Phytochemistry, 1975, Vol. 14, pp. 1425–1428. Pergamon Press. Printed in England.

AEROBIC OXIDATION OF INDOLE-3-ACETIC ACID WITH BISULFITE*

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(*Received* 15 *August* 1974)

Key Word Index—Indoleacetic acid; bisulfite oxidation; manganous ion; (2-sulfoindole)-3-acetic acid; dioxindole-3-acetic acid; O_2 and HO· radicals.

Abstract—Indole-3-acetic acid was rapidly destroyed in the presence of Mn^{2+}, oxygen and bisulfite. (2-Sulfoindole)-3-acetic acid and dioxindole-3-acetic acid were isolated and identified as the two major products. A chemical mechanism accounting for the formation of the products is presented in which O_2^- and HO· radicals, produced during the aerobic oxidation of bisulfite, function as oxidizing agents.

INTRODUCTION

Indole-3-acetic acid (IAA) is a natural plant hormone, and SO_2 is an atmospheric pollutant which causes serious damage to vegetation. IAA has been shown to be rapidly destroyed by bisulfite in the presence of Mn^{2+} and oxygen at neutral pH [1]. The destruction of IAA was dependent on the aerobic oxidation of bisulfite. Tracer studies indicated that IAA was converted into at least three compounds. Despite the advances made in understanding the free-radical chain mechanism for the aerobic oxidation of sulfite to sulfate [2–6] the environmental and physiological significance of the reaction has remained elusive. In this paper we describe the structure of two of the reaction products formed when IAA is reacted with the sulfite-O_2 system, and the possible pathways accounting for the formation of the products.

RESULTS

Changes in the UV spectrum of the IAA reaction mixture were observed during the aerobic oxidation of sulfite in the presence of Mn^{2+} at pH 6. The conversion of IAA into oxindole compounds was indicated by the increase of absorption at 240–250 nm concomitant with the decrease of indole absorption at 270–280 nm. After apparent complete conversion of IAA, as shown by a negative Salkowski test, absorption at 270–280 nm remained at a low but constant level during a prolonged period of time. This is taken as evidence that an indole compound other than IAA was present. The double peak at 247 and 253 nm, characteristic of methylene-oxindole [7] was not apparent.

Identification of compound **1**

Compound **1**, isolated as the cyclohexylammonium salt, showed, by electrophoresis, a COOH and a SO_3H[1] group. The IR has bands (1520, 1220 and 745 cm^{-1}) indicating the presence of $-CO_2^-$, and $-SO_3^-$ and an *ortho*-substituted benzene ring. The UV spectrum has two maxima, 274 nm (ϵ = 9240) and 218 nm (ϵ = 39 000). The 274 nm region is similar to those of 2-sulfoindoles [8]. The PMR spectrum shows a multiplet at δ 6·8–7·4 ($C_6\underline{H}_4$), a singlet at 3·72 ($C\underline{H}_2$), a broad multiplet at 2·63 ($C\underline{H}$), and two groups of broad multiplets at 1·35–1·00 ppm (methylenes of cyclohexyl groups) with relative intensity of 4:2:2:20. Thus, all the available data suggest that **1** is (2-sulfoindole)-3-acetic acid. This was further confirmed by IR, NMR, and UV comparison with an authentic sample.

* Supported by Grant R 801338 from the U.S. Environmental Protection Agency.

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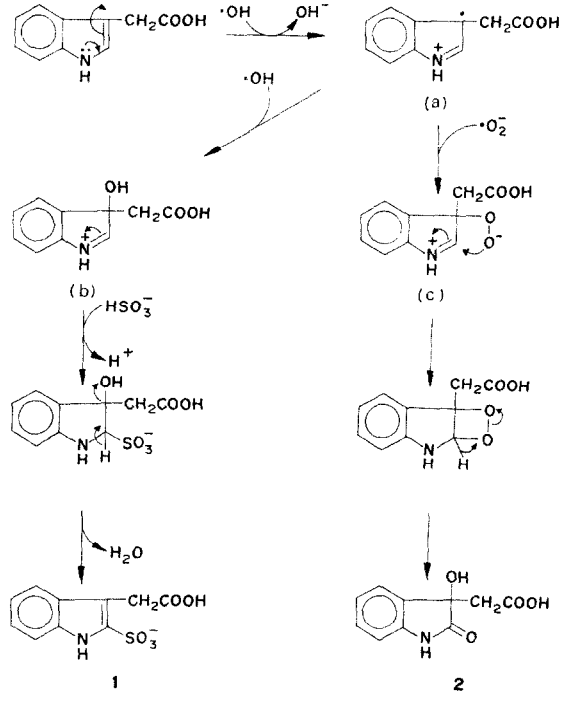
Identification of compound **2**

Although recrystallization of **2** and its methyl ester was unsuccessful, the purity of **2** was indicated by paper radiochromatography, paper electrophoresis and GLC. The IR spectrum of **2** has an NH absorption at 3390 cm^{-1} and two different OH absorption bands at 3200 and 3100 cm^{-1}. A broad absorption band at 1670–1725 cm^{-1} indicates the presence of two different but unresolved C=O groups. This broad absorption band was resolved into two bands at 1715 cm^{-1} (CO) and 1520 cm^{-1} (CO_2^-) when **2** was converted into a salt. An absorption band at 1615 cm^{-1} confirms the presence of a NHCO group. The *ortho*-substituted benzene ring is evidenced by the absorption at 750 cm^{-1}. The PMR spectrum of **2** in $(CD_3)_2SO$ exhibits a singlet at δ 10·2 ppm (NH) which is exchangable with D_2O and which is also present in the same intensity in the PMR spectrum of the methyl ester of **2**. The four aromatic protons on the benzene ring show as a complex multiplet at 6·8–7·4 ppm which was resolved into two sets of quartets on a 100 MHz instrument. The methylene protons appear as a sharp singlet at δ 2·95 ppm. The carboxylic proton and hydroxy proton were not observed due to their facile exchange with deuterated solvent. The PMR spectrum of the methyl ester of **2** has an additional singlet at 3·40 ppm (OCH_3). The UV spectra of both **2** and its methyl ester are comparable to that of *t*-butyl dioxindole-3-acetate [9] which shows three absorption maxima at 208 (ϵ = 25000), 252 (ϵ = 6150) and 292 nm (ϵ = 1400). These data are consistent with the view that **2** is dioxindole-3-acetic acid. Indeed, the UV, IR and NMR spectra and the retention time on GLC of the methyl ester of **2** are identical to those of the synthetic methyl dioxindole-3-acetate. That compound **2** is dioxindole-3-acetic acid was further confirmed by comparison of retention times and MS of methyl TMSi derivatives of **2** with those of synthetic dioxindole-3-acetic acid. A M^+ of 293 and the following major fragmentation peaks were observed at *m/e* 278, 250, 234, 220, 218, 204, 172, 89, 75 and 73. These peaks may be interpreted as follows: *m/e* 73 is Me_3Si^+ and CH_2COOMe, 75 is $[Me_2SiOH]^+$, 89 is $[Me_3SiO]^+$, 172 is $M^+ - [89(Me_3SiO) + 32(MeOH)]$, 204 is $M^+ - [89(Me_3SiO)]$, 218 is $M^+ - [43(NHCO) + 32(MeOH)]$, 220 is $M^+ - [73(Me_3Si \text{ or } CH_2COOMe)]$, 234 is $M^+ - [49(COOMe)]$, 250 is $M^+ - [43(NHCO)]$, and 278 is $M^+ - [15(Me \text{ from } Me_3SiO)]$.

Compound **3**, which accounted for about 10% of the reaction products based on radioactivity, was a decarboxylated product, since it was observed only when IAA[methylene-^{14}C] but not IAA [carboxyl-^{14}C] was employed as substrate. Chemical identification of **3** was not attempted.

DISCUSSION

The changes in the UV spectrum which occurred during the destruction of IAA through the co-oxidation of sulfite to sulfate are similar to those previously reported by Meudt [10], but differ from those obtained from enzymatic oxidations of IAA, in which the typical double peaks at 247 and 253 nm for 3-methyleneoxindole were clearly observed [7, 11]. The presence of oxindoles was indicated by the pattern of spectral changes, and was confirmed by the isolation of compound **2**. The isolation and identification of (2-sulfoindole)-3-acetic acid provides an explanation for the persistence of the indole absorption at 270–280 nm, even when the reaction mixture gave a negative Salkowski test.



It has been generally recognized that O_2^-, HO· and HSO_3· radicals are generated during the aerobic oxidation of sulfite, and that these maintain, through chain-propagating reactions, the aerobic oxidation of sulfite to sulfate [2–4, 6]. One possible mechanism which accounts for the formation of compounds **1** and **2** is shown above. The first step of this scheme is a one-electron oxidation of the indole ring in which either the O_2^- or HO· radical may function as a one-electron oxidant yielding radical (a). This is in keeping with earlier proposals [7] that the first step in the peroxidase-catalyzed oxidation of IAA is a one-electron oxidation at the 3-position of the indole ring which has a high electron density. Subsequent oxidation with HO· or O_2^- yields 3-hydroxyindolenine (b) or 3-hydroperoxyindolenine (c). It is also compatible with the opinion that the first intermediate in the reaction of an indole with a variety of oxidizing agents is a 3-hydroxy- or 3-hydroperoxyindolenine [12, 13]. In the next stage of reaction, two paths are possible. Rearrangement of the hydroperoxide with addition to the indolenine double bond, and concomitant ring opening, is a common route. The enzymic cleavage of tryptophan to *N*-formylkynurenine [14] and the peroxidase-catalyzed oxidation of indole-3-acetaldehyde to 4-hydroxyquinoline in the presence of sulfite have been formulated in this way [15]. However, ring cleavage may not occur to a significant extent if addition of another group to the indolenine double bond can compete with rearrangement of the hydroperoxide. Nucleophilic addition of bisulfite to (b), followed by dehydration, yields compound **1**, (2-sulfoindole)-3-acetic acid, while an intramolecular nucleophilic addition by a pendant hydroperoxide anion of (c), followed by rearrangement, should yield compound **2**, dioxindole-3-acetic acid, as depicted in the above scheme.

It should be noted that (2-sulfoindole)-3-acetic acid (compound **1**) was not formed when IAA was incubated with sulfite in the absence of Mn^{2+}; under such conditions aerobic oxidation of sulfite, as assayed by the oxygen electrode [1], did not occur. These observations indicate that the formation of compound 1 was not accomplished by a simple substitution of sulfite to indole-3-acetic acid, but was dependent on the aerobic oxidation of sulfite which was initiated by Mn^{2+}. The dependence of the formation of compound **1** on sulfite oxidation may be explained on the basis that cooxidation of sulfite is necessary for the generation of HO· radical, which in turn is essential for the formation of intermediate **2**, as shown in the scheme. This scheme is in agreement with the previous observation that the percentage yield of compound **1** relative to compound **2** increased as the concentration of sulfite was increased [1].

The reactions described above are rare examples of indole-3-acetic acid oxidation in which decarboxylation does not occur. They are also rare examples of indole oxidation in which the pyrrole ring is not cleaved. It should be noted that neither (2-sulfoindole)-3-acetic acid nor dioxindole-3-acetic acid has any auxin activity as assayed by their ability to induce ethylene production in mungbean hypocotyl segments (O. L. Lau, A. J. Horng and S. F. Yang, unpublished results).

It appears that the oxidation of IAA, like that of many other molecules of biological significance [6, 15–20], can be achieved through cooxidation of sulfite to sulfate. It remains to be determined, however, whether sulfite may inflict biological damage *in vivo* through such an oxidative mechanism.

EXPERIMENTAL

Materials. IAA[carboxyl-^{14}C] and IAA[methylene-^{14}C] were obtained from Amersham/Searle. C_5H_5N–SO_3 complex was supplied by Professor G. F. Smith. Methyl dioxindole-3-acetate was synthesized from methyl indole-3-acetate with 2 equivalents of *N*-bromosuccinimide followed by hydrolysis with $NaHCO_3$ [9]. (2-Sulfoindole)-3-acetic acid was prepared from IAA and C_5H_5N–SO_3 complex [8] and was isolated and recrystallized as the cyclohexylammonium salt.

Methods. Oxidation of IAA with bisulfite, determination of IAA, paper radiochromatography and paper radioelectreophorsis of the oxidation products were carried out as described previously [1]. IR spectra were recorded using KBr pellets. PMR spectra were recorded at 60 and 100 MHz. Chemical shifts (δ) were recorded in ppm downfield from TMS. UV spectra were obtained in 95% EtOH or H_2O. GLC was carried out on a FID instrument equipped with 152 cm × 3 mm columns.

Isolation and purification of the reaction products. The reaction was conducted in a 2 l. graduated cylinder equipped with a magnetic stirrer and an air inlet tube. To 1·7 l. of H_2O containing 0·2 mol of KPi, 100 ml each of 0·1 M IAA [methylene-^{14}C] (10 μCi), 0·1 M $MnSO_4$ and 0·4 M $NaHSO_3$ were added. IAA and $NaHSO_3$ were added 10 ml at a time, to allow the destruction of IAA to go to completion before the next portion was added. Thus, the conc of IAA in the reaction mixture was kept below 0·5 mM. Air was introduced to the reaction mixture and the pH was maintained at 5·6–5·8 throughout the course of the reaction. The destruction of IAA was monitored by measuring the change of absorption at 245 nm, as well as by the Salkowski test [21]. At the end of the reaction, the mixture was extracted

with Et_2O. Paper radiochromatographic analysis indicated that the Et_2O extracts contained compound **3** (R_f = 0·92), and that the aq. phase contained compounds **1** and **2** (R_f = 0·05 and 0·20, respectively). The aq soln was evaporated under red pres at 50°. The solid residue was taken up in EtOH and was then acidified with HCO_2H. The inorganic salts were removed by filtration. The EtOH extract was concentrated to 10 ml, and applied to a 2 × 30 cm cellulose column which was then eluted with 100 ml of BuOH–NH_3–H_2O (10:1:9) followed by 100 ml of BuOH–HOAc–H_2O (10:1:9). The eluate was collected in 2-ml fractions, which were monitored by liquid scintillation counting. Two radioactive compounds were isolated: compound **2** in Fractions 4 through 28, and compound **1** in Fractions 55 through 87. They were further purified and characterized as follows. Compound **1**, which was not eluted from the cellulose column by BuOH–NH_3–H_2O, but was eluted with BuOH–HOAc–H_2O, gave only a single radioactive spot after PC in either acidic or basic solvent systems (R_f = 0·45 and 0·05, respectively) or after paper electrophoresis. It is probable that the compound thus obtained was fairly pure. After removal of the solvent, the residue was dissolved in 25 ml of H_2O and applied to a cationic ion exchange resin (Dowex-50, H^+ form) column, from which it was eluted with 100 ml of H_2O. The resulting acidic effluent sol was titrated with cyclohexylamine to pH 10 and was then evaporated to dryness. About 0·5 g of white solid was obtained. This was recrystallized several times from H_2O–Me_2CO to constant specific radioactivity. Compound **2**, which was eluted from the cellulose column with BuOH–NH_3–H_2O (10:1:9) gave only one radioactive spot after either PC (R_f = 0·20) or paper electrophoresis. After removal of solvent, a reddish-yellow powder (about 0·5 g) was isolated. Attempts to further purify the compound by recrystallization were unsuccessful. Treatment of **2** with excess CH_2N_2 yielded a single derivative, as indicated by paper radiochromatography (R_f = 0·96) and by GLC with three different columns: SE-30, QF-1 and OV-225. The crude methyl ester of **2** was then converted to the TMSi derivative, which was subjected to GC–MS at 165° on a column packed with 2% SE-33.

Acknowledgments—We thank Professor G. F. Smith for his generous gift of C_5H_5N–SO_3 complex and Professor J. MacMillan for the GC–MS analysis and interpretations. We also thank Dr B. R. Miller for his helpful discussion.

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