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ISOLATION OF ULTRAVIOLET LIGHT-INDUCED
PYRIMIDINE DIMERS FROM ENZYMATIC
HYDROLYSATES OF DNA¹

Running title: Pyrimidine Dimers from DNA

by

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INTRODUCTION

The formation of cyclobutane-type pyrimidine dimers in DNA has been shown to be an important cause of the lethal and mutagenic effects of ultraviolet light (1). The usual method for the isolation of these photoproducts is acid hydrolysis of thymine labelled-DNA followed by chromatographic separation. However, not all cells will efficiently incorporate exogenous thymidine or thymine. For example, all pyrimidine precursors are poorly incorporated into the DNA of the green algae Chlamydomonas (2) and Euglena (3). However, the DNA of these organisms can be labelled highly with ^{32}P -phosphate. An enzymatic procedure that yielded cyclobutane-type pyrimidine dimers of the form $\text{Up}\overset{\wedge}{\text{U}}^2$ as well as other photoproducts from irradiated tobacco mosaic virus-RNA has been reported (4). This paper presents an enzymatic method for the isolation of pyrimidine dimers of the form $\text{Tp}\overset{\wedge}{\text{T}}$ from irradiated DNA, thus making it possible to use ^{32}P -labelled DNA for the study of ultraviolet light-induced pyrimidine dimers.

METHODS

Materials

✓ E. coli alkaline phosphatase (21U/mg), snake venom phosphodiesterase, and spleen phosphodiesterase were purchased from Worthington Biochemical Corporation, and units are as defined by Worthington.

Thymidine-methyl-³H (6.7 c/mMole) and ³²P-phosphoric acid were obtained from New England Nuclear Corporation.

TpT was purchased from Sigma. Thymine dimer was prepared by irradiating a frozen solution of thymine (5). [△]TpT was prepared by the irradiation of a solution of TpT (20 µg/ml) with a germicidal lamp until about 30% of the absorbance at 260 nm disappeared.

Preparation of DNA

For tritium labelled DNA, E. coli Hfr OR11 (T⁻Pyr⁻) was grown aerobically for 8 hrs at 37° in minimal media (6) supplemented with sodium citrate (0.5 g/l), uracil (10 mg/l), cytosine (10 mg/l), and thymidine 2 mg/l. Thymidine-methyl-³H (6.7 c/mMole) (New England Nuclear) was present (250 µc/l).

✓ For ³²P-labelled DNA, E. coli B was grown aerobically for 8 hrs at 37° C in minimal media supplemented with sodium citrate except that the orthophosphate concentration was reduced to 1x10⁻⁴ M and 20 µc/ml of carrier free ³²P added.

The DNA was isolated by a modification of the method of Muria (7). A 6 hr incubation with pronase (250 µg/ml, final concentration) was included following the incubation with

pancreatic RNase and RNase T₁, followed by a phenol deproteinization step.

The concentration of DNA was estimated from the absorbance at 260 nm by assuming an absorbance of 1.0 corresponds to 45 µg/ml of DNA.

The specific activity of the ³H-labelled DNA was 1.75×10^4 cpm/µg. The specific activity of the ³²P-labelled DNA was 5.3×10^5 cpm/µg. Radioactivity was determined with a Packard Tri-carb, Model 3003.

Irradiation of DNA

DNA at concentrations ranging from 18 µg/ml to 60 µg/ml in 0.015 M NaCl was irradiated with a germicidal lamp (Mineralight, Model R-51, principal emission at 253.7 nm) while the solution was constantly stirred by rotation.

Enzymatic Hydrolysis

The DNA was denatured by heating for 10 min at 100° and cooled in ice prior to hydrolysis. This step deaminates any cytosine containing pyrimidine dimer to a uracil analog (8). The enzymatic hydrolysis mixture contained in 0.4 ml: 30 µmoles of Tris HCl, pH 8.8; 0.5 µmole MgSO₄; 25 µg of snake venom phosphodiesterase; 50 µg of bacterial alkaline phosphatase. After 8 hrs incubation at 37°, the mixture was either chromatographed on Bio-Gel P-2 or further hydrolyzed with spleen phosphodiesterase. In the latter case, the pH was adjusted to 5.8 with 1 N acetic acid and 0.26 unit of spleen phosphodiesterase added. The mixture was incubated for 10-12 hrs at 37°.

Isolation of Pyrimidine dimers from an Acid Hydrolysate of DNA

DNA was hydrolyzed in 35% HClO₄ and the dimers isolated by ion-exchange chromatography according to the method of Sekiguchi *et. al.* (9).

Bio-Gel P-2 chromatography

Bio-Gel P-2, 100-200 mesh (Bio-Rad Laboratories), was allowed to swell in 10^{-3} M Tris HCl, pH 7.6 for at least 3 hrs. The gel was packed by gravity into a column 1.2x57 cm and equilibrated with 10^{-3} M Tris-HCl, pH 7.6. Nonradioactive thymidine was added as a standard to the sample to be chromatographed. The column was eluted with 10^{-3} M Tris-HCl, pH 7.6. Fractions of about 2 ml were collected.

Paper chromatography

System A: Descending chromatography, for 14 hrs with 76% ethanol as the solvent using Whatman #1 previously dipped in 10% saturated (NH₄)₂SO₄, pH 7.0, and dried.

System B: Descending chromatography using Whatman #1 (water washed) and n-butanol: acetic acid: H₂O, 80:12:30 as solvent for 16 hrs.

Radioactive peaks were located by cutting the paper into 1x4 cm strips, placing them into scintillation vials with 0.5 ml of H₂O, and adding 10 ml of scintillation solution. The scintillation solution contained 4 g of Omnifluor (New England Nuclear Corp.) per liter of toluene and 80 ml of Bio-Solv BBS-3 (Beckman).

Isolation of pyrimidine Dimers from an Enzyme Hydrolysate of DNA

Irradiated DNA was hydrolyzed with snake venom phosphodiesterase and phosphatase followed by spleen phosphodiesterase as described above. The enzyme hydrolysate was diluted to 4 ml with H₂O and transferred to Dowex 1-x8, formate, columns, 1x11 cm. The columns were washed with 110 ml of 0.02 N HCOOH (thymidine fraction), 50 ml of 0.2 N HCOOH, 80 ml of 4 N HCOOH (dimer fraction), and 100 ml of 4 N HCOOH-0.1 N NH₄COOH (P_i fraction). The dimer fraction was evaporated to dryness and dissolved in 1.0 ml of water. Aliquots were taken for radioactivity determination and characterization.

RESULTS

Most of our experiments were done with ³H-thymidine labelled DNA in order to facilitate a comparison of acid and enzyme hydrolysis. Incubation of unirradiated DNA with snake venom phosphodiesterase and alkaline phosphatase results in the complete hydrolysis to nucleosides as evidenced by only a single peak corresponding to thymidine in Fig. 1, panel A. Digestion of irradiated DNA results in larger undigested fragments (peak I) as shown in Fig. 1, panel B. This peak presumably consists of trinucleoside diphosphates of the type XpT[^]pT, as observed by Setlow, Carrier, and Bollum (10). If these undigested fragments are incubated with spleen phosphodiesterase and then rechromatographed on the Bio-Gel column, the result is the pattern shown in Fig. 1, panel C. Two peaks are evident, peak III is thymidine as verified by paper chromatography in system A,

and most of peak II co-chromatographs with $\widehat{\text{TpT}}$ as shown in Fig. 2. The identity of the smaller radioactivity peaks is not known. Thus, a sequential treatment of irradiated DNA with snake venom phosphodiesterase and alkaline phosphatase followed by spleen phosphodiesterase results in the complete hydrolysis to nucleosides and dimers of the type $\widehat{\text{TpT}}$.

Since orthophosphate elutes from the Bio-Gel P-2 column in the same region as $\widehat{\text{TpT}}$, it became necessary to devise another separation technique that would be feasible for the isolation of dimers from ^{32}P -labelled DNA. Fig 3 shows the resolution of thymidine, dimers of the form $\widehat{\text{TpT}}$ and orthophosphate by chromatography on Dowex 1. To further substantiate the identification of the material eluted with 4 N HCOOH (dimer fraction), we hydrolyzed the pooled fraction in 97% HCOOH at 175° for 30 min and chromatographed the product on Whatman #1 using system B (See methods). As shown in Fig. 4, the only significant radioactive peaks correspond to thymine dimer and thymine-uracil dimer. The assignment of the smaller of the two peaks to thymine-uracil dimer is based on the R_f as reported in the literature (5).

The next important point is the quantitative comparison between the isolation of pyrimidine dimers from an acid hydrolysate and an enzymic hydrolysate. Fig 5 shows that the quantitative agreement is quite good. Somewhat more radioactivity is found in the dimer fraction isolated from enzymic digests than from acid hydrolysates after large doses of irradiation.

If one wishes to isolate pyrimidine dimers from the DNA of cells labelled with ^{32}P , one must be certain of eliminating all RNA because ultraviolet light also induces the formation of uracil containing dimers in RNA (4). Hydrolysis with alkali followed by precipitation of the DNA with acid would appear to be the most efficient route for the removal of RNA. Table 1 shows that treatment of irradiated DNA in 0.3 N KOH for 5 hrs at 37° did not destroy the dimers.

Finally, Table 2 shows the application of this method for the isolation of pyrimidine dimers from ^{32}P -labelled DNA. The percent ^{32}P found in the dimer fraction is approximately what one would predict based on the dose used. This can be estimated in the following way. At a dose of 2.9×10^4 ergs/mm² we find 7.8% of the thymine converted to dimers (e.g. Fig 5). Since E. coli DNA is composed of 25% thymine and since the thymine dimer as isolated contains two thymines to one phosphorus, we would calculate $7.8/8$ or about 1% of the phosphorus would be associated with dimer. However, some of the dimers are of the form $\widehat{\text{TpU}}$ and probably some $\widehat{\text{UpU}}$. Thus, we would obtain a number somewhat higher than 1% depending on the exact proportion of uracil containing dimers.

DISCUSSION

The stepwise hydrolysis of irradiated DNA with snake venom phosphodiesterase and alkaline phosphatase followed by spleen phosphodiesterase and the subsequent isolation of pyrimidine

dimers by ion-exchange chromatography has been shown to yield quantitatively the same results as isolation of total pyrimidine dimers from an acid hydrolysate. The isolation of pyrimidine dimers from enzymatic digests described offers several advantages over the isolation from acid hydrolysates. First it affords a means of studying these photoproducts in organisms that do not efficiently incorporate exogenous thymine or thymidine into DNA but will use ^{32}P -orthophosphate. Secondly, the dimers are isolated as the structures TpT , TpU and UpU . Since the latter type dimer is not detectable in DNA labelled only in the thymine base, the present method presents a means of evaluating all dimers.

It should be noted that if one wished to further fractionate the pyrimidine dimers as obtained in the 4N HCOOH eluate from the ion-exchange column, one must use a method of concentration such as freeze-drying that would minimize hydrolysis of the glycosidic linkages in the dimer.

A third advantage of this method of hydrolysis is it presents the opportunity to look for labile photoproducts that are destroyed during the drastic acid hydrolysis that is usually employed.

At high doses of irradiation we consistently obtain somewhat more radioactivity in the dimer fraction isolated from enzyme hydrolysates than acid hydrolysates (Fig 5). A possible explanation for this is that an acid and alkali labile photoproduct(s) is formed in significant amounts at high doses of irradiation which is isolated with the dimer fraction from enzyme hydrolysates. Consistent with this hypothesis is the finding that treatment of the irradiated DNA with alkali prior to enzyme hydrolysis, which would

destroy the labile photoproduct, results in a complete agreement in the amount of dimer isolated by the two methods (Table 1). However, we have no direct evidence for such a labile photoproduct.

Finally, a comment on the apparent stability of the pyrimidine dimers to alkali. As long as the conditions are not too drastic, the ring system undergoes a reversible opening in alkali. For example, Balckburn and Davies (11) showed that thymine dimer can be treated with 10N NaOH to give the disodium salt of a bis-ureidocarboxylic acid. This compound reverts to the dimer upon solution in acid. A similar reversible reaction was observed with $\hat{\text{UpU}}$ by Brown, Freeman, and Johns (12). On the other hand, under very drastic conditions (2N KOH, 100°) thymine dimer is destroyed with a half life of 100 min (13). Hence, we conclude that our conditions (0.3N KOH, 37° 5 hrs) is not drastic enough to lead to significant loss of dimers.

SUMMARY

A method has been developed for the isolation of ultraviolet light - induced pyrimidine dimers from enzymatic hydrolysates of DNA. The dimers are isolated in the form $\hat{\text{TpT}}$, thus making it possible to use ^{32}P -labelled DNA for the study of ultraviolet light-induced pyrimidine dimers.

ACKNOWLEDGMENT

We wish to acknowledge the very competent technical assistance of Mrs. Soojae M. Zeon. We also wish to thank Dr. James N. Adams for the E. coli strains. RBS is a recipient of a NDEA pre-doctoral fellowship.

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LEGENDS TO FIGURES

FIGURE 1: Bio-Gel P-2 chromatography of DNA hydrolysates.

x-----x cpm per 0.2 ml; o-----o A_{267} .

Panel A: 6 μ g of unirradiated ^3H -DNA (100,000 cpm) was digested for 8 hrs with snake venom phosphodiesterase and alkaline phosphatase.

Panel B: 6 μ g of ^3H -DNA (100,000 cpm) that had been irradiated with ultraviolet light (2.9×10^4 ergs/mm²) was digested for 8 hrs with snake venom phosphodiesterase and alkaline phosphatase.

Panel C: Peak I from panel B was evaporated to dryness. 20 μ moles of ammonium succinate, pH 5.5 and 0.4 unit of spleen phosphodiesterase was added in a final volume of 0.4 ml. Incubation was carried out for 12 hrs at 37°.

FIGURE 2: Paper chromatography of Peak II from Bio-Gel.

Peak II from the Bio-Gel chromatography (Figure 1, panel C) was chromatographed for 14 hrs using system A. Irradiated TpT and thymidine were added as standards.

FIGURE 3: Isolation of pyrimidine dimers from an enzyme hydrolysate of irradiated DNA using Dowex 1 (formate).

o-----o Tritium cpm, unirradiated DNA hydrolysate;

o-----o Tritium cpm, irradiated DNA hydrolysate;

x-----x ^{32}P cpm.

^3H -DNA samples were subjected to hydrolysis with snake venom

phosphodiesterase, alkaline phosphatase, and spleen phosphodiesterase as described under Methods. 870,000 cpm of ^{32}P -orthophosphate was added to the unirradiated DNA hydrolysate as a marker. The irradiated DNA was subjected to a dose of 2.9×10^4 ergs/mm². The arrows indicate changes in eluting solvents. A: 0.02 N HCOOH; B: 0.2 N HCOOH; C: 4 N HCOOH; D: 4 N HCOOH - 0.1 M NH₄COOH.

FIGURE 4: Paper chromatography of 4 N HCOOH eluate from Dowex 1 column after acid hydrolysis.

The 4 N HCOOH eluate from Figure 3 was evaporated to dryness and hydrolyzed with 0.5 ml 97% HCOOH for 30 min at 170°C. The hydrolysate was chromatographed using system B for 16 hrs. Thymine dimer was added as standard.

FIGURE 5: Isolation of pyrimidine dimers from acid and enzyme hydrolysates of irradiated ^3H -DNA. o, cpm in dimer fraction isolated from acid hydrolysates; o cpm in dimer fraction isolated from enzyme hydrolysates. ^3H -DNA was irradiated at a concentration of 42 µg/ml, containing 750,000 cpm/ml, at a dose rate of 4.8×10^3 ergs/mm²/min. 0.1 ml aliquots were removed at appropriate times, denatured, and hydrolyzed with either acid or enzymes. Dimers were isolated as described in the Methods. Values are corrected for unirradiated DNA (265 cpm for enzyme hydrolysis, 144 cpm for acid hydrolysis).

TABLE 1

Effect of 0.3 N KOH on the Isolation of Pyrimidine
Dimers from Irradiated DNA

6 μ g of ^3H -DNA (100,000 cpm) was irradiated with 2.9×10^4 ergs/mm 2 of ultraviolet light. The base treated samples were incubated in 1 ml of 0.3 N KOH at 37° for 5 hrs. The DNA was precipitated by the addition of 0.1 ml of bovine serum albumin (10mg/ml) plus 1 ml of 10% trichloroacetic acid. After 10 min at 0°, the DNA was centrifuged, washed once with 1 ml cold absolute ether, and digested with enzymes as described in Methods. Values are corrected for unirradiated DNA.

Treatment	cpm in Dimer
1. Enzyme Hydrolysis	7087
	7307
	<hr/>
	Ave 7197
2. Acid Hydrolysis	6337
	5937
	<hr/>
	Ave 6137
3. KOH treated DNA, Enzyme Hydrolysis	5967
	6472
	<hr/>
	Ave 6219

TABLE 2

Isolation of Pyrimidine Dimers from ^{32}P -labelled DNA

0.9 μg of ^{32}P -DNA (400,000 cpm) was irradiated with 2.9×10^4 ergs/ mm^2 , digested with enzymes, and the pyrimidine dimers isolated by ion-exchange chromatography as described in the Methods.

	cpm in Dimer fraction	% phosphate as Dimer	Corrected for unirradiated
1. Unirradiated	698	0.17	
2. Irradiated	5091	1.27	1.1

FOOTNOTES

1. Supported by contract AT(11-1) 1793 of the Atomic Energy Commission.
2. $\overset{\wedge}{\text{UpU}}$ and $\overset{\wedge}{\text{TpT}}$ are the cyclobutane-type dimers of the corresponding parent compound.

100000

1000 94

1000 2A