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Purification and partial characterization of two biologically active compounds from the inflorescence of *Sauromatum guttatum* Schott (Araceae)

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The active principle triggering the metabolic outburst of the appendices of certain arum lily species has been purified and partially characterized. Although in the past it has been thought of as a single factor ("calorigen"), both paper chromatography and DEAE-Sephadex ion exchange chromatography reveal that there are two compounds (calorigen I and II) with calorigen activity. These can act alone or together, but show no synergism. They are low-molecular compounds which appear to be common constituents of the inflorescence of *Sauromatum guttatum* Schott. Calorigen II is also present in the spadix of *Arum maculatum* L., *Arum dioscoridis* Sibth. & Sm., and *Dracunculus vulgaris* Schott. Calorigen I absorbs UV at 242, 267 and 278 nm, calorigen II at 229 and 295 nm. No spectral changes are noted in alkaline environment, whereas at low pH-values there is a shift of the absorption peaks of both compounds towards longer wavelengths. Calorigen II displays a strong blue fluorescence and has a maximal emission at 410 nm when activated at 295 nm. The possible mechanism of calorigen action is discussed.

A prominent feature of the mature spadix of *Sauromatum guttatum* Schott is the naked, dark-purple, sterile appendix, which in some cases approaches half a meter in length (Fig. 1). Soon after the inflorescence opens the appendix tissue begins to display dramatic metabolic changes. Among its most striking characteristics at that time are an elevated respiration-rate, warming of the tissue, and exhalation of a strong, foul odor.

According to van Herk (*1*, *2*), the metabolic outburst in *Sauromatum* is triggered by a water-soluble, non-proteinaceous agent produced in the mature staminate flowers, which are borne on the spadix just below the appendix. In conversation, he referred to the active principle as calorigen, a term emphasizing the warming of the appendix. On the basis of a series of extirpation- and amputation-experiments he concluded that calorigen begins to leave the staminate flowers to move into the appendix approximately 22 hr before blooming. Calorigen, in his view, acts as a chemical messenger in the sense that it is synthesized in one part of the plant and acts in another. In later studies (*3*, *4*), it was found that the environmental factor "darkness" is a key element in controlling the blooming and the concomitant metabolic changes. The bunched staminate flowers are the receptive site for the

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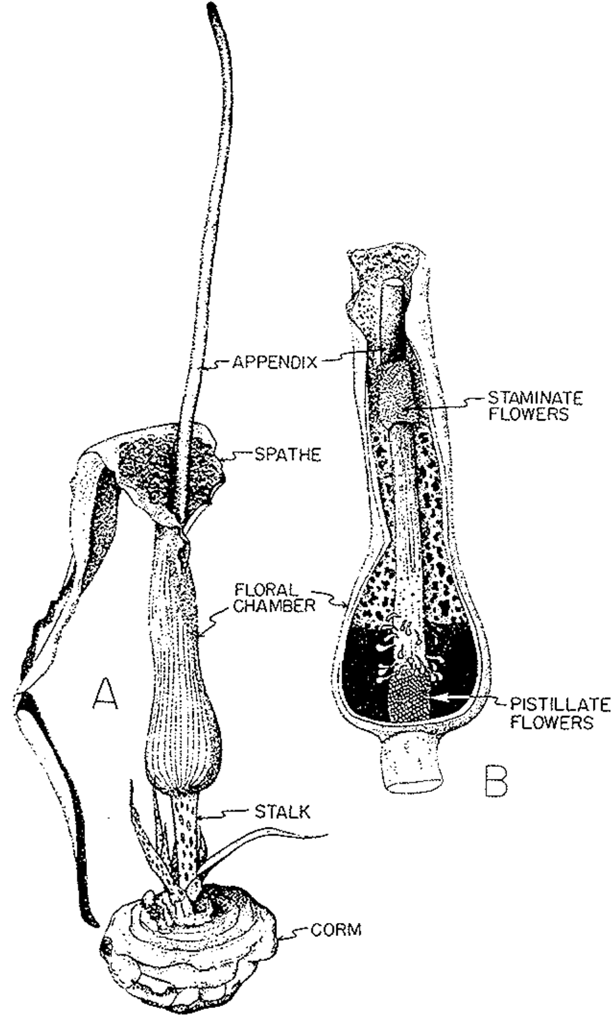

Fig. 1. *Drawing of* Sauromatum guttatum *inflorescence*. A. The inflorescence in bloom. B. Longitudinal section through the floral chamber, exposing the spadix.

dark treatment. The evidence for their role in the blooming of *Sauromatum* has been further strengthened by the discovery of a calorigen-like factor in the staminate flowers of several other arum lily species (*5*, *6*).

Since the discovery of calorigen, extensive studies on the calorigen-induced events have been made. The odoriferous substances, whose function presumably is the attraction of insect pollinators, are a mixture of ammonia, indoles and amines (*7*, *8*). The respiratory climacteric and the production of heat are believed to be

closely connected with the functioning of the cyanide-insensitive alternate pathway of respiratory electron transport; since this pathway is *not* characterized by much oxidative phosphorylation, most of the energy locked up in the storage starch appears within a short time-span in the form of heat (*9*). The identity of calorigen and its mode of action remain unsolved. This unsatisfactory state of affairs may be due to the limited supply of plant materials and the lack of a reliable assay system for calorigen. Recently, we developed a simple and sensitive bioassay procedure based on the ability of calorigen to induce the formation of indole in young appendix sections (*6*). In the present study we have exploited this bioassay method to establish a procedure for the purification of calorigen. Evidence is presented to show that calorigen activity is an effect caused by two low-molecular compounds which can act together or separately and are present at all ages in all parts of the inflorescence.

Materials and methods

Plant materials

Sauromatum guttatum Schott and the other species investigated were grown as described previously (*8*). The various floral parts were harvested on the day of blooming (D-day), or on a specified day a number of days prior to or after D-day, as reported earlier. For the preparation of calorigen, the materials were either used fresh or after storage at −20°C.

Detection of calorigen

In the course of the purification, calorigen was present either in solution or in dry form on a chromatogram. In both cases the bioassay procedure for calorigen described earlier (*6*) was followed. The organic solvent, if present in the fraction, was removed by evaporation, and the aqueous phase was neutralized before use for bioassay. For determination of calorigen on thin-layer plates or filter paper, the air-dried chromatogram was first viewed under a UV lamp to reveal any fluorescent compounds. It was then cut into sections of approximately 1 cm^2, each of which was dipped into the assay solution for the test. At later stages of purification, when some characteristics of calorigen had become known, calorigen was routinely detected by its fluorescence under a short wavelength UV lamp.

Instrumentation

The UV absorption spectra were recorded on a Cary 15 spectrophotometer. A "Turner Spectro 210" fluorospectrometer was used for measurement of the fluorescence spectra.

Results

I. Isolation of calorigen

The procedure for the isolation of calorigen is shown in the flow sheet of Fig. 2. Unless otherwise stated, all manipulations were performed at room temperature.

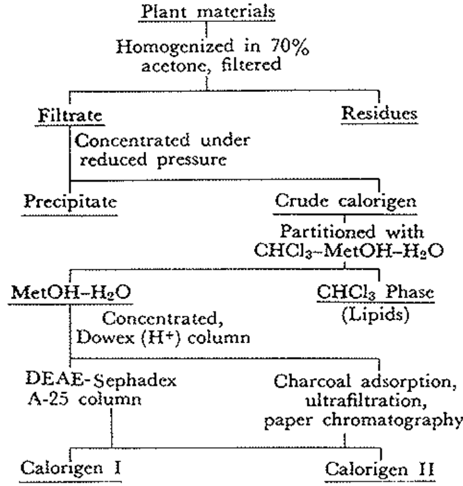

Fig. 2. *Isolation procedure for calorigen I and calorigen II.*

Preparation of crude calorigen

The fresh plant material was ground in a Waring blendor and was then homogenized in an excess amount of chilled acetone in a VirTis 45 high speed homogenizer. The slurry was allowed to stand in the cold for 2 hr and was filtered through a Büchner funnel. The residue was left resuspended in cold 70% acetone for 2 hr and was filtered; the filter cake on the Büchner funnel was washed once with cold acetone. The filtrates were combined, and the acetone was removed by flash evaporation. The remaining aqueous fraction was further concentrated under reduced pressure at 40°C to a small volume. The insoluble materials were removed by centrifugation. At this stage, the preparation has a pH of 6.5 and can be used for bioassay without further treatment.

Removal of lipids

The crude calorigen preparation was fractionationed with a mixture of chloroform-methanol (*10*). After centrifugation, the mixture was partitioned into two layers. Calorigen activity was found exclusively in the methanol-water phase. Methanol was removed by evaporation.

Dowex treatment

The calorigen solution was passed onto a column of Dowex 50W resin (H^+), 50–100 mesh. For the extract from one gram of tissue, one ml of resin bed was used. Upon application of the sample, the column bed turns pink-red due to the presence of anthocyanins. This serves as an indicator during the elution. The column was washed with water until the eluate was colorless. The eluate was neutralized and was concentrated by evaporation. The material was finally taken up in phosphate buffer (pH 7.0) and adjusted to a concentration of 0.01 M with respect to phosphate.

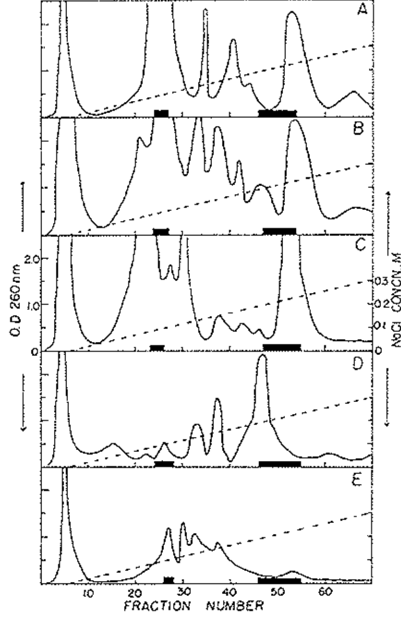

Fig. 3.

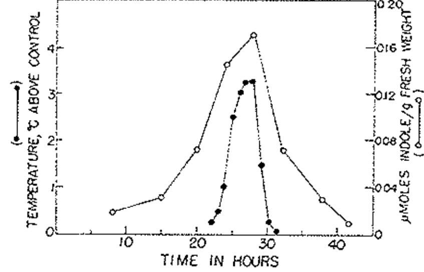

Fig. 4.

Fig. 3. *Elution profile of calorigens from a column of DEAE-Sephadex A-25.* Crude calorigen was prepared from A, appendix; B, the male flowers; C, sterile tissue; D, the female flowers; E, the stalk of *Sauromatum* inflorescences. Fractions with calorigen activity are indicated by a solid bar. The first peak is designated calorigen I; the second peak, calorigen II.

Fig. 4. *Calorigen-induced temperature changes and indole formation in young appendix sections of* Sauromatum. The calorigen was prepared from female flowers of the plant.

Ion-exchange column chromatography

The calorigen extract was chromatographed on a DEAE Sephadex A-25 column (1.5×27 cm) equilibrated with 0.01 M phosphate buffer at pH 7.0. The column was first washed with the buffer until 66 ml (6 fractions) of the eluate had been collected, and was then eluted with a linear gradient of 0 to 0.4 M NaCl in the buffer with the aid of 2 vessels of 500 ml each. The flow rate was 45–50 ml per hour. Since the tissues under study contain a considerable amount of nucleotides (*11*) the UV absorption at 260 nm for each fraction was also recorded for reference. Aliquots of 0.5 ml of each fraction were taken for detection of calorigen activity. The presence of NaCl in the fraction does not interfere with the bioassay. The elution profiles of calorigen from the column are presented in Fig. 3. From the results it is evident that calorigen activity appears in two fractions. The first peak will hereafter be designated as calorigen I; the second peak, as calorigen II.

The fractions with calorigen activity were pooled and charcoal (Nuchar 1.5 g/100 ml) was added. The absorbed materials were subsequently eluted with 15% pyridine; the organic solvent was removed from the eluate by extraction with

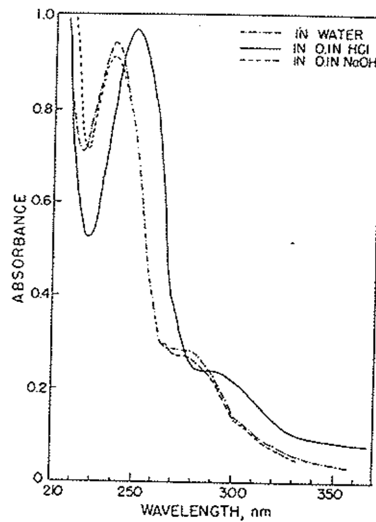

Fig. 5.

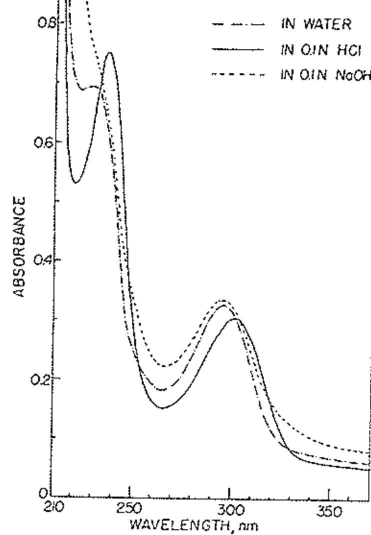

Fig. 6.

Fig. 5. *Ultraviolet absorption spectra of calorigen I.*
Fig. 6. *Ultraviolet absorption spectra of calorigen II.*

for calorigen studies. In order to determine the identity of the active principle in other aroids, extracts of staminate flowers were prepared from *Arum dioscoridis* Sibth. and Sm., *Arum maculatum* L., and *Dracunculus vulgaris* Schott. After removal of lipids, these extracts were put on paper side by side with the *Sauromatum* calorigens and developed with the butanol-acetic acid-water system. When viewed under UV light, a blue fluorescent band with an R_f equivalent to that of *Sauromatum* calorigen II was notable in each sample. The chromatogram was later assayed for calorigen activity. It was found that only one area shows the activity, i.e., causes indole formation in the *Sauromatum* appendix sections. The R_f for the active compound coincides with that of the blue fluorescent band, thus providing evidence that the compound is identical with calorigen II of *Sauromatum*. The other component, calorigen I, was not detectable in the *Arum* and *Dracunculus* species.

Spectral analysis

The UV absorption spectra of calorigen I and II obtained after the second TLC step are shown in Fig. 5 and 6, respectively. The spectra were measured in alkaline, near neutral and acidic conditions. It was found that in water and in alkaline conditions calorigen I has the same absorption pattern. It exhibits a sharp absorption peak at 242 nm and has absorption "shoulders" at 267 and 278 nm. In acid, the major peak shifted to 250 nm, while now a single shoulder at 295 nm was noted. In the case of calorigen II, the absorption peaks are very

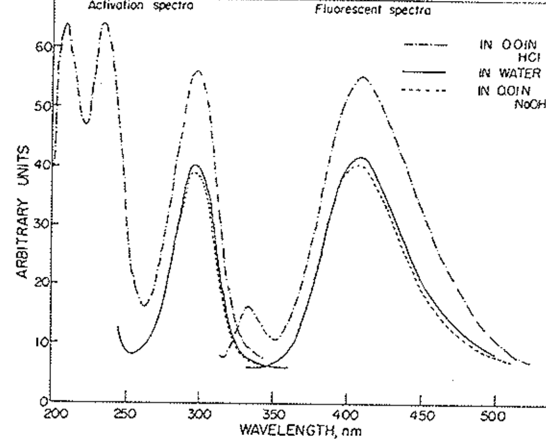

Fig. 7. *Activation and fluorescence spectra of calorigen II.* The spectrum in acid has been magnified 10 times.

distinct. Again, the light-absorption patterns under neutral and alkaline conditions are very similar. There is a peak at 229 nm, reduced to a shoulder at high pH, and another one at 295 nm. In acid, both peaks shift toward a longer wavelength to give absorptions at 236 and 301 nm. It should be mentioned that in both calorigen components the shifts of the absorption peak in acid are reversible by the addition of NaOH.

The activation- and fluorescent spectra of calorigen II are shown in Fig. 7. The same preparations as used for UV analysis above were used for the study. It is clear from the results that the maximal excitation coincides with the spectral range of UV absorption. The maximal emission occurs at 410 nm at all pH's tested. However, in acid the magnitude of emission was greatly reduced. Calorigen I shows no significant emission outside the UV range.

Other properties of calorigens

The calorigens are low-molecular compounds. They are dialyzable and pass through a membrane filter which retains substances with a molecular weight greater than 1000. In contrast to an earlier report (*2*), the calorigens are very stable. Repetitive pH changes, solvent treatments, and autoclaving for 15 min fail to destroy the calorigen activity. In dry form (on a paper chromatogram) calorigens are still active after more than two years at room temperature. Both calorigens are very soluble in water and other polar solvents, and insoluble in chloroform or ether. On a filter paper chromatogram run with the butanol-acetic acid-water system the R_f for calorigen I is 0.58 and for calorigen II 0.92. When the acid component is replaced by ammonia, both calorigens remain on the startline.

Discussion

The methods described here permit, for the first time, the isolation of calorigen of high purity. Small modifications of the procedure enabled us to prepare

calorigen from minimal amounts of plant material. Our isolation attempts have also improved our knowledge of the biochemistry of the arum lily inflorescence. For example, it is demonstrated here that, in *Sauromatum*, calorigen-effects are actually mediated by *two* compounds, tentatively designated as calorigen I and calorigen II. Furthermore, "calorigen", which until now was considered to be present only in the mature "male" flowers, appears to be a common constituent of the *Sauromatum* inflorescence: both calorigen I and calorigen II were found in all parts of the inflorescence at various stages tested. Calorigen II was also present in the spadix of the three *Arum*-species studied.

The metabolic changes induced by the calorigen compounds, (the development of a respiratory climacteric, and the production of heat, indole and stench) are identical. In each case, a lag time of 7 to 8 hr is required for the formation of indole by the tested appendix sections, and the peak of heat production (or, at least, the highest temperature) is reached approximately 27 hr after the start of the assay. In terms of the amount of indole formed, the two calorigen components show no synergistic effects. Since the metabolic changes observed in vitro are indistinguishable from the normal processes occurring on the day of blooming, it can be assumed that the two calorigen components that were isolated are functional in the living inflorescence.

The present finding that calorigens are common constituents of various parts of the *Sauromatum*-inflorescence contradicts an earlier report (*2*) in which it was concluded that calorigen is formed only in the staminate flowers and is, at the time of maturity, transported to the appendix tissue for action. The discrepancy can plausibly be ascribed to the fact that in the present study a sensitive bioassay was used. The evidence indicating that the two calorigens isolated here are, in combination, identical with van Herk's "calorigen" is satisfactory. However, one may legitimately ask why the young appendix tissue, which (according to our data) itself contains calorigen, is responsive only to added calorigen and fails to develop the unique metabolic flare-up at any time before blooming. A clue to the answer can perhaps be provided by considering certain observations we have made. Normally, the entire inflorescence, including the spathe, shows a "post-pollination phenomenon" in the form of senescence (wilting, shriveling-up) two days after blooming, even when no pollination has been forthcoming. This senescence seems to be linked with the exhaustive breakdown of fuel (in *various* parts of the inflorescence!) during the metabolic outburst. Conversely, senescence can be used as indicative of the development of a metabolic outburst in the spathe and the floral chamber. In our investigations, we have noticed that the spathe and the floral chamber of an inflorescence with aborted spadix or with aborted staminate flowers and the appendix remain physiologically healthy (so that the cells retain their turgidity) many days after blooming. Amputation of the appendix from a normal inflorescence does not delay the senescence of other parts. If the spadix is cut off at a level between the staminate and the pistillate flowers and the cut end is put in water, the development of heat and smell in the appendix as well as the withering of the spathe and the floral chamber are as usual if the operation is done within one day before blooming; if done earlier (e.g., 37 hr) before expected blooming-time the senescence of spathe and floral chamber is delayed and the appendix still displays the metabolic flare-up at the expected time. These findings indicate that the development of a complete, normal sequence leading to senescence of the entire inflorescence is dependent on the presence of intact staminate flowers—which have also been shown to be instrumental in mediating the light- (or rather, dark-) effects on the behavior of the inflorescence. However, in view of the fact that according to the bioassay calorigen activity can be extracted from *various* parts of the inflorescence, at different developmental stages (and not only from the staminate flower primordia), it seems plausible to assume that in young tissue calorigen is present in an inactive or bound form and that it becomes active, or available for action, only upon extraction. To explain the role the male flowers play, one can then go one step further and assume that the male flowers, in response to an environmental factor or their own physiological condition, signal or release a messenger to *all* other parts of the inflorescence about 22 hr before normal blooming-time. This would fit the original theory proposed by van Herk (*1*), except that calorigen per se is not involved in a transport system and that another messenger might be involved.

It appears that the *Sauromatum*-system discussed here could serve as a biological model for the study of regulatory mechanisms. It involves a rapid breakdown of protein and starch, and the formation of a series of intermediates involved in amino-acid and indole formation. Studies of the *Sauromatum*-system can best be done with calorigen of high purity, which is now available. Complete chemical identification of the calorigen should prove to be of particular interest.

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References

(*1*) Van Herk, A. W. H.: Die chemischen Vorgänge im *Sauromatum*-Kolben II. *Proc. Kon. Akad. Wet.* (Amsterdam) 40: 607–614 (1937).

(*2*) Van Herk, A. W. H.: Die chemischen Vorgänge im *Sauromatum*-Kolben III. Ibid. 40: 709–719 (1937).

(*3*) Meeuse, B. J. D. and R. G. Buggeln: Time, space, light and darkness in the metabolic flare-up of the *Sauromatum* appendix. *Acta Bot. Neerl.* 18: 159–172 (1969).

(*4*) Buggeln, R. G., B. J. D. Meeuse and J. R. Klima: The control of blooming in *Sauromatum guttatum* (Araceae) by darkness. *Can. J. Bot.* 49: 1025–1031 (1971).

(*5*) Buggeln, R. G. and B. J. D. Meeuse: Hormonal control of the respiratory climacteric in *Sauromatum guttatum* (Araceae). Ibid. 49: 1373–1377 (1971).

(*6*) Chen, J. and B. J. D. Meeuse: Induction of indole synthesis in the appendix of *Sauromatum guttatum* Schott. *Plant & Cell Physiol.* 13: 831–841 (1972).

(*7*) Smith, B. N. and B. J. D. Meeuse: Production of volatile amines and skatole at anthesis in some arum lily species. *Plant Physiol.* 41: 343–347 (1966).

(*8*) Chen, J. and B. J. D. Meeuse: Production of free indole by some arum lilies. *Acta Bot. Neerl.* 20: 627–635 (1971).

(*9*) Wilson, R. H. and B. N. Smith: Uncoupling of *Sauromatum* spadix mitochondria as a mechanism of thermogenesis. *Z. Pflanzenphysiol.* 65: 124–129 (1971).

(*10*) Bligh, E. G. and W. J. Dyer: A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911–917 (1959).

(*11*) Meeuse, B. J. D., R. G. Buggeln and S. N. Summers: Nucleotide levels in the appendix of *Sauromatum* and *Arum* (Araceae) during the flowering sequence. XI Intl. Bot. Cong., Seattle, Washington, p. 144 (1969).