T H E S I S

STUDIES ON THE OCCURRENCE AND PHYSIOLOGICAL ACTIVITY OF THE SEX HORMONES IN PLANTS

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TABLE OF CONTENTS

Acknowledgments - - - - - - - - - - - - - - - - - - - 2

Introduction - 3

Review of Literature - - - - - - - - - - - - - - - - 4

Chemical nature of the female sex hormone - - - 8

Physiological activities of the female sex hormone - 9

Methods and Materials - - - - - - - - - - - - - - - 10

Experimental Data - - - - - - - - - - - - - - - - - 16

Conclusions - 23

Bibliography - 25

This is to certify that Mr. George Plumb has translated for me assigned passages of technical French bearing upon his graduate Botanical work.

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by

G. W. Plumb

INTRODUCTION

Although some work had been published, at the time this study was begun, on the occurrence of an esterogenic substance in plants, there was some question as to the exact nature of this substance, and as to whether or not the sex hormones play a physiologically significant role in the plant kingdom. This work was undertaken to verify the reports of the occurrence of the material in plant substances, especially some of the fungi other than yeasts, and to determine whether or not the sex hormones from animals would show any physiological effect on plants.

HISTORICAL

The cyclic nature of the activities of the female reproductive organs of both humans and lower animals has been known for centuries. It was also learned from the changes resulting from castration, that the ovaries exerted considerable influence not only over the sex cycle, but also over growth, body form, and metabolism. Beyond these facts, nothing was discovered until the last part of the 19th century.

Berthold's (5) implantation experiments with cockerel gonads in 1848 received little attention and were soon forgotten. The spectacular experiments, however, of Brown-Sequard (9) in 1891, although his results have never been substantiated, in their appeal to the dramatic instincts of man, have inspired research in every branch of endrocrinology.

Halban (22) and Knauer (27) 1900, following Berthold's style of attack, proved definitely that the control exerted by the ovary was due to an internal secretion. In these experiments, ovarian tissue was severed from all blood and nerve connections, and transplanted to the abdominal wall of the animal. The sex cycle in such animals continued normally, and the secondary sex characters did not undergo the changes

characteristic of castrate animals.

Following this discovery, much work was done in injecting ovariectomized animals with extracts of ovaries. Probably the most important discovery was that of Iscovesco (25) in 1912, who found that the active principle was soluble in lipoid solvents. He also discovered that the lipoid extract caused hypertrophy of the uterus of castrate female animals.

Research in endrocrinology, as well as in other branches of science, was materially slowed up by the war. The period immediately following, however, saw a greatly increased activity in all fields of scientific endeavor. Hormone experiments were undertaken in laboratories throughout the world. Among the work turned out in this period was the new biological method of assay, introduced in 1923, by Allen and Doisey (2).

This simple and comparatively rapid test greatly facilitated the study of the hormone. The next problem was to find a cheap, convenient source of the material. The active principle had already been extracted from ovarial follicular fluid, and corpora lutea, and attention was turned to the other materials. The hormone was found in placenta and in blood and milk of animals in heat.

The most abundant and satisfactory source of the hormone found up to the present time, is the urine of pregnant women and pregnant mares. The high concentration of the hormone in this readily available material was reported in 1926 by Ascheim and Zondek (4).

Among the more recent reports is that of Zondek (50) who finds the concentration of estrin in the urine of the stallion to be higher than that of the urine of pregnant mares.

A considerable amount of work has been done in the plant kingdom on hormones. By far the greater part of this work, however, has been done on a plant growth hormone.

An active principle has been extracted from growing plants by Bonner (7 and 8) and others, (31, 34, 36, 43, 46, and 49) and called "Rhizopin" because it was produced in large quantities by the saprophytic fungus, Rhizopus suinus.

This plant growth hormone apparently is produced in rapidly dividing cells, and diffuses back to the older cells, stimulating their development (49). Hykes (23) reports the effects of various hormones on portions of plants, but does not mention the sex hormones.

The female sex hormone was isolated from several

types of plant material by various workers. Dohrn, Faure, Poll, and Blotevogel (15) in 1926 found the material in sugar beet seeds, potato tubers, parsley roots, the pericarp of cherries and plums, and in yeast. Fellner (11) in the same year, isolated the active principle from meal, oatmeal, and rice. Loewe, Lange and Spohr (30) in 1927, found an esterogenic substance in the catkins of willow, stems of Impatiens parviflora, the ovaries of Nuphar luteum, and a plant without blossoms,(Althea rosea).

Glimm and Wadehn (19) in 1928 studied different types of yeast with regard to their estrin content.They found that a strain of beer yeast produced more of the active principle than a strain of compressed yeast. Frank and Goldberger (17) in 1928 also isolated an estrus-producing substance from yeast.

Walker and Janny (48) found an esterogenic material in both male and female willow and alder catkins, rhubarb and alder leaves and oats. They found a higher concentration in germinating oats than in dormant seed. They obtained negative results from extracts of apples, beets, cabbage, carrots, corn, grapes, peaches, plums, potatoes, spinach, sweet potatoes, swiss chard, and tomatoes.

Much (32) in 1931 reports active extracts of

elder, linden, and nettle blossoms.

Butenandt and Jacobi (10) in 1933 isolated the active principle from corn, tomato, onion. They also crystallized 18 mg. of a substance from 50 kg. palm nuts. This substance was identified by them as identical with crystalline theelin.

Skarzynsky (38) also reports the preparation of a crystalline material from female willow catkins. This preparation was very similar to theelol in its physical properties. Its biological activity, however, was only about one-fourth that of theelol.

In addition to the discovery of these materials as sources of the female sex hormone, an active substance has been isolated from soft coal, petroleum, asphalt, peat, brown coal and coal tar. (Ascheim and Hohlweg,1933) (3).

CHEMICAL NATURE OF THE FEMALE SEX HORMONE

Considerable work has been done on the chemistry of the female sex hormone (1). The crystalline material originally isolated from crude extracts has been found to be a mixture of two compounds, generally known in this country as theelin and theelol. Theelin is an unsaturated ketomonatomic alcohol. Its molecular weight is approximately 270 and its emperical formula $C_{18}H_{22}O_2$.

The hydroxy group is phenolic in character. Theelin is physiologically active only when injected in to the animal (1, 21).

Theelol, on the other hand, differs from theelin only by the addition of a molecule of water to the theelin molecule, making the formula of theelol $C_{18}H_{24}O_3$. This compound is a try-hydroxy compound and is considered by some workers to be absorbed from the alimentary tract when administered orally (1, 21). Theelol has been converted to theelin by dehydrating it with fused potassium bisulphate and sublimation at .02 mm. pressure and 18°C. (1). The activity of theelol has been reported as about half that of theelin (11).

PHYSIOLOGICAL ACTIVITIES OF THE FEMALE SEX HORMONE

Although a complete physiological significance of the female sex hormone is not completely worked out as yet, it has been definitely proven that it controls the sexual cycle, and that it is of prime importance in governing the development of the mammary glands and the secondary sex characters.

One of the most outstanding of these characters is the color of the plumage in fowls (26, 27). Theelin has also been found to terminate pregnancy in animals when too great a concentration of the hormone is present. (12, 35, 39).

Cultures were grown in 9 cm. petri dishes, the medium being prepared in a manner similar to that suggested by Blakeslee (6). The medium in the plate was divided into two parts by a groove 5 mm. wide thru the center of the plant. The plus strain of the organism was placed on one side of the groove, and the minus strain on the other. The two strains of the fungus grow toward each other, and thus conjugate along the groove, which, being free from agar makes the fusion easier to observe.

The number of conjugations occurring in the center 15 mm. of the groove was recorded as an index of the effect of the hormone on the sexual cycle of the organism.

The male hormone used in these experiments was obtained from Dr. R. G. Gustavson of the Denver University, and was prepared by E. B. Womack of the University of Chicago from beef testicles (24, 49).

The female sex hormone was prepared by Gustavson from pregnancy urine (14), and was of such concentration that five gamma represented one Coward-Burns rat unit. The Coward-Burns rat unit of estrin (13), which is the unit used throughout this work, represents a considerably larger quantity of the active principle than does the Allen-Doisy unit. The Coward-Burns unit is about 10 or 12 times as large as the Allen-Doisy unit

(13, 37, 40).

The Coward-Burns unit was used chiefly because of the greater convenience of the single injection as compared to the series of injections used in the Allen-Doisy assay.

Theelin, prepared by Parke-Davis, and standardized at the University of St.Louis was also used to eliminate the possibility that some impurity in the crude preparation used might be responsible for the reactions observed.

Extractions were made from Rhizopus nigricans to determine whether or not the active principle is secreted by this organism. Extracts were made from the plus strain, the minus strain, and from cultures of both the plus and minus strains.

Cultures for extraction were grown in 9 cm. petri dishes in liquid media, on filter paper, according to the method described by Bonner (8). A sheet of filter paper was placed in the bottom of the dish, to provide a solid substrate for the organism and 15 cc. of the culture medium added to the plate.

A medium consisting of 1% dextrose, 1% peptone, .01% potassium acid phosphate, and .01% ferrous sulphate was used. This is the medium which Bonner found most satisfactory for the productionof the plant growth hormone, "rhizopin", by Rhizopus suinus (8).

The dishes were then sterilized in the autoclave, and inoculated with the organism. The plates were incubated for a week at 25°C. and then the cultures were extracted.

The filter papers with the mat of mycelium was squeezed dry, and the expressed liquid extracted with chloroform in an automatic, continuous extractor consisting essentially of a reflux condenser attached to a large flask. A layer of chloroform was poured into the bottom of the flask. The material to be extracted was acidified with acetic acid and poured on top of the chloroform. A reflux condensor was attached to the flask, and the apparatus set on an electric hot-plate. The chloroform boils off, is liquified in the condensor, and drops back thru the solution, carrying with it the fat soluble fraction of the aqueous solution. The whole apparatus was blown of glass, to minimize loss of chloroform in distilling, and to make a more compact apparatus.

As the boiling point of chloroform is relatively low, the heat required to distil it is not sufficient to break down estrin, and as the oxygen in the apparatus is replaced by chloroform vapor, oxidation is reduced to a minimum.

This extractor was devised for these experiments by the author, although it was found later that the same type of apparatus was used by Womack at the University of Chicago (21).

The culture medium was extracted for 24 hours in this apparatus. The chloroform was then separated from the aqueous solution and evaporated by dryness. The residue was taken up with ether.

The filter paper and mycelium were extracted three times with acetone, and the three extractions combined. The acetone was evaporated nearly to dryness, and extracted with benzene. The benzene was distilled off, and the residue taken up with ether. This ether was then combined with the ether fraction from the culture medium. The material was again evaporated to dryness and the residue taken up with a small quantity of ether. This ether was then washed with 350 cc. of an 8.5% solution of $NaHCO_3$. The soda solution was extracted by shaking three times with ether. These ether fractions were combined with the original ether solution. Most of the ether was then distilled off, and 10 cc. of olive oil was added.

The remaining ether was distilled off, under reduced pressure, and the resulting oil solution assayed on castrate female white rates, according to the method

of Coward and Burns, used by Gustavson and his co-workers (13).

For each assay ten rats were injected subcutaneously, the dose being one cc. each. Vaginal smears were taken after 40 hours, and at three subsequent four-hour periods. Smears were made according to the method recommended by Loewe (29). A swab made of cotton twisted on the end of a toothpick was saturated with physiological saline solution, and inserted into the vagina of the animal. The wad was withdrawn and pressed out into about 0.2 cc. of physiological saline solution on a microscope slide. This smear was examined under the microscope for the presence of leucocytes and cornified epithelial cells. A smear in which leucocytes predominate is considered negative. One in which the cornified cells replace the leucocytes, however, is considered positive (13).

The animals used for assay were kept in galvanized wire cages, and fed on a diet consisting of ground wheat, powdered milk, alfalfa meal, and salt. They received fresh meat once a week and lettuce or carrots twice a week. No diseases of any sort have developed in the colony.

In addition to the extracts made of Rhizopus some higher plants were tested for the occurrence of the

hormone. Willow catkins, sunflower ovaries, young apples, and corn were extracted.

The plant material was ground in a food chopper and extracted with 3 volumes of 97% alcohol for three days. The alcohol was evaporated down to an aqueous sludge under reduced pressure. This sludge was then extracted with an equal volume of benzene. The benzene was evaporated to dryness, and the residue taken up in a small volume of ether. Ten volumes of acetone was then added to precipitate the phospholipides. The material was filtered and ampulled in olive oil. It was then assayed according to the Coward-Burns method, as described above.

EXPERIMENTAL DATA

The strains of R. nigricans used were found to begin to conjugate normally from 28 to 35 hours after they were transferred to the culture medium. The time varied somewhat with the temperature and the age of the original culture. To eliminate this factor, during the course of the experiments, check cultures were always grown with the treated cultures, and the two sets of plates compared.

Only six dozen cultures were treated with the male sex hormone, due to the fact that a sufficient quantity

of the hormone could not be obtained. In all of the treated cultures, the formation of zygospores was delayed from eight to sixteen hours, compared with the check cultures. There was also a tendency toward an inhibition of the asexual sporangia of the plus strain and a stimulation of the sporangia production in the minus strain. A concentration of the male hormone of two bird units per plate was used in this experiment. The bird unit being that of Gallager and Koch, is defined as "the amount of the hormone which, when injected for five days, yields an average increase in length and height of the combs of at least five Brown Leghorn capons." (18)

A larger number of cultures of the organism were treated with the female sex hormone, as a plentiful supply of the material was available.

No significant effect of the hormone was noted on the sporangia formation of Rhizopus. The conjugation, however, was inhibited in the same manner as with the male hormone. The process was not delayed for as long a period of time with estrin as with the male hormone, the delay being only from four to eight hours.

The inhibition was noted in every plate to which estrin was added, whether it was incorporated in the agar

at the beginning of the experiment or added later, dissolved in sodium bicarbonate solution.

The conjugation was arrested almost immediately, in normal cultures, when the hormone was added.

In no case was any difference observed in the amount of mycelium produced by the check and the treated cultures. A total of more than 1,000 cultures were treated with the female sex hormone in these experiments.

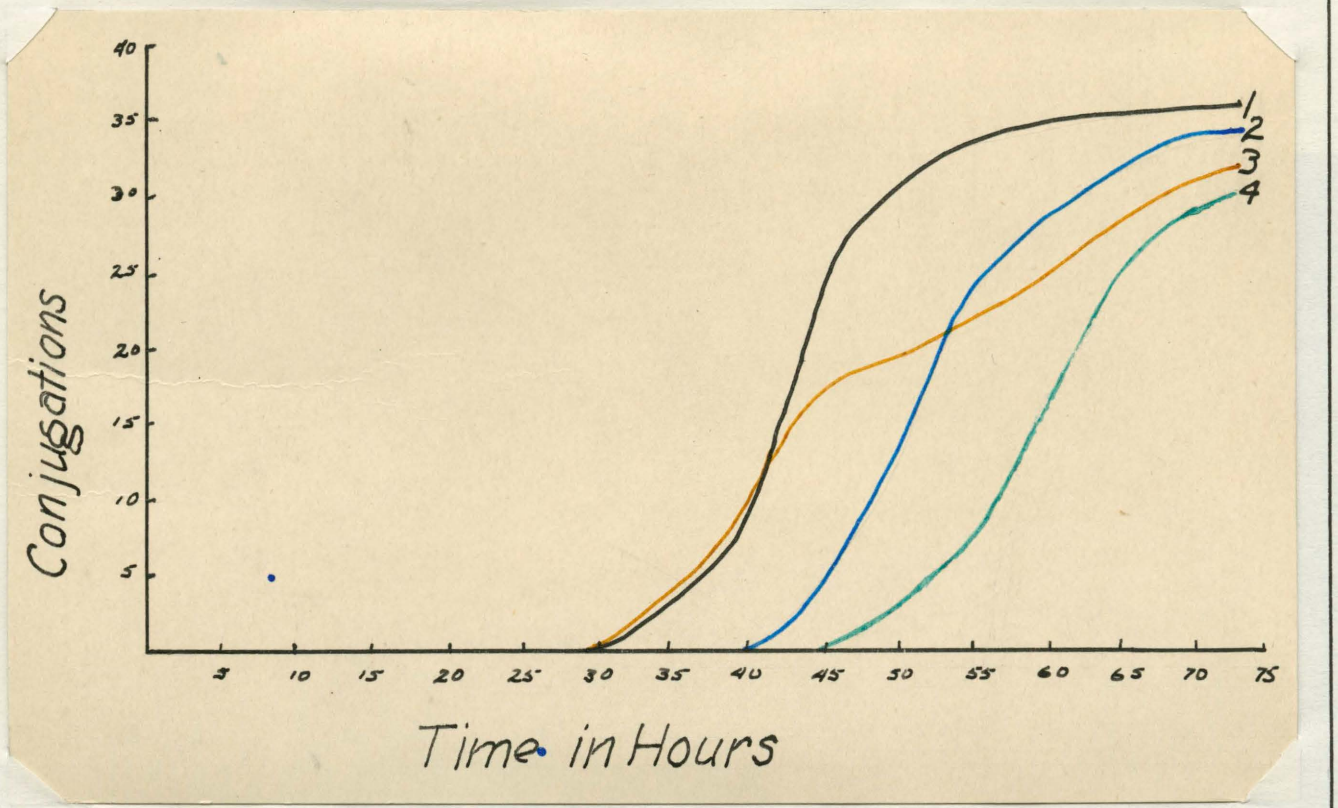
Varying concentrations of the hormone were used (from one rat unit to ten). It was found that the length of time the zygospore formation was delayed increased up to the concentration of five rat units, but above that concentration there was little nr no noticeable effect. By continuing the application of the hormone to the agar, however, the conjugation was delayed for longer periods of time; in some cases for 48 hours.

The results of these experiments are shown both graphically and in tabular form (Table 1.).

Table 1. Influence of Estrin on Conjugation of *Rhizopus nigricans*

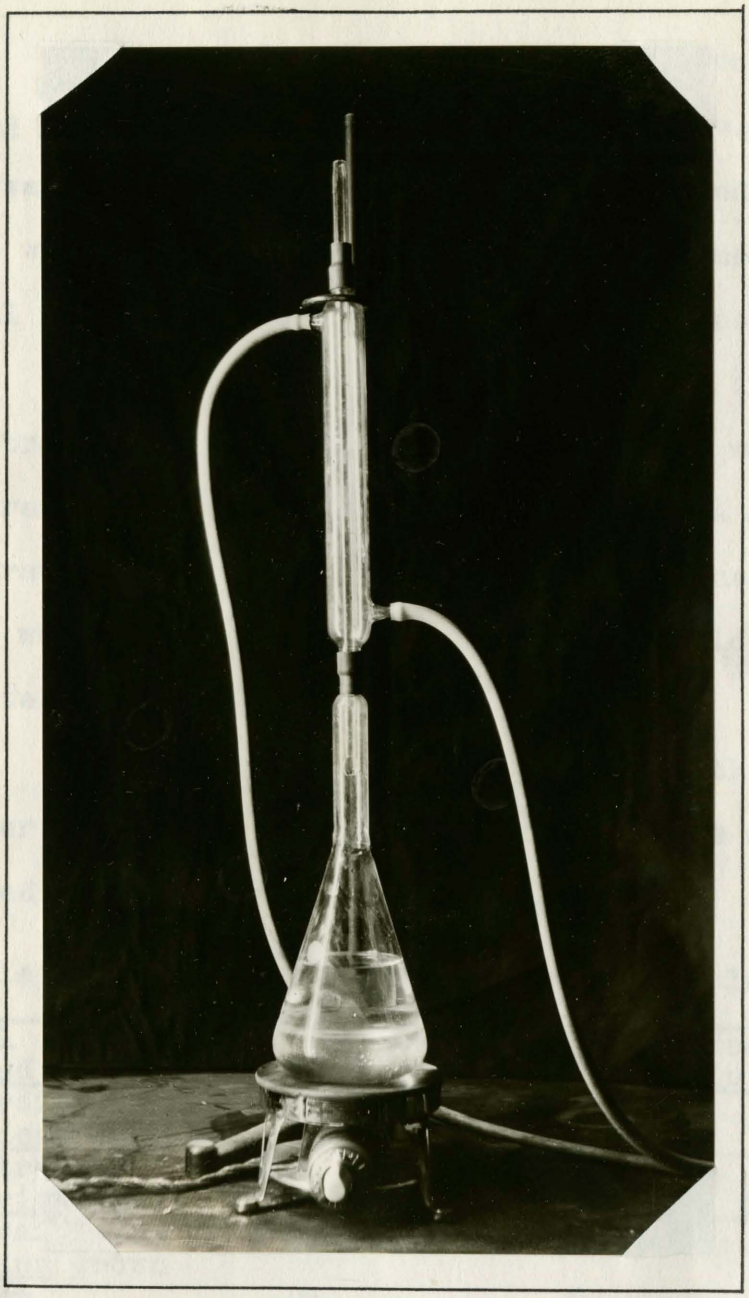
Treatment Number	Culture Medium	Time in Hours												
		24	28	32	36	40	44	48	52	56	60	64	68	72
		Number of conjugations												
1	Standard potato dextrose agar	0	0	1	4	7	23	31	34	35	35	36	36	37
2	P.D. agar + acetone and autoclaved	0	0	1	3	8	20	30	31	34	35	35	35	36
3	P. D. agar + estrin in acetone and autoclaved	0	0	0	0	0	3	9	18	25	29	31	33	34
4	P.D. agar + $NaHCO_3$ and autoclaved	0	0	2	5	9	24	30	35	36	37	37	37	38
5	P.D. agar + estrin in $NaHCO_3$ and autoclaved	0	0	0	0	0	2	7	19	22	28	32	33	35
6	P. D. agar + estrin in acetone and autoclaved. Estrin in $NaHCO_3$ added at end of 32 hours.	0	0	0	0	0	0	1	3	7	17	24	29	32
7	P. D. agar, estrin in $NaHCO_3$ added at end of 44 hours.	0	0	1	5	8	18	18	18	20	24	29	30	31

These figures are the average number of conjugations taking place in the 15m.m. portion of the groove in one hundred plates of each treatment. Numbers 2, 3, 4, 5 and 6 were sterilized with the hormone in the medium. No. 7 had the hormone added after conjugation had begun.



Effect of Estrin on Cultures of R. nigricans

1. P.D.Agar
2. P.D.Agar + estrin in acetone
3. P. D.Agar + estrin after conjugation had begun
4. P. D. Agar + estrin before and after inoculationwith organism.



Continuous automatic chloroform extractor.

In the extraction work, six dozen cultures of Rhizopus nigricans were extracted at one time. The medium was inoculated, and the fungi permitted to grow for one week at 20°C. At the end of this time the material was extracted and assayed as described above.

No esterogenic substance was found in the minus strain of the organism. The substance was found to be present in the plus strain cultures. A higher concentration was produced by the organism when both strains were grown in the same plate, and allowed to conjugate.

Estrin was also found in willow catkins, sunflower ovaries and corn, but not in apples as indicated in accompanying data, Table 2.

Table 2. Occurrence of Estrin in Materials Tested.

Material Extracted	Number of animals	Number in estrus	Number not in estrus
Plus strain R. nigricans	10	4	6
Minus strain R. nigricans	10	0	10
Both strains R. nigricans grown together	10	9	1
*Willow catkins	9	6	3
Sunflower ovaries	5	4	1
**Apple	3	0	3
Corn	5	3	2

*Assayed by Dr.R.G. Gustavson, Denver University, Denver, Colorado.

**Only 1 kg. of young apple extracted.

Summarizing, then, a substance which produced estrus in castrate female white rats was isolated from willow catkins, sunflower ovaries, and corn. A similar substance was also found in the plus strain of Rhizopus nigricans, and in increased concentration in conjugating cultures of the same organism. No activity was found in an extract of the minus strain of R. nigricans, or of 1 kg. of young apples. Both the male and female sex hormones inhibited the sexual cycle of R. nigricans.

CONCLUSIONS

From the variety of plant materials from which an esterogenic substance has been extracted, it seems safe to assume that some such substance is probably produced by all plants. The data obtained by Loewe and his co-workers (30) would seem to indicate that the substance is formed in the greatest quantity by the ovaries of the plants. Much of the work, however, indicates that the substance may be formed in the vegetative parts of the plant, as well as in the sexual organs.

It is interesting to note, in connection with the work of Zondek (51), that Walker and Janny (48) found an esterogenic substance in the male willow and

alder catkins, as well as in the female flower.

These results would tend to raise the question as to whether the material actually functioned sexually in plants. The question is made more interesting by the fact that while Butenandt (10) reports the crystalline substance prepared from palm-nuts to be identical with theelin, Skarzinsky (38) finds that the material crystallized from willow catkins has more nearly the structure of theelol, with only about one-fourth the activity of theelol.

Apparently, then, different species of plants may produce different types of esterogenic compounds.

The fact that no activity was found in the extract of the minus Rhizopus strain and that the concentration of the substance was higher in the conjugating cultures than in the plus strain, indicates that the hormone is formed in connection with the sexual cycle of this organism.

The inhibition of the sexual cycle by estrin, apparently paralleling the termination of pregnancy in animals, by large dosages of the hormone, also indicates a connection between the hormones and the sexual processes of the fungus.

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