INACTIVATION OF PARAMECIN ("KILLER" SUBSTANCE OF PARAMECIUM AURELIA 51, VARIETY 4) AT DIFFERENT HYDROGEN ION CONCENTRATIONS AND TEMPERATURES*

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Sonneborn (1) has described a character (the "killer" character) in varieties 2 and 4 of Paramecium aurelia. These stocks secrete into the medium an antibiotic substance. The killer that has been most fully studied is stock 51 of variety 4 of Paramecium aurelia. When sensitive stocks are exposed to the culture fluid containing the antibiotic substance secreted by the killer stock 51, they develop certain characteristic changes (2). A slight hump appears after several hours on the aboral surface near the hind end of the body. This hump gradually enlarges while the anterior end of the body wastes away and the posterior part is pushed into the humped region. The animals then become smaller and spherical and finally die. In any 5 hour interfission period, the amount of paramecin liberated into the medium by the killer animals is such that the usual relation between the numbers of the killer animals that had lived in the culture fluid for that period and the number of sensitive animals killed by the fluid is 1:1 (3). This indicates that in this period 1 unit of paramecin is released by one killer animal and that 1 unit is sufficient to kill a sensitive animal. More paramecin can be released when the animals are disintegrated by repeatedly forcing a suspension of Paramecium aurelia through a narrow gage injection needle (4).

The manifestation of the killer character is dependent upon both a cytoplasmic and a chromosomal factor (5). Mating tests proved that the latter is a single dominant gene, designated K. Sensitive clones (KK) exist which, without the cytoplasmic factor, do not manifest the killer character, but acquire this when the factor K is introduced. Thereafter the clones reproduce true to form. When the cytoplasmic factor is introduced into clones homozygous for the recessive allele (kk), the killer character does not manifest itself; nor is the cytoplasmic factor perpetuated. In about half of the known varieties of Paramecium aurelia a cytoplasmic factor intervenes between the genes at each locus and their phenotypic manifesta-

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tions. Examples of hereditary traits depending upon the presence of a cytoplasmic factor are those of mating type and antigen formation (6). There exist important resemblances between the properties of the genes and the cytoplasmic factors; both are self-reproducible under appropriate conditions and both control characters. The cytoplasmic factors for different characters are discrete and separable (7). However, there is no necessary correlation between the rates of reproduction of the genes and the cytoplasmic factors (8).

Cytoplasmic factors may be of general significance. L'Héritier and co-workers (9, 10) analyzed the carbon dioxide resistance in Drosophila which presents in many respects a striking parallel to the results obtained on Paramecium aurelia. Darlington (11) and Haddow (12) emphasized the rôle played by plasma genes in animals, plastogenes in plants and viruses. These cytoplasmic systems, while probably dependent upon a chemical or even a physiological equilibrium, have an important function in cell differentiation. They constitute that part of the genetic system that is not associated with any visible bodies in the cell, although it is related to the genes. Several other instances of cytoplasmic inheritance can be cited. Rhoades (13) described a gene-induced transmissible plastid difference in maize. Experimental work by Woods and DuBuy (14, 15) suggests the possibility, as pointed out by these authors, that some phytopathogenic viruses might be related in origin to certain constituents of the cytoplasm, the mitochondria. Heston, Deringer, and Andervont (16) and Heston (17) pointed out that the gene-cytoplasmic relationship in Paramecium aurelia resembles in many respects the gene-milk agent relationship in mammary tumor development in mice.

Paramecin, besides being the end-product of a gene-cytoplasmic factor relationship, is the first antibiotic known to be produced in an animal cell. It will be of great value for the understanding of its mode of production and its mode of action to have this compound identified as to its chemical constitution. It will then be possible to investigate the relationship between paramecin and the cytoplasmic factor and ultimately the whole hereditary system of gene-cytoplasmic factor-paramecin.

We wish to report here some preliminary investigations on the nature of paramecin.

EXPERIMENTAL

Method of Testing. Preparation of Test Solutions—Paramecium aurelia 51, variety 4, were grown in Erlenmeyer flasks on a lettuce infusion, inoculated with the bacterium Aerobacter aerogenes (18), at the rate of two fissions per day. When about 2000 animals per cc. were present, the concentration was increased approximately 10-fold by filtering the culture through a Berkefeld filter after clarification by filtering through a Gooch crucible.
lined with a loose layer of cotton (19). At this point the number of animals per cc. was determined by counting the *Paramecium* present in 1 cc. of an appropriate dilution. Final concentration was achieved by centrifuging the suspension in an angle centrifuge at 2000 R.P.M. for 10 minutes. The animals present in 1 cc. of the supernatant liquid were counted and this figure subtracted from the original count. This figure, multiplied by the number of cc. of suspension that had been centrifuged, was taken to represent the number of animals used for the determination. The packed animals were then suspended in 10 cc. of phosphate buffer* of pH 7 and

*The buffer was prepared by mixing 6 volumes of 0.25 M disodium phosphate solution with 0.25 M monosodium phosphate solution, until a pH of 7 was obtained when diluted to 0.01 M.

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**Fig. 1.** Numbers of sensitive *Paramecium aurelia* killed by three different preparations of a killer paste at increasing dilutions of each paste. ○, initial activity of the paste 20,000 units of paramecin per cc., ●, initial activity of the paste 70,000 units of paramecin per cc., and □, initial activity of the paste 270,000 units of paramecin per cc.
subsequently disintegrated by forcing the suspension through a narrow gage (No. 27) injection needle. Ten to fifteen passages through the needle were usually sufficient for a complete disintegration of all the animals present. The resulting paste was always subjected to a careful examination under a low power microscope and any non-disintegrated animals which might be present were removed. Two 4 cc. portions of this paste were pipetted into test-tubes, standing in a constant temperature water bath. The pH in one of the tubes was adjusted to the desired value with hydrochloric acid

The Test—Immediately after the volume of the paste in the second test-tube was adjusted, the first sample was removed from both tubes. This

<table>
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<th>No. of determinations</th>
<th>0 min.</th>
<th>10 min.</th>
<th>20 min.</th>
<th>30 min.</th>
<th>40 min.</th>
<th>50 min.</th>
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</table>

* Per cent activity ± standard error.
† The figures in parentheses represent the range.
time was arbitrarily taken as zero time. The sample (0.05 cc.) was immediately diluted to 25 cc. with the original buffer solution. Each 10 minutes a sample was removed, the last one being taken 60 minutes after zero time. Occasional checks did not show a change of pH in any of the determinations at the end of 1 hour. The pH of the dilutions was not significantly different from the original pH of the buffer solution. The activity of the dilutions was tested as follows. A suspension of sensitive animals (Paramecium aurelia 31, variety 8) was grown in the same manner as that described for Paramecium aurelia 51, variety 4. The solution was concentrated by filtration till about 5000 animals were present per cc. From this suspension approximately 0.5 cc. was added to depression slides. Ten depressions were used for a single determination. To each depression was added 0.1 cc. of the solution to be tested. The depression slides were stacked in glass moist chambers and incubated at 27°. After 48 hours, the dead and affected animals in each depression slide were counted by removal with a micro suction pipette. The number of animals killed in ten depres-
sions represents the activity of 1 cc. of the tested dilution. A wide variation in the activity of the untreated paramecin paste was always encountered. For this reason a control at pH 7.0 was included in every determination. In order to compare the results the actual counts were recalculated on a percentage basis. Where possible, the data were analyzed statistically according to the methods of Fisher (20).

Results

The experiments and the conclusions derived from them are valid only if there exists a direct proportional relationship between the dilution of the solution containing the killer principle and the number of animals killed. Austin (3) and Sonneborn and coworkers (4) have presented evidence that the number of sensitive animals killed is directly proportional to the concentration of paramecin. Their experiments, moreover, have established the
fact that one single particle of paramecin can kill a sensitive Paramecium, thereby permitting computation of the concentration of paramecin in the solutions. We have again confirmed the direct proportionality between the concentration of paramecin and the number of sensitive animals killed under the conditions of our experiments. These results are given in Fig. 1. The number of sensitive animals killed is directly proportional to the dilution of the three reported preparations which differ greatly in their paramecin content.

Fig. 4. The inactivation of crude paramecin preparations at 40°, expressed as per cent activity left at each time interval. •, pH 7.0, ○, pH 8.0.

It is clear from Table 1 and Fig. 2 that paramecin (in the crude extract) has a narrow pH stability range. Even at the pH where the compound appears to be most stable, about 15 per cent of its initial activity is lost by an exposure to 30° for 1 hour. When the data of Table I are plotted in such a manner that \[ \log_c C = kt \] (first order reaction), as is done in Fig. 3, it appears that the data fit a first order reaction curve very closely. It may be concluded here that the inactivation of paramecin at the different pH values is a first order reaction.
The inactivation of paramecin at 40° was investigated for only two hydrogen ion concentrations, pH 7.0 and pH 8.0. The mean values of three different determinations are represented in Fig. 4. The data also fit a straight line closely when log₀C is plotted as a function of time. The reaction constant (first order reaction) at this temperature for the two hydrogen concentrations is pH 7, \( K_{40} = 0.0359 \); pH 8, \( K_{40} = 0.0127 \). From the velocity of destruction at 30° and at 40° the activation energy for the inactivation of paramecin \((\mu = \Delta H - RT)\) has been calculated to correspond to 126,000 calories per mole at pH 7, and 169,000 calories per mole at pH 8, which are typical values for enzymes and proteins.

Unexpected results were obtained when the rate of inactivation of paramecin was investigated at 20°. These results are reported in Table II. There is an apparent increase in activity during the first 45 minutes

<table>
<thead>
<tr>
<th>pH</th>
<th>Per cent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min.</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>90 min.</td>
</tr>
<tr>
<td>7</td>
<td>131.3</td>
</tr>
<tr>
<td>8</td>
<td>112.6</td>
</tr>
</tbody>
</table>

* Two determinations only.

Thereafter the activity drops slowly and attains a logarithmic rate after about 120 minutes. At present no explanation can be given for this phenomenon. It is conceivable that the observed increase in activity is due to a release or dissociation of paramecin from the bound state in which it is present in the cell. The inactivation proceeds simultaneously. After 45 minutes this process becomes the determining reaction.

**DISCUSSION**

It is evident from the experiments reported here that paramecin is an unstable compound. It may be concluded from the experiments that the inactivation at different hydrogen ion concentrations can be represented by a first order reaction. This is comparable to the results obtained by Chick and Martin (21), and those reported by Lauffer and Price (22). The former showed that the heat denaturation reactions of hemoglobin
and of egg albumin are first order reactions, while the latter showed that this was also the case for the thermal denaturation of tobacco mosaic virus. The average value for the activation energy for the inactivation of thirty-four different enzymes ($\mu = \Delta H - RT$) was found to be $\mu = 68,600$ calories per mole (23). The values have a range from 22,000 calories per mole to 198,000 calories per mole. Recent work (24) also indicates that the energies of activation are similar for enzyme inactivation and protein denaturation. The experimentally obtained values for the energy of activation for the inactivation of paramecin ($\mu = 126,000$ calories per mole at pH 7, and $\mu = 169,000$ calories per mole at pH 8) are within the limits for those reported for enzymes and proteins. It may be concluded then that paramecin belongs to either of the two classes, or both. The inactivation reaction must proceed at a much higher speed than the release of paramecin from the cell material, for it is surprising indeed that no evidence for a release of paramecin could be found at 30\(^\circ\) or at 40\(^\circ\). It is possible that the release of paramecin is due to an enzymatic mechanism with a very low temperature optimum. It should, a priori, be possible to find an inhibitor for the inactivation reaction, thereby opening a way for obtaining a relatively richer source of paramecin. Investigations pertaining to this problem are in progress and will be reported at a later date.

SUMMARY

Paramecin, the killer substance of Paramecium aurelia 51, variety 4, is instantaneously inactivated at hydrogen ion concentrations ranging from pH 1.0 to 5.0 and pH 9.5 to 11.0. It is moderately stable in the pH range 7.0 to 9.0. Even in this range inactivation proceeds rapidly at the temperatures investigated. The activation energy for the inactivation of paramecin has the typical value for that of an enzyme or protein.

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