THE CONVERSION OF DEOXYURIDINE 5'-MONOPHOSPHATE TO DEOXYURIDINE 5'-TRIPHOSPHATE IN NORMAL AND REGENERATING RAT LIVER*

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Although DNA† is devoid of dUMP, except in rare instances (Takahashi and Marmur, 1963), dUTP can be incorporated readily into DNA by the DNA polymerase system (Bessman et al., 1958). As a result of this finding, it has been tacitly assumed that the inability of dUMP to serve as a precursor of DNA results from the apparent absence of a phosphotransferase capable of converting dUMP to dUTP (Bessman et al., 1958; Friedkin and Kornberg, 1957). The validity of this view was placed in doubt when it was found that dUMP could be converted to dUDP by a heat-stable extract from rat liver (Maley et al., 1967). We have now found that unheated extracts from normal and regenerating rat liver can effectively convert dUMP to dUTP, a situation that presents somewhat of a paradox in view of the apparent absence of dUMP in rat liver DNA. Other explanations must, therefore, be sought for the limitation in dUTP formation. These appear to involve, as will be shown, an active dUTP phosphatase, possibly similar to the pyrophosphatase found previously in chick embryo (Bertani et al., 1963), and the inhibition of dUMP phosphorylation by UMP and CMP. In some instances, however, dUMP may be

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† Abbreviations are: d-, deoxy; UMP, UDP, UTP, uridine 5'-mono, di-, and triphosphate, respectively; TMP, thymidine 5'-phosphate; CMP, cytidine 5'-phosphate; DNA, deoxyribonucleic acid.

actually incorporated into DNA and methylated by methionine to dTMP, as suggested by the recent findings of Scarano et al. (1967).

MATERIALS AND METHODS

Synthesis of dUMP-2-^{14}C - This compound was prepared by the treatment of dCMP-2-^{14}C (Schwarz BioResearch, Inc.) with a deoxycytidylate deaminase purified from T2-infected Escherichia coli (Maley et al., in press). The dUMP-2-^{14}C was obtained by elution from a Dowex 1-formate column (6 X 1 cm) with a convex formic acid gradient (4N formic acid reservoir + 250-ml mixing chamber), followed by lyophilization to remove the formic acid.

Assay for dUMP Phosphorylation - The reaction mixture contained the following components: 0.25 μmole of dUMP-2-^{14}C (2.7 X 10^5 cpm/μmole); 5 μmoles of dithiothreitol; 3 μmoles of $MgCl_2$; 25 μmoles of potassium phosphate (pH 7.5); 0.1 ml of a supernatant fraction from a 30-per-cent isotonic KCl rat liver homogenate centrifuged at 144,000 X g for 30 min. The total volume of the reaction mixture was 0.28 ml. The mixture was incubated at 37° and at various times 10-μl aliquots were removed and spotted near one end of a Whatman DE-81 anion exchange paper strip (10 X 1 cm). The other end was attached to a horizontal glass rod with Scotch tape and the end containing the radioactive spot was dipped into a solution of 4N formic acid + 0.1 N ammonium formate (Ives et al., 1963). (Numerous reactions can be measured simultaneously since this method of assay is limited only by the number of chromatographic strips that can be attached to a single glass rod.) The entire chromatographic system was enclosed. With this solvent system, dUMP migrated upward while dUDP and dUTP remained essentially at the origin. The latter region, after removal of the dUMP, was cut from the paper and placed in a scintillation vial with a phosphor containing 0.5-per-cent 2,5-diphenyloxazole and 0.03-per-cent p-bis [2-(5-phenyloxazolyl)] benzene in toluene. The radioactivity was determined in a Nuclear Chicago 702 scintillation counter.

dUTPase Activity - The rate of formation of dUMP from dUTP (α-^{32}P) (International Chemical and Nuclear Corp.) was measured by placing 10-μl aliquots on Whatman 3 MM paper at 3- and 6-min intervals from the following reaction mixture: 0.25 μmole of dUTP-α-^{32}P (1.4 X 10^6 cpm/μmole); 5 μmoles of dithiothreitol; 50 μmoles of potassium phosphate (pH 7.5); 0.1 ml of the rat liver supernatant fraction described above or the extracts described in Table I. The mixture was incubated at 37°. The unchanged dUT^{32}P was separated from dUM^{32}P by descending paper chromatography in ethanol-1M ammonium acetate, 5:2 (pH 3.8). The dUM^{32}P area was excised from the chromatogram and counted in a scintillation counter as described above.

RESULTS AND DISCUSSION

It is immediately apparent from Fig. 1 that the initial rate of dUMP phosphorylation exceeds that of dTMP by 20-30-fold. The presence of fluoride does not influence the initial rate significantly, but does increase appreciably the extent of dUTP formation, a result suggesting the presence of phosphatases. Fluoride does not appear to increase the total dUTP formed in regenerating liver extracts as in the case of the normal. A similar lack of effect of fluoride is obtained with dTMP phosphorylation, as seen in Fig. 1. Of interest is the finding that dUTP formation in regenerating liver extracts is only 30-40 per cent of that in normal liver extracts containing fluoride.

To establish the nature of products formed, a reaction mixture incubated for 2 hrs at 37°, similar to that described in Materials and Methods, was chromatographed on Dowex 1-formate. As revealed in Fig. 2, the radioactive areas coincide exactly with the ultraviolet-light absorbing regions corresponding to dUMP, dUDP, and dUTP, respectively. The dUTP region was desalted by passage through a Dowex 50-H^+ column, then lyophilized to remove formic acid. Treatment of the concentrated dUTP region with snake venom (Crotalus adamanteus), followed by ascending paper chromatography (Whatman 3 MM) in sec-butanol saturated with water, revealed that the radioactivity migrated with deoxyuridine. However, attempts to demonstrate deoxyuridine nucleotides

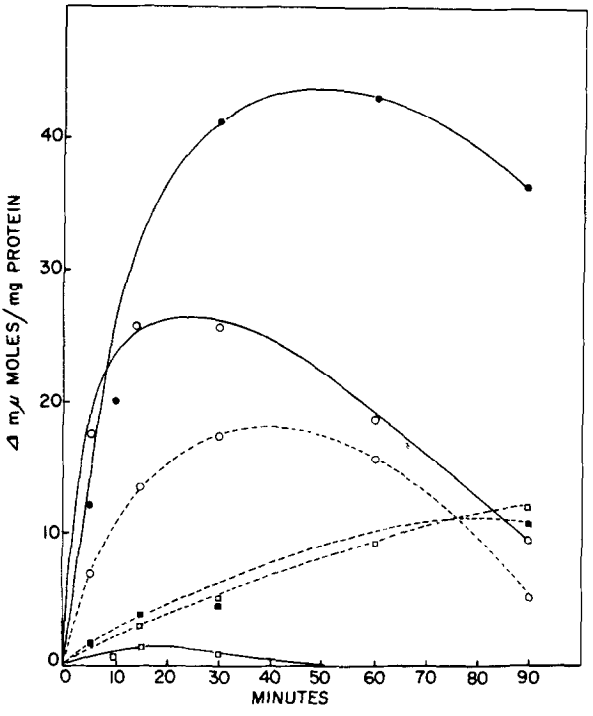

Fig. 1. Phosphorylation of dUMP (o) and dTMP (□) by normal (—) and 48-hr. regenerating liver (---) extracts. The presence of fluoride (0.3M) is indicated by (● and ■). For assay details, see Materials and Methods.

in partially hepatectomized rat liver following an intra-portal injection of deoxyuridine-2-^{14}C were unsuccessful, a result anticipated in view of the apparent absence of dUMP in rat liver DNA. A clue to at least one reason for the block in dUTP formation, despite the presence of deoxyuridine and dUMP phosphotransferases in regenerating liver, is evident from the data in Fig. 1 which suggest the presence of an active phosphatase. Other tissues, such as rat embryo and thymus and chick embryo, were investigated for the ability to phosphorylate dUMP, with the finding that although UMP phosphorylation could easily be shown, dUMP phosphorylation could not be demonstrated. Similar results were obtained with extracts of E. coli.

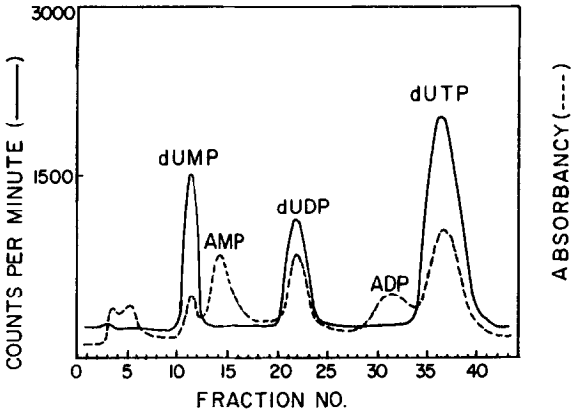

Fig. 2. Ion-exchange separation of the products resulting from dUMP phosphorylation by a normal rat liver extract. The reaction mixture, described in Materials and Methods and the text, was passed through a Dowex 1-formate column (10 X 1 cm). The column was eluted with a convex gradient formed by the addition of 1M ammonium formate to a 250-ml water reservoir. The pattern presented was obtained by simultaneously recording the ultraviolet light absorbing material and radioactivity eluted, as detected with a Gilson U.V. analyzer and a Nuclear Chicago scintillation flow cell system.

A reason for the apparent inability of tissue extracts to form dUTP is evident in Table I, where the rate of dUT^{32}P breakdown by various tissues is presented. The only tissue extracts that could affect dUTP synthesis were those with the lowest dUTPase activity, normal and regenerating liver.

TABLE I

Breakdown of dUTP-α^{32}P to dUMP-2^{32}P by various tissue extracts*

Enzyme source	Specific activity** (mμ moles/min/mg protein)
Rat liver (normal adult)	0.22
Regenerating rat liver (24 hr)	0.40
Rat embryo (15-day)	5.80
Rat liver (newborn)	2.57
Rat thymus	5.36
Escherichia coli B (mid-log)	8.85
Chick embryo (7-day)	18.7

* For details of assay, see Materials and Methods.

** dUTP converted to dUMP.

When the data in Fig. 1 are compared with those in Table I, it is seen that the rate of synthesis of dUTP is greater than its rate of breakdown. That the breakdown of dUTP is due to a specific pyrophosphate cleavage enzyme, as shown in chick embryo by Bertani et al. (1963), is not known, but such an enzyme is strongly suggested from the chromatographic data which revealed only two major reaction products, dUTP and dUMP. If the enzyme was present in rat liver, at the level found in chick embryo (Table I), it is doubtful that dUMP phosphorylation could be measured. The latter reaction, however, could be clearly shown in chick embryo extracts when purified free of phosphatase activity (Labow). The dUMP phosphotransferase activity could then easily be shown in a Gilford Multiple Absorbance Recorder by employing the coupled pyruvophosphokinase-lactic dehydrogenase assay (Maley and Ochoa, 1958). The ratio of UMP to dUMP activity was found to be about 100:1. Similar results will probably be obtained in tissue and possibly bacterial extracts containing UMP phosphotransferase activity and a high dUTPase activity. Recent studies (Labow) with a highly purified UMP phosphotransferase preparation from E. coli containing only a trace of dUTPase yielded similar results to those obtained with the purified UMP phosphotransferase from chick embryo.

It is of interest to note that the dUTPase activity of regenerating rat liver is greater than in normal rat liver, but still not sufficient to prevent the formation of dUTP in vitro. Another factor must, therefore, contribute to the impairment of dUTP formation in rat liver in vivo. Preliminary studies have revealed UMP and CMP to be effective inhibitors of the phosphorylation of dUMP at equivalent concentrations. Undoubtedly, the normal tissue ratio of UMP and CMP to dUMP is much greater than that employed in the above experiments. None of the other naturally occurring nucleoside and deoxynucleoside 5'-phosphates was found to inhibit, a result that enhances the regulatory significance of this inhibitory reaction.

SUMMARY

The above studies reveal for the first time that dUMP can be

converted to dUTP in rat liver, a reaction that may be more universal than originally believed. Naturally occurring mechanisms, such as dUTP breakdown and the inhibition of dUMP phosphorylation by CMP and UMP, may prevent this reaction from assuming a more significant physiologic role.

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