A CHEMICAL AND PHYSIOLOGICAL STUDY OF TRAUMATIN, A PLANT WOUND HORMONE

JAMES BONNER AND JAMES ENGLISH, JR.

(WITH THREE FIGURES)

Introduction

The first tentative formulation of the "wound substance" concept was made by WIESNER some 50 years ago. In his "*Elementarstruktur*" (**25**) he suggested that it might well be that *substances* formed or produced by wounded cells pass from these cells to neighboring uninjured tissue and there bring about such phenomena as callus formation and regeneration; bring about, in short, a resumption of meristematic activity by cells apparently mature. Sound experimental evidence in support of the wound substance hypothesis was obtained by HABERLANDT (**5–9**). His classical potato experiment should be particularly stressed. He found that discs cut from a potato tuber showed cell divisions leading to periderm formation only if (a) phloem and (b) "wound hormone" were present. This wound hormone appeared to come from contents of injured cells at the cut surface of the disc. In a number of different ways HABERLANDT demonstrated that both the influence coming from the phloem and that coming from the surface are diffusible chemical substances and that the interaction of the two is necessary for renewed division of the mature parenchymatous cells of the potato tuber, as well as of the kohlrabi root (**8**). In a later publication (**8**) HABERLANDT showed that in other cases also, division of mature cells may be induced by the cooperation of "lepto-hormone" and "wound hormone." By judicious dissection of the leaves of succulents, surfaces of uninjured cells could be exposed. The cells of these uninjured surfaces were capable of responding to the application of tissue juice from other leaves with a vigorous renewal of cell division activity.

REICHE (**19**) confirmed the results of HABERLANDT in another way. She injected the petioles and stems of various plants (Nymphaeaceae, *Solanum*, *Gratiola*) with dilute tissue extracts and found that cell divisions were induced wherever the *uninjured* cells of the stem or petiole came in contact with the extract of *injured* cells. She was inclined to the view, however, that the activity of the extracts resided in suspended cell fragments rather than in soluble substances. WEHNELT (**23**), whose work with the parenchymatous lining of bean pods will be discussed more extensively later, found that these intact parenchymatous cells react rather to a water soluble, heat stable substance present in tissue extract. Another favorable object for the demonstration of wound hormone activity was found by WILHELM (**26**) in the parencyhmatous lining of the hollow stem of *Vicia faba*.

It may be concluded that it is a well established fact that when a plant is injured, substances are formed or liberated which are capable of causing other uninjured cells to resume active growth. These substances may well be called wound hormones since they are carriers of correlation between one portion of the plant and other portions. They appear in some cases to be also "cell division substances." It is equally well established that in many cases the wound hormone acts only in conjunction with a second factor contained in the phloem. This factor, however, is less well known and is less amenable to study than is the wound hormone itself.

The chemical nature of the wound hormone has not been studied previously in any detail. In fact, its very existence as a chemical individual might be considered as thrown into doubt by the work of WEHNELT and WILHELM, who have shown that the most diverse and ill assorted substances possess some typical wound hormone activity. The chemical properties of the active principle of tissue extract are also in question since it has been reported by HABERLANDT that it is heat labile, by WEHNELT that it is heat stable, and by REICHE that it is not soluble in water but is heat stable. In this connection it should be remembered that different test objects may actually respond to different wound hormones.

That the wound hormone possesses considerable interest both from the theoretical and the practical points of view is perhaps obvious. In the wound hormone we have a substance which is capable, under suitable circumstances, of bringing about renewed growth activity of otherwise mature cells. The rôle of wound hormone in wound healing, parthenogenesis, adventive embryony, callus formation, etc., has been discussed in detail by HABERLANDT (**9**) and need not be gone into here. Attention has been called more recently to the rôle of wound hormone in the culture *in vitro* of plant tissues (**2**). In the present work, an attempt has been made to work out *one quantitative* test for wound substance activity, and, using this test, to purify the active principle. The work, therefore, has been confined to one test object. The test was developed from that suggested by WEHNELT (**23**). Since the work was undertaken in the fall of 1935, several papers bearing on the subject have appeared (**13, 15, 17, 20, 22**). These will be discussed later as the appropriate connections arise.

Experimentation

PRINCIPLE OF THE TEST

The use of the immature bean pod for the demonstration of the action of wound hormone is due to WEHNELT (**23**), who worked out a qualitative activity test and determined a number of facts concerning the physiology and chemical properties of the active principle.

The immature bean pod merely need be slit lengthwise along suture and

midrib, and the unripe seeds removed, to expose the layer of uninjured parenchymatous tissue which lines each seed chamber. If this layer of tissue is then injured, for example, by a prick with a needle, the cells in the region of the wound divide and enlarge so that a small "neoplasm" or intumescence projecting above the level of the surrounding tissue is formed. A much more striking result may be obtained by the application of juice from crushed beans. If a drop of such bean juice is applied to the uninjured surface it is absorbed in about 24 hours. Under the point of application of the drop and before it is completely absorbed, a cylindrical intumescence begins to arise, which may attain a height of as much as 3 mm. in the course of 48 hours. Histologically the intumescence consists of cylindrical parenchymatous cells elongated at right angles to the surface of the seed chamber.

The change in wall structure of these cells during the course of the reaction is a point of some interest. The normal parenchyma of the seed chamber consists of approximately isodiametric cells and these possess the foliar arrangement of cell wall micellar units which is typical of such cells (**1**). They are isotropic when viewed in tangential section between crossed nicols of the polarizing microscope. During the course of the wound hormone reaction the shape of these cells changes to cylindrical due to the extensive cell elongation which takes place. The elongating cells are anisodiametric and they show the corresponding anisotropy of cell wall structure which is typical of elongating cells. The micellar units are arranged in a direction which is, statistically, perpendicular to the axis of elongation (**1**).

Normal mitotic cell divisions occur in the new growth, their frequency depending upon the species and variety of bean used. In a given variety of bean the frequency of cell division depends upon the concentration of the applied extract as does the height of the intumescence. With increasing size, the total number of cell divisions increases. The intumescence is thus a *product* of simultaneous cell division and cell enlargement. In our experience those varieties of beans which give large intumescences in response to the addition of wound hormone also show extensive cell division, whereas those varieties which give intumescences of limited size may show few or no cell divisions. As far as has been observed during the present work, cell divisions appear to be essential to the formation of large amounts of new growth. Previous authors who have investigated the wound hormone response of the bean pod have interested themselves chiefly in this cell division activity (**23, 13**). The quantitative estimation of the latter, however, is at best tedious and hence difficult of application to large scale routine testing. Furthermore, investigators who have *confined* themselves to estimation of division frequency, as will be shown later, have been misled on important points. In the present investigation the *height* (size) of the intumescence, a readily and quantitatively determinable quantity, has been chosen as the criterion of wound substance activity.

THE QUANTITATIVE TEST

It was found quite possible, with the observance of a number of precautions, to place the "bean test" upon a quantitative basis. A large and constant supply of beans was essential. This was obtained through the cooperation of the Lake Farm Produce Co.[1] The beans (Kentucky Wonder) to be used on any given day were selected from a fresh, high grade stock (one pound chosen from 50 to 150 pounds). Only uniformly firm, immature (not rounded out by the developing seeds) pods of dark-green color were selected. On those days when fresh beans were not available, stock which had been stored at 35° F. was used. Such pods, however, gave less reliable results, apparently owing to the fact that they were extensively dried out. Tests were made at the same hour each day in order to avoid, among other things, effects of diurnal fluctuation such as are found in the *Avena* test (**14**).

The pods were slit lengthwise along suture and midrib (fig. 1), opened, and the seeds removed. The individual seed chambers were then cut from the pod and arranged in Petri dishes upon moistened absorbent paper. The apical chamber and the basal one from each pod were rejected since these are less reactive than the others. The "cups" were arranged in five columns of six cups each. Each column consisted of chambers from a single bean; each row, of chambers from different beans. In each Petri dish there were, therefore, cups from five different beans, six cups from each bean, and in each dish it was possible to test six different solutions on five different beans. Because five beans did not suffice in general to give an accurate measure of activity, due to individual variations, ten beans were used in most determinations. In tests requiring special accuracy (as, for example, molecular weight determinations by the diffusion method (**4, 24, 12**) twenty or more beans were used. The solutions to be tested were diluted with distilled water instead of nutrient solution, since it was found that the use of a balanced salt solution made no difference in the results. Drops of the test solution (approx. 0.01 cc. per drop) were placed in the center of each cup with a micropipette. The tests were then placed in an incubator at 25° C. At the end of the test period (in general 48 hours, see below), each cup was sectioned through the reacting portion and the height of intumescence measured under a low power binocular microscope.

COURSE OF THE REACTION WITH TIME

Figure 2 gives the course of the reaction with time for a series of dilutions of the same stock solution. The first reaction appears between 8 and 16 hours, before the drops have been completely absorbed. Each concentration

[1] The authors are deeply indebted to Mr. K. WADA of the Lake Farm Produce Co. Only through his constant cooperation and assistance was it possible to obtain the supply of fresh high quality beans needed for the routine testing.

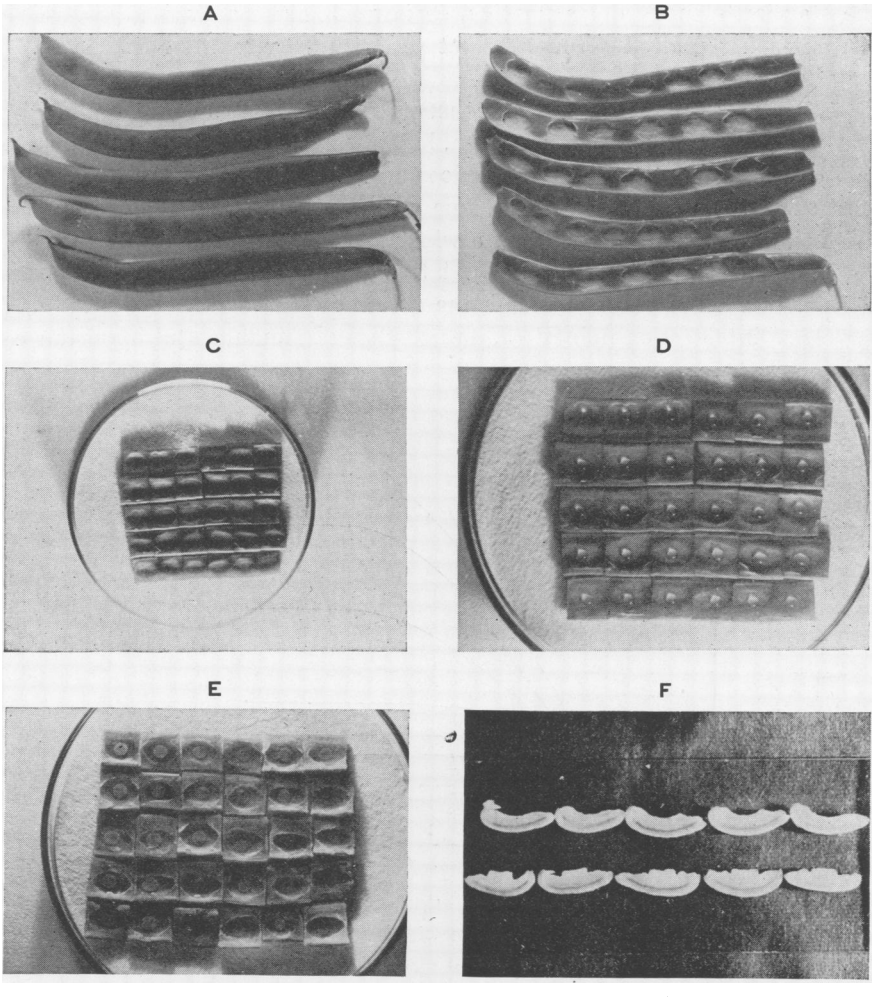

FIG. 1. Steps in the execution of the "bean test": A, fresh beans; B, beans opened and seeds removed; C, individual seed chambers arranged in Petri dish; D, drops (containing traumatin) in place; E, characteristic reaction to traumatin, after 48 hours; F, cross section through the seed chamber after 48 hour test. The top row is a typical control, and the bottom row a reaction which is shown in the linear portion of fig. 3.

gives a typical S-shaped growth curve, the curves from tests with smaller concentrations having lesser slopes and earlier maxima than those from tests with the higher concentrations. The highest concentration, for example, attained its maximum only after 64 hours while the lowest concentration attained its maximum after 40 hours. Since, for routine testing, it was desirable to have as short a test period as possible, 48 hours was chosen as

the standard length of incubation. Figure 3 gives a typical example of concentration plotted against height of intumescence after 48 hours. It may be seen that:

1. Height is proportional to concentration between approximately 0.20 and 0.67 mm. Over this range, then, height of intumescence, less the correction due to the fact that the curve does not pass through the origin, may be used as a measure of wound hormone activity. If a longer time is chosen, the linear relationship holds over a wider range.

2. The curve does not pass through the origin. If the main linear portion is extrapolated, it passes through the height axis at about 0.18 mm.

This "intersection point" (I, on fig. 3) is of fundamental importance to

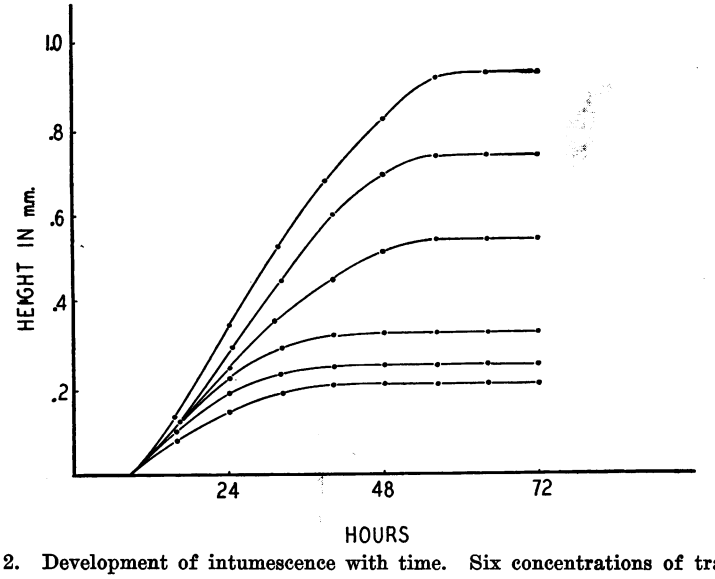

FIG. 2. Development of intumescence with time. Six concentrations of traumatin, each one-half as great as the preceding, are represented.

the understanding of the test. The height of intumescence corresponding to the intersection point varies from day to day, but on any given day it is equal to the maximum response which is given to any of the "non-specific" agents which are discussed below. It is to be noted, however, that non-specific agents may cause a response *smaller* than this; thus water causes a response which is several times smaller than the *maximum* non-specific response which is possible. Above this non-specific portion of the response is superimposed the major effect of the added hormone. Only those substances which in some concentration elicit a response greater than the non-specific portion, can be considered as possessing true wound hormone activity. The failure in the past to distinguish sharply between specific and non-spe-

cific response may have been due to the much smaller accuracy of the cell division frequency determinations which have been made. In any case it has led to the present confusion as to the specificity of the hormone, a question which will be discussed later.

The wound hormone itself is capable of causing the non-specific as well as the specific response. That this is so, is clear from the activity-concentration curve (fig. 3) in which one is superimposed on the other. The non-specific

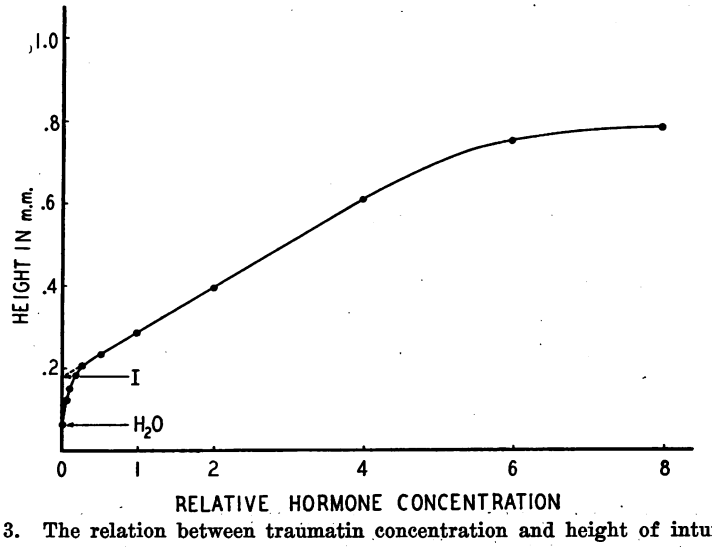

FIG. 3. The relation between traumatin concentration and height of intumescence. I, "intersection point" (see text); H_2O, the reaction to pure water.

portion is brought about by much lower concentrations of wound hormone than is the specific response, and also it is caused by much smaller concentrations of wound hormone than of the various non-specific agents. This fact suggests a second measure for wound hormone activity, namely, the lowest concentration which is just capable of causing full non-specific response. This measure of activity does *not* give relative activities for two wound hormone preparations identical with those obtained by the first method (from large intumescences), as will be shown later.

THE NON-SPECIFIC REACTION

It has already been mentioned, that WEHNELT (23) found a number of agents other than the juice of beans, capable of causing a reaction in the bean test. WEHNELT was often troubled with a considerable reaction caused by the application of drops of plain water. He also found that sugars, neutral salts, and even agar regularly caused reaction. It is desirable to make clear at once the relationship between these non-specific reactions

and those due to the application of wound hormone. Water alone gives a reaction particularly if the test tissue is relatively dry. The standard "fresh" beans[2] which have been used in this investigation give, in general, either no response or a negligible one to the application of pure water (0.07 mm. is a value obtained on many days). If such beans are allowed to dry out, however, they react more vigorously to water. Sugars and neutral salts give, with these beans, reactions greater than that of water only when used in high concentrations. Thus 10 per cent. glucose gave a reaction four times as great as that of water, while 1 per cent. gave only the reaction of water alone. The more permeable substance, urea, in equivalent concentrations gave reactions only half as great as those due to glucose. The effects of these substances are clearly osmotic ones. Another class of non-specific responses includes those due to wounds or to the application of markedly toxic substances. In either of these cases there is a circular zone of response around the dead or badly injured portion, and a crater-shaped intumescence is formed.

It is clear that the non-specific causative agents may be classified under two general headings: (1) Those causing slight if any visible injury such as hypo- and hyper-tonic solutions; (2) those causing severe injury, either mechanical (pricks, etc.) or chemical ($HgCl_2$, strong acid or alkali, etc.). In these cases it is clear from the crater shape of the intumescence that the injured cells have liberated wound hormone which caused adjacent cells to react.

The causative agents which superficially appear to be non-specific are probably all based upon the liberation of wound hormone within the tissue itself. The maximum amount thus liberated by agents which are not visibly toxic is then just sufficient to cause a maximum intersection point non-specific response. Intumescences caused in this way are not to be confused with those caused by the addition of wound hormone from outside. The average maximum response due to the former is 0.10 to 0.30 mm., that due to the addition of wound hormone, 1.9 mm. or more.

Reactivity of different varieties of beans

A number of leguminous pods other than those of *Phaseolus* were tested as to their suitability for the bean test. Pods such as those of pea, lima bean, and chinese pea, which have only rudimentary parenchymatous linings of the seed chambers give no reaction whatsoever. This is not surprising since the reaction is carried out by the parenchymatous lining of the bean. Different varieties of the string bean itself also differ in their response to wound hormone. The maximum average height of intumescence for five varieties

[2] By this is meant fresh market beans. Beans fresh from the plant were found to be improved by a slight amount of drying (2).

is recorded in table I. Kentucky Wonder, brown seed, is at least four times more reactive than is Kentucky Wonder, white seed, and excels the other varieties still more. The brown seed Kentucky Wonder was, therefore, used exclusively for the quantitative test outlined above.

TABLE I

MAXIMUM HEIGHT OF INTUMESCENCE GIVEN BY DIFFERENT VARIETIES OF BEANS

VARIETY	MAXIMUM AV. INTUMESCENCE HEIGHT
	mm.
Kentucky Wonder, brown seed	1.9
Kentucky Wonder, white seed	0.45
Florida Black Valentine	0.3
Golden Wax	0.15
Green Pod	0.00

Some investigation of the reasons underlying this varietal difference in reactivity has been made. In table II are given the results of reciprocal tests between Kentucky Wonder brown seed, and Florida Black Valentine. The

TABLE II

RECIPROCAL TESTS BETWEEN KENTUCKY WONDER AND FLORIDA BLACK VALENTINE BEANS

EXTRACT USED	HEIGHT OF INTUMESCENCE	
	KENTUCKY WONDER BEANS	BLACK VALENTINE BEANS
	mm.	*mm.*
Kentucky Wonder	0.65	0.30
Black Valentine	0.51	0.30
Kentucky Wonder + Black Valentine	0.66	0.30
Free Acid Conc.	0.70	0.0

latter contains wound hormone which is highly active on Kentucky Wonder but only slightly active on itself. Kentucky Wonder juice also has only slight activity on Valentine. A mixture of the two juices shows that Valentine juice contains no inhibitor of the reaction. The fraction of the juice which is active on Kentucky Wonder and which was purified during the course of the present work possesses little, if any, activity on Valentine. A possible explanation is that the interaction of several factors is necessary for the response and that we have purified only one of them. The others, apparently, are present in the Kentucky Wonder bean in considerable amount, but are lacking in the Valentine. A complete elucidation of the reasons for the difference must however await a further investigation.

Distribution of the wound hormone

That the wound hormone is widely found in nature has been indicated by the work of Wehnelt (**23**) as well as that of others. A more extensive investigation of its distribution has been made during the course of the present investigation and a portion of the results are presented in table III.

TABLE III

Occurrence of the wound hormone

Source	Activity	Source	Activity
Bean pod	++	Corn meal	−
Brussels sprouts	++	Soy bean meal	−
Sweet potato	+	Wheat germ	+
Potato	+	Molasses	+
Orange	++	Yeast, bakers'	−
Lemon	++	Yeast, brewers'	+
Tomato	++	Vitamin B concentrate	−
Lettuce	++	Urine, human	−
Spinach	+	Urine, cow	−
Pea plant, etiolated	++	Peptone, Difco	−
Pea plant, green	++	Beef extract	−
Pea seed	−	Liver extract	−
Hay, alfalfa	+	Milk	−
Malt	+	Egg albumin	−
Rice polishings	−	Serum	−
Cabbage	+	Emulsin	−

Each source was tested over a wide range of concentrations. A substance listed as inactive failed to cause any response other than non-specific in any concentration. Of the animal tissue and extracts examined, none was found to possess any marked activity. The reaction to serum obtained by Wehnelt was, presumably, merely non-specific in nature. The urine of cows is of particular interest. Alfalfa hay is a relatively rich source of the hormone (table III). The urine from cows fed this hay, however, is completely inactive. Wound hormone behaves, in this respect, quite differently from auxin a, which is ingested by the animal and excreted in the urine. The "sporogenes vitamin" (**18**) also is found in the urine of herbivores. Wound hormone, however, either is destroyed by the flora of the gut, or is metabolized further by the cow.

The richest sources were found to be green beans themselves, leaves in general, dry brewers' yeast, and the juice of oranges and tomatoes. Attention might be called to the fact that various samples of brewers' yeast were all inactive, in marked contrast to dry brewers' yeast. Although green portions of the plant are rich in wound hormone, the latter is not confined to

chlorophyll-containing parts. Thus, it is to be found in fruits, in potatoes, and in wheat germ. It is not present in any considerable amount in the pea seed, but is formed after germination and during growth of the seedling. Seedlings grown in darkness contained as much wound hormone per unit dry weight as did those grown in the light.

Table IV gives a quantitative comparison of a few of the more promising

TABLE IV

A QUANTITATIVE COMPARISON OF SOURCES RICH IN WOUND HORMONE

METHOD OF EXTRACTION	SOURCE	RELATIVE ACTIVITY PER MG. DRY WT. OF EXTRACT
Water extraction	Bean pod	100
	Pea plant	77
	Orange	50
	Yeast, brewers'	31
	Alfalfa hay	15
Absolute alcohol extraction	Bean pod	410
	Yeast, brewers'	380
	Alfalfa hay	233
	Wheat germ	143

sources of the hormone. In no case was a source found which was richer than the bean pod itself. This is in accord with the findings of SILBERSCHMIDT and KRAMER (**20**), that the wound hormone content of a plant tissue, as measured by the bean test, is greater the closer the relationship of the plant in question to the bean. However, brewers' yeast which is certainly systematically far removed from the bean yields a very active alcoholic extract.

It might be mentioned here that a number of different methods of extraction of the wound hormone have been compared. The results of a typical experiment are presented in table V. The hormone is heat stable and the

TABLE V

A COMPARISON OF DIFFERENT METHODS OF EXTRACTING WOUND HORMONE FROM BEAN PODS

METHOD OF EXTRACTION	PREPARATION OF MATERIAL	RELATIVE ACTIVITY	YIELD: % DRY MATTER/DRY WT.	TOTAL AMOUNT OF HORMONE
Water extraction	Fresh: ground	1	41	41
	Ground: dried	1	44	44
	Dried: ground	1	48	48
Alcohol extraction	Ground: dried	4.1	4	16.4
	Dried: ground	4.1		

fresh beans may be dried and extracted without loss of activity. It is of no advantage to grind the tissue (thus to wound it severely by mechanical means) before drying and extracting. Of the organic solvents used for this initial extraction, absolute ethyl alcohol was found to be the most effective. The extract which is obtained by extraction with hot alcohol is more *concentrated* in wound hormone than is the corresponding water extract, although a smaller *total amount* of hormone is obtained, even if the alcohol extraction is apparently complete. The alcohol extraction possesses other advantages (**4**), and has been used in the subsequent procedure for purification.

Specificity of the reaction

A number of pure substances, known to possess physiological activity upon plants, were tested during the course of the work. A partial list of these and other pure substances tried is given in table VI. None of the com-

TABLE VI

Pure substances found to be inactive in the bean test

Indole-3-acetic acid	Cystine
Aneurin, (Vitamin B_1)	Cystine
Lacto-flavin	Alanine
Ascorbic acid	Glycine
1–2–5–6–di-benzanthracene	Methionine
Folliculin	Tyrosin
Pantothenic acid*	Histidine
i-Inositol	Proline
Biotin†	Valine
	Tryptophane
	Leucine
Lecithin	Asparagin
Tannic acid	Aspartic acid
Hesperidin	Glutamic acid
Narinigen	Ornithine
Colchicine	Lysine
Allantoin	Serine
	Betaine

* Obtained through the generosity of Prof. R. J. Williams, Corvallis, Oregon.

† Tested on different but reactive beans by Prof. F. W. Went. Biotin supplied through the courtesy of Prof. F. Kögl, Utrecht.

pounds elicited any response other than the non-specific or that due to marked toxicity in the higher concentrations. Of particular interest are the following:

Hetero-auxin, mentioned by Jost (**13**) as possessing activity in the bean test. Jost, however, used very high concentrations (1:1000) which apparently were toxic. Hetero-auxin was inactive in the present test.

Aneurin (vitamin B_1), a growth factor for other plant tissues (**3**) but inactive in the bean test.

Biotin, pantothenic acid, and *i-inositol,* substances of the yeast "bios" group. LAIRD and WEST (**15**) have found a "bios 2b concentrate" to be active upon their beans. Pure or nearly pure "bios 2b" (biotin, pantothenic acid), however, were inactive in the present test. Prof. R. J. WILLIAMS, furthermore, has very kindly tested our fraction VII-b (below) under a number of conditions and found it to have only negligible "bios" activity.

Tyrosin, said by ORSOS (**17**) to be the active wound hormone principle in the kohlrabi test of HABERLANDT. It was inactive in the present test, as were all of the amino acids tested, both alone and in combination.

Ascorbic acid, which, according to HAVAS (**10**) plays some rôle in abnormal plant growth. It was inactive in the bean test.

Colchicine, said by HAVAS (**11**) to promote abnormal meristematic activity. It was inactive in the bean test.

PURIFICATION OF THE WOUND HORMONE

The quantitative test was developed with the particular view of using it as a tool in the isolation and chemical identification of the wound hormone. Each step in the purification was arrived at only after much preliminary groping with this physiological assay as a guide. The evidence for the value of each step cannot be given here. Since a detailed account of the procedure and a discussion of the chemical properties of the products may be found elsewhere (**4**), only a brief résumé is presented in this paper.

The activities of the various fractions are given below in two ways: (a) that determined from the concentration needed to give a large intumescence (0.30 to 0.70 on the scale of fig. 3), given as activity per mg. relative to the activity per mg. of the initial alcohol extract as unity; and (b) that determined from the minimum concentration giving an intersection point response. This is called the minimum active concentration.

Three separate lots of beans, 100 pounds in all, were extracted. The summary given in table VII is for the extraction of one of these lots, having an initial wet weight of 20 pounds.

It was impossible to increase significantly the activity of VII–b by further extractions or precipitations or even by preparation and distillation of the methyl ester. This free acid concentrate of VII–b is a yellow, extremely hygroscopic, amorphous solid, which is insoluble in ether, chloroform, etc., but readily soluble in water, alcohol, pyridine, etc. It is heat, acid and alkali stable, and contains basic nitrogen although not as a primary amine. The molecular weight as determined by titration, diffusion (**4, 12**), and by other methods, is in the neighborhood of 220. The ester product of this "free acid concentrate" is a yellow oil which dis-

TABLE VII

RÉSUMÉ OF STEPS IN THE PURIFICATION OF THE ACTIVE PRINCIPLE OF THE WOUND HORMONE

PROCEDURE	AMOUNT	ACTIVITY PER MG. RELATIVE TO I–b	MINIMUM ACTIVE CONCENTRATION
I. Extraction of dried beans with absolute alcohol			
a. hot water extract of residue	.....	0.0	.....
b. water soluble of alcohol extract	92 gm.	1.0	1:400
II. Adsorption of I–b with charcoal and elution with pyridine			
a. charcoal filtrate	42 gm.	0.0	.....
b. combined eluates	52 gm.	1.5	1:1600
III. Fractional ether precipitation of II–b from pyridine			
a. ether precipitate	22 gm.	0.0	.....
b. ether filtrate	30 gm.	1.4	1:4500
IV. Extraction of III–b with ethyl acetate			
a. ethyl acetate insoluble	17 gm.	0.3	.....
b. ethyl acetate soluble	12 gm.	2.0	1:12800
V. Conversion of IV–b to its barium salts, alcohol extraction, and regeneration of the free acids			
a. alcohol soluble	3.8 gm.	1.6	.....
b. alcohol insoluble	6.4 gm.	6.3	1:36000
VI. Precipitation of V–b with mercuric acetate and subsequent regeneration with H_2S			
a. mercuric acetate filtrate	3.0 gm.	2.8	.....
b. mercuric acetate precipitate	1.62 gm.	10.8	1:100,000
VII. Extraction of VI–b with acetone			
a. acetone insoluble	0.216 gm.	10.3	.....
b. acetone soluble	1.406 gm.	13.8	1:100,000
VIII. Preparation and high vacuum distillation of the methyl ester of VII–b			
a. unchanged free acid	25% of VII–b	13.1	.....
b. main fraction of ester distillate (after hydrolysis)	15% of VII–b	14.4	1:100,000

tilled well and could be redistilled repeatedly without change of activity. It should be noted that the ester can be tested only after hydrolysis, since it is water insoluble. The products of the three separate extractions gave identical analytical results, the composition of the redistilled ester agreeing approximately with the formula $C_{11}H_{17}O_4N$. It seems probable that this product is not far from pure although it has not as yet yielded a single crystalline derivative.

From the 100 pounds of fresh bean which were extracted a total of approximately 500 mg. of distilled ester were obtained. This amount has been too small to permit of further investigations, but it is planned to carry out the extraction upon a larger scale in the near future.

Discussion

The procedure used for the purification resulted in an enrichment of the active principle of about 250 times over that of the initial alcohol extract, as measured by the minimum active concentration. As measured by the concentration required to produce a given large size of intumescence, the enrichment, however, was only 14 times. Although no portions containing any significant amount of activity were discarded during the procedure, nevertheless the enrichment as measured in this way was much less than the enrichment of material, which was 65 times between alcohol extract and product VII–b. At least two possible explanations suggest themselves:

1. The wound hormone may be continuously altered to a closely related inactive substance.

2. The cooperation of a second substance or substances may be necessary for the production of large intumescences. Such substances should need to be inactive alone, but should augment the effect of the fraction which we have purified.

With regard to the first possibility, it has been impossible to demonstrate that spontaneous inactivation of the wound hormone occurs, although this has been looked for repeatedly. The second possibility also has been borne in mind constantly. At each step of the procedure the two resulting fractions were tested not only separately but *in combination*. In no case was it possible to find any synergistic action of the combined fractions. The free acid concentrate was also tested in combination with other fractions or substances, for example, with charcoal filtrate of fresh extract, fraction II–a, sugar, aneurin, lactoflavin, and hetero-auxin. The slightest evidence for any co-wound hormone activity was never detected.

UMRATH and SOLTYS (**22**) in a paper which has appeared since the present work was undertaken, have also used the bean test (although in a less quantitative fashion) as an aid for the enrichment of wound hormone. They obtained, from alfalfa, a product having an activity of 1:50,000, or one only slightly less active than that obtained here. Chemically, however, the products differ, that of UMRATH and SOLTYS containing less nitrogen and much more oxygen than that reported here, and apparently being more closely related to the "Mimosa substance" of SOLTYS and UMRATH (**21**). It seems possible that the apparent difference in composition of the two substances may be due only to a difference in their respective purities.

As we have mentioned in the introduction, HABERLANDT (**8**) found that

a substance coming from the phloem (lepto-hormone) interacts with the wound hormone in the induction of cell divisions, at least in certain cases such as in the potato tuber. In the case of the bean test, it may be supposed that the lepto-hormone, if it is necessary, may be supplied by the vascular tissue of the pod. Over the range in which response is proportional to applied wound hormone concentration, it is in any case clear that wound hormone rather than lepto-hormone is the limiting factor in the normal bean test. Tests were therefore carried out in which it was attempted to make lepto-hormone the limiting factor. The seed chamber was completely freed of vascular tissue and tests with high concentrations of wound hormone were carried out on the isolated parenchyma. Removal of the vascular tissue actually did reduce the response greatly. If the isolated parenchyma was placed upon agar containing sugar, it responded much more vigorously, although still not as well as the intact controls. One might be tempted to conclude that lepto-hormone, in the case of the bean, consists principally of nutrient sugars. However, the situation is actually more complicated and can be worked out fully only when larger supplies of pure wound hormone are available.

With the isolation of an active wound hormone, even though it may not as yet be in a completely pure state, it is proper to propose a chemical name for the substance. The entire background of the subject from HABERLANDT, in particular, to the present, would seem to make "traumatin" particularly appropriate. It should be stressed again that there may be many wound hormones, that different test objects respond to different chemical substances, and that the present investigation has been concerned with traumatin, *a* plant wound hormone, rather than with traumatin, *the* plant wound hormone.

Summary

1. The origin and development of the wound hormone concept have been reviewed briefly. Of the several available methods for the demonstration of wound hormone activity, that of WEHNELT (**23**) was chosen for the present work.

2. A quantitative assay of wound hormone activity has been described. In this test, *size* of the new growth rather than frequency of cell division is measured.

3. The test was shown to be specific for this wound hormone.

4. With the aid of the quantitative assay it was possible to isolate a substance, apparently not far from pure, and possessing typical wound hormone activity. For this substance the name traumatin is proposed.

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WILLIAM G. KERCKHOFF LABORATORIES OF THE BIOLOGICAL SCIENCES
GATES AND CRELLIN LABORATORIES OF CHEMISTRY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

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