ON THE METABOLIC ROLE OF THYMIDINE 2'-HYDROXYLASE (THYMIDINE, 2-OXOGLUTARATE: OXYGEN OXIDOREDUCTASE, EC 1.14.11.3) IN *NEUROSPORA CRASSA**

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1. Introduction

The 2'-hydroxylation of thymidine was first described by Shaffer et al. [2] in the course of a study of thymine metabolism in cell extracts from a *Neurospora crassa* strain. The product of this reaction is ribosylthymide [3]. Studies of a mutant strain from *Neurospora crassa* devoid of thymine 7-hydroxylase activity [4] as well as protein fractionation studies [4,5] demonstrated that thymidine 2'-hydroxylase (thymidine, 2-oxoglutarate: oxygen oxidoreductase, EC 1.14.11.3) was separate from thymine 7-hydroxylase (thymine, 2-oxoglutarate: oxygen oxidoreductase, (7-hydroxylating), EC 1.14.11.6), an enzyme which catalyzes the sequential oxygenation of thymine to 5-carboxyuracil [6–13]. Both enzymes belong to the group of 2-oxoglutarate-dependent oxygenases, in which oxidative decarboxylation of 2-oxoglutarate is coupled to a hydroxylation [5,10,14–17], a reaction similar to the oxygenation of 4-hydroxyphenyl-pyruvate to homogentisate [18,19].

Thymine 7-hydroxylase appears to be of importance in the sparing of the pyrimidine ring from thymine derivatives in *Neurospora crassa*. However, the biological role of thymidine 2'-hydroxylase has not been defined. *Neurospora crassa* lacks thymidine kinase [20] and thymidine is therefore metabolized solely by degradation. Thymine may be formed from thymidine by a lyase activity, but may also be formed from ribo-sylthymine, the product of thymidine 2'-hydroxylation. No attempt has been made to assess the quantitative importance of thymidine nucleosidase and thymidine 2'-hydroxylase in thymidine degradation [21,22]. Apart from being an intermediate in thymidine degradation, ribosylthymine might be utilized for e.g. nucleotide formation. Thymidine 2'-hydroxylase would then play a biosynthetic role.

A series of mutants with different blocks in the metabolism of thymidine have been characterized by Williams and Mitchell [20]. One of these, the *uc-2* mutant, had been selected on the basis of inability to degrade thymidine. It was inferred that the lacking enzyme was thymidine nucleosidase. In the previous study of thymidine 2'-hydroxylase [3] we remarked on the varying yields of thymidine nucleosidase from different batches of a wild *Neurospora crassa* strain. Of 20 batches grown on different occasions, 7 had low or no detectable thymidine nucleosidase activity. In the other 13, the activity was similar to that of ribosylthymine nucleosidase which was constantly present. This finding raised some doubt as to the biological importance of thymidine nucleosidase, and lack of this enzyme as the cause of low thymidine degrading capacity in the *uc-2* mutant would appear less probable.

We now report data showing that thymidine and ribosylthymine are hydrolyzed by two different enzymes. The metabolic defect in the *uc-2* mutant is, however, low thymidine 2'-hydroxylase activity. The results obtained in the present study indicate that 2'-hydroxylation is the main metabolic pathway for thymidine in *Neurospora crassa*.

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*A preliminary account of this work has been given [1].

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2. Experimental procedures

2.1. Materials

Materials were obtained from the following sources: sodium ascorbate from Dr Theodore Schuchardt GmbH, Munich, West Germany; 2-oxoglutaric acid and catalase from Boehringer und Soehne GmbH, Mannheim, West Germany; dithiothreitol from Calbiochem, Inc., Los Angeles, Calif.; thymine, ribosylthymine, thymidine and thymidylic acid from Cyclo Chemical Division, Travenol Laboratories, Inc., Los Angeles, Calif.; ATP and AMP from P-L Biochemicals, Inc., Milwaukee, Wisc.; EDTA (Titriplex II) from BDH Chemicals, Ltd., Poole, England; bathophenanthroline from G. Frederick Smith Chemical Company, Columbus, Ohio; 1,10-phenanthroline, diethyldithiocarbamate, kieselgel G and GF 254 from E. Merck AG, Darmstadt, West Germany; Sephadex G-25 (coarse) and DEAE-Sephadex A-50 from Pharmacia Fine Chemicals, Uppsala, Sweden; hydroxylapatite and Dowex 2-X8 [Cl$^-$] from Bio-Rad Laboratories, Inc., Richmond, Calif.; [7-$^{14}C_1$]thymine (60 Ci/mol), [2-$^{14}C_1$]thymidine (50 Ci/mol) and [U-$^{14}C_{10}$]thymidine (52 Ci/mol) from The Radiochemical Centre, Amersham, Bucks., England; ribosyl [2-$^{14}C_1$]thymine (59 Ci/mol) from Schwarz/Mann, Orangeburg, N.Y.

2.2. Chromatography

Thin-layer chromatography was carried out on glass plates coated with kieselgel GF 254 or kieselgel G, which had been heated at 105°C for one hour. The mobile phases were, I, benzene/glacial acetic-acid/96% ethanol (4:1:2), and, II, tert-butyl alcohol/ethyl-methyl-ketone/water/25% aqueous ammonia (4:3:2:1). The R_f values for thymine, thymidine, ribosyl-thymine and thymidylate were 0.79, 0.54, 0.48 and 0.06, respectively, in phase 1 and 0.78, 0.68, 0.60 and 0.09 in phase II. The plates were scanned for light absorption at 260 nm in a Zeiss chromatogram scanning photometer M4QII and for radioactivity with a scanner LB 2722 (Labor. Professor Dr Berthold, Wildbad, West Germany).

The 100 000 X g supernatant fractions of mycelial homogenates were desalted on columns of Sephadex G-25 and chromatographed on hydroxylapatite and DEAE-Sephadex A-50 as described previously [4].

2.3. Enzyme preparation

Neurospora crassa strain STA 4 (FGSC 262 A, Fungal Genetic Stock Centre, Humboldt State College, Arcata, Calif.) and the uc-2 and uc-3 mutant strains of Neurospora (obtained from Dr L. G. Williams) were cultured in a salt medium [10]. In the incubations of whole cells with labeled thymidine, small amounts of mycelia were inoculated into 10 ml of culture medium in 25 ml Erlenmeyer flasks, containing 1.2 μCi (20 nmol) of [2-$^{14}C_1$]thymidine and different amounts (0–1 mM) of unlabeled thymidine. The flasks were shaken for 4 days at 26°C. Samples (100 μl) of the medium were taken for determination of radioactivity and for thin-layer chromatography with about 12 h intervals.

When intracellular hydroxylase and nucleosidase activities were studied the mycelia were inoculated into 10 ml medium in 25 ml Erlenmeyer flasks and grown with shaking for 1–4 days at 26°C. The mycelia were harvested and homogenized as described previously [10,12].

2.4. Enzyme assay

Thymine 7-hydroxylase was assayed as described previously [4]. Thymidine 2'-hydroxylase was assayed in the presence of 0.5 mM ferrous sulfate and 5 mM dithiothreitol, otherwise as in [4]. The concentration of thymine and of thymidine were 0.1 mM, i.e. about the value of the apparent K_m. This low concentration was used because of the low activity of thymidine 2'-hydroxylase in the uc-2 mutant.

In the nucleosidase assays, the incubation mixture contained, for thymidine nucleosidase, enzyme (about 0.5 mg of protein from the 100 000 X g supernatant fraction), 1 mM (0.2 μCi) [2-$^{14}C_1$]thymidine and 100 mM potassium phosphate, pH 6.5. For ribosyl-thymine nucleosidase assay, the incubations contained enzyme (about 0.5 mg of protein), 1 mM (0.2 μCi) ribosyl [2-$^{14}C_1$]thymine and 100 mM potassium phosphate, pH 7.5. The total volume was 0.2 ml. After 30 min at 37°C the incubations were stopped by adding 0.4 ml of 96% ethanol in the cold. The labeled thymine was separated from the nucleosides by thin-layer chromatography and the plates were then scanned for radioactivity.

In the studies of phosphate stimulation of the nucleosidase activities, cell extracts were dialyzed over night at 4°C against a suspension of Dowex 2-X8 [Cl$^-$] in 25 mM Tris–HCl buffer, pH 7.5. Potassium phosphate buffers at pH 6.7 and 7.5 respectively were

added to the incubations in concentrations up to
500 mM.

In the attempts to demonstrate a 'ribosylthymine
kinase', the 100 000 $\times$ g supernatant fraction of
homogenized mycelia of the wild *Neurospora* strain
was desalted on columns of Sephadex G-25 (coarse)
in 25 mM Tris–HCl, pH 6.5. The incubation mixture
contained desalted cell extract (about 1 mg of protein),
0.02 mM (0.1 μCi) ribosyl [2-^{14}C$_1$]thymine, 5 mM
ATP, 2.5 mM magnesium chloride and 50 mM Tris–
HCl, pH 8.0, in a total volume of 250 μl. The incuba-
tion mixture for the assay of a 'ribosylthymine phos-
photransferase' contained AMP (5 mM) instead of
ATP, and the pH of the Tris buffer was 7.0. The incu-
bations were stopped after 15 min by heating the test
tubes in boiling water for 3 min. After centrifugation,
25 μl of the supernatant fraction were applied to
thin-layer plates coated with silica gel G, which were
developed in the two systems described above. The
plates were scanned for radioactivity. Detection limit
of product formation was about 1% of the substrate.

3. Results

3.1. Thymidine and ribosylthymine nucleosidases

Fractionation of *Neurospora crassa* strain STA 4
mycelial extracts, which contained both thymidine
and ribosylthymine nucleosidases, by hydroxylapatite
chromatography, fig.1, resulted in complete separation

of the two enzymes. Optimum assay conditions for
thymidine and ribosylthymine nucleosidases were
studied after partial purification by this technique.
The pH-optimum was 6.5 for thymidine nucleosidase
and 7.5 for ribosylthymine nucleosidase. The apparent
K_m values, determined in two experiments were
0.6–0.8 mM and 0.2 mM, respectively. Addition of
magnesium sulfate as well as of a number of transition-
metal salts had no effect on enzyme activities, nor had
the addition of any of the metal ion chelators, EDTA,
1,10-phenanthroline, bathophenanthroline or diethyl-
dithiocarbamate. The enzyme activity was constant
during 30 min of incubation at 37°C. The enzymes were
fairly heat stable, no significant decrease in enzyme
activity was noted when the cell extracts had been
kept at 42°C for 60 min before assay.

Thymidine nucleosidase was stimulated 4-fold by
100 mM phosphate. The stimulation of ribosylthymine
nucleosidase by phosphate was only slight, about 20%.
Incubations with uniformly labeled thymidine were
assayed for the presence of labeled deoxyribose phos-
phate in order to clarify whether the enzyme catalyzing
cleavage of thymidine was a phosphorylase or a
hydrolase. Only labeled deoxyribose was found.

3.2. Thymidine degradation in the uc-2 mutant

Results from a series of incubations of cells from
the *uc-2* mutant strain as well as from the wild strain
together with labeled thymidine confirmed the results
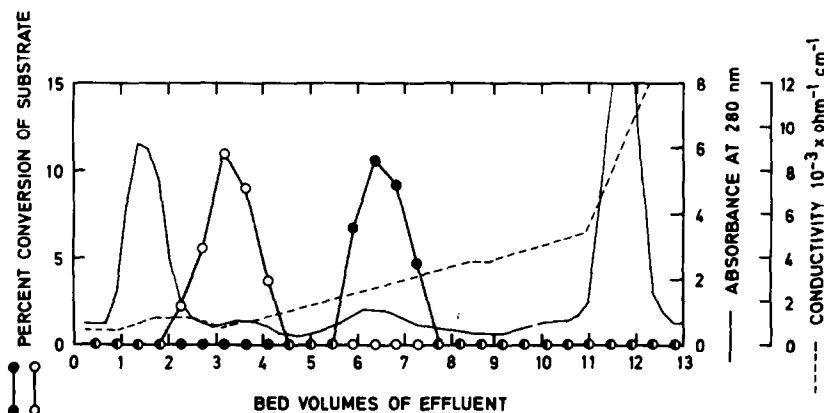obtained by Williams and Mitchell [20] that the *uc-2*

Fig.1. Chromatography of thymidine (●——●) and ribosylthymine (○——○) nucleosidase from 100 000 $\times$ g supernatant of mycelial
homogenates from *Neurospora crassa* STA 4 on a column (32) of hydroxylapatite. The column was eluted with a linear gradient
of 1–80 mM potassium phosphate buffer at pH 6.5, followed by a gradient of 80–500 mM of the same buffer.
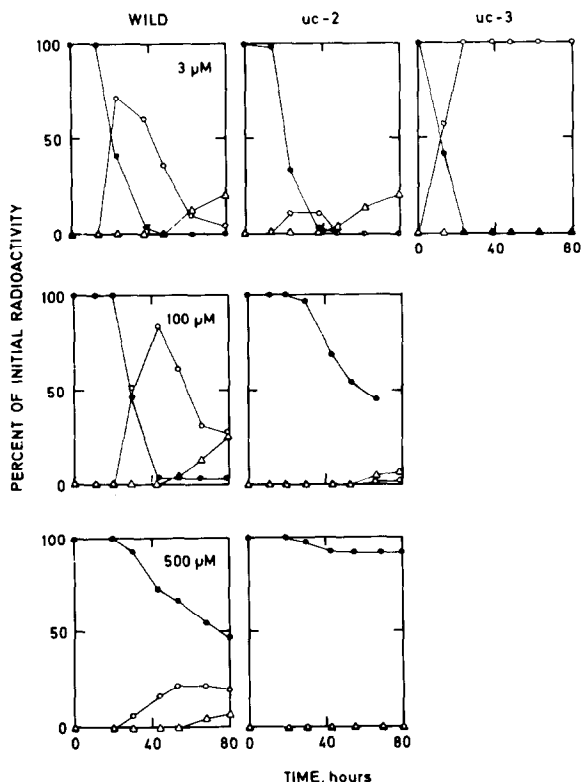
Fig.2. Incubations of whole cells from the wild strain of
Neurospora the *uc-2* and the *uc-3* mutants. The cells were
incubated with labeled thymidine in the following initial
concentrations: 3 μM (upper part), 100 μM (middle part)
and 500 μM respectively (lower part). The medium was
analyzed for labeled thymidine (●——●), thymine (○——○)
and polar metabolites (△——△) (See Experimental Procedures).

mutant had decreased capacity for thymidine degra-
dation. The medium was analyzed for labeled thymi-
dine, ribosylthymine, thymine and for polar meta-
bolites. Figure 2 shows some of the results obtained.
No difference between the incubations of the wild
strain and those with the *uc-2* mutant was demonstrat-
ed in the rate of decrease of thymidine concentration
when low concentrations of thymidine were used.
However, the amounts of thymine in the medium were
much higher with the wild strain than with the *uc-2*
mutant. After 45 h of incubation of the wild strain
with 100 μM [2-^{14}C$_1$]thymidine, about 5% of the initial
amount of radioactivity was recovered in thymidine

and 85% in thymine. After 45 h with the *uc-2* mutant
strain, 70% of the initial amount of radioactivity was
recovered in thymidine, whereas no thymine was
found. After 80 h of growth, the amount of radio-
activity presumed to be nucleotides or polynucleotides
was 4 times higher with the wild strain than with the
uc-2 mutant. A progressive decrease in thymidine
degradation was noted with higher concentrations of
thymidine in the growth medium. Labeled thymine
could be demonstrated in the medium only in the
incubations with the wild strain. No labeled ribo-
sylthymine was found in the medium in any of these
experiments.

In incubations of thymidine with the *uc-3* mutant,
which lacks thymine 7-hydroxylase activity [4], there
was a complete conversion of thymine. This was the
only labeled compound found in the medium after
80 h.

3.3. *Thymidine 2'-hydroxylase activity in wild and mutant* Neurospora *strain*

In a series of experiments, the wild strain and the
uc-2 mutant strain were grown for one to four days.
Cells were then extracted and assayed for activity of
thymine and thymidine hydroxylases as well as for
thymidine and ribosylthymine nucleosidases. There
was no difference between the two strains as regards
pH in the medium, weight of mycelia formed or
amount of protein in the mycelial extracts. Table 1
shows the results obtained. The specific activity of
thymine 7-hydroxylase in the wild strain was essentially
independent of the time of culture, whereas it
increased in the *uc-2* mutant. Ribosylthymine nucleo-
sidase activity which was higher in the *uc-2* mutant,
decreased with age of the culture. In none of these
experiments could thymidine nucleosidase activity be
demonstrated. There was a pronounced difference in
the activity of thymidine 2'-hydroxylase between the
two strains: it was lower, but significant, in the *uc-2*
mutant. The same difference was obtained when the
cell extracts after 4 days of growth were assayed after:
(1) dialysis against 10 mM potassium chloride, pH 6.5
(2) hydroxylapatite chromatography (3) DEAE
Sephadex chromatography and (4) isoelectric focusing
in sucrose gradients [3]. The results indicate that
there was no inhibitor responsible for the low
thymidine 2'-hydroxylase activity in the *uc-2* mutant
strain.

Table 1

Activities of thymine and thymidine hydroxylases and of thymidine and ribosylthymine nucleosidases in mycelial extracts (100 000 × g supernatant fractions of homogenates) of Neurospora crassa strain STA 4 and uc-2

Neurospora strain	Time of culture	pH of medium	Mycelia wet wt	Alkali soluble protein	Extract protein concn	Thymine 7-hydroxylase	Thymidine 2'-hydroxylase	Thymidine nucleosidase	Ribosylthymine nucleosidase
	Days		g	mg	g/l	Enzymic activity nmol.min^{-1}.mg^{-1}			
STA 4	1	4.8	7.4	85	29	0.21	0.07	< 0.09	3.4
(Wild)	2	4.3	23	233	36	0.12	0.22	< 0.07	1.7
	3	4.1	35	235	25	0.27	0.37	< 0.11	1.9
	4	4.1	39	192	21	0.29	0.53	< 0.13	1.6
uc-2	1	4.8	6.4	106	36	0.09	0.01	< 0.07	8.4
(Mutant)	2	4.2	17	225	45	0.10	0.01	< 0.06	4.5
	3	3.9	37	223	21	0.30	0.07	< 0.13	2.2
	4	4.0	44	337	18	0.36	0.07	< 0.15	1.8

The respective labeled substrate was incubated with enzyme and cofactors as described under Experimental Procedures. After deproteinization, the incubation mixture was fractionated by means of thin-layer chromatography on silica gel G. The plates were scanned for radioactivity. No thymidine nucleosidase activity was found; thymidine nucleosidase activity was of the same order of magnitude as that of ribosylthymine nucleosidase in those batches from the wild strain which demonstrated both these activities. The figures given are calculated from the detection limit of 1% for product formation.

3.4. *Attempts to demonstrate formation of ribosyl-thymine phosphate*

These experiments were carried out under the conditions used by Bresnick [23] for thymidine kinase with ATP as phosphate donor and by Arima et al. [24] for nucleoside phosphotransferase with AMP as donor. About 25% of the ribosylthymine was hydrolyzed during these experiments. There was no formation of labeled metabolites other than thymine from labeled ribosylthymine of high specific radioactivity.

4. Discussion

Thymidine nucleosidase activity could be demonstrated in a number of cultures of the wild *Neurospora* strain. It differed from ribosylthymine nucleosidase in chromatographic properties and pH optimum. Both nucleosidases were stimulated by phosphate, but the stimulation was more marked in the case of thymidine nucleosidase. In neither case was there an absolute requirement for phosphate, indicating that the reactions are hydrolytic and not phosphorolytic. However, in other cultures of the same strain, no or low thymidine nucleosidase activity was found.

We cannot afford any explanation for the different yields of thymidine nucleosidase. However, thymidine degrading capacity was significant also when no thymidine nucleosidase was found. In the experiments carried out with the *uc-2* mutant, we found no thymidine nucleosidase: an explanation for the low capacity for thymidine degradation in this mutant is, however, afforded by the finding of a low thymidine 2'-hydroxylase activity, about one-tenth of that found in the wild strain. That this enzymic reaction became rate limiting in the metabolism of thymidine is indicated by the finding of much lower content of labeled thymine in the medium from the *uc-2* incubations than from those with the wild strain.

In whole-cell experiments, thymine, but not ribosylthymine was found as an intermediate in the degradation of thymidine. The absence of ribosylthymine is probably a result of the highly active ribosylthymine nucleosidase. As discussed above, higher formation rate of thymine rather than lower degradation rate is the reason for the much higher content of thymine in the incubations with the wild strain than in those with

the *uc-2* mutant. Attempts to demonstrate formation of a nucleotide from ribosylthymine were unsuccessful. The available data thus indicate that ribosylthymine is solely an intermediate in the break-down of thymidine.

The metabolic 'block' in the *uc-2* mutant of thymidine 2'-hydroxylation was a moderate one permitting a low but significant rate in this metabolic step. In the incubations of the *uc-3* mutant strain with labeled thymidine, thymine accumulated in the medium. This finding is in good agreement with the finding of absence of thymine 7-hydroxylase activity in this mutant [4].

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