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Polyacrylamide gel polymerization under non-oxidizing conditions, as monitored by capillary zone electrophoresis

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ABSTRACT

It has been established that, during peroxodisulphate-catalysed polyacrylamide gel polymerization, there is a concomitant oxidation of all amino buffers present in the mixture, with the formation of N-oxides. This is deleterious in conventional isoelectric focusing and immobilized pH gradients because, even after discharging excess of peroxodisulphate at the anode, a residual oxidizing power remains in the matrix. When focusing proteins under mildly alkaline conditions, a redox reaction occurs during migration, and free SH groups are oxidized to –S–S– bridges, giving rise to spurious bands. The kinetics of riboflavin-catalysed gel polymerization have been reinvestigated and conditions were elucidated that allow >95% conversion of monomers into the growing chains, full control of the lag phase and short polymerization times, as typical of peroxodisulphate catalysis. It was demonstrated, by capillary zone electrophoresis and direct NMR analysis, that there is an additional unique advantage in photopolymerization, namely that even in a large excess of catalyst, no traces of oxidizing power can be found. The gel matrix thus generated is unable to induce any oxidation of proteins during gel electrophoresis.

INTRODUCTION

Peroxodisulphate-catalysed polyacrylamide gel polymerization has been a standard protocol since the inception of gel electrophoresis, as first reported by Raymond and Weintraub [1]. A notable exception is the use of riboflavin for gelation of the sample and spacer gel segments in disc electrophoresis, as proposed by Davis [2]. The reasons for using photopolymerization, however, were stated [2] to be only the convenience in controlling the onset of polymerization (as light exposure is required) and because "the pore size of riboflavin catalysed gel is larger than that of a peroxodisulphate catalyzed gel". However, the situation is more complex than that. In 1967 Fantes and Furminger [3] observed a loss of biological activity of interferon and ribonuclease under the influence of peroxodisulphate. Similar peroxodisulphate-induced inactivation was reported by Mitchell [4] in clostridial peptidase B, while Brewer [5] observed both inactivation and formation of protein cleavage products in yeast enolase. In the last report, peroxodisulphate attack of sulphydryl groups and of tyrosine and tryptophan residues was also proposed. In order to eliminate such peroxodisulphate-induced artifacts, Brewer [5] suggested addition of thioglycolate to the cathodic chamber, so as to have a reducing buffer boundary migrating in front of the proteins.

Our group has been recently able fully to verify and quantify this hypothesis of a potential oxidizing power of peroxodisulphate. When working with immobilized pH gradients (IPG) (the most powerful variant of isoelectric focusing, IEF) [6], we detected

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a pH-dependent oxidation of the four alkaline Immobilines (the acrylamide weak acids and bases used as buffers for maintaining the pH gradient in the gel) by peroxodisulphate during the polymerization process, with the formation of N-oxides. These $R-N^+O^-$ species are in turn strongly oxidizing and attack Cys residues in proteins by transforming them into -S-S- bridges. For alkaline proteins, this often results in spurious, additional bands existing in two possible equilibria: -SH and -S-S-. In the case of human α-globin chains, the formation of an inter-chain -S-S- bridge produces an intense band with a higher isoelectric point [7]. Not even the carrier ampholytes (the soluble, amphoteric buffers guaranteeing the pH gradient in conventional IEF) [8] are immune from this attack: they are also oxidized by peroxodisulphate, generating N-oxides able to oxidize -SH groups in proteins [9]. Hence the precaution of pre-running the gel for discharging harmful peroxodisulphate to the anode is ineffective, as new oxidizing species (N-oxides) remain in the gel, either because they seek an isoelectric point or because they are grafted to the matrix, as in the IPG technique. In a model system, where free Cys was incubated with isoelectric IPG beads at pH 9.0, 100% oxidation to cystine could be demonstrated in a 12-h period [10]. The situation appeared to be so dramatic that we even endeavoured to synthesize new types of acrylamido buffers for IPG resistant to oxidation by peroxodisulphate; one such a chemical was acryloylhistamine [2-(4-imidazolyl)ethylamine-2-acrylamide] [11].

We have recently reinvestigated riboflavin-catalysed gel polymerization, a technique which has not had many followers. In fact, in 1981, when we performed the first studies on photopolymerization, our conclusions were negative, because during the standard time allotted for polymerization (1 h at room temperature) barely 60% conversion of monomers into the growing chains could be obtained [12]. Given the fact that free, unreacted monomers are neurotoxic and can easily add to free -SH groups [13], we pursued the use of peroxodisulphate catalysis, because an efficiency of >90% can be easily elicited [14]. However, in a recent investigation [15], we established conditions allowing an efficiency of >95%, full control of the lag time and short polymerization times, as typical of peroxodisulphate catalysis. As is reported here, photopolymerization appears to offer another unique advantage, namely the complete absence of oxidizing power, even at higher catalyst levels than are customary.

EXPERIMENTAL

Aminopropylmorpholine and acetic anhydride were obtained from Aldrich-Chemie (Steinheim, Germany), pyridine, hydrogen peroxide and sodium dodecyl sulphate (SDS) from Merck (Darmstadt, Germany), riboflavin 5'-phosphate from Carlo Erba (Milan, Italy), ammonium peroxodisulphate from Bio-Rad Labs. (Richmond, CA, USA) and Immobiline p*K* 9.3 buffer from Pharmacia–LKB, (Uppsala, Sweden).

Capillary zone electrophoresis (CZE)

CZE was performed on a Waters Quanta 4000 (Millipore, Milford, MA, USA) in a 50 cm × 75 μm I.D. capillary from Polymicro Technologies (Phoenix, AZ, USA). All runs were made at 25°C in 50 m*M* phosphate buffer (pH 7.0) containing 50 m*M* SDS and 10% methanol (micellar electrokinetic chromatography) [16]. All runs were in the cathodic direction at 10 kV and 66 μA. Samples were loaded for 10 s by the "hydrostatic injection" method. Sample zones were revealed at 214 nm.

Nuclear magnetic resonance (NMR) spectroscopy

^1H and ^{13}C NMR spectra were recorded with a Bruker AC-200 instrument in the Fourier transform mode with tetramethylsilane as internal standard and D_2O as solvent.

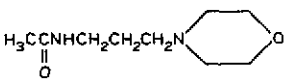
Thin-layer chromatography (TLC) and column chromatography

TLC was performed on silica gel $60F_{254}$ plates from Merck, using chloroform–methanol (7:3, v/v) as eluent. The spots were revealed with 3.5% molybdophosphoric acid in ethanol. Preparative chromatography was performed in columns of silica gel 60 (230–400 mesh) from Merck (with chloroform–methanol (9:1) as eluent.

Synthesis of N-Acetylamino-n-propylmorpholine

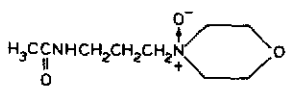
In order to study the capability of photo- or peroxodisulphate polymerization to induce N-oxides,

analogues of the Immobiline buffers, lacking the double bond, were prepared, so that during exposure to these catalysts no production of a gel phase would ensue. An analogue of the p*K* 7.0 Immobiline was prepared as follows: 7.2 g (7.34 ml, 0.05 mol) of 4-(3-aminopropyl)morpholine were dissolved in 28 ml of pyridine. After adding 14.14 ml (0.13 mol) of acetic anhydride, the reaction was allowed to proceed overnight at room temperature. A 50-ml volume of a 10% solution of K_2CO_3 was then added while keeping the pH above 8.5 and then the mixture was extracted with ethyl acetate (3 × 50 ml). The organic phase, dried over Na_2SO_4, was evaporated *in vacuo*, then the excess of pyridine was eliminated by two treatments with toluene (the latter being then eliminated *in vacuo*). A yellow, oily product was recovered (3.9 g, 41% yield), which was then purified on a silica gel column (with a 1:40 ratio of product to silica) and eluted with chloroform–methanol (9:1). ^{1}H NMR (D_2O): δ 1.45 (m, 2H), 1.4 (s, 3H), 2.18 (m, 2H), 2.30 (bt, J = 5.0 Hz, 4H), 2.92 (t, J = 7.0, 2H), 3.50 ppm (bt, J = 4.5 Hz, 4H). ^{13}C NMR (D_2O): δ 21.15, 27.26, 39.93, 54.8, 57.7, 68.42 ppm. The formula is thus



Synthesis of N-acetylamino-n-propylmorpholine-N-oxide

In order to prove the formation of N-oxides with peroxodisulphate catalysis, we synthesized such compounds to be used as reference standards according to VanRheenen *et al.* [17]. To 0.7 g (0.03 mol) of acctylamino-*n*-propylmorpholinc, stirred in a flask, were added dropwise 0.426 ml (0.03 mol) of H_2O_2 (30% solution) during 2 h. The reaction temperature was kept at 70–75°C for 4 h with the aid of a thermostat. After cooling to room temperature, the flask was kept under stirring for 24 h. After adding 10 ml of ice-cooled ethyl acetate, a hygroscopic precipitate was formed. After elimination of the solvent, the precipitate was dissolved in 2 ml methanol and 59 mg of activated charcoal (Darco) and 59 mg of Celite were added. After filtering, methanol was evaporated; the colourless oil remaining was dissolved in hot acetone and precipitated by adding hexane (95:5). A 200-mg amount of a white oil was recovered. ^{1}H NMR (D_2O): δ 1.70 (s, 3H), 1.80 (m, 1H), 2.85–3.35 (8H), 3.52 (d, J = 12 Hz, 2H), 3.86 ppm (t, J = 12 Hz, 2H). ^{13}C NMR (D_2O): δ 23.56, 24.18, 33.82, 63.55, 65.69, 70.38 ppm. The formula is thus



Oxidation of N-acetylamino-n-propylmorpholine by ammonium peroxodisulphate

Oxidation was carried out in three different ways, as follows.

(a) To a 100 m*M* solution of acetylamino-*n*-propylmorpholine in 200 m*M* phosphate buffer (pH 8.0) was added ammonium peroxodisulphate to a final concentration of 1.2%.

(b) The same solution as in (a) was first degassed for 20 min with a water pump prior to the addition of peroxodisulphate.

(c) To a 100 m*M* solution of acetylamino-*n*-propylmorpholine in 200 m*M* borate buffer (pH 9.0) was added ammonium peroxodisulphate to a final concentration of 1.2%.

In all three solutions, on progress of the reaction for 1 h at 50°C, the pH decreased spontaneously, to 6.2 in (a) and (b) and to 7.5 in (c). In order to quantify the reaction products, the above three solutions were diluted 1:10 in 50 m*M* phosphate buffer (pH 7.0), 0.25 m*M* Immobiline p*K* 9.3 was added as internal standard and the mixture was analysed by CZE in the Waters Quanta 4000 instrument.

Incubation of N-acetylamino-n-propylmorpholine with riboflavin 5′-phosphate

This was carried out in three different ways, as follows. (a) To a 200 m*M* solution of acetylamino-*n*-propylmorpholine in 300 m*M* phosphate buffer (pH 8.0) was added riboflavin 5′-phosphate to a final concentration of 1 m*M*. (b) To a 200 m*M* solution of acetylamino-*n*-propylmorpholine in 300 m*M* phosphate buffer (pH 8.0) was added riboflavin 5′-phosphate to a final concentration of 8 m*M*. (c) The same solution as in (b) was first degassed for 20 min with a water pump prior to the addition of riboflavin 5′-phosphate.

All the solutions were then irradiated at 70°C with a 12-W neon lamp for 1 h in spectrophoto-

metric cuvettes of 5 mm thickness. No difference in reactivity was found between glass and quartz cuvettes. In order to quantify the reaction products, the above three solutions were diluted 1:20 in 50 m*M* phosphate buffer (pH 7.0), 1 m*M* Immobiline p*K* 9.3 was added as internal standard and the mixture was analysed by CZE in the Waters Quanta 4000 instrument.

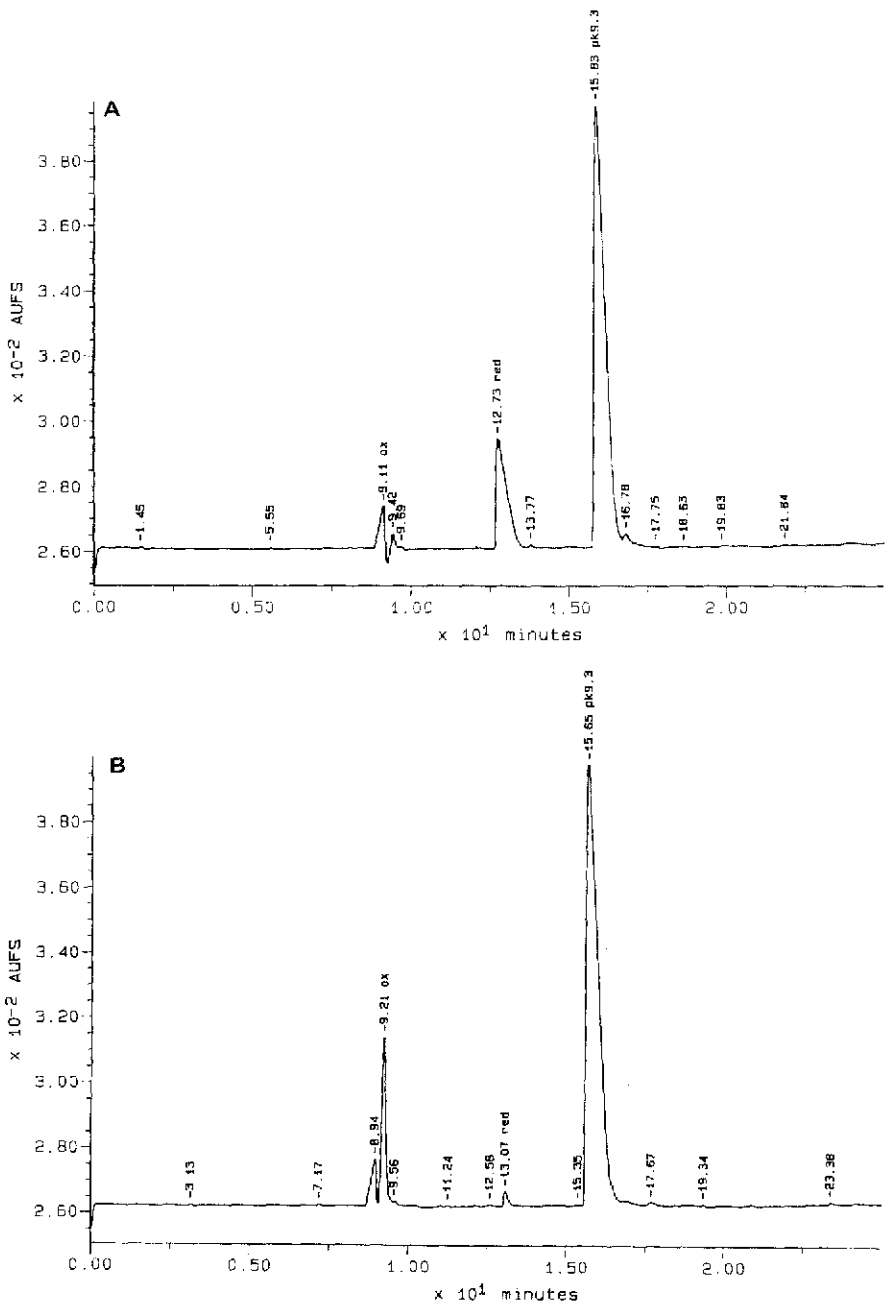


Fig. 1.

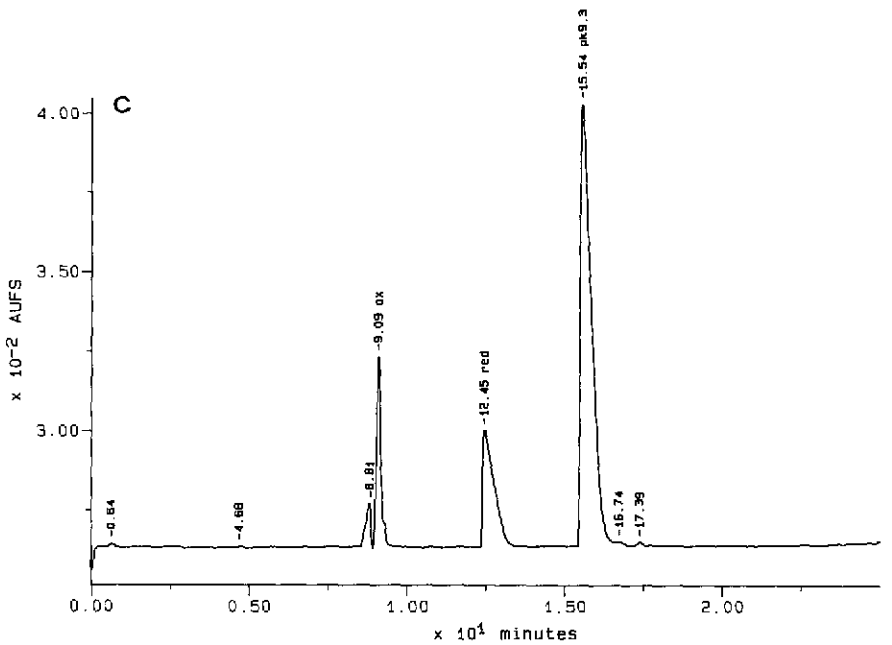


Fig. 1. CZE analysis of reduced and oxidized acetylamino-*n*-propylmorpholine. CZE was performed with a Waters Quanta 4000 instrument in a 50 cm × 75 μm I.D. capillary. All runs were at 25°C in 50 m*M* phosphate buffer (pH 7.0) containing 50 m*M* SDS and 10% methanol. Migrations were in the cathodic direction at 10 kV and 66 μA. Samples were loaded for 10 s by the "hydrostatic injection" method and were revealed at 214 nm. A = control, reduced sample with p*K* 9.3 internal standard added; B = oxidized, synthetic acetylamino-*n*-propylmorpholine with internal standard; C = mixture of reduced and oxidized species and of p*K* 9.3 standard.

RESULTS

Fig. 1 shows typical electropherograms of CZE separations of N-acetylamino-*n*-propylmorpholine and its derivatives. Fig. 1A shows an analytical run with a mixture of this compound with the internal standard (p*K* 9.3 Immobiline). When the corresponding N-oxide, synthesized as described above, is run instead, the peak of the reduced species disappears and a faster migrating component appears (Fig. 1B). When a 1:1 mixture of the reduced and oxidized standards is run (together with the internal standard), the peaks appear in the expected positions, indicating the capability of the CZE system to separate these two compounds. As the separation is done by micellar electrokinetic chromatography, such behaviour suggests that the N-oxide is more hydrophilic than the reduced species, and as such it is less incorporated in the SDS micelles.

Fig. 2 shows the results of incubating N-acetylamino-*n*-propylmorpholine with ammonium peroxodisulphate, the most common catalyst for inducing polyacrylamide gel polymerization. As shown in Fig. 2A, on incubation for 1 h at 50°C at pH 8.0, the reduced species appears to be in equilibrium with the N-oxide form (the latter representing 13%). When the same compound is incubated under the same conditions but at pH 9.0, the conversion to the N-oxide is of the order of 25% (Fig. 2B), but a number of other minor peaks are now also visible. They are tentatively identified as products of β-elimination of the N-oxide, as described in the literature (see Discussion) [18].

We next investigated the fate of the N-acetylamino-*n*-propylmorpholine standard when incubated with riboflavin 5'-phosphate under light irradiation, as in photopolymerization. Fig. 3A shows the CZE separation of the above product in presence of 1 m*M* riboflavin 5'-phosphate; no N-oxide peaks are visible. Even when the product is incubated in 8 m*M* riboflavin 5'-phosphate (a large excess in comparison with the 1 m*M* concentration required for

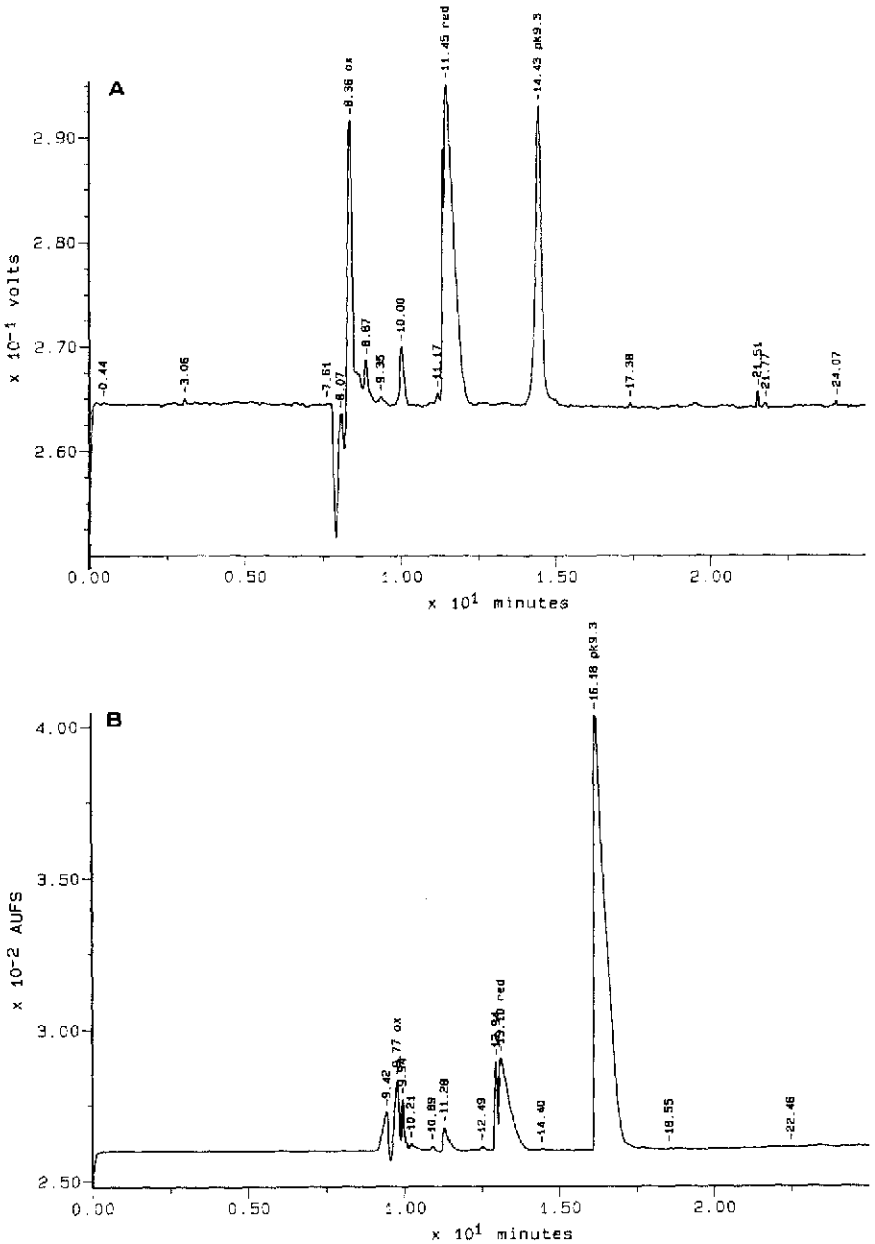


Fig. 2. CZE analysis of acetylamino-*n*-propylmorpholine incubated with peroxodisulphate. A = after incubation with 1.2% ammonium peroxodisulphate for 1 h at 50°C and pH 8.0; B = after incubation with 1.2% ammonium peroxodisulphate for 1 h at 50°C and pH 9.0 (borate buffer). Note the additional, minor peaks, attributed to products of β-elimination. All other experimental conditions as in Fig. 1.

photopolymerization) again no peaks resulting from oxidation are visible (Fig. 3B). When the latter mixture is degassed for 20 min prior to exposure to light and then analysed by CZE, the N-oxide peak is still absent (Fig. 3C).

We repeated the above experiments with light irradiation in the presence of riboflavin 5'-phosphate and with peroxodisulphate treatment and analysed all the reaction products also by TLC. Fig. 4 summarizes the results: in Fig. 4A, a chromatogram of

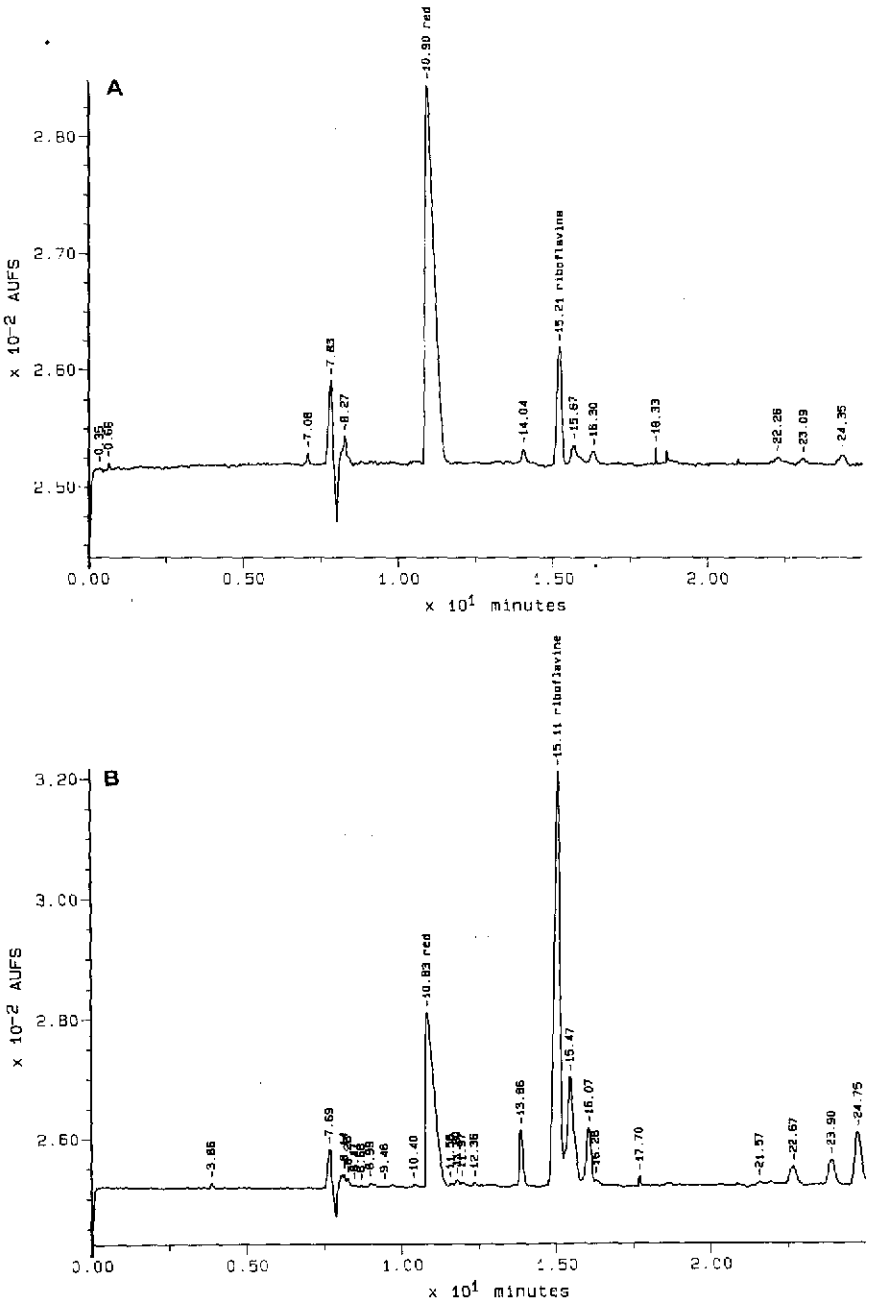


Fig. 3.

(Continued on p. 294)

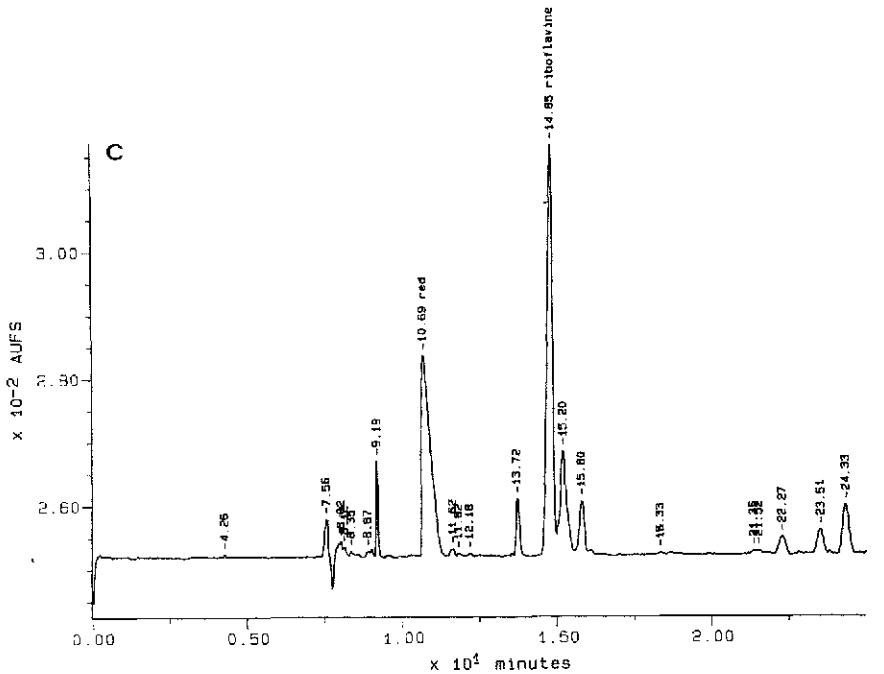


Fig. 3. CZE analysis of acetylamino-*n*-propylmorpholine incubated with riboflavin 5′-phosphate. A = reduced product incubated with 1 m*M* riboflavin 5′-phosphate; B = same as A but in the presence of 8 m*M* riboflavin 5′-phosphate (undegassed sample); C = same as B, but after degassing for 20 min with a water pump. Note in all instances the absence of the N-oxide peak.

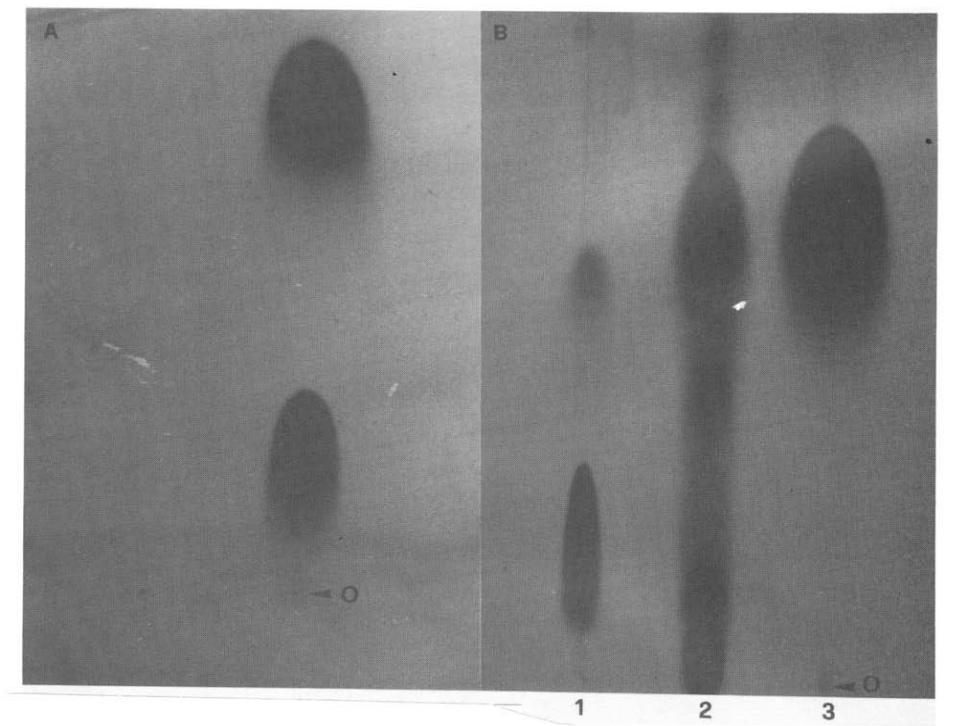


Fig. 4. TLC of reduced and oxidized acetylamino-*n*-propylmorpholine. A = 1:1 mixture of reduced (upper spot) and oxidized (lower spot) species; B: track 1 = purified N-oxide (note traces of reduced acetylamino-*n*-propylmorpholine with higher R_F); track 2 = peroxodisulphate-oxidized sample (1 h at 50°C in the presence of 3.2% peroxodisulphate); track 3 = purified, reduced acetylamino-*n*-propylmorpholine. The horizontal arrow heads with the letter O indicate the sample application line. The solvent front line is not visible on the upper part as this photograph is a close-up of the thin-layer plate.

an equimolar mixture of acetylamino-*n*-propylmorpholine and its N-oxide is shown. In Fig. 4B, the tracks are (1) the pure N-oxide (which is seen to contain traces of the reduced species), (2) the reduced compound treated with ammonium peroxodisulphate (a mixture of reduced and N-oxide species is clearly visible, with a few minor components tentatively identified as products of β-elimination) and (3) the standard of N-acetylamino-*n*-propylmorpholine.

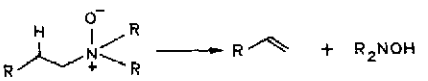
DISCUSSION

The results indicate univocally, for the first time, the oxidizing power of peroxodisulphate during gel polymerization and, in contrast, the lack of such oxidizing power in photopolymerization with riboflavin and light. Whereas in previous papers we had clearly indicated the presence of N-oxides in all four Immobiline chemicals (p*K* 6.2, 7.0, 8.5 and 9.3), such a presence had been inferred from UV visible spectra and indirect evidence. Here, in contrast, such species were identified from NMR spectra of the synthetic N-oxide product, followed by separation of the reduced and oxidized forms using both, CZE and TLC. We feel that this is an important advance as it finally clears years of debate on whether peroxodisulphate could or could not oxidize and modify proteins and what, on the contrary, would be the role of riboflavin in photopolymerization. From the present data, it is now clear that photopolymerization is totally unable to induce the formation of N-oxides, even when used in large excess and even in the presence of O_2 in the polymerizing solution (undegassed samples). In the light of the present results, photopolymerization now appears to be a very attractive alternative to peroxodisulphate catalysis. In fact, whereas formerly we had found poor conversion efficiencies in photopolymerization [12], we have recently reported very high conversions (>95%), obtained by either photopolymerizing at 70°C for 1 h with the standard 12-W neon lamp, or by photopolymerizing at room temperature but with a 105-W UV-A source.

In recent work [15], we also investigated the role of pO_2 during the polymerization process; oxygen acts as a retarder in photopolymerization, whereas it plays the role of a true inhibitor in peroxodisulphate catalysis. Even with very high levels of dissolved oxygen (pO_2 > 900 mmHg) one can still obtain a final product, in photopolymerization, identical with gels photopolymerized in only 35 mmHg of pO_2. Conversely, in peroxodisulphate polymerization, such high levels of oxygen in the gelling solution produce an irreversible inhibition of gelation.

It is of interest to note the coincident profiles of peaks in TLC and CZE: the spectrum of zones obtained in Fig. 2B is essentially identical with the series of spots in Fig. 4B (track 2). This further underlines the concept of Terabe [16] that in micellar electrokinetic chromatography the primary separation parameter is a chromatographic one, the electric field applied being merely a stronger field than a hydraulic pressure, and thus simply accelerating the separation process. From this point of view, it would appear that CZE would rather compete with high-performance liquid chromatography than with conventional electrophoretic techniques in gel slabs, such as isoelectric focusing and immobilized pH gradients.

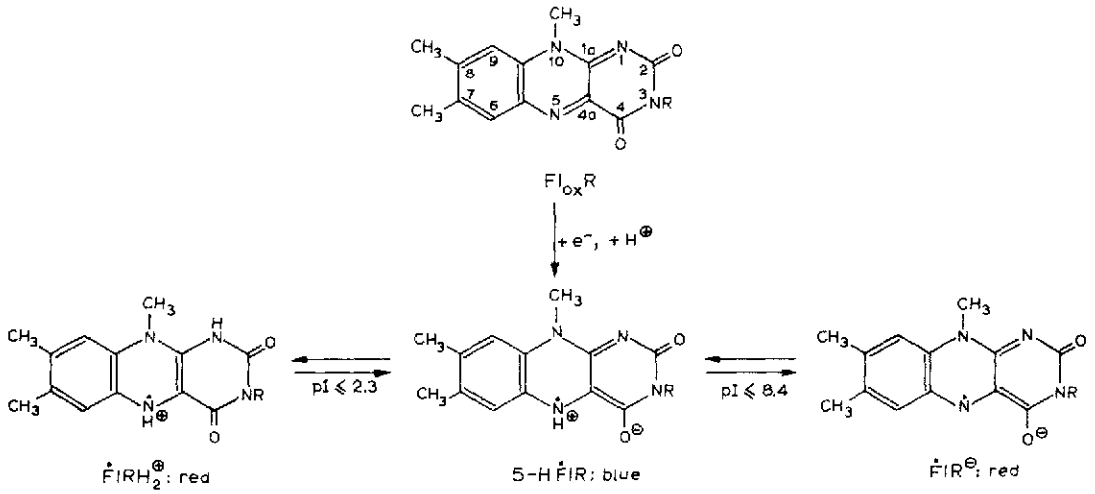
When performing peroxodisulphate incubation at pH 9.0, we had assumed that this would increase the formation of N-oxide of N-acetylamino-*n*-propylmorpholine, as we had demonstrated [7] that such an oxidation is strongly pH dependent, as it depends on the availability of a free electron pair on the nitrogen atom. When such a nitrogen is extensively protonated (*e.g.*, at *ca.* 2 pH units below the p*K* value), oxidation is completely abolished [7]. However, a series of new peaks appear in both the CZE profile (Fig. 2B) and in TLC (Fig. 4B). Tentatively, we assume that these additional minor components are products of β-elimination, by which a double bond is formed at the N-oxide site, according to the following reaction:



Although we have no direct proof of this, in previous studies we had noted a marked increase in UV chromophores after peroxodisulphate oxidation [7], in agreement with formation of UV-absorbing double bonds. Such a process of β-elimination has been well described [18]. Unfortunately, the amount of product was so minute as to defy any attempt at purification and further chemical characterization.

Chemistry of flavin radicals

The behaviour of flavin radicals has been elegantly elucidated by Hemmerich's group [19]. Basically, free flavin can produce radicals (by simultaneous abstraction of a proton and an electron) according to the scheme depicted below:



At the pH prevailing during gel polymerization (in general pH 6–8), the blue radical is formed, with a high absorption maximum (560 nm). This radical is also zwitterionic, as it bears a proton on N-5 and has the adjacent oxygen ionized. If the pH in solution is lowered, a red, cationic radical is produced (pK = 2.3, absorption maxima at 470, 400 and 375 nm). At alkaline pH, a new red radical is produced (pK = 8.4), characterized by being anionic (one net negative charge). At neutral pH, it is the zwitterionic radical that initiates and propagates the chain growth by adding to the acrylamide double bond. What is important, in riboflavin polymerization, is that, during the process of radical formation, there is no production of oxygen radicals which, in addition to propagating chain growth, act as oxidizing agents. Thus, the lack of oxidizing power is a unique feature of photopolymerization, whereas peroxodisulphate catalysis is simultaneously an oxidation event, as nascent oxygen radicals are produced by decomposition of peroxodisulphate.

CONCLUSIONS

Photopolymerization appears now to be a well controlled and highly efficient process: very high conversion rates (>95%) can be obtained by either carrying the process at 70°C for 1 h with a standard 12-W neon bulb or by using a high wattage source (105 W UV-A lamp) at room temperature. A unique feature of photopolymerization appears to be the lack of oxidizing power, always present in peroxodisulphate-initiated chain growth.

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