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Psoralen-DNA Photoreaction: Controlled Production of Mono- and Diadducts with Nanosecond Ultraviolet Laser Pulses

Abstract. *Samples of DNA which contain adducts of a new psoralen derivative, but no cross-links, have been prepared by irradiating mixtures of DNA and the derivative with single, 15-nanosecond pulses of laser light. Succeeding pulses introduce cross-links. The ability to rapidly and selectively create monoadducts and cross-links may allow the use of psoralens as probes of dynamic processes in living cells.*

Psoralens are linear tricyclic heterocycles which can intercalate between the base pairs of double-stranded nucleic acids, forming covalent adducts to pyrimidine bases upon absorption of long-wave ultraviolet light. If there is a second pyrimidine adjacent to a psoralen-pyrimidine monoadduct and on the opposite strand, absorption of a second photon can lead to formation of a diadduct which functions as an interstrand cross-link. This property has made psoralens useful probes of the secondary structure of DNA, either in isolated form (*1*, *2*) or in biological structures such as chromatin (*3*).

It is advantageous to be able to selectively create monoadducts, as distinguished from cross-links, for certain biological investigations. For example, monoaddition to DNA in vivo may well be a mutagenic event, but attempts to assay for mutagenicity of trimethylpsoralen have been complicated by the high lethality of the cross-linking which normally accompanies monoadduct formation. Moreover, rapid monoaddition of psoralens to replicating or transcribing DNA could label regions of the DNA which are accessible to intercalation and photoreaction without interfering with strand separation, and perhaps without blocking the action of polymerases or other functional entities. Labeled regions could be located later by purifying the DNA, exhaustively irradiating it to convert as many adducts as possible to cross-links, and then locating the cross-links by electron microscopy of denatured spreads of the DNA.

Two approaches are possible for the exclusive production of monoadducts. One is to use a very low light dosage during the photoreaction, so that it becomes highly unlikely that any single psoralen molecule would receive the two successive photons apparently necessary for cross-link formation. The difficulty with this procedure is that the concentration of monoadducts one can obtain is quite low. The other approach is to present the light in such a short pulse that there is no time for a second photon to be absorbed. It is believed that an excited triplet state is an essential intermediate for the photoreaction (*4*); thus, if the rate-limiting step is reaction from the triplet state, the light pulse need only be short compared to the triplet lifetime. We have utilized a frequency-doubled (347 nm), *Q*-switched ruby laser having a pulse length of approximately 15 nsec to produce exclusively monoadducts in this manner. A potential bonus of using such short irradiation times is the possible use of psoralens as fast probes of molecular dy-

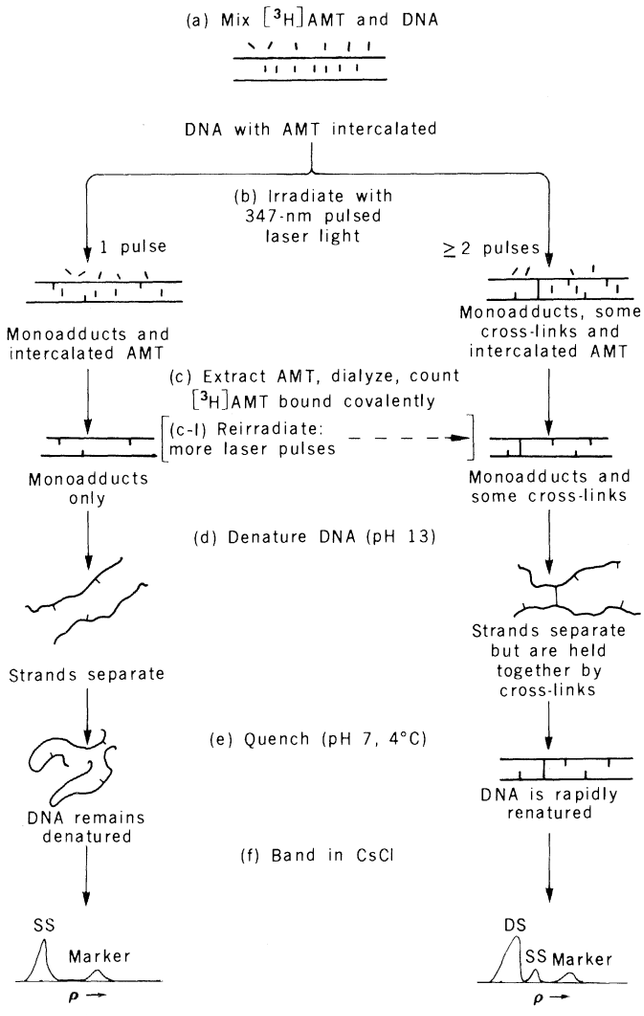

Fig. 1. Experimental scheme. (a) The T4 DNA was isolated by the procedure of Bautz and Dunn (*7*); AMT had a specific tritium activity of 1.42×10^5 count/min per microgram. Typically, we irradiated a 0.5- or 1.0-ml cylindrical quartz cell (path length, 1 or 2 cm) containing 50 μg of DNA and 5.6 μg of AMT per milliliter of a buffer solution containing 0.01*M* sodium phosphate (*p*H 7.0) and 1 m*M* EDTA (b). The beam almost completely filled the cell, although there were regions of greater and lesser intensity in the cross section. To average out these effects, the sample was mixed between successive pulses whenever more than one pulse was applied. The pulse energy was 50 to 100 mjoule. Free AMT was removed by making the sample 1*M* in NaCl and extracting two or three times with phenol (saturated with the above buffer), followed by dialysis through Spectrapore membranes against phosphate-EDTA buffer 1*M* in NaCl until a control sample which was unirradiated showed no residual tritium (c). The DNA concentration was then determined by ultraviolet absorbance at 260 nm, and 0.5 to 1.0 μg of DNA was mixed with 1 μg of *Micrococcus lysodeikticus* DNA (used as a buoyant density marker, ρ_{native} = 1.733 g/cm^3). The mixture was brought to *p*H 13 with NaOH (d), allowed to stand for 10 minutes at room temperature (22°C), and then cooled to 4°C, whereupon it was neutralized by addition of 0.2*M* NaH_2PO_4 (e). A 0.5-ml portion of CsCl was then added to give a final solution density of 1.720 g/cm^3. These samples were spun at 42,000 rev/min in a Beckman model E analytical ultracentrifuge equipped with an ultraviolet scanner (f), using double-sector titanium centerpieces. Abbreviations: *SS*, single-stranded; *DS*, double-stranded.

namics in biological systems, as in the aforementioned application to DNA replication.

For these experiments we used a new tritiated derivative of psoralen, 4′-[^{3}H]aminomethyl-4, 5′, 8-trimethylpsoralen (AMT), recently synthesized by Isaacs *et al.* (*5*) in our laboratory. This new compound possesses superior characteristics of water solubility and binding to DNA compared to commonly used psoralen derivatives.

The experimental design is shown in Fig. 1. DNA and AMT in the ratio of one AMT molecule to four base pairs were mixed in the dark (step a) and subjected to one or more pulses of laser light (step b). The AMT not covalently bound to DNA was then removed by phenol extraction and dialysis (step c). Total covalent binding (monoadducts plus cross-links) was determined by use of the tritium label. To distinguish DNA molecules which had no cross-links from those which had at least one cross-link, the samples were denatured by alkali and then quickly neutralized at 4°C (steps d and e). This "quenching" to *p*H 7 did not allow uncross-linked molecules conditions adequate for the renaturation of DNA, and they remained single-stranded. On the other hand, molecules which were held together by a cross-link would immediately "zipper up" to the native form on quenching.

The single-stranded and the renatured DNA were separated by equilibrium ultracentrifugation in a CsCl density gradient: denatured DNA bands at a higher buoyant density than native DNA (step f). This assay requires DNA of homogeneous length for the unambiguous counting of cross-links and of rather high molecular weight to keep the density bands sharp and well resolved. We used DNA isolated from the *Escherichia coli* phages T5, T7, and T4. All three showed the same effect, but T4, having the highest molecular weight (113×10^6), gave the clearest results.

The results for T4 DNA are shown in Fig. 2. From 1.7 to 2.8 AMT molecules were covalently added per 1000 base pairs with each pulse, the variations being probably due to irregularities in the laser beam. Samples which had received one pulse of laser light banded as denatured DNA, at the same density as controls which had seen either no ultraviolet light or no AMT. Two pulses of laser light produced a dramatic change: 67 percent of the DNA now had one or more cross-links and banded as double-stranded material. With five pulses, essentially all the DNA contained cross-links. [Apparently the DNA had some

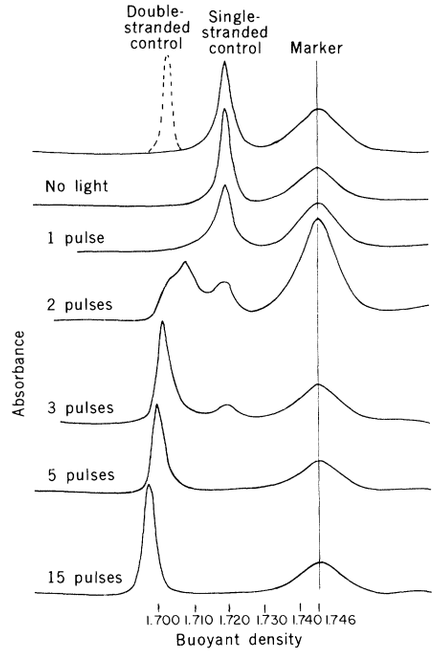

Fig. 2. Results of CsCl banding, where multiple-pulse samples were irradiated without removing the AMT after the first pulse. The top line shows the banding positions of T4 DNA which was not exposed to AMT or ultraviolet light. Succeeding traces show DNA treated with AMT and light exposures as indicated. The marker DNA peaks are broader here than in Fig. 3 because the marker in this case was heat-denatured, causing some breakage of the molecules, while in Fig. 3 it was alkali-denatured.

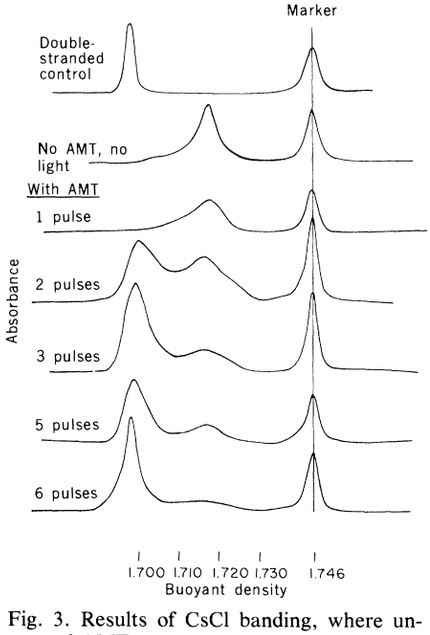

Fig. 3. Results of CsCl banding, where unreacted AMT was removed by extraction and dialysis after exposure to one laser pulse. This pure monoadduct sample was then further irradiated to produce the samples receiving two through six pulses, as in step c-1 of Fig. 1. The more leisurely decline of the denatured peak and the nearly constant buoyant density of the native peak are both due to the lack of new monoadduct being created after the first pulse (see text).

single-strand nicks, leaving the renatured material with single-stranded tails when there were few cross-links to hold the separate pieces of DNA together during the denaturation step. This would account for the heavier-than-normal density of most of the renatured peak in the two-pulse trace, an effect not seen in a later experiment (see Fig. 3).] Assuming that the cross-links were distributed according to a Poisson process, the proportions of native and denatured DNA for the two-pulse sample indicate that, on the average, each molecule contained 1.1 cross-links (*6*).

Does this result really show that no cross-links occur with a single pulse, or is there simply not enough energy in one pulse for the effect to be seen? To answer this we must consider the kinetics for monoadduct and cross-link formation, given the requirement that cross-links can be formed only by subsequent photoreaction of monoadducts. The concentration of monoadduct, A, is presumably proportional to the amount of energy absorbed per unit concentration, E

$$A = k_1 CE$$

where k_1 is a rate constant and C is the concentration of intercalated AMT. For the low extent of photoreaction in these experiments, we can consider C to be a constant and neglect the amount of A lost due to cross-link formation. The rate of cross-link formation is then

$$\frac{dX}{dt} = k_2 A \left(\frac{dE}{dt} \right) = k_1 k_2 CE \left(\frac{dE}{dt} \right)$$

where k_2 is a second rate constant. So the concentration of cross-links goes as the square of the energy absorbed

$$X = k_1 k_2 C \frac{E^2}{2}$$

Thus, if the pulse duration is unimportant and two pulses yielded 1.1 cross-links per molecule, one pulse, having half the energy, should yield (1.1)/4 = 0.275 cross-link per molecule. This corresponds to 76 percent of the molecules remaining single-stranded and 24 percent having a cross-link. This is clearly not the pattern we see with one pulse; we conclude that no cross-link is created with one pulse.

To make sure that the AMT remaining intercalated but unreacted after one pulse played no part in forming the cross-links observed with two pulses, the experiment was repeated with one change: after the DNA-AMT sample received one pulse of laser light, the unreacted AMT was removed. This sample was then further irradiated in the laser

beam (step c-1 of Fig. 1), and portions were removed after every additional pulse for analysis in the ultracentrifuge. Very similar results were obtained (Fig. 3), except that there remained a small residual denatured peak, apparently due to a small volume of solution which was outside the laser beam during the first pulse, and which therefore received few or no monoadducts.

A decrease in buoyant density of the double-stranded DNA peak with increasing light exposure is apparent in Fig. 2. This is due to the increasing number of adducts in the DNA. The effect is linear, and corresponds to a decrease in density of 0.271 g/cm^3 per AMT/base pair. This compares with a value of 0.182 reported for trimethylpsoralen (*2*). No comparable shift occurred in the series in which the unreacted AMT was removed after one pulse (see Fig. 3).

The psoralen-DNA system may be further characterized by measuring the quantum yields of the first and second photoreactions, and it should be possible to measure the lifetime of the putative triplet intermediate by using two laser pulses separated by an adjustable time delay, to determine the time interval required for the appearance of cross-links. We feel that the ability to separate the two photoreactions in psoralen cross-linking which we have demonstrated here will be especially valuable in applications to living systems.

BRIAN H. JOHNSTON
MARK A. JOHNSON
C. BRADLEY MOORE
JOHN E. HEARST

Department of Chemistry, University of California, Berkeley 94720

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Reye's Syndrome: Patient Serum Alters Mitochondrial Function and Morphology in vitro

Abstract. *A direct relationship between a putative Reye's syndrome "serum factor" and generalized mitochondrial damage has been demonstrated in vitro. The clinical features of Reye's syndrome may be secondary to disrupted mitochondrial structure and a consequent impairment of energy-linked functions involving many organs.*

Reye's syndrome (RS) is a grave, enigmatic illness of childhood (*1*), often preceded by an otherwise uneventful presumably viral illness (*2*). The course of RS has been subdivided (*3*) into five clinical stages (I to V), beginning with intractable vomiting and lethargy, followed by progressive rapid deterioration of neurological function. Of 349 cases reported in a 1974 epidemic in the United States, only 207 survived (*4*).

Patients with RS have high concentrations of serum ammonia, high serum transaminase activity, and prolonged prothrombin times, which have suggested a hepatic basis for the concomitant encephalopathy (*5*). Morphological studies of liver biopsies from RS patients by Partin *et al.* (*6*) revealed alterations in mitochondrial structure well correlated with the severity of the illness, but the etiology of the mitochondrial damage remained unexplained. The suggestion that an accumulating metabolite or an exogenous toxin in the serum may be a causative factor has been made repeatedly. Using a modification of the *Limulus* assay, Cooperstock *et al.* (*7*) were able to demonstrate endotoxin-like activity in the serum of RS patients, but the relation (if any) of this activity to the cause of the illness was obscure.

In searching for an etiological "serum factor," it seemed logical to test the effect of RS serum directly on mitochondria in vitro. With this approach, it was possible to demonstrate an effect of RS serum on both the respiratory function and the ultrastructure of isolated rat liver mitochondria. These results may provide an advance toward understanding this puzzling illness. Furthermore, the effects of RS serum on animal mitochondria in vitro may be potentially useful in combination with other clinical indicators for diagnosis and evaluation of treatment of RS patients.

The 24 control and RS serums used in this study were obtained from three widely separated geographical locations (*8*) and had been stored at −20° or −70°C for periods varying up to 2 years. All the serums were lyophilized and reconstituted to one-fifth the original volume in distilled water. Microliter portions of the concentrated serums were then tested for an effect on mitochondria in vitro. Nine of the 24 samples were tested without prior knowledge of the clinical state of the donors.

Respiratory activity of isolated rat liver mitochondria (*9*) (1 mg of protein in a 1-ml assay) was assessed polarographically at 30°C by monitoring O_2 consumption with a Clark electrode (Yellow Springs Instruments). The assay medium consisted of 0.225*M* sucrose, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* $MgCl_2$, 15 m*M* KCl, 15 m*M* NaH_2PO_4 buffer, and 50 m*M* tris-HCl, *p*H 7.5. The substrate used was 10 m*M* glutamate. Several parameters of mitochondrial respiration (*10*) were followed, including state 3 and state 4 oxidation rates, ADP/O ratio (ADP, adenosine diphosphate), and respiratory control ratio (RCR), the latter being the most important criterion of functional integrity. The course of a typical assay in the absence of added serum is shown in assay a in Fig. 1. Normal human serum had little effect on respiratory activity (assay b in Fig. 1), but serum from a deeply comatose RS patient (clinical stage IV) doubled the state 4 oxidation rate (assay c in Fig. 1) and consequently reduced the RCR. Changes in other parameters were not as readily apparent although the state 3 oxidation rate usually was increased somewhat in the presence of RS serum.

The magnitude of the effect of RS serum on RCR was dependent on the amount of serum added to the assay. Lower RCR's in the presence of RS serum were not restored with 3 percent fat-free bovine serum albumin in the assay, which suggests that the effect was not nominally due to free fatty acids (*11*). The effect of added RS serum could not be reproduced by adding ammonia (50 μl of 5 m*M* NH_4Cl) or salt (10 μl of 5× concentrated 0.9 percent NaCl) solutions to the assay. Nor was the effect diminished by prior deamination of the serum.

Ten microliters of concentrated serum from comatose RS patients (clinical stage III-IV) was sufficient to decrease