

EXTRACTION OF A MATING REACTION INHIBITING AGENT
FROM *PARAMECIUM CALKINSI**

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Since interaction of mating-type substances at the surfaces of conjugating paramecia may initiate the physiological changes of fertilization in these animals,¹ a detailed study of the nature and manner of interaction of these substances is warranted. So far all attempts to obtain an active extract of mating substance from paramecia have failed.^{2, 3} Furthermore all mating reactivity disappears when these animals are thoroughly broken up. This apparent disappearance of mating substance activity from both extract and residue suggests the release of some mating substance inhibiting agent. Such an agent has now been obtained from homogenized *Paramecium calkinsi*. The presence of this agent can account for disappearance of mating substance activity in certain, but not in all, extraction procedures.

Preparation of the Agent.—*Paramecium calkinsi* were grown in a 2/5 sea water-baked lettuce infusion which had previously been inoculated with *Aerobacter aerogenes*. To prepare paramecium extracts the animals in four to six liters of paramecium culture were concentrated to 25–50 ml. by passing the culture through a porous-grade Berkefeld filter (the senior author's method has been described fully by Sonneborn).⁴ The animals were further concentrated by centrifugation, washed in 2/5 sea water and taken up in one to three ml. of 2/5 sea water. These concentrated animals were then homogenized or were lyophilized⁵ and later taken up in distilled water and homogenized. The crude homogenate was then centrifuged or passed through a sintered glass filter to give an opalescent supernatant or filtrate.

The most practical method for detecting any mating-type substance inactivating agent is to treat reactive dead paramecia with the agent, wash the dead animals free of the agent and then test them for specific mating reactivity with living animals. When testing paramecium homogenates for mating substance inactivating action the use of reactive dead animals in preference to living animals is essential because living animals may be killed by a non-mating-type specific heat labile auto-toxin frequently present in homogenates (Metz, unpublished). This introduces a complicating factor which cannot be controlled. If living animals are not killed by the homogenate, they can feed upon it with consequent loss of mating reactivity due to overfeeding.

The dead reactive paramecia used in these experiments were prepared

by treating cultures of *P. calkinsi* with formalin or picric acid (19 or 15 volumes of culture to one volume of formalin or saturated picric acid solution, respectively) for one hour. After the treatment the animals were washed in 2/5 sea water and suspended in this saline. Properly selected cultures of reactive Type I *P. calkinsi* that have been killed in this manner give very strong mating reactions with living Type II animals. These dead animals will even induce meiosis, macronuclear breakdown and pseudoselfing pair formation in living Type II animals.³ Formalin-killed Type II animals at best give only moderately strong reactions with living Type I animals, whereas picric acid-killed Type II animals can give very strong reactions.

To test for inhibiting action one to several volumes of the opalescent extracts were mixed with a given volume of strongly reactive dead paramecia (usually two drops containing roughly 1000 dead animals). After an exposure of one to two hours the extract was withdrawn from the treated dead animals. These were then washed in 2/5 sea water and tested for mating reactivity by mixing with living reactive animals of the same and opposite mating type. Generally such extract-treated dead animals failed to give mating reactions or at best gave only weak transitory reactions as compared to control dead animals buffered at the pH (5.5) of the extract.

Relation of the Inhibiting Agent to the Mating Substances.—Apparently the mating substance inhibiting agent of the extracts is not the mating substance of the extracted animals. This follows from the fact that the inhibiting agent is non-specific. Thus the extract prepared from mating Type I *P. calkinsi* inhibited dead reactive animals of both Type I and Type II. Moreover *P. calkinsi* I extract inhibited formalin-killed reactive variety 4 (Types VII and VIII) *Paramecium aurelia*. Extracts prepared from Type II *P. calkinsi* were also non-specific in action. If the inhibiting agent were the mating substance of the extracted animals, it should show mating type specificity. It would be expected to react with and inhibit only animals of the complementary mating type, not animals of the mating type from which the extract was prepared.

In view of the non-specific character of the inhibiting effect, one system, namely the action of Type I extract on Type I animals, was selected for further study.

Mode of Action of the Agent.—The inhibiting action of homogenates was not altered by repeated centrifugation or by passage through a sintered glass filter. Thus the inhibiting action cannot be attributed to a simple mechanical factor such as clogging of the cilia of the treated dead animals with debris. Therefore it is concluded that inhibition results from chemical combination of the mating substance with some agent in the extracts.

Apparently the non-specific mating substance inhibiting agent does not

act by combining with the mating substance of dead animals in antigen-antibody-like fashion. This follows from the fact that all attempts to absorb the inhibiting agent of Type I extracts by living or dead Type I paramecia have failed, and suggests that the agent may inactivate the mating substance by enzymatic action. Furthermore the agent acts slowly and only in low dilution. This is indicated by the experiment presented in table 1. The extract used in this experiment was prepared by concentrating six liters of culture (0.8 cc. packed paramecia) and homogenizing the animals in 3 cc. of 2/5 sea water after lyophilization.

Unfortunately there is no convenient objective assay method available for determining the amount of active mating substance present on dead or living paramecia. However, the relative degree of reactivity as indicated subjectively in table 1 shows that little if any inactivation occurred during the first 20 minutes of exposure to extract even in the highest extract concentration. Sixty minutes' exposure to the extract were required for

TABLE 1
EFFECT OF EXTRACT CONCENTRATION ON RATE OF INHIBITION OF FORMALIN-KILLED
PARAMECIUM CALKINSI TYPE I BY P. CALKINSI TYPE I EXTRACT

TIME:	20 MIN.	40 MIN.	60 MIN.
Extract dilution			
5/7	++++	+	±
2.5/7	++++	+++	++
1.25/7	++++	++++	+++
0.65/7	++++	++++	++++
pH 5.5 buffer	++++	++++	++++

nearly complete inactivation of the formalin-killed Type I animals. The reactivity of the test animals in the fourfold (0.65/7) extract dilution was not significantly different from that of controls in buffer solution even after a 60-minute treatment with the extract.

It is apparent from this experiment that the inhibiting agent acts slowly even at the lowest dilutions used and that the rate of inactivation is a function of the concentration of the agent.

Properties of the Agent.—Preliminary attempts to characterize the inhibiting agent of Type I animals show that it is heat labile to the extent that its action is rapidly destroyed at 100°C. It is non-dialyzable and evidently stable in absolute acetone since paramecia which have been lyophilized, treated with absolute acetone, dried and finally extracted yield active preparations of the inhibiting agent. Extracts of formalin-killed Type I animals, however, do not inhibit Type I or Type II animals. Attempts to salt out the agent with ammonium sulfate have failed. The $(\text{NH}_4)_2\text{SO}_4$ precipitates and supernatants were inactive both separately and when combined. Probably the agent was inactivated in the process of removing the salt.

These fragmentary data indicate that the inhibiting agent is a rather labile substance of fairly high molecular weight and suggest that it may be a protein. This is in accord with the view that its action is enzymatic.

Discussion.—As mentioned previously, the mating substance inhibiting agent cannot be the mating substance of the extracted animals. This follows from the non-specific character of its action, from the fact that it cannot be extracted from formalin-killed reactive *Paramecium calkinsi* and that it can be obtained from animals which are not in mating condition. The presence of this agent can account for failure to obtain active extracts of mating substance from homogenized living or lyophilized animals. However, it cannot account for disappearance of mating substance activity in both extract and residue of homogenized animals that have received sufficiently harsh treatment (i.e., formalin) to destroy the inhibiting agent without destroying the mating substance. Disappearance of mating substance activity under these conditions cannot be explained readily except by assuming that essential structural relations of the cell surface are destroyed by the mechanical extraction procedure used. At present there is no evidence that any such gross steric factors are essential for mating substance activity.

This study suggests that the mating substance inhibiting agent is enzymatic in action. If the agent should prove to be an enzyme and if the chemical nature of its action could be demonstrated with a well-defined artificial substrate, knowledge of the nature of the mating substance might be considered advanced. Such an enzyme might be concerned specifically with the characteristic appearance and disappearance of mating reactivity in normal living animals in accordance with their nutritional state. It is also possible that the agent is in the nature of a "digestive" enzyme which bears no physiological relation to the mating substance. Either of these possibilities is of sufficient interest to warrant further investigation of the mating substance inactivating agent.

Summary.—Extracts of *Paramecium calkinsi* which inhibit the mating reactivity of formalin or picric acid-killed *Paramecium* are described. The extracts are not species or mating type specific in action. Therefore it is concluded that the active agent in the extracts is not the mating substance of the extracted animals. Since the inhibiting agent is not absorbed by paramecia it is suggested that it may be an enzyme. The possible relation of the inhibiting agent to the mating substance is discussed.

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¹ Metz, C. B., and Foley, M. T., *J. Exptl. Zool.* 112, 505 (1949).

² Metz, C. B., *Anat. Record*, 94, 347 (1946).

³ Metz, C. B., *Am. Naturalist*, 82, 85 (1948).

⁴ Sonneborn, T. M., *J. Exptl. Zool.* (in press) (1950).

⁵ Metz, C. B., and Fusco, E. M., *Biol. Bull.*, 97, 245 (1949).