INCORPORATION OF 5,6-DIHYDROURIDINE TRIPHOSPHATE INTO RIBONUCLEIC ACID BY DNA-DEPENDENT RNA POLYMERASE

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Received June 16, 1965

Animal tissues have been reported to contain an enzyme catalyzing the interconversion of 5,6-dihydro-UMP and UMP (Mokrasch and Grisolia, 1959). Recently Carr and Grisolia (1964) obtained evidence that dihydro-UMP is incorporated into microsomal RNA and sRNA in cell-free preparations from animal tissues without prior conversion to UMP. These data, in context with the recent report that dihydro-UMP is a minor constituent of sRNA from yeast (Madison and Holley, 1965), suggest that dihydrouracil may be a ubiquitous constituent of RNA.

Pretreatment with ribonuclease in cell-free systems results in a marked loss of incorporation of dihydro-UMP into RNA (Carr and Grisolia, 1964). This may imply that incorporation of the hydrogenated pyrimidine is not due to DNA-dependent RNA polymerase. However, since alternate explanations for the ribonuclease effect are possible, such as increased competition of nucleotides for nucleotide kinases, it was of interest to determine the effectiveness of dihydro-UTP as a substrate for DNA-dependent RNA polymerase. The results show that isotope from dihydro-UTP-C^{14} is incorporated into RNA to a significant extent in the presence of DNA, UTP, CTP, GTP, and ATP by RNA polymerase from Escherichia coli.

MATERIALS AND METHODS

DNA-dependent RNA polymerase used in these studies was pre-

pared from Escherichia coli, strain W, by procedure B of Furth et al. (1962). An equal volume of glycerol was added to the final enzyme preparation and stored at -20°. The enzyme was completely dependent upon the presence of DNA and all four ribonucleoside triphosphates. A modification of the method of Cohn and Doherty (1956) was used for preparation of 5,6-dihydro-UTP-C^{14} and 5,6-dihydro-UDP. The preparation of dihydro-UTP-C^{14} is described below in detail. Calf thymus DNA, UTP, CTP, ATP and GTP were obtained from Calbiochem. UTP-2-C^{14} was purchased from Schwarz BioResearch, Inc. 5% Rhodium on alumina was purchased from Engelhard Industries, Inc., Newark.

Preparation of dihydro-UTP-C^{14}: 33.91 mg UTP (48 μmoles) and a solution of 50 μc (2 μmoles) UTP-2-C^{14} were placed in a small hydrogenation tube. The solution was dried in a vacuum desiccator and dissolved in 5 ml aqueous hydrochloric acid, pH 4.8. Rhodium catalyst, 10 mg, was added and hydrogen was passed continuously through the solution at 0° and atmospheric pressure until light absorption at 260 mμ was reduced to zero. The time required was about 5 hours. Catalyst was removed by centrifugation and dihydro-UTP-C^{14} was separated from small amounts of radioactive contaminants by gradient elution on DEAE-cellulose (bicarbonate) with 0.1 M triethylammonium bicarbonate in the reservoir and one liter mixer volume of water. The presence of dihydrouracil-containing compounds in eluate fractions were detected by absorption of ultraviolet light at 230 mμ. The identity of the major product, containing 60% of the radioactivity originally present in UTP, was established as dihydro-UTP. The product was lyophilized and passed through Dowex 50 to convert it to the sodium salt.

The presence of the 5,6-dihydrouracil moiety was established by use of a reagent for detection of dihydropyrimidines devised by Fink et al. (1956). The molar ratio of acid-labile phosphorus to acid-stable organic phosphorus was found to be 1.96. The acid-stable P was calcu-

TABLE I. Effectiveness of Dihydro-UTP as a Substrate for DNA-Dependent RNA Polymerase

Experiment No.	Nucleoside triphosphates added	mμMoles ribonucleotide incorporated[+]
1	UTP* + CTP + ATP + GTP	4.87
2	Same as Expt. 1 (-DNA)	0.05
3	Dihydro-UTP* + CTP + ATP + GTP	0.22
4	Dihydro-UTP* + UTP + ATP + GTP	0.10
5	Dihydro-UTP* + UTP + CTP + GTP	0.06
6	Dihydro-UTP* + UTP + CTP + ATP	0.06
7	Dihydro-UTP* + UTP + CTP + ATP + GTP	0.61
8	Same as Expt. 7 (-DNA)	0.02
9	Same as Expt. 7 (-enzyme)	0.02
10	Same as Expt. 7 (+RNAse, 5 μg)	0.04

Reaction mixture (0.5 ml) contained: each ribonucleoside triphosphate (40 mμmoles), calf thymus DNA (0.90 optical density units at 260 mμ), $MnCl_2$ (1.0 μmole), $MgCl_2$ (2.0 μmole), β-mercaptoethanol (2.0 μmoles), spermidine (1.0 μmole), Tris buffer, pH 8.0 (25 μmoles) and 8.0 μg of RNA polymerase. After 60 minutes at 38°, the reaction mixture was chilled in ice and 2 ml 5% TCA solution at 0° were added. The solutions were filtered through millipore filters (Millipore Filter Corporation, HA 0.45 μ, white, plain, 25 mm) and each reaction tube rinsed four times with 2 ml each of 1% cold TCA and filtered through the millipores. The filters were dried under an infrared lamp and glued to planchets. Radioactivity was measured in a micromil end-window gas flow counter.

[+]The presence of dihydro-UMP residues in the acid-insoluble product formed by the polymerase in the presence of dihydro-UTP-C^{14} was indicated by the following experiments. DNA was removed by treatment with DNase and the acid-insoluble residue was treated with purified snake venom diesterase. Carrier dihydro-UMP was added, and the acid-soluble components separated by paper chromatography and electrophoresis. The only radioactive component recovered was associated with the fraction corresponding to carrier dihydro-UMP. There was, however, no separation of UMP from the dihydro derivative.

*UTP-2-C^{14} (610 cpm/mμmole), dihydro-UTP-2-C^{14} (630 cpm/mμmole).

lated by subtracting acid-labile P from the total inorganic P formed on ashing. Purity of the product was established by paper chromatography in ethyl alcohol-0.5 M ammonium acetate, pH 7.5 (5:2 v/v) and by electrophoresis at 2500 volts in 0.075 M sodium acetate, pH 4.0. In

both systems the product migrated as a single radioactive component to about the same position as UTP. Absorption of light at 260 mu was zero, indicating the absence of contamination by UTP in the product.

RESULTS

Isotope from dihydro-UTP-C^{14} is incorporated into acid-insolubles in the absence of UTP with an efficiency of only about 4% as compared to controls containing UTP-C^{14}, ATP, CTP and GTP (Table I). Even smaller amounts of incorporation occur when dihydro-UTP-C^{14} is substituted for CTP, ATP or GTP. Similar results (not shown in Table I) are obtained when ATP-C^{14} is used as the labeled substrate in place of dihydro-UTP-C^{14}. Thus, dihydro-UTP is a poor substitute for UTP, CTP, GTP or ATP in the DNA-primed RNA polymerase reaction.

Incorporation of dihydro-UTP-C^{14} is almost tripled when UTP is present in addition to CTP, ATP and GTP (Table I). This stimulatory

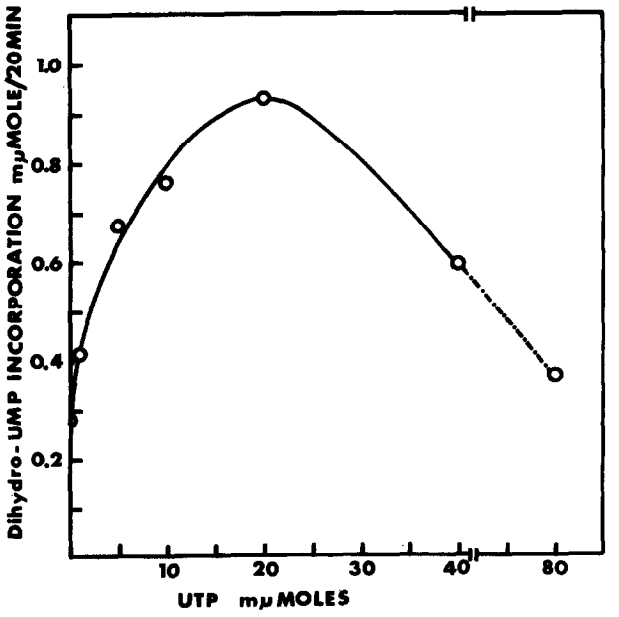

Figure 1. Effect of UTP concentrations on incorporation of isotope from dihydro-UTP-C^{14} into RNA. The reaction mixture (0.5 ml) contained 40 mumoles each of ATP, GTP, CTP and dihydro-UTP-C^{14}. UTP concentration was varied from 0 to 80 mumoles. Conditions are described in Table I.

effect of UTP was investigated in more detail by addition of increasing amounts of UTP in the presence of GTP, ATP, CTP and dihydro-UTP-C^{14}. UTP enhances incorporation of label from dihydro-UTP-C^{14} to a maximum at a molar ratio of dihydro-UTP to UTP of 2 (Fig. 1). Higher concentrations of UTP repress incorporation of dihydro-UTP in a manner consistent with the ability of the former to act as a preferred substrate for the polymerase. The stimulatory effect of UTP remains unexplained.

A similar stimulatory effect of UTP-C^{14} in the presence of increasing amounts of dihydro-UTP does not occur (Fig. 2), instead a progressive inhibition occurs. A 40% inhibition of UMP incorporation occurs in the presence of 40 mumoles dihydro-UTP and 40 mumoles each of UTP-C^{14}, GTP, ATP and CTP, as compared to a control value in the

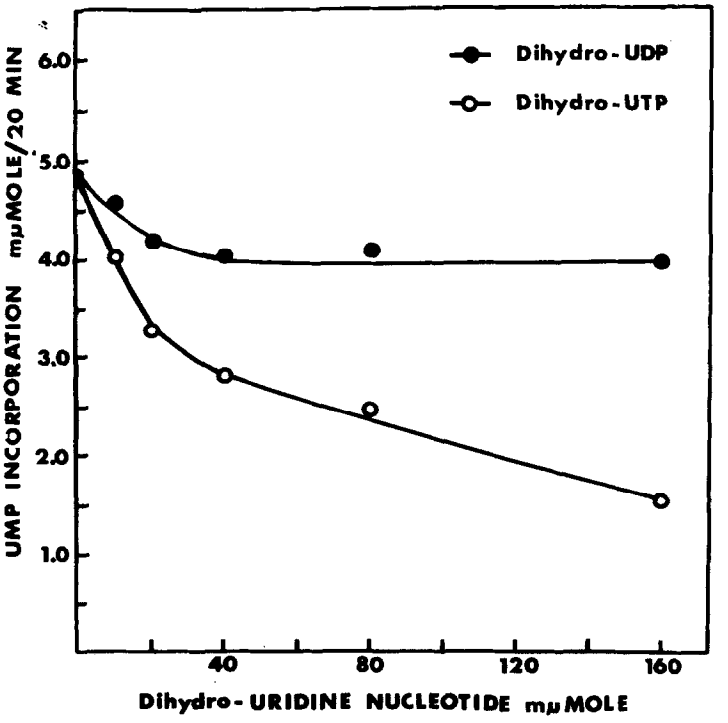

Figure 2. Effect of dihydro-UTP and dihydro-UDP on incorporation of UMP into RNA. The reaction mixture (0.5 ml) contained 40 mumoles each of ATP, GTP, CTP and UTP-C^{14} and variable concentrations of dihydro-UDP and dihydro-UTP. Other conditions were the same as described in Table I except that the incubation time was decreased from 60 minutes to 20 minutes.

absence of the hydrogenated nucleotide. Dihydro-UDP also represses incorporation of UTP-C^{14} into RNA but to a much lower extent than dihydro-UTP (Fig. 2).

Fig. 3 shows the inhibitory effect of dihydro-UTP on UMP-C^{14} incorporation into RNA in the presence of various concentrations of UTP-C^{14}. Lineweaver-Burk plots of the data are irregular.

The data show that dihydro-UTP is incorporated into RNA by DNA-primed RNA polymerase to a significant extent in the presence of UTP, CTP, ATP, and GTP. Incorporation is sufficiently high to establish that DNA-dependent RNA polymerase is a possible mechanism for incorporation of 5,6-dihydro-UMP into RNA, particularly when the present data are considered in context with the known incorporation of dihydro-UMP into RNA as demonstrated by Carr and Grisolia (1964). However, it is difficult to explain non-random distribution of dihydro-UMP in RNA (Holley et

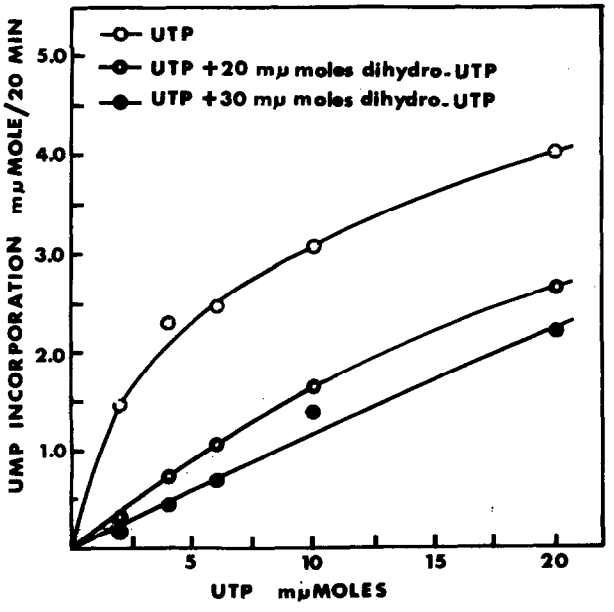

Figure 3. Inhibition by dihydro-UTP of UMP incorporation in the presence of various concentrations of UTP. The conditions were as described in Fig. 2 except that the concentration of UTP-C^{14} was varied as indicated and dihydro-UTP was present at 20 mumoles or 30 mumoles concentrations.

al., 1965) by the classical H-bonding assumed to occur in the polymerase reaction unless a new or additional mechanism is involved. Non-random distribution of the hydrogenated pyrimidine may be explained more readily by a process of enzymatic hydrogenation at the polynucleotide level. The possibility of this latter mechanism has not been excluded.

ACKNOWLEDGEMENTS

The authors are indebted to Mrs. Ildiko Berty for excellent technical assistance. This research was supported by grant No. CA 02373 from the U. S. Public Health Service.

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