Cytological and Cytogenetical Studies on *Paramecium polycaryum*, II. Cytological Responses of Paramecia to Mitomycin-C, Azan, MEPA and Podophyllin

By

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(With 16 Text-figures and 7 Tables)

Paramecium polycaryum was reported to be unstable in the number of micronuclei by Woodruff and Spencer (1923) who described this species. Hayashi and Takayanagi (1961) have studied the variation in number of micronuclei in laboratory cultures. Lloyd (1947) reported that *Paramecium caudatum* showed a variation in number of micronuclei under the effect of hexachlorocyclohexane. Bimicronucleate animals of *P. caudatum* were observed by Miyake (1956) after treatment of urea. Watanabe (1959) reported that amicronucleate animals appeared under the influence of colchicine on *P. caudatum*.

The present study was undertaken in order to examine the effects of Mitomycin-C, azan, MEPA and podophyllin on *Paramecium polycaryum*, with special reference to the variation in number of micronuclei.

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Material and Methods: *Paramecium polycaryum* used in the present study was derived from foul waters in the city of Sapporo. Isolation cultures were made several times. Paramecia were cultivated at 20–21°C in a lettuce infusion enriched with food bacteria, *Aerobacter aerogenes*. The culture method according to Sonneborn (1950) was principally employed in this study. The culture medium was regulated with $CaCO_3$ at pH 7.0. For examination of the micro- and macronuclear conditions, Schaudinn's mixture and Bouin's solution in combination with the Feulgen reaction with light green were employed. Toluidine blue and methyl green were used to observe the length of the macronucleus and diameter of cells. Out of a number of clones established in the author's laboratory, those numbered 10, 11 and 19 were used. Chemicals used for this experiment were Mitomycin-C, azan, MEPA and podophyllin. The concentrations were 100 γ per ml for Mitomycin-C, 80 γ per ml for azan, 100 γ per ml for MEPA, and 3/5 saturated solution for podophyllin. Animals in good condition were transferred to culture media in which the above chemicals were dissolved, to the number of about 500 animals per ml. They remained for 10 days in each experimental medium.

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As a control, the variation of micronuclear number of each clone was investigated before treatments. The results are given in Table 1.

Table 1. Distribution of the number of micronucleus in untreated clones

Clone	Unimicro-nucleate	Bimicro-nucleate	Trimicro-nucleate	Tetramicro-nucleate
10	—	—	6.0	94.0
11	6.0	94.0	—	—
19	—	7.4	88.9	3.7

Results

I. Effect of the Chemicals on Micro- and Macronucleus

a) Effect of Mitomycin-C

1. *Micronucleus*: Calculation of micronuclei was made every 5 days. The results were summarized in Table 2. Clone 11, a bimicronucleate strain,

Table 2. Variation in number of the micronucleus in Mitomycin-C-treated clones

Clone	Day	amacro-nucleate				Diffused macronucleate					Regular macronucleate					
		0	I	II	III	0	I	II	III	IV	0	I	II	III	IV	V
10	5	—	—	—	—	—	—	—	—	—	0.9	5.0	11.3	35.8	44.1	0.9
	10	29.0	1.1	—	—	3.3	3.9	5.5	2.7	0.5	6.6	13.6	17.0	12.3	5.5	—
11	5	—	—	—	—	—	—	—	—	—	1.0	18.0	81.0	—	—	—
	10	17.7	0.4	—	—	3.8	0.4	0.4	—	—	20.3	29.5	27.5	—	—	—
19	5	—	—	—	—	—	—	—	—	—	1.3	1.3	30.3	65.7	1.3	—
	10	6.2	—	0.6	—	2.1	6.9	1.5	0.9	—	11.0	19.7	32.3	24.6	—	—

O; showing amicronucleate cells (%). I; showing unimicronucleate cells (%).
II; showing bimicronucleate cells (%). III; showing trimicronucleate cells (%).
IV; showing tetramicronucleate cells (%). V; showing pentamicronucleate cells (%).

showed a high stability in number of micronuclei. After the treatment with Mitomycin-C for 5 days, bimicronucleate specimens rapidly decreased in number with an increase of unimicronucleate specimens. In 10 days' treatment, amicronucleate animals appeared at 20.3 per cent, with unimicronucleate ones at 29.5 per cent, while bimicronucleate animals were 27.5 per cent. Many animals having a diffused macronucleus, no micronucleus or no macronucleus, or so-called

ghost animals having neither macro-, nor micronuclei, were observed in the samples of 10-day-treatment. In most animals, micronuclei showed swelling or diffused condition, being weakly stained with the Feulgen stain (Figs. 5 and 6).

2. *Macronucleus*: Animals placed in the culture medium containing Mitomycin-C exhibited a remarkable change in size of macronuclei. As shown in Figures 1 and 2, the longitudinal diameter of the macronucleus rapidly increased after treatment. The maximum value (35.8 μ) was observed after exposure for 6 days. Then, a sudden decrease was observed after 8 days (Fig. 1) On the

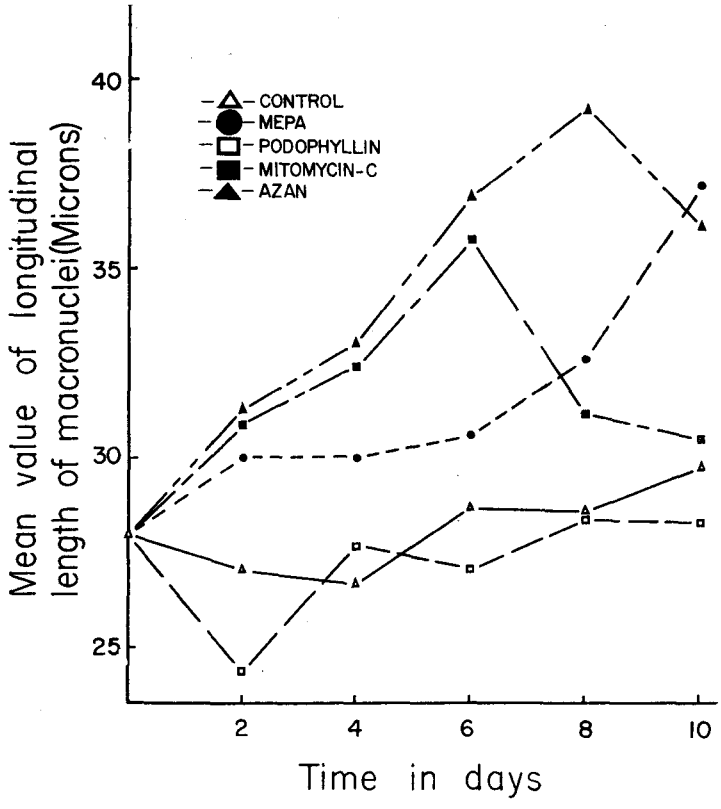

Fig. 1. Longitudinal length of the macronucleus in clone 11 treated with four chemicals.

other hand, the short diameter of the macronucleus was the largest (23.5 μ) on the 4th day with a gradual decrease with the passage of time (Fig. 2). After 8 days' treatment, the macronucleus was deeply stained with the Feulgen reaction. Also, some macronuclei were observed broken into two or more fragments. After 10 days' treatment, the diffusion of the macronucleus was remarkable in many

animals (Figs. 7, 8 and 9). The macronuclear diffusion was observed in 15.9 per cent of the animals in clone 10, 4.6 per cent in clone 11 and 5.4 per cent in clone 19. Ghost cells were found in 29.0 per cent of the animals in clone 10, 17.7 per cent in clone 11, and 6.2 per cent in clone 19 (Table 2).

Longitudinal and transverse lengths of cells changed after chemical treatments as shown in Figures 3 and 4. On the basis of the results, it is apparent that

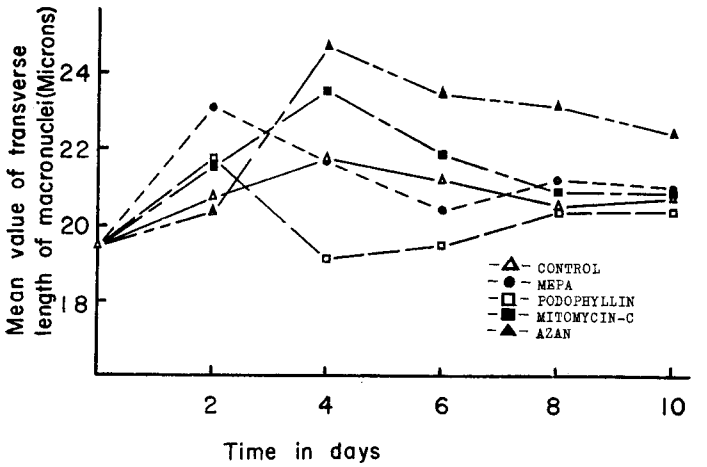

Fig. 2. Transverse length of the macronucleus in clone 11 treated with four chemicals.

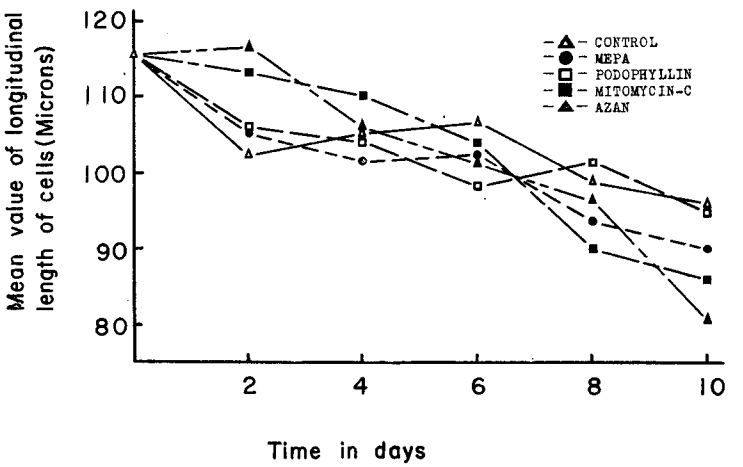

Fig. 3. Longitudinal length of the cell body in clone 11 treated with four chemicals.

the animal cells are more variable in longitudinal length than in transverse one. Animals gradually decreased in cell size for 6 days subsequent to exposure to chemicals. Then, the majority of animals showed a remarkable decrease in longitudinal length (Fig. 10). Most animals exhibited little or no motility; they lay on the bottom of the culture dish.

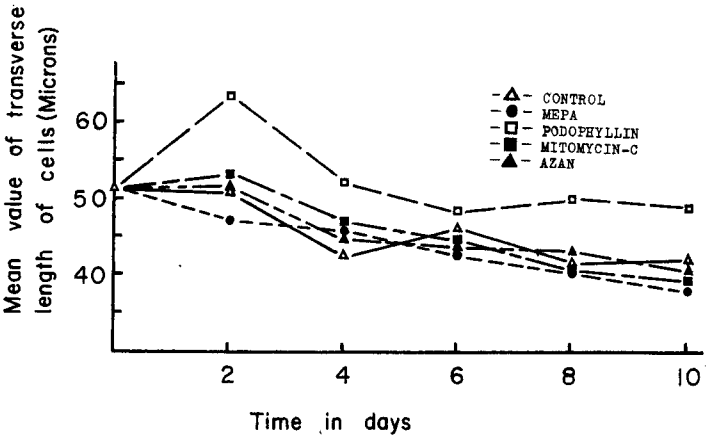

Fig. 4. Transverse length of the cell body in clone 11 treated with four chemicals.

b) Effect of azan

1. *Micronucleus*: As seen in Table 3, under the influence of azan the micronuclei showed a tendency to decrease numerically in each clone examined, with similar features to those observed in the Mitomycin-C experiment.

Table 3. Variation in number of the micronucleus in azan-treated clones (%) (cf. Table 2)

Clone	Day	Amacronucleate				Diffused macronucleate					Regular macronucleate				
		0	I	II	III	0	I	II	III	IV	0	I	II	III	IV
10	5	—	—	—	—	—	—	—	—	—	0.8	1.6	16.5	24.0	57.1
	10	2.2	0.8	0.8	0.8	3.8	0.8	3.0	1.5	—	—	4.5	20.4	36.3	25.0
11	5	—	—	—	—	—	—	—	—	—	3.7	37.9	57.5	0.9	—
	10	12.6	0.5	0.5	—	7.5	6.4	3.4	—	—	6.4	28.4	30.9	2.9	—
19	5	—	—	—	—	—	—	—	—	—	0.9	2.7	19.1	75.6	1.8
	10	7.9	0.8	0.8	—	5.3	0.8	1.6	0.8		10.6	17.7	28.3	25.4	0.8

2. *Macronucleus* : A more remarkable increase in the longitudinal diameter of the macronucleus was shown with this chemical than with Mitomycin-C, MEPA and podophyllin. This will be discussed later. The results are shown in Figure 1. The macronucleus generally showed the largest value of diameter on the 8th day after exposure (Figs. 1 and 11). In the extreme case, the macronucleus was about 1.3 times as long as the control group under the same condition. The transverse length of the macronucleus was however less varied than the longitudinal one; it was 1.1 times that of the control one at exposure for 4 days (Fig. 2). Some macronuclei were observed in a diffused condition in the 10 days' sample (Fig. 12). Ghost cells were also observed in that sample (Table 3 and Fig. 13).

Both longitudinal and transverse lengths of cell body gradually decreased with the passage of time. Sometimes, large vacuoles one to two in number, were observed in cytoplasm of cells (Fig. 14). Most animals lay on the bottom of dish.

c) Effect of MEPA

1. *Micronucleus* : After a drastic treatment the number of micronuclei remarkably decreased with a similar tendency to that observed in the Mitomycin-C and azan experiments (Table 4).

Table 4. Variation in number of the micronucleus in MEPA-treated clones (%) (cf. Table 2)

Clone	Day	Amacronucleate				Diffused macronucleate					Regular macronucleate					
		0	I	II	III	0	I	II	III	IV	0	I	II	III	IV	V
10	5	—	—	—	—	—	—	—	—	—	—	3.9	15.9	33.3	46.9	—
	10	1.0	—	—	—	—	—	1.0	—	—	1.0	9.8	22.9	34.8	—	—
11	5	—	—	—	—	—	—	—	—	—	1.0	13.0	85.0	1.0	—	—
	10	—	1.1	—	—	—	1.1	—	—	—	11.4	29.8	56.6	—	—	—
19	5	—	—	—	—	—	—	—	—	—	0.8	5.7	28.8	62.9	1.6	0.8
	10	1.6	0.5	—	—	1.6	—	3.5	1.1	0.5	2.4	9.5	36.3	42.5	0.5	—

2. *Macronucleus* : Under the effect of this chemical, the longitudinal length of the macronucleus showed a gradual increase for 6 days and then a rapid increase. On the 10th day, it showed a maximum value (Figs. 1 and 15). The transverse length of the macronucleus showed a maximum value 2 days after exposure, then it gradually decreased as shown in Fig. 2. The macronucleus showed fragmentation in the 10 days' sample. One to two, rarely three, large macronuclear fragments were observed in the central part of the animal at a frequency of 26.4 per cent. As in the Mitomycin-C and azan experiments, ghost cells and those having a diffused

macronucleus (Fig. 16) were observed in the 10 days' sample.

The variations in longitudinal and transverse lengths of the cell body observed every two days were shown in Figs. 3 and 4. It is evident that both lengths showed a gradual decrease with time.

d) Effect of podophyllin

As given in Table 5, the micronucleus slightly decreases after the exposure to podophyllin. It is remarkable that under the influence of the drug the macronucleus did not show elongation but became spherical; it was deeply stained with

Table 5. Variation in number of the micronucleus in podophyllin-treated clones (%) (cf. Table 2)

Clone	Day	0	I	II	III	IV
10	5	—	—	2.4	23.2	74.2
	10	—	2.3	14.2	23.8	59.7
11	5	1.0	11.1	88.0	—	—
	10	6.8	17.9	73.7	0.8	—
19	5	0.8	16.4	81.9	0.8	—
	10	8.8	27.6	0.6	—	—

the Feulgen's reaction. There were no animals showing diffused macronucleus or ghost condition in the samples so far observed. There were a few animals having cytoplasmic vacuoles in the sample after exposure for 10 days. By increase in their transverse length they became spherical in form.

II. Effect of Chemicals on the Fission Rate

To examine the effects of same chemicals Mitomycin-C, azan, MEPA and podophyllin, on the fission rate of animals, experiments were carried out with

Table 6. Fission rates of animals under the influence of chemicals

Day	Control	Azan	MEPA	Mitomycin-C	Podophyllin
1	2.04	0.80	1.95	0.90	0.70
2	2.20	1.15	2.02	0.70	0.85
3	2.00	0.20	0.16	0.30	0.90
4	2.06	0.00	0.85	0.30	0.70
5	2.20	0.30	0.20	0.40	0.30
6	2.04	0.30	0.20	0.10	0.20
7	2.00	0.00	0.10	0.00	0.00
8	2.02	0.00	0.00	0.00	0.00
9	2.06	0.00	0.00	0.00	0.00
10	2.22	0.00	0.00	0.00	0.00

clone 11. The specimens for study were daily reisolated and placed in moist chambers in which depression slides were placed with three drops of the culture medium containing chemicals. Mean value of the fission rate was observed after thirty animals, the results being shown in Table 6. The fission rate of control animals was found to be about 2.0. The fission rate of experimental animals dropped soon after chemical treatments, and showed no increase with time (Table 6). After the 7th day of exposure, cell division was completely inhibited by the agents used here.

III. Effect of Mitomycin-C on Division of Micronuclei

Animals treated with Mitomycin-C for 5 days were transferred into a fresh culture medium. Leaving them for 24 hours, animals in the course of division are fixed for examination of the aberrant division of the micronucleus. In the untreated group, the aberrant division of micronuclei was found in animals at the rate of 3 per cent. In treated group, 25 animals showing an unequal division were found among 80 animals which were in process of division (Table 7). Further,

Table 7. Frequency of specimens showing equal and unequal division of micronuclei after the treatment with Mitomycin-C

Micronuclear division	Untreated		Treated	
1 : 0	0	0 (%)	17	21 (%)
1 : 1	6	5	6	8
2 : 0	2	2	1	1
2 : 1	1	1	6	8
2 : 2	105	92	49	61
3 : 1	0	0	1	1
Total	114		80	

a number of specimens were observed which had undivided micronuclei and a divided macronucleus. It is of interest that Mitomycin-C does not induce irregular division of the macronucleus.

Discussion

There have been published many reports on the effects of chemicals and antibiotics, but they deal mostly with metazoan cells. The results of the present experiments on the effects of some chemicals have revealed that in many animals from three clones, the number of micronuclei decreased extremely within 10 days after treatment. The chemicals used in the experiments showed a nearly similar pattern of effect except podophyllin. Kobayashi (1959) reported irregular coagulation of chromatin materials and nuclear fragmentation after treatment with

Mitomycin-X in mammalian cells *in vitro*. It was observed in the present study that Mitomycin-C, azan and MEPA affected micronuclei resulting in reduction in their number and decreased staining, and reduced the swelling and induced the degeneration of the macronucleus. Comparatively, Mitomycin-C was found to exert more effect on the nuclear material than other chemicals used here.

It was shown that podophyllin exerted less remarkable effect on the variation in number of the micronuclei than the other drugs used, and that it induced a shrinkage into a spherical form of the cell body and macronucleus. Working with living ascites tumor cells of rats, and using the same drug, Makino and Nakanishi (1955) reported that the tumor cells were damaged by blebbing and vacuolization of the cytoplasm, followed by a pycnotic condensation of the nucleus.

Based on the results obtained in the present study, it seems most probable that the reduction in number of micronuclei may be due to their unequal distribution in the course of binary fission together with their degeneration under the effects of the chemicals used here. Unequal distribution of micronuclei was observed rather frequently in dividing specimens. Probably the micronuclear number may decrease through repeated aberrant binary fissions.

Summary

1. The present study deals with the effects of Mitomycin-C, azan, MEPA and podophyllin on *Paramecium polycaryum*, with special reference to the variation in number of the micronuclei.

2. The number of micronuclei showed a remarkable decrease under the influence of these chemicals, except podophyllin which was less effective on the variation of the micronuclear number (Tables 2, 3, 4 and 5).

3. The fission rate of animals dropped soon after chemical treatments, and then cell division was completely inhibited (Table 6).

4. Aberrant division of the micronuclei of animals in process of binary fission was frequent under the effect of Mitomycin-C (Table 7).

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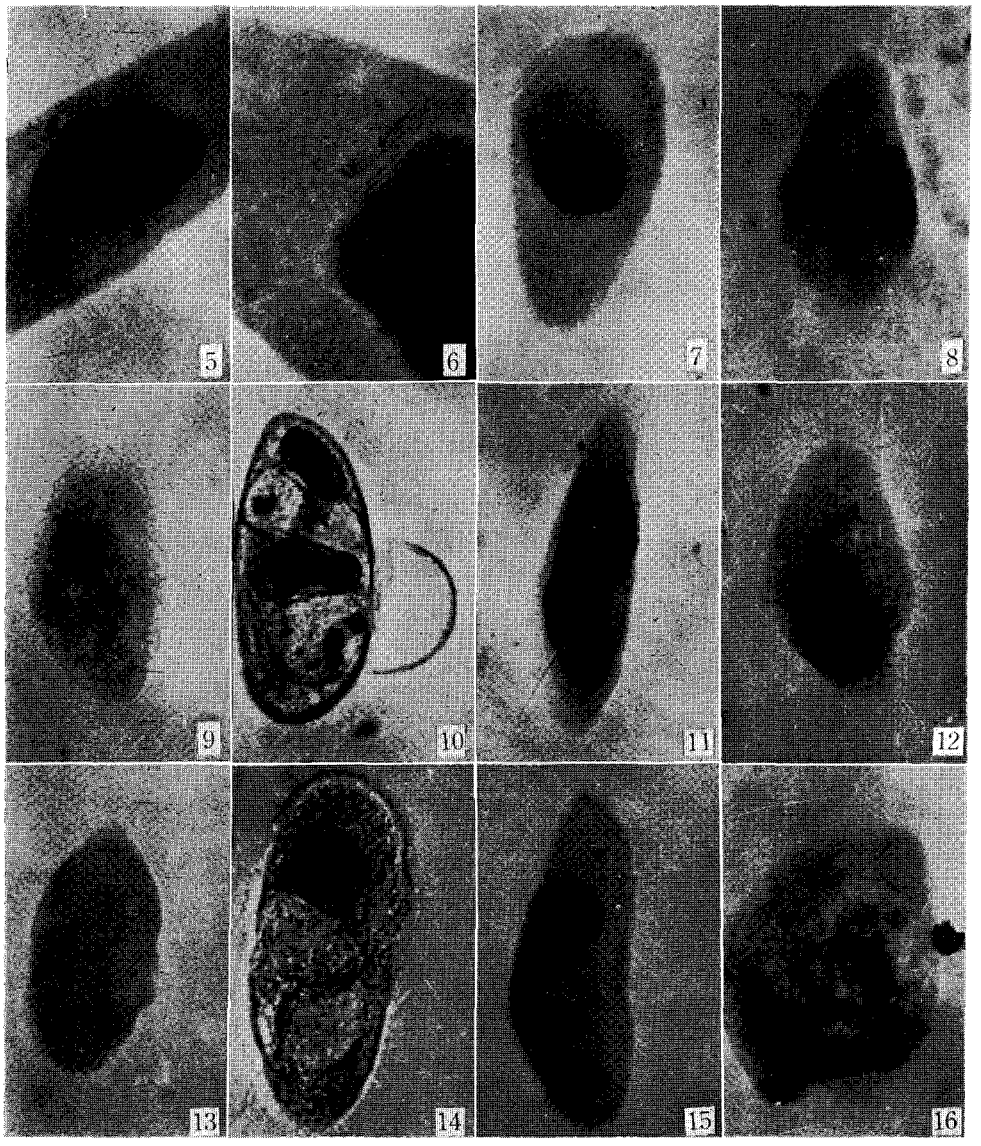
Explanation of Plate IX

Figs. 5–10. Photographs showing specimens treated with Mitomycin-C. Figs. 5 and 6. Diffused micronucleus, stained with Feulgen reaction, ×480, ×1000. Figs. 7, 8 and 9. Various patterns of diffused macronuclei, stained with Feulgen reaction, ×480. Fig. 10. Large vacuoles and blister, stained with methyl green, ×480.

Figs. 11–14. Photographs showing specimens treated with azan. Fig. 11. Enlarged macronucleus stained with Feulgen reaction, ×480. Fig. 12, Diffused macronucleus, stained with Feulgen reaction, ×480, Fig. 13 "Ghost" cell, ×480. Fig. 14. Large vacuoles stained with methyl green, ×480.

Figs. 15–16. Photographs showing specimens treated with MEPA. Fig. 15. Elongated macronucleus, stained with Feulgen reaction, ×480. Fig. 16. Diffused macronucleus, stained with Feulgen reaction, ×480.



T. Takayanagi: Cytological Responses of Paramecia to Chemicals