The chemical composition of the indigoid dyes derived from the hypobranchial glandular secretions of *Murex* molluscs

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The composition of the indigoid dyes and their precursors derived from the hypobranchial glandular secretions of *Murex trunculus* and *Murex brandaris* have been determined by high-resolution mass spectrometry. *M trunculus* secretions yielded three dyes: 6,6′-dibromoindigotin (DBI; CI 75800), indigotin (CI Natural Blue 1; CI 75780) and 6-bromoindigotin (MBI), the latter reported here for the first time. Indirubin (CI Natural Blue 1; CI 75790) constituted a relatively small amount of the final dye product. No statistically significant sexual differentiation in the final dye colorations was observed when the secretions were fully developed in light. When developed in the dark, male glandular secretions were more purple than those of pseudohermaphroditic females, implying that male glands contain less of the unsubstituted indoxyl precursors, which oxidatively couple in air to form blue indigotin and MBI. *M brandaris* yielded solely DBI and its precursor the 6-bromo-2-methylthioindoxyl (tyrindoxyl); no methylsulphone precursor was observed, although reported by earlier investigators. Moreover, exposure to light was found to be critical for the formation of the greenish-coloured intermediate 2,2′-bis-substituted 6,6′-dibromo-2,2′-di-indoxyl, not only for the latter's decomposition to DBI.

INTRODUCTION

In antiquity royal purple (DBI, structure III in Figure 1) and related indigoid dyes were derived from the hypobranchial glandular secretions of certain marine molluscs. While peoples from around the globe, including the ancient Chinese and Peruvians, discovered dyeing processes that employed the molluscan secretions [1–3], the Phoenicians of the first millennium BC are most intimately associated with purple in history and legend [4,5]. They traded textiles, which had been dyed in their homeland of Lebanon, throughout the Mediterranean, and further promoted the industry by setting up dye factories in their colonies, most notably along the coasts of Tunisia, Malta, Sicily, and Spain [6]. The socio-political and religious significance of the dye in the ancient Mediterranean world, as exemplified by kings and priests arrogating to themselves the wearing of purple-dyed attires, can be explained by the fact that the molluscan glandular secretions are the only known natural source, and that as many as 10 000 animals are required to produce 1 g of the dye from *Murex brandaris* [7,8].

The precursors in the molluscan glandular secretions form indigoid dyes through established sequences of reactions (Figure 1), which appear to be quite comparable for Old and New World molluscan species alike [2,3]. The dye precursors (structures I and V in Figure 1) have been identified as the sulphate esters of indoxyl (I, X = H), 6-bromoindoxyl (I, X = Br) and derivatives of these indoxyls which are substituted in the 2-position with methylthio (V, X = H or Br, R = SCH_3) or methylsulphonyl (V, X = H or Br, R = SO_2CH_3) groups [9,10]. The hydrolysis of these esters to their respective indoxyls (steps 1 and 1a in Figure 1) is catalysed by the enzyme purpurase. Precursors that are unsubstituted in the 2-position then form indigoids directly by oxidative coupling in air (step 2). If substituted in the 2-position, the 2,2′-bis-substituted 2,2′-di-indoxyl is formed (compound VIII in Figure 1), which is green in colour [11]. A photochemical reaction (step 2c) then converts this intermediate to the indigoid dye.

EXPERIMENTAL

Collection of *Murex* molluscs

The two Mediterranean species of most importance to the Phoenicians, *Murex trunculus* and *M brandaris*, were collected between 14 and 28 May 1989 at the marine biological station, Le Laboratoire Arago, which is located in the town of Banyuls-sur-Mer on the Gulf of Lion of the Mediterranean Sea, close to the French–Spanish border. Several hundred live molluscs were collected during morning scuba diving expeditions in small bays along the rocky coast between Banyuls and Porte Vendres, about 10 km to the north-east. *M brandaris* were recovered at depths of 45–50 m, and *M trunculus* at a depth of about 5 m. Molluscs were kept in tanks of circulating sea water for no more than two days before their hypobranchial glands were extracted. The *M brandaris* specimen group was supplemented by 30 live specimens of unknown provenance, which were purchased at the Euromarché supermarket in nearby Perpignan. These molluscs were kept in a container with sea water, until they could be transferred to the tanks at the marine station. They were similar in shell size and internal anatomy to the Banyuls–Porte Vendres specimens, but

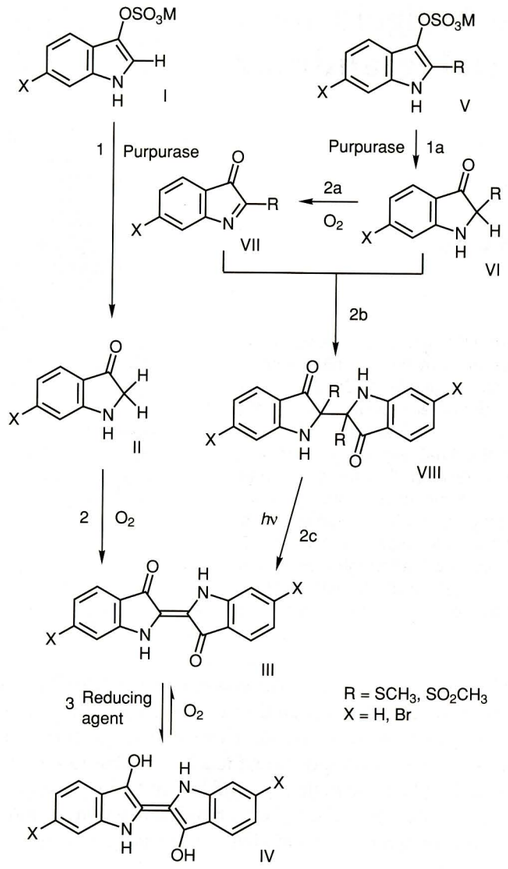

Figure 1 Chemical pathways for the production of indigoid dyes from *Murex* hypobranchial glandular secretions

were free of fouling on the exterior shells and were more easily broken (see below).

Except for the purchased specimens of *M brandaris*, the molluscs came from well defined habitats. As *M trunculus* had just entered its mating season, large groups of males were observed to gather separately on the bottom of the bays [12], later to be joined by groups of females. Consequently groups comprising exclusively males or females could easily be collected. Individuals in the same groups had very similar outer shell colorations (most often orangish or purplish), due to fouling by algae, barnacles, and sponges [12]. Very probably animals in the same group had developed in similar habitats and had comparable diets. On the other hand, only individual *M brandaris* were observed and collected during dives, possibly because they had not yet begun to mate; an outer shell of blackish coloration attested to a similar habitat and development.

The sizes of the *M trunculus* shells, which correspond to the ages of the molluscs (larger shells belonging to older animals and vice versa) could be roughly classified as small (length of approx. 4 cm and maximum diameter of approx. 3 cm), medium (approx. 6 × 4 cm), and large (approx. 8×5 cm). Medium-sized animals constituted the vast majority of the collection. No size difference by sex was noted. *M brandaris* shells were generally about 8–9 cm in length, with a maximum diameter of 4–5 cm (excluding the spines).

Extraction of the hypobranchial gland secretions and sexing

After the molluscs were transferred to 100 × 30 × 40 cm aquaria with circulating sea water in a laboratory of the marine station, the hypobranchial gland of each specimen was extracted by breaking with a hammer the upper part of the shell, several whorls above and to the right of the aperture [13,14]. The gland was clearly visible as a dark-coloured sac attached to the mantle and protruding into the mantle cavity [12,15]. It was surrounded by varying amounts of fatty tissue. The gland was dissected from the mantle with surgical scissors and placed in the middle of a 5 or 10 cm diameter circular filter paper and its liquid contents squeezed onto the paper. The latter operation was carried out either in the laboratory in subdued light or in a photographic darkroom under total darkness.

The reproductive organs of the mollusc, which are located along the outside of the visceral mass, were usually visible after the shell had been broken; sometimes it was necessary to pull the visceral mass out of the upper whorls of the shell to expose the gonads fully and to sample the tissue with a pair of tweezers. In general, testes and ovaries were sexually differentiated by colour: most often greyish for males and yellowish or pinkish for females, but occasionally exceptional colours (e.g. blackish ovaries in *M brandaris* and light-coloured testes in *M trunculus*) were observed. A small sample of gonadal tissue, placed under a cover glass, was examined at 30 × and 100 × magnification with a Wild Heerbrugg microscope in order to identify sperm or eggs and thereby the sex of the animals.

The presence of a penis, a J-shaped organ at the base of the right eye, is not unequivocal evidence for a male. Female *M trunculus* can develop a penis and sperm ducts [16], a biological process referred to as imposex. Females of related molluscan species (*Ocenebra erinacea* and *Ilyanaas obsoleta*) have also been shown to undergo imposex, due to exposure to tributyltin compounds found in antifouling marine paints [17–21]. Animals can also lose penises by predation or parasitic castration [22]. *Murex* has sometimes been described as hermaphroditic or protandric [23], but the recent studies of imposex suggest that males and females retain their sex throughout life. While females can develop male sexual organs (which are often smaller than those of a similarly sized male), they continue to produce eggs and can function as females, unless imposex has reached an advanced stage in which oviducts or other essential parts of the reproductive system have been deformed. In this paper females with penises will be denoted 'pseudohermaphroditic females' [16,18,19,21], and females lacking penises, which were rare, will be described as 'true females'.

Mass spectrometric procedure

The instrumental details of high-resolution mass spectrometry have been described in an earlier paper [24]. All samples except one were prepared as residues from dimethyl sulphoxide (DMSO) extracts as follows:

(a) Several confetti-sized pieces of dyed filter paper (with a maximum dimension of 0.5 cm) were cut out
(b) These pieces were soaked in water at room temperature for several days to remove extraneous glandular materials
(c) The dyes were extracted with DMSO (in sufficient quantity to cover the pieces of paper) by heating briefly to the boiling point (189°C at 760 mm, 86°C at 20 mm)
(d) The hot solution was transferred to a sample tube, with an outer diameter of 0.15 mm, using a hypodermic syringe with a pre-heated needle, filling about a 4–5 cm length of the tube and carefully avoiding the formation of bubbles
(e) Finally the solvent was removed by placing the tube in a vacuum chamber and slowly heating the chamber from room temperature to about 60°C.

The one sample that was not extracted with DMSO, i.e. secretions from an *M brandaris* which were examined for their precursor content, was introduced into the mass spectrometer as filter paper scrapings. Experiments [25] have shown that additional MBI can form from DBI by debromination in the spectrometer in the presence of various materials, including paper. No erroneous conclusions were drawn from the *M brandaris* spectrum, however, since only precursors were of interest.

Methylphenylsulphone was synthesised from commercial methylphenyl sulphide according to Price and Hydock [26].

RESULTS AND DISCUSSION

Murex trunculus dyes

The precursors in the hypobranchial glandular secretions of *M trunculus* are indoxyls and 6-bromoindoxyls, some of which are substituted in the 2-position. Consequently this animal produces both DBI and indigotin (CI Natural Blue 1; CI 75780). Somewhat surprisingly, the product of cross-reaction, 6-bromoindigotin, had never been unequivocably identified. Bouchillaux and Roche isolated pure indigotin from *M trunculus* secretions, while their bromo-substituted indigoid was obviously impure, as shown by a lower than theoretical bromine content for DBI [27]. An indirubin (CI Natural Blue 1; CI 75790) impurity, which was proposed to explain this discrepancy, was never confirmed, although its greater solubility should make it readily separable. Friedländer identified DBI in *M trunculus* secretions, but was unable to identify a blue-coloured component, apparently because of separation difficulties [28,29]. The chromatographic procedures used did not address the issue because the precursors were separated from one another and thus did not cross-react [2,9,10].

According to our mass spectrometric results, *M trunculus* secretions (Figure 2) do indeed contain a significant amount of MBI. The spectrum shows the MBI doublet *M/Z* (mass/charge ratio) of 340 and 342, in addition to the DBI triplet (*M/Z* 418, 420 and 422) and the indigotin band (*M/Z* 262). The multiplets represent the different bromine isotope combinations. The absence of significant MBI bands in the spectra of *M brandaris* secretions, which were processed and analysed by identical procedures, and in a mixture of synthetic indigotin and DBI is convincing evidence that its presence in the *M trunculus* secretions is not a result of processing or analysis [25]. The colour of pure MBI has not been described in the literature. However, it can be inferred from the product of partial debromination of DBI to MBI, i.e. a blue colour very similar to that of indigotin [24].

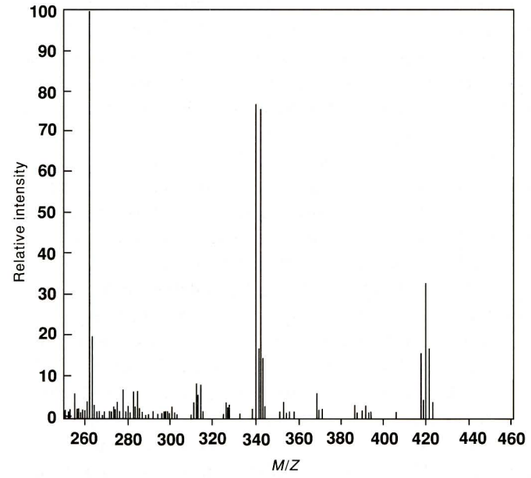

Figure 2 Mass spectrum of *Murex trunculus* glandular secretions, after full exposure to sunlight, recovered from DMSO solution

Indirubins: a minor component of *Murex trunculus* secretions

The presence of indirubins, isomers of the indigoids, in the *M trunculus* dye mixture was also considered. Indirubins cannot be distinguished by mass spectrometry alone but, in conjunction with u.v.–visible light spectrometry, their relative concentration can be assessed as follows. Indirubins are more soluble than their corresponding indigoids [7,8,30,31], and both have similar extinction coefficients at 568 and 596 nm respectively [9,10]. A dye smear from *M trunculus*, containing DBI, MBI, and indigotin (as based on mass spectrometry), was extracted with boiling chloroform. The extract, which was bluish in colour, gave a u.v.–visible spectrum that

indicated the presence of both indirubins and indigoid dyes in about equal amounts. Because of indirubin's greater solubility, however, similar concentrations of indirubin and indigoids in the molluscan secretions should have been reflected in significantly stronger absorption bands for indirubins. A contrary result implies that indirubins are only minor components in *M trunculus* secretions.

Sexual differentiation of final dye products of *Murex trunculus*

Earlier investigators have suggested that the relative proportions of blue and purple dyes, and hence the coloration of fully developed *M trunculus* secretions, are related to sex. The males produce more blue dye and the females more purple dye according to one researcher [32] or vice versa according to another [13]. Recently Elsner [23] reported that a sex dependency of final dye coloration is unlikely.

Our results also show no statistical correlation between final dye coloration and sex. It was noted that the blue coloration developed first on the periphery of the glandular smear. After full development of dye coloration in light (requiring 50 min in subdued morning Mediterranean light, and 10 min in direct sunlight), the secretions of 13 male *M trunculus* were predominantly purple, whereas 12 other male animals yielded mixtures of purple and various amounts of blue. In three of the latter instances the final coloration was predominantly blue. Three pseudohermaphroditic females gave primarily purple, and five showed a greater admixture of blue (although none were predominantly blue). One true female gave a mixture of purple and blue.

The final colorations of samples collected and stored in the dark are probably differentiated by sex. Pseudohermaphroditic female *M trunculus* secretions were predominantly blue (four out of seven animals; three specimens gave a mixture of purple and blue), whereas the male secretions were predominantly purple (17 out of 18 animals; one specimen gave a mixture of purple and blue). These results are statistically significant for the male molluscs, but more female samples need to be studied. Compared with those of the pseudohermaphroditic females, it may be proposed that male glands contain relatively less of the completely unsubstituted indoxyl precursor, which oxidatively reacts in air to form blue indigotin and MBI.

Murex brandaris precursor and dye

The primary precursor in the hypobranchial glandular secretions of *M brandaris* is a 2-substituted 6-bromoindoxyl (structure V in Figure 1). By a series of reactions (1a and 2a–2c in Figure 1) in the presence of light and air, this precursor is converted to DBI [7,8]. The mass spectrum of the *M brandaris* derived dye (Figure 3) has the DBI triplet (*M/Z* 418, 420 and 422). A second mass spectrum (Figure 4), emphasising the lower molecular mass regions, has bands at *M/Z* 257 and 259, which correspond either to the

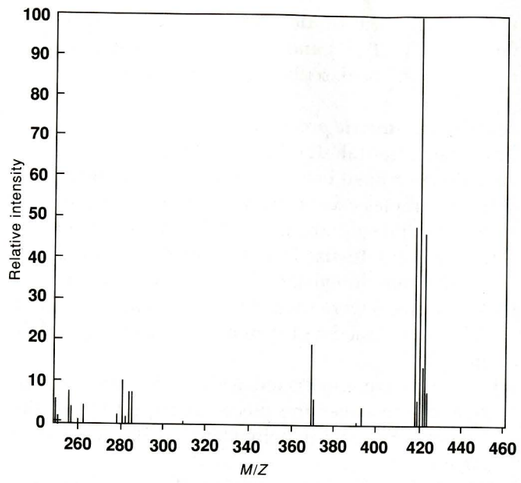

Figure 3 Mass spectrum of *Murex brandaris* glandular secretions, after full exposure to sunlight, recovered from DMSO solution

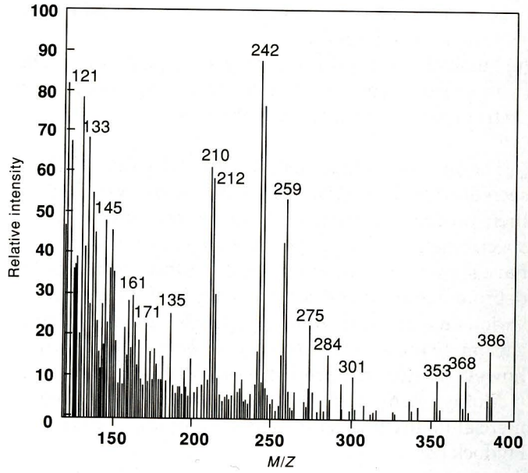

Figure 4 Mass spectrum of *Murex brandaris* glandular secretions, which were incompletely developed and analysed as filter paper scrapings

2-thiomethyl-6-bromoindoxyl (tyrindoxyl) precursor or to a fragment derived from the 2-methylsulphonyl derivative in which the oxygen has been lost. The sulphone was the primary precursor identified by Fouquet and Bielig [9,10]. Yet the fact that the methylphenylsulphone does give a sulphone molecular ion with an *M/Z* of 156 (Figure 5), but that no sulphone precursor appeared in the *M brandaris* spectrum (Figure 4), supports the view that the precursor tyrindoxyl is present in *M brandaris* glandular secretions. Other bands in the spectrum, *M/Z* 242 and 244 and *M/Z* 210 and 212, correspond to the loss of the methyl and thiomethyl groups from tyrindoxyl respectively during the mass spectrometric analysis. Since the mass spectrum in Figure 4 was derived from paper scrapings, it contains bands resulting from glandular

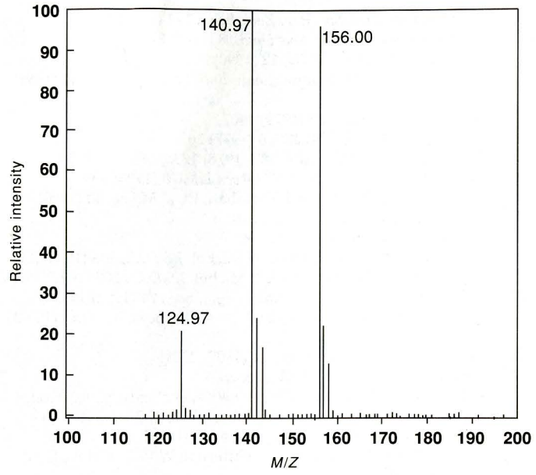

Figure 5 Mass spectrum of methylphenylsulphone

constituents other than the precursor. Cholesterol, for example, is represented by bands at *M/Z* 386 and 368, the latter being its dehydration product.

The precursor molecules in the *M brandaris* secretions convert to the greenish-coloured 2,2′-bis-substituted 6,6′-dibromo-2,2′-di-indoxyl in about 1 min in direct sunlight (Philadelphia, July 1990, 10 a.m.). Conversion to DBI required another 4 min. The unconverted tyrindoxyl in the final product could be due to the briefness of sunlight exposure and/or a screening effect by molluscan residues (in particular, a heavy mucoid substance produced by the hypobranchial gland) or by the dye that has already formed.

We have previously proposed that *M brandaris* secretions might have been processed by ancient dyers in subdued light. This might have been achieved by covering the container with a lid, or by screening out light by the accumulation of molluscan residues on the surface or the formation of a dye bloom [4]. The textile could then have been dipped in the dyebath containing precursors, and a fast dye developed by exposure to sunlight. This hypothesis has greater credence now that it has been confirmed that precursors are still present after exposure of the secretions to light.

Photochemical reactions in *Murex brandaris* secretions

Previous investigators were aware of the fact that the whole series of colour transformations in the secretions of *M brandaris* is initiated by light. In particular, the photochemical formation of indigoid dyes from a 2,2′-bis-substituted 2,2′-di-indoxyl has been emphasised [11]. In our studies the secretions, which had been smeared on filter paper in the dark, wrapped in aluminum foil, and kept in a light-tight black photographic box, were still colourless after storage for about a year. On exposure to light, the greenish-coloured 2,2′-bis-substituted 6,6′-dibromo-2,2′-di-indoxyl formed and gradually developed throughout the smear. This result implies that the photosensitive intermediate, which decomposes to DBI (step 2c in Figure 1), is itself a product of a photochemical reaction, most likely the oxidation of an indoxyl to an indoleninone (structure VII). According to Baker and Dube [33,34], the indoleninone forms from indoxyl during the chromatography of a solution of the latter on alumina. These researchers make no mention of excluding light during the procedure.

CONCLUSIONS

The chemical composition of the dyes derived from the hypobranchial glandular secretions of two historically important Mediterranean molluscan species, *Murex trunculus* and *Murex brandaris*, have been presented, to the extent that they can be determined by the mass spectrometric technique employed. Although considerable chemical and biological information is already available for these species, refinements and even corrections in the understanding of the chemical species and pathways leading to the fully developed dyes have emerged from our investigation. Admittedly only a relatively small sample of animals from a small area of the world, collected during only two weeks of the year, has been studied. Yet the results achieved thus far, using a precise instrumental technique and explicit collection procedures for the molluscs (by season of the year, habitat, diet and sex), define a set of parameters and hypotheses that can be further tested by sampling and analysing secretions from animals of the same species under different conditions. Precursor composition and conversion to the dye (including mechanisms, wavelength sensitivities of photochemical reactions, etc.) should also be investigated in greater detail.

Similarly rigorous analytical and collection procedures can now be applied to the investigation of other Old World species (the Mediterranean *Purpura haemastoma*, which was not available at Banyuls in May 1990; the western Pacific *Rapena bezoar*; etc.) and New World species (e.g., *Purpura patula*, which is found in both the Caribbean Sea and the Pacific Ocean), which were also exploited by ancient and modern peoples.

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The extraction of the *Murex* glandular secretions at Le Laboratoire Arago in Banyuls-sur-Mer, France, which was carried out by one of the authors (PEM), was underwritten by a research grant from the American Philosophical Society. The marine station provided a ship and scuba divers, under the supervision of M Jean Mabit, for gathering molluscs in the Mediterranean Sea, a well equipped laboratory for extracting the hypobranchial glands and sexing the animals, and a dark room for developing glandular secretions in total darkness. The staff at Banyuls, in particular Dr Jean Michel Amouroux and then-director Dr Jacques Soyer, were most helpful in arranging an expeditious and comfortable stay, as well as

in sharing their biological expertise. R Robertson advised us on malacological matters, including recent research on imposex. We are most appreciative to A Bolinski for his help in the mass spectrometric analyses, and to E Matthews for the u.v.–visible spectra.

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Flash photolysis of azo dyes in aqueous solutions of biacetyl

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Irradiation by microsecond flash of aqueous aerobic solutions of biacetyl (2,3-butanedione) and a number of different azo dyes resulted in oxidative fading of the dyes. The fading kinetics of all tautomeric dyes could only be described properly by assuming three oxidative species. The first one is believed to be singlet oxygen. Due to its short lifetime in aqueous systems, the dye fraction consumed by singlet oxygen was confined to a maximum of about 30%. Second order reaction rate constants for the second oxidator were in the range of $1.4–4.7 \times 10^8$ l/(mol s). A radical mechanism is proposed, in which the adduct of oxygen and triplet biacetyl is the second oxidising species. A third oxidator formed from the second was necessary to describe adequately the fading curves of four azo dyes with slow fading rates. This oxidator could be an acylperoxy-radical. The non-tautomeric dye CI Acid Red 37 was recycled partially after very fast fading during the first 0.5 ms.

INTRODUCTION

Flash photolysis is a powerful technique for obtaining kinetic information about photochemical processes [1]. For example, it has been applied to the study of intermediates formed during photofading of azo dyes in solution [2] and in a polymer matrix [3]. In a previous publication we described the biacetyl-sensitised photo-oxidation of some tautomeric azo dyes in aerobic aqueous solutions as a model for the fading of azo dyes on non-proteinic substrates in the presence of oxygen [4]. This oxidative fading was very efficient, with quantum yields ranging up to 0.44 mol/einstein. Fading was inhibited by phenol and other hydrogen donors that quench the intermediate triplet state of biacetyl [5]. Like other authors [6–8], we assumed an oxidation mechanism with singlet oxygen as the oxidative species formed by energy transfer from triplet biacetyl to ground-state molecular oxygen. We now report results from flash photolysis experiments conducted on the system in an attempt to elucidate further the mechanism of the photo-oxidation.

EXPERIMENTAL

Materials

The origin and the analysis of all the materials used have been described previously [4,5]. The structures of the dyes investigated are given in Figure 1.

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