SEXUAL HORMONES IN ACHLYA

IV. PROPERTIES OF HORMONE A OF ACHLYA BISEXUALIS

By JOHN R. RAPER* and A. J. HAAGEN-SMIT

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena)

(Received for publication, December 6, 1941)

The sexual reproductive process of *Achlya ambisexualis*, a heterothallic form belonging to a well known and widely distributed genus of saprophytic aquatic fungi, is initiated and coordinated by means of specific substances (Raper, 1939, *a*). Four of these substances have been demonstrated (Raper, 1939, *b*, 1940) in the rôles of initiators and coordinators of the various stages in the sexual process.

The entire process is initiated by the secretion from the female plant of hormone A which brings about the formation of male sexual organ initials, thin thread-like filaments or antheridial hyphae, on the male plant. The antheridial hyphae then secrete the second of the specific substances, hormone B, which causes the formation of female sexual organ initials, oogonial initials, on the female plant. The oogonial initials, when fully formed, secrete the third substance, hormone C, which has two specific actions on the antheridial hyphae of the male: (1) It causes a directional growth of the antheridial hyphae, so that they grow to and become applied to the oogonial initial, the locus of the production of hormone C. (2) Once the tip of an antheridial hypha becomes applied to the walls of an oogonial initial, hormone C is necessary for the differentiation of the small male sexual organ, the antheridium. Finally, after they are differentiated from the antheridial hyphae, the antheridia secrete a fourth specific substance, hormone D, which brings about the differentiation of the female sexual organ, the oogonium, from the oogonial initial. The differentiation of gametes and the process of fertilization follow to form oospores (zygotes).

The discovery of this mechanism pointed to a number of possible lines of investigation. Of these possibilities, that relating to the chemical nature and identity of the several specific substances was particularly interesting to the authors of the present account. The first of the four

* Report of work carried out with the aid of Works Progress Administration, Official Project No. 165-1-07-172, Work Project No. N-12165. The authors wish to acknowledge the valuable assistance of Mr. Harvey W. Tomlin in the culturing and testing necessary to the work.

This investigation was made during the tenure of a National Research Fellowship in Botany.

specific substances, hormone A, has been the subject of this work for three reasons. (1) Of the four hormones it alone is secreted by the entire vegetative plant and not by specialized organs; hence probably in the greatest quantity. (2) Its activity in controlling the initial reaction in the sexual process makes it of first importance. (3) A method of quantitative determination, biological assay, for this substance has already been worked out (Raper, 1942).

The present paper reports the results of the work done on the problem of the properties of hormone A during the stay of the senior author in these laboratories. The work is being continued at present in these laboratories.

Materials and Methods

It is known from previous work that two species of *Achlya* in which hormones have been demonstrated, *A. ambisexualis* and *A. bisexualis*, are incompatible. Incompatibility results from specificity of certain of the hormones secreted by the two plants. Hormone A of either species brings about the production of antheridial hyphae on the male plants of both species. However, the male plant of *A. ambisexualis* is the more vigorous of the two and it reacts much more strongly in the filtrate from the female plant of *A. bisexualis* than in that from the female of the species to which it belongs. Whether the greater activity brought about by the filtrate of *A. bisexualis* female is due to minor differences in the chemical nature of the specific substances secreted by the two species or to the greater quantity produced by this plant is an interesting question, for which the answer is not known. In either case the quantitative test for hormone A of *A. bisexualis* is more sensitive than for the corresponding hormone of *A. ambisexualis*, since the method of assaying depends on the intensity of the reaction induced in the male plant.

The female plant of *Achlya bisexualis*, therefore, has been used exclusively as the source of hormone A in the present study. It is the opinion of the writers that the initial hormones of the two plants will eventually be shown to differ slightly from each other in chemical composition.

The male plant of *Achlya ambisexualis* has been used in all cases in testing for hormone A.

Technique of Culturing Female Plant—A large number of natural products of plant and animal origin have been tested with the male test plants in the hopes of finding a source of the hormone other than the female mycelium. In all cases the results have been negative. Preliminary work also showed that the quantity of hormone secreted by the female plant was exceedingly small. As a consequence large amounts of the fungus have of necessity been grown in the laboratory to furnish the material necessary for extended work.

Bottles of 20 liters capacity, such as are used to distribute distilled or mineral water, have been found satisfactory as culture vessels. 18 liters of distilled water containing low concentrations of several salts (KH_2PO_4, M/3 × 10^4; $MgSO_4$, M/8 × 10^4; $ZnSO_4$, M/10^7; $CaCl_2$, M/10^5; and $FeCl_3$, M/10^6) were poured into each bottle. The bottles and the dilute salt solution were then sterilized in a large horizontal drum with live steam at atmospheric pressure for 5 hours. Upon cooling, 100 gm. of halved hempseed[1] and 250 cc. of water in which they had been boiled were added to each bottle. At the same time a sterilized solution of 350 to 400 mg. of malonic acid was added to each bottle, the addition of this acid in the concentration of 20 to 30 mg. to each liter of culture medium having been shown to increase greatly the production of hormone A by the female plant (Raper, 1942). The culture was then inoculated by adding to each bottle a fully matured mycelium of the female plant grown on a half hempseed in a small Erlenmeyer flask in 10 cc. of water. Such a mycelium has a large number of sporangia and chlamydospores which within a few hours liberate millions of zoospores, each capable of germinating to form a mycelium.

A sterile aeration tube was placed in each bottle with sterile cotton forming a plug. A piece of pressure tubing was used to connect the aeration tube to an outlet of the compressed air line. The upper portion of the aeration tube was packed with glass wool to entrap any microorganisms blown into it from the air line. For the first 2 or 3 days of incubation only a small stream of air was blown through the culture and the hempseed remained at the bottom of the bottle and became uniformly covered with the young germlings. A strong stream of air was then blown into the culture until the somewhat matted mass of seed and fungus was broken up into individual seeds and their attached mycelia. The cultures were then incubated for 10 to 12 additional days with a sufficient stream of air bubbled through them to keep the mycelia separated and in constant motion. The air stream method of stirring also served the necessary function of keeping the water well aerated, a constant supply of oxygen being required for the vigorous growth of the organism. At the end of the incubation period the mycelia and seeds were removed from the liquid by filtering through a pad of cotton, the filtrate containing 500 to 1500 units of hormone A per cc.

From the standpoint of hormone A content of these large cultures sterilization of the medium and the culture of the plant under aseptic conditions

[1] The seeds were neatly split in quantity by a machine designed and built by Mr. Tomlin. Seeds boiled until the seed coats burst were found to be very inferior to split seed boiled only long enough to effect sterilization. On halved seeds the mycelia grew readily and vigorously.

are not absolutely necessary. When these precautions are not taken the fungus gets well started before bacterial contamination becomes apparent and the vigor of mycelial growth is only slightly affected. The hormone content of such a culture is decreased by a negligible amount. However, the filtrate from contaminated cultures is so unpleasant to work with that the extra time and effort required to grow the source plant in pure culture are well spent.

Biological Assay—The method of quantitative determination of hormone A has been described in a previous paper (Raper, 1942). The intensity of the initial male reaction, as measured by the relative number of antheridial hyphae produced on a male plant, is directly proportional to the concentration of hormone A. A physiological unit of hormone A has been defined as that amount of hormone per cc. of water which will bring about the production of an average of ten antheridial branches per 3 mm. of hyphal tip on 72 hour male tests plants, at 25°, at pH 6.0, when the concentration of dissolved electrolytes does not exceed 0.001 M, and at the time of maximal male sensitivity. Light has not been shown to affect the reaction.

One modification has been made in the test. It was shown that the sensitivity of the male test plants to hormone A varies greatly at different times. This variation is not a strict diurnal rhythm and no means has been found whereby it can either be eliminated or stabilized at 24 hours. It has been found, however, that plants left in a hormone A solution for 24 hours produce no greater number of antheridial hyphae than are produced in the same solution in a 2 hour period at the time of maximal sensitivity. After 24 hours in the test solution those antheridial hyphae produced first are extensively branched but only the original branches (arising directly from the parent vegetative hyphae) were counted. Even so there was still slight variation from day to day, but this variation seldom exceeded 25 per cent deviation from the mean. To correct for this variation, controls containing hormone A in the concentrations of 0.6, 6.0, and 60 hormone A units per cc. were tested simultaneously with each unknown sample. These control solutions were respectively 10^{-5}, 10^{-4}, 10^{-3} dilutions of the acetone-soluble material from concentrated female filtrate. This standard solution, used throughout the work, contained 6×10^4 hormone A units per cc. Each sample was tested in a series of at least five dilutions, a factor of 10 between the concentration in the members of the series. From the counts thus obtained the amount of hormone A (in physiological units) contained in any sample could be calculated.

Properties of Hormone A

A number of attempts have been made during 2 years to isolate hormone A as a chemically pure compound. While this objective has not been at-

tained, many pertinent facts about the properties of the hormone have been determined. Those most likely to be of help in future work on isolation are given below.

Solubilities—The solubilities of the active substance in the more common organic solvents are as follows: (1) very soluble in methyl alcohol, ethyl alcohol, acetone, diethyl ketone, ethylpropyl ketone, dioxane, acetic acid, acetic anhydride, methyl acetate, pyridine, and chloroform; (2) slightly soluble in ethyl acetate, propyl alcohol, amyl alcohol, ethyl ether, carbon tetrachloride, toluene (90–100°), and water (100°); and (3) insoluble in toluene (cold), water (cold),[2] petroleum ether, ligroin, benzene, and carbon disulfide.

Metallic Precipitation—Hormone A is not precipitated by ions of heavy metals. Since the active substance is not soluble in water, a modification of the usual procedure has been tried. To an alcoholic solution containing the active substance was added an equal volume of saturated solution of the acetate of the metal in alcohol. This mixture was then diluted with 3 times its volume of distilled water and cooled to 0–5° for 24 to 48 hours. When this procedure was used, the acetates of cadmium, lead, copper, barium, and mercury, as well as phosphotungstic acid, all gave precipitates. In all cases, however, the greater percentages of activity remained in the filtrates. Only in the case of phosphotungstic acid precipitation (in the presence of sulfuric acid) did recovery approximate 100 per cent. The loss of activity in the other instances was apparently due to adsorption of the active compound on the precipitates which were formed. The only satisfactory eluting solvents, alcohol and acetone, however, dissolved a large portion of the precipitates. Thus of the heavy metals tried only phosphotungstic acid can be used to advantage in the purification of the hormone.

Adsorption—The active substance can be adsorbed on a number of materials. Adsorption on norit is very strong from water (very low concentration of the hormone) and ethyl ether. However, acetone elutes only about 25 per cent of that adsorbed, and with a mixture of acetone-water-ammonium hydroxide a maximum of 50 per cent is recovered. Adsorption is also strong on aluminum oxide from water, ether, chloroform, and carbon tetrachloride. From this material the activity can be recovered quantitatively by elution with acetone. On silica hydrate adsorption is slight from ether and carbon tetrachloride and the recovery is complete. There was no adsorption on calcium carbonate from any of the solvents used. Considerable enrichment of the active principle could be attained by ad-

[2] Hormone A is physiologically active in exceedingly low concentration and, of course, it is sufficiently soluble in cold water to give a solution of the necessary concentration for maximal physiological activity.

sorption on aluminum oxide. On this material the active and a large portion of the inert material was adsorbed at the same level in a Tswett column and enrichment depended almost entirely on the fact that the active material can be eluted with acetone, whereas a stronger eluting agent is required to remove the inactive material.

When a Tswett column of aluminum oxide on which the material had been adsorbed was examined under ultraviolet light, no fluorescence could be detected.

Acid and Base Relations—Hormone A is stable to both acid and base. When solutions containing the active principle were treated with 10 per cent H_2SO_4 and 10 per cent KOH for 24 hours at 25°, no decrease in activity resulted in either case.

Likewise the hormone is soluble neither in dilute acid, nor in dilute base. The active principle is neutral, since it cannot be extracted from ether solution either by repeated partitions with 5 per cent KOH or with 5 per cent HCl.

Ketone and Aldehyde Reactions—When an alcoholic solution containing the active substance is treated with trimethylacethydrazide ammonium chloride (Girard and Sandulesco, 1936) according to the procedure of Petit and Tallard (1939), only 30 to 40 per cent of the activity could be extracted from the alcoholic reaction mixture (non-ketone fraction). However, after hydrolysis for 1 hour with 0.5 N HCl, the remaining 60 to 70 per cent of the active material was extractable in ethyl ether (ketone fraction). When the non-ketonic fraction was treated a second time with the reagent, practically all of the activity was again found in the non-ketonic fraction, the activity in the ketonic fraction being negligible.

These results indicate two possibilities: (1) Two compounds, one ketonic, the other non-ketonic, each having activity in inducing antheridial hyphal formation are secreted by the female plant. (2) A single active compound, a ketone, is present but its separation was incomplete.

The second of the two possibilities seems likely in view of the fact that no other indication of the presence of two active compounds has been encountered elsewhere in the work.

Another indication of the ketonic nature of hormone A is that complete inactivation can be brought about by reaction with 2,4-dinitrophenylhydrazine. A gummy precipitate is formed with this reagent, but neither the precipitate nor the filtrate (from which the excess reagent had been removed) gave any effect when tested with male plants.

Inactivation—Two means have been found of inactivating hormone A in addition to that mentioned above. Temperatures in excess of 130° bring about complete inactivation. In attempts to purify the material by micro distillation *in vacuo* (0.01 mm. of Hg), fractions distilling over below

this temperature were inactive, and at 125–130° the residues were likewise inactive.

A more thermostable derivative was desired and a reaction with diazomethane was carried out. Complete inactivation resulted and no means was found of recovering the activity.

TABLE I

Procedure of Chemical Fractionation of Female Filtrate for Enrichment of Hormone A

Treatment	Active fraction	Dry weight	Total activity, hormone A units × 10^6	Hormone A units per mg.	Inactive fraction
		gm.			
	Original filtrate	393	1872	4.7×10^3	
Vacuum-distilled	Crude concentrate	393	1872	4.7×10^3	Distillate
Acetone	Filtrate	163	1840	1.13×10^4	Ppt.
Saturated NaCl	Acetone	53	1834	3.45×10^4	Water
Petroleum ether extraction	Water	48	1834	3.75×10^4	Petroleum ether
Ethyl ether extraction	Ether	4.5	1830	4.0×10^5	Water
5% HCl wash	"	4.1	1830	4.5×10^5	HCl
5% KOH "	"	0.554	1830	3.3×10^6	KOH
Toluene, 100°	Soluble		1774		Residue
Chilled to 0°	Ppt.	0.113	1700	1.54×10^7	Filtrate
Phosphotungstic acid	Filtrate	0.063	1625	2.6×10^7	Ppt.
Adsorbed on Al_2O_3	Adsorbed		1600		Filtrate
Eluted with acetone	Eluate	0.013	1575	1.21×10^8	Residue
Dissolved in chloroform, ether-petroleum ether added at −80°	Ppt.	0.0042	700	1.66×10^8	Filtrate
Dissolved in H_2O at 100°	Water	0.002 (*Ca.*)	700	3.5×10^8	Residue

Chemical Fractionation

A representative chemical fractionation will be given to illustrate the procedure followed in attempts to isolate the active compound. The procedure is outlined in Table I.

The filtrate from eighty 18 liter cultures of the female plant (1440 liters) was collected over a period of 2 months. The hormone content varied between 1000 and 1500 hormone A units per cc. of filtrate with an average of 1300 hormone A units per cc. The starting material thus contained 1.872×10^9 units of hormone A.

Concentration—Each week's collection of filtrate (180 liters) was concentrated in a large metal still (with continuous feed) at 25 to 30 mm. pres-

sure to approximately 1/200 its original volume. The solid material in the combined concentrates of the 8 weeks weighed 393 gm. and no activity was lost during concentration.

Acetone Precipitation—To the 7 liters of concentrate was added an equal volume of redistilled acetone and the temperature of the mixture was lowered and kept at 0° for a week. A precipitate of 230 gm. contained 3.2×10^7 units. The filtrate contained 163 gm. of solid material which had a total activity of 1.84×10^9 units, an enrichment of 2.5 times.

Acetone-Water Partition—The acetone-water filtrate was then saturated with NaCl and the mixture allowed to separate into acetone and water layers. The water layer was shaken with two additional washes of acetone and these were combined with the original acetone layer. The acetone solution thus obtained contained 53 gm. of solid material with 1.834×10^9 units.

Extraction with Petroleum Ether—The acetone solution was boiled down and the residue suspended in 2 liters of water. This was transferred to a continuous extractor and extracted exhaustively (2 days) with petroleum ether (boiling range 40–60°). The activity remained quantitatively in the water layer, whose solid content was reduced to 48 gm.

Extraction with Ethyl Ether—The water suspension was then exhaustively extracted with ethyl ether for a week. (The ether was purified by standing over $CaCl_2$ for a week, filtered, and allowed to stand over metallic sodium from which it was distilled. Ether obtained by other methods of purification caused a great loss of activity.) This ether extract contained 4.5 gm. of solid material and activity of 1.83×10^9 units.

Acid Wash—The ether extract (2 liters) was then washed with six 100 cc. portions of 5 per cent HCl followed by two 100 cc. portions of water. 400 mg. of completely inactive material were thus removed, the active material remaining in the ether.

Alkali Wash—The ether extract was then washed with six 100 cc. portions of 5 per cent KOH. Again the activity remained in the ether fraction. The ether was boiled off and the reddish residue weighed 554 mg. and had activity of 1.83×10^9 units. The active material in this residue was then 700 times more concentrated than in the original material.

Solution in and Precipitation from Toluene—The residue was then heated on a water bath at 90–100° with three 10 cc. portions of toluene. Practically all of the active material was soluble. The toluene solution was concentrated to about half its original volume and then placed in an ice-salt bath at 0°. A reddish yellow gelatinous precipitate weighing 113 mg. with activity of 1.7×10^9 units was obtained. At this time the active material had been enriched more than 3000 times.

Phosphotungstic Acid Precipitation—The precipitate from cold toluene

was then taken up in 5 cc. of purified ethyl alcohol. (The alcohol used was refluxed with powdered zinc for 10 hours, distilled, refluxed with CaO for 12 hours, and again distilled. Alcohol obtained by other treatments caused loss of activity.) To the alcoholic solution were then added 5 cc. of a 20 per cent solution of phosphotungstic acid in alcohol. To this were added 20 cc. of 5 per cent sulfuric acid in water, and the mixture kept at 0° for 24 hours. The precipitate was removed by centrifugation, dissolved in alcohol, and reprecipitated. Materials in the combined filtrates and in the precipitate were then recovered by the methods described by Kögl and Tönnis (1936). The filtrate contained 63 mg. of material with activity of 1.625×10^9 units. The active substance had now been concentrated more than 5000 times.

Adsorption on Aluminum Oxide—After the solvent was removed from the filtrate, the residue was dissolved in 10 cc. of chloroform. To this was added 1 gm. of finely pulverized aluminum oxide which had been heated strongly for 5 hours immediately before use. The flask containing the chloroform solution and the alumina was kept in constant motion for 12 hours. At this time the aluminum oxide was filtered off. The chloroform filtrate contained no activity. The alumina was then shaken with three 10 cc. portions of acetone. 13 mg. of material were recovered from the adsorbing agent in the acetone and its activity assayed at 1.57×10^9 units. Additional elution with acetone-water-ammonium hydroxide yielded 45 mg. of inert substances. The residue of the acetone eluate contained the active material enriched about 25,000 times.

Precipitation from Chloroform and Ether—The yellowish residue was dissolved in 0.2 cc. of chloroform. To this was added 0.8 cc. of a mixture of ethyl ether and petroleum ether. The solution was then placed in an alcohol-carbon dioxide bath at $-80°$ for 6 hours. The solvent mixture was then filtered off, leaving 4.2 mg. of a creamy white flocculent precipitate. In this particular case approximately equal amounts of activity, 7×10^8 units, went into the two fractions. The greater part of the activity in the filtrate was recovered by repetition of the precipitation, but subsequent precipitates contained a much higher percentage of inert material. At other times a much higher percentage of the active material had been precipitated out in the initial chilling. Even so the concentration of active material in the precipitate was 35,000 times that in the original material.

Solution in Hot Water—The active substance was then dissolved in three 1 cc. portions of boiling water. After the water was boiled off, the filtrate of partially crystallized creamy white material weighed approximately 2 mg. with activity of 7×10^8 units.

The active material in this residue had been enriched slightly more than 70,000 times as compared with the dry weight of the starting material and

the residue caused the male test plant to produce antheridial hyphae in the concentration of 10^{-12}.

Crystallization from water and from toluene yielded two crystalline substances (m.p. 155°, 218°) both of which were inactive, the activity in both cases remaining in the mother liquor.

SUMMARY

1. The hormonal coordinating mechanism of the sexual process in *Achlya* is briefly reviewed.

2. A technique is described for culturing the female plant of *Achlya bisexualis* in sufficient quantity to furnish material for the chemical study of hormone A.

3. A modification of the biological assay for hormone A is described.

4. Many of the properties of hormone A have been determined: (*a*) solubilities in common organic solvents, (*b*) adsorption, (*c*) stability, (*d*) inactivation, and (*e*) reactions with certain reagents.

5. A procedure is described whereby enormous enrichment of the active principle has been achieved.

BIBLIOGRAPHY

Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19,** 1095 (1936).

Kögl, F., and Tönnis, B., *Z. physiol. Chem.*, **242,** 43 (1936).

Petit, A., and Tallard, S., *Rev. chim. ind.*, Paris, **48,** 226 (1939); *Chem. Abst.* **34,** 716 (1940).

Raper, J. R., *Science*, **89,** 321 (1939, *a*); *Am. J. Bot.*, **26,** 639 (1939, *b*); **27,** 162 (1940); **29,** 159 (1942).