

짚신벌레의 성물질 합성에 대한 연구

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**Studies on the mating type substance in *Paramecium aurelia***

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**ABSTRACT**

Sexual reproduction of paramecia have been accomplished through conjugation between individuals which have opposite mating type substances on their cilia when they were starved. Using selfing clone in which mating takes place, I examined whether a mating type change in individual cells required new protein and new mRNA synthesis or not and also whether there is a precursor relationship between both of the complementary mating type substances in their synthetic pathway. I found that

1. Mating type change needs new protein(s) and new mRNA synthesis.
2. Mating type substances are synthesized sequentially from mating type XIII to XIV.
3. There might be a common precursor pool from which the mating type XIII substance is synthesized and then complementary mating type XIV is formed by addition of small group to the mating type XIII substance.

**INTRODUCTION**

Since mating types in *Paramecium aurelia* were discovered by Sonneborn(1937), mating types have been found in most species of *Paramecium* and also in other ciliated such as *Euplotes* and *Tetrahymena* etc. Thanks to much extensive studies, some information on the mechanism of mating type inheritance is now available for further investigation.

It is known that mating occurs because of the presence of cells of complementary mating type in a cell mixture. Cells became sexually reactive in early starvation phase of the growth cycle.

The chemical nature of the mating

type substance is known to be a glycoprotein in yeast (Crandall *et al.*, 1968) and *Chlamydomonas* (Forster *et al.*, 1956) and thought to be a protein in *Paramecium* (Cohen and Siegel, 1963). Two results in *P. aurelia* suggest that the odd mating type (eg. XIII-type) substance may be in some way a precursor of the even substance (XIV-type); (1) in studies of gene mutation affecting mating type determination in syngens 1, 4, and 7, only mutations restricting cells to the odd mating type have been found (Butzel, 1955; Taub, 1963; and Byrne, unpublished) (2) selfing cultures of syngen 7 contain cells which change mating type unidirectionally from XIII to XIV (Taub, 1966).

According to this "precursor hypothesis", cells synthesize first XIII mating type substance, but, if the cells are XIV type, XIV mating type substance is synthesized instead from XIII mating type substance or in immediate precursor. The mating type substances are on the cilia which is located only at ventral region and perhaps on this outer cell membrane. It was known that mating type substance give cells primary specificity of mating type and mating reactivity (Mezt, 1954).

Occasionally mating takes place within a clone in *Paramecium*. This unusual conjugation is called "selfing". In this case, cells of both of the complementary mating types are found within single cultures.

Unidirectional mating type changes in cells of selfing clones of syngen 7, *P. aurelia*, has been shown by Taub(1966): XIII→(hemaphrotypic state)→XIV. The mating type change occurs within one cell generation. In other words, certain cells express first mating type XIII, and after few hours these cells show mating type XIV. The change never occurs in the reverse direction. From these facts we could suppose that there may be a mechanism which controls an ordered, sequential genic expression in this system. From all of the data on mating type inheritance in *Paramecium*, *Tetrahymena* and *Euplotes* it has been shown that mating type substances are synthesized under the control of genes but the expression of these gene is dependant on cytoplasmic and environmental factors.

As for the molecular level of gene action and of interaction between gene and cytoplasm, we have little information: How does the interaction between gene and cytoplasm and also between allelic

genes regulate the expression of gene action to determine mating type. Why do the gene(s) controlling mating type "turn off" in the growth period and become active only at the time of starvation?

Studies on mating type changes in selfers within one cell generation may give some helpful information in explaining the gene action controlling mating type expression. The main purpose of this study was to elucidate the mechanism regulating genic expression for mating type in selfing clones of syngen 7 of *P. aurelia*.

For attacking this problem the effects on the synthesis of mating type substance after treatment of antibiotics (actinomycin D and puromycin) were observed.

The first experiments were designed to examine whether the mating type change (XIII→XIV) in individual cells requires new protein synthesis or not. The second experiments were done to investigate the precursor hypothesis for the mating type substances. And the third experiments were aimed at studying the effects of heat shock on the synthesis of mating type substance. Finally, experiments were concerned with the study of nutritional effects on regulation of the synthesis of mating type substance. Amino acids were chosen as nutrients.

## MATERIALS AND METHODS

Selfing clones were obtained from the cross between stock 227 XIV and 227 XIII. Syngen 7 of *P. aurelia* was used for this study. Culture methods were those used by Sonneborn.

When cells were split after mating, each exconjugant cells were transferred into a

fresh medium (0.2 ml) separately. Following the first cell division each exconjugant's daughter cells called "sister caryonide" were also separated into 1 ml of fresh medium and then maintained at 25°C by daily isolation.

After cells of each generation were grown for  $2\frac{1}{2}$  days, medium was exhausted. As cells begin to starve, cells became reactive in mating. Each caryonide clones were examined for the appearance of selfing pairs. Selfing clones were selected for material to study.

From 2-day-old selfing cultures in depression slides (1 ml) cells from expressing initially mating type XIII were subcultured in 100 ml of fresh medium in a plastic bottle (400 ml) and grown for  $2\frac{1}{2}$  days.

#### 1. Preparation fo Split Pair

Just before selfing began cells were concentrated by centrifugation and stained for later identification with Nile blue sulfate 0.5 ml of stained, selfing culture was transferred to depression slides, and under the binocular microscope the loose mating pairs were isolated and split part into two cells by pushing in and out through micro-pipette repeatedly.

#### 2. Selection of Reactive Mating Type XIII Cells from Spiit Pairs

The two cells from each split pair were moved to separate drops of exhausted medium. In order to select the XIII-type cell from each the split pair, XIII-type reactive testers were added to both cells. Cells not reacting with XIII-type testers were moved to fresh exhausted medium. XIV-type reactive tester cells then were added to these cells to confirm that they were reactive XIII. The cells then were immediately used.

#### 3. Preparations of Stable Cells for Experiments

Washed stock cells were separately grown in depression slides for  $2\frac{1}{2}$  days. Clones were then transferred to 100 ml of fresh medium as in the case of selfing cultures. After mating activity was expressed, cells were stained and mixed with opposite mating type testers. Loose pairs were isolated and the stained cells were used.

#### 4. Preparation of Exhausted Medium

Cell-free exhausted medium was prepared by centrifuging each line of selfers or stable cells own culture medium at 400g for 5 minutes and filtering the supernatant through a 0.45 micron milipore filter.

#### 5. Treatment with Antibiotics

The concentration of actinomycin-D was 2.5–3.5  $\mu\text{g/ml}$  and that of puromycin was 30  $\mu\text{g}$ –100  $\mu\text{g/ml}$ . Antibiotics were dissolved into distilled water as stock solution. Final concentrations were adjusted in the exhausted medium. Each XIII cell from a split pair and stable cell was treated with antibiotics individually. Cells were moved individually to 0.1 ml of antibiotic treated medium. According to experimental design the length of treatment and the concentrations of antibiotics differed.

#### 6. Heat Shock

The selected cells as previously described were put into 1 ml of exhausted medium which was preincubated for several hours at 42°C. After  $6\frac{1}{2}$  minutes of heat treatment, cells were transferred into exhausted medium at room temperature (23–25°C).

#### 7. Amino Acid Solution

Nineteen L-amino acids were dissolved

separately into distilled water. Among the 20 amino acids only cystine was not used because it does not dissolve well in water. In some experiments the concentration of each amino acid was  $0.25\mu\text{g/ml}$ , the total concentration of amino acid applied to cells was  $5\mu\text{g/ml}$ ; and in other experiments  $2.5\mu\text{g/ml}$  for each amino acid,  $50\mu\text{g/ml}$  for total amino acid. Each concentrated source of amino acids was adjusted to  $0.25\mu\text{g/ml}$  or  $5\mu\text{g/ml}$  in the total sample volume.

### 8. Staining Method

Nile blue sulfate dye (0.0000125%) was used for distinguishing between selfer cells and standard (tester) cells. Nile blue sulfate dye was dissolved into distilled water as stock solution. Before staining cells, final concentrations (0.0000125%) was made in exhausted medium.

### 9. Testers

Highly reactive mating type XIII and XIV cells of stock 227, *P. aurelia* were used as standard testers.

### 10. Mixed Culture

In order to eliminate differential growing conditions in the stable cell lines, a mixed culture method was used. Washed stock cells of complementary mating type were grown separately in depression slide for  $2\frac{1}{2}$  days. Before each clone became reactive in mating activity the two mating type clones were subcultured together in 100ml of same fresh medium. Cells were stained just before mating appeared within the cultures. When mating occurred loose pairs were isolated and split. These split pairs are used for experiments as described above.

### 11. Mass Culture Experiment

Selfing clones or stable clones were cultured for 3 day in 1000ml in a flask.

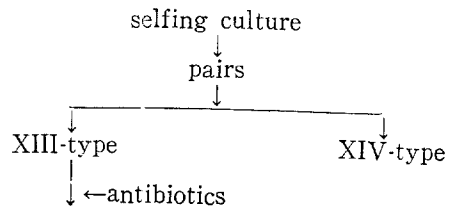
Before mating activity appeared cells were concentrated into exhausted medium or dryl's salt solution. Samples were taken from this concentrated cell source. The concentration of antibiotics was adjusted to each sample volume.

### 12. Method for Testing Mating Type after Treatment with Antibiotics

Split pair members were divided into two groups. In order to confirm the mating type of selfer cells one group was tested first with XIII reactive testers and the other group with XIV-testers. Following the first test each group was again secondly with the opposite mating type tester to each previous ones.

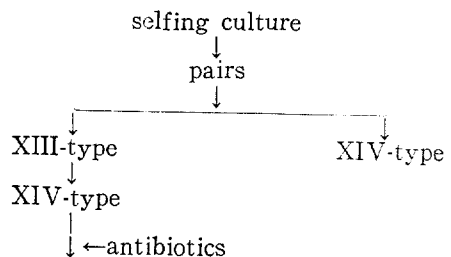
### 13. Antibiotic Treatments before Mating Type Change in Split Pair Member (Cell's Having XIII-type)

Fig. 1



### 14. Antibiotics Treatments after Mating Type Change (Cell Having XIV-type)

Fig. 2



## RESULTS

### 1. Antibiotics inhibit the mating type change from mating type XIII to XIV in the split pair numbers.

Experiments were designed at the individual cellular level to examine whether

or not mating type change requires a new mRNA and protein synthesis. Before doing these experimentally I measured the average time at which mating type change occurs after isolation of the type XIII split pair number. The average time of mating type change is dependent on cell lines but usually at 2.5 hrs but sometimes at 3.5-4 hrs after isolation. The lengths of antibiotic treatment were based on this average time of mating type change. The actinomycin-D (2.5  $\mu\text{g}/\text{ml}$ ) and puromycin (30 $\mu\text{g}/\text{ml}$ ) treatments were done before mating type change occurred, as shown in Fig. 1. In the case of actinomycin-D, treatment was done for 2.5 and 6 hrs. Cells were treated with puromycin for 6 hours because the puromycin effects are not as strong as that of actinomycin-D, as will be shown later.

In the cases of 6 hrs of treatment with puromycin (30  $\mu\text{g}/\text{ml}$ ) 27 were mating type XIII and 6 were XIV from a total of 33 treated cells as compared with 9 XIII and 19 XIV out of a total of 28 of non-treated cells. In another experiment with puromycin, 18 cells were XIII type, 15 cells XIV type. In the non-treated 37 cells, 8 were XIII cells and 29 XIV.

In the 6 hrs of treatment with actinomycin-D (2.5  $\mu\text{g}/\text{ml}$ ), 25 cell were mating type XIII, none were XIV but 21 cells not reactive. Among the 42 non-treated cells, 17 cells were XIII type and 25 were XIV type. In the second experiment using 2.5 hrs of treatment with actinomycin-D (2.5  $\mu\text{g}/\text{ml}$ ) 21 cells were mating type XIII, 9 XIV from the 29 cells as compared with 9 XIII and 23 XIV type out of 32 non-treated cells (Tables 1 and 2).

**Table 1.** Effects of puromycin on mating type change in selfing cultures  
6 hr treatment of puromycin (30  $\mu\text{g}/\text{ml}$ )

		Expt.		Cont.	
1st test					
Testers	XIV	XIII	XIV	XIII	
No. of mating cells					
With test after 6hr treatment	15+(17)	4+(17)	7+(17)	11+(14)	
2nd test					
Testers	XIII	XIV	XIII	XIV	
No. of mating cells					
With testers	3+(17)	13+(17)	9+(17)	3+(14)	
( ) : No. of total cells tested					

**Table 2.** Effects of actinomycin-D on mating type change in selfing cultures.  
6 hr treatment of actinomycin-D

		Expt.		Control	
1st test					
Testers	XIV	XIII	XIV	XIII	
No. of reacting cells with tester after 6hr treatment	9(19)	0(27)	11(23)	15(21)	
2nd test					
Testers	XIII	XIV	XIII	XIV	
No. of reacting cell with tester	0(19)	16(27)	10(23)	6(21)	

The chi-square tests for the effects of antibiotics on the mating type change were highly significant: 15.454 and 8.099 for the puromycin treatment and 19.8 and 9.23 for actinomycin-D. These tests show that the treatment of actinomycin-D and puromycin inhibits the mating type change from mating type XIII to XIV. This result may indicate the mating type change requires a new mRNA and a new protein(s).

Antibiotics also cause of mating rea-

ctivity. As shown in table 2 of actinomycin-D treatment (2.5  $\mu\text{g}/\text{ml}$ ), there were 21 non-reactive cells among 46 cells total as compared with only 2 non-reactive cells from 44 control cells. In 6 hrs of treatments with puromycin (30  $\mu\text{g}/\text{ml}$ ) the number of non-reactive cells was same as the control groups. But in the increased concentration (50  $\mu\text{g}/\text{ml}$ ) the number of non-reactive cells also increased to 16 out of 40 cells instead of 4 cells out of 33 control cells. This loss of mating reactivity may be due to the inhibition of further synthesis of mating type substance by antibiotics.

## 2. No differential effects of antibiotics on mating types.

Before concluding the effects of antibiotics on mating type change we should think about the possibility of differential effects of antibiotics on the two different mating types. In other words, if the antibiotics had more drastic effects on

Table 3. Effects of actinomycin-D (2.5  $\mu\text{g}/\text{ml}$ ) on the mating type

3 hr treatment			
	NR	R	
XIV	2	20	22
XIII	6	12	18
Total	8	32	40
P=0.0654 nonsignificant			
6 hr treatment			
	NR	R	
XIV	13	5	18
XIII	10	2	12
Total	23	7	30
P=0.04028 nonsignificant			

NR: non-reactive cells

R: reactive cells

the XIV mating type, those XIV mating type cells, which arise from XIII type cells would become non-reactive or die, giving misleading results. To test this possibility experiment was done with stable mating type XIII and XIV cells for 3 hr and 6 hr-treatments with actinomycin-D (Table 3).

In the stable XIV cells treated with actinomycin-D (2.5  $\mu\text{g}/\text{ml}$ ) for 3 hrs, 20 cells were reactive, 2 cells non-reactive from a total of 22 as compared with 12 reactive, 6 non-reactive in the 18 stable XIII cells. In the 6 hrs of treatment with actinomycin-D, 13 cells became non-reactive and 5 cells were reactive from 18 active XIV cells while 12 of the stable XIII cells, 10 cells were non-reactive and only 2 cells reactive. Fisher's exact tests for the differential effects of antibiotics between mating type XIII and XIV were done. The P values for 3 hr actinomycin-D treatments were 0.07 and 0.40 respectively. This value indicates that the differential effects of actinomycin-D were nonsignificant. Therefore we can eliminate these differential effects of actinomycin-D on mating types.

## 3. The effect of these antibiotics is seen in the early period of synthesis of mating type substance.

Experiments were carried out to find out the stage at which antibiotics affect mating type change or mating reactivity in the synthetic pathway of mating type substance. Stable XIV clones in mass culture were treated with the two antibiotics separately. Before expressing their mating reactivity, cells were sampled at certain intervals, treated with antibiotics, and then their mating activity was observed. As shown in Table 4 treat-

**Table 4.** Actinomycin-D effects

a) 2 hr treatment of actinomycin-D (3.5 µg/ml) before mating type change

No. of control cell	9			9			17			20			24		
No. of changed to XIV type after 2.5hrs in control Experiments	5			8			12			15			19		
No. of treated cells	Expt. 1			Expt. 2			Expt. 3			Expt. 4.			Expt. 5		
No. of XIII after treatment	11			10			17			22			16		
No. of XIV after treatment	2			4			6			9			9		
No. of NR	9			5			11			8			6		
Mating type show first among NR	XIII	XIV	XIV	XIII	XIV	XIV	XIII	XIV	XIV	XIII	XIV	XIV	XIII	XIV	XIV
Time at which mating type become reactive (hrs)															
1.5				1			1			1			1		
2.5	3						4								
3.5										1			1		
4.5				1			1			1			3		
5.5	1			1			1			1			1		
6.5 hrs	1			2			1			2			1		
Mean time regaining reactivity (hrs)	2.5	6	6.18	3	5.5	5.5	2.3	5.3	1.5	4.62	3.5	4.5	6.5	5.15	

XIV: became through mating type XIII.

ments at an early stage strongly inhibited mating activity but the treatments approaching the time of mating type expression do not inhibit it much. Further, the inhibiting effect of Act.-D is stronger than that of puromycin. The effect of puromycin diminishes with time. Similar experiments were done with selfing cultures. Generally the results were the same as that of stable cells. Mating type changes were inhibited by the two antibiotics.

**4. Sequential mating type changes of non-reactive cells, which were formed by Act.-D treatment before mating type change in selfing clone.**

As shown in Fig. 1, some cells were treated with Act.-D (3.5 µg/ml) before mating type change took place. After 2 hrs of treatment with Act.-D,

cells were transferred to exhausted medium and immediately tested for mating type.

The results appear in Table 4. Among the treated cells some had still mating reactivity, but a few cells lost mating reactivity and are called "non-reactive cells" (NR). These nonreactive cells (NR) were subjects for observation. As before, Act.-D also inhibited the mating type change from XIII to XIV in this experiment.

13 NR cells express mating type XIII first when they regain mating activity in non-Act.-D medium. And 9 NR cells also show first mating type XIV. Generally NR cells become reactive first as mating type XIII within 3 hrs in non-Act.-D treated medium. On the other hand NR cells express mating type XIV after

**Table 5.** Actinomycin-D effects on selfing cultures (II)a) Actinomycin-D (3.4  $\mu\text{g/ml}$ ) treatment after mating type change.

No. of treated cells	Expt. 1			Expt. 2			Expt. 3		
	XIII	NR	XIV	XIII	NR	XIV	XIII	NR	XIV
Time at which mating type become reverse or to be NR									
1.5(hrs)				1					
2							2	1	
2.5									1
3	1								
3.5				1					
4	1						1	1	1
4.5			1			1			
5							2		
6		2			3	1			
6.5	1							2	
7		2			2			1	
8			1		1				
9		1							
9.5		1			1				
10		1							
Mean time of mating time change after become to be NR (hrs)	4.5	7.8	6.25	2.5	7.01	5.25	3.6	5.2	3.25

XIV: became through mating type XIII

4.6-6 hrs in the non-Act.-D treated medium. Furthermore these 12 NR cells become reactive first as mating type XIII and change later to mating type XIV. This indicates that mating type changes occur in sequence; NR cell  $\rightarrow$  mating type XIII  $\rightarrow$  mating type XIV. The results are consistent with precursor hypothesis.

**5. Changed mating type XIV cells could be reverted to their original mating type XIII in actinomycin-D medium.**

Actinomycin-D (3.5  $\mu\text{g/ml}$ ) treatment was begun after cells changed mating type to XIV as shown in Fig. 2. Treatment was continued until most cells became non-reactive (NR) or XIII. During and after treatment cells were

tested for mating type every one hour.

As shown in Table 5 a few cells still have mating activity in the Act.-D treated medium for 10 hrs or more. Many cells, however, became NR after about 7 hrs of treatment. A small fraction of cells following post-mating type-change treatment with actinomycin-D came back to their original mating type (XIII-type) in the Act.-D treated medium and then changed to XIV-type in non-treated medium. As shown in Table 5 in this experiment, the revertants to XIII express mating type at an earlier time (at 2.5 hrs-4.5 hrs) comparing with becoming NR cells.

**II. 1. A delayed loss of mating reactivity was caused by heat treatment in stable mating type XIII and XIV and selfing**



**Table 6.** Time difference between loss and regaining of mating reactivity according to mating types of stable cell lines

Time between loss and regaining mating reactivity (hrs)	Stable cell lines	
	mating type XIII	mating type XIV
	No. of cells	No. of cells
1	16	1
2	29	12
3	2	17
4		9
5		8
6		1
7 hrs		1
Total	47	49
Mean time between loss and regain (hrs)	1.702 hrs	3.367 hrs

#### cultures.

Experiments were attempted to observe the effect of heat treatment on mating reactivity. Stable mating type XIII and XIV and selfing clones were used. After stable and selfing clones were treated to heat for  $6\frac{1}{2}$  min, cells still had mating reactivity but at around 4 hrs after heat shock most of treated cells lost mating reactivity.

#### 2. Time difference between loss and regaining of mating reactivity caused by heat shock in the stable cell lines.

Experiments were designed to observe whether there are differential behavior depending on mating type when cells regain mating reactivity. As summarized in Table 6, mating reactivity was lost around 4 hrs after heat shock and then regained about 2 hrs later in the stable mating type XIII cells. On the other hand in the mating type XIV cells this loss occurred at 4 hrs and was regained about 3 hrs later. The mean time difference between loss and regaining of mating reactivity was 1.702 hrs for

mating type XIII and 3.367 hrs for mating type XIV. T-test for the time difference between loss and regaining of mating activity between stable lines of mating type XIII and XIV was highly significant ( $t=8.49$ ,  $P<0.0005$ ).

These results are the same as in Act.-D treated experiments. It takes significantly longer to regain mating type XIV than to regain mating type XIII after loss of mating activity by heat shock.

#### 3. Heat shock does not affect mating type specificity.

In the case of selfing clone experiments with heat shock before mating type change (Tables 7 and 8), most of cells regained mating type XIV, three cells as hemaphrotype and one cell as mating type XIII. In this case cells also regained mating type XIV at a late time as in stable cell experiments. When the cells were treated with heat after mating type change (to XIV) all cells regained mating type XIV. In the stable cells treated by heat all cells regained their original mating type after loss of mating reactivity. Therefore in both of stable and selfing clones mating specificity was not affected by heat shock. Furthermore, the mating type change did occur after heat treatment. This result indicates that heat shock did not affect this switching mechanism.

#### III. 1. Amino acids do not cause the loss of mating reactivity.

These experiment were designed to observe whether or not amino acids causes loss of mating reactivity as bacterized medium does and, if so, what kind of amino acids are responsible for the effects. 19 combinations of amino acids were mixed so that each pool contained 18 and

Table 7. Heat shock experiment

- a) XIII mating type cells from selfing clone  
 b) temperature: 42°C  
 c) exposure times: 6  $\frac{1}{2}$  minute  
 d) heat treatment before mating type change

Mating reactivity					
Individual cells	Loss at hrs after heat shock	Mating type before loss	Time difference between loss and regain	Regain at hrs after heat shock	Mating type after regain
1	4.5	XIV	4	8.5	XIV
2	4.5	XIII	4	8.5	XIV
3	3.5	XIII	3	6.5	XIV
4	2.5	XIII	4	6.5	XIV
5	4.5	XIII			
6	3.5	XIII	3	6.5	hemaph
7	4.5	XIII	4	8.5	XIV
8	3.5	XIII	4	8.5	XIV
9	4.5	XIII	4	6.5	XIV
10	4.5	XIII	2	6.5	hemaph
11	4.5	XIII	2	6.5	hemaph
12	3.5	XIII			
13	3.5	XIII			
14	3.5	XIII	3	7.5	XIII
15	4.5	XIII	3	7.5	XIV
16	4.5	XIII	4	8.5	XIV
mean (hrs)	4		3.85	7.75	

Table 8. Heat shock experiment

- a) temperature: 52°C  
 b) exposure time: 6  $\frac{1}{2}$  minute  
 c) heat treatment after mating type change (XIV)

Mating reactivity					
Individual cell	Loss at hrs after heat shock	Mating type before loss	Time difference between loss and regain	Regain at hrs after heat shock	Mating type after regain
1	2.5	XIV	4	6.5	XIV
2	4.5	XIV			
3	3.5	XIV			
4	3.5	XIV	3	6.5	XIV
5	4.5	XIV	1	5.5	XIV
6	3.5	XIV	2	5.5	XIV
7	2.5	XIV			
8	3.5	XIV	2	5.5	XIV
9	3.5	XIV			
mean	3.5		2.0	6.90	

a different one was deleted from each combination. Cells were then treated with each combination.

Stable mating type XIII and XIV cells were used. None of amino acid inhibits mating reactivity. The addition of 3 ml of bacterized medium to 20 ml of cell samples was enough to cause the loss of mating reactivity. These results may permit use of labeled amino acids in order to study the internal synthetic mechanism of mating type substances by isotopic techniques or autoradiographic study.

### DISCUSSION

The inhibition by actinomycin-D and puromycin of the mating type change was shown in individual cellular level and in mass cultures experiments in the selfing clone (Tables 1, 2, 4 and 6). It can be concluded that the mating type changes may need a new mRNA and protein synthesis.

Antibiotics (actinomycin-D and puromycin) affect cells in two aspects; the inhibition of mating specificity change (XIII type to XIV type), and the loss of mating reactivity. The loss of mating reactivity occurred only after rather long treatment with actinomycin-D. It suggests that antibiotics do not affect mating type substances themselves but their internal synthetic system. It is thought that actinomycin-D and puromycin prevent mating type changes by inhibiting synthesis of mRNA and proteins which will be involved in the change of mating type specificity. In view of sequential synthesis reaction for mating type substances, which will be discussed later, the inhibition of mating type change

caused by antibiotics occurs in the stage between mating type XIII substance and mating type XIV substance. On the other hand, in the mass culture experiment antibiotics were able to inhibit mating type change and mating reactivity at an early stage—perhaps before a certain substance was synthesized but had no effects at later stage—when a certain substance had been synthesized. Combining this above fact with the result that long treatment of antibiotics cause the loss of mating reactivity but do not cause immediately, these antibiotics may affect somewhere an early stage before mating type XIII substance synthesizes under the assumption of the sequential synthetic pathway of mating type substance. Based on these results alone we cannot tell about the nature of this protein and new RNA. But it can be assumed that (1) this protein could be an entirely new protein for XIV mating type, (2) certain enzyme molecules which catalyze the reaction(s) from XIII mating substance to XIV mating substance, or (3) a small protein molecule which may attach to XIII matingtype substance to make mating type XIV substance.

It was known that actinomycin-D was inserted into G and C rich region of DNA, and somehow it inhibited the RNA polymerase actions and consequently prevented mRNA synthesis. Therefore actinomycin-D affects on the transcriptional level. On the other hands puromycin inhibits the protein synthesis by incorporating into the synthesizing polypeptide chain and ending immature polypeptides. Thus, the puromycin affects on the translational level. From these different action levels of the two antibiotics, it

may be possible to distinguish the inhibitions of these two antibiotics. But the differential effects in terms of time of these two antibiotics could not be distinguished. This failure may be due to the inaccurate method of measuring mating activity. In the mass culture experiments, effects of both antibiotics were not complete, perhaps because of the low concentrations of antibiotics which were applied.

In the observation of the NR cells formed by actinomycin-D treatment before mating type changes in selfing clones (Table 4) some non-reactive cells after removal from actinomycin-D, expressed initially their XIII mating type when they first became reactive and then changed to XIV-type. These NR cells were derived by the same actions of antibiotics as described above. If the gene(s) responsible for synthesis of XIV-type substance are expressed after expression of gene for XIII type and both mating type substances are in a sequential relationship in the same reaction, treatment with actinomycin-D would block transition from XIII-type to XIV-type substance. Furthermore, NR cells from treated group should begin with XIII-type. A few NR cells appeared to change directly to XIV, but it is possible that the short XIII-type stage in these cells was missed. Most NR cells showed the first mating type (XIII-type). From these results, it can be supposed that there is a sequential relationship between the XIII- and XIV-mating type substance. When NR cells regain their mating type XIII first appear at an earlier stage than those expressing first mating type XIV (shown in Table 4).

It is very important to discuss the reasons why mating type XIII cells are expressed early and why mating type XIV cells later from NR cells. It may be assumed that some differential processes are involved for each synthesis of mating type substances.

In the experiments in which cells were treated with actinomycin-D after mating type changes as shown in Fig. 2 (Table 5) a small fraction of cells came back as their original mating type (XIII-type) in the actinomycin-D treated medium and then changed to XIV-type in actinomycin-D free medium. Among these 12 reverted cells, 10 cells returned to XIII-type directly and 2 cells became XIII-type through a non-reactive stage. These results are unexpected and it is very difficult to explain these facts with the simple scheme, that mating type XIII substance converts to mating type XIV substance directly. If this scheme is true the cells treated with actinomycin-D after mating type change should remain mating type XIV which was expressed before treatment. To explain the above contradictory results we need assume a hypothetical "switch" which may permit or prevent the mating type XIII substance to continue through the final synthetic pathway toward XIV mating type substance. The same kind of experiments were done with stable mating type XIV. All of the NR cells after treatment of actinomycin-D became reactive first as mating type XIV. None of NR cells reverted to mating type XIII as selfing clone did.

From the heat shock experiments with stable cells (Table 6), it has been shown that there is a clear difference in the

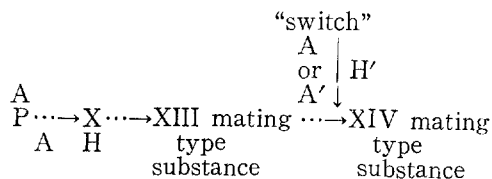
mean time of regaining mating reactivity after the loss between stable mating type XIII cells and XIV cells. Stable mating type XIV cells have a longer mean time. When cells regain mating reactivity all cells expressed their original mating type.

In case of heat shock to selfing clones before and after mating type change (Tables 7 and 8), cells lost their mating reactivity as stable cells did. Most cells exposed to heat before mating type change expressed mating type XIV when they became reactive. Only one cell was mating type XIII and three cells were hemaphrotypic, when cells treated after mating type change regained mating activity all of cells returned to their original mating type XIV. In both experiments with heat shock in stable and selfing clones the mating specificity was not changed after heat treatment and also mating reactivity was not lost immediately. This indicates that heat treatment also does not affect the mating type substance itself. Instead, it may act on the internal synthetic mechanism. In other words heats inhibit the synthesis of mating type substance at an early stage before mating type XIII synthesize as same as in cells treated with actinomycin-D. Therefore no further mating type substance supply to cilia and consequently cells lose mating reactivity. We also suppose that heat shock may prevent only the transport of mating type substances from internal site to cell surface (cilia and outside membrane). If this mechanism is true one would expect the same mean time between loss and regaining mating reactivity from both mating types. The result of the differential mean time between loss and regaining

of mating reactivity in the stable cell of mating type XIII and XIV suggests to us that this kind of effect of heat shock could not be possible.

A few important facts could drive from the above experiments. First, a delayed loss of mating type reactivity occur after actinomycin-D and heat treatment. Second, the mating type XIII was expressed earlier than mating type XIV when the NR cells regained mating reactivity. Third, mating type XIV cells from selfing cultures only could be reverted to mating type XIII in the actinomycin-D-treated medium. Fourth, mating type changes unidirectionally. Fifth, differential mean time in regaining mating activity after loss was seen between mating type XIII and XIV cells.

It is thought that there may be three steps in order to synthesize mating type substances: 1) a signal 2) common synthetic pathways 3) a "switch". As a signal such as starvation comes to cells, cells response to this signal and begin to synthesize the mating type substance through the common synthetic pathways. As soon as the XIII mating type substance have synthesized the "switch" may operate or not depending on cells stocks. Based on the assumption



where P: a pool X: intermediate

A: sensitive stage to actinomycin-D

H: sensitive stage to heat

H': resistant stage to heat

A': resistant stage to actinomycin-D of "switch" and our above results we

can put a hypothetical scheme for the synthetic pathway of mating type substances.

The "switch" may be cytoplasmic factor and exist in two states: 1) unstable state → sensitive to actinomycin-D 2) stable state → resistant to actinomycin-D.

As cells stabilize as XIV mating type or the amount of cytoplasmic factors is increasing to the threshold of its stabilizations in the selfing cultures the unstable state of "switch" become a stable state. In the stable XIII mating type cells this "switch" may never operate while in the stable XIV type cells this "switch" may always function, but this change from unstable state to stable state occurs within extremely short time. In the case of selfing clone this "switch" may not first operate but late begin to function and the change of states from unstable to stable occurs slowly.

According to the above synthetic scheme and the assumption on the "switch" the fact of reversion to mating type XIII by actinomycin-D could be explained easily: immediately after cells changed from mating type XIII to XIV the "switch" will be in unstable state. If cells

in this stage were treated with actinomycin-D, this unstable sensitive "switch" may turn off its function which permit cells to synthesize a protein determining mating specificity. Therefore the synthetic reaction now stop at stage of XIII mating type and cells could express XIII mating type. In my result, these revertants appeared at earlier times in the actinomycin-D medium than becoming NR cells. This indicate that actinomycin-D affected this "switch" or a certain stage between XIII mating type and XIV mating type substance, but not earlier stages in the synthetic pathway. These results, the earlier expression of XIII mating type than that of XIV mating type and longer mean time between loss and regaining mating reactivity for XIV mating type than that of XIII mating type, are quite natural under the above scheme: compared to the XIII mating type synthesis which followed to the same synthetic pathways, the synthesis of XIV mating type substance has more synthetic steps including activation of the "switch" after synthesis of the XIII mating type substance. Therefore it could take more time for XIV type expression than XIII type expression.

### 摘 要

질신벌레의 유성생식은 서로 상보되는 성물질을 세포에 가지고 있는 개체 사이의 접합에 의하여 일어난다. 본 실험에서는 한 개체내에서 서로 상보적인 두 종류의 성물질을 합성할 수 있는 clone을 택하여 이들 성물질의 생합성에 있어 선구물질적인 관계가 있는가를 규명하기 위하여 항생물질을 각 개체별로 처리하고 성물질의 합성과정을 검토한 결과 질신벌레의 XIII 성물질은 그 상보적인 XIV 성물질의 선구물질이며 이들 두 성물질의 합성에 있어 공통되는 전물질이 체내에 있다는 것을 암시하였다.

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