THE CELL-FREE CONVERSION OF A DEOXYRIBONUCLEOSIDE TO A RIBONUCLEOSIDE WITHOUT DETACHMENT OF THE DEOXYRIBOSE

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A cell-free preparation has been obtained from Neurospora crassa which catalyzes the conversion of thymidine to thymine riboside. This oxidation occurs at the deoxyribonucleoside level and is dependent on the presence of Fe(II), ascorbate and α-ketoglutarate.

While the enzymatic reduction of ribonucleoside diphosphates and triphosphates to their corresponding deoxyribonucleoside phosphates has been much studied (1), little is known of the reverse reaction. Results obtained in experiments with intact Ehrlich ascites cells have suggested that there is some direct transformation of deoxyuridine into the uridine of RNA without detachment of the deoxyribose (2). In substrate specificity studies with partially purified preparations of thymine 7-hydroxylase it was found that the methyl groups of neither thymidine nor 5-methylcytosine were hydroxylated (3). However, when incubation mixtures containing thymidine as substrate were incubated for longer periods of time or with more concentrated enzyme preparations, the formation of thymine riboside was observed. This paper concerns the identification of thymine riboside and characterization of the reaction generating it.

Materials and Methods. Thymidine-2-C^{14} and deoxyuridine-2-C^{14} were purchased from New England Nuclear Corporation, and each was adjusted to a specific activity of 3mc per mmole. Thymidine which was uniformly enriched with respect to C^{14} (specific activity, 3 mc/mmole) and which contained H^3 only in the pyrimidine ring (specific activity, 6 mc/mmole) was prepared from thymidine-6-H^3(n) [code number, TRA.61] and thymidine-C^{14}(U) [code number, CFB-77] both of which were purchased from Nuclear-Chicago. Nonradioactive thymine riboside, 5-hydroxymethyldeoxyuridine, other nucleosides and pyrimidines were obtained from Calbiochem.

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The calcium phosphate gel fraction was prepared from Neurospora crassa strain 1A (wild type) as previously described (4). A saturated aqueous solution of ammonium sulfate was rapidly stirred into this fraction until it was 75% saturated with respect to ammonium sulfate, and the resulting precipitate was immediately removed by centrifugation at 10,000 x g for 10 min. The precipitate was dissolved in a volume of 0.05M Tris-HCl (pH 8.0) so that the protein concentration was 5 mg per ml. In some of the experiments discussed in this paper the calcium phosphate gel fraction or the ammonium sulfate preparation was subjected to Sephadex G-25 chromatography as previously described (5) using a column which was equilibrated with 0.05M Tris-HCl (pH 8.0) containing 0.05 mM EDTA. The temperature range at which the above procedures were carried out was 0 to 4°.

The standard incubation system was prepared by adding 0.1 ml of the enzyme preparation to a 10 mm x 75 mm test tube containing 0.1 ml of 0.05 M Tris-HCl (pH 8.0). This aliquot of buffer contained radioactive thymidine, 50 mμmoles; $FeSO_4$, 100 mμmoles; α-ketoglutarate, 100 mμmoles; and ascorbate, 200 mμmoles. In the assay procedure the mixture was shaken in a Dubnoff incubator at 30° in the presence of air. The reaction was stopped by adding 4 volumes of absolute ethanol. Following removal of the coagulated protein by centrifugation, chromatography markers were added to an aliquot of the supernatant fluid which was subjected to two-dimensional ascending chromatography (6). When two phase solvent systems were used, the aqueous phase was placed in a 20-ml beaker within the chromatographic jar. The solvent systems (7) most used in the present study were "Form/EtAc," "EtAc/form," "S & T/Bu" and "Bor/s-Bu" (8): 200 ml of 5% boric acid was equilibrated with 200 ml of sec-butyl alcohol; 50 ml of the resultant upper phase was poured into the chromatography jar; the 20-ml beaker contained 10 ml of the lower phase plus 1 ml of 2.5 M ammonium hydroxide. This system was allowed to equilibrate overnight prior to developing the chromatogram. The "Bor/s-Bu" solvent was the initial one employed for both dimensions of the chromatography. However, thymidine and thymine had identical R_f values in this system, i.e., 0.76 at 28°, and thus the spot containing these components was cut out, extracted with 80% ethanol and rechromatographed. Good separation of these compounds was achieved using "Form/EtAc" in the first dimension and "EtAc/form" in the second. The resulting thymine riboside spot (R_f 0.39) occasionally contained some radioactive 5-formyluracil (R_f 0.43) or D-ribose (R_f 0.27). Extraction and rechromatography in two dimensions utilizing the "S & T/Bu" solvent yielded thymine riboside with no detectable radiochemical impurity.

Simultaneous measurements of H^3 and C^{14} were carried out in a Packard Tri-Carb scintillation counter. A portion of the filter paper chromatogram

containing the radioactive compound was cut out and dropped into a sample vial containing 3 ml of water. The vial was shaken and 14 ml of a dioxane, scintillator solution (9) was added. The mixture was inverted several times and placed in the counter at 0° with the filter paper lying flat on the bottom of the vial. Counting efficiency of the system was 38% for C^{14} and 3.2% for H^3 beta particles. The quenching effects of the samples were insignificant as judged by remeasurement of each sample after addition of a H^3 standard and again after the addition of a C^{14} standard. Prior to the cutting out of the chromatographic spots, C^{14} measurements were also made with a thin-windowed Geiger tube as previously described (10). Duplicate assays of a given enzyme preparation usually agreed within ±10% of the mean. The position of C^{14} labeled compounds was determined by radioautography.

Thymine riboside was hydrolyzed with 12N perchloric acid under the conditions described by Marshak and Vogel (11).

Results and Discussion. When thymidine-2-C^{14} was incubated in the standard incubation system containing the calcium phosphate gel fraction for 2 hr, the formation of radioactive thymine and 5-hydroxymethyluracil was observed, but a large discrepancy was noted by comparing the amounts of thymidine utilized and products formed. Radioautography revealed that the difference was explained by the formation of a new radioactive compound which was eventually identified as thymine riboside. Chromatography of this new compound in 10 diverse solvent systems (7) always resulted in its radioactivity coinciding exactly with the authentic thymine riboside added as a marker. These solvent systems separated thymine riboside from other potential intermediates such as 5-hydroxymethyldeoxyuridine, 5-hydroxymethyluridine and dihydrothymidine. Preliminary kinetic studies indicated that the standard incubation system initially converted thymidine to thymine riboside which was in turn hydrolyzed to thymine and ribose; the thymine was then converted to 5-hydroxymethyluracil which, if the incubation period were long enough, was oxidized to 5-formyluracil. No thymine riboside was formed in control incubation mixtures in which the enzyme preparation was replaced with buffer or denatured with heat or acid as previously described (4).

The identity of the new compound was further investigated utilizing the product of the standard incubation system which contained the calcium phosphate gel fraction and thymidine-2-C^{14} as substrate. After 2 hr of incubation the reaction mixture contained 50% of its radioactivity as thymine riboside, 37% as thymine, 13% as 5-hydroxymethyluracil and no detectable thymidine or 5-formyluracil. Authentic, nonradioactive thymine riboside was added to the deproteinized solution and the mixture of the radioactive, enzymatically produced and authentic thymine riboside was purified by paper chromatography

utilizing the "Bor/s-Bu" solvent. The isolated thymine riboside contained no detectable radiochemical impurity when chromatographed in the "EtAc/form" and "Bor/s-Bu" solvent systems. Upon hydrolysis of this isolated product with perchloric acid, it was found that over 90% of the radioactivity of the thymine riboside was contained by thymine. Approximately 8% of the radioactivity was found in an unidentified compound. The identity of this radioactive thymine was checked in 10 diverse solvent systems (7) and radioautography showed that the authentic thymine marker always coincided exactly with the radioactivity.

Since the capacity to oxidize thymidine was not lost by enzyme preparations which had been selectively adsorbed on and eluted from calcium phosphate gel, concentrated in ammonium sulfate and passed through a Sephadex G-25 chromatography column, it appeared unlikely that the formation of thymine riboside involved detachment of the deoxyribose moiety. Additional support for the conversion occuring at the deoxyribonucleoside level was obtained utilizing incubation mixtures containing thymidine as substrate which was uniformly labeled with C^{14} and enriched in position 6 of the pyrimidine moiety with respect to H^3. Typical results of these studies are shown in Table I. That the ratio of the specific activity of C^{14} to that of H^3 was the same for thy-

Table I. Retention of C^{14} and H^3 in the thymine riboside produced.

	Specific Radioactivity (curies per mole)		
	C^{14}	H^3	C^{14}/H^3
Thymidine[a]	3.0	6.2	2.1
Thymine riboside[a]	3.1	5.9	1.9
Thymidine[b]	3.0	6.0	2.0
Thymine[a]	1.7	6.3	3.7

a Isolated, after 25 min of incubation, from standard incubation system containing the Sephadex-treated ammonium sulfate preparation (2.4 mg protein per ml). The amounts of thymine riboside and thymine produced were 5.0 and 2.0 mμmoles, respectively.

b From stock solution of thymidine (uniformly labeled with respect to C^{14} and only in the pyrimidine ring with H^3) which was the source of the substrate for the standard incubation system.

Table II. Requirements for α-ketoglutarate, ascorbate and Fe(II).

Omission in standard incubation system[a]	Thymine riboside produced (mμmoles)
None[b]	27.5
α-Ketoglutarate	0.3
Ascorbate	2.3
Fe(II)[b]	3.7
α-Ketoglutarate, ascorbate and Fe(II)	0

a The standard incubation system containing the Sephadex-treated calcium phosphate gel fraction (1.4 mg protein per ml) and thymidine-2-C^{14} as substrate. The incubation period was 2 hr.

b In addition to thymidine and thymine riboside, the incubated system with no omissions also contained a total of 4.1 mμmoles of thymine and 5-hydroxymethyluracil. In the system in which iron was omitted the total mμmoles of thymine and 5-hydroxymethyluracil was 2.0. In none of the other systems was the formation of thymine or 5-hydroxymethyluracil detected.

mine riboside as it was for the substrate strongly argues against an endogenous ribose derivative being the precursor of the ribose moiety of the synthesized ribonucleoside. In other experiments nonradioactive potential intermediates were added to the standard incubation system containing the doubly labeled thymidine as substrate. It was found that when incubation mixtures were made 2 mM with respect to nonlabeled thymine, D-ribose, 2-deoxy-D-ribose, α-D-ribose-1-phosphate or 2-deoxy-α-D-ribose-1-phosphate, the ratios of the specific activities of C^{14} to H^3 of the synthesized thymine riboside were still the same as that of the substrate. Moreover, it should be noted that the ratio of the specific activities would not be the same for substrate and product if the latter contained a lesser number of carbon atoms or was a derivative of barbituric acid.

Table II shows that the formation of thymine riboside is dependent on the presence of α-ketoglutarate, ascorbate and Fe(II) in the incubation mixture. The specifity of these requirements has not been examined. Preliminary experiments have shown that no thymine riboside is formed when the air of the standard incubation mixture is replaced by nitrogen.

In studies with mycelial pads, Fink and Fink (9) demonstrated that

Neurospora most likely does not directly phosphorylate either thymidine or deoxycytidine for selective incorporation into DNA and that these compounds probably undergo conversion to the corresponding ribonucleosides prior to their utilization for both RNA and DNA synthesis. Our results suggest that in the transformation of thymidine to the pyrimidines of RNA and DNA by Neuorospora, this deoxyribonucleoside, initially, is directly converted to thymine riboside. Although no studies have yet been made with deoxycytidine, in preliminary experiments it has been demonstrated that when radioactive deoxyuridine was substituted for thymidine in the standard incubation system, radioactive uridine was formed. Since the addition of either 2-deoxy-D-ribose or 2-deoxy-α-D-ribose-1-phosphate to the standard incubation system, in the experiments described above, did not decrease the amount of thymine riboside produced, it appears unlikely that these compounds can serve as substrates in this system.

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