abalone sperm appears simply to be concerned in the penetration part of the fertilization process.

Summary.—A lytic agent having the property of dissolving the egg membrane has been extracted from sperm of the giant keyhole limpet (*Megathura crenulata*). The lysin is non-dialyzable and readily inactivated by heat. It precipitates upon saturation of the extract with ammonium sulphate. The precipitate gives the usual protein tests and pure proteinases inactivate the lytic agent. Abalone (*Haliotis cracherodii*) sperm also yield a lysin which acts on the eggs of the same species. Cross-lysis between limpet and abalone does not occur; nor does cross-fertilization. Limpet sperm extracts also contain a substance (anti-fertilizin) capable of neutralizing the sperm agglutinin (fertilizin) obtained from eggs. Heat inactivation of the lysin leaves the anti-fertilizin unaffected.

[1] J. Loeb, "Artificial Parthenogenesis and Fertilization," The University of Chicago Press (1913).

[2] F. R. Lillie, "Problems of Fertilization," The University of Chicago Press (1919).

[3] M. M. Sampson, *Biol. Bull.*, **50**, 301 (1926).

[4] T. H. Morgan, "Experimental Embryology," Columbia University Press (1927).

[5] E. E. Just, *Protoplasma*, **10**, 300 (1930).

[6] A. Tyler and J. Schultz, *Jour. Exp. Zoöl.*, **63**, 509 (1932).

[7] H. Kupelwieser, *Arch. Entw.-mech.*, **27**, 434 (1909); *Arch. f. Zellforsch.*, **8**, 352 (1912).

[8] R. Chambers, *Jour. Exp. Biol.*, **10**, 130 (1933).

[9] J. A. Frank, *Biol. Bull.*, **76**, 190 (1939).

[10] The crystalline proteinases were very kindly supplied by Dr. J. H. Northrop.

THE WOUND HORMONES OF PLANTS II. THE ISOLATION OF A CRYSTALLINE ACTIVE SUBSTANCE

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Introduction.—The existence of plant "wound hormones" has been recognized since the formulation of the wound hormone concept by Wiesner[1] and the demonstration by Haberlandt[2] of the diffusible nature of the active principles. It was shown by these early investigators that there are formed or liberated at injured surfaces of plant tissues water-soluble substances which are capable of evoking renewed growth activity in mature uninjured cells or tissues. In the present paper the isolation from plant

material of a crystalline substance possessing typical "wound hormone" activity will be described.

Materials and Methods.—Numerous methods for the demonstration of wound hormone activity have been suggested.[3] For the present work the parenchymatous mesocarp lining the seed chambers of the string bean pod has been used as the test material. It has been found by Wehnelt[4] that if drops containing the water extract of bean are placed on the uninjured cells of the mesocarp, a vigorous proliferation and elongation of the underlying cells results, whereas drops of pure water are without such an effect. A detailed description of the adaptation of this reaction to the quantitative estimation of wound hormone activity has been presented elsewhere.[5] Whereas others have attempted to utilize as a measure of wound hormone activity the frequency of cell divisions in the resulting intumescences, we have utilized the total volume of new tissue formed. This volume, as determined by the height of the intumescence, is not only linearly proportional to the concentration of wound hormone solution applied, but is also easily determined and well adapted to large scale routine bioassays. In brief, the method is as follows: Young pods of Kentucky Wonder string bean (*Phaseolus vulgaris*) are slit longitudinally, the seeds removed, sections containing the seed chamber excised and arranged in Petri dishes, 30 sections per dish. Uniform drops (0.01 cc.) of each solution to be tested are placed on 20 sections from 20 individual beans. Each of the 20 individual beans is also treated with two concentrations of a standard bean extract.[6] This solution of standard activity is tested daily on every set of beans and activities of the unknowns referred to it. The test beans are then incubated for 48 hours at 25°C. At the end of this time cross-sections through the seed chamber and intumescence are cut and the total height of each intumescence measured under a low-power binocular equipped with an eyepiece micrometer. It has been found readily practicable to assay thus 30 solutions per day, each solution tested on 20 beans, together with the above-mentioned standard solutions.

In following wound hormone activity through a series of fractionations of the initial crude material it has been found desirable in the present work to make use of an arbitrary "unit" of activity. A *unit* of wound hormone activity will be defined as that amount which when present in 1 cc. of solution will, when this solution is tested, give a reaction equal to that of the standard bean extract.

During the course of the procedure outlined below for the isolation of a crystalline wound hormone it was found that there are one or more "cofactors," themselves inactive or essentially so, which have, however, the property of greatly increasing the potency of the more active fraction. This can be made clear by an example of the distribution of activities before and after fractionation of concentrated extract with chloroform, as is

shown in table 1. Only a small amount of activity remained in the water layer after extraction with chloroform. The chloroform-extractable material contained, when tested, alone, only one-fourth of the total amount of activity present in the starting material. If, however, the two fractions were recombined the mixture was found to possess a total number of units of activity essentially equal to that of the starting material. This behavior would be in accordance with the view stated above that there remained in the chloroform-insoluble material a substance (or substances) inactive in itself but capable of enhancing the activity of the chloroform-soluble fraction. A similar behavior was noted at other stages of the fractionation, notably at steps 2 and 3 below. For this reason, in the experiments reported here the active material was invariably tested in the presence of a mixture of two of the discarded inactive fractions, including the acetone and ethyl acetate insoluble portions.[7]

TABLE 1

DISTRIBUTION OF ACTIVE MATERIALS BETWEEN $CHCl_3$ AND WATER

	MG.	UNITS/GM.	TOTAL UNITS
Starting material	201	170	34.2
$CHCl_3$ extract (continuous extraction)	16	500	8.0
Water layer	185	30	5.5
Combination	201	150	30.2

Procedure. 1. One hundred pounds of fresh bean pods were ground and immediately extracted twice with water at 50–60°C. for one-half hour. The suspension was filtered and the clear solution (about 100 liters) was evaporated at once under reduced pressure under nitrogen at a bath temperature of 60°C. The evaporation was continued until a thick syrup (85% solids) was obtained.

Yield—2750 gm. 48 units/gm. 132,000 units

2. The syrup thus obtained was acidified with concentrated hydrochloric acid to pH 2 and extracted repeatedly with acetone. The bulk of the activity was found in the acetone layer.

a. Acetone layer	554 gm.	170 units/gm.	94,000 units
b. Water layer	2200 gm.	0 unit/gm.	0 unit
c. Combination	2750 gm.	48 units/gm.	132,000 units

3. The acetone was evaporated off in nitrogen at 60°C. and the residue taken up in water (2000 cc.) and adjusted to pH 2. This solution was exhaustively extracted with ethyl acetate. All tests were carried out in cofactor mixture.

a. Ethyl acetate layer	67.4 gm.	2000 units/gm.	135,000 units
b. Water layer	487 gm.	35 units/gm.	17,000 units

4. The ethyl acetate was evaporated as before and the oily residue dissolved in dilute sodium hydroxide, adjusted to pH 10–11 and extracted with chloroform. The water layer was then acidified to pH 2 and extracted again with chloroform. At this point an oil material separated which was insoluble in both chloroform and water. In view of its comparatively low activity this was set aside. All tests were carried out in co-factor mixture.

a. Chloroform from alkaline solution, 4.0 gm.; 650 units/gm.; 2600 units
b. Chloroform extract from acid solution, 7.3 gm.; 6800 units/gm.; 50,000 units
c. Oily material, 8.2 gm.; 1450 units/gm.; 12,000 units
d. Water layer, 39 gm.; 0 unit/gm.; 0 unit

5. Fraction 4*b* (which contains organic acids) was dissolved in 0.1 normal sodium hydroxide after evaporation of the chloroform and a solution of barium chloride added. There resulted a dark, oily precipitate which was separated by centrifuging and washed with water. Both filtrate and precipitate were treated with dilute hydrochloric acid and the acidified solutions exhaustively extracted with ethyl acetate. The regenerated precipitate was a very dark, oily material and the filtrate was a light brown viscous oil which partially crystallized on standing.

a. Insoluble barium salts	4.2 gm.	2200 units/gm.	9000 units
b. Soluble barium salts	3.1 gm.	2900 units/gm.	9000 units

6. The crystals of 5*b* were separated by the addition of cold peroxide-free isopropyl ether and filtration of the thick syrup on a sintered glass filter. The crystalline mass thus obtained was pressed on a porous plate and then recrystallized from absolute alcohol to constant melting-point and activity. In this way there were obtained 18 milligrams of crystalline material, m. p. 165.5–166°. By petroleum ether extraction of the oily mother liquors, further active fractions were obtained including an additional small amount of this crystalline material with identical properties and activity.

Analysis.[8] Found: % C, 63.37; % H, 9.01. Calculated for $C_{12}H_{22}O_4$: % C, 63.15; % H, 8.77. Calculated molecular weight, 228. Equivalent weight by titration, 118.

Preparation of the Methyl Ester.—Fifteen milligrams of crystalline material, m. p. 163–164°, recovered from mother liquors of recrystallization were dissolved in ether and treated with an excess of an ethereal diazomethane solution at 0°. After standing for an hour, the ether was distilled off and the residual oil distilled *in vacuo*. At 140°C. bath temperature a nearly colorless oil distilled which crystallized on cooling. The crude ma-

terial (m. p. 29–31°) was recrystallized from a mixture of ether and petroleum ether to a constant m. p. of 30–31°.

Analysis. Found: % C, 65.21; % H, 9.18. Calculated for $C_{14}H_{24}O_4$: % C, 65.55; % H, 9.40. Calculated molecular weight, 256. Molecular weight, by m. p. depression in camphor, 267.

On hydrolysis in alcoholic KOH the acidic starting material was regenerated, as judged by m. p. and activity.

Proof of Purity.—In order to ascertain whether the activity was due to the crystalline material itself or to small amounts of adsorbed impurities, 15 mg. of the substance were subjected to chromatographic analysis on aluminum oxide from $CHCl_3$ solution. No alterations in melting point or activity of the material could be effected in this way.

Nine milligrams of crystalline material were sublimed *in vacuo* (0.1 mm. of Hg) at a bath temperature of 160°C. The sublimate possessed the same m. p. and activity as the starting material.

Activity of the Pure Material.—The pure crystalline material has been assayed for its activity 60 times in the bean test in the presence of the standard co-factor mixture. The average activity has been found to be 4800 units per gm. or about 100 times that of the starting material. In the absence of the co-factor mixture the activity of the crystalline substance varied greatly in successive daily tests, with a maximum of 4800 units/gm., a minimum of 0 unit/gm. (one test) and a mean activity of 2700 units/gm. The fluctuations in activity of the pure material when tested alone would be in accordance with the hypothesis that co-factors are to some extent present in the test beans themselves, but that their amount varies from day to day due to unidentified and uncontrolled variations in environmental circumstances.

Numerous pure substances have been tested for co-factor activity in the presence of the crystalline factor. Attention was first given to sugars and amino acids since these were found to constitute a large portion of the initial water extract and were also known to constitute a considerable part of the acetone and ethyl acetate insoluble fractions. When the crystalline factor is tested together with 0.5% sucrose (which is itself completely inactive) the activity is increased from 4800 to 6600 units/gm. Of the amino acids tested glutamic acid was found to exert a marked co-factor activity. Glutamic acid at high concentrations is itself slightly active in the standard bean test (approximately 100 units/gm.). At a concentration of 0.25%, however, the intumescence evoked by glutamic acid is sufficiently small to be disregarded. When the crystalline factor is tested in the presence of 0.25% glutamic acid the activity of the former is enhanced to approximately 45,000 units/gm., or almost ten times that obtained with the standard co-factor mixture. The crystalline factor at a concentration of 10 gamma per

cc. and in the presence of 0.25% glutamic acid can evoke intumescences even higher than those evoked by the standard bean extract.

Discussion.—The crystalline wound hormone possesses detectable activity at a concentration of 10 mg. per liter in the presence of 0.25% glutamic acid. At this concentration, 0.1 gamma would be present in each of the 0.01 cc. drops which are applied in the standard bean test. This substance is thus comparable in potency to other of the physiologically active substances.

The substance isolated can account for at most 10% of the total wound hormone activity present in the initial extract. This may be attributable either to the presence of other as yet unrecognized co-factors in the initial extract, or to the presence in this extract of still further highly active substances. With relation to the first possibility, there is a highly labile co-factor, itself inactive, present in the water layer of step 4*d*. It has been impracticable because of its instability to incorporate this co-factor in the standard co-factor mixture. Because of its high activity the discarded fraction (5*a*) from the barium salt separation could not be incorporated into the standard co-factor mixture. In these two fractions, then, there may well be inactive co-factors capable of increasing still further the activity of the substance which has been isolated.

With relation to the second possibility, that there may be still further highly active wound hormones present in the initial extract, it has been shown that glutamic acid possesses real although low activity. The non-crystalline material (traumatin) whose isolation has been previously reported[5] also appears to differ both in chemical properties and in activity from the crystalline substance reported here. The nature of other possible active materials will be the subject of future communications.

It is of interest to know in how far the present crystalline material functions in the regulation of cell division. Preliminary histological investigations have shown that under the influence of the pure substance, in the absence of added co-factors, both cell division and cell enlargement take place in the tissue of the bean mesocarp.

Summary.—The isolation from fresh beans of a crystalline substance possessing typical wound hormone activity has been described. The substance, a dibasic acid, the analysis of which corresponds to $C_{12}H_{20}O_4$, is capable of evoking renewed cell division and cell extension activity in the mature parenchymatous cells which compose the mesocarp of the string bean pod.

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[1] J. Wiesner, *Die Elementarstructur....*, Wien (1892).

[2] G. Haberlandt, Summary and Review in *Biol. Zent.*, **42**, 145 (1922).

[3] G. Haberlandt, loc. cit.; H. Reiche, *Zeit. Bot.*, **16**, 241 (1924); A. Wilhelm, *Jahrb. wiss. Bot.*, **72**, 203 (1930).

[4] Wehnelt, *Jahrb. wiss. Bot.*, **66**, 773 (1927).

[5] J. English and J. Bonner, *Jour. Biol. Chem.*, **121**, 791 (1937); J. Bonner and J. English, *Plant Physiol.*, **13**, 331 (1938).

[6] For the preparation of the standard bean extract, 200 mg. of a dried, finely ground bean powder are boiled for 1 minute in 10 cc. of water, filtered and the filtrate made up to 10 cc. The resulting solution contains approximately 10 mg. of solid material per cc. By testing both this 1% solution and a solution diluted to 0.5%, the slope of the intumescence height vs. concentration curve is obtained daily. One cubic centimeter of the standard 1% solution is defined as containing 1 unit of activity. The dried bean powder has been used over a period of 2 years without detectable loss of activity.

[7] The ethyl acetate insoluble fraction of step 3*b* possessed a small activity. This activity was completely removed by further extraction with ethyl acetate and the deactivated portion used as a component of the standard co-factor mixture. The water layer of step 2*b* and the water layer of step 3*b* were mixed in the proportions of 4.5 to 1 and the resulting solution used in a final concentration of 1% as a diluting medium for the unknown solutions.

[8] Microanalyses by G. A. Swinehart.

THE CHEMICAL INDUCTION OF GENETIC CHANGES IN FUNGI

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Biochemical and technical studies of Aspergilli began about the middle of the 19th century. By 1930, Tamiya and Morita cited 2424 numbers in their bibliography of the genus, and many have been added since. Of all the molds which have caught man's fancy as experimental material the black Aspergilli—under the name *A. niger* van Tieghem—have had the greatest share of attention starting with the basic assumption that they were a single species, hence easy to identify.

A few outstanding references to *A. niger* will show some of the reasons for our study. As far back as 1867, van Tieghem described the use of a black Aspergillus in fermenting tannins toward gallic acid. That factory process is still in use. In 1891 Wehmer showed that his strain produced oxalic acid from sugars. In 1916, Thom and Currie tested twenty such strains for oxalic acid production and found variations as great as ten to one in the amount of oxalic acid produced by different strains. From this series, Currie selected one as a citric acid fermenter and developed production upon a factory scale which is said to have reached half of the citric acid used in the United States. The basis of this fermentation at that time was granulated sugar and the agent was a black Aspergillus originally iso-