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A Study of Ageing of Gomori Aldehyde Fuschin Stain

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ABSTRACT

The photochemical stability of Gomori Aldehyde Fuschin stain samples of different ages was investigated by thin layer, column and high performance liquid chromatographic techniques and the component dyes isolated. The dyes obtained were individually and sequentially irradiated with simulated sunlight and the photodegradation products isolated and characterised by comparison of infra red spectra with those of authentic samples. Pararosaniline was found to undergo progressive methylation leading eventually to the formation of Crystal Violet which was subsequently ethylated to Methyl Green. Methyl Green was found to undergo disproportionation to Malachite Green. The histochemical consequences of these reactions are discussed. © *1997 Elsevier Science Ltd*

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INTRODUCTION

Gomori Aldehyde Fuschin stain was introduced in 1950 as an elastic tissue stain [1]. The solution comprised a mixture of pararosaniline (C.I. 42500, Basic Red 9), paraldehyde, hydrochloric acid and ethanol which liberates acetaldehyde *in situ.* [1], resulting in condensation of the acetaldehyde with the primary aromatic groups of the dye. The binding of the stain to elastin is regarded as being non ionic. In addition to elastic fibres and laminae,

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aldehyde fuschin stains the sites of half sulphate esters and sulphonic acid or aldehyde groups are artificially introduced into the tissue sample [2]. In the case of sections treated with an oxidant prior to staining, they will show coloured carbohydrate moieties due to oxidation of alcohols to aldehydes; areas of high concentration of disulphide groups will also be stained due to cystine being oxidised to cysteic acid which reacts with the stain. This effect is demonstrated in the pancreatic islets containing insulin, which is rich in cystine. The stain solutions need to be aged for 2–7 days at room temperature before use [1]. It has been suggested that the staining properties on elastic tissue deteriorated on prolonged ageing. The objective of the present work is to investigate the effect of light on Gomori Aldehyde Fuschin stain by an established method [3].

MATERIALS AND METHODS

Thin layer chromatographic separation of dyes

Pre-prepared Silica 60 t.l.c plates (20 cm × 20 cm) were spotted with 2 μl aliquots of Gomori Aldehyde Fuschin stain (Pararosaniline 1.0 g, paraldehyde, 2 ml; concentrated hydrochloric acid, 1 ml; absolute ethanol, 60 ml; purified water (Millipore) 40 ml) which had been irradiated by a previously described method [3], and samples taken at time intervals for a period of up to ten weeks. Chromatograms were eluted with chloroform: methanol (80:20). The dye spots were recorded as were the Rf values (Table 1). The individual dye spots were scraped off the chromatographic plates, the adsorbant washed with ethanol, the washings combined, and evaporated under reduced pressure to give dye samples. The infra red (i.r.) spectra of the dyes isolated were compared with those from authentic samples of dye.

TABLE 1
Thin Layer Chromatography of Dyes Isolated From Irradiated Gomori Aldehyde Fuschin Stain

Dyes isolated	*Rf. values*	*Eluting solvent mixture*
Pararosaniline	0.30	Water:methanol 25:75
Rosaniline	0.05	Water: methanol 25:75
Crystal Violet	0.38	Water: methanol 50:50[a,b]
Methyl Violet 2B	0.46	Water: methanol 50:50[b]
Methyl Violet 6B	0.23	Water: methanol 25:75[a,b]
Methyl Green	0.14	Water: methanol 50:50[b]
Malachite Green	0.93	Water: methanol 50:50[b]

[a]Ammonia (S.G. 0.90) 25 μl/2 ml.
[b]Concentrated Hydrochloric Acid 25 μl/2 ml.

Photochemical decomposition of Gomori Aldehyde Fuschin stain

Samples of Gomori Aldehyde Fuschin stain were irradiated with simulated sunlight using an established method [3], and samples taken at time zero and initially at hourly intervals and then at daily and weekly intervals. The ultra violet (u.v.)/visible spectrum and hplc assay were undertaken on each sample.

Preparation and photodegradation of reference samples of dyes

Commercial samples of those dyes isolated by t.l.c, after irradiation of Gomori Aldehyde Fuschin stain with simulated sunlight, were purified. A column (60 cm long, internal diameter 4 cm), fitted with a glass sinter, was three-quarters filled with a slurry of Silica 60 (Merck) in the eluting solvent (Table 2). The solvent was run off until the level was just at the top of the adsorbant column and then eluted with the appropriate solvent. A solution of each commercial dye (30 mg in 15 ml) was applied to the column and, after separation and evaporation under reduced pressure, followed by freeze drying of a concentrated aqueous solution, a pure sample of each dye was isolated. The purity of the samples was checked by i.r. spectra and t.l.c. and compared with the i.r. spectra and t.l.c. of authentic samples.

The u.v./visible spectra of the purified dyes (25 mg in 25 ml) in the other components of the Gomori Aldehdye Fuschin stain as a stock solution were run, the absorption maxima found and Beer Lambert plots created from a range of concentrations of 0.001, 0.002, 0.003., 0.004., 0.005 mg ml^{-1}, absorbance measurements being taken at the wavelength of the maximal absorbance in the visible region of the spectrum.

Each of the purified dyes (1 mg ml^{-1}) were dissolved in the solvent system of the Gomori Aldehyde Fuschin stain and irradiated [3], and the u.v./visible

TABLE 2
Physical Data of Purified Dyes

Name of dye	*Chromatographic solvent water: methanol*	*λ max nm.*	*Beer Lambert plot linear regression coefficient*	*C.I. No.*
Crystal Violet	1:1 + Ammonia/HCl	590	0.9998	42555
Ethyl Violet	1:1 + HCl (2 M)	639	0.9999	42600
Malachite Green	1:1 + HCl (2M)	622	0.9998	42590
Methyl Green	1:3 + Ammonia /HCl	592	0.9975	42585
Methyl Violet 2B	1:1 + HCl (2M)	579	0.9996	42535
Methyl Violet 6B	1:3 + Ammonia/HCl	579	0.9975	42536
Pararosaniline	1:3 + Ammonia /HCl	547	0.9960	42500
Rosaniline HCl	1:1 + Ammonia /HCl	547	0.9999	42510

Commercial dyes were supplied by Aldrich Chemical Co. Ltd.

spectrum monitored weekly for 18 weeks, measurements being taken at the absorbance maximum in the visible range of the spectrum.

High performance liquid chromatographic study of irradiated Gomori Aldehyde Fuschin stains

The work was undertaken on Waters 501 h.p.l.c pump, Waters 484 Tuneable absorbance detector, Reverse phase C 18 ODS column, Flow rate 1 cm min^{-1}. Attenuation 16, wavelength 547 nm, Solvent: methanol:water (50:50) with ammonia solution added to give a pH of 10. Stain samples were diluted 1:1 with Millipore water and filtered through a 0.2 μM filter. Samples of the irradiated solutions were chromatographed at time intervals over a period of 18 weeks.

Samples of Gomori Aldehyde Fuschin stain which had been exposed to daylight for periods of time of up to two years were also examined chromatographically.

RESULTS AND DISCUSSION

The initial hplc study of Gomori Aldehyde Fuschin stain samples was carried out using a previously reported method [4] which was unsuccessful. Modification of the technique by using an alkaline aqueous mobile phase gave a better separation of the component dyes, but the results were insufficiently consistent to be acceptable. Consequently, the mixture of dyes in the stain samples were separated by t.l.c and column chromatography. The samples of purified dyes isolated from the Gomori Aldehyde Fuschin stain were characterised by comparison of i.r. spectra with those of authentic material. In order to investigate the photochemical degradative pathway of Gomori Aldehyde Fuschin stain, commercial samples of the individual dyes isolated by chromatography were purified by column chromatography and individually and sequentially irradiated by simulated sunlight [3] in the solvent system of the stain, and the products isolated by column chromatography and characterised in the manner described above (Table 3). The postulated photochemical reaction pathway is shown in Fig. 1.

The main reaction was found to be progressive methylation of the basic centres of the dyes, and in one case ring methylation adjacent to a basic centre; Pararosaniline is methylated to give a mixture of Rosaniline and Methyl Violet 2B. The latter dye undergoes progressive methylation to give Methyl Violet 6B which in turn yields Crystal Violet. It was noted that irradiation of pure Crystal Violet under the conditions of test gave some Methyl Violet 6B and Methyl Green. In the formation of Methyl Green, ethylation

occurred and it is thought that the ethyl group was probably derived from the ethanol used as a solvent in the Gomori Aldehyde Fuschin stain. Methyl Green subsequently undergoes disproportionation, involving loss of a dimethylamino group, on continued irradiation with simulated sunlight to give Malachite Green. The methylation reactions could be expected from irradiation of a system containing paraformaldehyde under acidic conditions,

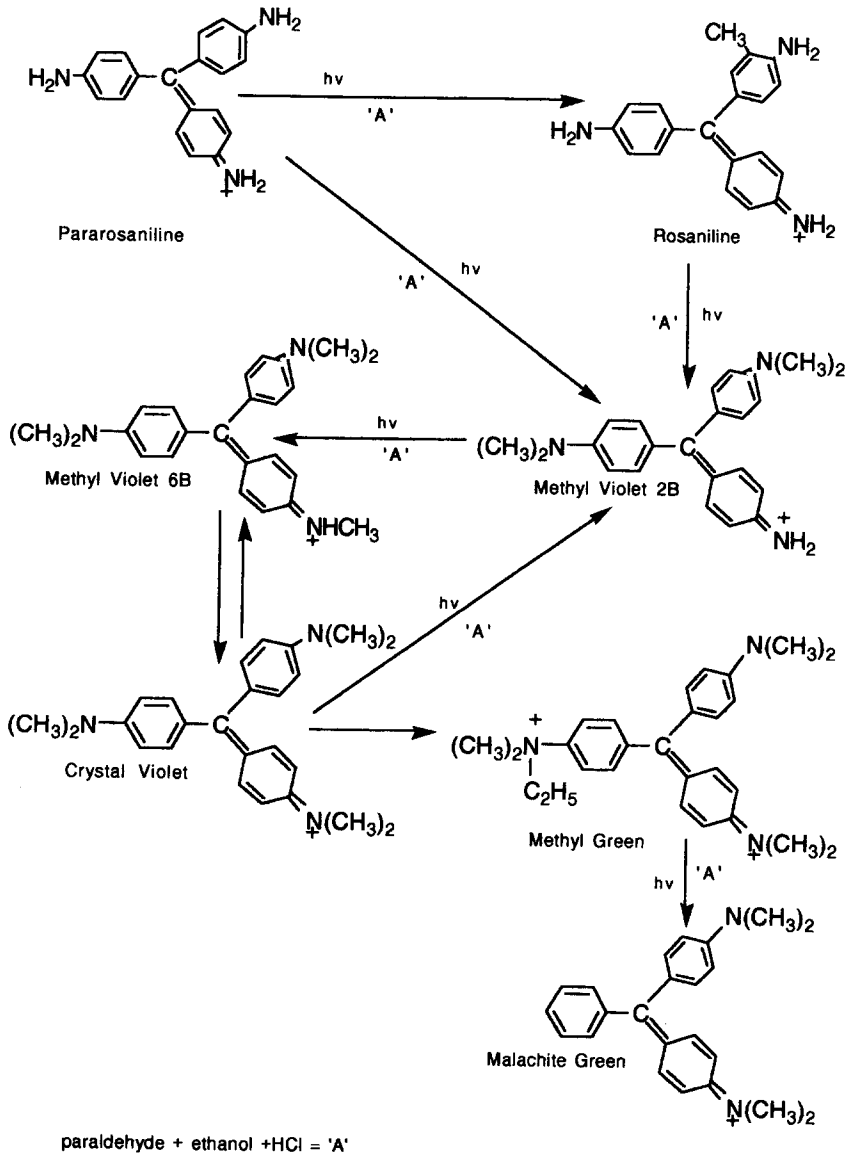

Fig. 1. Photodegradation pathway of Gomori Aldehyde Fuschin stain.

TABLE 3
Sequential Irradiation of Dyes Isolated From Gomori Aldehyde Fuschin Stain

Dye irradiated	*Major products isolated*
Pararosaniline	Rosaniline + Methyl Violet 2B
Rosaniline	Methyl Violet 2B + Methyl Violet 6B
Methyl Violet 2B	Methyl Violet 6B
Methyl Violet 6B	Crystal Violet
Crystal Violet	Methyl Violet 2B + Methyl Violet 6B + Methyl Green
Methyl Green	Malachite Green

since heating an amine with paraformaldehyde under acid conditions gives progressive methylation of the amine (Clarke–Eschweiler Synthesis) [5]. Photochemical disproportionation in triarylmethane dyes has been previously reported [6]. as have the photochemical oxidation, reduction and dealkylation of triphenylmethane dyes [7].

Previous studies [8] of the effect of ageing Gomori Aldehyde Fuschin stain have shown that the stain produces a strongly staining purple material, which is shown to be a Schiff's base derived from Pararosaniline. The formation of this compound is the first stage of the Clarke–Eschweiller synthesis leading to methylation of the dye. These authors also reported the presence of ethylated products, as were found in this present work.

The consequences of using old stocks of Gomori Aldehyde Fuschin stain in histochemical applications are that misleading results may be obtained. There are reports of some of the dyes produced by the photochemical degradation of Pararosaniline in Gomori Aldehyde Fuschin stain having other histochemical applications; for example, Methyl Green has been used to bind to AT-rich regions of DNA [9, 10]. Crystal Violet has application in staining amyloid cells and nuclei red violet, and is a component of Gram's stain for bacteria [11] and many other micro-organisms [12].

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