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SEXUAL HORMONES IN ACHLYA. V. HORMONE A', A MALE-SECRETED AUGMENTER OR ACTIVATOR OF HORMONE A

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The rôle of specific sexual hormones in the sexual reaction of two heterothallic species of *Achlya* has been described in earlier papers of this series.[1] Four specific substances were postulated, two secreted by the ♀, bringing about specific reactions in the ♂, and two secreted by the ♂, inducing specific responses in the ♀. On the basis of evidence available at that time it seemed that the entire sexual reaction was initiated wth the secretion of hormone *A* by the vegetative mycelium of the ♀, which induced the formation of sexual organ initials, antheridial branches, on the ♂. In the course of a more detailed analysis of the response of the ♂ plant to hormone *A* from the ♀,[2] it was found that the vegetative mycelium of the ♂ secreted some factor which profoundly influenced its response to hormone *A* from the ♀. This factor, produced by the vegetative ♂ plant independently of any response to the ♀, will hereafter be referred to as hormone *A'* (*A* prime).

The species and sexual strains used in this work are those previously employed in the work on sexual hormones in *Achlya: Achlya bisexualis* ♀ for the production of hormone *A*,[3] and *Achlya ambisexualis* ♂ as test plants for the assay of hormone *A* and for the detection of hormone *A'*. The method of testing is that previously described[2] and the standard hormone *A* solution is the same as that used throughout the work on the characterization of this hormone.[4] Any modifications of previously described methods or techniques will be described in the body of the paper.

The discovery of hormone *A'* came about quite by accident. In the analysis of the response of the ♂ plant to hormone *A*, a series of experiments was performed in an attempt to determine whether the hormone was used up, i.e., removed from the test solution during the course of the reaction, or whether it produced its effect through the catalytic activation of some existing physiological system, the hormone thus remaining un-

changed and unconsumed in the test solution. The first of these experiments was the repeated quantitative determinations of the hormone *A* content of a 10-ml. sample of standard (6 units/ml.) test solution at successive times with new ♂ plants. The results were unexpected, for instead of the response remaining constant or decreasing there was an *apparent* increase in the amount of hormone *A* during the first day of the experiment. This increased activity reached approximately 300% of that of the sample at the beginning of the experiment and of that of a control series of new samples of hormone *A* solution tested at the same intervals. Toward the end of the second day the reaction decreased somewhat, probably because of the dilution of the hormone *A* solution by water unavoidably carried in with each new pair of ♂ plants. The results of these tests could not be explained at the time.

A second series of experiments to determine the ultimate fate of hormone *A* was then performed. Into 10-ml. samples of standard hormone *A* solution in Petri-plates were placed ♂ test plants with two plants in the first plate, four in the second and so on up to twenty plants in the tenth plate. At the end of two hours the average number of antheridial branches per vegetative hypha was determined for the plants in each sample. On three successive days, October 7, 8 and 9, 1940, the results were the same. In each series the intensity of the response varied directly with the number of test plants in the 10-ml. sample of standard hormone *A* solution. The average responses for the three series are given in table 1.

TABLE 1

MASS EFFECT OF ♂ PLANTS ON THEIR RESPONSE TO HORMONE *A*

Number of plants in tests	2	4	6	8	10	12	14	16	18	20
Average number of antheridial branches	5.2	5.8	9.7	9.8	10.6	13.1	13.7	12.5	13.9	14.1

The results from these tests agree strikingly with those of the previous experiment when, in effect, additional plants were added to the sample.

From these experiments it was obvious that the ♂ plants themselves exerted some mass effect on their response to hormone *A*, and that the intensity of their response depended on some factor in addition to that of hormone *A* concentration. The most probable explanation of this increase seemed to be the secretion by the ♂ plant of a factor which augmented the effect of hormone *A*, or which behaved as an activator for hormone *A*. The secretion of such an active substance, hormone *A'*, has been repeatedly confirmed by experiments extending over a period of two years.

The first tests designed to demonstrate the secretion and activity of an active substance from the ♂ were made as follows. The liquid from a number of 48-hour cultures, each containing ten ♂ plants in 10 ml. of water, was filtered, and this filtrate was used in making up a series of test

solutions containing undiluted ♂ filtrate plus concentrations of hormone *A* over the range of 0–6 units/ml. A second series, similar to the first but made with distilled water instead of ♂ filtrate, served as controls. Into 10-ml. samples of each of these solutions were placed two ♂ test plants which were washed in several changes of distilled water immediately before use. At the end of two hours counts of the antheridial branches were made. The results are given in table 2. A number of facts are apparent when these data are analyzed: (1) The filtrate from the vegetative ♂ plants contains an active substance, hormone *A'*. This sub-

TABLE 2

EFFECT OF ♂ FILTRATE ON THE REACTION OF ♂ PLANTS TO HORMONE *A*. REACTION INTENSITY IS EXPRESSED AS AVERAGE NUMBER OF ANTHERIDIAL BRANCHES

Hormone *A*, units/ml.	0	1	2	3	4	5	6
Control (no ♂ filtrate)	0	0.6	1.7	2.7	3.0	3.6	4.2
♂ filtrate added	0	11.8	12.6	13.1	12.4	10.4	12.5

stance (*a*) in the absence of hormone *A* is inactive in inducing the formation of antheridial branches, but (*b*) in the presence of hormone *A* activates this hormone or augments its activity. (2) The ♂ filtrate, in the presence of hormone *A* within the range of concentrations here used, determines the intensity of the ♂ reaction.

Comparison of the results in the foregoing experiments with those to follow will show that the amount of activation of ♂ filtrate varies appreciably among different samples of ♂ filtrate. Preliminary attempts to standardize the conditions of hormone *A'* production and to establish a quantitative test for its relative concentration have been unsuccessful. The experiments to follow, however, will show something of the activity of hormone *A'* and its relation to the rôle of hormone *A* in inducing the formation of antheridial branches on the ♂ plant.

The interaction of hormones *A* and *A'* is best shown in the following experiment. Two series of test solutions were prepared, and all the samples were tested simultaneously with carefully washed ♂ plants. In the first series hormone *A* in concentrations of 0.0, 0.006, 0.06, 0.6, and 6.0 units/ml. were added to 10-ml. samples of undiluted ♂ filtrate as prepared in the preceding experiment. The second series consisted of 10-ml. samples each containing 6.0 units/ml. hormone *A* plus 100, 50, 10 and 0% of ♂ filtrate. Average antheridial branch production (reaction intensity) for each solution was determined at the end of two hours. The results in figure 1 and table 3 clearly show that the number of antheridial branches produced by the ♂ plant varies in direct proportion to the concentration of both hormones *A* and *A'*, and that for a greater than minimal reaction both hormones must be present in adequate quantity. Thus a deficiency in either results in a low intensity reaction. The absolute necessity of

hormone A (from the ♀) for the production of antheridial branches on the ♂ plant is again demonstrated by these results.

TABLE 3

INTERACTION OF HORMONE A AND HORMONE A' IN THE PRODUCTION OF ANTHERIDIAL BRANCHES

Per cent conc. ♂ filtrate	←——100——→				50	10	0
Conc. hormone A, units/ml.	0.0	0.006	0.06	0.6	←——6.0——→		
Reaction intensity	0	2.9	6.7	11.0	27.5	20.6	11.5	7.5

The fact that the ♂ plant in the solution in which no ♂ filtrate was added still gave a reaction cannot be interpreted as meaning that the factor secreted by the ♂ plant is not equally essential. It will be recalled from the first two experiments reported above that the ♂ plants secrete sufficient quantity of hormone A' *during the two-hour testing period* to affect significantly the intensity of their reaction.

A marked rhythmic variation in the response of ♂ plants to a standard solution of hormone A has been described in an earlier paper.[2] No means have been found to change or eliminate this variation through rigid control of the conditions under which the plants are grown and tested. The substance secreted by the ♂ plant was naturally suspected of being responsible for this variation. Since hormone A' increases the activity of hormone A, is secreted by the ♂ plant and can limit the reaction within a critical concentration range, it seemed entirely possible that such variation resulted from the elaboration and secretion into the test fluid of varying amounts of the hormone at different times.

The results of a preliminary experiment seemed to justify this belief. At four-hour intervals carefully washed ♂ plants were placed in 10-ml.

TABLE 4

RELATION OF HORMONE A' TO THE RHYTHMIC VARIATION OF THE RESPONSE OF ♂ PLANT TO HORMONE A, EXPRESSED AS AVERAGE NUMBER OF ANTHERIDIAL BRANCHES

	TIME (NOV. 18–19, 1940)				
	10 A. M	2 P. M.	6 P. M.	10 P. M.	2 A. M.
Standard hormone A, 6 units/ml.	24.7	5.9	12.5	14.1	16.1
Standard hormone A + ♂ filtrate	24.9	21.7	..	21.8	21.7

samples of (1) standard hormone A solution (6 units/ml.) and (2) ♂ filtrate to which had been added hormone A in equal concentration. Counts of antheridial branches were made at the end of the four-hour intervals. These tests were performed under the conditions earlier described for the biological assaying of hormone A.[2, 4] The average number of antheridial branches produced in each of the solutions at four-hour intervals is given in table 4. In the series using only hormone A the coefficient of variation

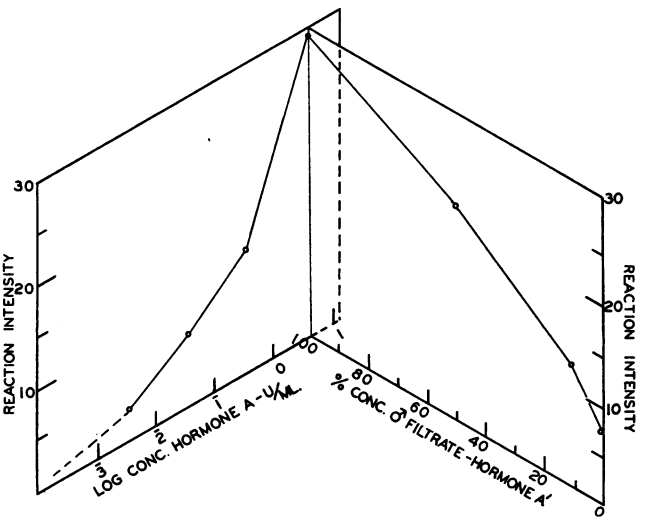

FIGURE 1

Interaction of hormones A and A' in the production of antheridial branches.

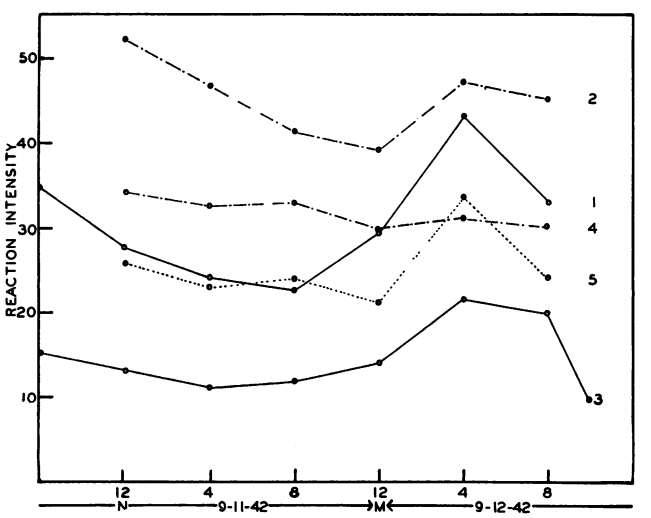

FIGURE 2

Effect of male filtrate on the variation in response of male plants to hormone A. The curves represent the reaction intensities of male plants in the following solutions: (1) 60 units of hormone A/ml. in distilled water, (2) 60 units hormone A/ml. in ♂ filtrate, (3) 6 units hormone A/ml. in distilled water, (4) 6 units hormone A/ml. in ♂ filtrate, and (5) 6 units hormone A/ml. in samples of ♂ filtrate collected at the times indicated.

in the reaction of the ♂ plants was 50.5 ± 4.25%. In the series containing added ♂ filtrate the coefficient of variation was reduced to 25.1 ± 2.1%.[5]

Later a more extensive and more adequately controlled experiment was conducted to retest the results obtained in the preliminary experiment above. Four solutions were made up as follows: (1) 60 units of hormone *A*/ml. in distilled water, (2) 60 units of hormone *A*/ml. in undiluted filtrate from ♂ plants as described above, (3) 6 units hormone *A*/ml. in distilled water and (4) 6 units of hormone *A*/ml. in ♂ filtrate. Two carefully washed ♂ plants were placed in a 10-ml. sample of each of these solutions, and at the end of four hours counts were made of the antheridial branches on the ♂ plants in each solution. This procedure was repeated at four-hour intervals during 24 hours. In addition to the four series thus established, a fifth series (5) was set up as follows: At the beginning of the experiment twenty ♂ plants were carefully washed and placed in 10 ml. of distilled water. At the end of each four-hour period the liquid was poured off these plants and replaced by fresh distilled water. The samples which were poured off were saved, and at the end of the 24-hour testing period hormone *A* was added to each in a concentration of 6 units/ml. The six samples, comprising series 5, were then tested with ♂ plants simultaneously with a standard (6 units/ml.) hormone *A* solution as control. The results of these five series are plotted in figure 2. It is obvious on examination of these data that under these conditions the variation of ♂ response is not eliminated by the addition of hormone *A'*, but in each of the series containing added ♂ filtrate the variation is considerably less than in the corresponding control series lacking ♂ filtrate. The mean and the coefficient of variation in each of the different series are presented in table 5. In series 2, containing ♂ filtrate, the amplitude of the variation is only half that of series 1, which contains the same concentration of hormone *A*. The amplitude of the variation in series 4 is approximately one-fourth that in the corresponding control series lacking added ♂ filtrate. These results taken with those in table 3 demonstrate that the variation of the ♂ response is due at least in part to the quantity of some active factor

TABLE 5

HORMONE *A'* CONTROL OF RHYTHMIC VARIATION IN RESPONSE OF ♂ PLANTS TO HORMONE *A*

SERIES	MEAN	COEFF. OF VARIATION—%
1. 60 units/ml. *A*	30.2±0.81	31.6±2.08
2. 60 units/ml. *A* + ♂ filtrate	45.6±0.76	18.2±1.21
3. 6 units/ml. *A*	14.9±0.52	42.6±3.42
4. 6 units/ml. *A* + ♂ filtrate	31.9±0.47	16.5±1.11
5. 6 units/ml. *A* + filtrate from 20 ♂ plants at 4-hour intervals	25.4±0.72	30.5±2.28

secreted by the ♂ mycelium. The rôle of the ♂ secretion is even more strikingly shown in the results of series 5. The curve of the intensity of the reactions induced in the four-hour samples of ♂ filtrate follows rather closely the curves of series 1 and 3, to which no ♂ filtrate was added. The agreement in shape among these curves demonstrates conclusively that the ♂ plant secretes at different times varying quantities of hormone A', and that this variation in amount of secretion results in the rhythmic variation in the response of the plants to a constant concentration of hormone A.

Nothing is known as yet about the nature of the mechanism of hormone A' effect. Elucidation of the nature of this effect will be difficult, since the only means of detecting the presence and activity of the substance lies in the reaction of the plant which secretes it. Nor is it possible at the present time to say whether or not the substance is indispensable for the formation of antheridial hyphae. It seems entirely possible that it is essential, since carefully washed ♂ plants give a reaction of very low intensity at the time of low hormone A' productivity. Before these problems can be solved some means must be found either to inhibit the production of A' or to block its effect. Working out a method for the quantitative determination of the active substance will be similarly handicapped. On the other hand, it is difficult to explain the tremendous variation in response of ♂ plants to hormone A on the basis of quantitative differences of a single substance, and it is entirely possible that more than a single active factor is secreted into the medium by the ♂ plant.

Summary.—In addition to the four specific substances earlier described as initiating and coördinating the sexual reaction in two heterothallic species of *Achlya*, a fifth active factor, hormone A', secreted by the vegetative ♂ mycelium, has now been demonstrated. The activity of this substance has been shown in the following ways: (1) the response of the ♂ plant to hormone A increases (*a*) with successively introduced ♂ plants into a single sample of hormone A solution, (*b*) with the number of ♂ plants per unit volume of hormone A solution, (*c*) in the presence of ♂ filtrate and (*d*) with increasing concentration of ♂ filtrate; and (2) the variation in the response of ♂ plants to hormone A depends upon the rhythmic variation in the quantity of hormone A' produced by the ♂ plant.

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[1] Raper, John R., *Science*, **89**, 321 (1939); *Amer. Jour. Bot.*, **26**, 639 (1939); *Ibid.*, **27**, 162 (1940).

[2] Raper, John R., *Amer. Jour. Bot.*, **29**, 159 (1942).

[3] Hormone *A* from *Achlya bisexualis* ♀ has been used in these studies for convenience and in order to obtain as nearly reproducible solutions as possible. Tests have been made with hormone *A* from *Achlya ambisexualis* ♀ with entirely comparable results.

[4] Raper, John R., and Haagen-Smit, A. J., *Jour. Biol. Chem.*, **143**, 311 (1942).

[5] In this and the following experiment the coefficient of variation has been calculated from the total number of individual counts rather than from the averages given in table 4 and figure 2. Each of these values represents the average number of antheridial branches on twenty hyphae chosen at random.

OXIDIZED COTTON, AN IMMUNOLOGICALLY SPECIFIC POLYSACCHARIDE

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The immunologically specific polysaccharide of Type III pneumococcus, to which the type-specificity and virulence of this pathogenic microörganism are due,[1] is made up of a chain of aldobionic acid,[2] more particularly, cellobiuronic acid,[3] units. The corresponding capsular carbohydrate of the Type VIII pneumococcus contains the same aldobionic acid unit,[4] and in addition, roughly two glucose molecules for every such unit.[5] The close chemical relationship of these polysaccharides is reflected in their serological behavior: each polysaccharide not only precipitates antisera to the homologous pneumococcus type, but the Type III polysaccharide also precipitates a portion of the antibodies in Type VIII antiserum and the Type VIII polysaccharide throws down a portion of the antibodies in Type III antiserum.[6] Evidence has been given that this cross-reactivity is due to some multiple of the repeating unit containing cellobiuronic acid in common.[5]

It has recently been shown that cotton may be oxidized by means of nitrogen dioxide to products containing varying percentages of carboxyl.[7] These products would therefore contain cellobiuronic acid units, separated by glucose, at intervals in the long cellulose chain and might not unreasonably be expected to react specifically with Type VIII antipneumococcus horse serum, or even with the Type III antiserum. Samples of the oxidized cotton containing 16 and 21 per cent of $-CH_2OH$ oxidized to $-COOH$ were kindly supplied by Drs. Yackel, Unruh and Kenyon of the Eastman Kodak Laboratories. While it was possible to fractionate the material to some extent, it was most convenient to dissolve weighed quantities in excess *N* $NaHCO_3$ solution, a slow process in the case of the sample of lower $-COOH$ content, neutralize the excess of bicarbonate and dilute, first with