On the Nature of a Sparing Effect by Thymidine on the Utilization of Deoxycytidine*

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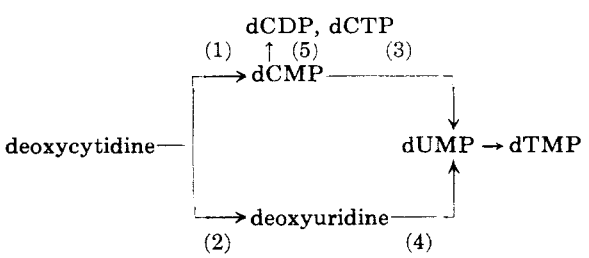
It was demonstrated in studies with rat embryo extracts that dTTP[1] (deoxythymidine triphosphate) is an effective inhibitor of deoxycytidylate deaminase and deoxyuridine kinase. None of the other deoxynucleoside triphosphates tested inhibited these enzymes. In addition, dCTP was found to inhibit the phosphorylation of deoxycytidine, but did not inhibit deoxycytidylate deaminase or deoxyuridine kinase. In conjunction with the above *in vitro* studies, deoxycytidine-2-C^{14} was found to be more readily incorporated into the thymidine of rat embyro mince DNA than into the deoxycytidine. However, in the presence of unlabeled thymidine, the incorporation into the deoxycytidine of the DNA was enhanced. The most likely explanation for this effect appears to originate in the inhibition of deoxycytidylate deaminase and deoxyuridine kinase by dTTP. Deoxythymidine triphosphate can thus regulate its own synthesis by sparing the contribution of deoxycytidine to the thymidine pool. From these studies, it was concluded that the most probable pathway of metabolism of deoxycytidine in rat embryo occurs via deamination to deoxyuridine followed by phosphorylation to dUMP.

Recent studies on chick embryo mince (Maley and Maley, 1962) have revealed deoxycytidine-2-C^{14} to be more efficiently incorporated into the thymidine of DNA than into the deoxycytidine. Since these results were at variance with previously reported studies on extracts (Maley and Maley, 1961b) and minces (Reichard, 1958; Maley and Maley, 1960b), experiments were undertaken to delineate the pathways available to deoxycytidine as a precursor of DNA. The initial steps in the utilization of deoxycytidine involve either a deamination to deoxyuridine or a phosphorylation to dCMP. Subsequent reactions of these compounds in embryo extracts yield dUMP by either a deamination of dCMP (Maley and Maley, 1959) or a phosphorylation of deoxyuridine as indicated in the scheme below.



Because of the presence of deoxycytidylate deaminase in rapidly growing tissues (Maley and Maley, 1959; Scarano, 1960; Maley and Maley, 1961a), it would not be illogical to assume that the major pathway of dUMP formation from deoxycytidine is via reactions (1) and (3). A recent study by Schneider and Rotherham (1961) supports this view. However, there are a number of factors that mitigate against this assumption, some of which are the subject of this report.

EXPERIMENTAL

Materials and Methods.—The nucleoside mono- and triphosphates were purchased from the Sigma Chemical Company, St. Louis, Mo. The tritiated deoxynucleosides were purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. Deoxyuridine-H^3 was purified by paper chromatography in secondary butanol and deoxycytidine-H^3 was purified by chromatography on Dowex 50-H^+. dCMP-2-C^{14} was prepared as previously described (Maley and Maley, 1960a) and deoxycytidine-2-C^{14} by treatment of the nucleotide with snake venom (*Crotalus adamanteus*) followed by ion-exchange chromatography.

Tritium was measured in a Nuclear-Chicago liquid scintillation counter (Model 701). The scintillation fluid was the same as that previously described (Maley and Maley, 1960b), but the sample aliquot was 0.5 ml. Quenching correction factors were determined with known samples of tritiated deoxynucleosides.

For the measurement of deoxynucleoside kinase activity, the following reaction conditions and chromatographic isolation procedures were employed: each reaction vessel contained the following compounds (in μmoles): deoxycytidine-2-C^{14}, as indicated, or deoxycytidine-H^3, 0.26 (1.12×10^6 cpm/μmole), or deoxyuridine-H^3, 0.26 (2.17×10^6 cpm/μmole); Tris buffer, *p*H

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[1] The abbreviations used are those suggested by the *Journal of Biological Chemistry*.

8.0, 100; ATP, 5.0; $MgCl_2$, 5.0; NaF, 15; and 0.4 ml of a rat embryo (14–16 days old) extract prepared by homogenizing in isotonic KCl and centrifuging at 35,000 × *g* for 30 minutes. The final volume of the reaction mixture was 1.00 ml. After incubation at 37° for 30 minutes or at the indicated time intervals, 0.1 ml of 60% $HClO_4$ was added to the reaction mixture followed by 2 ml more of 1 N $HClO_4$. The resulting suspension was hydrolyzed at 100° for 30 minutes, cooled to 0°, and the perchlorate precipitated with 50% KOH until neutrality was attained.

In order to isolate dCMP and dUMP the supernatant solutions, after centrifugation of the $KClO_4$, were passed through columns (4 cm long × 1 cm in diameter) of Dowex-1-formate-X-8 (200–400 mesh). The reaction tubes were washed with 2 ml of ice-cold water, which was added to the columns, followed by 10 ml more of water. The columns were then eluted as follows: two 10-ml fractions of 0.01 N formic acid; two 10-ml fractions of 1 N formic acid; two to four 10-ml fractions of 4 N formic acid. The water effluent and the two 10-ml 0.01 N formic acid fractions were combined and passed through a column of Dowex 50-H^+-X-10 (200–400 mesh), 4 cm × 1 cm, to obtain deoxyuridine. The first 1 N formic acid fraction yielded most of the dCMP, whereas the second and third 4 N formic acid fractions yielded most of the dUMP. Zero time samples were found to be necessary when the tritiated nucleosides were used. The C^{14}-deoxynucleosides gave less of a correction factor, but the assay was less sensitive with these compounds.

Deoxycytidylate deaminase and thymidylate synthetase were assayed with radioactive substrates, as previously described (Maley and Maley, 1960a).

Results

Phosphorylation of Deoxycytidine.—In an attempt to determine which of the two pathways of formation of dUMP predominates in rat embryo extracts, the products resulting from the reaction of deoxycytidine-2-C^{14} in the presence of ATP were isolated (Table I). To facilitate the interpretation of the data, as well as the isolation of the nucleotides, the reaction mixtures were hydrolyzed with 1 N perchloric acid to yield the nucleoside monophosphates. It is seen that the total deoxycytidine nucleotides formed with time (presented as dCMP) is less than the dUMP. If the incubation mixtures were not hydrolyzed, very little dCMP could be detected, most of it being present as deoxycytidine di- and triphosphates. It is not possible to conclude from these data that dUMP is not derived primarily from reactions (1) and (3), since the high activity of deoxycytidylate deaminase as compared with that of deoxycytidine deaminase (about 100:1) could be responsible for the low concentration of dCMP in the unhydrolyzed samples. However, if deoxycytidine deamination (reaction 2) is a rate-limiting reaction, so is the phosphorylation of deoxycytidine (reaction 1).[2] It will be demonstrated below that the phosphorylation of deoxyuridine is very rapid (reaction 4) and thus bears the same relationship to reaction (2) that reaction (3) does to reaction (1). Unlike the formation of dUMP via reactions (1) and (3), wherein there is a competition for the intermediate dCMP, there is no competition for any intermediates during the formation of dUMP via reactions (2) and (4).

Table I

Deoxycytidine-2-C^{14} Utilization by Rat Embryo Extract in the Presence of ATP[a]

Time (min.)	Amount Converted to dCMP (mμ-moles)	dUMP (mμ-moles)	Deoxyuridine (mμ-moles)
20	3.46	4.23	0.16
40	6.37	9.75	3.16
60	7.38	12.1	4.46
60 (+dTMP)	7.00	3.00	14.1

[a] 0.365 μmole of substrate (1.45 × 10^5 cpm/μmole) was present in each reaction mixture. The enzyme source was an extract from 12–14-day-old embryos. The compounds were determined after acid hydrolysis of the reaction mixture. For details, see Materials and Methods.

When reaction (4) was blocked with dTMP (Maley and Maley, 1959) so as to obtain a measure of the contribution of reactions (2) and (4) to the formation of dUMP, it was observed that dUMP formation was apparently inhibited, but the phosphorylation of deoxycytidine was not. Also noted was the marked accumulation of deoxyuridine, suggesting the inhibition of deoxyuridine phosphorylation by dTMP or a derivative. This is borne out in Table II, which presents a measure of deoxyuridine phosphorylation. The above results with deoxycytidine-2-C^{14} have been verified a number of times with a more sensitive assay using deoxycytidine-H^3.

Phosphorylation of Deoxyuridine.—Table II presents a measure of deoxyuridine-2-C^{14} phosphorylation. The experiment in the column at the right employed the same rat embryo extract as used in the experiment shown in Table I. Whereas less than 10% of the deoxycytidine was utilized in 60 minutes, almost all of the deoxyuridine was phosphorylated; this observation emphasizes the difference in rates of metabolism of these compounds. The experiment in the column at the left reveals the phosphorylation to be linear to almost complete reaction. The further utilization of dUMP is of little consequence, since dUMP does not appear to be phosphorylated in rat embryo extracts (this is in contrast to recent bacterial studies [Greenberg and Somerville, 1962]) or converted to dTMP in the absence of

[2] A more detailed account of these studies is in preparation.

TABLE II

DEOXYURIDINE-2-C^{14} PHOSPHORYLATION BY RAT EMBRYO EXTRACTS

Time (min.)	dUMP Formed[a] (mμmoles)	
10	77.4	
20	138	91.9
40	223	216
40 (+dTMP)	28.8	
60		269
60 (+dTMP)		46.1

[a] In the middle column, 0.270 μmole deoxyuridine-2-C^{14} was used as substrate in each reaction mixture. The enzyme source was the same as that employed in Table I. The dTMP concentration was 1.92 μmoles. For details on the assay, see Materials and Methods. Right-hand column similar to middle column except that an extract from 16-day-old embryos was used as an enzyme source.

TABLE III

DEOXYURIDINE-H^3 PHOSPHORYLATION IN THE PRESENCE OF NUCLEOSIDES AND NUCLEOTIDES

Addition	Amount Added (μmoles)	dUMP Formed[a] (mμmoles)	
None		59	111
Thymidine	2.20	0	
Uridine	1.96	61	
dTMP	2.00	17	36.9
dUMP	1.88	46	
dTTP	0.24		0.9

[a] The two columns at the right represent results obtained with different embryo extracts. The incubation time in each case was 30 minutes. For substrate concentration and assay procedure, see Materials and Methods.

added hydroxymethyl tetrahydrofolate. If dTMP was formed to any extent, it would effect the rate of phosphorylation of deoxyuridine, as indicated by the results with added dTMP. Other compounds have been tested as inhibitors, and, though thymidine (Table III) appears to be the most inhibitory, a number of extraneous factors contribute to this effect, one of the most important being the possibility that deoxyuridine kinase and thymidine kinase are the same enzyme. Uridine has no effect, which is expected in view of the presence of separate uridine and deoxyuridine kinases (Reichard *et al.*, 1962). Since dTMP, unlike dUMP, is phosphorylated in the presence of ATP, the possibility that dTTP was responsible for the inhibitory effect was tested and, as seen in Tables III and IV, this appears to be the case. As can be seen in Table IV, none of the other deoxynucleoside triphosphates tested affected deoxyuridine phosphorylation, except dTTP. Since this compound was present at one-tenth the concentration of dTMP used in the experiments described in Tables II and III and still caused complete inhibition, most if not all of the inhibitory effect caused by dTMP appears to

TABLE IV

EFFECT OF DEOXYNUCLEOSIDE TRIPHOSPHATES ON DEOXYURIDINE-H^3 PHOSPHORYLATION[a]

Additions	Amount Added (μmoles)	dUMP Formed (mμmoles)
None		99.8
dCTP	0.40	103
dGTP	0.15	93.2
dATP	0.38	98.0
dTTP	0.16	1.71

[a] For substrate concentration and assay procedure, see Materials and Methods.

TABLE V

DEOXYCYTIDINE-H^3 UTILIZATION IN THE PRESENCE OF DEOXYNUCLEOTIDES[a]

Additions	Amount Added (μmole)	Compound Formed: dCMP (mμmoles)	dUMP (mμmoles)	Deoxyuridine (mμmoles)
None		3.06	7.07	2.36
dTMP	0.50	3.53	3.11	4.61
dTTP	0.24	3.23	0	7.92
dCTP	0.32	0	6.88	1.99

[a] For details on substrate concentration and assay procedure, see Materials and Methods.

result from its conversion to dTTP. A similar effect on thymidine phosphorylation in extracts of regenerating liver and Novikoff hepatoma was recently reported by Ives *et al.* (1962), and this fact would further support the contention that the deoxyuridine and thymidine kinases are the same enzyme. It should be noted that the results of Ives *et al.* have been confirmed with rat embryo extract as an enzyme source.

Inhibition of Deoxycytidylate Deaminase by dTTP.—Since dTTP so effectively inhibited deoxyuridine phosphorylation, it was believed that a measure of dUMP formation via reactions (1) and (3) could be obtained by a study similar to that described in Table I. Surprisingly, no dUMP formation could be measured in the presence of dTTP (Table V), though deoxyuridine did accumulate as expected (reaction 2). As a result, it was concluded that either little or no dUMP was formed by the deoxycytidylate deaminase pathway or that this enzyme, like deoxyuridine kinase, was inhibited by dTTP. That the latter is indeed the case is shown in Figure 1, which presents a measure of the inhibition of deoxycytidylate deaminase with increasing dTTP concentration. The inhibition appears to be quite effective, with 50% inhibition being encountered at a ratio of dTTP to dCMP of 1:10; dTDP was found to be as inhibitory as dTTP. This is in contrast to the results obtained with dTMP, where a ratio of dTMP to dCMP of 1:1 was required for 50% inhibition. The possible

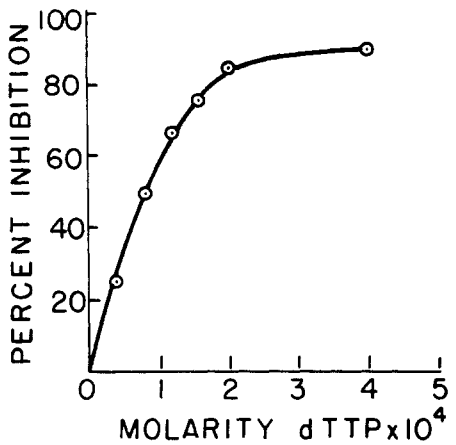

FIG. 1.—Inhibition of deoxycytidylate deaminase by dTTP. The reaction mixture contained the following compounds (in μmoles): dCMP-2-C^{14}, 0.45 (2.00×10^5 cpm/μmole); Tris, *p*H 8.0, 50; NaF, 15; dTTP as indicated; 0.05 ml. of an extract from a 50% homogenate of 14–16 day old rat embryos. The final volume of the reaction mixture was 0.50 ml. After incubation at 37° for 10 minutes, the reaction was stopped by heating at 100° for 2 minutes. Assay as described previously (Maley and Maley, 1960).

regulatory role of dTMP and dUMP was previously alluded to because of the ability of these compounds to inhibit deoxycytidylate deaminase (Maley and Maley, 1959), but they do not appear to be nearly as effective as dTTP. In addition, the triphosphates of deoxyadenosine, deoxyguanosine, and deoxycytidine, tested at a concentration sufficient to give almost complete inhibition with dTTP, were found not to affect the deamination of dCMP. However, it will be noted in Table V that dCTP apparently inhibits the phosphorylation of deoxycytidine, and as a result the total dUMP formed would presumably come from reactions (2) and (4). Since the amount of dUMP formed in this case does not differ significantly from that found in the absence of dCTP, it would appear that the dUMP results primarily from the above-indicated reaction pathway, deoxycytidine → deoxyuridine → dUMP. Because almost all of the dUMP can be accounted for by this reaction sequence, the alternate pathway reactions (1) and (3) are probably of minor significance.

Incorporation of Deoxycytidine-2-C^{14} into Rat Embryo Mince DNA.—Evidence in support of an alteration of deoxycytidine utilization by thymidine derivatives came initially from studies with chick embryo mince (Maley and Maley, 1962).[2] Similar results were obtained with rat embryo mince, and these are presented in Table VI. As a precursor of DNA pyrimidine deoxynucleosides, deoxycytidine-2-C^{14} is incorporated more effectively into the thymidine of DNA than into the deoxycytidine of DNA. In the presence of thymidine, the total incorporation of deoxycytidine into DNA is decreased, which could be attributed to a dilution effect; however, it will be noted that the incorporation into deoxycytidine is about doubled. In view of the inhibitory effect of dTTP on the synthesis of dUMP by both pathways (reactions 3 and 4), it would appear that the elevated incorporation of the deoxycytidine in the presence of thymidine results from a sparing effect on deoxycytidine utilization. The possibility that this effect is a consequence of a stimulation of DNA synthesis by thymidine has been ruled out by Pi^{32} incorporation studies.[2]

TABLE VI

INCORPORATION OF DEOXYCYTIDINE-2-C^{14} INTO THE DNA OF RAT EMBRYO MINCE[a]

Additions	DNA (cpm/mg)	Cytosine (cpm/μmole)	Thymine (cpm/μmole)
None	990	520	1000
Thymidine	690	1000	20

[a] Each reaction mixture contained 0.73 μmoles deoxycytidine-2-C^{14} (145,000 cpm/μmole), 1.0 ml Krebs-Ringer phosphate, 0.8 g of 12-day rat embryo mince, and, where indicated, 2 μmoles thymidine. Incubation was at 37° for 2 hours with rapid shaking. The reactions were stopped with 0.2 ml of 10 N $HClO_4$ and the DNA was isolated as described (Maley and Maley, 1960).

DISCUSSION

Numerous studies have implicated dTMP formation and phosphorylation as a rate-controlling step (Canellakis and Mantsavinos, 1958; Bollum and Potter, 1959; Flaks and Cohen, 1959; Maley and Maley, 1960a; Weissman *et al.*, 1960; Hiatt and Bojarski, 1961), if not the ultimate step, in the initiation of DNA synthesis. In addition to this vital role, it appears from the studies presented here that dTMP can regulate its own rate of synthesis and in the process make another precusor of DNA, dCMP, more available as a substrate. This is apparently accomplished by the conversion of dTMP to dTTP, which acts as an effective inhibitor of deoxycytidylate deaminase and deoxyuridine kinase. The nature of the inhibition by dTTP has been studied in crude extracts, and because of complicating side-reactions it was impossible to evaluate the data. Purification of the enzymes free from contaminating phosphatases will be necessary before an answer to this problem can be obtained. Little or no effect of dTTP has been observed on the next enzyme in the sequence, thymidylate synthetase.[3]

It might also be proposed that the thymidine stimulation of deoxycytidine incorporation is only an apparent one resulting from an inhibition of the ribose to deoxyribose conversion by dTTP, as recently proposed by Reichard *et al.* (1960). This inhibition would have the effect of raising

[3] Maley, F., unpublished observations.

the specific activity of the deoxycytidine by virtue of a decrease in the size of the deoxycytidine pool. However, this appears to be unlikely in view of results demonstrating the absence of an effect of thymidine on the incorporation of cytidine-2-C^{14} into the cytosine of chick embryo mince DNA (Maley and Maley, 1960b).

In studies on the synthesis of DNA in chick embryo extracts (Maley and Maley, 1961b), it was demonstrated that $dCMP^{32}$ in the presence of dGTP and dATP is a precursor of both the dCMP and the dTMP of DNA. The incorporation into the dTMP, though, was not as great as could be expected in view of the high levels of deaminase activity in these extracts (Maley and Maley, 1959). One explanation for the low dCMP → dTMP conversion was available from evidence indicating the presence of dTTP in the crude extracts, which, as indicated in Figure 1, would have the effect of impairing the synthesis of dTMP from dCMP. Upon supplementation of these extracts with dTTP, the $dCMP^{32}$ incorporation into the dCMP of the DNA was markedly stimulated, which is in support of the results obtained here with rat embryo mince and also with chick embryo mince.[2]

Additional evidence emphasizing the difference in metabolism of deoxycytidine and dCMP comes from incorporation studies with cytidine-2-C^{14} (Reichard, 1958; Maley and Maley, 1960b), where it was demonstrated that the deoxycytidine of DNA was more heavily labeled than the thymidine. These results contrast with those obtained here with deoxycytidine-2-C^{14} and are more closely akin to the *in vitro* studies with $dCMP^{32}$ (Maley and Maley, 1961b). The above observations are thus in support of the experiments of Reichard (1960) demonstrating that the ribose-deoxyribose conversion occurs at the nucleotide level.

ADDENDUM

Since the submission of this paper, dCTP has been found to reserve the inhibition of deoxycytidylate deaminase by dTTP and to reactivate the enzyme after exposure to treatments that reduce its activity (Maley, G. F., and Maley, F., *J. Biol. Chem.*, in press).

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