Journal of Chromatography, 79 (1973) 267–273

CHROM. 6655

THE FLUORESCENCE PROPERTIES OF POLYACRYLAMIDE GELS

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(Received February 12th, 1973)

SUMMARY

A study has been made of the fluorescence properties of a range of polyacrylamide gels typical of those used in electrophoretic methods, or in the immobilisation of macromolecules. In many cases the fluorescence is of sufficient intensity to interfere with the fluorimetric scanning of gel electrophoresis separations. Factors affecting the gel fluorescence are described, and several methods for minimising it are suggested.

INTRODUCTION

Polyacrylamide gels are widely used in a number of techniques in analytical biochemistry: they are easily formed, stable and hydrophilic, and their pore sizes can be controlled by polymerising appropriate concentrations of the monomers, acrylamide and methylene bisacrylamide. High-resolution electrophoretic separations of proteins and other macromolecules can be obtained using short columns[1] or thin layers[2] of suitable gels, and similar arrangements are used in isoelectric focusing experiments[3]. In all these cases, the separations obtained can be evaluated by staining the molecules with an appropriate dye, or by direct transmission densitometry of the gels[4,5]. Recently, however, it has been shown that certain protein separations can be monitored fluorimetrically, utilising either the native fluorescence of the proteins[6], or the fluorescence of an appropriate label molecule, added to the sample either before[7,8] or after[9] the separation. The great sensitivity of fluorimetric measurements can thus be used to study nanogram quantities of proteins.

Polyacrylamide gels are also appropriate media for the immobilisation of antibodies and enzymes, either by physical entrapment of the macromolecules in the gel pores[10] or by covalent attachment of the protein to beads of a chemically-modified gel[11]. In the latter case the beaded gels used in gel chromatography are often suitable starting materials. It has recently been demonstrated that fluorimetric methods can be used to study the properties of immobilised proteins, including their susceptibility to denaturation[12]. During attempts to study the fluorescence of enzymes entrapped in polyacrylamide gels it was found that the gels themselves exhibited considerable fluorescence. The present paper describes the fluorescence properties of a range of gels, and indicates how such fluorescence can be minimised.

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MATERIALS AND METHODS

Acrylamide and methylene bisacrylamide were recrystallised using LOENING's methods[4]. Gel formation was normally catalysed using ammonium persulphate and tetramethyl-1,2-diaminoethane (TEMED). In some experiments no bisacrylamide was incorporated in the gel: the product was then sufficiently fluid to allow other reagents to be added some hours after polymerisation had begun. 0.1 *M* tris(hydroxymethyl)methylamine–HCl (Tris–HCl) buffer, pH 8, was the solvent in most experiments, but was occasionally replaced by 0.1 *M* sodium bicarbonate, pH 8.4 or 0.1 *M* phosphate–citric acid buffers, pH 8.0 and 6.0. The persulphate content of the gels was determined using BENNICK's benzidine acetate method[13]. Bio-Gel P300 polyacrylamide gel beads, wet particle size 50–100 mesh, were obtained from Bio-Rad Ltd,. St. Albans. Dithiothreitol was a product of Koch-Light Ltd., Colnbrook; all other reagents were products of B.D.H. Ltd. Water was distilled three times from an all-glass apparatus.

Uncorrected fluorescence emission and excitation spectra were obtained using a "Fluoripoint" spectrofluorimeter (Baird Atomic Ltd., Braintree) equipped with a 150 W xenon discharge source and motor-driven monochromators, and connected to a Bryans 27000 recorder (Bryans Ltd., Mitcham). A spectral bandwidth of 8 nm was used throughout and instrumental fluctuations were corrected using the Raman scatter signal of water, excited at 330 nm, as a reference[14]. Polyacrylamide gels were formed and examined in 1-cm square spectrosil cells (Optro Ltd., Hornchurch) except when an electrophoretic pre-run was employed (see below). Suspensions of polyacrylamide gel beads were studied using the "front-face" optical accessory of the spectrofluorimeter.

Electrophoresis experiments were performed in a Shandon disc electrophoresis apparatus (Shandon Southern Instruments Ltd., Camberley). Columns (6 cm) of polyacrylamide gel were contained in quartz tubes of 6 mm I.D., and their fluorescence was monitored by placing the tubes directly in the fluorimeter sample compartment. Tris–HCl buffer, 0.1 *M*, pH 8.0 was used in both electrode compartments.

RESULTS

Fig. 1a shows the development of fluorescence during the polymerisation of a 12/5 acrylamide gel*. The gel began to form about 10 min after the addition of the initiators, TEMED and ammonium persulphate each at 2.65 m*M* concentration. It was slightly turbid, and a very strong signal due to Rayleigh and Tyndall light scattering partially overlapped the fluorescence band (λ_{max} = 415 nm) even when the exciting wavelength was 300 nm (Fig. 1b). Whereas the scattered light signal reached a maximum intensity within 1 h, the fluorescence, initially negligible, continued to increase in intensity for about 24 h.

The intensity of scattered light could be reduced by lowering the proportion of methylene bisacrylamide in the monomer mixture. The 8/2 gel whose fluorescence properties are shown in Fig. 2 only scattered approximately one-thirtieth of the light of the 12/5 gel. In these conditions the gel fluorescence was more clearly seen,

* A 12/5 acrylamide gel has a total monomer concentration (acrylamide + methylene bisacrylamide) of 12 g/100 ml, of which 5% (0.6 g/100 ml) is methylene bisacrylamide.

and showed the same pattern of development. The fluorescence intensities of the gels were approximately equal, being some thirty times that of the Raman scattering signal obtained from the pure solvent, and approximately equal to the fluorescence at 350 nm of a typical protein solution of *ca.* 0.2 mg/ml concentration.

Polymerisation of a 2% acrylamide solution in the absence of methylene bisacrylamide produced a very viscous fluid, whose fluorescence properties were again similar to those of the above gels, but which gave a negligible scattered light signal. It was thus possible to obtain the excitation spectrum of the fluorescence,

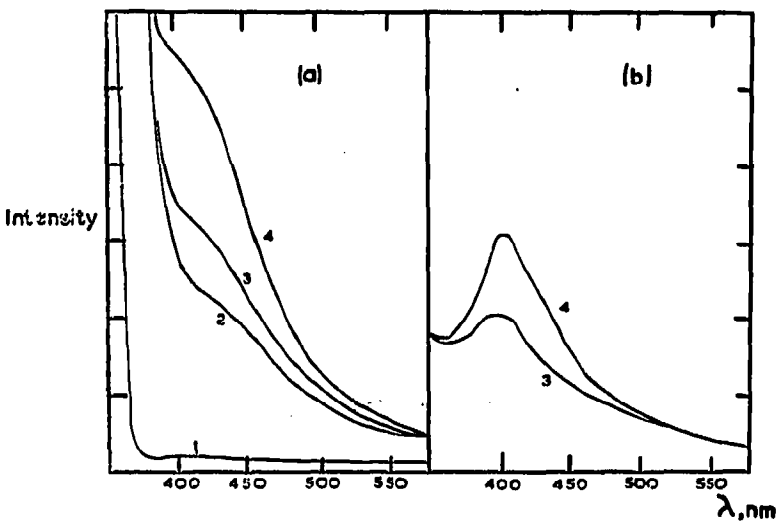

Fig. 1. Development of fluorescence in a 12/5 polyacrylamide gel. (a) Excitation wavelength 350 nm, (b) excitation wavelength 300 nm. Spectra were obtained 5 min (1), 1 h (2), 3 h (3), and 22 h (4) after initiation.

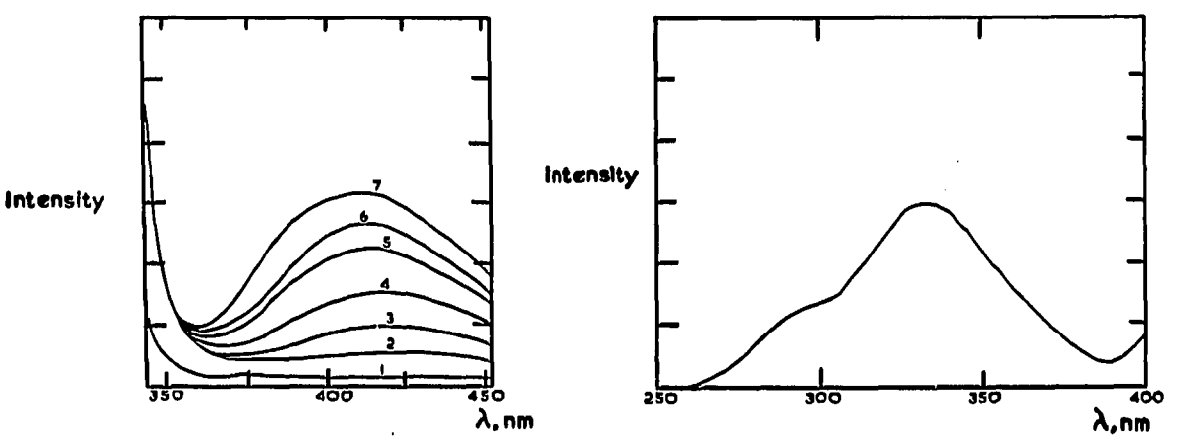

Fig. 2. Development of fluorescence in an 8/2 polyacrylamide gel. Spectra were obtained 5 min (1), 30 min (2), 1 h (3), 2 h (4), 4 h (5), 6 h (6) and 25 h (7) after initiation. The excitation wavelength was 330 nm.

Fig. 3. Excitation spectrum of a 2% polyacrylamide solution, 24 h after initiation: the fluorescence emission wavelength was 415 nm.

which had a maximum at 333 nm with a pronounced shoulder at shorter wavelengths (Fig. 3.).

The intensity of the gel fluorescence was found to be independent of total monomer concentration in the range 1–8 %, but no fluorescence was shown by mixtures containing no monomers, *i.e.* buffered solutions of the initiators. To test for possible photochemical effects, some measurements were made on gels polymerised in the dark, but this produced no reduction in fluorescence. Gels polymerised in daylight and then exposed to 333 nm radiation for 1 h lost approx. 50 % of their fluorescence intensity. None of the buffer solutions used was intrinsically fluorescent, but it was found that at pH 6.0 the formation of the gel and the development of the fluorescence were both slower than at alkaline pHs.

Increasing the concentrations of the initiators produced increased gel fluorescence at first, but no further effects were observed at initiator concentrations above *ca.* 15 m*M* (Fig. 4). Similarly, when extra initiators were added to 24-h-old polymers the fluorescence increased further (Fig. 5a), after an initial decrease that may have been caused by inhomogeneities in the very viscous mixture. The addition of extra ammonium persulphate in the absence of TEMED (Fig. 5b) had a similar effect, but the effect of extra TEMED alone (Fig. 5c) was negligible. When a persulphate/sulphite initiator system was used (gels formed in this way are non-fluorescent, see below) and TEMED added after 24 h polymerisation, gel fluorescence developed rapidly.

Further evidence for the participation of persulphate in the formation of gel fluorescence was obtained by the use of BENNICK's test[13]. Gels tested within 4 h of the onset of polymerisation invariably gave a positive reaction for persulphate, gels 4–8 h old gave variable results, and all 24-h-old gels were apparently persulphate-

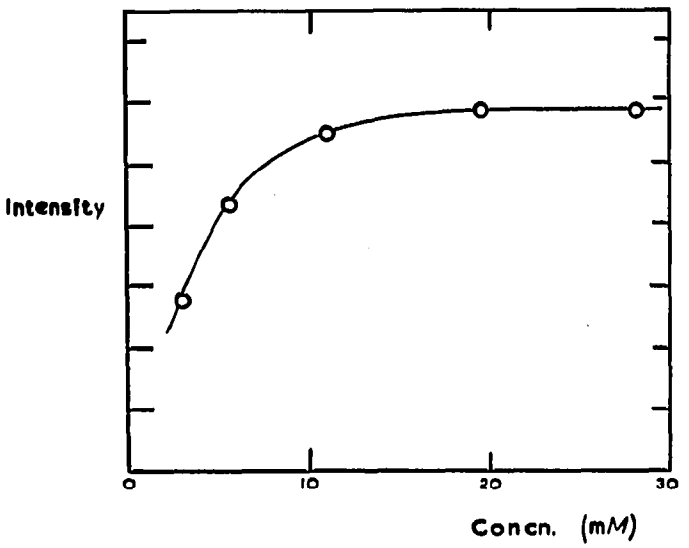

Fig. 4. Effect of initiator concentrations (equimolar amounts of TEMED and ammonium persulphate) on the fluorescence of 2% polyacrylamide solutions. Fluorescence intensities were measured 24 h after initiation using excitation and emission wavelengths of 330 nm and 420 nm, respectively.

free. The development of polymer fluorescence was thus paralleled by a fall in the persulphate content of the gels.

The beaded acrylamide gel, Bio-Gel P300, exhibited a quite intense fluorescence with the same wavelength characteristics as those cited above.

A number of attempts were made to reduce or abolish the gel fluorescence.

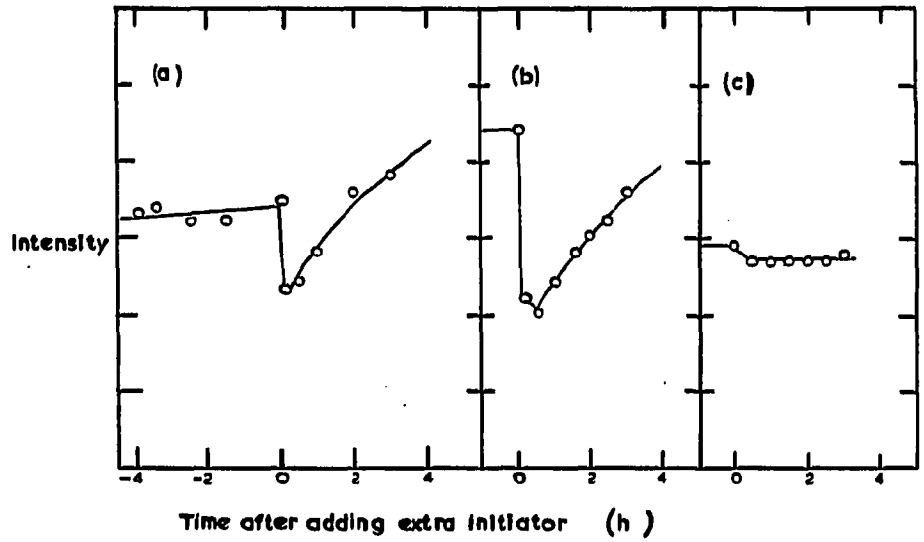

Fig. 5. Effect of additional initiators (equal in concentration to those originally used) on the fluorescence of 24-h-old 2% solutions of polyacrylamide. Excitation wavelength 350 nm, emission wavelength 420 nm. (a) Addition of persulphate + TEMED, (b) addition of persulphate alone, and (c) addition of TEMED alone.

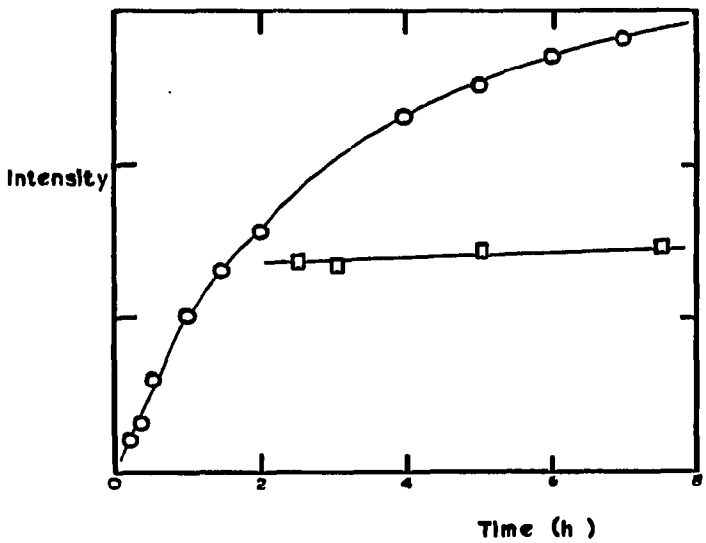

Fig. 6. Effect of a 2-h electrophoresis step at 5 mA on the fluorescence intensity of an 8/2 polyacrylamide gel. Excitation wavelength 330 nm, emission wavelength 420 nm. ○, Fluorescence of an untreated gel; □, fluorescence of a gel after electrophoresis.

The effect of submitting a newly-formed 8/2 gel to an electrophoresis step, recommended by MITCHELL[15] as a method for removing persulphate residues prior to protein analyses, is shown in Fig. 6. A 2-h electrophoresis removed all the persulphate from the gel, as judged by BENNICK's test[13]. The fluorescence of the gel was reduced but by no means abolished: however, hardly any increase in fluorescence was observed after the electrophoresis.

The addition of 5 m*M* dithiothreitol to a 2% acrylamide solution before polymerisation prevented the development of any fluorescence over a period of at least 72 h. The rate of polymerisation and the persulphate content were both unaffected.

When the commonly-used alternative method of polymerising acrylamide with riboflavin was employed, the situation was complicated by the native fluorescence of the riboflavin (λ_f = 520 nm). However, when a 2% acrylamide solution containing 2.65 m*M* TEMED and 0.006 m*M* riboflavin was polymerised using 356 nm radiation the characteristic gel fluorescence still developed, though its rate of increase and the rate of polymerisation were both less than in persulphate-containing gels. The omission of TEMED resulted in a further slowing of both processes.

It was found that non-fluorescent polyacrylamide gels could be produced by using an initiator system containing equimolar amounts of ammonium persulphate and sodium sulphite. At 2.65 m*M* initiator concentrations polymerisation was slow, but a ten-fold increase in these concentrations produced rapid gel formation. In such gels, persulphate ions survived for much longer periods than usual.

DISCUSSION

The results obtained show that the polyacrylamide gels commonly used in biochemical work normally possess a considerable fluorescence. This fluorescence can be excited both at *ca.* 280 nm and at *ca.* 340 nm, close to the respective excitation wavelengths of native proteins and of proteins labelled with 1-dimethylaminonaphthalene-5-sulphonyl chloride (Dansyl-chloride) or 1-anilinonaphthalene-8-sulphonic acid (ANS). The wavelength of maximum fluorescence (λ_f = 415 nm) differs considerably from those of such molecules (λ_f = *ca.* 340 nm and 460–520 nm, respectively) but the broad fluorescence bands overlap to a noticeable extent. Thus the gel fluorescence will cause an elevated background signal when electrophoretic separations are monitored fluorimetrically, and will also interfere when attempts are made to obtain the fluorescence spectra of proteins immobilised in or on polyacrylamide matrices.

The fluorescence develops most rapidly when the persulphate–TEMED initiating system is used, and more slowly in riboflavin-polymerised gels. In persulphate-polymerised gels, the development of fluorescence can apparently be correlated with the disappearance of persulphate ions. A 2-h electrophoretic pre-run removed the persulphate, but a limited fluorescence developed before this removal was complete. Electrophoretic removal of persulphate has been recommended as a means of avoiding artefacts during the electrophoresis of proteins[15], but since this ion is not detectable in 24-h-old gels, and may indeed disappear within 8 h or less, it is doubtful if it is responsible for such effects. KING has noted that electrophoretic artefacts may not be wholly avoided by an electrophoretic pre-run[16].

Non-fluorescent gels can be produced by adding dithiothreitol to the monomer mixture, or by replacing TEMED by sulphite. The former method does not affect the polymerisation process, but in the latter much higher initiator concentrations are necessary if gel formation is to proceed at a practicable rate. The reasons for the abolition of the fluorescence are uncertain: it is known that mercaptans are anti-oxidants and good transfer agents for radical reactions[17], and sulphite has been shown to prevent the participation of hydroxyl radicals in polymerisation reactions[18]. Both procedures may be of limited value in protein studies. Quite low concentrations of dithiothreitol are used to reduce the disulphide bonds of proteins[19], so this method of producing non-fluorescent polyacrylamide may only be of use in sodium dodecyl sulphate-gel electrophoresis when reducing agents are normally present in any case[20]. Sulphite has been used to cleave disulphide bonds by oxidative sulphitolysis, although transition metal ions may also be present in such reactions[21].

The results presented here, together with additional evidence, have enabled us to draw some conclusions concerning the nature of the fluorescent species in polyacrylamide gels: these will be the subject of a separate publication[22].

ACKNOWLEDGEMENTS

D. H. G. is grateful to Mount Allison University, New Brunswick, Canada, for granting sabbatical leave and for financial assistance from the Marjorie Young Bell Faculty Fund, to Loughborough University of Technology for providing research facilities, and for a Commonwealth Universities Travel Fellowship.

J. N. M. is grateful to Mr. L. FITZER of Baird-Atomic Ltd., for the loan of spectrofluorimeter accessories.

REFERENCES

1 H. R. MAURER, *Disc Electrophoresis*, De Gruyter, Berlin, 1971.
2 M. NANDI AND G. P. LEWIS, *J. Clin. Pathol.*, 23 (1970) 727.
3 H. HAGLUND, *Methods Biochem. Anal.*, 19 (1971) 1.
4 U. E. LOENING, *Biochem. J.*, 102 (1967) 251.
5 J. E. WATKIN AND R. A. MILLER, *Anal. Biochem.*, 34 (1970) 424.
6 D. M. EASTON, H. LIPNER, J. HINES AND R. C. LEIF, *Anal. Biochem.*, 39 (1971) 478.
7 D. N. TALBOT AND D. A. YPHANTIS, *Anal. Biochem.*, 44 (1971) 246.
8 I. P. GRIFFITH, *Anal. Biochem.*, 46 (1972) 402.
9 S. T. NERENBERG, C. GANGER AND L. DEMARCO, *Anal. Biochem.*, 43 (1971) 564.
10 P. BERNFIELD AND J. WAN, *Science*, 142 (1963) 678.
11 P. CUATRECASAS, *J. Biol. Chem.*, 245 (1970) 3059.
12 D. GABEL, I. Z. STEINBERG AND E. KATCHALSKI, *Biochemistry*, 10 (1971) 4661.
13 A. BENNICK, *Anal. Biochem.*, 26 (1968) 453.
14 C. A. PARKER, *Photoluminescence of Solutions*, Elsevier, Amsterdam, 1968.
15 W. M. MITCHELL, *Biochim. Biophys. Acta*, 147 (1967) 171.
16 E. E. KING, *J. Chromatogr.*, 53 (1970) 559.
17 C. WALLING, *J. Amer. Chem. Soc.*, 70 (1948) 2561.
18 P. GHOSH, S. C. CHADHA AND S. R. PALIT, *J. Polym. Sci. A*, 2 (1964) 4441.
19 R. M. E. PARKHOUSE, B. A. ASKONAS AND R. R. DOURMASHKIN, *Immunology*, 18 (1970) 575.
20 A. K. DUNKER AND R. R. RUECKERT, *J. Biol. Chem.*, 244 (1969) 5074.
21 I. M. KOLTHOFF AND W. STRICKS, *Anal. Chem.*, 23 (1951) 763.
22 D. H. GRANT, in preparation.