important factor in the utilization of carbohydrates.

Data presented on the nature of starch with respect to amylose moiety and results of earlier studies on the in vitro digestibility of carbohydrates in these pulses and in vivo experiments in children clearly indicate that both the carbohydrate makeup and their utilization in different pulses are different and that legumes whose amylose has a longer chain length are difficult to digest.

Murphy (1963) implicated low molecular weight oligosaccharides such as raffinose and stachyose contained in beans as being responsible for flatulence. The pulses investigated here also have these oligosaccharides, but their concentrations are essentially similar in Bengalgram and greengram (Nigam and Giri, 1961) and these oligosaccharides by themselves cannot account for the higher amounts of flatus produced following the ingestion of Bengalgram (Narayana Rao et al., 1973).

These observations may be interpreted as suggesting that among the factors that can cause flatulence is the extent of digestibility of carbohydrates in pulses. It is, however, premature to conclude that poor digestibility may cause flatulence, although present observations on pulses and earlier work in the field of carbohydrate utilization point to such a possibility.

ACKNOWLEDGMENT

The author gratefully acknowledges C. Gopalan, Director-General, Indian Council of Medical Research, New Delhi, S. G. Srikantia, Director, National Institute of Nutrition, Hyderabad, and B. S. Narasinga Rao, Deputy Director, for their valuable suggestions during the conduct of this investigation and Vinodini Reddy, Assistant Director of the National Institute of Nutrition for her cooperation in carrying out in vivo studies in children.

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Received for review March 9, 1976. Accepted June 4, 1976.

Fluorescence Behavior of Sterigmatocystin

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The fluorescence and absorbance spectra of sterigmatocystin and its *O*-methyl derivative were determined in buffered aqueous solutions and concentrated sulfuric acid solutions. The excited state species of sterigmatocystin undergoes phototautomerism prior to emission resulting in a zwitterionic structure. This mechanism was supported by the acidity dependent spectral shifts of sterigmatocystin and *O*-methylsterigmatocystin. Fluorescence intensity in sulfuric acid solutions was enhanced due to carbonyl protonation. Analysis in 60% sulfuric acid solution was demonstrated to have a sensitivity limit of 0.01 μg/ml.

Sterigmatocystin (I), a mycotoxin structurally and biosynthetically (Hsieh et al., 1973) related to the aflatoxins, has been demonstrated under laboratory conditions to be a causative agent in production of hepatocellular carcinoma in rats (Purchase and Van der Watt, 1970). Additionally, hepatotoxic effects have been observed with vervet monkeys (Van der Watt and Purchase, 1970). The fungus *Aspergillus versicolor* will produce sterigmatocystin under optimum conditions (Holzapfel et al., 1966), and the mycotoxin has occasionally been detected in agricultural commodities (Scott et al., 1972; Purchase and Pretorius, 1973).

Analytical methodology available for quantitation of sterigmatocystin levels includes a semiquantitative thin-layer chromatographic (TLC) method (Reiss, 1975) and a gas chromatographic assay (Manabe et al., 1971). The mycotoxin is reported to exhibit weak fluorescence on silica gel which may be magnified by an aluminum chloride treatment (Stack and Rodricks, 1971). This report details the fluorescence behavior of sterigmatocystin in various aqueous media and describes a fluorescence assay capable of detecting levels expected in contamination of agricultural commodities.

EXPERIMENTAL SECTION

Apparatus. Fluorescence measurements were obtained on a Turner, Model 430, recording spectrofluorometer equipped with a xenon light source. Absorption spectra were recorded using a Coleman 124 spectrophotometer.

Materials. Sterigmatocystin was obtained from cultures of *Aspergillus versicolor* (Vuill.) Tiraboschi, American Type Culture Collection no. 18643. The cultures were grown on Czapek Dox Agar for a period of 15 days. The mycelial mats along with the agar were extracted with

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Table I. Long-Wavelength Absorption (λ_a) and Fluorescence (λ_f) Maxima of Sterigmatocystin and *O*-Methylsterigmatocystin

Compound	60% H_2SO_4, $H_o = -5.83$		0.1 N HCl		pH 7.0		2 N NaOH	
	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm
Sterigmatocystin	377	520	340	570	340	570	380	520
O-Methylsterigmatocystin	374	510	318	450	318	445	318	445

three 100-ml portions of boiling chloroform; the extracts were combined, reduced in volume, and placed on a silicic acid column. The sterigmatocystin was eluted from the column with 5% chloroform in hexane. The elution procedure was monitored by TLC (Sullivan et al., 1976). Purification of the eluted sterigmatocystin was accomplished by repeated recrystallizations from methanol. Positive identification was obtained by comparison of uv, NMR, ir, and mass spectra with those of an authentic sample of sterigmatocystin. The sterigmatocystin was found by TLC to be chromatographically pure. *O*-Methylsterigmatocystin was obtained from Dr. H. W. Schroeder of USDA, Market Quality Research Division, College Station, Tex.

Analytical reagent grade sulfuric acid from Mallinckrodt Chemical Works, St. Louis, Mo., was diluted with distilled deionized water to prepare solutions of varying acidity for fluorometric and absorptiometric measurements. A corrected Hammett acidity scale (Jorgenson and Hartter, 1963) was employed for the concentrated sulfuric acid solutions. All other reagents and solvents were at least of reagent grade quality.

Procedures. Stock solutions approximately 10^{-2} M for sterigmatocystin and its derivative *O*-methylsterigmatocystin were prepared using dioxane–methanol as the solvent. The compound was predissolved in dioxane and then diluted tenfold with methanol. The final solutions were prepared by dilution of the stock solution (1:100) immediately prior to taking the spectra. For analysis in sulfuric acid solutions, the spectra were obtained at exactly 3 min after final solution preparation in a concentration range of 0.06–0.36 μg/ml. The standardized time was used due to slow fading of fluorescence intensity for solutions containing greater than 50% sulfuric acid.

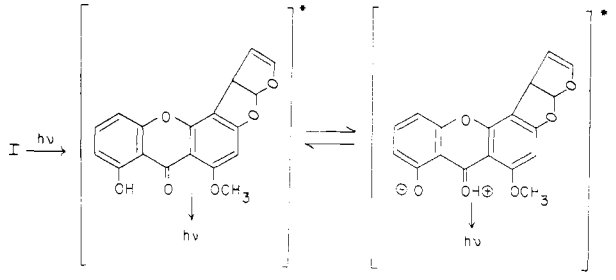
RESULTS AND DISCUSSION

Sterigmatocystin fluoresces a brick-red color on silica gel TLC plates whereas structurally related compounds fluoresce blue using the same excitation wavelength (Sullivan et al., 1976). This anomalous behavior is similar to the long wavelength fluorescence observed with salicylic acid (Weller, 1956). For salicylic acid it was shown that emission from the excited state is preceded by a prototropic transfer from the phenolic group to the carbonyl oxygen.

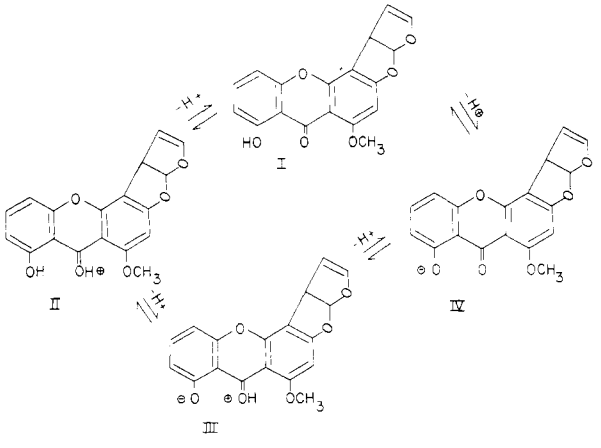
Scheme I depicts the comparable phototautomerism for sterigmatocystin showing both tautomers as possible emitting species. Since both carbonyl protonation and phenolic deprotonation result in stabilization of the excited state relative to the ground state, the zwitterion would exhibit anomalously long-wavelength fluorescence compared to its tautomer (Weller, 1961).

Later studies with salicylic acid and derivatives (Kovi et al., 1972) demonstrated that media effects on fluorescence and absorbance behavior could be used to delineate ground-state and excited-state structures. Solvent effects, throughout the pH and Hammett acidity regions, on the electronic transitions of sterigmatocystin and *O*-methylsterigmatocystin are given in Table I. The two possible deprotonation pathways available for sterigmatocystin, going from concentrated acid to strong base

Scheme I



Scheme II



solution, are shown in Scheme II.

The differences in the two paths occur on the first deprotonation reaction. If the path (II → I → IV) is operative in the transition from strong acid solution to concentrated base solution, then a shift to a shorter wavelength for absorbance and fluorescence determinations would be expected for production of the uncharged molecule (I). Subsequently, deprotonation leading to the phenolate ion would produce a shift to longer wavelength. The second path (II → III → IV) would show opposite shifts. Formation of the zwitterion would show a long-wavelength shift, followed by a shift to shorter wavelength for phenolate production. Table I details the sequence of shifts observed for the fluorescence and long-wavelength absorbance band of sterigmatocystin. This latter band shows a shift sequence compatible with the pathway (II → I → IV), that is, a blue shift followed by a red shift. However, fluorescence shifts are opposite directionally. Decreasing acidity in the Hammett acidity region produces a shift to longer wavelength. In the higher pH region, a shift to shorter wavelength is encountered when the spectra are determined in pH 7.0 aqueous solution and 2.0 N NaOH. For these shifts, the deprotonation path (II → III → IV) is implicated.

Thus, it is apparent that the ground-state prototropic reactions are dissociation of protonated carbonyl oxygen with subsequent ionization of the phenolic proton. Excitation of the neutral sterigmatocystin (I) results in a reorientation of the electron distribution. The charge-transfer acceptor properties of the carbonyl enhance the basicity of its oxygen atom while the phenol, being a

Table II. Relative Fluorescence Intensity for Various Concentrations of Sterigmatocystin in 60% H_2SO_4 ($H_0 = -5.83$)

Concn, μg/ml	Rel fluorescence intensity
0.060	6.93 ± 0.60
0.121	13.93 ± 0.51
0.181	19.10 ± 1.35
0.242	26.63 ± 0.93
0.311	32.70 ± 0.79
0.362	39.18 ± 0.88

charge-transfer donor in the excited state, becomes a stronger acid. The intramolecular acid–base reaction occurs within the lifetime of the excited singlet state and the zwitterion becomes the emitting species. Support for this sequence of events is found in the spectroscopic data for the *O*-methyl derivative. Without the phenolic proton, a path comparable to (II → I) would be expected in the media transition from strong acid to strong base leading to a single shorter wavelength shift in spectroscopic determinations. As reported in Table I, both the long-wavelength absorbance and fluorescence measurements show the predicted blue shift. Additionally, the 510-nm emission in strong acid is comparable to the 570-nm sterigmatocystin zwitterion emission observed in the neutral pH region indicating a similar electron distribution for the two species.

The fluorescence intensity for both compounds is weak in the normal pH region. However, the singly positive charged species produced in the Hammett acidity region shows enhanced fluorescence. For example, relative fluorescence intensity was increased approximately 200-fold in going from 20 to 60% sulfuric acid. As H_0 is progressively decreased from −1 to −10, the protonated sterigmatocystin is formed with a pK_a in the region −4 to −6. At H_0 values lower than −6, facile chemical transformation of sterigmatocystin, as indicated by fluorescence intensity fading, becomes a problem from an analytical viewpoint. Aflatoxin B_1 has been reported to undergo hemiacetal formation by acid-catalyzed hydration of the nonconjugated double bond of the difurano portion of the molecule (Pons et al., 1972). Since this analogous bond in sterigmatocystin is not in direct conjugation with the absorbing and emitting chromophore, the observed fluorescence fading cannot be attributed to hemiacetal formation. Additionally, there is no evidence to refute or support appreciable hemiacetal formation during absorptiometric and fluorometric determinations. If the reaction does occur prior to obtaining the spectrum, it apparently does not affect the measurement, and thus should not interfere with a fluorescence detection of sterigmatocystin. The solvent chosen for fluorescence analysis was 60% sulfuric acid (H_0 = −5.83). At this Hammett acidity value the sterigmatocystin exhibits strong fluorescence intensity and fluorescence fading is slow enough to be compensated analytically by standardization of the time of fluorescence measurements. All fluorescence measurements for analyses were taken at exactly 3 min after final solution preparation. The standard solutions (0.06–0.36 μg/ml) were prepared and fluorescence measured in 60% sulfuric acid. Triplicate determinations were made at each concentration and are reported in Table II along with standard deviations. Good linearity was observed over this concentration range (correlation coefficient $r = 0.999$). The best fit straight line for the calibration curve possessed a slope of 105.2 fluorescence units μg^{-1} ml^{-1} and a *y*-intercept of 0.67 fluorescence unit. The sensitivity of the assay (fluorescence response 2× background) was calculated at 0.01 μg/ml.

The sensitivity is comparable to that of 0.02 μg reported for the gas–liquid chromatographic assay. The extraction and cleanup procedures used for this latter assay, coupled with the alternative fluorometric assay, may provide a rapid and sensitive detection of sterigmatocystin.

ACKNOWLEDGMENT

The authors wish to thank H. W. Schroeder for a sample of *O*-methylsterigmatocystin and Sandra Sugarek for technical assistance. Helpful discussions with Leonard Friedman of the FDA are also acknowledged.

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Received for review March 8, 1976. Accepted May 10, 1976. This investigation was supported in part by FDA Contract 223-74-2219, Department of Health Education and Welfare.