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RAPID QUANTITATION OF ULTRAVIOLET-INDUCED THYMINE-CONTAINING DIMERS IN HUMAN CELL DNA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Rapid and efficient separation of all three types of cyclobutyl pyrimidine dimer (Pyr<>Pyr) species induced in cellular DNA by far-ultraviolet (UV) light (chiefly 254 nm) has been achieved by reversed-phase high-performance liquid chromatography using octadecylsilyl stationary phases. The order of elution is: (Ura<>Ura) < (Ura<>Thy) < (Thy<>Thy) <Thy. The determination of Pyr<>Pyr species in DNA from UV-irradiated, [^{3}H]thymidine-labelled human skin fibroblasts in tissue culture is demonstrated for far-UV fluences as low as 10 J/m^2. The ability to measure specifically individual dimer types allowed demonstration of comparable kinetics of repair for two labelled dimer species (Ura<>Thy and Thy<>Thy).

INTRODUCTION

Cyclobutyl pyrimidine dimers (Pyr<>Pyr)** constitute the major class of structural alterations induced in the DNA of living cells by exposure to far-UV light[1]. Pyr<>Pyr are also formed, albeit less efficiently, by the action of near-UV light[2,3] and ionizing radiation[4]. Generalized assays are currently available for monitoring various hallmarks of the enzymatic DNA excision repair processes that deal with such lesions (reviewed in refs. 5 and 6); these assays usually follow the insertion of repair patches into damaged DNA. This hallmark, DNA repair synthesis, can be quantitated, for example, as (1) radioactive nucleotides incorporated into pre-existing damaged DNA

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** Cyclobutyl dimers formed between adjacent intrastrand pyrimidines. The pyrimidine may be either thymine (Thy) or cytosine (Cyt).

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after its separation from newly synthesized DNA in isopycnic gradients[7], (2) autoradiographic silver grains over nuclei in non-S phase cells[8], and (3) strand breaks induced in bromouracil-containing repair patches by near-UV light[9,10]; a major drawback to all three is their inability to identify the particular DNA lesions undergoing repair. Other assays, however, do monitor Pyr<>Pyr lesions directly. One method uses purified bacterial UV endonucleases to convert dimer-containing sites in DNA into single-strand breaks which are subsequently measured by velocity sedimentation in alkaline sucrose gradients[11], by alkaline agarose gel electrophoresis[12], or by alkaline elution[13].While offering superb sensitivity, such enzymatic assays, as well as other immunofluorescent ones[14,15], fail to differentiate among the three types of dimers (Thy<>Thy, Thy<>Cyt, and Cyt<>Cyt) formed in UV-exposed DNA. (The treatment used to hydrolyse DNA to the free bases deaminates cytosine in dimers to uracil [Ura], so the three dimers in DNA are actually analysed ultimately as Thy<>Thy, Thy<>Ura and Ura<>Ura.) Various chromatographic methods, for example using paper[16], cellulose thin layer[17,18], or anion-exchange columns[19] as supports, have been developed in order to achieve this resolution. Unfortunately, at physiologically significant sublethal UV fluences these methods suffer from high background noise due primarily to tailing of thymine into the dimer-containing regions[20].

Despite the extensive application of high-performance liquid chromatography (HPLC) for separation of nucleic acid components, this technology has seen only limited use in the measurement of Pyr<>Pyr[21,22]. Its application to the estimation of dimers in hydrolysates of DNA from UV-exposed cells, however, using cation-exchange HPLC, failed to resolve the two labelled (Thy<>Thy and Ura<>Thy) dimer species[22]. The potential of reversed-phase HPLC for such purposes was first illustrated by the resolution of the four stereoisomers of Thy<>Thy[23]; this report concluded that the chromatographic separation reflected primarily the different accessibility of the two methyl groups of this dimer species. Such a result suggested to us that within the *cis-syn* series of dimers formed in UV-irradiated DNA, separation of the three species Ura<>Ura, Ura<>Thy, and Thy<>Thy should be possible (based on their possession of zero, one, and two methyl moieties, respectively) on a reversed-phase support. This achievement was first reported in preliminary communications[24,25]; detailed examination of chromatographic behaviour of different reversed phase materials is reported here, using authentic standards. In addition, we describe the novel application of this procedure in the quantitation of the incidence of *cis-syn* dimers of both Thy<>Thy and Thy<>Ura induced in the DNA of [^{3}H]thymidine-labelled cultured human cells by far UV-light; to illustrate the utility of our procedure, we display both the induction and subsequent repair of these two dimer types in normal cells.

The use of reversed-phase HPLC for dimer separation has also been reported recently by two other laboratories[26,27]. One of these studies used 254 nm light on labelled *E. coli* DNA irradiated *in vitro* instead of in living cells, and was able to achieve quantitation of only the Thy<>Thy species[26]. The other report dealt with exposure of human fibroblasts, but at 313 nm, not at 254 nm[27]; again, the induction of Ura<>Thy was not detected. In contrast, we have accomplished analysis of both labelled dimers by *in vivo* exposure of human cells at 254 nm.

MATERIALS AND METHODS

Cell cultivation and radioactive labeling of DNA

Practising conventional cell cultivation methodology, monolayer cultures of GM 38, a diploid skin fibroblast strain derived from a clinically normal donor, were grown to late logarithmic phase in fortified Ham's F12 medium (detailed in ref. 28). Cellular DNA was labeled by incubating cultures for 72 h in growth medium containing 1.0 μCi/ml [^{3}H]dThd (specific activity of stock, 7 Ci/mmole; New England Nuclear, Boston, MA, U.S.A.). The radioactive medium was then replaced with fresh non-radioactive growth medium, and the cells were incubated for one additional day to deplete the endogenous pools of [^{3}H]dThd.

Far-UV exposure

Upon aspirating the growth medium, the cultures were washed twice with PBS and exposed to various fluences of germicidal light (chiefly 254 nm) at a fluence rate of 1.4 J/m^2/sec, as calibrated by potassium ferrioxalate actinometry. If repair were to be followed, fresh prewarmed growth medium was added and the cultures were incubated for the indicated times. After withdrawing the medium, the cultures were washed with PBS and stored frozen.

Isolation and acidic hydrolysis of DNA

The cells were lysed on plates by addition of 1 ml of lysis solution (4-aminosalicylic acid, 1.5 g; triisopropyl naphthalene sulfonic acid, 0.25 g; 2-butanol, 1.5 ml; water, 23.5 ml). After collecting the lysate, 1 ml of freshly distilled phenol (saturated with 0.05 *M* Tris–HCl buffer, pH 8.0) was added and the mixture was then shaken for 30 min. After centrifugation (5000 *g*, 10 min) and withdrawal of the aqueous phase, the phenol phase was re-extracted with 1 ml of water, and, after centrifugation, this phenol phase was collected and combined with the first. The aqueous phases were dialysed overnight against water, after which DNA was precipitated by addition of cold trichloroacetic acid (5% w/v, final concentration). The DNA pellet was dissolved in 0.5 ml of 98% formic acid, transferred to thick-walled Pyrex tubes, sealed, and hydrolysed at 180°C for 40 min. Finally, the hydrolysate was evaporated to dryness under reduced pressure.

Chemicals and reagents

All purchased chemicals were reagent grade. Thymine and uracil were obtained from Sigma (St. Louis, MO, U.S.A.) Published procedures were followed for preparation of *cis*-5,6-dihydroxy-5,6-dihydrothymine[29], the *cis-syn* Thy<>Thy[30,31], and the *cis-syn* Ura<>Ura[32]. The *cis-syn* Ura<>Thy was prepared by UV-irradiation (254 nm) of a frozen 1:1 aqueous mixture of Ura and Thy[33,34], and shows IR and ^{1}H NMR spectroscopic properties identical with those for the product of 10 % HCl hydrolysis of the two *cis-syn* dUrd<>dThd dimers[35].

The distilled water which was used as the mobile phase for HPLC was filtered through Millipore type HA filters (pore size, 0.45 μm; Millipore, Bedford, MA, U.S.A.) prior to use.

Instrumentation

The high-performance liquid chromatograph used for separation of DNA hy-

drolysates consisted of a custom-designed pneumatic (nitrogen pressure) pump operating with a constant-pressure-head device (Atomic Energy of Canada Ltd.), a sample valve with 100-μl injector loop capacity (Valco, Houston, TX, U.S.A.), and a variable-wavelength detector (Model 837, Dupont Instruments, Wilmington, DE, U.S.A.) equipped with a 8-μL flow cell. Signals from the UV spectrometer were recorded by a Farrand Model 100 recorder (Heath, Benton Harbor, MI, U.S.A.) at a chart speed of 0.2 cm/min.

A second HPLC system was employed for the separation of authentic samples of the different Pyr<>Pyr species. This consisted of a Waters M6000A dual-piston pump and U6K universal injector (Waters, Milford, MA, U.S.A.), with a Cecil Model CE 212 variable-wavelength detector (Cecil Instruments, Cambridge, U.K.). A Searle Mark III liquid scintillation counter (Searle Analytic, Chicago, IL, U.S.A.) was used for ^{3}H counting.

Chromatographic conditions

Prepackaged octadecylsilyl ODS-2 and ODS-3 columns (25 × 0.46 cm I.D.), both with a particle size of 10 μm, were obtained from Whatman (Clifton, NJ, U.S.A.). In addition, a stainless-steel column (25 × 0.47 cm I.D.) was prepared with a C_{18} Nucleosil reversed-phase packing; this consists of porous silica gel (mean diameter 10 μm) coated with a chemically bonded monolayer of octadecylsilane (Macherey-Nagel, Düren, F.R.G.). Packing was accomplished by the "non-balanced" slurry procedure[36], using 1-propanol as dispersant with a constant-pressure Haskell pump system (Chromatém, Paris, France) operating at a delivery pressure of 6000 p.s.i. For samples from DNA hydrolysates, a guard column (5 × 0.46 cm I.D.) was used between the injector and the ODS-2 column; the guard column was packed with pellicular Co:Pell ODS bonded stationary phase (Whatman) by the "tap fill" technique.

In a typical analysis of Pyr<>Pyr content in UV-damaged DNA, the evaporated DNA hydrolysate was dissolved in 0.20 ml of water and then applied to a DEAE-Cellulose column (Whatman DE52, 4 × 0.4 cm I.D., prewashed with 5 ml of water). This DEAE-Cellulose column was eluted with 1.8 ml of water; 50 μg of thymine (internal standard) was added to this flow-through, which was then evaporated to dryness under reduced pressure. (The DE52 step removed non-radioactive material which otherwise decreased resolution of rapidly eluting material.) The resulting residue was dissolved in 0.20 ml of water and 0.10 ml was applied to the HPLC column. The flow-rate was *ca.* 1.2 ml/min. The column effluent was monitored continuously at 220 nm, using a full scale deflection of 0.5 A. Fractions (*ca.* 0.5 ml) were collected every 25 sec directly into scintillation vials carried in a Model 1320 Bio-Rad fraction collector (Richmond, CA, U.S.A.).

RESULTS AND DISCUSSION

HPLC separation of authentic samples of Pyr<>Pyr

The three chromatograms in Fig. 1 show HPLC separations on different reversed-phase column materials for the three Pyr<>Pyr dimer species in relation to that for thymine and *cis*-5,6-dihydroxy-5,6-dihydrothymine ("thymine diol", a self-irradiation product of ^{3}H-labelled thymine [see below]). The relative order of elution is the same on the three different columns: (Ura<>Ura) < thymine diol < (Ura<>Thy)

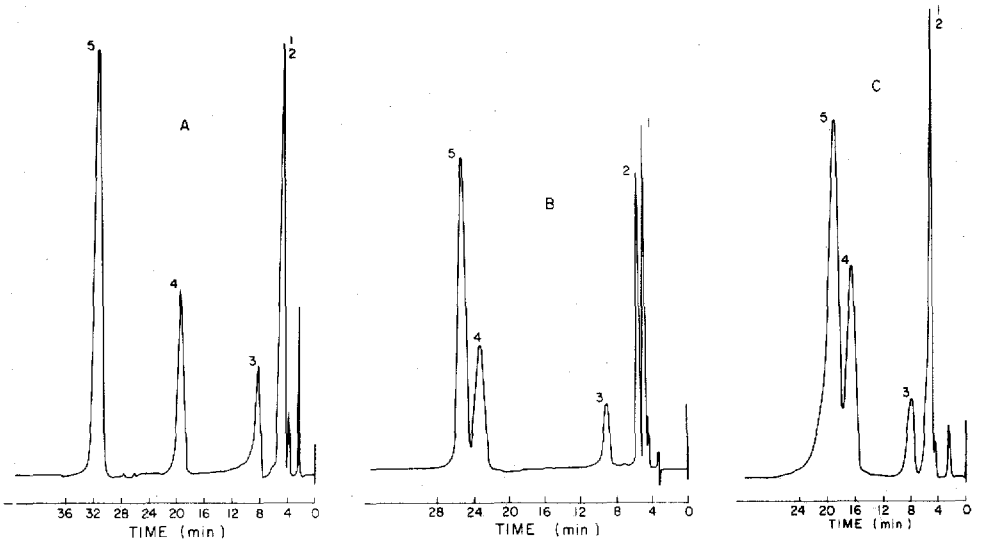

Fig. 1. Separation by HPLC of authentic samples (see Materials and methods) of the three pyrimidine dimers (in their deaminated form, where applicable), thymine, and the thymine diol formed as a consequence of radio-decomposition of labelled thymidine. The three reversed-phase columns used are: (A), ODS-2; (B), ODS-3; (C), C-18 Nucleosil. Elution, from right to left, was monitored by the absorbance at 220 nm as shown by the tracing. Water was used as the mobile phase. The numbers on each graph identify the peaks as the following: 1 = *cis*-5,6-dihydroxy-5,6-dihydrothymine; 2 = *cis-syn* Ura<>Ura; 3 = *cis-syn* Ura<>Thy; 4 = *cis-syn* Thy<>Thy; 5 = thymine.

< (Thy<>Thy) < thymine. These separations can be expressed in terms of the capacity factors (k'; Table I) of the three ODS packings for these species.

The mechanisms responsible for retention of solute with alkyl groups chemically bonded to silica gel have been postulated to involve mainly partition[37] and adsorption processes[38], although a role for dispersive interactions between solute and the non-polar part of the stationary phase has also been suggested[39,40]. The retentive capacity of a reversed-phase system is assumed to be primarily determined by solvophobic in-

TABLE I

CAPACITY FACTORS OF PYRIMIDINE DIMERS, THYMINE, AND THYMINE DIOL ON DIFFERENT ODS COLUMNS*

Compounds	*Octadecylsilyl reversed-phase packing material*		
	ODS-2	*ODS-3*	*C_{18} Nucleosil*
cis-syn Ura<>Ura	0.61	0.47	0.85
cis-5,6-Dihydroxy-5,6-dihydrothymine	0.61	0.68	0.85
cis-syn Ura<>Thy	1.87	1.81	1.81
cis-syn Thy<>Thy	5.97	6.23	4.94
Thymine	10.16	6.71	5.91

* ODS columns are eluted at 1 ml/min with 0.01 *M* potassium phosphate aqueous solution (pH 5.5) as the isocratic eluent. Similar results are obtained if water is used as the mobile phase.

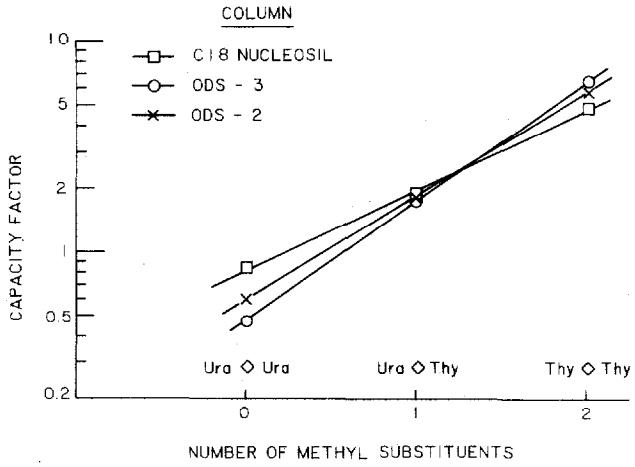

Fig. 2. The logarithm of the capacity factor *versus* number of methyl substituents, for the three Pyr<>Pyr species separated on the reversed-phase column materials used in Fig. 1.

teractions between the polar mobile phase and the non-polar part of the solute[41]. The linear plots obtained for log (capacity factor) *versus* the number of methyl moieties (Fig. 2) indicate that within the Pyr<>Pyr series, separation is based entirely on the hydrophobicity of the methyl substituents, for all three reversed-phase columns.

The relatively high retention capacity exhibited by both ODS-2 and ODS-3 columns (Table I) is consistent with previous observations[40,42] indicating that solutes tend to be more extensively retained on the bulky modified form of ODS stationary phase (*i.e.* ODS-2 and ODS-3) than on the monosubstituted ODS chains (C_{18} Nucleosil). The almost complete silanization of the silanol groups in the ODS-3 packing material does affect, albeit slightly, the retention behaviour of the 5,6-saturated pyrimidines (Table I; Fig. 1B *vs.* Fig. 1A and C). The accessible unchanged silanol groups of the ODS-2 phase, which behave as weak cation exchangers[43], may possibly interact with 5,6-unsaturated-2,4-dioxopyrimidines. If so, the ODS-3 column may be of particular utility in the separation and identification of Ura<>Ura dimer, when analyzing hydrolysates of DNA labeled with a generalized pyrimidine precursor.

The selectivity coefficient (Table II; $\alpha = k'_a/k'_b$) in separation of *cis-syn* Pyr<>Pyr species increases (and the efficiency of the columns decreases) in the following order:

TABLE II

SELECTIVITY COEFFICIENTS (α) FOR THE VARIOUS PYR<>PYR DIMERS AND THYMINE ON THE THREE DIFFERENT REVERSED-PHASE HPLC COLUMNS

Packing material	*Selectivity coefficients* ($\alpha = k'_a/k'_b$)		
	$\frac{Ura<>Thy}{Ura<>Ura}$	$\frac{Thy<>Thy}{Ura<>Thy}$	$\frac{Thy}{Thy<>Thy}$
ODS-2	3.07	3.19	1.70
ODS-3	3.85	3.44	1.08
C_{18} Nucleosil	2.13	2.73	1.20

ODS-3 > ODS-2 > C_{18} Nucleosil. By itself, this would seem to decree use of the ODS-3 column for DNA repair studies. However, most cellular studies on induction and repair of pyrimidine dimers utilize radioactively labelled thymidine incorporated via the salvage pathway; this of course results ultimately in labelling of the Ura<>Thy and Thy<>Thy dimers only. The vast majority (generally much more than 99 %) of label remains in Thy after acid hydrolysis, and it is of paramount importance to separate this cleanly from Thy<>Thy (the nearest labelled dimer). The absence of complete separation between Thy<>Thy and Thy on either the fully silanized silica gel (ODS-3: see Fig. 1B and Tables I and II) or the C_{18} Nucleosil column (Fig. 1C; Tables I and II) precludes the use of these stationary phases for the quantitative estimation of thymine-containing dimers in the DNA of living cells exposed to UV light. The markedly improved selectivity coefficient for Thy/(Thy<>Thy) separation (Table II: α = 1.70) on ODS-2 *versus* the other two materials makes it the material of choice for such biological studies. Furthermore, the selectivity of ODS-2 for separation among dimer species closely rivals that for ODS-3 (Table II).

It should be noted that in our reversed-phase HPLC separation system, unlike many other chromatographic systems for examining pyrimidine dimers[16–19], thymine is the *last* radioactive material to elute. This effectively eliminates any problem of decreased resolution or high background due to tailing from this main peak of radioactivity.

Estimation of thymine-containing dimers in human cells exposed to far-UV (254-nm) light

HPLC elution profiles obtained on the ODS-2 column are shown for formic acid hydrolysates of DNA obtained from human fibroblast cells exposed to UV fluences of either 10 J/m^2 or 50 J/m^2 (Figs. 3 and 4, respectively). Results for unirradiated cells are

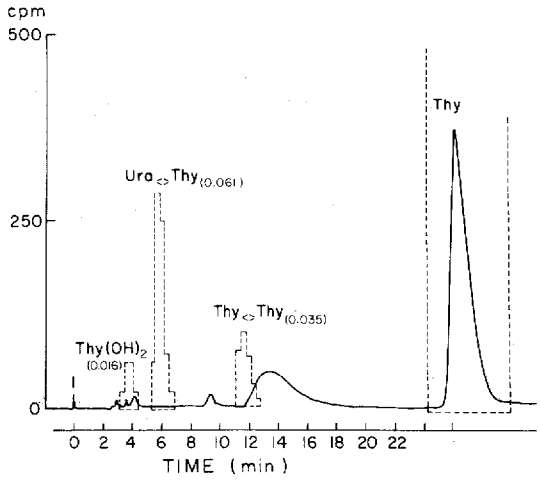

Fig. 3. HPLC separation on an ODS-2 column of the acid hydrolysate of DNA isolated from UV-irradiated (254 nm; fluence=) 10 J/m^2 GM 38 human fibroblast cells labelled using [^{3}H]thymidine. Counts per minute of each fraction *versus* elution time. The identification of the major peaks of radioactivity is described in the text. The number in brackets after each listed compound is the percentage of the total recovered radioactivity obtained in the region of the chromatogram attributable to that product.

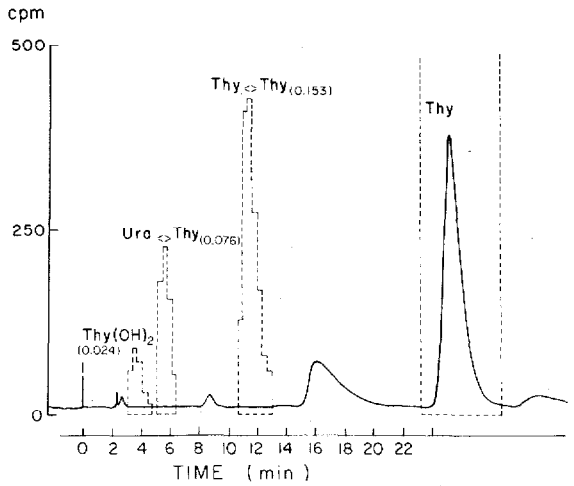

Fig. 4. As for Fig. 3 except that the UV fluence was 50 J/m^2.

depicted in Fig. 5. The three main radioactive species other than thymine have been identified, as indicated in both Figs. 3 and 4, as thymine diol and the *cis-syn* dimers of Ura<>Thy and Thy<>Thy. This identification of the materials induced by UV light was made by their co-chromatography with authentic standards both on ODS-2 columns and on thin-layer chromatograms[23]; additional confirmation of the dimeric structure of the two main photoproducts was obtained by photochemical reversal[44], yielding thymine as the only radioactive molecule, after exposure to 239 nm light (data not shown).

The thymine diol, which is also present in the control experiment (Fig. 5), arises from self-irradiation of [^{3}H]thymine involving radical degradation processes[45]. The

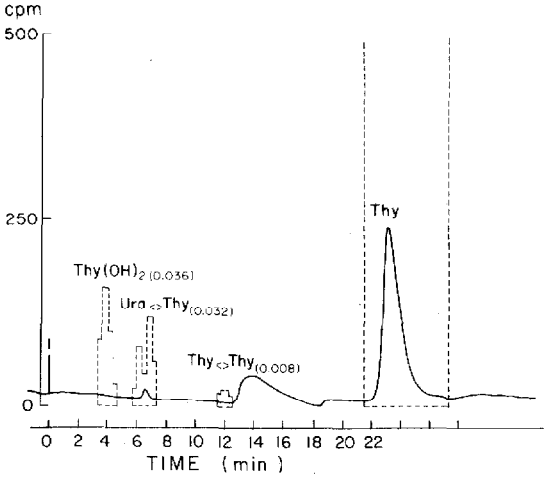

Fig. 5. As for Fig. 3 except that no UV exposure was given. The materials chromatographing in the region of the products of interest are labelled as in Figs. 4 and 5, but the chemical identity of the product in the region labelled Ura<>Thy appears not to be the dimer (see text).

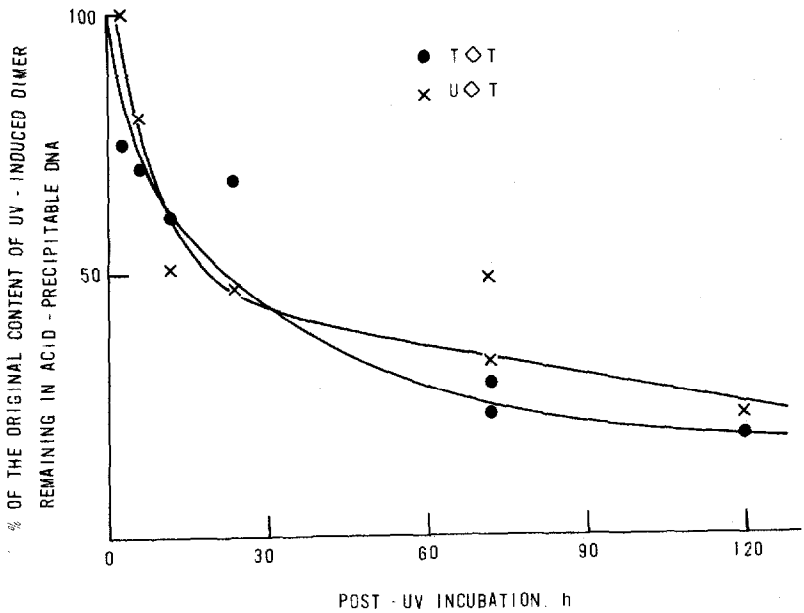

Fig. 6. Kinetics of repair in GM 38 fibroblast cells for the two thymine-containing dimer species. The percentage of the initial dimer content (induced by 40 J/m^2 of 254-nm UV exposure) that remains in trichloroacetic acid-precipitable DNA is plotted as a function of time of post-irradiation incubation.

presence of a second material, which exhibits chromatographic behaviour on ODS-2 similar to that for Ura<>Thy (see Fig. 5, unirradiated cells) results in significant background in this region (*e.g.* 0.032 % of total counts); a fluence of 10 J/m^2 is required to double the incidence of radioactivity chromatographing in this region (Fig. 3) and may be considered a practical lower exposure limit for detection of Ura<>Thy. In contrast, a very low background is observed in the Thy<>Thy region (Fig. 3), thus permitting estimation of Thy<>Thy at even lower fluences than shown here. By allowing specific determination of individual dimer types, this HPLC technique should be adaptable to novel studies on the induction and repair of UV-induced pyrimidine dimer damage at biologically significant exposures.

Kinetics of repair of thymine-containing dimers in normal human cells

Fig. 6 depicts the kinetics of repair in human fibroblasts for the two thymine-containing dimers, after exposure to a UV-fluence of 40 J/m^2 (254 nm). The dimer contents in Fig. 6 are expressed as a percentage of the initial yield of UV-induced (*i.e.* above the background levels in unirradiated control cells) dimers; if one chooses a doubling of the background value as a limit of resolution, these dimer measurements are valid for following the progression of repair down to a level of *ca.* 30–40 % (for Ura<>Thy) and less than 10 % (for Thy<>Thy) of the level initially induced. This preliminary experiment indicates comparable rates of excision for the two dimer species, with 50 % removal occuring in *ca.* 20 h; the two dimer species appear to be recognized in proportion to their content in DNA, and may therefore be acted upon by a common excision repair process. More extensive experiments are in progress on both normal and repair-deficient (*Xeroderma pigmentosum*) cell lines.

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