THE ACTION OF X-RAYS ON EUPLOTES TAYLORI AND ASSOCIATED BACTERIA*

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In the first two papers of this series (1, 2) the results of preliminary investigations on the rôle of bacteria in the nutrition of protozoa were reported. Apart from demonstrating a curious dependence of *Euplotes taylori* upon two strains of living bacteria these introductory studies centered around the problem of obtaining bacteria-free protozoa. Two methods of sterilization were examined in detail, and shown to be experimentally feasible,—repeated immersion in sterile media, and the killing of bacteria with appropriate bactericidal agents. Through the use of soft x-rays we have now been able to develop a third method of considerable promise, which, along with related observations on the lethal action of x-rays, we propose to discuss in this communication.

Apparatus

The x-ray equipment used in these investigations was designed by Dr. Harry Clark. High ionization intensity has been obtained by a short target-to-specimen distance (3 cm.), silver anode, thin aluminum window (0.003 inches) and moderately high power (30 ma. at 50 kv.). The intensity as measured by an ionization chamber was maintained at 2110 Roentgen units per second in the experiments of Part I and at 2530 Roentgen units per second in the later studies.

PART I

The Action of X-rays on Euplotes taylori

Throughout the investigation we employed thriving cultures of *Euplotes taylori* which had been maintained for many generations in

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1:1 artificial sea water on a mixture of two strains of bacteria (*Pseudomonas fluorescens* and *Bacillus coli* K_{13})[1] in approximately equal proportions. 48 hours after the last feeding the protozoan culture was divided into two portions, the first for irradiation of the organisms in the motile state and the second for the preparation and irradiation of the encysted forms. For this purpose the organisms were caused to encyst by gradual evaporation of the medium from a partly open watch-glass at room temperature. Encystment was complete within 15 hours. Fifteen to forty-five of the organisms or cysts suspended in approximately 0.01 cc. of medium[2] were transferred to a sterile, shallow, spherically ground slide. This was provided with a moist chamber consisting of a circular trough ground into the concave depression near the edge, into which water could be placed. The whole was sealed with a sterile cover-slip of mica[3] held in position with vaseline. The slide was then inserted in the holder and irradiated for a stated time as indicated in Table I. The organisms were observed immediately after irradiation, 15 minutes later, and finally after 5 hours. In the case of the motile forms, special attention was given to any abnormalities in the movements of the organism (cessation of motion, spiral movements, marked changes in rate of movement), vacuolation, and disintegration. In many of the experiments the organisms were inactive and seemingly dead 15 minutes after irradiation but were found actively swimming at the end of 5 hours or later. In the case of the cysts attention was given to vacuolation and the rate of encystment. As a rule those cysts with many vacuoles were found to be dead. Marked vacuolation and failure to excyst in 1:1 artificial sea water were accepted as evidence of death.

[1] For a description of the organisms and culture medium, *cf.* the first paper of this series (1). Organism A, mentioned therein, has since been identified as *Pseudomonas fluorescens*.

[2] The motile organisms were suspended in 1:1 artificial sea water, and the cysts in 1:1 artificial sea water which by evaporation had been reduced to 1/20 of its initial volume.

[3] It was necessary to measure accurately the thickness of all mica cover-slips and to employ correction factors for variations therein. Mr. Hugo of the Stanford Gauge Laboratory, was good enough to make the measurements. In preliminary experiments where the slips were only approximately similar, appreciable differences in absorption were observed.

On completion of the 5 hour microscopic examination, ten to twenty of the organisms,[4] either as cysts or in the motile state, were transferred to 1:1 artificial sea water and fed with the appropriate bacteria. On the 2nd day the numbers of actively motile and apparently normal organisms were counted, the values so obtained serving as an index of viability. These results together with intermediate counts made upon the motile forms are entered in Table I.

Finally, sterility tests upon the irradiated organisms were also run. For this purpose the entire contents of the slide, after removal of ten to twenty organisms for the viability test, were transferred to nutrient agar. We were interested to observe that brief periods of irradiation, even of 10 to 30 seconds, were sufficient to sterilize the protozoa. It is this observation which has permitted us to prepare bacteria-free *Euplotes* with comparative ease. Although the ciliate suffers no immediately recognizable injury when irradiated for brief periods, there is evidence which will be presented later in the paper, that irradiation for 60 seconds materially reduces its longevity and power of continued reproduction.

It should be pointed out that all experiments were rigidly controlled by the use of parallel cultures of motile forms and cysts which were transferred to depression slides, subjected to microscopic examinations, and ultimately run through the viability and sterility tests. They differed from the experimental ones only in not being irradiated. In the sterility tests run upon these controls, numerous colonies appeared in the nutrient agar within a day or two.

The results of the experiments are presented in Table I.

Examination of the table indicates that 190 seconds of irradiation were sufficient to kill the encysted organisms. The motile forms required exposure for 220 seconds. Due to the different conditions of irradiation (*cf.* footnote 2) we are not disposed to conclude that these values are indicative of any fundamental difference in the resistance of encysted and excysted forms to death by x-rays. To investigate this possibility it will be necessary, presumably, to suspend the organisms in identical media and to maintain the bacterial content

[4] It should be mentioned that some of these organisms at the time of transfer were inactive and, in some cases, seemingly dead due to the curious effect of x-rays in bringing about a temporary suspension of ciliary movement.

TABLE I

The Action of X-Rays on Euplotes taylori

Time of irradiation	State	No. of experiments	Total No. of organisms irradiated	No. of organisms active* after irradiation Hours after exposure				Sterility of irradiated *Euplotes*. No. of pour-plate colonies
				0	½	5	48†	
sec.								
0	Cysts	5	147				272	Many
0	Motile	8	219				377	Many
10–30	Motile	6	199	199	199	197	375	0
30–45	Motile	7	218	218	218	220	373	0
60	Motile	5	152	152	152	152	242	Few
76–90	Motile	4	133	133	133	133	233	0
91–105	Cysts	6	201				218	0
91–105	Motile	7	189	177	156	171	264	0
106–120	Cysts	6	206				211	0
106–120	Motile	7	173	161	129	142	223	0
130	Cysts	5	153				188	0
130	Motile	3	78	67	56	55	96	Few
135–150	Cysts	16	495				156	0
135–150	Motile	15	376	317	184	214	416	1
151–165	Cysts	4	102				72	0
151–165	Motile	5	136	100	95	118	132	Few
166–180	Cysts	6	174				40	0
166–180	Motile	7	169	128	7	54	145	5
190	Cysts	2	55				0	0
190	Motile	2	34	6	1	5	24	0
196–210	Cysts	4	118				0	0
196–210	Motile	5	185	84	1	8	70	2
220	Cysts	6	171				0	0
220	Motile	5	127	0	0	0	0	0
230	Cysts	1	31				0	0
230	Motile	1	16	0	0	0	0	4
240	Cysts	3	89				0	0
240	Motile	4	116	0	0	0	0	0
260–270	Cysts	3	92				0	0
260–270	Motile	3	91	0	0	0	0	0

* Ciliary activity and swimming.

† 43 hours earlier, a known number of the organisms,[4] approximately one-half, had been placed in 1:1 artificial sea water and fed with the appropriate bacteria. The values in this column are calculated for the entire number actually irradiated.

approximately constant. Other experiments which we have in progress indicate that the presence of much bacterial substance decreases the toxicity of x-rays towards protozoa.

Accepting the higher value of 220 seconds as the lethal exposure time for *Euplotes*, calculations may be made of the energy required to kill a single organism. If it be assumed that the absorption coefficient of cytoplasm is the same as that of water the amount of radiation absorbed in 220 seconds by 1 cc. of irradiated substance would be 220 $\times$ 2110 Roentgen units. This equals 4.6×10^7 ergs ($220 \times 2110 \times 6.1 \times 10^{13} \times 1.591 \times 10^{-12}$). From the values published by Garnjobst (3) for the linear dimensions of *Euplotes taylori* its volume may be calculated as approximately 2.1×10^{-7} cc. The energy required to kill a single protozoon by the use of x-rays is therefore 9.7 ergs. This calculation assumes that radiation absorbed by the surrounding medium is not productive of toxic or protective substances, and that the absorption coefficient of cytoplasm equals that of water. The former assumption is of doubtful validity.

It is also clear from the table that all bacteria associated with *Euplotes* were killed with but a few seconds of exposure. This marked difference in the resistance of the protozoa and bacteria to death by x-rays suggested that similar differences might exist between the two species of bacteria. This possibility led us to the experiments reported in Part II.

PART II

The Action of X-Rays on Pseudomonas fluorescens and Bacillus coli, K_{13}

The purpose of this inquiry was to determine the lethal dose of x-rays for the two strains of bacteria used as nutrients in the cultivation of *Euplotes taylori*. Approximately 0.01 cc. of a suspension of the appropriate organism, washed from an agar slant, was transferred to a depression slide, covered with mica, and irradiated for some stated time. 1:1 artificial sea water was used as the suspension medium. After irradiation the contents were transferred quantitatively to nutrient broth and tested for sterility. The results are presented in Table II. From these it may be concluded that approximately 15

seconds of exposure (3.8×10^4 Roentgen units) are sufficient to kill *Pseudomonas fluorescens*. *Bacillus coli*, K_{13}, requires approximately 45 seconds (9.4×10^4 Roentgen units). If we consider $0.4\mu \times 1.4\mu$ and $0.5\mu \times 1.5\mu$ to be the linear dimensions of *Pseudomonas fluorescens* and *Bacillus coli* respectively, and 3.7×10^6 ergs to be the energy per cc. of the absorbed radiation it follows that 5.9×10^{-7} and 29×10^{-7} ergs represent the lethal energy per organism for *Pseudomonas fluorescens* and *Bacillus coli*, respectively. These values may be

TABLE II

Lethal Dose of X-Rays for Pseudomonas fluorescens and Bacillus coli, K_{13}

Time groups	*Pseudomonas fluorescens*			*Bacillus coli, K_{13}*		
	No. of slides	Viable	Sterile	No. of slides	Viable	Sterile
sec.						
2.5– 7.5	4	4	0	4	4	0
7.5–12.5	10	7	3	4	4	0
12.5–17.5	14	0	14	5	5	0
17.5–22.5	4	0	4	5	5	0
22.5–27.5	2	0	2	1	1	0
27.5–32.5	4	0	4	5	5	0
32.5–37.5	0	0	0	10	6	4
37.5–42.5	3	0	3	7	3	4
42.5–47.5	0	0	0	1	0	1
47.5–52.5	1	0	1	5	0	5
52.5–57.5	0	0	0	0	0	0
57.5–62.5	2	0	2	3	0	3
over 62.5	1	0	1	1	0	1

compared with that of 2×10^{-4} ergs reported by Coblenz and Fulton (4) as the minimum energy required to kill a bacterium of *B. coli* with ultraviolet light (1700–2700 Å. u). A value somewhat less than 2×10^{-4} ergs may be deduced for staphylococci (5). With electrons, Wells (6) reports the relatively high value of 0.65 ergs as the energy required to kill a single staphylococcus. In these cases, however, the amounts of absorbed radiation remained undetermined. The significance of the calculations is therefore not clear.

Our observation that *Euplotes taylori* is killed less readily than bacteria agrees with the early work of Hertel (7) who studied the lethal

action of ultraviolet light on bacteria, protozoa, coelenterates, annelids, molluscs, amphibia, and plant cells.

Here it should be mentioned that investigations on *Colpidium campylum* carried out by others in this laboratory, suggested that it would be highly desirable to determine whether death by x-rays might not be largely due to the production of toxic products in the medium. With this possibility in mind we irradiated twelve slides of nutrient broth and twelve of yeast autolysate for 9 to 35 minutes (2.5×10^3 Roentgen units per second). After irradiation, six of each received approximately 0.001 cc. of a suspension of *Pseudomonas fluorescens* and the remainder, 0.001 cc. of a suspension of *Bacillus coli*. Pour-plates were made and examined 48 hours later. Growth was equally luxuriant irrespective of whether the plates were prepared from organisms in irradiated or unirradiated media. There was no evidence that irradiation of the medium produced substances of appreciable toxicity to these bacteria.

Eight similar experiments on the effect of irradiated media (1:1 artificial sea water and $F_1 + K_{13}$)[5] on *Euplotes taylori* also gave negative results. Although the medium was irradiated for upwards of 30 minutes it failed to be appreciably toxic towards the ciliate. The action of various sensitizers and protective substances on the lethal dose of x-rays for the protozoa and its associated bacteria remains for investigation. It is apparent from theoretical principles and from observations made with x-rays and other sources of radiation (8, 9, 10, 11) that certain changes in the composition of the medium influence materially the lethal dose of radiation. Nevertheless, with *Euplotes taylori* and the bacteria studied here an ordinary medium when irradiated by itself does not become lethal to the organisms.

PART III

An Attempt to Cultivate Euplotes taylori on Bacteria Killed by X-Rays

In our earlier work (1) we have shown that *Euplotes taylori* which will thrive on a mixture of two varieties of living bacteria, cannot be

[5] F_1 = *Pseudomonas fluorescens*.
K_{13} = *Bacillus coli*, strain K_{13}.

maintained upon these same organisms previously killed by heat, toluene, autolysis, phage lysis, or upon sterile dialysates of these bacteria. Since the physical state and chemical composition of bacteria are likely to be the factors which determine their nutritive value for protozoa, it is apparent that the manner of killing is likely to have substantially different effects upon the nutritive value of the bacteria, inasmuch as the end results upon the physical state, enzyme distribution, and chemical composition will be widely different. Recognizing, therefore, that bacteria killed by x-rays would be substantially different from bacteria killed by toluene, heat, lysis, or other means, we were tempted to investigate the possibility of maintaining *Euplotes* upon irradiated bacteria.

In the experiments reported upon in Table III the organisms of Groups 1, 4, and 5 were fed at 3 day intervals upon the same product—equal proportions of F_1 and K_{13}, suspended in artificial sea water, and subjected to 67 seconds irradiation[6] (2.5×10^3 Roentgen units per second). In Group 4 the irradiated bacteria were also washed twice by centrifuging, the object being to remove water-soluble toxic products produced during irradiation. The protozoa used in these tests were sterilized prior to cultivation in two ways,—by washing (Groups 1 and 4) and by irradiation for 58 seconds (Group 5).

Finally three sets of controls were devised: irradiated protozoa fed upon normal unirradiated F_1 and K_{13} (Group 7); washed protozoa similarly fed (Group 2); and normal untreated *Euplotes* fed upon normal F_1 and K_{13} (Group 3). It should be mentioned that the *Euplotes* used in this last control were drawn from a stock laboratory culture which had been maintained for many months in pure line on F_1 and K_{13}.

The irradiated bacteria and the sterilized protozoa were tested routinely for sterility by the use of heavy inoculations into nutrient broth. All preparations which failed to be sterile were discarded. The table reports only upon cultures which gave satisfactory sterility tests.

In earlier experiments employing very low doses of x-rays (a few seconds exposure) we were interested to notice that the broth media

[6] Note that 15 seconds are sufficient to kill F_1 (*Pseudomonas fluorescens*) and 45 seconds to kill K_{13}.

used for sterility tests revealed the phenomenon of delayed growth. In a few cases the tubes remained clear for so long as 9 or 10 days (room temperature) and showed such faint cloudiness that we could not be certain for perhaps 48 hours longer whether there was growth. In control experiments on normal unirradiated protozoa the broth used in the sterility tests showed heavy clouding within 24 hours. This phenomenon may be comparable to that displayed by irradiated ciliates (*cf.* Table I) in which sublethal doses of x-rays brought about a marked but temporary cessation of activity.

TABLE III

Irradiated Bacteria as Food for Euplotes

Group	No. of cultures tried	Total No. of protozoa used	Initial state of protozoa	Initial state of bacteria ($F_1 + K_{18}$)	Life of culture (extremes of entire group)
					days
1	16	16 × 6	Washed	Irradiated	11–23
2	15	15 × 6	Washed	Normal	7 died out within 4 weeks. Remainder in normal condition.
3	22	15 × 6 7 × 10	Normal	Normal	2 cultures died out within 4 weeks. Remainder continued normal
4	16	16 × 6	Washed	Irradiated and washed	13–23
5	30	30 × 10	Irradiated	Irradiated	12–28
6	7	7 × 10	Irradiated	Normal	12–25

Two conclusions follow from the data of Table III. (*a*) *Euplotes taylori* may not be maintained upon bacteria killed by irradiation (Groups 1 and 5). (*b*) Even sublethal doses of x-rays cause an insidious damage to the protozoan, as indicated by an ultimate failure in reproduction (Group 7). This injury is not immediately manifest.

SUMMARY

1. The minimum lethal dose of x-rays for *Euplotes taylori* was determined. Under the conditions of this investigation a 220 second exposure (2110 Roentgen units per second) was required to kill the protozoon. Much less exposure was sufficient to kill the associated

bacteria. This difference in resistance permits the sterilization of protozoa with comparative ease.

2. Irradiation of *Euplotes* for 100 to 220 seconds caused a complete but temporary cessation of ciliary activity in many of the organisms, the percentage so affected increasing with the length of irradiation.

3. Pure cultures of *Pseudomonas fluorescens* and *Bacillus coli*, K_{13}, separately irradiated, were found to be killed much more readily than protozoa,—the former in 15 seconds exposure (2530 Roentgen units per second) and the latter in 45 seconds.

4. The death of these organisms by irradiation was not due to the action of toxic products in the medium since separately irradiated media were not found to be toxic.

5. Irradiated bacteria were found unsatisfactory for the nutrition of *Euplotes*, previously sterilized either by irradiation or washing.

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Note Added to Proof.—Schepman and Flecke (*Klin. Woch.*, 1926, **5**, 1608) irradiated agar-plate cultures of five strains of bacteria with soft x-rays. Although the intensity of the radiation was low (36.1 Roentgen units per second) the lethal dose was of the same order of magnitude as we have reported here. They obtained values ranging between 6500 and 100,000 Roentgen units, which compare favorably with the values of 38,000 and 114,000 observed by us.