

RECOVERY OF THE ABILITY TO SYNTHESIZE DNA IN SEGMENTS OF NORMAL SIZE AT LONG TIMES AFTER ULTRAVIOLET IRRADIATION OF HUMAN CELLS

STEVEN N. BUHL, R. B. SETLOW, *and* JAMES D. REGAN

From the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

ABSTRACT DNA synthesized in human cells within the first hour after ultraviolet (UV) irradiation is made in segments of lower molecular weight than in nonirradiated cells. The size of these segments approximates the average distance between pyrimidine dimers in the parental DNA. This suggests that the dimers interrupt normal DNA synthesis and result in gaps in the newly synthesized DNA. However, DNA synthesized in human cells at long times after irradiation is made in segments equal or nearly equal to those synthesized by nonirradiated cells. The recovery of the ability to synthesize DNA in segments of normal size occurs in normal human cells, where the dimers are excised, and also in cells of the human mutants xeroderma pigmentosum (XP), where the dimers remain in the DNA. This observation implies that the pyrimidine dimer may not be the lesion that causes DNA to be synthesized in smaller than normal segments.

INTRODUCTION

UV radiation makes dimers between adjacent pyrimidines in DNA. These dimers may be removed either by photoreactivation, which does not occur in mammalian cells except those of marsupials (Cook and Regan, 1969; reviewed by Cook, 1970) or by excision repair (reviewed by R. B. Setlow, 1968). Cells of different mammals excise dimers at different efficiencies, normal human cells being the most efficient and cells of the human mutants XP among the least efficient (Setlow et al., 1972). Individuals with XP (Cleaver, 1968) are sensitive to sunlight, as indicated by the appearance of skin cancers at a very early age, and have a greatly reduced ability to excise pyrimidine dimers.

Newly synthesized DNA from UV-irradiated *Escherichia coli* cells is made in segments that are smaller than normal (Rupp and Howard-Flanders, 1968), and the

size of these segments approximates the average distance between pyrimidine dimers in the parental strands. This finding suggests that the pyrimidine dimers interrupt normal DNA synthesis, resulting in the formation of gaps or discontinuities in the daughter DNA. Upon incubation of the irradiated cells, the gaps disappear as the short segments of DNA are elongated and joined to form high molecular weight DNA (postreplication repair) (Rupp and Howard-Flanders, 1968).

DNA synthesized within the first hour after UV irradiation of Chinese hamster, mouse L5178Y cells, and both normal human and XP cells is also made in segments that are smaller than those synthesized by nonirradiated cells; and upon incubation the short segments are elongated and joined to form high molecular weight DNA (Cleaver and Thomas, 1969; Rupp et al., 1969; Buhl et al., 1972 *b*). The size of the short segments synthesized by mouse L5178Y and human cells approximates the average distance between the pyrimidine dimers in the parental DNA (Lehmann, 1972; Buhl et al., 1972 *b*), but the length of the short segments synthesized by UV-irradiated Chinese hamster cells is greater than the distance between dimers (Rauth et al., 1973). DNA segments synthesized by UV-irradiated mouse L cells are equal in size to those synthesized by nonirradiated cells (Chiu and Rauth, 1972).

UV-irradiated Chinese hamster CHO cells which synthesize DNA in segments smaller than normal when pulse labeled within the first hour after irradiation recover the ability to synthesize DNA in normal-sized segments at long times after irradiation (Meyn and Humphrey, 1971). (While this manuscript was in preparation we learned of similar findings in mouse L5178Y cells).¹ On the other hand, Chinese hamster B14 cells which also synthesize DNA in segments smaller than normal within the first hour after irradiation do not recover the ability to synthesize DNA in normal-sized segments at long times after irradiation (Meyn and Humphrey, 1971).

We now report that UV-irradiated normal human and XP cells, which synthesize DNA in segments equal to the distance between dimers within the first hour after irradiation, recover the ability to synthesize DNA in segments of normal size at long times after irradiation. We suggest that this represents a separate and new repair system.

MATERIALS AND METHODS

Cell Lines and Media

The cell lines used are listed in Table I. Cells were normally grown in minimum essential medium (Eagle, 1959) supplemented with 10% fetal calf serum and the nonessential amino acids at 37°C in a water-saturated atmosphere of 2% CO₂ in air. 1 or 2 days before each experiment the cells were trypsinized (0.25% trypsin) and plated in minimum essential medium supplemented with 10% calf serum and the nonessential amino acids on plastic Petri

¹ Lehmann, A. R., and S. Kirk-Bell. 1972. *Eur. J. Biochem.* 31:438.

TABLE I
CELL LINES USED

Name	Origin
HS-WP	Normal human fibroblasts
JP-XP2	deSanctis-Cacchione syndrome
WY-XP3	Clinically diagnosed as XP but having normal repair replication (Burk et al., 1971)
SG-XP4	Uncomplicated XP

dishes (Permanox, Lux Scientific Corp., Thousand Oaks, Calif.) at approximately 20,000 cells per dish. For some experiments cells were grown in air without CO₂ at temperatures other than 37°C for at least 2 h before initiation of the experiments. The temperature was increased to 37°C 30–60 min before pulse labeling at 37°C in air.

Pretreatment, UV Irradiation, Pulse Labeling, and Incubation

All the dishes were incubated in medium containing 1 mM hydroxyurea for at least 2 h before initiation (UV irradiation) of the experiments to stop semiconservative DNA synthesis (Pfeiffer and Tolmach, 1967). The hydroxyurea was removed and the cells were washed with warm medium 0.75–1 h before pulse labeling. Each dish of cells was washed with warmed phosphate-buffered saline (Dulbecco and Vogt, 1954) and exposed to UV radiation from a germicidal lamp at a rate of 10 ergs/mm²·s. The cells were then washed and incubated in warmed medium for various times. The DNA was pulse labeled by addition of 12–50 μCi/ml [*methyl-³H*]thymidine (sp act, 20 Ci/mmol) for a prescribed time (usually 30 min).

Sedimentation of DNA in Alkaline Sucrose

The cells were washed with and suspended in an ice-cold isotonic EDTA solution (Setlow et al., 1969), sedimented in a refrigerated centrifuge (Sorvall RC-2B, Ivan Sorvall, Inc., Newtown, Conn.), and resuspended in cold EDTA solution. They were then irradiated with 2-krad gamma rays from a ⁶⁰Co source at a rate of 3.65 krad/min. The gamma irradiation causes a few single strand breaks in the DNA, permitting sedimentation without excess entanglement of the DNA strands (Lehmann and Ormerod, 1970). The cells were then immediately lysed in a 0.2% sodium dodecyl sulfate solution on top of a 3.6-ml gradient of 5–20% sucrose, 0.1 M NaCl, and 0.1 M NaOH in a 4-ml polyallomer tube.

The gradients were centrifuged at 40,000 rpm for 75–100 min at 20°C in the SW56 rotor of a Spinco model L3-50 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Aliquots (0.125 ml) were collected through a hole punched in the bottom of the tube onto Whatman no. 17 paper strips (Carrier and Setlow, 1971). The strips were washed once in 5% trichloroacetic acid, twice in ethanol, and once in acetone (Bollum, 1966), dried, cut, and placed into vials with a toluene–2,5-bis-(5-*tert*-butylbanzoazolyl)thiophene scintillator, and the radioactivity was counted in a liquid scintillation spectrometer. The molecular weights were calculated by computer, using the relationship observed by Abelson and Thomas (1966) and confirmed by R. B. Setlow (unpublished results), with single strands of T4, λ, and φX174 DNA's as standards (Freifelder, 1970). The relationship found was that single strand molecular weights are proportional to the 2.62 power of the distance sedimented.

RESULTS

DNA profiles from alkaline sucrose gradients of normal human cells pulse labeled and harvested within the first hour after UV irradiation show more slow-sedimenting DNA than do profiles of cells pulse labeled 6 h after irradiation or profiles of nonirradiated cells (Fig. 1). We first thought that the ability to synthesize DNA in segments near normal size 6 h after UV irradiation resulted from the excision of pyrimidine dimers, which were thought to cause the short DNA by interrupting DNA synthesis. To test this notion the same experiment was done with XP cells, human cells that have a greatly reduced ability to excise pyrimidine dimers. Fig. 2 shows results from cell line SG-XP4. Note that DNA profiles from cells pulse labeled 24 h after UV irradiation indicate that DNA is made in segments of normal size. Fig. 3 is a summary of pulse label experiments with normal cells and XP cell line JP-XP2. We conclude that the ability to synthesize DNA in normal-sized segments is recovered during a 24-h period in human cells whether the dimers have been excised or not.

In an effort to determine the nature of the recovery of the ability to synthesize DNA in normal-sized segments we measured the effect of temperature on the phenomenon to determine whether the recovery results from enzymatic or chemical

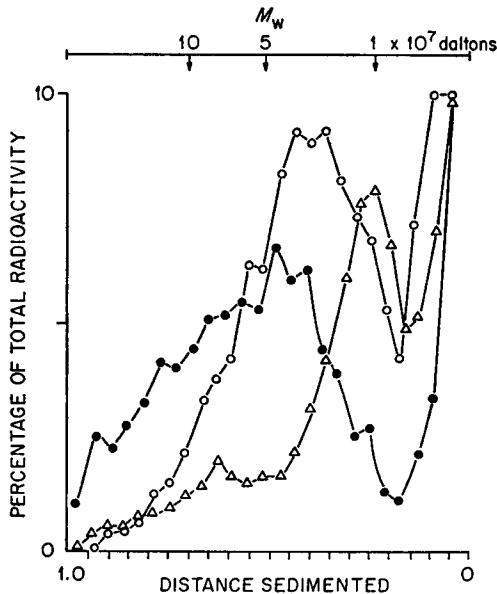


FIGURE 1 Alkaline sucrose gradient profiles of DNA from cell line HS-WP, which were nonirradiated and pulse labeled (●), UV irradiated (70 ergs/mm²) and pulse labeled after 30 min (Δ), or UV irradiated (70 ergs/mm²) and pulse labeled after 6 h (○). Pulse labeling was for 30 min. Sedimentation was for 75 min at 40,000 rpm. Maximum: Δ, 9.75%; ●, 6.6%; ○, 7.9%. M_w is the weight average molecular weight.

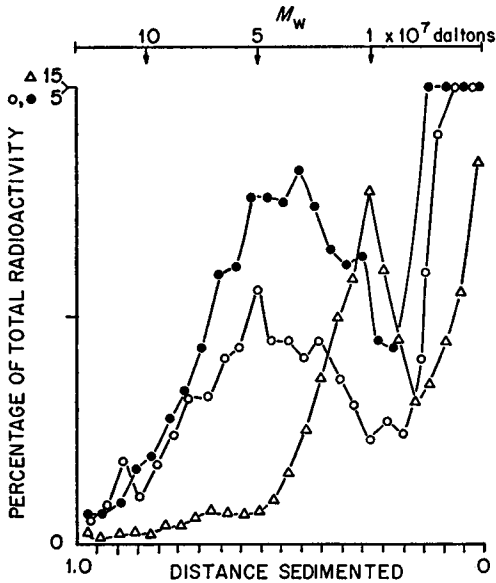


FIGURE 2 Alkaline sucrose gradient profiles of DNA from cell line SG-XP4, which were nonirradiated (○), UV irradiated (70 ergs/mm²) and pulse labeled after 30 min (△), or UV irradiated (70 ergs/mm²) and pulse labeled after 24 h (●). Pulse labeling was for 30 min. Sedimentation was for 90 min at 40,000 rpm. Maximum: △, 11.55%; ○, 2.7%; ●, 4.1%; M_w is the weight average molecular weight.

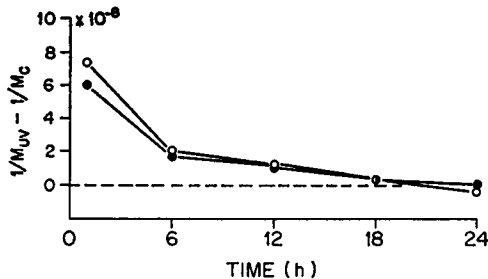


FIGURE 3 Differences between the reciprocals of the weight average molecular weights of DNA of nonirradiated and UV irradiated (○) HS-WP (100 ergs/mm²) and (●) JP-XP2 (70 ergs/mm²) pulse labeled for 30 min at various times after irradiation.

change. Fig. 4 shows the effect of incubating irradiated cells at reduced temperatures for 6 or 18 h before pulse labeling at 37°C. Recovery continues at 27–37°C but rapidly decreases below 27°C and is completely arrested at 19–20°C. These data indicate that the recovery system is enzymatic.

Recovery of the ability to synthesize DNA in normal size segments can be explained by removal of only a few dimers in select regions of the DNA and the use of these regions as the templates for the DNA we observe at long times after irradiation.

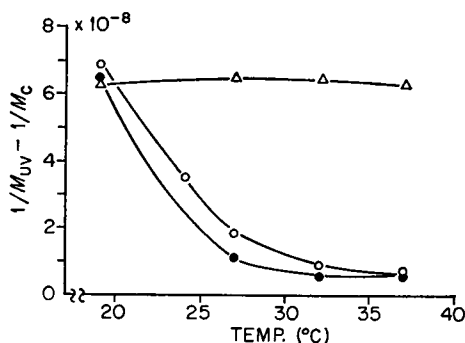


FIGURE 4 The effect of temperature on recovery of the ability to synthesize DNA of normal size. HS-WP cells were incubated at various temperatures for 2 h before irradiation with 75 ergs/mm² and then incubated for 0, (Δ), 6 (○), or 18 (●) h before being warmed to 37°C for 30–60 min before pulse labeling for 30 min and sedimentation. The nonirradiated cells (1/M_c) were incubated for the same lengths of time at the reduced temperatures as were the irradiated cells. 1/M is the reciprocal of the weight average molecular weight.

ation. This notion can be dismissed by analyzing DNA profiles from alkaline sucrose gradients obtained during excision repair studies (Regan et al., 1971). The profiles are single modal indicating random repair of the dimers rather than bimodal which would indicate repair of select regions in the DNA. Additionally, Meyn (1973) reports that although UV irradiation reduces the rate of DNA replication it does not delay initiation of replication. He also reported that replication occurs at times after irradiation from DNA which contains dimers.

The observation of the recovery of the ability to synthesize DNA in normal size segments could be an artifact if (a) the pulse-labeled DNA we observed at long times after irradiation was made from normal DNA templates synthesized between irradiation and pulse labeling, or (b) limitations of our techniques did not allow us to observe the formation of the gaps which are left presumably opposite the dimers. The gaps or the short segments of DNA would not be observed if the gap-filling process increased in rate compared with normal DNA synthesis which is interrupted by the presence of lesions in the template DNA. As time increased after irradiation the gaps would be filled nearly as rapidly as they are formed. This would make the point of DNA replication very near the point of nucleotide insertion into the gap, and our present alkaline sucrose gradient technique could not resolve the small difference that would exist.

We have eliminated these two possibilities in the following manner. Previously we had shown that the segments of daughter DNA synthesized within the first hour after UV irradiation were elongated and joined by exogenous nucleotide insertion (Buhl et al., 1972 *a*) and that this process as well as semiconservative replication (Pfeiffer and Tolmach, 1967) were inhibited by hydroxyurea. The data in Figs. 1–4 were obtained from cells treated with 1 mM hydroxyurea as indicated in the Materials and Methods. Identical results were obtained without

hydroxyurea. Although the previous reports indicate the effectiveness of hydroxyurea we further evaluated its effectiveness in preventing DNA synthesis and to determine whether the DNA synthesized at long times after irradiation is made from DNA synthesized between irradiation and pulse labeling by performing the following experiment. Cells were irradiated and incubated in 1 mM hydroxyurea and 0.1 mM 5'-bromodeoxyuridine (BrdUrd) for 12 h, washed with warm medium, pulse labeled with $[^3\text{H}]$ thymidine for 1 h, and sedimented in CsCl. For the light-light marker we used DNA bulk labeled with $[^{14}\text{C}]$ thymidine, and for the heavy-light marker we used cells that had DNA bulk labeled with $(^{32}\text{PO}_4)^{-3}$ and BrdUrd. We observed $[^3\text{H}]$ thymidine-labeled material only in the light-light region of the gradient and concluded that hydroxyurea prevented BrdUrd incorporation into DNA and that the DNA synthesized at long times after UV irradiation was made from the DNA template that had been irradiated.

To test the second possibility we irradiated the cells, incubated them for 18 h, irradiated them again, and finally pulse labeled them. Fig. 5 shows typical results from these experiments. The profile from cells that received the double exposure shows small DNA segments equal to the distance between the dimers produced by the second exposure. If there had been a change in the relative synthesis rates as

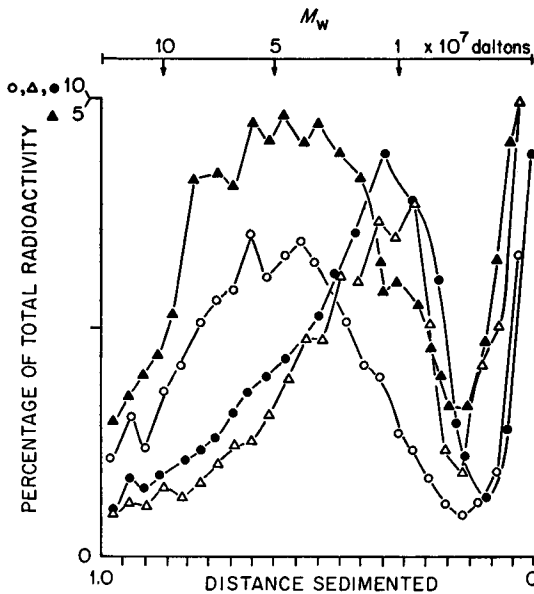


FIGURE 5 Alkaline sucrose gradient profiles of DNA from cells of line WP-XP3 that were nonirradiated and pulse labeled (○), UV irradiated (70 ergs/mm²) and pulse labeled after 30 min (●), UV irradiated (70 ergs/mm²) and pulse labeled after 18 h (▲), or UV irradiated (70 ergs/mm²), incubated for 18 h, UV irradiated (70 ergs/mm²), and pulse labeled after the second irradiation (△). Pulse labeling was for 30 min. Maximum: ●, 8.8%; △, 7.7%; ○, 6.9%; ▲, 4.8%. M_w is the weight average molecular weight.

supposed above, we would have seen DNA of normal size. Similar results were obtained with each of the cell lines in Table I. Since the DNA segments are shorter than normal, we cannot invoke changes in the relative rates of gap filling and normal synthesis to explain these results. We conclude that the lesions causing the formation of the gaps or synthesis of the shorter DNA segments as a result of the first UV irradiation were repaired and that we observed only the shorter segments resulting from the lesions produced by the second irradiation.

DISCUSSION

We have extended to human cells the observation that DNA synthesized in Chinese hamster cells (Meyn and Humphrey, 1971) at long times after UV irradiation is made in segments equal to those synthesized by nonirradiated cells. The recovery of the ability to synthesize DNA in segments of normal size at long times after irradiation occurs in normal human cells, where the dimers are excised, and in XP cells, where the dimers are not excised. We have also shown that this repair system

TABLE II
DNA REPAIR SYSTEMS

Cells	Dimers excised	Approximate size of DNA synthesized shortly after UV relative to the distance between pyrimidine dimers	Recovery of the ability to synthesize DNA of normal size at long times after UV	DNA chain elongation and joining
	%			
Human				
Normal XP	80	equal	yes	yes
SG-XP4	2	equal	yes	yes
JP-XP2	2	equal	yes	yes
WY-XP3	not measured	equal	yes	yes
Hamster				
CHO	24	greater	yes	yes
B14	8	greater	no	yes
Mouse				
L5178Y	10	equal	yes	yes
3T3	10	equal	yes	yes
L	not detectable	greater		yes
<i>E. coli</i>				
AB2487 rec ⁻ uvr ⁺	0	equal	not determined	no
AB2499 rec ⁺ uvrB5	0	equal	no*	yes
AB2497 rec ⁺ uvr ⁺	90	equal	yes	yes

* Photoreactivable.

is present in the atypical XP cell line WY-XP3, the exact biochemical defect of which has not yet been determined.

The nature of this repair system, which operates before DNA replication and is reflected in the recovery of the ability to synthesize DNA in segments of normal size, is not known; however, our data indicate that it is temperature sensitive and therefore probably enzymatic.

The various repair systems and the above observations in different mammals and in *E. coli* are summarized in Table II. There are several possibilities for the damage acted on by this repair system. The first is pyrimidine dimers. This possibility is supported by the observations that the size of the short, newly synthesized DNA segments in irradiated mouse L5178Y (Lehmann, 1972) and human (Buhl et al., 1972 *b*) cells approximates the distance between the dimers in the parental DNA. In irradiated *E. coli*, where the short segments also equal the distance between the dimers (Rupp and Howard-Flanders, 1968), recovery of the ability to synthesize DNA in segments of normal size can be accomplished by photoreactivating or excising the pyrimidine dimers (Smith and Meun, 1970). However, the possibility that the dimer is not the lesion for this repair system but that the lesion is an unknown photodamage is supported by the observations in Chinese hamster (Meyn and Humphrey, 1971) and mouse L (Chiu and Rauth, 1972) cells, where DNA synthesized shortly after irradiation is made in segments that are longer than the distance between the dimers, or equal to normal size.

If the pyrimidine dimer were the lesion acted on by this repair system, then a model (Fig. 6) to explain our data would require a repair system that monitored and corrected conformational anomalies in DNA, so that dimers could be circumvented or ignored and normal DNA replication could occur. The repair would have to occur without breaking the primary DNA chain, because no decrease in molecular weight of the parental DNA has been observed in XP cells after UV irradiation (Setlow et al., 1969). No incision into the DNA would be required if a

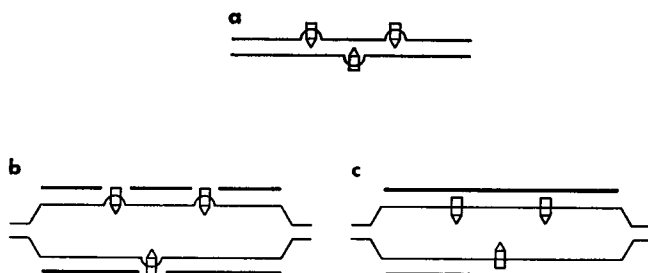


FIGURE 6 Recovery of the ability to synthesize DNA in normal size segments. (a) UV-damaged DNA contains pyrimidine dimers. (b) DNA synthesized from damaged DNA contains gaps opposite the pyrimidine dimers. (c) DNA synthesized from "repaired" DNA does not contain gaps. The dimers are still present and can be detected chromatographically, but normal DNA replication can occur.

physical reorientation were accomplished by adjustment of charges or modification of chemical bonds. On the other hand, repair of an undescribed lesion might involve direct removal of the lesion. Removal could occur without breaking the DNA chain if the lesion involved an abnormal protein—DNA interaction.

As in the case of the excision-repair system, different mammals would have different efficiencies for the repair system described here. Mouse L cells would be most efficient and could tolerate low doses of UV with no interruption of DNA synthesis although at higher doses, where the repair enzymes would be saturated, DNA replication would be partially disrupted. The Chinese hamster CHO cell line would be intermediate, and humans would be the least efficient. An interesting qualitative correlation is that the efficiency is inversely proportional to excision ability. Chinese hamster B14 and mouse L5178Y mutants would have a totally or partially impaired recovery system. However, different efficiencies of repair would not be needed to account for the variable size of the DNA synthesized if the lesion occurred with a different frequency per UV dose in each different cell line (although this notion seems an unlikely explanation).

It may be that the short DNA segments synthesized shortly after irradiation cannot be elongated and joined until this repair system operates; however, this does not seem likely because Chinese hamster B14 (Meyn and Humphrey, 1971) cells do not recover the ability to synthesize DNA in normal-sized segments but do elongate and join the short DNA segments synthesized within the first hour after UV irradiation.

This repair system, as reflected by the recovery of the ability to synthesize DNA in segments of normal size, has also been observed in human cells treated with methyl methanesulfonate; however, the recovery is much more rapid after the chemical treatment than after UV irradiation, requiring only 1.5 h after the maximum reduction in the size of the DNA observed after a 1-h treatment (Buhl and Regan, 1973).

This research was jointly sponsored by the National Cancer Institute and by the United States Atomic Energy Commission under contract with the Union Carbide Corporation.

Steven N. Buhl is a predoctoral trainee supported by grant GM 1974 from the National Institute of General Medical Sciences.

Received for publication 13 March 1973 and in revised form 30 May 1973.

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