PRODUCTION OF FREE INDOLE BY SOME AROIDS

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SUMMARY

The production of free indole by several arum lily species (*Araceae*) was studied. The compound was identified by paper chromatography and thin-layer chromatography in several solvent systems, colour reactions with various reagents, enzymatic conversion to tryptophan, UV absorption spectrum, and formation of a complex with polynitrofluorenone.

The presence of indole is restricted to certain floral parts and is correlated with the flowering sequence of these plants. In general, indole is present for not more than one day during the flowering cycle. In the case of *Sauromatum guttatum* Schott, production of indole by the appendix starts on the eve of flowering between 6 and 8 p.m. and ends about 24 hours later. At the peak of the metabolic flare-up period, about 0.3 μmoles indole is present per gram fresh tissue.

There is a close connection between the indole cycle and the production of heat and stench in the respiratory flare-up. Some of the indole formed is released as a constituent of the stench.

1. INTRODUCTION

The spectacular metabolic flare-up during the flowering of certain arum lilies has been the subject of several studies (VAN HERK 1937 a & b; JAMES & BEEVERS 1950; SMITH & MEEUSE 1966; HESS & MEEUSE 1968a & b; MEEUSE & BUGGELN 1969). Considerable changes in respiration rate, and a transient production of much heat and stench are among the most striking phenomena that can be observed, e.g., in the appendix of *Sauromatum guttatum* Schott, the voodoo lily.

The mixture of volatile, odoriferous substances produced by the appendices of 5 arum lily species belonging to the *Aroideae* and *Lasioideae* has previously been shown to contain amines and ammonia and, in some cases, skatole (SMITH & MEEUSE 1966). The presence in free form of simple indoles such as skatole and indole in higher plants is rather limited, as indicated in a review by STOWE (1959). In this report we present evidence for the production of free indole by *Sauromatum guttatum* and a few related species.

2. MATERIALS AND METHODS

2.1. Plant materials

Sauromatum guttatum Schott was grown in the greenhouse where the inflorescences were allowed to develop under natural conditions until sacrificed, or until the time they were transferred to the laboratory for observation and ex-

perimentation at room temperature. The sojourn in the laboratory does not alter the natural flowering sequence (SMITH 1964).

For convenience, an arbitrary timescale was used to define certain stages of flower development. The day of female flower anthesis, accompanied by unfolding of the spathe and production of heat and stench, is referred to as D-day. Stages corresponding with a given number of days before and after this day are referred to as D-1, D-2, etc., and D+1, D+2, etc., respectively. In order to determine retroactively the various D-minus stages, a stump of the appendix was left attached to the inflorescence. Production of heat and stench by the stump on D-day, and pollen shedding on D+1 were then used as reference points.

Other aroids used were grown under natural conditions outside the greenhouse. The flowering sequence of these plants was followed daily during May and June. The inflorescences of *Lysichitum americanum* Hult. and St. John (Western skunk cabbage) were collected in the Seattle area in April.

2.2. Extraction and determination of indole

A weighed amount of plant material was cut into small sections; immediately after this it was rapidly and thoroughly ground in a mortar. The paste was then ground with 5 volumes of toluene. The toluene layer was decanted. The residue was re-extracted with an additional 2 volumes of toluene, and the toluene extracts were combined. This procedure recovers more than 98% of the total extractable indole.

Aliquots of the toluene extract ranging from 0.05 to 1.0 ml were taken for colorimetric determinations as described by CHEN & BOLL (1968). The presence of a small amount of impurities in the extract does not interfere with the determination.

2.3. Paper and thin-layer chromatography

One-dimensional descending chromatography was performed with Whatman No. 4 paper. The solvent systems applied were: (a) butanol-95% ethanol-water (8:1:1); (b) butanol-acetic acid-water (4:1:2); (c) chloroform-methanol-17% ammonia (4:1:2); and (d) isopropanol-ammonia-water (7:1:2). The chromatograms were allowed to run at room temperature for 5 to 7 hours. Indole was detected with Ehrlich's reagent (CHEN & BOLL 1968), p-dimethyl-aminocinnamaldehyde (DMCA; WIGHTMAN 1962), or ferric chloride-perchloric acid (GORDON & WEBER 1951).

Eastman pre-coated silica gel plates were used for thin-layer chromatography. After spotting, the plates were percolated with petroleum ether-methanol (100:4) for one hour at room temperature, and then sprayed with Ehrlich's reagent to show indole reaction.

3. RESULTS

Since this study deals mainly with *Sauromatum*, the results obtained with this plant will be dealt with first.

3.1. Identification of indole

Paper-chromatographic studies showed that the toluene extract contains an Ehrlich-positive substance which has an Rf identical to that of authentic indole in all of the solvent systems used. The Rf values are 0.91, 0.96, 0.90, and 0.92 in solvent a,b,c, and d, respectively. It should be mentioned that other simple indoles such as 2-methyl indole and skatole (which has been reported as a constituent of the odoriferous substance of some aroids: SMITH & MEEUSE 1966), have the same Rf as indole in solvents a and b. However, these indoles produce a colour with the above-mentioned reagents different from that displayed by indole In all cases the colours developed by the substance suspected to be indole were comparable to those produced by authentic indole, i.e., pink-red with Ehrlich's reagent, green with DMCA and red with ferric chloride-perchloric acid. The pink-red substance appearing as the result of development with Ehrlich's reagent is soluble in chloroform, a criterion emphasized by FELLERS & CLOUGH (1925). Furthermore, formation of a pink ring was observed in the Salkowski test (cf. FELLERS & CLOUGH 1925).

The substance was further tested in one of the tryptophan synthetase reactions, i.e., the condensation of indole and serine to tryptophan in the presence of pyridoxal phosphate (YANOFSKY 1960). Tryptophan synthetase from pea seedlings (*Pisum sativum* L. cv. 'Alaska') was partially purified and assayed according to the method of CHEN & BOLL (1968), except that the quantity of reaction mixture was 5 times as large. After completion of the reaction, the protein in the reaction mixture was precipitated with trichloroacetic acid at 10% strength. The soluble fraction was neutralized and freeze-dried. The residue was then taken up into 95% methanol for chromatographic studies.

It was found that a spot with an Rf identical with that of commercial tryptophan was produced by such methanol extracts when they were obtained from complete reaction mixtures, in contrast to control mixtures. The Rf's as located with Ehrlich's reagent (pink) or DMCA (violet) were 0.55 and 0.19 in solvents a and d, respectively. These facts indicate that tryptophan was enzymatically formed from the substance obtained from the appendix of *Sauromatum*.

Under the same conditions, 2-methyl indole was used to a lesser extent than indole. Skatole was found to be inactive, which can be ascribed to the presence of a methyl group at the third position of the indole nucleus (YANOFSKY 1960). With 2-methyl indole as the substrate, a substance different from tryptophan, presumably 2-methyl tryptophan, is formed.

For UV spectral studies, the toluene extract was brought to dryness and the residue was extracted with a small amount of hot water (55 °C) leaving out much of the yellow impurities. The aqueous fraction was then applied as a strip on a solvent-washed paper and chromatographed in solvent system a. The portion of

the paper corresponding with the position of indole was cut off and eluted with water. After concentration, the solution was read in a Beckman DB spectrophotometer. It was found that the absorption curve of the substance under study was identical with that of authentic indole.

During the course of this study, a method for the isolation of indoles as polynitrofluorenone complexes was reported by HUTZINGER & JAMIESON (1970). They proposed the use of their method for isolation of indoles from natural sources. The presence of suitable amounts of indole in the appendix of *Sauromatum* held out considerable promise for success of the new method; accordingly we have tried it out.

To the toluene extract prepared from 500 grams of fresh appendix tissue and containing approximately 10 mg indole, 30 mg of 2,4,7-trinitro-9-fluorenone was added. A red-brown complex was formed upon evaporation. This complex was recrystallized from a mixture of ethanol and chloroform. When heated, it became pale yellow as indole sublimated off. When 1.0 mg of the complex was examined for its infrared spectrum on a Beckman IR 5-A infrared spectrophotometer, it yielded a spectrum identical to that of the complex formed between authentic indole and 2,4,7-trinitro-9-fluorenone.

3.2. Temporal and spatial distribution of indole

The distribution of indole in various parts of the inflorescence was studied for the D-1, D-day and D+1 stages. In each case the same amount of tissue (12 grams), pooled from 5 inflorescences, was used, except that 5 grams were taken for the appendix and 25 grams for the spathe. The materials were harvested at noon. The D-1 stage was judged on the basis of our own experience, with an error of approximately one day. The toluene extracts of each of the samples tested were evaporated to a small volume and indole was tested for with a few drops of Ehrlich's reagent.

It was found that only the extract of D-day appendix showed a positive indole reaction. With other parts of the inflorescence, e.g., the stalk, the spathe, the male and female floral zones and the tissue in between, no indole reaction was observed at any stage. This was also true for leaf tissue.

Fig. 1 shows the vertical distribution of indole in D-day appendices harvested at 10 a.m. and 2 p.m. In both cases it is clear that the distribution is not uniform. The highest indole level, calculated on a fresh weight basis, occurs in a region 10 to 20 cm above the male floral zone.

As to the distribution of indole over the appendix tissues, it was found that the highest concentration occurs in the cortical part. This was demonstrated by a simple experiment. A freshly cut cross section or longitudinal section of the appendix was stamped on a piece of paper previously treated with either DMCA or Ehrlich's reagent. The characteristic indole reaction appeared within seconds. The high concentration of indole in the cortical tissues resulted in an intense ring of dark green (with DMCA) or pink-red (with Ehrlich's reagent) on the paper. The same colour also appeared on the part of the paper that had been in contact with the central pith, but here the colour was much less intense.

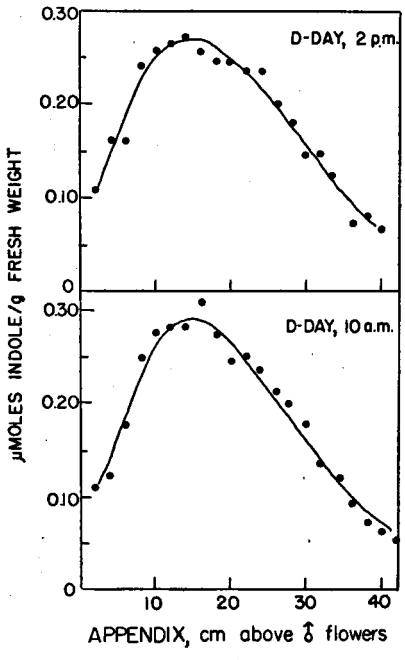

Fig. 1. Spatial variation of indole concentration in individual *Sauromatum* appendices harvested on the day of flowering at the time indicated.

3.3. Variation of indole level on D-day

The classical work of VAN HERK (1937a & b) and the investigations originating from our own laboratory (SMITH & MEEUSE 1966; HESS & MEEUSE 1968 a & b; MEEUSE & BUGGELN 1969) have documented some of the metabolic changes during the flowering of *Sauromatum*. From the results shown above it seemed justified to investigate whether or not indole production follows the same pattern. To do this we have chosen 3 batches, each composed of 5–13 appendices of comparable weight (20–30 g) for the test. The total indole present in each of the individual appendices, harvested at a definite time of flowering, was determined and the levels were then compared (*fig. 2*). Variations in the flowering cycle of such individuals are very small, which makes the comparison reasonable.

It is interesting to note from the results that indole production occurs only during a 24-hour period out of the flowering cycle. The process of indole synthesis starts on the eve of flowering, usually at about 6 to 8 p.m., and ends late on D-day. The peak hours were found to be around noon on D-day when extensive production of heat, CO_2 and an unpleasant odour take place. During this flare-up period, 0.2 to 0.3 µmoles of indole is present per gram of fresh material. This means there is an average of approximately 5 to 6 µmoles of indole per appendix. No indole was ever detected prior to 3 p.m. on D-1, or after 10 p.m. on D-day. Since the spathe unfolds early on D-day, one can claim that indole production starts about 12 hours before, and disappears about 12 hours after the opening of the spathe.

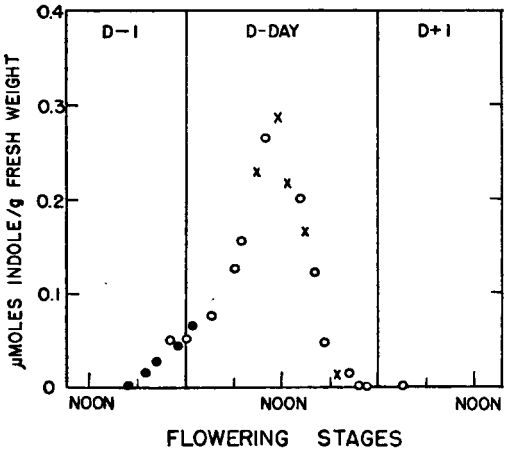

Fig. 2. Changes in the free indole content of the *Sauromatum* appendix. Each datum represents the amount of extractable indole per gram fresh weight of an individual appendix harvested at the time shown. Three batches of appendices, indicated by different symbols, were examined.

3.4. Indole as a constituent of the stench

The condensate of the volatiles from the appendix of *Sauromatum* was collected as described elsewhere (SMITH & MEEUSE 1966). The mixture showed a strong indole reaction upon the addition of a few drops of Ehrlich's reagent. Indole in this mixture was again demonstrated by paper chromatography. Apparently indole is released into the air, contributing to the stench. Indeed indole can easily be detected during the period of stench production by placing a piece of paper previously treated with DMCA or Ehrlich's reagent near the appendix for a few seconds.

For quantitative estimation of indole released from the appendix, the latter was mounted in a plexiglass cylinder and the volatile discharges were trapped into a coil of glass tubing embedded in dry ice. Very gentle suction was applied to ensure the continuous movement through the trap of the air surrounding the appendix. It was found that during the 6-hour peak period, i.e., between 9 a.m. and 3 p.m., a total of 0.436 μmoles of indole could be collected from an appendix weighing 29 grams. At the end of the experiment 5.005 μmoles of indole were extracted from the tissue. This indicates that only about one-tenth of the indole formed was released concomitant with the metabolic flare-up. The total disappearance of indole later on D-day must mean that it is being metabolized to some other substance(s).

3.5. Indole in other arum lilies

Our own studies as well as the work of SMITH & MEEUSE (1966) suggest that indole and skatole may also be present in the ill-smelling inflorescences of certain other members of the Araceae. Accordingly, we have made critical tests for these two simple indoles in several arum lily species at present in cultivation here:

Arum maculatum L. No part of the inflorescence of this plant shows a detectable indole reaction at any stage, although the D-day appendix has an odour reminiscent of decaying urine (KNUTH 1899).

Arum orientale Bierb. The appendix of this plant releases a rather pleasant, somewhat sulfureous, odour when it blooms. No indole reaction was observed in any part of the inflorescence on the day of flowering.

Arum dioscoridis Sibth. and Sm. The presence of skatole in the exhalations of appendices collected on D-day has been established (SMITH & MEEUSE 1966). With the aid of thin-layer chromatography, we have detected a trace amount of indole in addition to the skatole which is a main compound of the odour here. Both indole and skatole appeared only in the appendix at the day of flowering. No quantitative study of indoles was made for this plant, but the Ehrlich reaction at 4:30 p.m. when the appendix heats up is much stronger than that occurring at 9:30 a.m. of the same day. All other parts of the inflorescence show a negative indole reaction.

Dracunculus vulgaris Schott. The odour released by the appendix of this plant on the first day of blooming is comparable to that of rotting flesh (SCHMUCKER 1930). The amount of indole in D-day appendix, harvested at 2 p.m., is about 0.1 μmoles per gram fresh tissue. The appendix was found to be the only part to contain indole, which moreover was present only on D-day.

Helicodiceros muscivorus Engl. (= H. *crinitus* Schott). At the time of flowering the inflorescence of this Mediterranean species also produces a very unpleasant odour, but neither the appendix nor the male and female flowers show any indole reaction.

Lysichitum americanum Hult. & St. John. In contrast to the 5 species just discussed *L. americanum* lacks the specialized, naked, odour-producing appendix; it is largely the conspicuous yellow spathe that displays the osmophore function here. The individual, hermaphroditic, flowers reach maturity over a period of several days. The spathe which remains rigid and healthy during the whole blooming period contains a large amount of indole, up to 0.4 μmoles per gram fresh tissue. The basal part of the spathe contains less indole (0.1 μmoles/g) than the upper parts do. No indole can be demonstrated in the spathe before it unfolds and after it has softened. Neither is indole demonstrable (at any stage) in the thick spadix.

4. DISCUSSION

The experiments presented here clearly demonstrate that the inflorescence of some members of the *Araceae*, during anthesis, contains a considerable amount of indole. This finding adds new items to the limited list of higher plants known to contain free indole (STOWE 1959). It should be mentioned that in most other cases also, indole is found in association with the floral parts. Biologically this may be meaningful since indole makes smells linger (which is the reason why it is used in the perfume industry). Whether indole in arum lilies plays a governing role in the biochemistry of flowering remains to be seen. Its role in attracting potential pollinators is obvious.

In answer to the question why in several instances indole production in arum lilies occurs only during a one-day period, one can point out that it is correlated

with the well-known metabolic flare-up on the first day of flowering, as documented in this study. It has been reported (VAN HERK 1937a & b) that the sudden metabolic changes in the appendix of *Sauromatum* are triggered by a substance, calorigen, which begins to move upward from the male flowers some 20 hours prior to flowering. The activity of this substance has also been demonstrated in several other arum lilies (BUGGELN & MEEUSE 1971). Further studies on the characteristics of this proposed substance and its role in the induction of metabolic changes, including indole formation, are in progress.

Although indole is released during the metabolic flare-up period, the transpiratory loss cannot account for the total disappearance of indole later on D-day. Obviously a large portion of the indole formed is again metabolized, conceivably to tryptophan (cf. CHEN & BOLL 1968). We have evidence (unpublished) that the appendix of *Sauromatum* contains tryptophan synthetase and that the level of free tryptophan on D+1 is higher than on D-day or D-1. It should also be mentioned that indole can fall prey to the action of plant peroxidase (MANN & SMITHIES 1955).

The appendix of *Sauromatum* seems ideally suited for studies of indole-formation and -metabolism in higher plants because it is able both to form and to use indole within a very short time period.

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