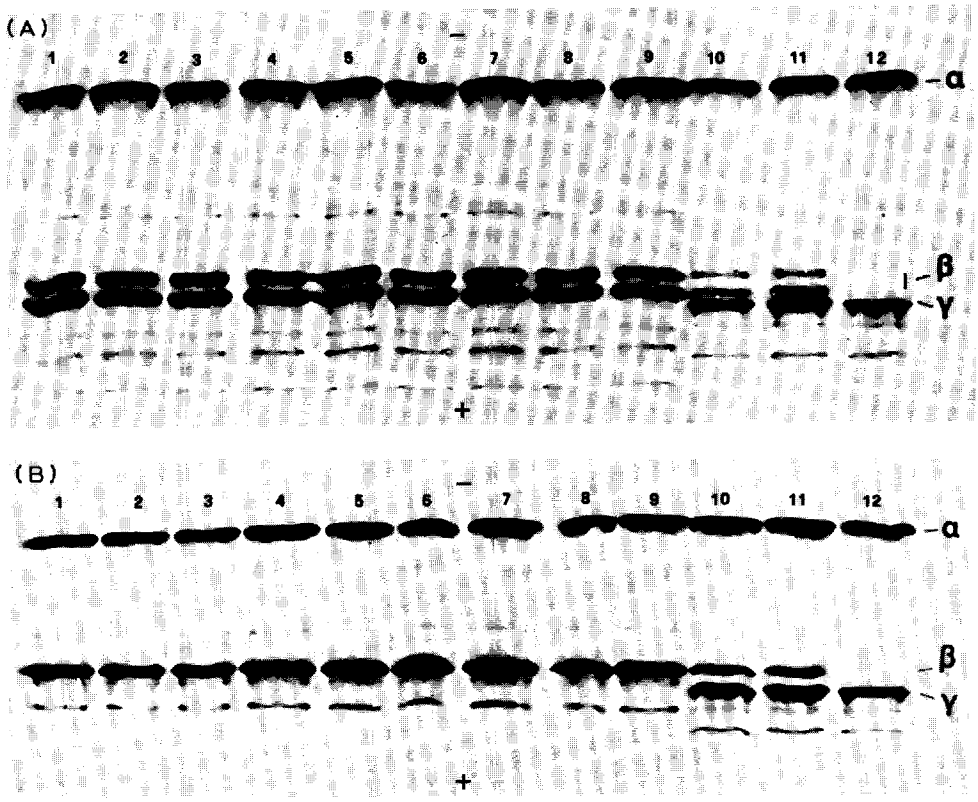


Fig. 3. Focusing of human globin chains. Gels as in Fig. 2, except that "empty" matrices were polymerized, washed once (45 min) with 100 m*M* ascorbic acid (pH 4.5) and then twice for 45 min with distilled water. After drying, the matrices were reswollen in the presence of 8 *M* urea and either 2.5% Ampholine (A) or 2.5% Pharmalyte (B) in the ratios given in Fig. 2. Other experimental conditions as in Fig. 2. Note the disappearance of oxidized α-chains in both instances. Samples 1–11 as in Fig. 2; No. 12 is from a homozygous β°-thalassemic.

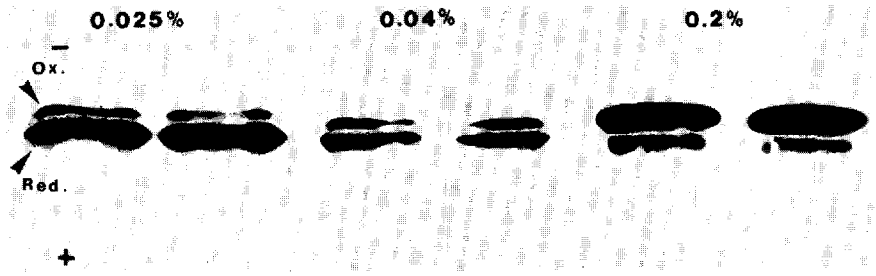


Fig. 4. Oxidation of α-globin chains as a function of persulphate levels during gel polymerization. Three different gels were polymerized (in the presence of Ampholine and urea) with either 0.025, 0.04 or 0.2% persulphate. Focusing and other experimental conditions as in Fig. 2. After staining, the ratio α_{ox}/α_{red} was evaluated by densitometry and found to be 15:85, 35:65 and 80:20, respectively.

investigated: containing 0.025% persulphate (AP) (the minimum level for obtaining a reasonable gel polymerization), or 0.04% persulphate (the amount routinely used in most laboratories) or 0.2% persulphate (definitely an excess, seldom used in routine work). The gels were then prerun for 45 min and reduced globin chains applied anodically. As shown in Fig. 4, the amount of oxidized α-globin chains is directly proportional to the initial amount of persulphate used in gel polymerization. At the lowest AP level (0.025%) the ratio α_{ox}/α_{red} is only 15:85; at the normal AP levels (0.04%) this ratio is increased to 35:65, whereas at the highest AP level, the oxidized α-chain clearly predominates (α_{ox}/α_{red} = 80:20).

DISCUSSION

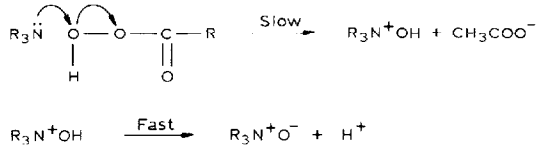
We had recently become aware, in our studies on immobilized pH gradients (IPG) (for a review, see Righetti and Gianazza[15]) that, when focusing in alkaline pH gradients, strange phenomena occurred with cysteine-rich protein molecules. For example, with pro-urokinase (a 46 kDa protein containing 24 Cys out of a total of 411 amino acids and exhibiting a p*I* of *ca.* 9.8), a size-homogeneous preparation would be resolved, in IPGs, into an extremely large number of bands (at least ten major and ten minor bands focusing in the pH range 7–10)[16]. Having ruled out common sources of polydispersity, such as different degrees of glycosylation or IEF artefacts, such as binding to carrier ampholytes or carbamylation by urea, we attributed this phenomenon to the coexistence among species in the –SH, –S–S– and SO_3^- states. This equilibrium could remain undetected in most instances, as two thirds of the known proteins have isoelectric points failing in the acidic portion of the pH scale[17]. Given the mildly alkaline p*K* of the SH group of Cys (p*K* = 8.3), the presence of an –SH rather than an –S–S– group would go undetected in acidic proteins, as neither would contribute to the surface charge. However, in urokinase-like molecules, owing to the high isoelectric point of the "native" forms and to the presence of an unusually large number of Cys, an equilibrium between –SH and –S–S– states would be immediately visible by producing a series of charge-altered species. For example, the disappearance of two –SH groups, with the formation of a single –S–S– bridge, would produce species with a net loss of two negative charges because, at the high p*I* (9.8) of the "native" protein molecules, such groups would be fully ionized. The high-p*I* pro-urokinase was

found to have almost no titratable SH groups (less than 1 mol/mol of protein), in agreement with literature data, whereas the low-p*I* components had a larger number of available SH groups. However, this number was much lower than the theoretically expected amount of 24–SH groups/mol of protein. As an answer we had proposed that oxidation of Cys could proceed to an irreversible state, *i.e.*, cysteic acid. From the point of view of charge, high-p*I* molecules carrying a free –SH group or its fully oxidized cysteic acid derivative should be indistinguishable, as both would carry a net negative charge. However, titration with Ellman reagent would reveal only the former and ignore Cys residues converted into cysteic acid and in fact lower p*I* pro-urokinases had very few extra SH groups that could be titrated.

Indeed, this phenomenon of Cys oxidation was described so long ago that it has probably been forgotten. Thus, as early as 1971, Jacobs[18,19] first reported the partial modification of Cys and Met to cysteic acid and methionine sulphoxide, on prolonged IEF, in bovine ribonuclease (also an alkaline protein). These oxidation phenomena could be largely suppressed by removal of oxygen from the IEF column (at that time most IEF experiments were run in a vertical column in a sucrose density gradient) and by addition of antioxidants, such as thiodiglycol and ascorbic acid. With the advent of open-face IEF gels, both of these remedies were abandoned as impractical (thiol groups are inhibitors of gel polymerization). However, with the modification proposed here, such remedies can easily be exploited again, as an "empty" IEF gel can be polymerized, washed and dried and can be reswollen in the solvent of choice (including any desired reducing agent).

More recently, when exploring the chemistry of the Immobiline chemicals, we discovered that these buffers also were easily oxidized by persulphate during the polymerization process[20]. In the light of these observations, it became clear that such redox reactions occurred during the IPG run itself and that the IPG matrix was directly responsible for oxidation of Cys residues in proteins[20]. The alkaline Immobilines themselves (oxidized during the polymerization process by persulphate) act as electron acceptors in this redox process. We therefore suggested that, during the IPG run, two reactive Cys residues in a protein would release two protons and two electrons which were captured by an oxygen atom bound to the tertiary amine, thus reducing the latter and forming a molecule of water. If this were the mechanism, it became clear that protection against oxidation should be obtained by direct reduction of the matrix. In fact, washing the matrix in 100 m*M* ascorbic acid, at pH 4.5, afforded full protection against oxidation of Cys residues in proteins[20].

What happens to the four alkaline Immobilines when they are exposed to ammonium persulphate could simply be the addition of oxygen to the tertiary amino group giving amine oxides ($R_3N^+O^-$). It is known that this reaction is facile and usually occurs readily at room temperature in water, alcohol or benzene solvents in presence of even dilute solutions of organic peracids[21]. The mechanism of tertiary amine oxide formation has not been studied in detail but, by analogy with primary amines, the reaction must involve attack by the electrophilic peroxidic oxygen on the amine lone pair, followed by anion elimination and proton loss, according to the following scheme:



In accord with this conclusion, the reactions are first order with respect to each reactant, and the protonated amine is unreactive.

Some comments remain to be made also about the oxidizing power of carrier ampholytes in conventional IEF. It is clear from the present data (see Fig. 4) that carrier ampholytes have an oxidizing power with respect to Cys residues which is even greater than that exhibited by Immobilines. When using high persulphate levels, in fact, almost all of the α-globin chains exist as oxidized species. We believe that this oxidation could occur on the Cys-104 residue of α-chains, according to the following equilibrium (at pH > 7.5):

$$2\alpha\text{-SH} \rightleftharpoons \alpha\text{-S–S-}\alpha$$

Are there any examples of such phenomena in the literature? Except for the data of Jacobs[18,19] (who, however, never postulated the existence of oxidized carrier ampholytes), not really; however, we can recall here that in the 1970s the Ampholine chemicals (at that time produced only by LKB) had a distinct yellowish colour (especially in the pH range 3.5–5) and produced peaks in the UV region, with maxima around 320 and 368 nm[22]. Moreover, ampholytes synthesized by us in the laboratory exhibited two chromophores in a pH-dependent equilibrium (maxima at 315 and 368 nm, isosbestic point at 335 nm) with an associated protolytic function having an apparent pK of 1.1. We had attributed this pK value to the sixth nitrogen atom in pentaethylenehexamine[23] and had hypothesized oxidation of these amines with the formation of nitrogen heterocyclic structures. Judging from the present results it must have been these species that were responsible for the oxidative phenomena reported by Jacobs[18,19].

A possible explanation for the observed α-chain oxidation is as follows: as the redox potential in reactions involving nitrogen is of the order of +0.5 V, whereas that of similar reactions involving sulphur is of the order of −0.5 V, it is clear that the N-oxides formed on the carrier ampholytes during gel polymerization will act as oxidizing agents, at alkaline pH values, on free –SH groups (possibly even on –S–S– bridges) producing species of higher oxidation level.

As a guideline for future developments in carrier ampholyte chemistry, we believe that Ampholines should be discontinued, as they are more extensively oxidized than Pharmalytes, thus producing larger amounts of oxidized protein bands during the IEF run. In addition, as a disturbing phenomenon, they appear, even in the reduced state, to produce a splitting of the β-chains into two bands (whose origin is not understood), whereas this splitting is never observed with Pharmalyte chemicals.

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