

An Improved Assay of UV-Induced Thymine-Containing Dimers in *Saccharomyces cerevisiae*

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DNA-Specific Labelling, Thymine Dimers, Thin Layer Chromatography

The method for assaying thymine-containing dimers in yeast is based on highly efficient ($[^3\text{H}]$ -deoxythymidine-5'-monophosphate) DNA-specific labelling and employs ascending thin layer chromatography. It allows satisfactory quantitative analysis down to UV-doses of 500 erg/mm².

Introduction

In yeast studies of DNA-repair after UV-irradiation at the molecular level have long been impeded. No (thymine) DNA-specific labelling is possible in this organism and methods of (5'-dTTP) DNA-specific labelling were poorly developed in the past^{1–7}. Thus the assay of thymine-containing dimers UV-induced in yeast-DNA *in vivo* was only possible after DNA-unspecific labelling and efficient removing of co-labelled RNA⁸. Meanwhile methods for economizing (5'-dTTP) DNA-specific labelling in the yeast *Saccharomyces cerevisiae* have been developed^{9,10}. This paper presents a method of assay of thymine-containing dimers UV-induced in yeast-DNA *in vivo* after efficient DNA-specific labelling with [^3H]deoxythymidine-5'-monophosphate.

Materials and Methods

Strains

Strain 211-1aMT6-425 (α)⁺ *ilv2 typ1 tlrC* ϱ^- of *S. cerevisiae* (= T6-425 in the text): isolated as a spontaneous *tlr* mutant of strain 211-1aMT6 according to Fäth *et al.*⁹. Strain 211-1aMT6-425 (α)⁺ *ilv2 typ1 tlrC tmp1-51* ϱ^- of *S. cerevisiae* (= T6-425 *tmp1-51* in the text): isolated as an EMS-induced *tmp* mutant of strain T6-425 according to Fäth *et al.*⁹ and Brendel and Fäth¹⁰. Strain MB1052 *tmp1-3*: isolated as a spontaneous *tlr* mutant of strain

MB1052 $\frac{\alpha \text{ ilv2 typ1 tmp1-3} + + + +}{\alpha \text{ ilv2 typ1 tmp1-3 ade arg4-17 his5-2 lys1-1}}$

according to Fäth *et al.*⁹. Strain T6-425 can optimally grow in liquid medium R containing 3 μg Na₂·5'-dTTP (Merck)/ml and in medium N. Strain T6-425 *tmp1-51* grows optimally in medium N containing 3 μg Na₂·5'-dTTP/ml and strain MB1052 *tmp1-3* does so in medium 2 \times N containing 6 μg Na₂·5'-dTTP/ml. The stationary titers each of the strains are approx. 1.5×10^8 /ml in these media.

Media and growth conditions

Medium N and R:^{11,9}; medium 2 \times N: doubly concentrated medium N. For standard growth conditions see Fäth and Brendel¹¹.

Labelling of cells

The strains were twice pre-grown to stationary phase in medium N (T6-425) for 24 h or in the corresponding medium described above (T6-425 *tmp1-51*; MB1052 *tmp1-3*). The second stationary culture each was diluted 10^{-2} into the labelling medium R (3 μg Na₂·5'-dTTP/ml; 40 μCi (NH₄)₂·[methyl- ^3H]-5'-dTTP/ml; Amersham, specific activity 25.6 Ci/mmol) (T6-425) or medium N (3 μg Na₂·5'-dTTP/ml; 40 μCi (NH₄)₂·[methyl- ^3H]-5'-dTTP/ml (T6-425 *tmp1-51*) or medium 2 \times N (6 μg Na₂·5'-dTTP/ml; 40 μCi (NH₄)₂·[methyl- ^3H]-5'-dTTP/ml) (MB1052 *tmp1-3*). Inocula were incubated for 3 days.

Abbreviations: 5'-dTTP, deoxythymidine-5'-monophosphate; Thy, thymine; Ura, uracil; Cyt, cytosine; UÜ, uracil-uracil dimer; ÜT, uracil-thymine dimer; TT, thymine-thymine dimer; PC, paper chromatography; TLC, thin layer chromatography.

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* The mating type is put in brackets as the strains have lost it, as judged by unsuccessful forced matings.



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UV-irradiation

The UV-source used was a low pressure mercury lamp (Osram, HNS 12, predominantly 253.7 nm). The incident flux at irradiation was 18.1 erg/mm² as determined from a UV-survival curve of bacteriophage T2r¹². Labelled cells were washed with ice-cold phosphate buffer (0.067 M, pH 7) in a Christ centrifuge and irradiated in 10 ml portions (2.5 × 10⁶ cells/ml) at 0 °C in glass petri-dishes (ϕ 86 mm) under vigorous stirring. Irradiated yeast cells (in total 10⁸ cells per dose) were immediately killed by a 15 min' incubation at 100 °C. After pelleting in a Sorvall centrifuge (10⁴ rpm, 0 °C, 10 min) the cells were transferred into Eppendorf microtubes for pre-hydrolysis treatment.

Pre-hydrolysis treatment

The extraction procedure was that of Unrau *et al.*⁸ (adapted from Williamson¹³). Sometimes the RNase + pronase step was omitted. An evaluation of both procedures for (5'-dTTP) DNA-specific labelling is given in Results.

Formic acid hydrolysis

This was done by incubating extracted samples in 200 µl of 98% formic acid (Merck) at 175 °C for 45 min in sealed evacuated hydrolysis tubes¹⁴.

In vitro preparation of pyrimidine dimers

This was essentially done as described by Beu-
kers and Berends¹⁵ and Unrau *et al.*⁸. For chromatographical separation ascending TLC with solvent system iso-propanol-H₂O (3:1; Greenstock and Johns¹⁶) was employed. The radionuclides used were [¹⁴C]uracil and [¹⁴C]thymine (Amersham; specific activity 1 Ci/mmol).

Chromatography

Ascending TLC on sheets of PEI-cellulose (20 × 20; Macherey & Nagel) was employed. The sheets were pre-washed by running them in H₂O dest., dried in the air and prepared for chromatography: They were cut to 18 cm width and divided into 6 cm bands by pencil. The starting line was drawn 1 cm off the lower edge of a sheet and 18 cm off the lower edge a front canal was scraped (well below the washed out PEI). Samples were spotted in a thin line (3 cm length with 1.5 cm distance to either border of a band) with 5 µl capillaries (Brand). Hydrolysate was never narrowed before spotting. The amount of radioactivity per single run was approx. 3 × 10⁶ dpm. As a standard solvent we used solvent system RB211⁸.

Liquid scintillation counting

After chromatography the sheets were dried in a stream of warm air. Each band was cut into 32 fractions of 0.5 × 4.5 cm and two fractions (enclosing starting line and front canal resp.) of 1.0 × 4.5 cm. Each fraction was further cut into three subfractions of 1.5 cm length and these were transferred into Packard plastic vials for a 15 min' elution with 1.5 ml H₂O dest. under permanent shaking in a New Brunswick Gyrotary shaker at stage 6. Then 15 ml of scintillator were added (120 g naphthalene (Merck, scintillation grade), 4 g PPO (Koch-Light), 50 mg POPOP (Koch-Light) in 1 l of 1,4-dioxane (Merck, reinst); Packard Instructions) were added per vial. Samples were counted in a Packard TRI CARB spectrometer (model 3380) for 5 min each. The external standard ratio (ESR) was co-determined and re-checked twice per sample. The cpm-data were corrected for quench *via* an ESR-counting efficiency calibration curve. The mean efficiency of counting was approx. 10%, the mean counting error for dimer region fractions was <5% and that for the thymine region was 0.2%. For quantitative evaluation of the chromatographical hydrolysate analyses the radioactivity per fraction (dpm) was converted to radioactivity as % of the total radioactivity per run (dpm) after correcting for actual standard background radioactivity (ASB). The ASB (determined from runs of unlabelled cell hydrolysate) was approx. 100 dpm per standard fraction.

For monitoring pre-hydrolysis extraction defined volumes of supernatant each were withdrawn and their dpm contents determined after counting them in 15 ml of the scintillator described. The ASB was determined by counting blank controls containing equivalents of water.

UV-splitting of dimers generated in vivo

2 × 10⁸ labelled cells of strain T6-425 were UV-irradiated in one batch with 10⁵ erg/mm² incident dose. The acid hydrolysate (prepared after employing the full extraction procedure) was routinely chromatographed by ascending RB211-TLC and then the fractions from $R_F = 0.10$ to $R_F = 0.38$ were eluted with 6 ml of H₂O dest. 5 ml of eluate were withdrawn and split into two portions of 2.5 ml. One was made 5% for ethanol and UV-irradiated with 10⁵ erg/mm² incident dose in an aluminium dish (ϕ 38 mm) under stirring; the second remained unirradiated. Both samples were then evaporated to dryness at 60 °C, the remainders each resolved in small amounts of 5% ethanol and the solutions analyzed by ascending RB211-TLC.

Mathematical

Mean values and standard errors as given in Table II were calculated by the formulae

$$\bar{x}_w = \frac{\sum x_i w_i}{\sum w_i} ; s_{\bar{x}_w} = \sqrt{\frac{\sum w_i (x_i - \bar{x}_w)^2}{(n-1) \sum w_i}}$$

for weighted means and for standard errors of weighted means¹⁷. The quotients

$$w_i = \frac{\text{total radioactivity per single run}}{\text{total radioactivity of the single run with highest dpm value}}$$

were used as weighting factors.

Results

In radiochromatographical assays of formic acid hydrolysate of thymine (Thy) labelled UV-irradiated bacterial DNA three significant peaks are found that were identified as $\hat{U}\hat{T}$, $\hat{T}\hat{T}$ and Thy¹⁸. Neither a cytosine (Cyt)-nor a uracil (Ura)-nor a $\hat{U}\hat{U}$ -peak is traceable. The same should be expected for 5'-dTMP labelled yeast. Many solvents have been employed for dimer assay by descending paper chromatography (PC) or ascending thin layer chromatography (TLC)^{8, 16, 19-22}. The solvent system obviously best qualified is solvent RB211⁸: 1. Dimers clearly migrate off the origin with this solvent employed. 2. RB211 clearly separates $\hat{U}\hat{U}$, $\hat{U}\hat{T}$, and $\hat{T}\hat{T}$ from each other. 3. RB211 moves the bulks of non-dimerized pyrimidine well in front of the dimers — with the Thy well separated from Cyt/Ura and distal to the fastest running dimer $\hat{T}\hat{T}$.

Unrau *et al.*⁸ employed RB211 for dimer assay after DNA-unspecific labelling of yeast. Under their conditions the bulk of radioactive Cyt + Ura (+ Thy) strongly trails into the dimer region. In a dimer assay after 5'-dTMP labelling such trailing should be minimal as only a radioactive Thy-peak should be found in front of the dimer-peaks. One could argue that a satisfactory quantitative dimer analysis in yeast not necessarily demands (5'-dTMP) DNA-specific labelling as this is equally well done after UV-unspecific Ura-labelling. But to get the net yield of UV-induced dimers makes necessary a correction for zero-dose background in the dimer region. And such a mathematical correction will find its limits sooner after (Ura) DNA-specific labelling than after (5'-dTMP) DNA-specific labelling. Thus DNA-specific labelling with 5'-dTMP was expected

to allow satisfactory quantitative analysis at UV-doses well below 1000 erg/mm², *i. e.* in a dose range usually employed in biological experiments with yeast.

Pre-hydrolysis treatment of 5'-dTMP labelled yeast

Two methods were evaluated (Materials and Methods). According to the data given in Table I an optimal extraction of non-DNA material⁸ obviously

Table I. Extraction of non-DNA bound radioactivity from 5'-dTMP labelled yeast. 5×10^7 cells of strain T6-425 each were extracted; labelling in medium R ($3 \mu\text{g Na}_2\cdot 5'\text{-dTMP/ml}$; $10 \mu\text{Ci (NH}_4)_2\cdot [\text{methyl-}^3\text{H}]\cdot 5'\text{-dTMP}$) gave 2.2×10^6 dpm per sample to be extracted. Numbers in column 1: step numbers according to Unrau *et al.*⁸.

Step	% radioactivity removed complete extraction	abbreviated extraction
2+3	4.1	3.6
4+5	0.9	—
6+7	0.9	—
8	<0.1	0.1
9+10	1.6	5.2
total	7.6	8.9

must not necessarily include the RNase + pronase step of Williamson¹³. However, Fig. 1 and Table II show that the complete extraction procedure is

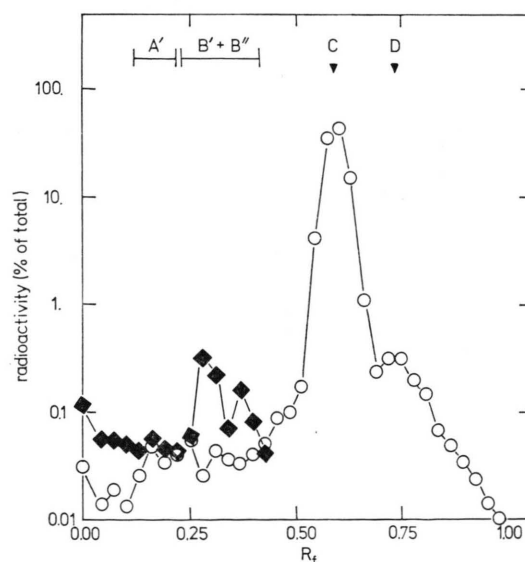


Fig. 1. Radiochromatogram of hydrolysate of unirradiated strain T6-425: Results after ascending RB211-TLC for the samples extracted according to Table I. —○—○—, complete and —◆—◆—, abbreviated pre-hydrolysis extraction. A', B', B'', C, D: see text.

Table II. Evaluation of complete and abbreviated pre-hydrolysis treatment. "c" or "a": complete or abbreviated extraction; index "D": UV-dose 1080 erg/mm², index "0": UV-dose 0 erg/mm². The values given in column 3 are means (Materials and Methods) each for three parallel assays with approx. 3.5×10^6 dpm/run. Columns 3a: Only the zero-dose values for strain T6-425 are measured. The values for the UV-doses 1080 erg/mm² were calculated according to $0.16 (0.57) + |[\text{UT}]_D - [\text{UT}]_0| (|[\text{TT}]_D - [\text{TT}]_0|)$ from columns 3c.

1 Strain	2 UV-irradiation [erg/mm ²]	3 % Radioactivity $\hat{\text{UT}}$ -region (=[UT])	3 % Radioactivity $\hat{\text{TT}}$ -region (=[TT])	4 [UT] _D /[UT] ₀	5 [TT] _D /[TT] ₀
		c	a	c	a
T6-425	0	0.12 ± 0.01	0.16 ± 0.02	0.14 ± 0.01	0.57 ± 0.06
	1080	0.24 ± 0.01	0.28	0.38 ± 0.03	0.81
T6-425 <i>tmp1-51</i>	0	0.10 ± 0.01		0.17 ± 0.01	
	1080	0.24 ± 0.03	0.30	0.48 ± 0.06	0.88
MB1052 <i>tmp1-3</i>	0	0.09 ± 0.01		0.14 ± 0.02	
	1080	0.20 ± 0.03	0.27	0.44 ± 0.06	0.87

superior to the abbreviated one as it more efficiently cleans the dimer region from zero-dose background. Nevertheless, even with an RNase+pronase step involved the region where dimers would be expected after RB211-chromatography is not totally void of radioactive material. Three small peaks can be observed banding there. All of them are reproducible and significantly surpass the standard background of blank runs (already corrected for in Fig. 1 and

Table II). The mean R_F -values found for these zero-dose peaks are $R_F = 0.17$ (A' in $\hat{\text{UT}}$ region), $R_F = 0.26$ and $R_F = 0.33$ (B' and B'' in $\hat{\text{TT}}$ -region). Fig. 2 demonstrates the limits of applicability for both extraction procedures (The values taken as a basis for the composing of Fig. 2 are given in Table II): 1. Employing complete extraction and demanding a ratio of $[\text{UT}]_D$ or $[\text{TT}]_D$ to $[\text{UT}]_0$ or $[\text{TT}]_0$ of 2 will allow working down to UV-doses near 750 erg/mm². Confining oneself to a ratio of 1.5 the limit is given by UV-doses near 500 erg/mm². 2. Employing abbreviated extraction increases the dose limits to approx. 1800 erg/mm² (for a ratio of 2) or to 1100 erg/mm² (for a ratio of 1.5). Here the limits given by Fig. 2b are mandatory. The shallower slope of the lower beam in Fig. 2b as compared to that of the lower beam in Fig. 2a reflects that omission of the RNase+pronase step leads to a stronger increase of peak $B' + B''$ than of peak A' (Fig. 1, Table II).

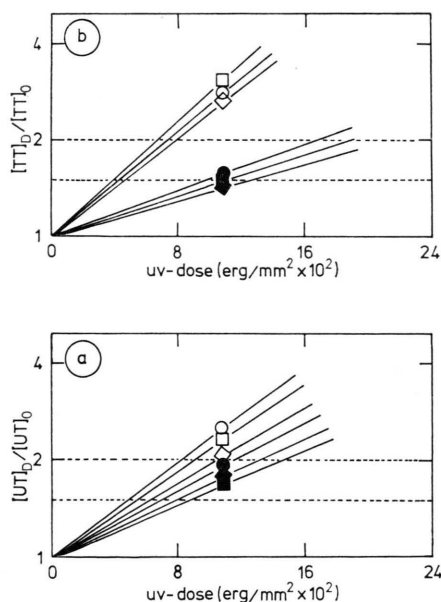


Fig. 2. Limits of applicability of complete or abbreviated pre-hydrolysis extraction. The data given in (a) ($[\text{UT}]_D/[\text{UT}]_0$) and in (b) ($[\text{TT}]_D/[\text{TT}]_0$) are those of Table II, columns 4 and 5 resp. \diamond (\blacklozenge), complete (abbreviated) extraction of strain T6-425; \circ (\bullet), analogs for strain T6-425 *tmp1-51*; \square (\blacksquare), analogs for strain MB1052 *tmp1-3*; broken horizontal lines: $[\text{UT}]_D/[\text{UT}]_0$ and $[\text{TT}]_D/[\text{TT}]_0 = 1.5$ and 2.0, resp.

Chromatography

We preferred to employ ascending RB211-TLC instead of descending RB211-PC as ascending TLC is known to be superior to descending PC at least as far as the time factor is concerned²³. The R_F -values for the components to be resolved were expected to be essentially the same as found by Unrau *et al.*^{8, 16}. Our results with ascending RB211-TLC are summarized in Fig. 3 and Table III: 1. Mostly less than 0.1% of the total radioactivity are found to band in the range from the origin up to the peak A region. Similar amounts each are found for zero-dose hydrolysates and hydrolysates of irradiated yeast. Such retained or very slow mi-

Table III. R_F value comparison of dimers and other pyrimidines for ascending RB211-TLC and descending RB211-PC. 1, ascending RB211-TLC: (a) data of dimer assays of 5'-dTMP labelled yeast; (b) data for dimers generated *in vitro*. 2, descending RB211-PC⁸: (a), data of dimer assays of Ura labelled yeast; (b), data for dimers generated *in vitro*. Asterisks in 1b, 2b: ¹⁴C-labelled moiety. For peak nomenclature see text.

	R_F value			
	1 a	1 b	2 a	2 b
U \hat{U}	—	0.10 (U \hat{U}_4^*)	0.12 (peak A)	0.05–0.12 (U \hat{U}^*)
U \hat{T}	0.20 (peak A)	0.20 (U \hat{T}^* , U \hat{T}^*)	0.22 (peak B)	0.14–0.24 (U \hat{T}^* , U \hat{T}^*)
T \hat{T}	0.31 (peak B)	0.28 (T \hat{T}_1^*)	0.34 (peak C)	0.25–0.36 (T \hat{T}^*)
Ura	—	0.46	—	0.41–0.52
Cyt	—	—	—	0.46–0.60
Thy	0.61 (peak C)	0.61	—	0.58–0.72
peak A'	0.17	—	—	—
peak B'	0.26	—	—	—
peak B''	0.33	—	—	—
peak D	0.74	—	—	—

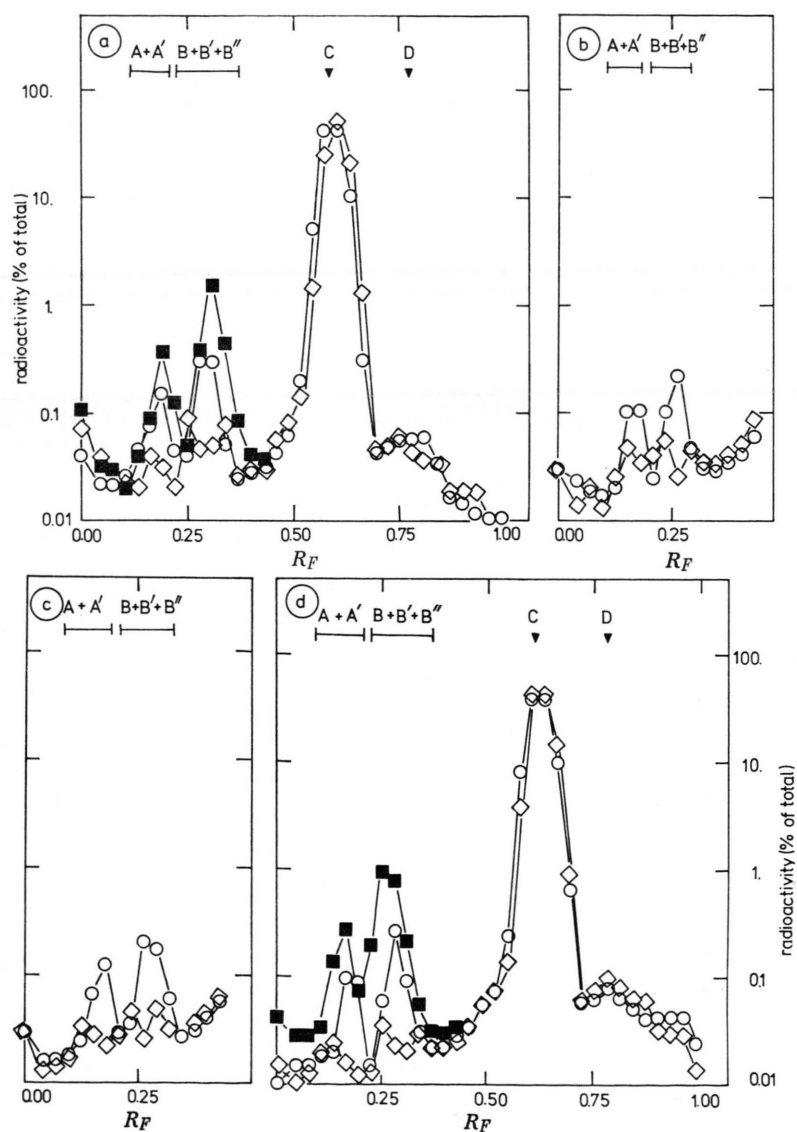


Fig. 3. Radiochromatograms of hydrolysate of UV-irradiated yeast. (a) strain T6-425: Labelling as in Table I, abbreviated extraction; (b), (c), (d) strain T6-425, strain T6-425 *tml-51* and strain MB1052 *tml-3*, resp.: standard labelling; complete extraction. \diamond — \diamond , unirradiated controls; \circ — \circ , UV-dose 2160 erg/mm² in (a) or 1080 erg/mm² in (b), (c), (d); \blacksquare — \blacksquare , UV-dose 8640 erg/mm² in (a) or 8100 erg/mm² in (d). A, A', B, B', B'', C, D: see text.

grating radioactivity is present irrelevant of whether RB211 is used with or without sodium tetraborate in the water-component. 2. The migrating material clearly bands in four peaks designed as A, B, C, and D. The R_F -values of the peaks A, B, and C correspond to those given by Unrau *et al.*⁸ for their peaks B (= $\hat{U}\hat{T}$), C (= $\hat{T}\hat{T}$), and E (= Thy), resp. 3. No peaks are detectable at $R_F=0.10$ or $R_F=0.46-0.53$ where $\hat{U}\hat{U}$ or Cyt/Ura band in descending RB211-PC. This is in agreement with what was expected for our dimer assay. 4. Peak D has an $R_F=0.74$ and is thought to be identical with the shoulder-like peak F close in front of the Thy-peak in the descending RB211-PC of Unrau *et al.*⁸ (The authors did not give an R_F -value for that material.). Setlow and Carrier¹⁸ — performing dimer assay with Thy-labelled bacterial DNA — employed a solvent yielding nearly the same sequence of pyrimidine dimers and non-dimerized pyrimidine as solvent RB211 does. However, it is not clear from their paper whether or not they found such peak D-like material in front of their Thy. As we find that the amount of peak D-material is practically the same both in zero-dose controls and in hydrolysate of UV-irradiated yeast cells (Fig. 3a, Fig. 3d) we think this material not to be any masked cyclobutane type dimer. Hence no attempt was made to further analyze it — nor was this done for the peaks A', B', and B'' found in zero-dose controls.

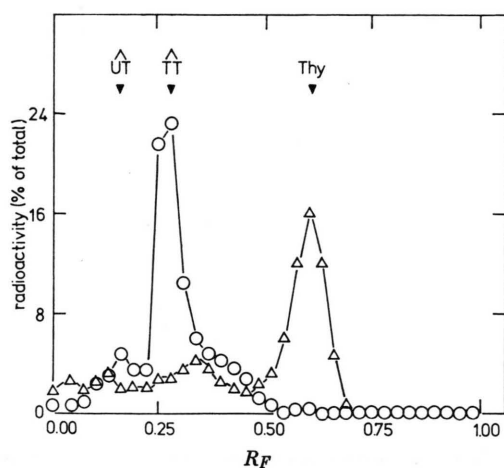


Fig. 4. UV-splitting of peak A+B material (putative $\hat{U}\hat{T}$ and $\hat{T}\hat{T}$). \circ - \circ , unirradiated eluate; \triangle - \triangle , irradiated eluate.

Proofs of dimers

1. A first proof of the peaks' A+B identity with the cyclobutane type dimers $\hat{U}\hat{T}$ or $\hat{T}\hat{T}$ is thought to be given by the above overall R_F -comparison for ascending RB211-TLC and descending RB211-PC.
2. Unrau *et al.*⁸ have shown that their peak B and peak C material is UV-splittable after elution and eliminable after photoreactivation treatment *in vivo*. The same was expected for our peak A and peak B material. Fig. 4 shows that UV-splitting is excellent for both peak A and peak B eluate. The radioactivity remaining in the dimer region for UV-irradiated eluate is thought to be mainly identical with that found in zero-dose control runs as depicted in Fig. 1 and Fig. 3. However, neither peak A- nor peak B-radioactivity is significantly reduced after photoreactivation treatment *in vivo*. This may be explained by the fact that all the strains used show rather a poor photoreactivability of UV-killing their photoreactivable sectors being approx. 0.30.
3. Formic acid hydrolysate of UV-irradiated frozen solutions of [^{14}C]Thy (Thy*) or [^{14}C]Ura (Ura*) or Thy*+Ura or Thy+Ura* were chromatographed in standard manner with solvent iso-propanol-H₂O (3:1; Greenstock and Johns¹⁶). The peaks banding at $R_F=0.20$ ($\hat{U}\hat{U}_4$), $R_F=0.28$ (supposed $\hat{U}\hat{T}$) and $R_F=0.40$ ($\hat{T}\hat{T}_1$) were eluted and re-chromatographed by ascending RB211-TLC. The supposed $\hat{U}\hat{T}$ — isolated as $\hat{U}^*\hat{T}$ or $\hat{U}\hat{T}^*$ — bands at an R_F -value identical with that of peak A; the $\hat{T}\hat{T}_1^*$ bands at an R_F -value congruent with that of peak B (Table III). In reciprocal tests peak A or B eluate were found to band at the R_F -values of the supposed $\hat{U}^*\hat{T}/\hat{U}\hat{T}^*$ or $\hat{T}\hat{T}_1^*$ when run in isopropanol-H₂O (3:1) or at the R_F -values of the $\hat{U}\hat{T}$ - or $\hat{T}\hat{T}$ -peak in solvent n-butanol-acetic acid-water (80:12:30¹⁸).
4. As a further proof the finding may hold that for UV-doses higher than 1000 erg/mm² the yields per dose of the supposed $\hat{U}\hat{T}$ or $\hat{T}\hat{T}$ are essentially the same as by Unrau *et al.*²⁴. Detailed data shall be given in a subsequent paper.

Discussion

Quantitative radiochromatographical assay of thymine-containing dimers UV-induced in (5'-dTMP) DNA-specific labelled yeast was found to be possible down to approx. 500 erg/mm² incident UV-dose. This dose barely allows sufficient resolu-

tion of $\hat{U}\hat{T}$ or $\hat{T}\hat{T}$ from zero-dose background radioactivity in the dimer regions at our standard conditions of dimer assay. Satisfactory quantitative analysis at UV-doses lower than 500 erg/mm² would require at least two alterations of our standard dimer assay: 1. The amount of hydrolysate radioactivity employed per chromatographical run should be adequately increased to maintain sufficiently low error limits at scintillation counting. This could be done either by increasing the specific activity of the 5'-dTTP offer in the labelling medium and/or by increasing the input of labelled hydrolysate per chromatographical run. 2. In addition a procedure of pre-hydrolysis extraction must be available which more efficiently than Williamson's¹³ extraction

procedure eliminates non-dimer radioactivity banding in the dimer regions.

It was shown by a series of control experiments that the radioactive material banding as peak A and B in ascending RB211-TLC is $\hat{U}\hat{V}$ and $\hat{T}\hat{T}$, resp. Unfortunately, the classical proof of pyrimidine dimers, *i. e.* dimer splitting by photolyase²⁵, is not possible *in vivo* with our strains as they show very poor photoreactivability of UV-killing. This characteristic is already typical for strain 211-laM (Fäth, unpublished results) and is not induced by either the *typ*, *tlr* or *tup* mutation.

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