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Detection and determination of the hydrazo and azo photoproducts of 4-aminobenzoic acid by high-performance liquid chromatography

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Abstract

The photochemistry of 4-aminobenzoic acid has been investigated using two validated reversed-phase HPLC methods. Up to nine photoproducts have been detected, with chromatographic evidence for the formation of 4,4′-azobenzenedicarboxylic acid and 4,4′-hydrazobenzenedicarboxylic acid. The synthesis and analytical characterization of 4,4′-hydrazobenzenedicarboxylic acid is reported.

Keywords: 4-Aminobenzoic acid; Azobenzenedicarboxylic acid; Azoxybezenedicarboxylic acid; Hydrazobenzenedicarboxylic acid; Photochemistry; Reversed-phase HPLC

1. Introduction

With the discovery of the nature of sunburn in 1922, the use of 4-aminobenzoic acid as a sunscreen developed [1]. Reports on the photochemical reactivity of 4-aminobenzoic acid appeared in 1935 [2] with the formation of 4,4′-azobenzenedicarboxylic acid and unidentified photoproduct(s) in 1942 [3], although the use of 4-aminobenzoic acid as a sunscreen was later reported to be effective and without toxicity [4,5].

Subsequently, 4-aminobenzoic acid and its esters were reported to cause allergic contact dermatitis [6,7]. A more recenty study reported the formation of *cis*- and *trans*-4,4′azobenzenedicarboxylic acid [8] and proposed a mechanism for their formation. An attempt to repeat this work suggested that the formation of this photoproduct was due to a minor pathway [9] and identified several other photoproducts. 4-Aminobenzoic acid is also a degradation product arising out of the hydrolysis or photochemical cleavage of procainamide, procaine and metoclopramide. In the course of studying the photochemical degradation of procainamide, it became apparent that the photochemical degradation of 4-aminobenzoic

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acid was central to the photochemical degradation profile of procainamide. Two previous studies used HPLC methods to detect 4,4′-azobenzenedicarboxylic acid [8,9] but no validation data for these methods were reported.

The study reported in this paper attempts to confirm the mechanism for the formation of 4,4′-azobenzenedicarboxylic acid in the photolysis of 4-aminobenzoic acid proposed by Gasparro [8]. This study involved the synthesis of the azo and azoxy diacids and the development of validated HPLC methods of analysis.

An attempt is made to correlate the synthesized photoproducts to compounds detected in photolysed solutions of 4-aminobenzoic acid. Additionally, in view of the reported formation of anilino radicals [10,11] and possible dimerization, an attempt to detect the presence of 4,4′-hydrazobenzenedicarboxylic acid was logical.

2. Experimental

2.1. Materials and methods

2.1.1. Reagents

All compounds of AnalaR grade (Merck), except where stated otherwise: dimethylformamide, diethyl ether, formamide, hydrochloric acid, methanol (HPLC grade, Labscan), 4-nitrobenzoic acid (GPR grade, Merck), phosphoric acid, potassium dihydrogenorthophosphate (Hipersolve grade, Merck), potassium hydroxide, sodium hydroxide, tetramethylammonium hydroxide (TMA) (Hipersolve grade, Merck) and zinc powder. Sterile distilled water was used to make up all aqueous reagents (Parkfields RSSU). 4-Aminobenzoic acid (Sigma Chemical) was assayed on receipt using the method in the British Pharmacopoeia 1988, without being dried and weighings adjusted accordingly. The pH of all buffers was adjusted using a Pye Unicam Model 204 pH meter and filtered through a 0.45 μm nylon filter (Gelman Nyflo) before use.

2.1.2. HPLC instrumentation

The Pye Unicam chromatography system consists of a Model 4011 dual-piston, low-pressure pump and Model 4020 gradient controller, Model 4040 variable-wavelength UV detector, range 200–450 nm, and Model 4810 computing integrator. A Knaur Model 6000 column oven with Model 6001 controller (20–100°C) was fitted to the system. The injector was a Rheodyne Model 7125 valve with a 20 μl loop. The HPLC column was LiChrosorb C_{18} 125-4 (10 μm), 12.5 cm, with a guard column.

2.1.3. HPLC mobile phases

Solvent A was potassium dihydrogenorthophosphate (0.5 M) with TMA (pH 6.0, 0.002 M), pH adjusted using phosphoric acid and solvent B was methanol. for method 1 A:B = 92:8 (v/v) and for method 2 A:B = 95:5 (v/v). The mobile phases were degassed under helium, maintained at 5 psi.

2.1.4. Solutions for HPLC

Initial stock solutions containing 1 mg ml^{-1} of 4,4′-azobenzenedicarboxylic acid, 4,4′-azoxybenzenedicarboxylic acid and 4,4′-hydrazobenzenedicarboxylic acid were prepared in 0.1 M sodium hydroxide. This solvent was necessary given the considerable insolubility of these compounds. Subsequent dilutions for HPLC analysis were prepared in the mobile phase to give concentrations between 100 ng ml^{-1} and 2 μg ml^{-1}.

2.1.5. Quantification by HPLC

Method 1 was used for the determination of 4,4′-azobenzenedicarboxylic acid and 4,4′-azoxybenzenedicarboxylic acid at 40°C, using a detection wavelength of 330 nm. Method 2 was used for the determination of 4,4′-hydrazobenzenedicarboxylic acid also at 40°C, using a wavelength of 290 nm. Sample chromatograms are shown in Figs. 1 and 2.

2.1.6. Photolysis experiments

The photoreactor cabinet consisted of a cylindrical arrangement of eight blacklight blue fluorescent tubes, with an output from 303 to 400 nm, maximum at 360 nm [12]. The energy output, determined by ferrioxalate actinometry [13], was 40 Wm^{-2} calculated with reference to a wavelength of 366 nm. Photolysis was carried in Drechsel bottles placed in the centre of the cabi-

net. The test solutions were not deaerated and were constantly stirred with the aid of a magnetic stirring bar. All experiments were carried out at ambient temperature (22–25°C).

2.1.6.1. Photolysis of 4-aminobenzoic acid. A 220 cm^3 volume of 0.1% (w/v) 4-aminobenzoic acid in 0.05 M phosphate buffer (pH 7.0) was pipetted into a 250 cm^3 Drechsel bottle and placed in the photoreactor cabinet. Samples of 0.5 cm^3 were withdrawn at 6 and 12 h and thereafter every 12 h up to 120 h. Neat samples were injected on to the HPLC column and analysed by method 1. Initially repeated analyses at 280, 314 and 330 nm were carried out, subsequently reduced to runs at 280 and 314 nm. Standards were analysed between sample runs. A sample chromatrogram, after 96 h of photolysis, is shown in Fig. 3, with data reported in Table 2.

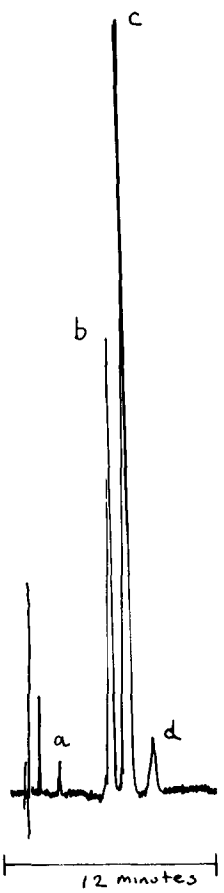

Fig. 1. HPLC trace for method 1. Peaks: a = 4-nitrobenzoic acid; b = 4,4′-azoxybenzenedicaboxylic acid; c = 4,4′-azobenzenedicarboxylic acid; d = azoxy photoproduct.

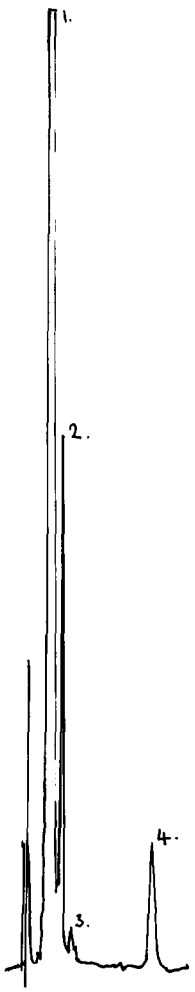

Fig. 2. HPLC trace for the photolysis of 4,4′-hydrazobenzenedicarboxylic acid after 1 h (method 2). Peaks: 1 = 4,4′-hydrazobenzenedicarboxylic acid; 2 and 3 = hydrazo photoproducts (not identified); 4 = 4,4′-azobenzenedicarboxylic acid (Table 1).

2.1.6.2. Photolysis of 4,4′-hydrazobenzenedicarboxylic acid. A 500 cm^3 volume of a 0.01% (w/v) solution of 4,4′-hydrazobenzenedicarobxylic acid in 0.05 M phosphate buffer (pH 7.0) was pipetted in to a 500 cm^3 Drechsel bottle, which was placed in the photoreactor cabinet. Samples of 0.5 cm^3 were withdrawn at 30 min and then hourly up to

12 h. Two analyses were carried out for each time period: (a) neat for the detection of photoproducts and the determination of 4,4′azobenzenedicarboxylic acid and (b) 1 in 100 dilution for the assay of 4,4′-hydrazobenzenedicarboxylic acid.

After 7 h, further dilutions were required for the assay of 4,4′azobenzenedicarboxylic acid. This allowed two determinations of 4,4′-hydrazobenzenedicarboxylic acid and one determination of photoproducts for each time period to be performed using HPLC method 2. A sample chromatogram, after 1 h of photolysis, is shown in Fig. 2, with data reported in Table 1.

2.2. Syntheses

2.2.1. 4,4′-Azobenzenedicarboxylic acid (CAS 568-91-4) and 4,4′-azobenzenedicarboxylic acid (CAS 582-69-4)

These were synthesized by the methods of Reid and Pritchett [14]. The azo compound was recrystallized from dimethylformamide (600 cm^3), but the azoxy compound was not soluble in dimethylformamide [15]. The synthesized materials were washed with diethyl ether and dried to constant weight at 105°C. TLC analysis (0.1% w/v) solutions in sodium hydroxide (0.1 M) did not detect any residual starting materials or side products. Subsequent TLC analysis did show slight degradation on storage.

Analysis: 4,4′-azobenzenedicarboxylic acid, yield 42% of dark orange powder, m.p. > 350°C; 4,4′-azoxybenzenedicarboxylic acid, yield 59% of fine canary yellow powder, m.p. ca. 347°C (decomp.).

2.2.2. 4,4′-Hydrazobenzenedicarboxylic acid (CAS 6237-30-4), mol. wt. 272.0

4-Nitrobenzoic acid (4.177 g, 0.025 mol) was dissolved in 30% (w/v) sodium hydroxide (50 cm^3) and the solution filtered. Zinc powder, 90% (w/w) (3.3392 g, 0.046 mol) was gradually added and the mixture was maintained at 80–85°C for 2 h with constant stirring. Water (200 cm^3) and zinc powder (5.12 g, 0.070 mol) were added and the mixture was refluxed under nitrogen for about 2 h, until colourless. The hot mixture was immediately suction filtered into iced 50% (v/v) hydrochloric acid under nitrogen. The pale pink precipitate was filtered off and recrystallized from methanol. The methanol-crystallized product was then recrystallized from formamide, washed with hot water and dried under vacuum for several days in the dark. As this compound has only been described previously by elemental composition and melting point [16], a full analytical characterization was carried out.

Analysis: yield of methanol-crystallized product 44% and of final product 7% as a pale orange powder, m.p. 295°C (decomp.). Found, C 62.06, H 4.7, N 10.5; $C_{14}H_{12}N_2O_4$ requires C 61.95, H 4.39, N 10.25%. IR (KBr disc): 3300, 2990, 2830, 2670, 2550, 1680, 1605, 1580, 1520, 1485, 1462, 1422, 1315, 1300, 1245, 1180, 1125, 1012, 935, 852, 790, 772, 720, 700, 622 cm^{-1}. UV (0.02 M sodium acetate, pH 5.5): 221 nm (ε 87 352), 292 nm (ε 8174). PMR (tetramethylsilane dissolved in dimethylsulfoxide giving a PMR reference value of 2.25): 6.75 (4H d $J = 9$ Hz), 7.75 (4H d $J = 9$ Hz), 8.05 and 8.2 (0.5H d, ? contaminant), 8.5 (2H s), 12.3 (2H); 8.5 and 12.3 disappear on shaking with D_2O. MS (m/z): M^+ 272, 227, 182, 137, 120, 114, 92, 65 and 39.

3. Results and discussion

The HPLC methods reported have been validated to comply with current best practice [17], using limits quoted in the British Pharmacopoeia [18]. Diode-array detection was not available to the authors; accordingly, a series of experiements were run at three different wavelengths in an attempt to detect possible co-eluting compounds. No different pattern of compounds was detected using this approach.

The HPLC detection of 4,4′-azobenzenedicarboxylic acid and 4,4′-azoxybenzenedicarboxylic acid is shown in Fig. 1 (method 1). This HPLC method also resolves the azo/azoxy compounds from 4,4′-hydrazobenzenedicarboxylic acid, which elutes at the solvent front and from the synthetic precursor 4-nitrobenzoic acid. HPLC method 1 was not able to resolve 4-nitrobenzoic acid from 4-nitrosobenzoic acid, another possible photoproduct of 4-aminobenzoic acid. Thin-layer chromatographic methods also failed to resolve

Table 1
Detection of photoproducts by HPLC method 2 on the photolysis of 4,4′-hydrazobenzenedicarboxylic acid

	Retention time (min)	(% RSD)	Retention time relative to compound 4	Detection wavelength	Time period (hours)
1	3.29	(1.1 n = 6)	0.282		All up to 8 hours
2	4.20	(3.3 n = 10)	0.358	290 nm used for	All up to 8 hours
3	5.00	(0.93 n = 7)	0.430	this experiment	All up to 6 hours
4	11.65	(8.1 n = 10)	1.000		All up to 8 hours

1 = 4,4′-hydrazobenzenedicarboxylic acid.
3 = 4,4′-azobenzenedicarboxylic acid (confirmed by standards run during experiment, high RSD due to chromatic drift).

4-nitrobenzoic acid and 4-nitrosobenzoic acid, and therefore the significance of peaks co-eluting with 4-nitrobenzoic acid is unknown.

In an attempt to investigate the possible formation 4,4′-hydrazobenzenedicarboxylic acid, this compound was synthesized. Tomlinson [16] described this compound but the characterization was inadequate by modern standards so a full analytical characterization was carried out. The HPLC detection is shown in Fig. 2 (method 2). The azo and azoxy compounds are also detected using this method, but long retention times result in poor repeatability and the method was not validated further for these compounds

The design of the photochemical experiments attempts to simulate clinical and pharmaceutical in-use conditions. Irradiation at wavelengths in the range 303–400 nm, of maximum intensity at 366 nm, simulates sunlight. This allows a comparison with the studies of Gasparro [8] at 313 nm and Shaw et al. [9] at >290 nm. Aqueous systems were used in all the work reported, but the pH varied from 11 [8] to 7.5 [9]; phosphate buffer was used in this work to maintain the pH at 7.0. All the studies discussed were carried out in solutions which were not deoxygenated.

The results from the photolysis of 4,4′-hydrazobenzenedicarboxylic acid are shown in Table 1. Chromatographic conditions limit the reporting of results to 8 h. Two major photoproducts (**2** and **4**) were detected, of which only **4** could be identified as 4,4′-azobenzenedicarboxylic acid, and one minor photoproduct (**3**) with a retention time of 5.0 min. Analysis of the stock solution, kept in the dark, showed that there was a significant degradation of 4,4′-hydrazobenzenedicarboxylic acid to 4,4′-azobenzenedicarboxylic acid over the experimental period. Insufficient data are available to follow the "dark" degradation pathway. However, photoproduct **3** was absent from the "dark" samples.

Fig. 3 and Table 2 show the results arising from the photolysis of 0.1% (w/v) 4-aminobenzoic acid in phosphate buffer (pH 7.0) after 96 h. All compounds were detected at 280 nm. A total of 25 compounds were detected in the course of the experiment. However only nine compounds meeting the following criteria are reported: (1) a minimum of five observations for definite existence; (2) an RSD of 2% or less for establishing retention time; and (3) present in at least two sample periods.

Despite the investigation of 38 TLC systems, the results were poor and no useful data to complement the HPLC observations was obtained.

Gasparro [8], in the first of the studies using HPLC for the detection of 4-aminobenzoic acid photoproducts, used an ion-exchange method, leading to the identification of two photoproducts. Fractions isolated from the chromotographic procedure were analysed by UV spectrophotometry, with reference to published data [14,15] and mass spectra, although only one mass ion value is quoted. The analytical work was not validated using reference substances.

While the formation of trans-4,4′-azobenzenedicarboxylic acid is supported by the reported data, the formation of the "*cis*" isomer is more doubtful. The *cis* isomer formation was based on an extrapolation of data on *p*-azophenyldiphenylcarbamyl chloride [19]. Photochemical *cis–trans* isomerization for azobenzene derivatives is well

established [20,21]. However, the isolation and analytical characterization of *cis*-4,4′-azobenzenedicarboxylic acid has not been described and an attempt to isolate the *cis* isomer of 4-azobenzenecarboxylic acid was not successful [20]. From the UV data quoted in the above study [8], the formation of 4,4′-azoxybenzenedicarboxylic acid appeared to be a possibility.

A mechanism based on the formation of an amino radical cation, with subsequent oxidation to 4-hydroxylaminobenzoic acid and disproportionation to the azo product, was also proposed in the above study [8] and from flash photolysis

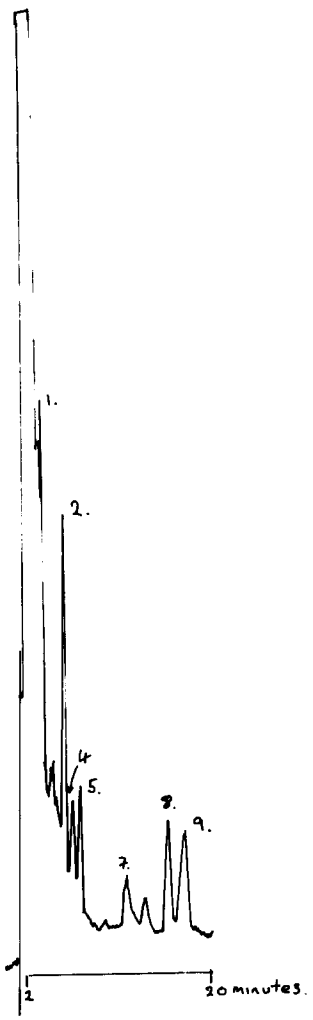

Fig. 3. HPLC trace for the photolysis of 4-aminobenzoic acid after 96 hours (method 1). Compounds as detailed in Table 2.

studies [11] (see Fig. 4). The formation of radical cations by flash photolysis has been reported for aniline in aqueous solution [22] and for 1,2-phenylenediamines [23], the products of the latter being azo compounds.

Gasparro [8] quoted unpublished work reporting the formation of a radical cation from 4-aminobenzoic acid. Clearly, an alternative mechanism for the formation of azo compounds is a direct reaction between anilino radicals, initially forming 4,4′-hydrazobenzenedicarboxylic acid, which then oxidizes to the azo compound.

Evidence for the formation of 4-hydroxylaminobenzoic acid, as an intermediate in the formation of 4,4′-azobenzenedicarboxylic acid in Fig. 4, presents difficulties. The formation of phenylhydroxylamine in the flash photolysis of aniline has been reported [24]. In the photolysis of nitrobenzene, phenylhydroxylamine is formed via a four-electron process [25] and is then oxidized to and coupled with nitrosobenzene to form azoxybenzene [26].

A similar mechanism is well established in the electrochemical reduction of 4-nitrobenzoic acid [27,28]. The 4-hydroxylaminobenzoic acid formed is oxidized to 4-nitrosobenzoic acid and reaction between these two compounds produces 4,4′-azobenzenedicarboxylic acid. Further reduction of azoxy compound produces the azo and hydrazo derivatives and eventually the corresponding amine [21,29].

In contrast to the mechanism proposed by Gasparro [8], the studies mentioned above involve a reducing environment, with the formation of the azoxy compound as a difinite product. While the condensation between an aromatic amine and a phenylhydroxylamine is an extablished preparative method for azobenzenes [21], no references supporting the photochemical mechanism in Fig. 4 were found.

In the experimental work described, evidence for the formation of 4,4′-hydrazobenzenedicarboxylic acid was found, together with the subsequent formation of 4,4′-azobenzenedicarboxylic acid. In a separate series of experiments, the ready formation of 4,4′-azobenzenedicarboxylic acid on photolysis of 4,4′-hydrazobenzenedicarboxylic acid was demonstrated.

Table 2
Detection of 4-aminobenzoic acid photoproducts by HPLC method 1 on photolysis of a 0.1% w/v solution in 0.05 M phosphate buffer at pH 7.0

	Retention time (min)	(% RSD)	Retention time relative to compound 7	Detection wavelength	Time period (hours)
1	2.46	(1.4 n = 6)	0.265	280 nm	24, 36, 60, 72, 96, 130
2	4.23	(0.65 n = 9)	0.456	280/330 nm	36, 48, 60, 72, 96, 130
3	4.69	(1.0 n = 8)	0.507	280/314 nm	36, 60, 72, 96
4	5.66	(0.77 n = 9)	0.611	280/314 nm	36, 60, 72, 96, 130
5	5.81	(0.3 n = 5)	0.627	280 nm	12, 48
6	6.27	(1.6 n = 6)	0.677	280 nm only	24, 36, 60, 72
7	9.26	(1.5 n = 6)	1.00	330 nm	48, 60, 96, 130
8	13.72	(0.46 n = 5)	1.482	280 nm	48, 60, 96
9	14.43	(0.97 n = 7)	1.56	280 nm	24, 36, 72

1 = 4,4′-hydrazobenzenedicarboxylic acid.
3 = 4-nitrobenzoic acid or 4-nitrosobenzoic acid.
7 = 4,4′-azobenzenedicarboxylic acid.

This work complements that reported in a recent study of the photochemistry of 4-aminobenzoic acid [9], where six photoproducts were identified. Equivalent compounds have been found in the flash photolysis of aniline [24]. These experiments were carried out at 254 nm and an unspecified wavelength, above 290 nm, which produced the same photoproducts. Shaw et al. [9] attempted to repeat Gasparro's study with no result.

However, using a higher concentration of 4-aminobenzoic acid, they isolated 4-amino-3-hydroxybenzoic acid and 4-aminophenol, with 4,4-azobenzenedicarboxylic acid as a very minor photoproduct. Two aspects of the above study may account for the latter observation: first, the samples for analysis were concentrated or evaporated before analysis. The methods of concentration were not stated, but such procedures could compromise the detection of relatively unstable photoproducts. In particular, any hydrazo compounds present would rapidly oxidize, as was found in the course of the synthesis of 4,4′-hydrazobenzenedicarboxylic acid and subsequent investigation of its photochemistry (Table 1). Second, the HPLC method employed uses trifluoroacetic acid as a pairing agent for amines. This would result in a very acidic pH, which would inhibit the elution of acidic compounds such as 4,4′-azobenzenedicarboxylic acid and some possible oxidation products. A significant observation from both the work of Shaw et al. [9] and that reported in this paper is the absence of oxidation products of 4-aminobenzoic acid. These would include 4-hydroxylaminobenzoic acid, 4-nitrosobenzoic acid and their coupling product 4,4′-azoxybenzenedicarboxylic acid. Had these been present, the HPLC methods reported in this paper are capable of detecting them.

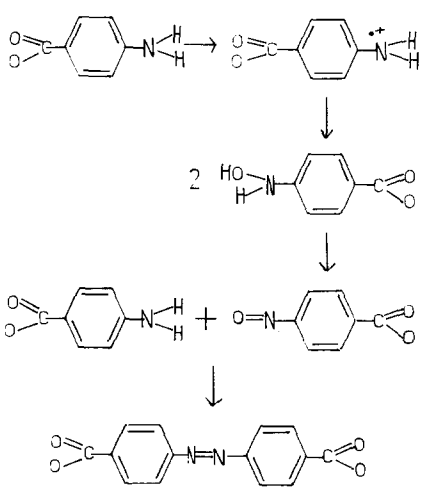

Fig. 4. Proposed Scheme for the formation of 4,4′-azobenzenedicarboxylic acid [8].

In the aqueous systems used, especially in the presence of phosphate buffer, an oxidizing environment was present. Reactive oxygen species

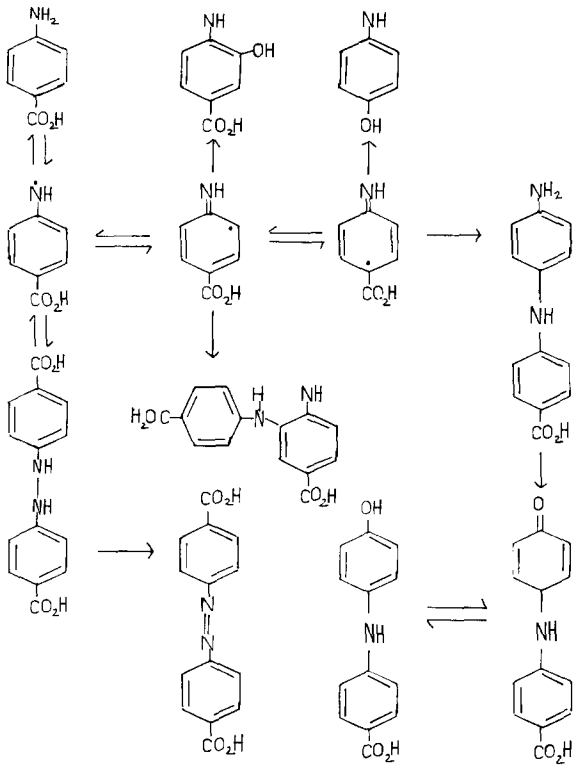

Fig. 5. Diagram showing the photochemical degradation pathways for 4-aminobenzoic acid, from the work of Shaw et al. [9] and reported in this paper.

would undoubtedly be present [30,31]. Phenylhydroxylamine has been observed by Subramanian et al. [24] in the photolysis of aniline and nitroso and nitro products on reaction with superoxide [32].

As a result, the mechanism proposed by Gasparro [8], Fig. 4, is not substantiated in the above studies. A diagram showing the photochemical degradation for 4-aminobenzoic acid discussed in this paper and the work of Shaw et al. [9] is shown in Fig. 5.

4. Conclusion

Recent studies on the photolysis of 4-aminobenzoic acid have revealed a complex photochemical degradation pattern, with up to nine photoproducts. Six photoproducts have been identified in recent published work and chormatographic evidence for the formation of 4,4′-hydrazobenzenedicarboxylic acid is now reported. A review of the literature and the results of the experimental work reproted in this paper do not support the mechanism proposed by Gasparro [8] for the photolysis of 4-aminobenzoic acid.

Further work into the formation and photochemistry of 4,4′-hydrazobenzenedicarboxylic acid is required, especially as there appears to be little published work on the photochemistry of hydrazo compounds in general [29,33,34].

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