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Kinetics of acrylamide photopolymerization as investigated by capillary zone electrophoresis

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ABSTRACT

Riboflavin-mediated photopolymerization of acrylamide had been investigated earlier and found to give a poor conversion of the monomer into the polymeric phase (barely 60% with standard 1-h light exposure at room temperature with a 12-W neon source). The kinetics of such a process were re-examined by using capillary zone electrophoresis and it was found that the efficiency could be augmented to >95% (even higher than in peroxodisulphate polymerization) under the following conditions: by conducting the photopolymerization process at 70°C for 1 h, instead of at room temperature, alternatively, by using a 105-W UV-A lamp, having a radiation spectrum extending also in the UV region, instead of the standard 12-W neon bulbs, and by increasing the amount of catalyst (riboflavin 5'-phosphate) from 6 to 12 ppm. In all instances, it was found that atmospheric oxygen would act as a retarder, the lag time being *ca.* 17 min at an oxygen partial pressure of 35 mmHg and increasing progressively to >70 min at >200 mmHg. However, even at a partial pressure as high as 900 mmHg (as obtained by oxygen insufflation from a tank), once the retarder had been consumed a conversion of monomers into the polymer chains of >92% was still obtained. In contrast, oxygen acts as an inhibitor in peroxodisulphate polymerization: from an incorporation efficiency of *ca.* 92% at 25 mmHg oxygen partial O_2 pressure, the conversion is lowered to only 40% at 900 mmHg.

INTRODUCTION

In the last few years, we have conducted an extensive investigation of the properties of Immobilines, the acrylamido weak acids and bases used for generating insolubilized pH gradients for isoelectric focusing (see ref. 1 for a review). Such research was prompted by the availability of capillary zone electrophoresis (CZE), which seems ideally suited for studying small molecules for which electrophoretic techniques in gel slabs do not have much to offer. We then continued such studies on a novel series of neutral acrylamido derivatives, both mono- and bifunctional, which we have recently synthesized [2]. In this work, we reinvestigated the kinetics of riboflavin polymerization of polyacrylamide gels.

The use of riboflavin for gel polymerization had been suggested as early as 1957 by Oster *et al.* [3] and was subsequently recommended by Davis [4] in disc electrophoresis for casting the two segments of sample and spacer gel. Later, the use of riboflavin 5'-phosphate (FMN) instead of riboflavin was proposed [5].

There are some distinct advantages in photopolymerization: riboflavin, which can be used at very low concentrations (6 ppm, as opposed to 400 ppm for peroxodisulphate), does not seem to oxidize or denature proteins entrapped in the sample gel [6],

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and allows ample control of the start of the polymerization, as gelation is only initiated by fluorescent light. Some aspects of photopolymerization have been elucidated. Thus, it appears that photopolymerization requires traces of oxygen in order to take place [7]. Photodecomposition of riboflavin, with the consequent reoxidation of leucoflavin and production of free radicals, can occur only if molecular oxygen is initially present. The standard technique for photopolymerization has been to subject the gelling solution to a 1-h light exposure with a 12- or 16-W neon bulb at level of 6 ppm riboflavin 5′-phosphate. However, when we followed the kinetics of this polymerization process [8], we found only 60% conversion under these conditions and reported that light exposure for at least 8 h was required to achieve >90% efficiency, as is customary in peroxodisulphate polymerization [9]. We had therefore favoured chemical initiation with the standard redox couple peroxodisulphate and N,N,N′,N′-tetramethylethylenediamine (TEMED) over the use of photopolymerization.

In this work, we studied photopolymerization again and report here conditions that allow conversion efficiencies of better than 95%, *i.e.*, even more favourable than in peroxodisulphate polymerization.

EXPERIMENTAL

Materials

Acrylamide, N,N′-methylenebisacrylamide (Bis), TEMED and ammonium peroxodisulphate were obtained from Bio-Rad Labs. (Richmond, CA, USA). The p*K* 9.3 Immobiline, used as an internal standard in CZE runs, was purchased from Pharmacia–LKB (Uppsala, Sweden).

Gel preparation

All gels were prepared at a concentration of 6%T and 4%C[a] in 5 m*M* phosphate buffer (pH 6.0). TEMED was always added at a concentration of 0.4 μl per ml of gelling solution. In photopolymerization, 6 μl/ml of a 1 mg/ml FMN solution were added, corresponding to $1.16 \cdot 10^{-5}$ mmol/ml of gel, whereas for chemical polymerization (peroxodisulphate) $1.4 \cdot 10^{-3}$ mmol/ml of gelling solution were used. Gel polymerization was conducted in spectrophotometric cuvettes of 5 mm thickness. No difference in incorporation efficiency was found between glass and quartz cuvettes. Photopolymerization was conducted either with a 12-W neon bulb (at a distance of 10 cm from the slab) or with a 105-W UV-A lamp. Standard conditions in both instances were 1 h at 25°C, but 50 and 70°C were also tried and some kinetic experiments were prolonged up to 24 h. In both instances, gelling solutions were either degassed with a mechanical or a water pump, or gassed for up to 120 s with pure oxygen. We also explored the effect of different levels of FMN in solution, from the standard value ($1.16 \cdot 10^{-5}$ mmol/ml of gel) up to 1.5× and 2× concentration.

Measurements of incorporation efficiencies

In all instances described above, 5 ml of acrylamide–Bis solution (6%T, 4%C) in 5 m*M* phosphate buffer (pH 6.0) were polymerized. The gel was then extruded, minced and extracted with an equal volume of methanol. After filtering the gel debris, the methanol was evaporated, the solution reconstituted to the original volume and p*K* 9.3 Immobiline was added as internal standard (2.0 m*M* final concentration). The amount of unreacted monomers was assessed by CZE, as described below, with a calibration graph consisting of eight dilutions of pure acrylamide covering the range 0.025–0.1 m*M*.

Capillary zone electrophoresis (CZE)

CZE was performed in a Beckman (Palo Alto, CA, USA) instrument (P/ACE System 2000) equipped with a fused-silica capillary (having an untreated inner surface) of 57 cm × 75 μm I.D. from Polymicro Technologies (Phoenix, AZ, USA). Runs were performed at 25°C in a thermostated environment in 0.1 *M* borate buffer (pH 9.0). In all instances the migration direction was toward the negative electrode. The samples were injected into the capillary by pressure (80 p.s.i.), usually for 10 s. The calibration graph for each acrylamido derivative analysed was constructed with the Beckman Gold integration system. In each run p*K* 9.3 Immobiline (2.0 mM) was used as an internal standard. Runs were usually performed at 15 kV and 50 μA with the detector set at 254 nm.

[a] C = g Bis/%T; T = g acrylamide + g Bis per 100 ml of solution.

Kinetics of riboflavin degradation

In order to follow the degradation kinetics of riboflavin during exposure to light for gel polymerization, a 2 m*M* solution of FMN in 5 m*M* phosphate buffer (pH 6.0) was irradiated with the 12-W neon bulb at time intervals up to 24 h at both 25 and 70°C. The irradiated samples were then analysed by CZE, as above, by using the p*K* 9.3 Immobiline (2.0 m*M*) as internal standard. The CZE analysis was done in a 57 cm × 75 μm I.D. capillary in 0.1 *M* phosphate buffer (pH 7.0) at 10 kV and 100 μA.

Inhibition/retardation curves

In order to check the effect of oxygen on riboflavin- *vs.* peroxodisulphate-polymerized gels, the two series of gels were prepared under the following conditions: (a) degassed for 30 min with a mechanical pump; (b) degassed for 30 min with a water pump; (c) control, non-degassed; (d) gassed with pure oxygen from a tank for 15, 30, 60 or 120 s. Polymerization was then conducted at 70°C and aliquots were collected at different time intervals. After extraction in methanol, the efficiency of incorporation was assessed by CZE as described above. The oxygen partial pressure in solution was determined by haemo-gas analysis in an ABL 330 instrument from Radiometer (Copenhagen, Denmark).

Polymerization lag

In order to check the effect of oxygen partial pressure on the lag time (*i.e.*, the time between the start of light exposure and onset of polymerization), a series of gels were photopolymerized at 25, 50 and 70°C, either under degassing conditions or after oxygen insufflation, as above. The onset of polymerization was assessed either by measuring the heat of polymerization with a differential thermocouple (copper–constantan with a sensitivity of 40 μV/°C) [9] or by shaking the gelling solution at regular time intervals.

RESULTS

Conversion efficiency as a function of time and temperature

For photopolymerization, it has been customary in the past to subject the gelling solution to a 1-h light exposure at room temperature [8], usually in front of neon lamps of 12 or 16 W, placed at a distance of *ca.* 10 cm from the gel cassette. However, under these conditions, the incorporation efficiency, as measured previously [8] by permanganate titration of unreacted double bonds, was barely 60%. We have repeated these measurements determining unreacted monomers, after extraction from the gel, by CZE. Fig. 1 gives an example of the kind of separations obtained; p*K* 9.3 Immobiline was always added as internal standard for quantification purposes. As shown in Fig. 2, the conversion at 1 h was *ca.* 70% and an 8-h exposure time was required to obtain an incorporation of *ca.* 95%, in agree-

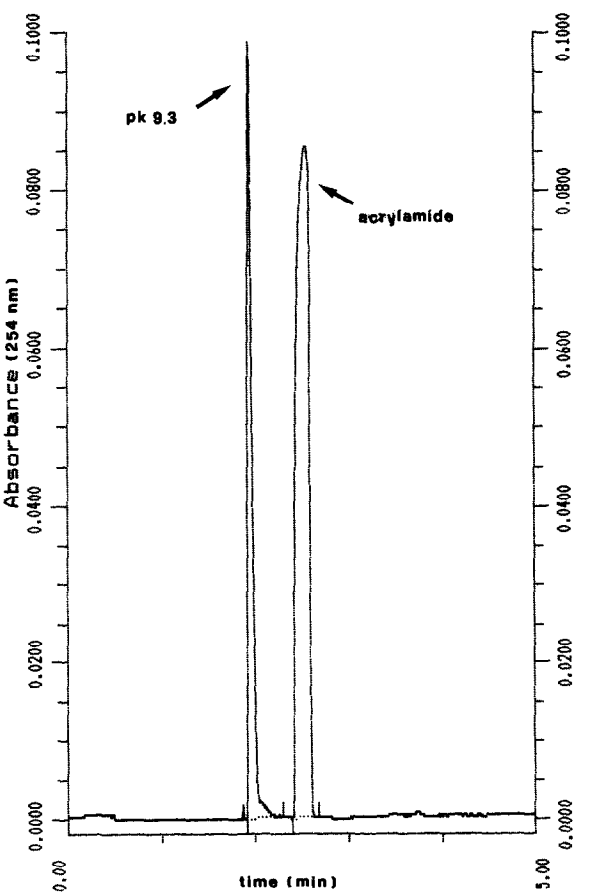


Fig. 1. Example of a CZE determination of unreacted acrylamide after gel polymerization. To the unreacted monomer, extracted from the gel, was added an internal standard (2.5 m*M* p*K* 9.3 Immobiline) and the mixture was run in a Beckman P/ACE 2000 at 15 kV and 50 μA in 100 m*M* borate buffer (pH 9.0) (cathodic migration). Capillary, 57 cm × 75 μm I.D.; detection at 254 nm.

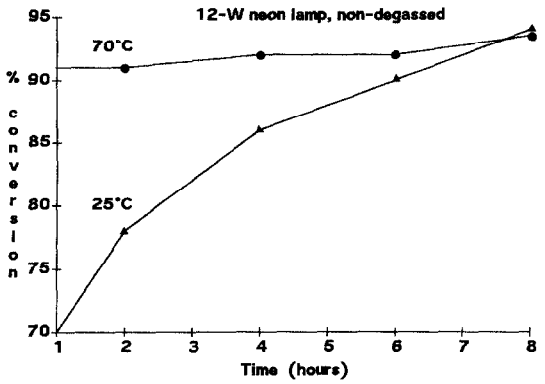


Fig. 2. Conversion efficiency as a function of time and temperature. Solutions of 6%T, 4%C monomers in 5 m*M* phosphate buffer (pH 6.0) were photopolymerized with FMN ($1.16 \cdot 10^{-5}$ mmol/ml of gelling solution) at either 25 or 70°C for up to 8 h. The solutions were not degassed and a 12-W neon bulb was used as a light source. At the given time intervals, 5-ml tubes were collected, the gels minced and extracted in methanol and ungrafted monomers determined after CZE in a P/ACE 2000 instrument in 100 m*M* borate buffer (pH 9.0) at 15 kV and 50 μA and 25°C. Capillary 57 cm × 75 μm I.D.; detection at 254 nm.

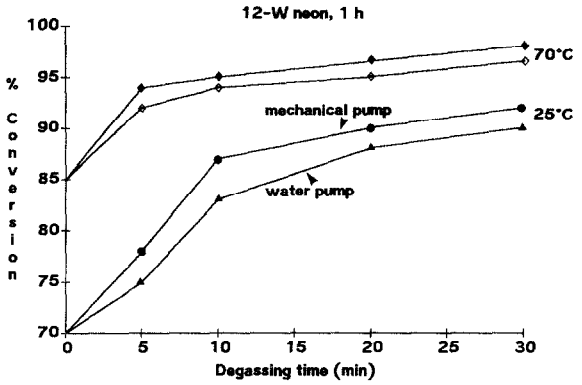


Fig. 3. Polymerization efficiency as a function of degassing method. Solutions of 6%T, 4%C monomers in 5 m*M* phosphate buffer (pH 6.0) were photopolymerized with FMN ($1.16 \cdot 10^{-5}$ mmol/ml of gelling solution) at either 25 or 70°C for 1 h in front of a 12-W neon bulb. The solutions were degassed for the times indicated either with a mechanical pump (giving a 35 mmHg oxygen partial pressure after 30 min) or with a water pump (65 mmHg). Ungrafted monomers were determined by CZE as in Fig. 2.

ment with the previous work [8]. However, in the past, there have been no reports on the effect of temperature on such a polymerization process. We had already reported in 1984 that, for casting Immobiline gels, good conversion in peroxodisulphate polymerization was only obtained by 1-h polymerization at 50°C [10]. Based on these observations, we tried the photopolymerization at 70°C; as shown in Fig. 2, at the end of the standard 1-h period, a conversion of >92% was obtained and essentially no "ripening" of the gel was noted if light exposure was prolonged up to 8 h.

Conversion efficiency as a function of oxygen partial pressure

The role of oxygen dissolved in solution has never been well elucidated in photopolymerization. We prepared different series of gels degassed either with a mechanical or with a water pump and polymerized them at either 25 or 70°C. As shown in Fig. 3, dissolved oxygen always inhibits, to some extent, the incorporation of monomers into the growing polymer, the effect being much less pronounced at 70 than at 25°C however. As shown in the graphs, a minimum degassing time of 10 min is required to minimize oxygen retardation. The oxygen partial pressure also has a strong effect on the lag time, *i.e.*, on the time of polymerization onset after starting light irradiation. As shown in Fig. 4, whereas at room temperature the lag time is *ca.* 30 min in the

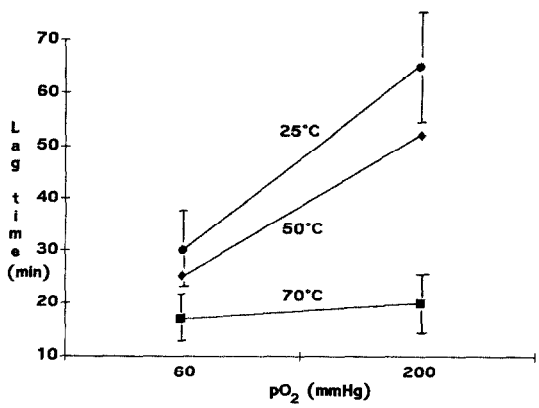


Fig. 4. Lag time of photopolymerization as a function of oxygen partial pressure (pO_2) and temperature. Solutions of 6%T, 4%C monomers in 5 m*M* phosphate buffer (pH 6.0) were photopolymerized with FMN ($1.16 \cdot 10^{-5}$ mmol/ml of gelling solution) at either 25, 50 or 70°C for 1 h in front of a 12-W neon bulb. The lag time was measured at two values of pO_2, corresponding to degassing with a water pump (65 mmHg) and to control, undegassed solutions (200 mmHg). The onset of polymerization was assessed either with a differential thermocouple (25°C) or by shaking the gelling solutions at regular time intervals.

presence of 60 mmHg of oxygen, it becomes 65–70 min at 200 mm Hg of oxygen. The lag time, however, is progressively decreased at gradually higher temperatures: thus, at 70°C, it is only 17 min at 60 mmHg and increases slightly to only 20 min at 200 mmHg oxygen partial pressure. There is, however, a large standard deviation, so that controlling the lag time still remains one of the most difficult tasks in photopolymerization.

What exactly the role of oxygen is in photopolymerization, as opposed to peroxodisulphate polymerization, is shown in Fig. 5. Both reactions were conducted at 70°C for a standard 1-h period, so as to minimize the lag time and ensure high conversions. In photopolymerization, it is seen that, on going from 200 mmHg (the oxygen partial pressure in control, undegassed solutions) to 900 mmHg (the oxygen partial pressure after gassing a solution for 120 s), the conversion efficiency is essentially unaffected, and remains at a constant, high level of >90% (the lag time will be strongly affected, however; see below). Conversely, with peroxodisulphate initiation, progressively increasing levels of oxygen are associated with a gradual decrease in conversion efficiency, from 94% at 200 mmHg down to barely 40% at 930 mmHg of oxygen (Fig. 5).

The different role played by oxygen in the two catalyst systems is further elucidated in Fig. 6; we

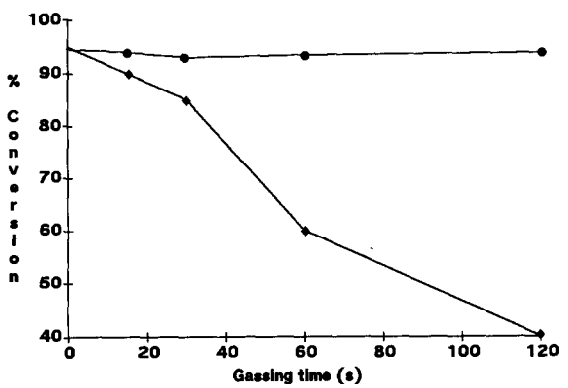


Fig. 5. Polymerization efficiency as a function of pO_2. Solutions of 6%T, 4%C monomers in 5 mM phosphate buffer (pH 6.0) were either photopolymerized with FMN ($1.16 \cdot 10^{-5}$ mmol/ml of gelling solution) at 70°C for 1 h in front of a 12-W neon bulb (●) or initiated with peroxodisulphate (1 h, 70°C) (◆). The solutions were either controls (200 mmHg pO_2) or gassed with pure oxygen from a tank (930 mmHg pO_2 at 120 s of gassing). Ungrafted monomers were determined by CZE as in Fig. 2.

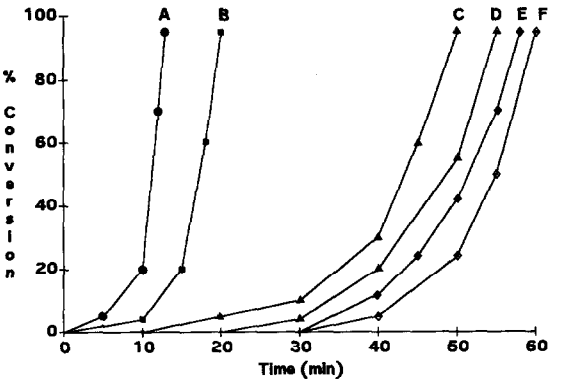


Fig. 6. Retardation curves as a function of pO_2. Solutions of 6%T, 4%C monomers in 5 mM phosphate buffer (pH 6.0) were photopolymerized with FMN ($1.16 \cdot 10^{-5}$ mmol/ml of gelling solution) at 70°C for 1 h in front of a 12-W neon bulb. The solutions contained from 35 mmHg pO_2 (curve A, degassed for 30 min with a mechanical pump) up to 930 mmHg pO_2 (curve F, gassed for 120 s with oxygen). Ungrafted monomers were determined by CZE as in Fig. 2.

have measured the lag time before polymerization onset as a function of oxygen partial pressure, from 35 mmHg (in solutions degassed for 30 min with a mechanical pump) up to 930 mmHg (in solutions gassed with pure oxygen for 120 s) (see Table I). It is seen that, in photopolymerization, oxygen has a strong influence only on the lag time, not on the final conversion efficiency, which is good (>90%) in all instances. In other words, whereas in photopolymerization oxygen acts only as a "retarder", with no effect on the final gel product, in perox-

TABLE I

OXYGEN PARTIAL PRESSURE IN DEGASSED, CONTROL AND GASSED GELLING SOLUTIONS

pO_2 measured in 5 mM phosphate buffer (pH 6.0)

Type of solution	pO_2 (mmHg)[a]
Degassed, mechanical pump (30 min)	35 ± 5
Degassed, water pump (30 min)	63 ± 12
Control	205 ± 4
15 s O_2 gassing	860 ± 39
30 s O_2 gassing	900 ± 35
60 s O_2 gassing	910 ± 40
120 s O_2 gassing	927 ± 45

[a] Mean values ± standard deviations for ten determinations.

odisulphate polymerization oxygen acts as an "inhibitor", impeding the incorporation of free monomers in the growing chains (see Discussion).

Another way to control the lag time (and the incorporation efficiency) is by using a stronger light source. When the gels are polymerized not in front of a 12-W neon source but in front of a 105-W UV-A lamp, the lag time, which at room temperature is 60 min, is reduced to barely 17 min (Fig. 7). The 105-W lamp also dramatically increases the incorporation efficiency: in fact, whereas at 25°C, with a 12-W bulb, barely a 70% conversion is achieved in a 1-h illumination period, under the same conditions the incorporation efficiency with the 105-W UV-A lamp is increased to >90% (Fig. 7), *i.e.*, to the same conversion that would have required, under the former conditions, an 8-h illumination period (see also Fig. 2).

Conversion as a function of riboflavin concentration

It has been customary, up to now, in photopolymerization to use a fixed level of FMN of the order of $1.16 \cdot 10^{-5}$ mmol/ml (standard conditions). We investigated whether a better conversion could be obtained by increasing the level. As shown in Table II, 1.5× and 2× concentration levels do offer a slight increase in incorporation efficiencies at all temperatures investigated. However, no effect was visible at higher concentrations (not shown). Therefore, a 2× FMN level is recommended in photopolymerization.

TABLE II

CONVERSION (%) AS A FUNCTION OF RIBOFLAVIN CONCENTRATION AND TEMPERATURE

Temperature (°C)	[Riboflavin][a]		
	1×	1.5×	2×
25	70 ± 12	70 ± 11	73 ± 15
50	76 ± 9	84 ± 9	88 ± 11
70	84 ± 6	88 ± 5	92 ± 8

[a] The standard riboflavin concentration (1×) was $1.16 \cdot 10^{-5}$ mmol/ml of gelling solution. Conversion results are mean values ± standard deviations for ten determinations.

The fate of riboflavin on illumination was investigated by light exposure at 25 and 70°C for up to 24 h. The sample was then injected into the CZE system and the remaining FMN peak was measured. As shown in Fig. 8, higher temperatures, at all times, produce a more rapid destruction of FMN; this could explain the much higher incorporation efficiencies at 70°C as opposed to 25°C.

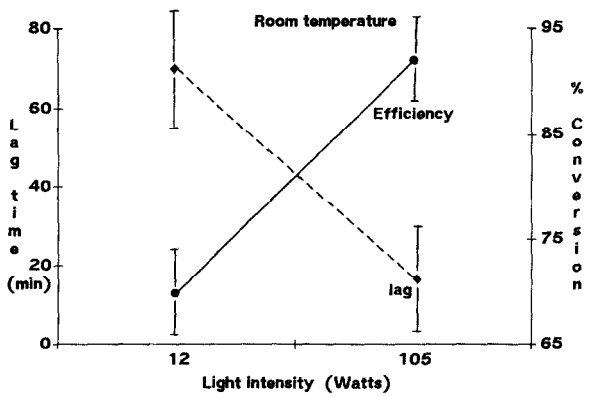


Fig. 7. Lag time and efficiency of incorporation as a function of wattage of the light source. Solutions of 6%T, 4%C monomers in 5 m*M* phosphate buffer (pH 6.0), were photopolymerized with FMN ($1.16 \cdot 10^{-5}$ mmol/ml of gelling solution) at 25°C for 1 h either in front of a 12-W neon bulb or by irradiation with a 105-W UV-A lamp. The onset of polymerization was assessed with a differential thermocouple, whereas the conversion efficiency was measured by CZE as in Fig. 2.

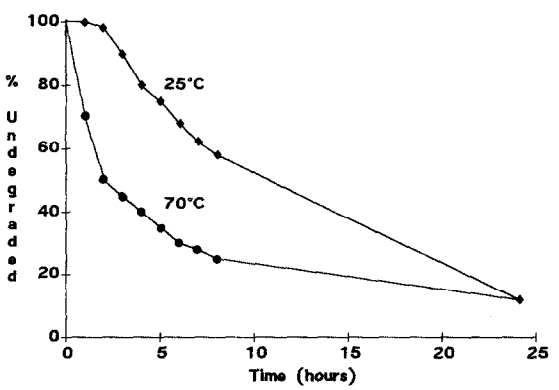


Fig. 8. Kinetics of riboflavin degradation as a function of temperature. FMN ($1.16 \cdot 10^{-5}$ mmol/ml) dissolved in 5 m*M* phosphate buffer (pH 6.0) was irradiated with a 12-W neon bulb at either 25 or 70°C. At the times indicated, aliquots were collected and analysed by CZE in 50 m*M* phosphate buffer (pH 7.0) at 100 μA and 10 kV (25°C).

DISCUSSION

Ten years ago, when we made our first thorough investigation of the kinetics of photopolymerization [8], we concluded that such a process was not attractive for producing polyacrylamide gels, because under standard conditions (1-h exposure, as also used in peroxodisulphate initiation), only 60% conversion could be achieved (*vs.* >90% with peroxodisulphate). In order to achieve the same incorporation efficiency, photopolymerization had to be protracted for at least 8 h, a much too slow process to be of practical interest in routine work. However, as we are planning a series of polymerization experiments in microgravity, photopolymerization became attractive, as the gel cassettes could be prepared on earth and polymerization activated in space simply by light irradiation: this basic need prompted the present investigation. We therefore established conditions which allow ample conversion of the monomers into the polymers within the standard 1-h exposure time by: (a) using a conventional neon bulb (12–16 W) but at 70°C or (b) using a strong light source (105-W UV-A, a domestic lamp used for indoor sun-tanning) at room temperature. The latter process is definitely preferable, because in the first instance both the light source and the gel cassette have to be placed in an oven. We have also demonstrated that it is possible to control the lag time by controlling the oxygen partial pressure in solution: when the latter is reduced to only 35 mmHg, the lag time is reduced from 60 min to only 15–17 min. It is tempting to suggest that, perhaps, in the total absence of oxygen, the lag time could be reduced to a vanishing value, but preparing a completely anaerobic solution (*e.g.*, by passing it through insolubilized ascorbic oxidase or another oxygen scavenger) would be too demanding in routine laboratory practice. There are, nevertheless, some important conclusions of our research which are discussed below.

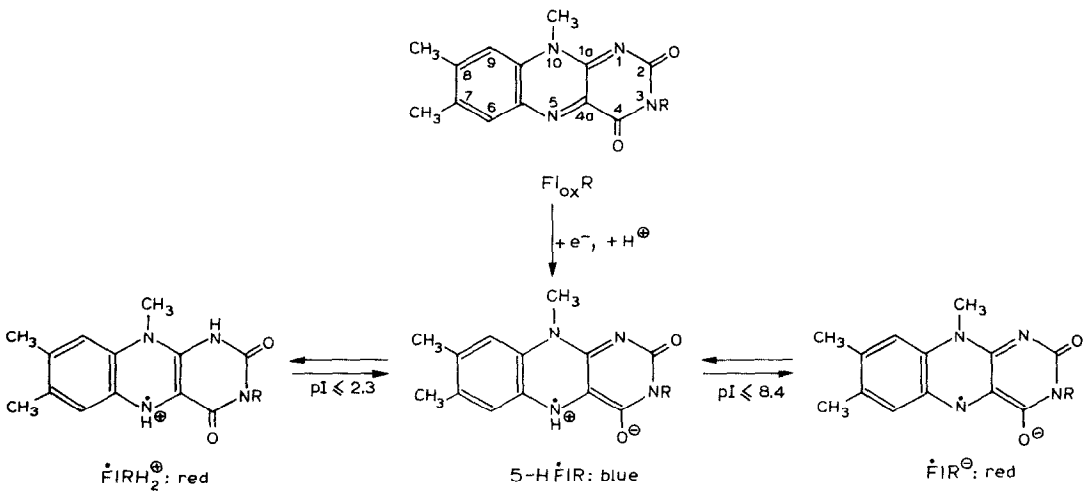
Inhibitors vs. retarders

Inhibitors and retarders are substances which, when added to polymerization reactions in very low concentrations, produce large decreases in rate by, *e.g.*, deactivation of initiating centres of interruption of propagating chains. The terms retardation and inhibition tend nowadays to be used interchangeably, an inhibitor being considered to be a powerful retarder that cuts off chain growth at a very early stage and so effectively prevents significant polymerization. In classic chain kinetics, however, the two terms have specific and different meanings: inhibitors react very rapidly with initial active centres and so prevent initiation, whereas retarders destroy propagating species and thus reduce the rate of reaction. Obviously the distinction becomes tenuous when the similarity in reactivity between initiating and propagating species is close, and this is the situation with many free-radical polymerizations. Nonetheless, the difference between these two concepts is important for kinetics reasons. It is clear from our data (see Fig. 5 and 6) that oxygen acts in a completely different manner during peroxodisulphate as opposed to riboflavin polymerization. In the former instance, oxygen behaves as a true inhibitor or terminator, *i.e.*, it brings about the cessation of growth of a propagating radical, by a process of chain transfer [11]. This phenomenon is easily appreciated in routine laboratory practice: when gels are polymerized in an open-ceiling cassette, the top liquid layer, in contact with atmospheric oxygen, never polymerizes, unless it is protected by, *e.g.*, a layer of butanol. Conversely, with retardation, as in photopolymerization, only the onset of polymerization is shifted along the time axis (see Fig. 5); when the retarder initially present is completely consumed by reaction with primary radicals, the rate of polymerization assumes its uninhibited value, and the final gel product is indistinguishable from the unretarded controls.

Chemistry of the flavin radicals

The behaviour of flavin radicals has been elegantly elucidated by Hemmerich's group [12]. Basically, free flavin can produce radicals (by simultaneous abstraction of a proton and an electron) according to the scheme depicted on the next page.

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. This is in fact what happens in peroxodisulphate polymerization: our group has demonstrated that, in all polymerization processes initiated by peroxodisulphate, oxidation of all buffer components (especially primary to tertiary amines) is a common event. In immobilized pH gradients gels, at an appropriate pH, the Immobiline chemicals form N-oxides [13]; in conventional focusing gels, the carrier ampholyte bufers are also oxidized [14]. These N-oxide species remain in the gel matrix (whereas excess of peroxodisulphate is discharged at the anode in a pre-run) and are able to readily oxidize –SH groups in proteins to cystine residues, thus introducing an artefactual heterogeneity [15]. This phenomenon (a residual oxidizing power inherent to peroxodisulphate-polymerized gels) is totally absent in photopolymerized matrices [16].

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. The role of oxygen as a retarder has been elucidated, and the lag time can be accurately controlled at low oxygen partial pressures (< 35 mmHg). A unique feature of photopolymerization appears to be the lack of oxidizing power, always present in peroxodisulphate-initiated chain growth.

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