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SUBSTRATE SPECIFICITY OF THE HYDROXYLASE REACTION IN WHICH THYMIDINE IS CONVERTED TO THYMINE RIBONUCLEOSIDE

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SUMMARY

1. A partially purified enzyme preparation from *Neurospora crassa*, which had previously been reported to convert thymidine to thymine ribonucleoside, catalyzes the conversion of deoxyuridine to uridine but not that of deoxycytidine, deoxyadenosine or deoxyguanosine to the corresponding ribonucleoside.

2. The conversion of deoxyuridine to uridine, like that of thymidine to thymine ribonucleoside, occurs without detachment of the deoxyribose moiety.

3. Since neither deoxyuridylic acid nor deoxyribose were hydroxylated, the enzymatic 2'-hydroxylation reaction appears to be specific for substrate at the nucleoside level.

4. A requirement for oxygen is suggested by the inhibition of the conversion of thymidine to thymine ribonucleoside by exclusion of air from the incubation mixture.

INTRODUCTION

A partially purified hydroxylase from *Neurospora crassa* converted thymidine to thymine ribonucleoside without detachment of the deoxyribose moiety[1]. The same enzyme preparation catalyzed the conversion of thymine to 5-hydroxymethyluracil, but these hydroxylations appear to be catalyzed by different enzymes[2]. Both enzymes may be involved in the pathway by which thymidine is converted to the pyrimidines of RNA, a pathway which is thought to include the oxidative demethylation of thymine to yield uracil[3]. Since deoxycytidine is incorporated into RNA to about the same extent as is thymidine[3] it was suspected that deoxycytidine would also serve as a substrate for the hydroxylase reaction. However, as described in a preliminary report[4], the partially purified enzyme preparation which hydroxylated thymidine did not hydroxylate deoxycytidine but instead deaminated it yielding deoxyuridine. The deoxyuridine thus produced was converted to uridine, which in turn formed uracil. That report is documented in the present paper.

Abbreviations: dUMP, deoxyuridylic acid; UMP, uridylic acid; dUrd, deoxyuridine; Urd, uridine; Ura, uracil; dThd, thymidine; Thd, thymine ribonucleoside; Thy, thymine; dCyd, deoxycytidine; Cyd, cytidine; hmUra, 5-hydroxymethyluracil; fUra, 5-formyluracil.

MATERIALS AND METHODS

Source of materials

The specific activities to which radioactive compounds were adjusted and the sources from which they were purchased are the following: [2-^{14}C]thymidine 3.0 mC/mmole, [2-^{14}C]-deoxyuridine 3.0 mC/mmole, [2-^{14}C]deoxycytidine 4.0 mC/mmole, [8-^{14}C]deoxyadenosine 3.0 mC/mmole, New England Nuclear; [5-^{3}H]dUMP 16.5 mC/mmole, [8-^{3}H]deoxyguanosine 16.5 mC/mmole, Schwarz Bioresearch, Inc.; [2-^{14}C]uracil 3.0 mC/mmole, [2-^{14}C]thymine ribonucleoside 3.0 mC/mmole, Calbiochem; 2-dexoy-D-[1-^{14}C]ribose 3.0 mC/mmole, Amersham/Searle. 5-Hydroxymethyluracil and 5-hydroxymethyldeoxyuridine, were purchased from Calbiochem, 5-hydroxymethyluridine from Cyclo Chemical.

The calcium phosphate gel eluant was prepared from *Neurospora crassa* strain 1A (wild type) by a procedure[2] which involved the selective adsorption of the 2'-hydroxylase activity on to the gel, the rinsing of the thus formed gel–protein mixture with water and the selective elution of the hydroxylase activity from the gel. The calcium phosphate gel eluant was concentrated with ammonium sulfate[2] and subsequently passed through a Sephadex G-25 chromatography column[1] which had been equilibrated with 0.05 M Tris–HCl, pH 8.0.

In the assay procedure a 0.1-ml aliquot of the enzyme preparation was pipetted into a 10 mm × 75 mm test tube which contained the substrate and cofactors in 0.1 ml of 0.05 M Tris–HCl, pH 8.0. The resultant standard incubation mixture contained 0.5 mM α-ketoglutarate, 1.0 mM ascorbate, 0.5 mM $FeSO_4$, 1 mM GSH and 0.25 mM radioactive substrate. The concentrations listed for the ascorbate and GSH concentrations in the standard incubation mixture do not take into account the amounts of these compounds which were added to enzyme preparations during the purification procedure. The testing of radioactive substrates for the hydroxylation reaction was carried out in the presence and absence of various nonradioactive compounds which were added to the incubation mixtures in order to detect the formation of possible transient intermediates. Each of these trapping agents were tested at a minimum of two concentrations which were usually 0.5 and 4 times the concentration of the substrate.

After addition of the enzyme preparation, the uncapped test tube was placed in a Dubnoff incubator and shaken at 32°. The enzymatic reaction was usually stopped by heating it to 100° for 3 min. Following removal of the coagulated protein, appropriate nonradioactive compounds were added as chromatographic markers to the supernatant fluid. Two dimensional paper chromatography[5] was carried out at 28° on an aliquot of this supernatant containing from 0.2 to 0.8 μmole of each of the marker compounds. When two-phase solvent systems were used to develop the chromatograms the aqueous phase was placed in a 20-ml beaker within the chromatography jar. The solvent systems used for development have previously been described[6].

The separated pyrimidines, purines and their derivatives on the developed chromatograms were outlined under ultraviolet light. The positions of ribose and deoxyribose were determined with an aniline hydrogen phthalate spray[7]. In order to make radioactivity measurements portions of the chromatograms which contained the separated compounds were either centered under a thin-windowed Geiger tube or cut out, added to scintillation fluid and placed in a Packard Tri-Carb scintillation

counter at 0° with the filter paper lying flat on the bottom of the vial[2]. Duplicate assays of a given enzyme preparation usually agreed within ±10% of the mean. The position of radioactive compounds was determined by radioautography.

To demonstrate the oxygen requirement the Thunberg technique was employed. The tubes were evacuated (2 mm Hg), filled (700 mm Hg) with either air, oxygen or nitrogen which had previously passed through a solution of 0.1 M vanadyl sulfate containing a Zn–Hg amalgam[8], evacuated again and refilled with the appropriate gas.

RESULTS

The time course of the conversion of deoxyuridine to uridine and its subsequent conversion to uracil by a calcium phosphate gel enzyme preparation is depicted in

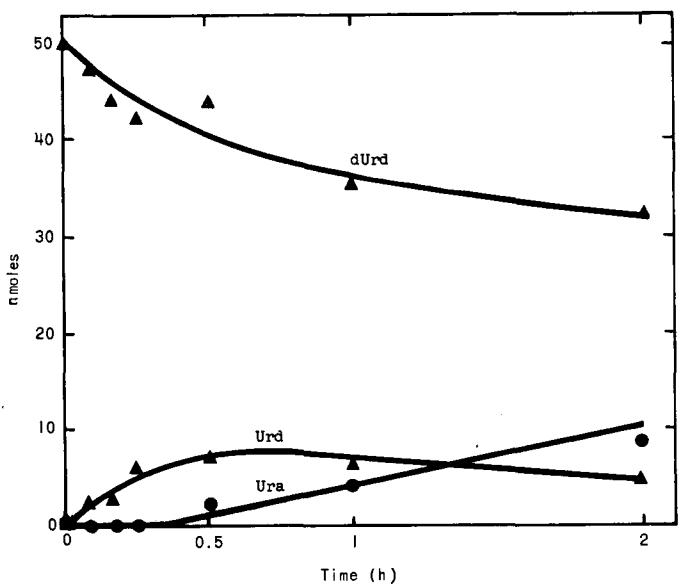

Fig. 1. Time course of deoxyuridine decomposition and product formation. The standard incubation mixture contained [2-^{14}C]deoxyuridine and a calcium phosphate gel enzyme preparation (1.8 mg of protein per ml of incubation mixture). The radioactive products of the deproteinized incubation mixture were separated with paper chromatography and their radioactivity was measured as described in MATERIALS AND METHODS.

Fig. 1. The inclusion of nonradioactive uracil in the incubation mixture at concentrations up to 10 times that of the substrate had no detectable effect on the hydroxylation reaction. Standard incubation mixtures which contained [2-^{14}C]uracil as substrate, in the presence and absence of nonradioactive deoxyuridine, formed no radioactive uridine when incubated for 1 h.

The results of kinetic studies in which [2-^{14}C]thymidine was used as substrate are shown in Fig. 2. The appearance of thymine ribonucleoside preceded that of

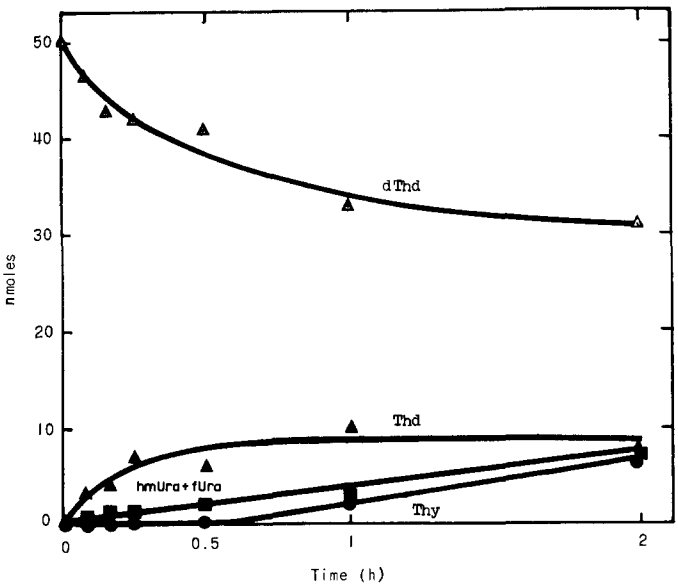

Fig. 2. Time course of thymidine decomposition and product formation. The standard incubation mixture contained [2-^{14}C]thymidine and a calcium phosphate gel enzyme preparation (1.8 mg of protein per ml of incubation mixture).

thymine*. That thymine was formed by the hydrolysis of thymine ribonucleoside and not of thymidine was also suggested by the observation that little or no thymine or thymine ribonucleoside was formed in standard incubation mixtures from which α-ketoglutarate had been omitted. Similarly the replacement of the air of the incubation mixture with nitrogen inhibited the production of both thymine and thymine ribonucleoside (Fig. 3). In control runs it was shown that replacement of the air of the incubation mixture with oxygen had no effect.

It appeared that the methyl group of the deoxyribonucleoside or of the ribonucleoside of thymine could not be hydroxylated by this enzyme preparation since incubation mixtures which contained these nucleosides as substrates yielded no detectable 5-hydroxymethyldeoxyuridine or 5-hydroxymethyluridine. To further test for the formation of these compounds [2-^{14}C]thymidine was incubated in the presence of nonradioactive 5-hydroxymethyldeoxyuridine, and likewise, [2-^{14}C]thymine ribonucleoside was incubated with nonradioactive 5-hydroxymethyluridine. No detectable 5-hydroxymethyldeoxyuridine or 5-hydroxymethyluridine was produced in these trapping experiments which were carried out with enzyme preparations highly active in catalyzing the conversion of thymidine to thymine robonucleoside and of thymine and 5-hydroxymethyluracil. While thymine ribonucleoside was not hydroxylated, it was readily hydrolyzed (Table I). As anticipated, this reaction was not dependent on the inclusion of α-ketoglutarate, ascorbate or Fe(II) in the incubation mixture. Seventy per cent of the hydrolase activity was recovered when the calcium

* While with some enzyme preparations 5-hydroxymethyluracil appeared to be initially formed at a rate which prevented thymine from accumulating, in experiments carried out with other calcium phosphate gel enzyme preparations no 5-hydroxymethyluracil was detected until thymine had accumulated in the reaction mixture.

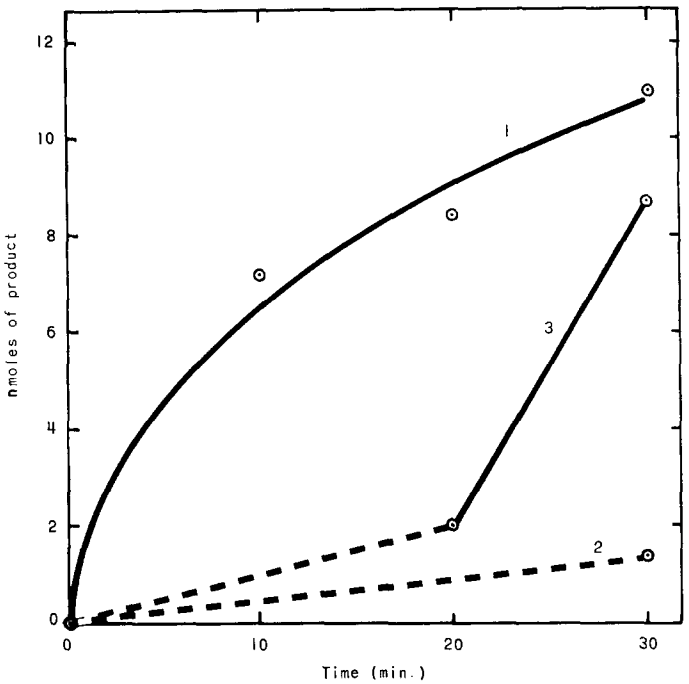

Fig. 3. The effect of air on the conversion of thymidine to product. The standard incubation mixture which contained [2-^{14}C]thymidine and a calcium phosphate gel enzyme preparation (2.5 mg of protein per ml of incubation mixture) was incubated in a Thunberg tube in the presence of air (Curve 1); in the presence of nitrogen (Curve 2); initially with nitrogen and then with air (Curve 3).

TABLE I

THE EFFECT OF COFACTORS ON INCUBATION MIXTURES WITH THYMINE RIBONUCLEOSIDE AS SUBSTRATE

The calcium phosphate gel enzyme preparation (4.0 mg of protein per ml of incubation mixture) was incubated with [2-^{14}C]thymine ribonucleoside for 30 min at 32°. A, standard incubation mixture except for the omission of α-ketoglutarate, ascorbate and Fe(II). B, standard incubation mixture.

Incubation mixture	*Product (nmoles)*		
	(a) Thymine	*(b) hmUra + fUra*	*(a + b)*
A	11.3	0.27	11.6
B	1.9	7.7	9.6

phosphate gel preparation was concentrated with ammonium sulfate and then passed through a Sephadex G-25 chromatography column. It is noteworthy that the crude extract was equilibrated with 1.0 mM EDTA prior to being subjected to the calcium phosphate gel fractionation procedure[2]. Unlike deoxyuridine and thymidine, deoxycytidine did not serve as a substrate for the hydroxylase reaction. When [2-^{14}C]deoxycytidine was incubated with the calcium phosphate gel enzyme preparation in kinetic

studies which were carried out in the presence and absence of pools of unlabeled cytidine, the formation of cytidine was not detected. It can be seen in Fig. 4 that deoxycytidine was deaminated to form deoxyuridine. This compound was then hydroxylated to yield uridine, which in turn was converted to uracil.

Incubation of [8-^{14}C]deoxyadenosine and [8-^{3}H]deoxyguanosine, at concentrations of 0.25 mM and 1.0 mM, with the calcium phosphate gel enzyme preparation for 2-h periods resulted in the formation of no radioactive adenosine or guanosine, respectively. Negative results were also obtained when the above incubations were repeated in the presence of the corresponding unlabeled ribonucleosides. While no hydroxylation of the purine deoxyribonucleotides was detected, it appeared that deamination occurred. After 2 h of incubation 8% of the deoxyadenosine was converted to a compound with the same R_F value as deoxyinosine. 10% of deoxyguanosine was converted after 2 h of incubation to an unidentified compound.

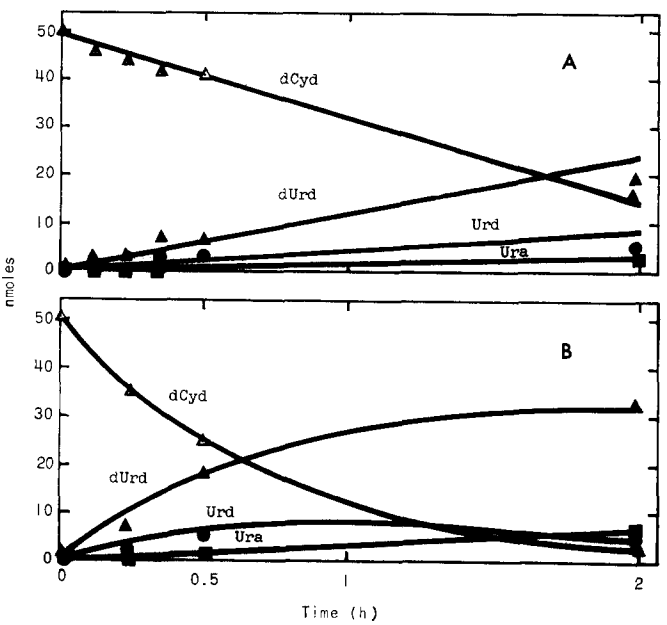

Fig. 4. Time course of deoxycytidine decomposition and product formation. The calcium phosphate gel enzyme preparation (1.8 mg of protein per ml of incubation mixture) was incubated with [2-^{14}C]deoxycytidine. A. Standard incubation mixture except for the addition of non-radioactive cytidine (2.5 mM). B. Standard incubation mixture.

To determine if deoxyribose could be hydroxylated by the calcium phosphate gel preparation, [1-^{14}C]deoxyribose was incubated in the presence and absence of nonradioactive ribose. No ribose was generated in these incubation mixtures. Similarly, to determine if a deoxyribonucleotide could serve as a substrate for the hydroxylase reaction, [5-^{3}H]deoxyuridylic acid was tested. No evidence for this reaction was obtained in experiments which included using unlabeled uridylic acid as a trapping agent.

DISCUSSION

The 2′-hydroxylation reaction occurs at the nucleoside level with only the uracil and thymine deoxyribonucleosides of the common pyrimidine deoxyribonucleosides and, apparently, not with purine deoxyribonucleosides. These substrate specificity requirements are in contrast to those observed for that which is in part the reverse of this reaction, the reduction of both purine and pyrimidine ribonucleoside diphosphates and triphosphates[9].

Even though the methyl group of thymine is readily hydroxylated[2], that of thymidine or of thymine ribonucleoside is not. This suggests that the pathway through which the oxidative demethylation of thymidine occurs[3] is one in which pyrimidines and not their nucleosides are intermediates. Enzymatic reactions which oxidize thymine[2], 5-hydroxymethyluracil[10] and 5-formyluracil[11] and decarboxylate uracil 5-carboxylic acid[12] have been demonstrated. However, except for thymine, the nucleosides of these compounds have not been tested as substrates nor has Neurospora been examined for additional enzymes which are not active in the purified enzyme preparations used.

The demonstration that oxygen is required as well as α-ketoglutarate, ascorbate and Fe(II)[1] is in accord with a mechanism[13–15] in which a peroxide anion of thymidine makes a nucleophilic attack on the carbonyl carbon of α-ketoglutarate so that succinate, CO_2 and thymine ribonucleoside are produced. The α-ketoglutarate dependent oxygenations of thymine[2,16], 5-hydroxymethyluracil[10] and 5-formyluracil[11] appear to proceed by similar mechanisms[17]. In preliminary studies of the enzymatic hydroxylation of thymidine, thymine ribonucleoside and CO_2 have been shown to be produced in a 1:1 molar ratio and succinate has been identified as a product.

ACKNOWLEDGEMENT

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