than pigment values for prediction of digestibility. On the other hand, the pigment results are significantly better than the methoxyl ones in two cases, one at the 1% level (experiment 460, forage) and one at the 5% level (total feces from sheep). The chromogen determination proved significantly better (1% level) than pigment in only one experiment (experiment 477).

Thus, the data in Table I show that the pigment determination is at least as good as the methoxyl or chromogen determinations for prediction of dry-matter digestibility.

In the case of experiments 408 and 460, the pigment results were calculated on an organic-matter basis and compared with the organic-matter digestibilities. A regression analysis was carried out, and the results are shown in Table II. In general, the results are similar to those obtained on the dry-matter basis.

Since many workers routinely carry out proximate constituent analyses on their samples, it would be desirable to include this crude pigment determination in the analysis of the proximates either instead of, or in addition to, the crude fat determination. In a further investigation using the samples of experiment 482, the pigment extraction with benzene–ethanol was carried out following a crude fat determination using petroleum ether (b.p. 30° to 60° C.). By this method, the crude fat-free pigment averaged 19.49 and 9.50% in the forage and feces, respectively, and the correlation coefficients with dry-matter digestibility were 0.901 and 0.872, the regression equations were $y = 27.09 + 2.18x$ and $y = 42.67 + 2.84x$, and the standard errors of estimate (8 and 27 degrees of freedom) were 3.603 and 3.849, respectively. Without this pre-extraction of the crude fat, the average pigment values were 21.49 and 13.87% for the forage and feces, respectively.

This result indicated that the pigment determination could be carried out along with the proximate constituent analysis, provided the crude fiber results were not altered. To test this, 12 samples of forage and 12 of feces were analyzed for crude fiber both in the normal way after extraction with petroleum ether and also following a further extraction with benzene–ethanol. An analysis of variance carried out on the results showed that there was no significant difference between the two methods. Thus, the crude fiber results were not affected by the prior extraction of pigment.

This means that if crude fat values are not required then the crude pigment determination can be substituted in the regular proximate constituent analysis. If, however, the crude fat values are wanted, then the pigment determination can be carried out following the extraction of the crude fat and preceding the crude fiber determination. The appropriate regressions would be used in each case.

Conclusion

The results obtained indicate that the gravimetric determination of pigment described here can be used to predict digestibility in much the same manner as chromogen and methoxyl values are used. The determination is readily carried out, requiring only an extraction apparatus, a vacuum oven, and a balance. Satisfactory results are obtained on dried samples, precautions such as must be taken with the chromogen method are not needed, and satisfactory correlations with digestibility are obtained using either forages or feces. The determination can, if desired, be carried out along with the regular proximate constituent analysis.

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CHEMICAL CONTROL OF FLOWERING

Concentration of a Floral-Inducing Entity from Plant Extracts

DARWIN L. MAYFIELD,[1] RICHARD G. LINCOLN, ROBERT O. HUTCHINS, and ALAN CUNNINGHAM

Departments of Chemistry and of Botany, Long Beach State College, Long Beach, Calif.

DURING the past 25 years, experimental evidence has been accumulating to support the hypothesis that a flowering hormone or class of hormones exists. It has been proposed that such a hormone is in effect the stimulus which signals differentiation of the cells of a growing plant site from the vegetative to the flowering state. A comprehensive and critical summary of current knowledge concerning the physiology and chemistry of the flowering processs is given by Hillman (*3*).

Chemical control of the flowering process, obviously, would be of great assistance in the elucidation of the mechanism of plant reproduction and would have applications in various specialized areas of agriculture. Attempts to isolate from the living plant and to identify chemically the hypothetical hormone, named florigen by Chailakhyan (*2*), have been vigorous but singularly unsuccessful. Lang and Reinhard (*4*) have shown that the various gibberellins have a function, perhaps indirect, in the process of flower formation in plants which are classified as long-day (*3*) as far as day-length or photoperiod requirements for flowering are concerned.

To date, no single chemical entity or small number of chemical compounds

[1] Present address: Centre National de la Recherche Scientifique, Gif-sur-Yvette, Seine-et-Oise, France.

A preparation was reported in 1961 of an extract of flowering *Xanthium strumarium*, a photoperiodically sensitive short-day plant, which would initiate flowering in other *Xanthium* (cocklebur) plants maintained under conditions of illumination such that flowering otherwise would not occur. The procedure for extracting the dried plant material has been modified. Techniques for rapid preparation of more than 100-gram quantities of the crude extract have been developed. *Xanthium* test plants grown under continuous illumination have been caused to flower by single applications of the crude extract. The active principle in the *Xanthium* extract, in mixture with varying amounts of other substances, has been concentrated by paper chromatography. The active entity is soluble in solvents of intermediate to strong polarity and has been concentrated also by utilizing aqueous acid-base solubility differences. Certain other chemical properties of the active principle are outlined.

acting in concert has been found which will reproducibly initiate flowering in plants of the short-day type in the photoperiod classification. Short-day plants are defined as those which require continuous dark periods of particular minimum durations for floral initiation to occur. Several reports have appeared in the literature concerning the floral-inducing capabilities of various plant extracts (*1*, *8–10*).

In the summer of 1960, an extract was prepared of flowering *Xanthium strumarium*, a photoperiodically sensitive short-day plant, which initiated flowering in other *Xanthium* (cocklebur) plants maintained under conditions of illumination such that flowering otherwise would not occur (*5*). It is the purpose of this paper to report continuing work on the chemical nature of the active entity in this *Xanthium* extract. The work is directed toward ultimate isolation and structural characterization of the chemical substance responsible for the observed flowering effect, but to date only a partial concentration of the crude extract has been achieved.

Experimental Methods and Results

Preparation of Crude Active Extract. Earlier procedures (*5*) for the preparation of an extract from *Xanthium*, which has floral-initiating activity, have been modified in certain respects. Since lack of reproducibility has characterized several preparations of plant extracts with reported activity, the experimental procedures for the preparation of this *Xanthium* extract will be described in detail.

Figure 1 summarizes the process for obtaining crude extract. The weight relations given are merely illustrative. Amounts of freshly picked plant material varying in weight from 100 grams to as much as 60 kg. have been worked up according to this general procedure.

The source has been the leaves of indigenous *Xanthium strumarium L. var. canadense* (Mill.) T. and G. growing within a radius of 20 miles of Long Beach, Calif. The plants were found in drainage ditches, in dry river beds, and in gullies carrying irrigation runoff. Only leaves branching within 4 cm. of a vigorous inflorescence (the individual staminate inflorescence ranged from 0.5 to 1.5 cm. in diameter) were picked. Stems and burrs were not used. Activity has been found in extracts from plants which flowered in any of the 5 months from June to October. No activity was found in extracts from the leaves of flowering *Xanthium* grown in the Botany Greenhouse at the University of California at Los Angeles and induced by several exposures to dark periods longer than the 8-hour, 40-minute critical length for *Xanthium*.

Leaves were extremely rapidly frozen in direct contact with liquid nitrogen with a delay of no more than 5 minutes after scission from the stalk. The stalks themselves were packed in wet sacks with leaves unsevered and rushed to the laboratory after being gathered from the field at night or during a cool part of the day.

The frozen leaves were mechanically fragmented and lyophilized to less than 2% water content in a laboratory freeze-dry apparatus or in commercial equipment. Sublimation of the ice required 2 to 4 days at −10° C. Several lyophilizations of 40 to 60 kg. each of frozen, fragmented leaf material have been performed. Dried leaf material was stored at −20° C. until extraction.

Leaf material to be extracted was powdered in a Waring Blendor, slurried in cold (0° to 10° C.) absolute methanol, and filtered in a Büchner funnel. The residue was extracted with additional portions of methanol. To extract 73.5 grams of dried leaf material (Figure 1), a total of approximately 600 ml. of methanol in three or four portions was necessary. The bright green filtrate was placed in a round-bottomed flask connected to a Rinco evaporator, and the methanol removed under reduced pressure. The rotating flask was immersed in a container of circulating cold water. When the volume had been reduced sufficiently, the filtrate was transferred to a two-piece resin flask from which the dark green tarry residue could be easily removed after evaporation was complete.

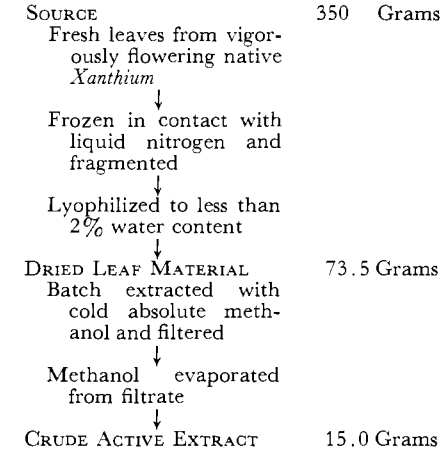

Figure 1. Flow diagram of preparation of crude active extract

This residue, the crude active extract, was stored in a deep freeze until used.

Bioassay Procedure. The bioassay, as described in this work, is strictly a qualitative assay of floral initiation. Plant extract to be tested for activity in such a physiological process was mixed to homogeneity with enough anhydrous lanolin to give a total of 1 to 2 grams of extract mixture for application to each test plant. Typically, for treatment of a single test plant, about 0.2 to 0.6 gram of extract was blended with 1 gram of lanolin. For test purposes, young *Xanthium* plants (Chicago type) with four to eight fully developed leaves, grown from seed under conditions of 20 hours (and in some cases continuous) illumination, 20° to 26° C. temperature, and with no additional nutrients other than those in the planting mixture, were used.

The lanolin-extract mixture to be tested was applied in a thin layer to the total underside surface of the uppermost four, fully developed leaves of the test plants. The treated plants, together with an equal number of control plants, were placed in growth chambers. Temperatures were maintained at 21° to 25° C. in the growth chambers, and illumination (500 foot-candles at the leaf surface) was either continuous or a precise 20-hour light, 4-hour dark regime. In 17 to 26 days after applica-

Table I. Flowering Response Due to Application of Crude Active Extract

Material Applied to Test Plants					Elapsed Time from Treatment to Dissection, Days	Flowering Response			
						Test plants		Controls	
Crude extract, grams	Lanolin, grams	Test Plants Treated	Controls Used	Illumination Conditions		Number flowering	%	Number flowering	%
25	60	40	40	4 Hrs. dark in 24 hrs.	21	9	22.5	0	0
2	10	10	10	Same	17	2	20.0	0	0
25	50	40	45	Same	21	8	20.0	0	0
20	30	25	25	Continuous (500 ft. ca.)	23	4	16.0	0	0

Table II. Flowering Response of Various Partially Concentrated Extracts

Experimental Sample[a]	Weight Extract Applied, Grams	Weight Lonolin Used, Grams	Test Plants Treated	Controls Used	Illumination Conditions	Elapsed Time from Treatment to Dissection, Days	Flowering Response			
							Test plants		Controls	
							Number flowering	%	Number flowering	%
A	0.30	10	10	20	4 Hrs. dark in 24 hrs.	26	2	20	0	0
B	15.3	20	19[b]	40	Same	20	7	37	0	0
C	11.7	25	25	40	Same	20	4	16	0	0

[a] (A) Fraction of crude *Xanthium* extract separated by paper chromatography at R_f 0.42 to 0.65.
(B) Aqueous base-soluble, aqueous acid-insoluble, ether-soluble portion of crude *Xanthium* extract.
(C) Aqueous base-insoluble fraction from B, chromatographed on paper and eluted from R_f 0.20 to 0.65 section.
[b] One test plant accidentally destroyed after treatment.

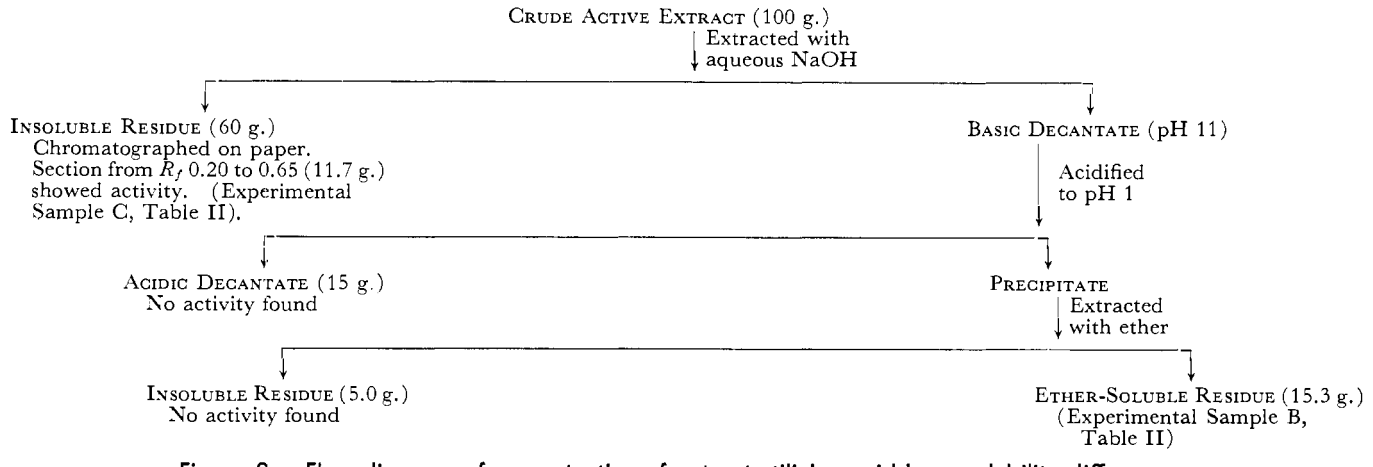

Figure 2. Flow diagram of concentration of extract utilizing acid-base solubility differences

tion, the plants were dissected, and the flowering response was recorded (*7*). Buds were judged to be flowering when, at minimum, the first morphological change to flowering in the stem apex was easily recognizable. All others were classed as vegetative. Controls, without exception, were vegetative in experiments reported here.

The number of plants treated in a particular experiment varied from 10 to 40. An individual test plant could not be too heavily loaded with a single application of extract without subsequent leaf damage. The upper limit appeared to be about 0.75 gram of crude extract per plant.

In addition to untreated controls, several experiments were conducted wherein test plants were treated with lanolin alone, with a lanolin-chlorophyll or lanolin-lampblack mixture, and with a lanolin-nonflowering cocklebur leaf extract. None of these treated controls flowered.

Concentration of Crude Extract by Filter Paper Chromatography. Six experiments have been performed employing filter paper chromatography to concentrate the active extract. In each case, active material has been eluted from an area located in the middle third of the developed length of the paper with no other active area located. The filter paper sheets were heavily loaded with extract (up to 0.6 gram per sheet of Whatman No. 1, 2.5 grams per sheet of Whatman No. 3 MM, and 6.0 grams per Whatman No. 17) to avoid large amounts of paper in subsequent elution steps.

In a typical experiment, 15.0 grams of crude *Xanthium* extract dissolved in 60 ml. of methanol was streaked on 24 sheets of Whatman No. 1 paper. The streaks were allowed to dry by evaporation, and the paper was equilibrated (24 hours) in solvent vapor and then developed (24 hours) at 25° C. using the upper layer of a mixture of 1-butanol, glacial acetic acid, and water (19:1:6 parts by volume) as the descending phase.

As a consequence of overloading the filter paper, the various visible bands, particularly those of the chlorophylls, were wide and wavy. The carotenes moved only short distances from the origin.

The paper, after drying, was cut into five strips based on the appearance under ultraviolet radiation. Each set was eluted with absolute methanol, the methanol was evaporated, and the residue tested in the usual way. The band in the R_f region 0.42 to 0.65 contained the active material while the other four bands showed no activity. The active band had no visible color and gave only a faint fluorescence in ultraviolet light.

Ninety per cent of the material applied to the paper was recovered. Two thirds of this was found in the lower third of the paper and only 2% (0.3 gram) in the active band. The active concentrate was a light tan, viscous oil and is listed as Experimental Sample A in Table II.

Extracts prepared from the indigenous sunflower, *Helianthus annuus*, a day-neutral plant in photoperiodic response, have been found to be capable of initiating flowering in *Xanthium* test plants (*6*). The *Helianthus* extract was prepared by methods identical to those used for *Xanthium*. Preliminary concentration experiments with the *Helianthus* extract using paper chromatography showed movement of the active material in the paper to be similar to that in *Xanthium*.

Concentration of Crude Extract Utilizing Acid-Base Solubility Differences. Crude *Xanthium* extract, shaken with 10 times its volume of water, gave an aqueous phase of pH 5.6. A total of 1.5 mmoles of sodium hydroxide and 15 ml. of water in contact with 1.0 gram of extract gave an aqueous phase of pH 9.6. Such properties suggested a separation scheme which is outlined in Figure 2 using as illustration the data from one of several such separations.

The initial basic extraction of the crude active extract was performed with three or four portions of aqueous base of such concentration that the pH of the combined decantates was in the range 11 to 12. Emulsion formation hindered clean separation of the insoluble residue, but centrifugation and addition of sodium sulfate solution partially alleviated this difficulty.

The insoluble residue in the experiment used as illustration was dissolved in ethyl acetate, applied in a stripe to 16 sheets of Whatman No. 3 MM chromatography paper, and developed with a descending phase of 1-butanol–acetic acid–water. The paper was cut into three sections; the upper contained the yellow and orange pigments, the lower the green pigments, and the middle (R_f 0.20 to 0.65) no visible colored material. Each section was eluted with methanol following which solvent was evaporated. Only the middle section showed floral-initiating activity (Experimental Sample C, Table II).

The aqueous basic decantate from extraction of the crude extract was acidified to pH 1 with concentrated HCl. A dark-brown, sticky precipitate separated which was partially soluble in ether. The ether-soluble fraction, on evaporation of the solvent, was viscous and brown in color and exhibited weak floral-initiating activity (Experimental Sample B, Table II).

Discussion of Experimental Results

Tables I and II summarize results of several typical experiments utilizing two general approaches to concentrating the crude active extract. The experiments selected are representative of many others and show comparable flowering response. In these many experiments, usual flowering response (if it has been exhibited at all) has been in the 10 to 40% range with a very few instances above 40% and none above 60%.

Since cocklebur is remarkably sensitive in its flowering response, these results are indicative of a definite active entity in the applied extract. Applications to test plants of colored materials such as bone black or chlorophyll mixed in lanolin have given no response. The consistently low flowering response suggests a threshhold requirement for the stimulus in *Xanthium*. To exceed this threshhold in 80 to 90% of test plants should require either a greater concentration of hormonal material applied to the leaves or a greater uptake of that already available than is afforded by the application method presently used. Interaction between flowering inhibitors and a flowering hormone may also be a factor.

The bioassay is primarily qualitative and does not indicate whether applied amount or degree of uptake or both are responsible for the experimental flowering response. A quantitative assay of florigen activity, either chemical or biological, is greatly needed. Dilution effects are currently being studied as are other methods of application of the active material (including multiple applications).

The absence of increased flowering response on application of partially concentrated extracts needs explanation. The removal of the major part of the yellow and the green pigments as well as considerable amounts of other plant substances from Experimental Samples A and B (Table II) is apparent. Perhaps florigen is being destroyed or lost, or possibly limited uptake is the explanation.

Certainly, the hormonal material appears to be reasonably stable. An essential step in the extraction process may be the rapid cooling of the leaf material to a temperature of −196° C. with consequent denaturation of enzymes which might promote degradation or inactivation of the active factor. In qualitative terms, the experiments previously described as well as related ones have shown that the active entity in the crude extract is:

Not deactivated by a few hours contact with aqueous solutions ranging in pH from 1 to 12.

Not particularly sensitive to exposure to normal light or to air.

Soluble in solvents of the approximate polarity of water, methanol, acetone, ethyl acetate, and ether, and much less soluble in petroleum ether.

Not destroyed on being maintained at a temperature of 45° C. for 3 hours.

Possibly composed of two or more distinct compounds with floral-initiating activity (Experimental Samples B and C, Table II).

No estimate of the actual amount of florigen necessary to cause a visible flowering response has been possible. West and Phinney (*11*) have shown that 0.001 μg. (3×10^{-12} moles) of pure gibberellic acid will produce a visible growth response in a single plant. If florigen is active in similar absolute amounts and is present in flowering plants at low levels, much remains to be done before the pure chemical entity is isolated. Standard methods, including paper, column, and ion exchange chromatography as well as counter current distribution, are presently being applied.

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