PYRIMIDINE DIMERS IN THE DNA OF *PARAMECIUM AURELIA*

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ABSTRACT The production and fate of thymine-containing pyrimidine dimers in *Paramecium aurelia* DNA was investigated in three experimental series: production of dimers by UV irradiation, fate of dimers in the dark, and "loss of photoreactivability of dimers." It is shown that cyclobutyl dimers are made by UV irradiation of *Paramecium* DNA in vivo, that because of cytoplasmic absorption the number of dimers made in DNA irradiated in vivo is much lower than in DNA irradiated in vitro, that dimers are lost from animals incubated in the dark after irradiation, and that all the dimers that remain in the animals can be destroyed by photoreactivating illumination. Since mutation induction is photoreactivable, these and previous photoreactivation data suggest that pyrimidine dimers are important in mutation induction in *P. aurelia*.

INTRODUCTION

Ultraviolet (UV) radiation (2200–3000 A) causes killing and mutation in a variety of organisms. Cyclobutane-type pyrimidine dimers have been implicated in the lethal effects of ultraviolet light in bacteria (see reviews by Wacker, 1963; Smith, 1964; J. K. Setlow, 1966; R. B. Setlow, 1964), and there is evidence that dimers produced in the DNA of bacteria are important in mutation induction (Witkin et al., 1963; Hill, 1965; Witkin, 1966; Kondo and Kato, 1966). However, it has not been possible to evaluate the role of dimers in mutation in higher organisms, since dimers and their fate and mutation production have not been investigated in the same eukaryote. *Paramecium aurelia*, a ciliate protozoon, is known to be amenable to mutation studies (Kimball and Gaither, 1951). Therefore, we have used *Paramecium* as the subject of three experimental series: the production of thymine-containing dimers in the range of biological doses, the fate of dimers in cells incubated in the dark after irradiation, and the "loss of photoreactivability" of dimers with time after irradiation. These experiments, together with data on photoreactivation in *Paramecium*, support the hypothesis that pyrimidine dimers in the DNA of *P. aurelia* play an important role in induction of mutations by ultraviolet radiation.

MATERIALS AND METHODS

Maintenance and Labeling. *Paramecium aurelia*, syngen 7, stock 57, were maintained routinely in *Aerobacter aerogenes*. The cell cycle was about 6 hr. The paramecia were labeled by letting them feed overnight on *Escherichia coli* 15 T^- which had been grown in medium containing high specific activity ^{3}H–methyl thymidine (Berger and Kimball, 1964). The paramecia were then fed unlabeled *A. aerogenes* for 2½ hr to allow digestion of the labeled bacteria in the food vacuoles. The animals were collected by centrifugation and suspended in Dryl's (1959) solution, which is transparent to UV. About 10^4 animals, with about 5×10^5 CPM in radioactivity, were used in each analysis. The procedure of Kimball and Perdue (1962) was followed for radioautography.

Irradiation. A germicidal lamp, emitting mainly at 2537 A, supplied all UV exposures at a rate of about 55 erg mm^{-2} sec^{-1}, as estimated from Jagger (1961) meter readings. About 10^4 animals were irradiated in 10.0 ml of Dryl's solution in a 6.0 cm diameter Petri dish. At this concentration the shielding of one animal by another is negligible. An exposure of 4000 erg mm^{-2} was used in excision and photoreactivation experiments as all single-cell isolates examined after 48 hr were alive (100% survival). Thus, during the experiments, the longest of which was 24 hr, the survival was 100%; however, assays taken later (R. F. Kimball, personal communication) indicated that many of the cells died between 72 and 96 hours. Two General Electric BLB black-light bulbs supplied photoreactivating light at a rate of 8000 erg mm^{-2} sec^{-1} as indicated by Jagger meter readings. A heavy glass plate between the cells and the black-light bulbs prevented transmission of wavelengths shorter than about 3000 A. Cells were killed either by freezing or by adding ice cold 5% trichloroacetic acid (TCA).

A Hilger quartz monochromator supplied the exposures of 2390 A radiation for in vitro dimer reversal. A calibrated Beckman photocell was used to determine dose rate.

Cesium Chloride Density Centrifugation. Cesium chloride density centrifugation was used to separate *Paramecium* DNA from *E. coli* and *Aerobacter* DNA. DNA was extracted from labeled cells by Marmur's (1961) procedure. The method of Flamm et al. (1966) was used for CsCl density centrifugation. From 25 to 100 μg of DNA extracted from the labeled paramecia-bacteria mixture, plus 8 μg of a ^{14}C-thymine–labeled *E. coli* 15 T^- DNA, were suspended in 4.5 ml of 7.7 M CsCl in 0.01 M Tris pH 8.0. The density of the solution was adjusted to 1.700 g cm^{-3}. The mixture was centrifuged for 62 hr at 20°C in a Spinco No. 40 rotor at 33,000 rpm. After centrifugation, approximately 80 fractions were collected through a hole punched in the bottom of the tube. The fractions were assayed for ^{3}H and ^{14}C acid-insoluble material by the paper disc method (Bollum, 1966) and for absorbance at 260 mμ.

Chromatography. Whole cells (about 10^4 per sample), TCA-soluble and -insoluble fractions, or extracted DNA's, along with calf thymus DNA to aid in chromatographic identification, were hydrolyzed in 97% formic acid at 175°C for 30 min. The hydrolysates were assayed for radioactivity in thymine and thymine-containing dimers by the following procedure. Thymine and thymine-containing dimers were separated by two-dimensional paper chromatography (*n*-butanol 86 ml:water 14 ml, followed by saturated ammonium sulfate 40 ml:isopropanol 1 ml:1 M sodium acetate 9 ml), and the radioactivity was eluted from the chromatogram and counted in a liquid scintillation counter in a dioxane-based system (Setlow et al., 1963). This chromatographic system does not separate the dimers thymine-thymine and uracil-thymine (derived by deamination of cytosine-thymine) (Setlow and Carrier, 1966).

Gel Filtration. Gel filtration was used to separate DNA from any oligonucleotides that might contain dimers. Cells were prepared for gel filtration as follows. The frozen paramecia were thawed, then packed by centrifugation; the cells were sonicated for 30 sec in 0.01 N NaOH in a Biosonik sonicator (Bronwill Scientific, Rochester, N.Y.) at setting 30, and a

yeast sRNA marker was added; the sonicates were neutralized, placed on a 20 × 1 cm column packed with Sephadex G-100 (Pharmacia Fine Chemicals), and eluted with 0.01 M ammonium bicarbonate. Thirty 1-ml fractions were collected and assayed for absorbance at 260 mμ and for tritium activity.

RESULTS AND DISCUSSION

Dimers in Paramecium DNA

Since bacteria were used to introduce ^{3}H-thymine label into paramecia, it was necessary to show that the label was in *Paramecium* DNA and that any labeled photoproducts in irradiated paramecia were really thymine-containing dimers from the *Paramecium* DNA. This was done in three series of experiments.

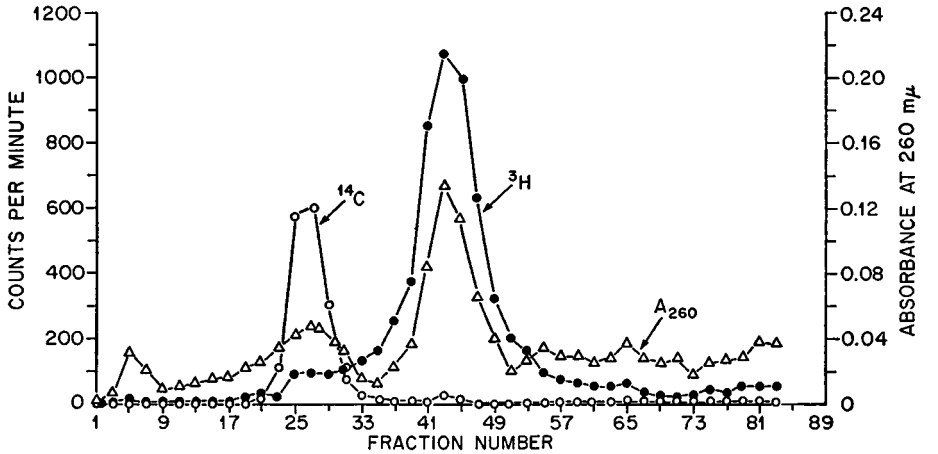

FIGURE 1 A typical isopycnic separation, in CsCl, of DNA extracted from *Paramecium aurelia* that had fed on *Escherichia coli* labeled with ^{3}H-thymine, and on unlabeled *Aerobacter aerogenes*. Solid circles, ^{3}H activity; open circles, ^{14}C activity; triangles, absorbance at 260 mμ. The ^{14}C activity represents ^{14}C-thymine-labeled *E. coli* DNA used as a density marker. The absorbance at fraction 5 corresponds to the density expected for *A. aerogenes*, 1.717 g cm^{-3} (Smith-Sonneborn et al., 1963). The peak at fraction 27 corresponds to *E. coli*, 1.710 g cm^{-3} (Schildkraut et al., 1962), and that at fraction 43 corresponds to *P. aurelia*, 1.699 g cm^{-3} (Smith-Sonneborn et al., 1963). About 25 μg of *P. aurelia* DNA were used in this experiment.

First, radioautograms of labeled animals showed that the macronucleus was heavily labeled, while the cytoplasm and surrounding medium were only lightly labeled.[1] These results indicated qualitatively that most of the ^{3}H label was in *Paramecium* DNA. Second, CsCl density gradients gave a quantitative estimate of the amount of ^{3}H label in *Paramecium* DNA and in bacterial DNA. Fig. 1 shows the results of the assay of a typical gradient for ^{14}C and ^{3}H activity, and for absorbance at 260 mμ. The densities of the DNA's at the fractions 5, 27, and 43 were calculated by comparison with the data of Flamm et al. (1966) for the density fraction relation

[1] B. M. Sutherland. Doctorate Dissertation, The University of Tennessee.

in a CsCl gradient in the Spinco 40 rotor. They were found to correspond to the densities expected for the DNA's of *Aerobacter aerogenes* (Smith-Sonneborn et al., 1963), *Escherichia coli* (Schildkraut et al., 1962), and *P. aurelia* (Smith-Sonneborn et al., 1963), respectively. About 5% of the ^3H activity appears in the *E. coli* region; the remaining 95% of the ^3H appears in the peak with density expected for *Paramecium* DNA.

Since only small percentages of thymine-containing dimers were observed in DNA irradiated in vivo as compared with values observed for DNA irradiated in vitro (see section on Production of Dimers by UV Irradiation, below), and since about 5% of the ^3H label was in bacterial DNA, the possibility arose that the dimers measured originated in bacterial DNA and that the apparent low levels of dimer production were due to the contribution of radioactive thymine, but of no or few dimers, by the *Paramecium* DNA. This was ruled out by the following experiment. DNA from about 10^4 cells exposed to 3000 erg mm^{-2} of 2537 A radiation was extracted and divided into two parts. One part ("unfractionated DNA") was frozen, and the other was centrifuged in a CsCl density gradient. "Unfractionated DNA" and the *Paramecium* peak from the gradient were assayed for radioactivity in thymine and thymine-containing dimers. Since the "unfractionated DNA" contained

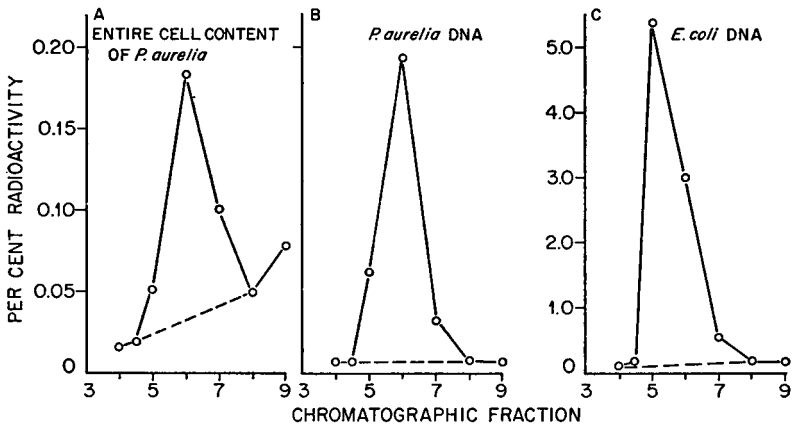

FIGURE 2 A comparison of the radioactivity in the pyrimidine dimer region of the second dimension of typical two-dimensional chromatograms of acid hydrolysates. The dashed lines are arbitrarily taken to indicate chromatographic streaking of thymine and unknown compounds, and the differences between them and the solid curves are used in subsequent discussions as the "radioactivity in the dimer region." In unirradiated DNA this activity was less than 0.03%. A, the entire cell contents (6.9×10^6 CPM) of about 10^4 *Paramecium aurelia* exposed to 4000 erg mm^{-2} of 2537 A radiation. B, the DNA (5.7×10^5 CPM) extracted from *P. aurelia* that had been exposed to 4000 erg mm^{-2} of 2537 A radiation. C, *Escherichia coli* DNA (4.0×10^4 CPM) irradiated with 4×10^4 erg mm^{-2} of 2800 A radiation. Although in A the nonphotoproduct radioactivity was high and the resolution was not as good, the dimer frequencies of such preparations agreed within experimental error with those made on DNA extracted from irradiated *P. aurelia*.

0.265% of its radioactivity in thymine-containing dimers, as compared with 0.269% in the *Paramecium* peak, about 95% of the ^{3}H-thymine–containing dimers were from *Paramecium* DNA.[2]

These results showed that the fraction of dimers observed in extracted DNA could be used as a good estimate of the fraction of dimers in *Paramecium* DNA. It was also found that hydrolysates of whole cells gave a good estimate of dimer formation in *Paramecium* DNA. Fig. 2 shows representative two-dimensional chromatograms of the dimer region of hydrolysates of extracted *Paramecium* DNA, whole paramecia, and, for comparison, extracted *E. coli* DNA. The tendency of *Paramecium* to incorporate radioactivity from ^{3}H-thymidine into materials other than DNA (Berger and Kimball, 1964) made chromatographic resolution poor in some experiments. In such groups of animals the level of nonspecific incorporation was so high that the entire experiment was discarded.

The photoproducts measured were identified as pyrimidine dimers by their chromatographic mobility (Smith, 1963) and by the kinetics of their splitting into thymine. The UV-induced photoproducts were isolated by paper chromatography from hydrolysates of *Paramecium* DNA. They were reirradiated at 2390 A and samples were taken after 0, 20,000, 40,000, and 60,000 erg mm^{-2}. These reirradiated samples were chromatographed, eluted, and assayed for radioactivity as a function of R_f in butanol:water followed by ammonium sulfate:isopropanol:sodium acetate. Virtually all the radioactivity was in either the dimer or the thymine region. The e^{-1} dose (40,000 erg mm^{-2}) for the splitting of dimers agreed within experimental error with the value obtained by Setlow and Carrier (1966) for the destruction of dimers formed from the irradiation of thymine in ice.

These three series of experiments show that almost all of the photoproducts measured were indeed cyclobutyl pyrimidine dimers from *Paramecium* DNA.

Production of Dimers by UV Irradiation

Labeled paramecia suspended in Dryl's solution were exposed to 0, 1000, 2000, 3000, 4000, 5000, 6000, 8000, or 10,000 erg mm^{-2} of 2537 A radiation. The animals were frozen immediately after irradiation, and later were assayed for radioactivity in the thymine and thymine-containing dimer regions. Fig. 3 shows the results of three typical experiments to determine dimer production on exposure of whole paramecia to UV radiation. The fraction of the total radioactivity in the dimer region increased approximately linearly with exposure. The differences between experiments are appreciably greater than the errors in individual experiments. This is

[2] Unfractionated DNA contained 0.265% of its radioactivity in thymine-containing dimers. Of the label in DNA, 95% was from *Paramecium*. Therefore, the maximum activity that could be in *Paramecium* dimers is 0.265%/0.95 = 0.279%. Since we observed 0.269%, at least 0.269/0.279 or 96% of the dimers in whole animals are in *Paramecium* DNA.

probably due to differences in cytoplasmic shielding from one group of animals to another (see next paragraph).

From Fig. 3 it can be calculated that roughly 6×10^{-5} per cent radioactivity in dimers was formed per erg mm^{-2} of 2537 A exposure to whole paramecia. This number is low in comparison with the value 4×10^{-4} per cent per erg mm^{-2} for

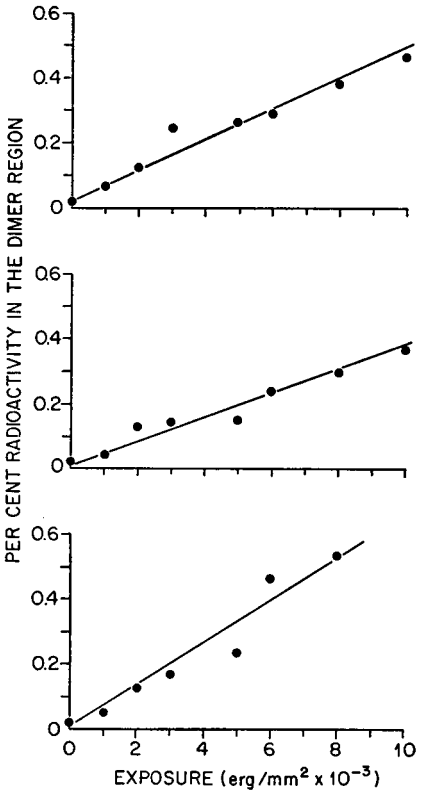

FIGURE 3 Per cent radioactivity in the dimer region of hydrolysates of three different groups of whole cells exposed to 2537 A radiation. The variation between groups is greater than that within a group.

dimer production in *E. coli* DNA at the same wavelength (Wulff, 1963). Three lines of evidence indicate that absorption of UV light by the cytoplasm is the major factor in reducing the effect of incident UV radiation on *Paramecium* DNA. First, microspectrophotometric measurements of single cells indicate that there is very little transmission of UV through the cytoplasm of whole paramecia (R. F. Kimball, personal communication). Second, analysis of extracted *Paramecium* DNA and whole cells, irradiated at the same time in Dryl's solution in separate Petri dishes, indicated that a UV exposure, 3000 erg mm^{-2}, produced 0.243% dimers in whole cells (of which at least 95% was from *Paramecium*, as mentioned previously) and 2.53% in extracted DNA. Third, very high UV exposures to whole paramecia pro-

duced as much as 10% dimers,[3] indicating that large numbers of dimers could be formed if the UV dose were great enough. These results indicate that cytoplasmic shielding rather than a UV-resistant configuration of DNA plays an important role in reducing the effect of UV on *Paramecium*.

The high cytoplasmic shielding, varying exponentially in magnitude with the size of the animal and the absorbance of the cytoplasm, can explain the big differences in the fractions of dimers observed among different groups of animals. Since intra-experimental variation was usually low (see Fig. 3), representative experiments give a more accurate representation of a result and its precision than data pooled from several groups of animals.

Fate of Dimers in the Dark

After exposure to 4000 erg mm^{-2} of 2537 A radiation, cells were suspended in non-radioactive medium containing *A. aerogenes* and grown in a light proof chamber. At several times after irradiation, a sample was centrifuged from the culture medium and killed by the addition of TCA. All samples were assayed for radioactivity in thymine and thymine-containing dimers. Fig. 4 shows the results of a typical experi-

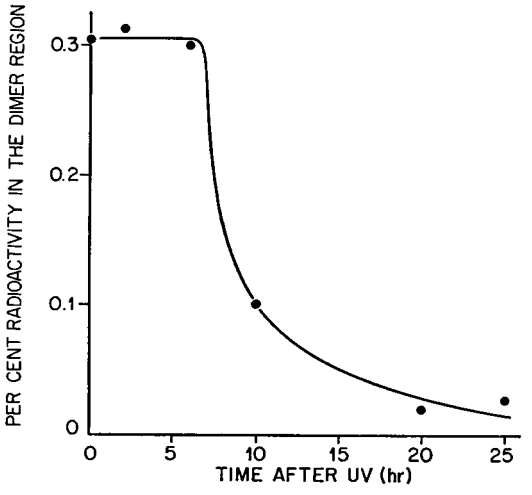

FIGURE 4 A typical experiment that shows the disappearance of dimers from the TCA-insoluble fraction of UV-irradiated paramecia incubated in the dark. The dimers do not appear in the TCA-soluble fraction.

ment designed to determine the fate of thymine-containing pyrimidine dimers in the DNA of paramecia incubated in the dark. No change in dimer content could be detected for about the first 6 hr after irradiation. After 6 hr the dimers disappeared from the TCA-insoluble fraction of the cells; they did not appear in the TCA-soluble fraction of the animals. Assays of radioactivity lost from the paramecia dur-

[3] On the basis of their buoyant densities in CsCl, the adenine-thymine content of *Paramecium* DNA is higher than that of *E. coli* DNA. Thus the number of thymine-thymine sequences would be higher and the number of dimers in *Paramecium* DNA would be greater than the value of 6.5% observed by Wulff (1963) for *E. coli* DNA.

ing incubation showed that about 0.5% of the total radioactivity was released from the cells. This is sufficient to account for the disappearance of dimers from *Paramecium* DNA; however, it indicates that net massive DNA breakdown did not occur within the 24-hr span of the experiment. *Micrococcus radiodurans* (Boling and Setlow, 1966) and *Bacillus subtilis* (Shuster, 1967), incubated in the dark after irradiation, lose excised dimer-containing oligonucleotides to the incubation medium. We have not examined the medium for the dimers lost from paramecia.

Two possible explanations for the lag before dimer excision are: (1) the cells were sufficiently damaged by the radiation and other procedures so that no excision occurred until the cells had overcome the effects of treatment; (2) the dimers were excised in pieces large enough to be precipitated by TCA, and only after intracellular nuclease action were the fragments too small to be found in the TCA-insoluble fraction. These possibilities were tested by fractionation (on Sephadex G-100) of sonicates of cells (1) killed immediately after irradiation, or (2) killed 6 hr after irradiation. No significant difference was detected in the dimer content of large molecular weight components of the zero time and 6-hr samples. These data imply

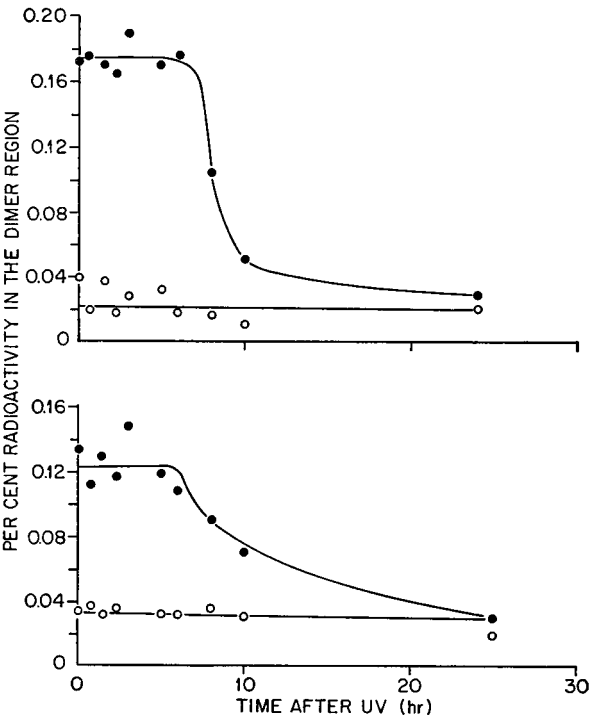

FIGURE 5 Two typical experiments that show the loss of dimers from UV-irradiated cells incubated in the dark. Samples (solid circles) were killed after various incubation times with no further treatment. Other samples (open circles) were exposed to 4×10^5 erg mm^{-2} of photoreactivating light after various times of incubation in the dark. In these experiments 0.02–0.03% of the radioactivity in the thymine of unirradiated cells was in the dimer region.

that there was no excision in large, TCA-precipitable oligonucleotides. Further, results of single-cell isolates indicated that many of the cells died between 79 and 96 hr (R. F. Kimball, personal communication). These two lines of evidence support the first alternative: that the cells were injured by the various treatments and the excision mechanism did not function for about 6 hr.

A lag in dimer removal has not been observed in bacterial. For example, *E. coli* 15 T^- excised dimers produced by exposure to 400 erg mm^{-2} of 2650 A radiation without a lag, although at a lower rate than those produced at 100 or 200 erg mm^{-2}.[4]

We have shown elsewhere (Sutherland et al., 1967) that dimers in UV-irradiated paramecia can be split by exposure to photoreactivating light (PRL). The exposure 3×10^5 erg mm^{-2} of 3000–4000 A radiation reduced the level of radioactivity in the dimer region to that measured in unirradiated controls. In the current studies, UV-irradiated cells were incubated in the dark, then exposed to 4×10^5 erg mm^{-2} of PRL. Fig. 5 shows that PRL destroys dimers equally well at all times after UV irradiation. Since the number of dimers in the paramecia decreases (after 6 hr), the number of dimers in the animals that are destroyed by PRL decreases with time, and thus there is less difference between photoreactivated and nonphotoreactivated samples. In this special sense, there is "loss of photoreactivability." The contrast between the extended period of photoreactivability of dimers and the rapid loss of the ability to photoreactivate mutational damage (R. F. Kimball, unpublished data) may be due to the more than 3-fold greater dose used in the present dimer experiments.

Quantitative comparison between the present dimer experiments and mutation experiments (Kimball and Gaither, 1951; Kimball, unpublished data) is not possible, since the experimental conditions were not the same. For example, the conditions of the mutation experiments were: a small number of cells in the same stage of the cell cycle, very gentle handling of the cells, and irradiation in a highly UV-absorbing medium containing bacteria. In contrast, in the present dimer studies the conditions were: mass cultures in mixed stages of the cell cycle, fairly rough handling (seven centrifugation procedures and four changes of medium), and irradiation in a salt solution transparent to UV. Also, UV irradiation studies on paramecia show large interexperimental variability, probably because of differences in cytoplasmic shielding. If mutation experiments were carried out under the conditions used in the dimer work, it would be possible to make quantitative comparisons of inhibition and kinetics of biological repair and of dimer excision during various treatments. However, qualitative comparisons may be made for the existing data as follows: First, we have shown that dimers are produced in the same dose range as mutations (Kimball and Gaither, 1951). Second, there is evidence for the repair of UV-induced permutational damage in *Paramecium* (see review by Kimball, 1966). It is possible that the disappearance of dimers seen in the current studies is an excision step lead-

[4] R. B. Setlow and W. L. Carrier. Unpublished data cited in R. B. Setlow, 1964.

ing to the repair of mutational lesions. Third, the effect of photoreactivating light on both dimers and mutations decreases with time after irradiation. Finally, mutational lesions in *Paramecium* are photoreactivable (Kimball and Gaither, 1951), and dimers in *Paramecium* DNA are photoreactivable in vivo (Sutherland et al., 1967). Moreover, the doses of photoreactivating light required for photoreactivation of dimers (Sutherland et al., 1967) and of mutations (R. F. Kimball, unpublished data) are of the same order of magnitude. These comparisons and the assumption that photoreactivation of mutation is direct rather than indirect (Jagger and Stafford, 1965) suggest that pyrimidine dimers in *Paramecium* DNA may indeed be an important fraction of the UV-induced lesions leading to mutation.

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