EVIDENCE FOR THERMAL REACTIONS FOLLOWING EXPOSURE OF DIDINIUM TO INTERMITTENT ULTRAVIOLET RADIATIONS*

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(Received for publication, May 31, 1956)

Many previous workers have found low intensity ultraviolet (UV) radiation less effective than high intensity UV in producing injury to various living organisms (Christensen, 1953; Coblentz and Fulton, 1924; Dreyer, 1903; Gates, 1929; Swann and del Rosario, 1932; Weinstein, 1930; Wyckoff, 1932). Experiments performed here on the protozoan *Didinium nasutum*, indicated an opposite, greater effect of low intensity UV. It appeared likely that a dark reaction succeeds the absorption of UV, so that quanta are supplied at high intensity more rapidly than they can be utilized; *i.e.*, saturation occurs. To investigate this possibility the UV was flashed and the dark period between flashes varied, with the expectation of increased injury with longer dark periods. Since a dark reaction is thermochemical, it should proceed more rapidly at high than at low temperature, and injury should therefore increase with temperature. Experiments were accordingly carried out at various temperatures. The expectations have been confirmed in the present study. The effects of variable light period and flash rate, and the comparison of flashed high intensity with continuous low intensity irradiation have also been explored.

Materials and Methods

Cultures of *Didinium nasutum* were grown in 4 mm. bore isolation tubes on concentrated suspensions of *Paramecium caudatum* in the manner previously described (Brandt *et al.*, 1955). Didinia selected at random for a sample were irradiated in a quartz cell, put into isolation tubes with food, one animal per tube, and the number in each tube was recorded three times daily, as a rule, until the fourth division was reached in all tubes. This stage was usually reached earlier in some tubes than in others, and the tubes were discarded as they attained it, since higher counts were difficult to make, and were likely to be biased because of rapid depletion of the food supply.

* Supported in part by contract AT(11-1)234 with the Atomic Energy Commission, in part, particularly the analysis of data, by Public Health Contract C-1799(C2).

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When observations for divisions had to be made for more than 48 hours, the didinia were gently centrifuged to the bottom of the isolation tubes, the supernatant fluid was removed under a dissecting microscope (× 6) with a mouth pipette, and a fresh suspension of paramecia was added. This is necessary because didinia deteriorate if fed on starved paramecia (Beers, 1926). All counts and observations were made in red light to avoid the possibility of photoreversal.

Intensity was varied by reducing the maximum intensity with one or more fine copper screens placed between the radiation source and the first lens of the monochromator. Each intensity was measured with the thermopile. Filters of cellophane were tried at first, but their fluorescence was a source of visible light, which introduced a considerable error in the determination of the intensity of UV, and also may have produced some photoreversal of the UV effects.

For most experiments UV of 2654 A from a quartz monochromator was used and the intensity was determined with a calibrated thermopile. Tests were made with dosages of 2000, 3000, and 4000 ergs/mm.^{2}/sec. and the dosage chosen in a particular experiment depended upon the tests to be made. For example, if long dark periods were used the largest dosage induced such a marked delay in division that it was not convenient for routine experimentation, although it gave some revealing information; a lower dosage would therefore be employed. Experiments with monochromatic UV were performed at room temperature (21–24°C.), but the didinia were grown at 27°C. in a dark incubator.

When the effect of temperature was being tested, a Westinghouse sterilamp which gives off about 85 per cent of its radiation at 2537 A was employed because it was impossible to bring the monochromator and ancillary equipment into the constant temperature rooms in which the experiments had to be performed.

It was not possible to obtain an accurate absolute measure of the intensity of the UV from the sterilamp under the conditions of operation, but measurement with a Hanovia UV meter made it possible to demonstrate that the intensity was constant during the period of experimentation; therefore the dosages were the same from experiment to experiment. A biological effect (the delay to the fourth division) was used as a criterion to set the initial dosage for the temperature experiments. This dosage or some fraction of it was then used in all subsequent experiments.

Temperature experiments were performed in constant temperature rooms at 5, 13, 25, and 30°C. All equipment, pipettes, and solutions were brought to the constant temperature room several hours before treatment and the didinia were brought in an hour before the experiment, since preliminary testing showed this time to be adequate for them to reach the temperature of the room. After an experiment the didinia were transferred to isolation tubes and put in the 27°C. incubator in the usual manner (see Brandt *et al.*, 1955). They were therefore exposed to the lower or higher temperatures only during the preexperimental incubation period and during irradiation, and for a short time before being transferred.

Flashing was performed by rotating a black paper disc with sectors cut out of it in front of the slit leading to the reaction cell. The disc was attached to a motor by a pulley. The speed of flashing was set at the start of an experiment with a strobotac.

In most experiments a sample of 10 didinia was taken for each test and for each control; smaller samples were occasionally necessary, and samples of 11 or 12 were

used at times. Data for a sample thus consisted of a series of counts obtained from each of about 10 tubes.

The first step in the statistical reduction of the data has been the computation, from each sample, of the mean time to the fourth division ($\bar{T}_4$), and the standard deviation (σ) of this time. The time to the fourth division in a given tube when the final number of animals was exactly 16 was taken as the time from isolation to that count; and when more than 16, was determined by interpolation between last and next to last counts, using a chart with logarithm of number of animals plotted against time. In a few instances, when the final count was less than 16, it was necessary to extrapolate from the last two counts.

In comparing results, $\bar{T}_4$ was always calculated using these conventions, and confidence limits for $\bar{T}_4$ were determined, or tests of significance of the difference between means made, using tables of Student's distribution (Dixon and Massey, 1951).

This procedure is subject to the general objection that only a fraction of the information obtained from each sample is used in the computed statistics. For this reason, special pains have been taken to study the data before its analysis, with a view to detection, follow-up, and reporting of any significant departures from normal patterns.

In many samples, one or more specimens failed to reach the fourth division. The procedure followed in such instances has been to compute mean and variance of the remainder of the sample, and to record the number of mortalities so as not to be misled by the statistics. The risk of bias is small when the sample is one with slight injury or none, for the dead specimens are then so far out of line with the rest of the sample that they may safely be regarded as gross errors, or as members of a different population (injured in manipulation, or contaminated, for instance). Occasionally a specimen which reached the fourth division, but much later than all others in the sample, would be excluded from the computation for the same reason, and with almost equally small risk. But the risk of bias is much greater when this procedure has to be followed in a sample with severe injury, for the dead specimens are then much less likely to represent gross errors, and should by rights be included. Yet they have an infinite T_4, and there is hence no choice unless the sample is thrown out entirely. Since valuable information would often be lost if the sample were thrown out, it was deemed the lesser of evils to follow the regular procedure, carefully noting mortalities and drawing no inferences without taking them into account.

Experimental Results

1. Nature of UV Injury.—The experiments performed here provide examples of UV injury which run the gamut from none (as in dark controls) to death before the first division in all specimens of a sample. The pattern of increase of numbers in a sample is not a simple exponential pattern, except in dark controls, and is even quite complicated in instances of severe injury. To report only $\bar{T}_4$ and its standard error is to report none of this complexity, and this is to exclude from consideration some features of general interest for the light which they throw on the nature of UV injury; moreover such procedure risks giving a false impression of simplicity of the raw data and adequacy of the

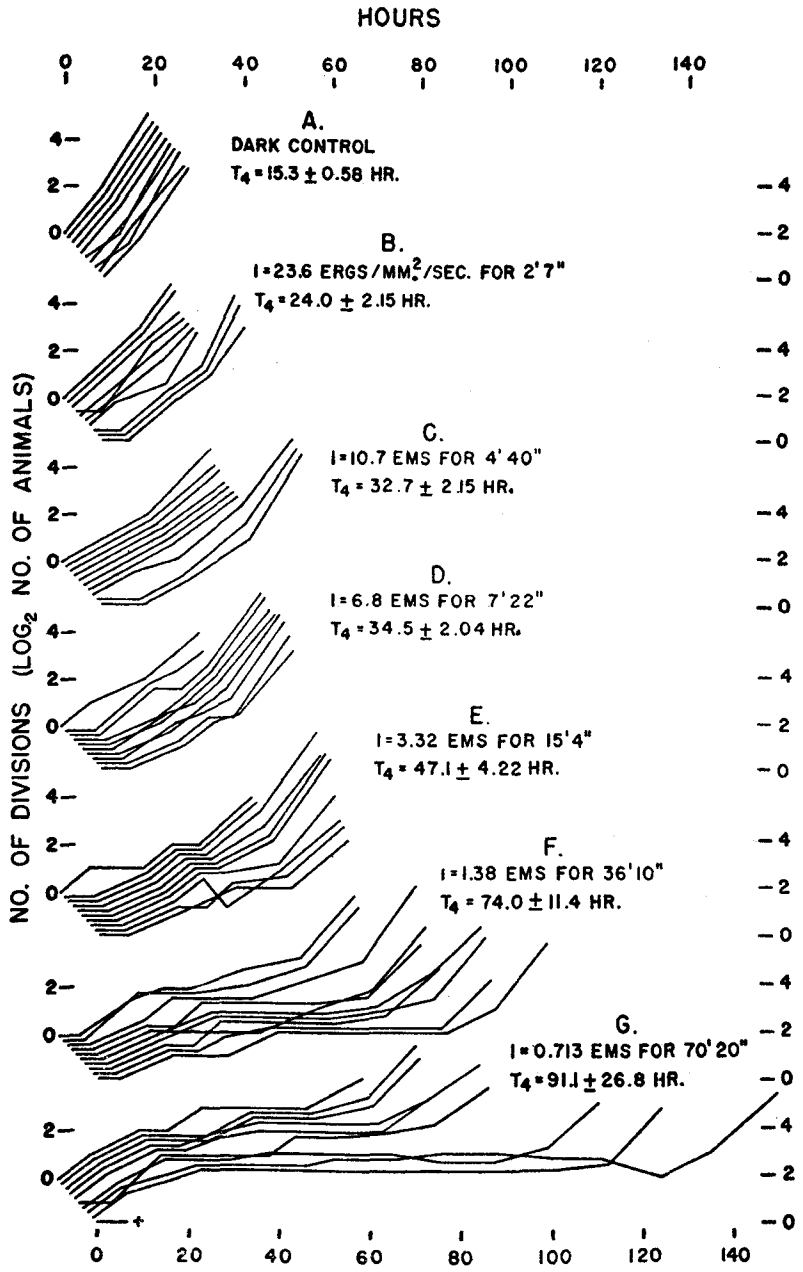

FIG. 1. Pattern of division delay induced by ultraviolet: graphs of a typical series of experiments, with dosage of 3000 ergs/mm.² at λ2654 A. The intensities decrease consecutively down the page (I = 23.6 to 0.713 ergs/mm.²/sec.). T_4 is the time to the fourth division after irradiation (or inoculation, in the dark control); it is represented by mean ($\overline{T}_4$) ± standard deviation (standard error of individual specimen). The curves are staggered for clarity, with two sets of coordinates given: one set at the top and left for the upper specimen in each sample; the other at bottom and right for the lower specimen in each sample. By interpolating between the given scales, the number of animals counted at a particular time can be accurately determined for any intermediate specimen.

statistics computed from them. A proper appraisal is facilitated by Fig. 1, in which representative samples are illustrated in such a way as to include all data from each specimen (tube), and to permit any given specimen to be followed through the entire course of its growth, from isolation through the fourth division.

The figure reveals the typical patterns of injury which have been found in scores of experiments with *Didinium*. Slight injury results in an immediate "slowdown" of division, with gradual and continuous recovery, *i.e.* the first division after irradiation is delayed longest and the normal rate is more closely approached with each subsequent division, being usually restored in full by the fourth division after irradiation.[1] This is the pattern for a short exposure at high intensity (Fig. 1 B). As intensity is lowered and exposure increased in duration to maintain a constant dosage, the slowdown increases somewhat (Fig. 1 C), depending on wave length.

But the greater injury which occurs with still lower intensity and longer exposure appears not as further slowdown, but as a "stasis" after the second division (occasionally after the first or third).[1] If the injury is not fatal, normal division rate is restored by about the fourth division, after a time in stasis which varies from a few hours to at least 100 hours, depending on the degree of injury (Figs. 1 D, E, F, G).

It will be noted that the more severely injured samples show much more variability. This increase of variability, which is reflected in the increase in standard deviation of T_4, appears to be attributable fundamentally to greater variability in time of stasis. Two specimens in the most severely injured sample (Fig. 1 G) show a decline in numbers before ultimate recovery (also one in Fig. 1 E, but this is most likely because of a mistake in counting), which obviously contributes to the larger variability of that sample; yet it is clear that the major contribution is from the variability of time in stasis.

2. The "Intensity Effect."—The increase of injury with decrease of intensity is evident in Fig. 1, which has been compiled from various experiments in which a dose of 3000 ergs/mm.^{2} was given at λ2654 A. Approximately 95 per cent confidence limits for $\bar{T}_4$ have been extracted from the data of Fig. 1 and plotted in Fig. 2, along with similar results from experiments with the same dose given at three other wave lengths.

Indications are that there is no intensity effect at intermediate and high intensities (6 to 36 ergs/mm.^{2}; 2654 A is exceptional). Injury rises rather abruptly at lower intensities, however, down to the lowest values which could be administered within the limits of accuracy of calibration of the arc. It is to be expected that at still lower intensities a peak of injury would be reached

[1] These characteristics were also found by Kimball, Geckler, and Gaither (1952) for *Paramecium aurelia*, except that injury was increased by raising the dose instead of lowering the intensity.

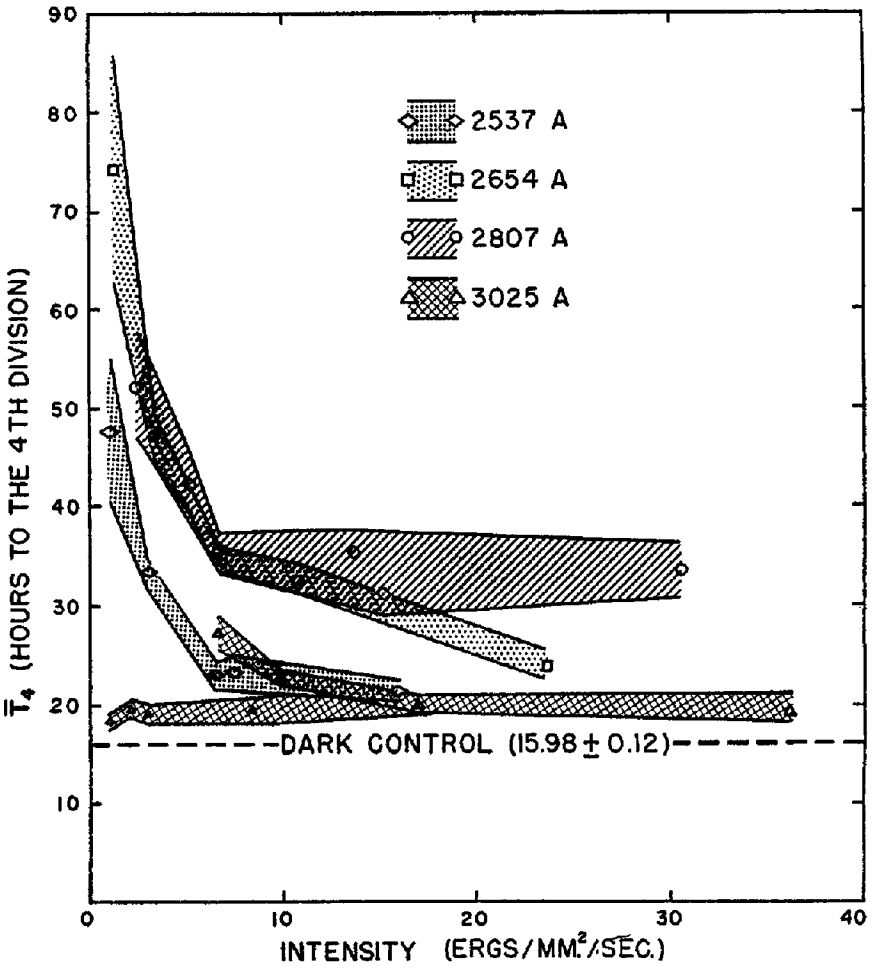

FIG. 2. The "intensity effect." Time to the fourth division of didinia after irradiation by each of four wave lengths of UV with the same dosage (3000 ergs/mm.²/sec.) delivered at different intensities. The points are the mean values of T_4 and the width of the bands indicates 95 per cent confidence limits at the points. At λ3025 A one series of experiments (April 24, 1954) suggests a slightly greater effect of low intensity than of high; a second more extensive series (September 24, 1955) performed at lower intensities does not; a third series (not illustrated) in which little injury occurred also showed no increase in effect at low intensities.

and passed, since repair of UV injury is probably occurring even during irradiation, or because the number of quanta of UV falling in unit time becomes insufficient to injure the cells. Unfortunately it was not practicable to investigate these lower intensities.

The occurrence of the confidence bands of the various wave lengths in pairs at different levels suggests an action spectrum of UV effect on division in *Didinium* with a maximum at the intermediate wave lengths. No systematic investigation of the UV action spectrum has yet been reported for division delay in *Didinium*, but the results provided by these experiments are in general accord with those obtained from more direct and extensive work on *Paramecium* and other forms (Giese, 1945; Kimball, 1955).

In the λ2537 and λ3025 A curves the level of $\overline{T}_4$ at high intensities is so near that of the dark control that nothing of interest is to be expected at still higher intensities. The $\overline{T}_4$ "plateau" at λ2807 A is at a much higher level, however, and it would be desirable to know the behavior of this curve as intensity is further increased. It would likewise be advisable to follow the λ2654 A curve to higher intensities. The high values obtainable with λ3025 A were not obtainable with the other wave lengths, however, because their spectral lines are much weaker.

Experiments performed to extend the range of intensities to lower values at λ3025 A have had the unexpected result that no intensity effect was shown at this wave length, perhaps because injury is not marked (Fig. 2). This phenomenon calls for further investigation, which it has not yet been feasible to undertake here. It suggests that the UV responsible for the "intensity effect" may have a rather narrow action spectrum in the range of short waves, from perhaps λ2500 to λ2800 A.

3. Intermittent Light.—If UV of intermediate and high intensity is less effective than UV of low intensity because the energy of quanta absorbed is not utilized in subsequent thermal reactions, flashing the light should increase its efficiency, as it allows time for the thermal reactions.[2] The data bear out this expectation, as can be seen by comparing results of continuous irradiation with those of flashed UV in Figs. 3 and 4, or in Fig. 5.

In the expectation that increasing the dark period would increase the injury, experiments were run using the largest possible range of dark periods within experimental limits. It proved to be impossible to obtain results over the whole range at a given dose and temperature; if any injury was to be obtained with the shorter dark periods, there would be too much with the longer ones, and the investigation of the longer dark periods was therefore accomplished by lowering the dose or the temperature.

Results are shown in Table I for experiments performed at 25°C. with the sterilamp at an intensity of 37 ergs/mm.^{2}/sec. In Table I A there is a slight increase of $\overline{T}_4$ with dark periods from 0.0039 to 0.0246 sec., and then a marked rise at 0.0532 sec. This pattern is at variance with the asymptotic increase of

[2] Studies at room temperature by Christensen (1953) indicated that intermittent UV is more effective than the same dose of continuous UV on *Tetrahymena*, suggesting thermochemical reactions in this case.

effect to be expected of a thermochemical reaction following a photochemical reaction (*cf.* Emerson and Arnold, 1932), but it may be due to a non-linearity of $\overline{T}_4$ as a measure of injury, rather than to anomalous behavior of the reactions themselves. The rise of $\overline{T}_4$ is not so steep at the still longer dark periods, as shown in Table I B from experiments with a lower dose. It was expected that a maximum would be approached, and the results suggest this possibility, although they cannot be considered conclusive.

Since time for thermochemical reactions is gained equally well by irradiating at a slower rate, one might assume that if light is delivered to the cell in about

TABLE I

Increase of UV injury with length of dark period between flashes: results of experiments at 25°C. with the sterilamp at an intensity of 37 ergs/mm.²/sec. The "40/90" disc means that a 40° sector was cut out of each 90° quarter, etc. The various discs were rotated at speeds such that the duration of flashes (light period) was constant at 0.0031 sec. Results on specimens exposed to continuous UV are included for comparison under disc, "none."

Dose, *erg/mm.²*	Disc	Dark period	$\overline{T}_4 \pm \sigma$, *hrs.*	
			First experiment	Repeat experiment
		sec.		
A. 6000	None	0	24.4 ± 2.3	27.3 ± 3.5
	40/90	0.0039	26.9 ± 2.1	28.0 ± 2.8
	20/90	0.0109	30.8 ± 2.2	36.0 ± 8.6
	15/90	0.0156	38.4 ± 6.2	33.5 ± 3.6
	10/90	0.0246	35.9 ± 4.0	34.1 ± 6.4
	5/90	0.0532	86.0 ± 34.7	74.3 ± 10.1
B. 4000	None	0	18.0 ± 1.9	
	5/90	0.0532	26.3 ± 3.3	
	5/180	0.1096	37.7 ± 6.3	
	5/360	0.222	39.7 ± 4.0	

the same time but in one case the light is flashed and in the other it is delivered continuously at lower intensity, the efficiency of the two treatments should be about the same. There were many experiments performed which provided suitable bases for comparison, in that the dose and over-all time of exposure were nearly identical. Of over 200 such experiments, 142 revealed instances in which flashed light was somewhat more injurious than continuous, 65 in which the opposite was true, and a few were ties. The probability of obtaining by chance such a deviation from equal numbers in each category is one in ten million. There is thus a good basis for the inference that flashed low intensity UV is slightly more effective than continuous low intensity UV delivered at the same average rate, though it is not easy to see why this is so.

A number of experiments were performed using the same disc with a favor-

able constant ratio of light to dark periods but varying the rate of rotation and hence of flashing (in the most extensive experiment, 100 to 12,896 cycles/min.; light period, 0.0126 to 0.000098 sec.; dark period, 0.1370 to 0.0011 sec., respectively). The expectation was that an optimum flash rate might be found for a given light-dark ratio. The results have not borne this out, however. In some experiments differences significant from the statistical standpoint have appeared, but no over-all pattern is discernible. Thus in one experiment the slowest flash rate may produce most injury, in another the fastest, and in a third, an intermediate rate. Often all flash rates produce nearly identical results. The most reasonable assumption is therefore that significant differences, when they appear, are attributable to factors other than flash rate. For this reason the experiments are not documented here.

One experiment was performed with practically constant dark period and variable light period. The UV injury was found to increase with decreasing light period; thus the T_4 was 26.4 $\pm$ 3.0 hours for 0.00333 sec. and 51 $\pm$ 11.7 hours for 0.00083 sec., from which it can be concluded that the time of the light reaction is less than the shortest light period used (0.0008 sec.), but it may well be much shorter than this. Equipment was not available to test shorter light periods, however.

4. Temperature and Dark Period.—If there is a thermochemical or dark reaction between UV absorption and the manifestation of injury, as suggested by the intensity effect, it should be possible to increase injury by raising the temperature as well as by flashing the light. Also, long dark periods should be more effective than short ones, regardless of temperature. Experiments were therefore performed at several temperatures using the same series of dark periods at each temperature, with results which confirmed these expectations: for a given dark period, the injury increased with temperature, and at a given temperature, the injury increased with dark period.

Figs. 3 and 4 represent the results of comparable parts of these experiments in full detail, in the same way as in Fig. 1. Each group of 10 specimens represents a sample treated at one of four temperatures and one of two dark periods (light period was constant at 0.0031 sec.). The samples of the shorter dark period are plotted in Fig. 3, along with the continuous light controls; those of the longer dark period are plotted in Fig. 4. The increase of injury with temperature is evident in both series, and so is the increase of injury with dark period, except at 5°C., where as expected only slight injury occurred in either case.

In Fig. 5 the values of $\overline{T}_4$ computed from these data are plotted as a function of temperature, showing the increase of injury with temperature more directly. The $\overline{T}_4$ values from a series of controls of zero dark period are also plotted (lower curve in the figure); these manifest only very slight injury at all temperatures, and their increase of injury with temperature is of questionable significance, though the trend is upwards. The increase of injury with dark period is

shown also by the fact that the higher curve corresponds to the longer dark period.

The difficulty of statistical reduction of the raw data can be appreciated from the study of Figs. 3 and 4. It is indeed open to question whether the adopted

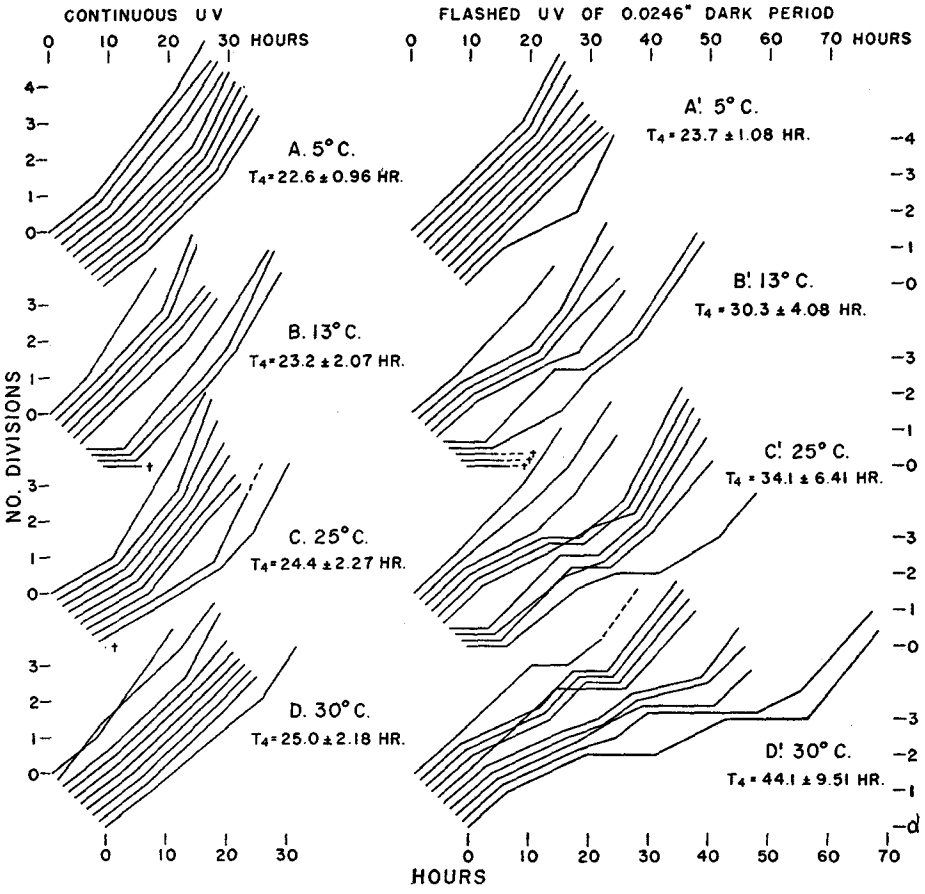

FIG. 3. Increase of UV injury with temperature, for continuous light and light flashed with dark period of 0.0246 sec. Results are plotted as in Fig. 1, with specimens going from highest to lowest in each sample in order of their speed of recovery, and with origins staggered both horizontally and vertically, to permit the clearest complete representation of the data.

measure of injury ($\overline{T}_4$) is adequate for the purpose. It is evident first of all, from the high degree of variability among the specimens of a sample, that $\overline{T}_4$ is subject to large error, particularly in those samples which show considerable injury. The standard errors of $\overline{T}_4$ were computed, and 95 per cent confidence

limits determined for each value by means of standard techniques (Dixon and Massey, 1951). There is general overlap of these limits at 5°C., but not at the higher temperatures. Thus statistical analysis confirms the unsophisticated impression from the raw data of significantly greater injury with longer dark

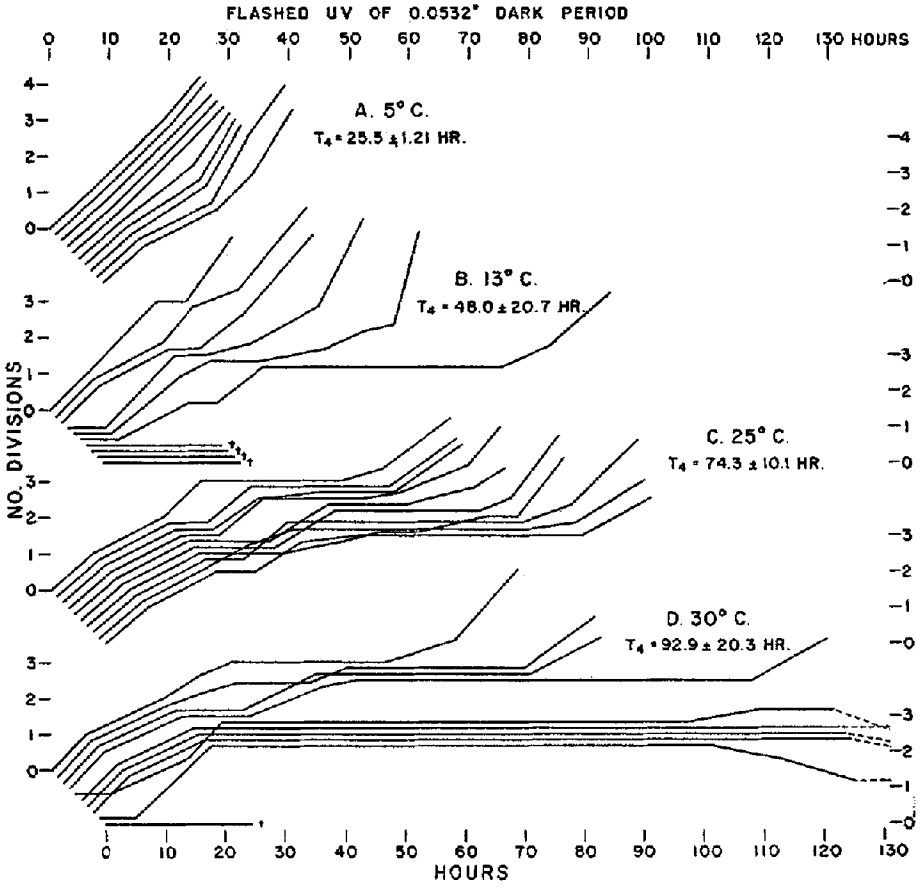

FIG. 4. Increase of UV injury with temperature for light flashed with dark period of 0.0532 sec. Figs. 3 and 4 both represent results of experiments with the sterilamp at an intensity of 37 ergs/mm.²/sec. Where the light was flashed, the light period was constant at 0.0031 sec. Time to the fourth division is given as mean ± standard deviation, as in Fig. 1.

period. The confidence limits of adjacent points at different temperatures on the same curve overlap in some cases, but those of alternate points do not, except the controls (lower curve), and there is therefore statistical support also for the impression of a real rise of injury with temperature.

There is doubt as to the validity of $\bar{T}_4$ and its standard error as a basis of comparison in a few instances. Specimens which never divided after irradiation were treated as gross errors in the sample, and therefore did not enter into the

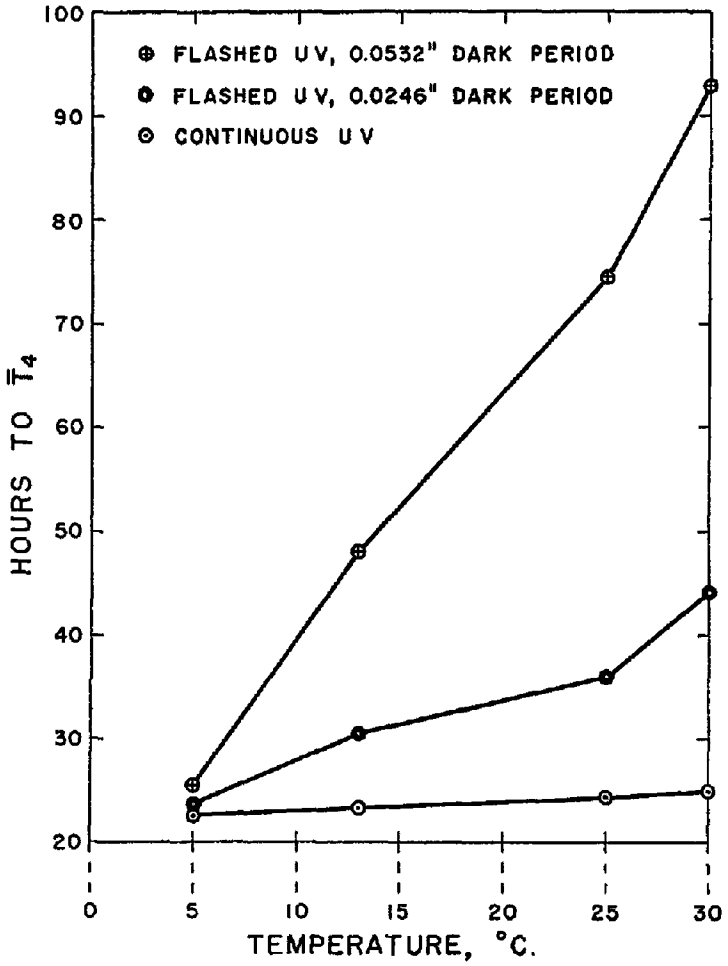

FIG. 5. Increase of UV injury with temperature, using values of T_4 extracted from the data of Figs. 3 and 4.

computation of $\bar{T}_4$. Since it has been our experience that specimens occasionally fail to divide even in non-irradiated controls, this procedure seems quite justified in the case of Fig. 3 B′. However, to have four failures in a control is rather rare; therefore, the procedure is subject to more doubt for Fig. 4 B. The exceptionally large variability of this sample may cast further doubt on its statistics. It is

clear, however, that the surviving specimens do show on the whole more injury than those at 5° and less than those at 25° of the same dark period, and also more than those at 13° of the shorter dark period.

It is a matter of certainty rather than of doubt that the statistics of Fig. 4 D are in error, since the five specimens which divided but which never reached the fourth division are hardly gross errors, The value of $\bar{T}_4$ is in this case unquestionably too low, since data for the infinite delay of the five specimens never reaching the fourth division are not included; but even so, it is the highest value among all the samples.

Thus there is nothing in the statistics to confute the conclusions which may be drawn by inspection of the data, that injury increases with temperature and dark period, and in fact they add some further support to these conclusions.

DISCUSSION

UV has three main effects upon the division pattern of didinia: (1) It decreases the rate of division so that the curve, plotting the logarithm of the number of individuals against time, declines in slope; (2) It induces a temporary cessation of division or stasis when the injury is marked, the length of the stasis depending upon the injury which in turn depends upon the dosage, the intensity, the temperature, the light period and the dark period; (3) It may kill the didinia if the stasis becomes so prolonged that recovery is impossible, or by inhibiting posttreatment division altogether.

At the present time no information is available to pinpoint the injuries which change the division pattern. It seems most likely that synthesis of some material essential for division (DNA?; see Kelner, 1953; Kimball, 1955) is blocked by UV and that so long as a store of this material is available divisions continue for a time after irradiation, but that stasis develops when this material has been consumed (Brandt *et al.*, data to be published). The retardation of the divisions preceding stasis might then be interpreted as resulting from injury of another sort, possibly to the cytoplasm, but in any case at a locus other than that producing stasis. That UV affects different components of the protozoan is demonstrated by the different types of action spectra observed for different effects on the same cell; for example, sensitization to heat approximates the absorption spectrum of cytoplasmic proteins, while division delay approximates that of nucleic acid (Giese, 1945).

The investigation of photosynthesis with flashing light (Emerson and Arnold, 1932) was based on the proposition that there was a single photochemical reaction which set the stage for a series of dark reactions. The dark reactions might actually be many and varied, and complexly interrelated, but their overall behavior would be like that of a single reaction which would go asymptotically toward completion at a speed that would vary with temperature and could be approximately determined experimentally with flashing light by increasing

the dark period between flashes until a maximum effect was obtained. The maximum rate of photosynthesis per unit dose of flashed light was shown to increase with temperature and to be approached asymptotically with increasing dark period, in accordance with this hypothesis.

UV injury to *Didinium* increases with temperature in a similar way. At a given temperature it also increases with dark period, but not asymptotically, as evidenced by the much greater steepness of the highest curve in Fig. 5. This may indicate a non-proportionality between degree of injury and amount of dark reaction, which offers difficulties of interpretation. It is more likely, however, that it is a reflection of the non-linearity of our measure of injury.

However that may be, it seems clear that thermochemical reactions follow in a very short period of time the absorption of UV by didinia. No reason exists to doubt that this conclusion is also applicable to other organisms.[2] It should therefore provide another tool for the analysis of UV effects on living matter.

SUMMARY

1. The nature of ultraviolet injury and its variation with the same dose given at different intensities and wave lengths have been investigated in the protozoan *Didinium nasutum*, using time to the fourth division as a measure of injury.

2. The injury has been found to consist of a "slowdown" of division rate, which always occurs, and a "stasis," usually at the second division after irradiation, which appears in varying degrees among more severely injured samples.

3. Injury was found to be almost independent of intensity at three wave lengths out of four studied over a wide range of intermediate and high intensities, but was found to rise sharply with lower intensity at all except the longest wave length.

4. Flashed UV of high intensity is much more effective than the same dose of continuous radiation at high intensity and shorter total time of treatment. It is also more effective than the same dose at low intensity and equal time of treatment, though only slightly so.

5. An increase of injury with rise of temperature and with increase of dark period clearly indicates that injury depends on thermochemical reactions following the absorption of UV in *Didinium*.

6. The most reasonable assumption is that a similar conclusion applies to other organisms as well, and that its general application may be useful in the investigation of UV effects on protoplasm.

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