EVIDENCE FOR THE PRESENCE OF CYTOSINE PHOTOHYDRATES IN UV IRRADIATED NUCLEIC ACIDS*

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Introduction

It is possible with the use of polymerase systems to demonstrate induced transitions in vitro (Ono et al, 1965; Phillips et al, 1965, 1966) which are similar to those predicted from genetic studies with phages (Drake, 1963; Tessman et al, 1966). For example, the effective reduction of the C_5-C_6 double bond of cytosine induced by ultraviolet irradiation (cytosine hydrate) of poly C and the addition of the elements of hydroxylamine and O-methylhydroxylamine at these same positions in such a homopolymer leads to the formation of a template which specifically directs the synthesis of poly GA as catalyzed by the RNA polymerase (Ono et al, 1965; Phillips et al, 1965, 1966). Similar transitions in "recognition" are also observed when the substrate CTP is catalyti-

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cally reduced to form 5,6-dihydroCTP (Grossman et al, 1966; Streeter et al, 1968).

Analogous modifications of uracil or thymine residues in polynucleotide templates brought about by ultraviolet light or direct $NaBH_4$ reduction lead to an irreversible block to transcription (Adman and Grossman, 1967). Moreover, reduction, hydration, or dimerization of UTP results in a loss of its ability to participate as a substrate by this enzyme (Grossman et al, 1966).

Although much of this evidence in vitro implies that the photohydrate of cytosine is the likely photoproduct responsible for the mutagenic effects of ultraviolet light, it has been virtually impossible to detect cytosine hydrates in irradiated DNA because of its extreme instability.

This communication will describe a sensitive and specific assay technique for cytosine hydrates; moreover, evidence will be provided that cytosine photohydrates constitute a significant proportion of the photoproduct population in irradiated DNA.

Experimental

Charcoal Assay for Cytosine Hydrates

Preparation of Norit Suspension - 150 grams of Norit A (Fisher) are suspended in 1 l of 6 N HCl, stirred and allowed to settle for 6 hours. The bright yellow supernatant fluid is removed by suction. An additional 4 l of 6 N HCl are added as before and the process repeated at least 3 times until the absorbance at 260 nm decreases to less than 0.05 absorbance units/ml. The acid washed charcoal is washed with 4 l batches of distilled water until the pH of the supernatant fluid reaches pH 4. The charcoal is resuspended in a

sufficient volume of distilled water so that a 20% (V/V) suspension is prepared.

A 50% (V/V) Celite suspension is prepared in distilled water and used without further treatment.

Samples of 5-^{3}H labeled cytosine containing compounds in volumes between 0.5 cc to 2.0 cc are treated with 1 cc of 0.1 N HCl, 0.5 cc of the 20% Norit suspension and 0.5 cc of the 50% Celite suspension and water to a total volume of 10 cc. The samples are shaken for 30 minutes at room temperature and placed on a 15 cc scintered glass funnel. The washes are collected under vacuum. Two additional washes of 2 cc each of 0.01 N HCl are collected and aliquots counted in a scintillation counter. A convenient and simple filtration apparatus consists of a miniature bell vacuum jar in which a rubber stopper is treated to support a 15 ml scintered glass funnel with the receiving test tube inside the jar.

Preparation of 5-^{3}H-poly C

Polynucleotide phosphorylase is purified from Micrococcus lysodeikticus after the modified procedure described by Thanassi and Singer (1966). The incubation conditions that are employed for the synthesis of 5-^{3}H-polycytidylic acid consist of 0.2 ml of 0.5 M Tris buffer, pH 9.5; 0.1 ml 0.1 M $MgCl_2$, 6.075 mgs unlabeled CDP, 3 μmoles 5-^{3}H-CDP (1 mc) (Schwarz BioResearch), 0.3 ml water and 0.4 cc polynucleotide phosphorylase. The mixture is incubated for 3.5 hours at 36° and the reaction terminated by the addition of 0.1 cc of 0.1 M EDTA and 36 mg of KCl. The polymer is purified by phenol treatment and dialyzed extensively against 0.5 M NaCl. A 28% yield of purified 5-^{3}H poly C is obtained in this manner having a specific activity of 0.316 μc/μg poly C and S_{20} = 1.5.

Preparation of 5-^{3}H cytosine labeled E. coli DNA

Escherichia coli 15 TAU$^-$ (provided by Dr. Phillip Hanawalt, Stanford University) is grown in the medium described by him (1961), supplemented with thymidine (2 μg/ml), arginine (20 μg/ml), uridine (3 μg/ml) and 5-^{3}H cytosine (8 μg/ml, 5 μc/ml) (Schwarz BioResearch). The DNA is isolated from mid log cells according to the procedures described by Marmur (1961) and has a final specific activity of 7.1 x 10^3 cpm/ mμmoles of dCMP equivalent. Formic acid hydrolysis and resolution of the free bases revealed that 99.8% of the radioactivity was located in cytosine with the remainder in thymine.

Ultraviolet Irradiation

Stirred solutions are irradiated in stoppered cuvettes with monochromatic ultraviolet light (1% dispersion) at about 10 ergs/mm^2/sec. The light is furnished by a 200 watt air cooled super-high pressure mercury arc lamp (Gates HB0200W), and diffracted through a grating monochromator (Bausch and Lomb). The light intensity is measured with a photometer (Eldorado Electronics, model 201) calibrated using the ferrioxalate actinometer (Hatchard and Parker, 1956).

Results and Discussion

From studies of the properties of dihydrocytosine and related compounds it was found that such pyrimidine analogs exhibit an exchange reaction of C-5 hydrogens with deuterium oxide (Wechter and Kelly, 1967; Shapiro and Klein, 1967; Brown and Hewlins, 1968). A similar exchange reaction has been reported by Chambers (1968) for the effectively reduced photohydrate of uracil rings. It can be assumed that no such exchange reaction should occur at the same position for uracil-uracil dimers or cytosine containing dimers since it is not

possible to have a double bond at a bridge carbon atom (Brecht, 1924) (See Figure 1).

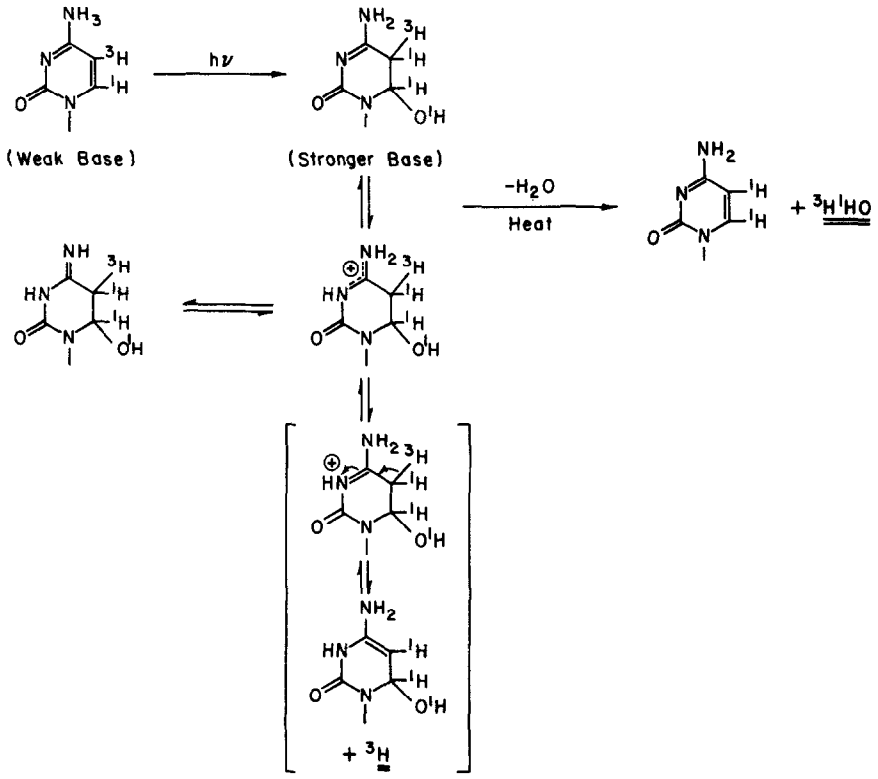

Fig. 1

By taking advantage of these observations, the detection of cytosine photohydrates is feasible by a combination of the exchange reaction of 5-^{3}H labeled cytosine compounds with the medium, together with the release of such a label under dehydration or reversal conditions. An assay has been devised the kinetics of which may be attributed to the following sequence of reactions (Figure 1).

Release of labeled hydrogen as a method of photohydrate detection is in principle a measure of the total population of cytosine photohydrates that have already existed and do exist at the time of the assay. Since the photosteady states

of cytosine hydrate formation allow for reversal to cytosine, there may be considerably more ^{3}H released than the number of photohydrates assayed spectrophotometrically.

The irradiation of ^{3}H-5-CMP-5' by monochromatic UV and the relationship of the number of photohydrates found to the number of ^{3}H atoms released is shown in Figure 2. It can be seen that the slope of 1 implies a quantitative release of label for each hydrate formed. This unexpectedly high value implies that the exchange reaction is extremely fast unless the dehydration reaction is stereospecific. It is not possible at this juncture to invoke any specific mechanism for ^{3}H release. Regardless of the precise mechanism, the extent of tritium release allows for quantitation for the formation of this specific photoproduct whereas no such release is observed with dimers of ^{3}H-5-UMP-5'.

Since the assay conditions require separation of the labeled water from the pyrimidine in charcoal which during the acid treatment should have been completely dehydrated, there was some question whether reduced pyrimidine might not be only partially adsorbed to the Norit. To show that the released label is not due to pyrimidine "leaking" through the Norit, a parallel experiment with ^{14}C-CMP-5' was carried out. It is evident from Figure 2 that little, if any, leakage has occurred.

The quantum yield for CMP-5' hydrate found in this assay, Table I, is in good agreement with the spectrophotometric data already published (Shugar and Wierzchowski, 1957, 1958).

Similar experiments were performed with labeled polycytidylic acid prepared from ^{3}H-CDP as described in the Methods section. The results, seen in Figure 3, show a quantative

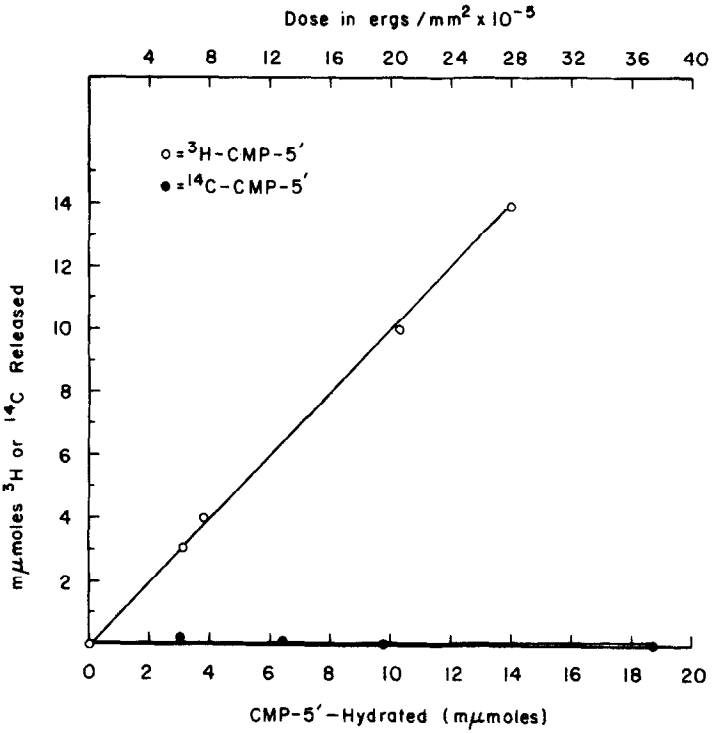
Dose in ergs / mm² x 10⁻⁵
4 8 12 16 20 24 28 32 36 40
o = ³H-CMP-5′
● = ¹⁴C-CMP-5′
mμmoles ³H or ¹⁴C Released
0 2 4 6 8 10 12 14
0 2 4 6 8 10 12 14 16 18 20
CMP-5′-Hydrated (mμmoles)

Fig. 2

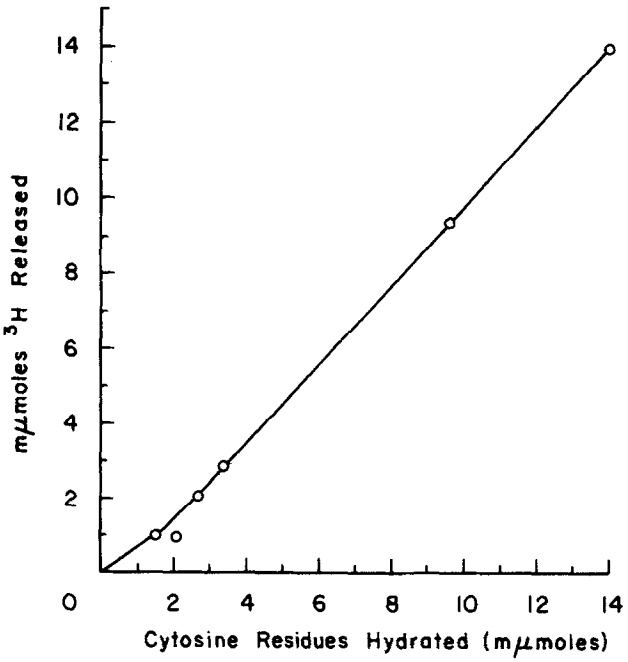
mμmoles ³H Released
0 2 4 6 8 10 12 14
0 2 4 6 8 10 12 14
Cytosine Residues Hydrated (mμmoles)

Fig. 3

Table I

Quantum Yield for Cytosine Photohydrate Formation

^{3}H-Cytosine Derivative	Quantum Yield (in moles/einstein x 10^3)
CMP-5'	16.2
Poly rC	6.8
Poly rC in 90% ethylene glycol	15.6
Poly rC:rI	2.7
E. coli 15 TAU$^-$ DNA-Native	1.6
E. coli 15 TAU$^-$ DNA-Denatured	5.1

release of the ^{3}H from position C-5 of the cytosine moieties. Irradiation was carried out at 5°C and the polymer diluted into 100% ethylene glycol for absorbance measurements (Fasman *et al*, 1967). The level of hydration was estimated from the absorbance decrease at 270 mμ and its subsequent restoration under dehydration conditions.

By providing ^{3}H-5-cytosine in the presence of uridine and thymidine for the growth of *E. coli* 15 TAU$^-$, it was possible to obtain DNA preparations almost exclusively labeled in the cytosine moieties. Since the C-5 hydrogen of cytosine is removed during metabolic deamination and methylation, there is sufficient guarantee for exclusive cytosine labeling. Chromatographic resolution of an acid hydrolysate of this DNA supported such a contribution since less than 2% of the tritium of cytosine was recovered in thymine.

There are a number of significant aspects to Table I which show that cytosine hydration, in fact, does exist and

its level is sufficiently high to warrant its implication in the biological effects of UV. In a population of photoproducts it can be expected that in the dose range of 10^4 ergs/mm^2 there would be approximately 3 cytosine hydrates for every 10 thymine-thymine dimer residues formed.

In addition, the level of cytosine hydration in polynucleotides is, as predicted by Setlow and Carrier's data (1965) with poly dC:dI and poly rC, conformation specific. It is of interest that the quantum yield for hydration of these cytosines in poly C dissolved in 90% ethylene glycol is of the same order as the monomer in keeping with the effects of this solvent on the "stacking forces" in polynucleotides (Fasman et al, 1960).

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