Biochemical Pharmacology, Vol. 19, pp. 2745–2756. Pergamon Press. 1970. Printed in Great Britain

STUDIES ON THE EFFECT OF TRIPHOSPHATES OF 5-AMINOURIDINE AND 5-HYDROXYDEOXYURIDINE ON RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID POLYMERASES*

S. ROY-BURMAN, P. ROY-BURMAN and D. W. VISSER

Department of Biochemistry, University of Southern California, School of Medicine, Los Angeles, Calif. 90033, U.S.A.

(*Received* 24 *January* 1970; *accepted* 10 *March* 1970)

Abstract—5-Aminouridine-5′-triphosphate (H_2N-UTP) was prepared by enzymatic phosphorylation of 5-aminouridine-5′-monophosphate (H_2N-UMP) and its effectiveness as substrate for RNA synthesis by *Escherichia coli* RNA polymerase was tested. The analog substituted specifically for UTP with an efficiency of about 40 per cent in the RNA polymerase reaction. H_2N-UTP was found to inhibit incorporation of UMP-^{14}C into RNA and the inhibition appeared to be competitive with respect to UTP. 5-Hydroxydeoxyuridine-5′-triphosphate (HO-dUTP) was synthesized and its effectiveness as substrate for DNA synthesis by *Micrococcus lysodeikticus* DNA polymerase was determined. HO-dUTP replaced specifically dTTP in the DNA polymerase reaction and was incorporated into DNA to about 37 per cent as compared to dTTP. The analog inhibited incorporation of dTMP into DNA and acted as a competitive inhibitor of dTTP in the DNA polymerase reaction. The substrate and inhibitory effects of HO-dUTP decreased markedly with increasing pH values. This behaviour was related to the low pK_a value of 5-hydroxydeoxyuridine.

THE URIDINE derivative, H_2N-Urd,† inhibits growth of bacteria, virus, fungi and tumors.[1–4] The analog undergoes most of the reactions of uridine, including conversion to aminouridine diphosphate sugar compounds and minor incorporation into RNA.[5] Its monophosphate derivative, H_2N-UMP, inhibits conversion of orotidylic acid to UMP by orotidylic acid decarboxylase.[5] Since H_2N-UMP is further phosphorylated to its triphosphate derivative, it was considered of interest to investigate the effect of H_2N-UTP on RNA polymerase.

HO-Urd and HO-dUrd are inhibitors of bacterial, viral and tumor growths.[2, 3, 6, 7] It was shown that HO-Urd is phosphorylated to its triphosphate derivative, HO-UTP, which acts as a strong inhibitor of RNA polymerase.[8, 9] These studies also suggested that the inhibitory effect of HO-UTP is more predominant when the 5-hydroxyuracil moiety exists in ionized form. In contrast, the effectiveness of the analog as substrate is markedly decreased when it exists in the ionized form. It was considered of interest to

*This investigation was supported by Grant CA 02373-14 from the United States Public Health Service, and in part by Dernham Senior Fellowship D 120 of the American Cancer Society, California Division, and Grant T-478 from the American Cancer Society

†Abbreviations used: H_2N-Urd, 5-aminouridine; H_2N-UMP, 5-aminouridine-5′-monophosphate; H_2N-UTP, 5-aminouridine-5′-triphosphate; H_2N-UDP-glucose, 5-aminouridine-5′-diphosphate-α-D-glucose; HO-Urd, 5-hydroxyuridine; HO-UTP, 5-hydroxyuridine-5′-triphosphate; HO-dUrd, 5-hydroxydeoxyuridine; HO-dUMP, 5-hydroxydeoxyuridine-5′-monophosphate; HO- dUTP, 5 hydroxydeoxyuridine-5′-triphosphate; dAT, deoxyadenylate-deoxythymidylate copolymer.

investigate the ionization effects of 5-hydroxyuracil on the reactions catalyzed by DNA polymerase.

This report describes the substrate and inhibitory effects of H_2N-UTP and HO-dUTP in the reactions catalyzed by *Escherichia coli* RNA polymerase (nucleoside-triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) and *Micrococcus lysodeikticus* DNA polymerase (deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7), respectively. Preparation of H_2N-UDP-glucose by UDP-glucose pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9) and the effect of the cofactor analog on UDP-glucose dehydrogenase (UDP-glucose: NAD oxidoreductase, EC 1.1.1.22) reactions are also reported.

EXPERIMENTAL PROCEDURE

Materials. CTP-2-^{14}C, ATP-8-^{14}C, dATP-8-^{14}C and dTTP-2-^{14}C were purchased from New England Nuclear Corp. Glucose 1-phosphate, NAD^+, UDP-glucose, phosphoenol pyruvate, phosphoenol pyruvate kinase, spermidine-HCl, calf thymus DNA, salmon sperm DNA, ribonucleotides and deoxyribonucleotides were obtained from Calbiochem. dAT copolymer was kindly supplied by Dr. A. Kornberg. Preparations of *M. lysodeikticus* DNA polymerase and *E. coli* W RNA polymerase were purchased from Miles Laboratories, Inc. Pancreatic ribonuclease was purchased from Worthington Biochemical Corp.

Enzyme preparations. RNA polymerase was prepared from *E. coli* W by procedure B as described by Furth *et al.*[10] and stored at −20° in 50% glycerol.[11] Crude UDP-glucose pyrophosphorylase was prepared from dried brewers' yeast (Anhauser-Busch, Inc., Van Nuys, Calif.), as described by Rabinowitz and Goldberg.[12] UDP-glucose dehydrogenase was purified from bovine liver by a modified procedure of Strominger *et al.*[13] A supernatant fraction (100,000 *g*) of a hyperdiploid strain of Ehrlich ascites tumor cells was prepared as described previously[8] except that the cells were disrupted in a French press.

Solvent systems. Two paper chromatographic solvent systems were used: Solvent A, ethanol–0·5 M ammonium acetate, pH 7·5 (5:2, v/v); and Solvent B, ethanol–0·5 M ammonium acetate, pH 3·8 (5:2, v/v).

Preparation of H_2N-UTP. H_2N-UMP was synthesized from H_2N-Urd as described previously.[5] H_2N-UTP was prepared by the enzymatic phosphorylation of H_2N-UMP using kinases from a soluble enzyme preparation of Ehrlich ascites cells. The reaction mixture (300 ml) contained 0·5 m-mole ATP, 2 m-moles phosphoglyceric acid, 3 m-moles $MgCl_2$, 15 m-moles Tris-HCl (pH 8·0), 0·1 m-mole H_2N-UMP and 100 ml (1·3 g) of 100,000 *g* supernatant from Ehrlich ascites cells. After incubation at 37° for 15 min, the reactions were terminated by heating the incubation mixture at 100° for 2 min. The precipitates were removed by centrifugation and the supernatant solutions were placed on a column of Dowex 1 (chloride), 2 × 40 cm. The products were eluted from the column with an ammonium bicarbonate gradient. Initially the mixer contained 1 l. water and the reservoir 0·5 M ammonium bicarbonate. After collection of about 2 l., the concentration in the reservoir was changed to 1·0 M ammonium bicarbonate. H_2N-UTP emerged from the column immediately prior to elution of ATP. The H_2N-UTP fraction was detected by its production of a yellow color with *p*-dimethylaminobenzaldehyde reagent.[14] The fractions containing H_2N-UTP were

collected, lyophilized and separated from impurities by descending paper chromatography in Solvent A on Whatman No. 3 MM filter paper. After elution from the paper and lyophilization, the dried product was dissolved in about 1 ml water and desalted on a column of Sephadex G-10, 1 × 90 cm. The solution containing H_2N-UTP was lyophilized and kept at −20° until used. The yield was 22 per cent (based on H_2N-UMP). The purity of the product was ascertained by paper chromatography with Solvents A and B. In each of these systems, the product migrated as a single ultraviolet-absorbing area which produced a yellow color with *p*-dimethylaminobenzaldehyde reagent. The molar ratio of acid-labile phosphorus to total phosphorus of the compound was 1·51. The products formed after treatment of H_2N-UTP with 1 N HCl at 100° for 10 min were chromatographed on Whatman No. 3 MM filter paper in Solvent A. A single ultraviolet absorbing spot was obtained which migrated to the same extent as synthetically prepared H_2N-UMP and produced characteristic yellow color with *p*-dimethylaminobenzaldehyde reagent.[14] Spectral characteristic of H_2N-UTP: $\lambda_{max}^{pH\ 2}$, 265 mμ; $\lambda_{max}^{pH\ 12}$, 282 mμ; A_{280}:A_{260} at pH 2, 0·62, at pH 12, 1·4.

Preparation of H_2N-UDP-glucose. H_2N-UDP-glucose was prepared from H_2N-UTP and glucose 1-phosphate with a crude preparation of UDP-glucose pyrophosphorylase from yeast.[12] The composition of the reaction mixture and the methods of purification used were similar to those described for the preparation of 5-hydroxyuridine-5′-diphosphate glucose,[15] except that the pH of the reaction mixture was 8·1. The yield of H_2N-UDP-glucose was 30 per cent (based on H_2N-UTP). The purity of the product was ascertained by paper chromatography with Solvents A and B. In each of these systems the product migrated as a single ultraviolet-absorbing area which produced a yellow color with *p*-dimethylaminobenzaldehyde reagent.[14] The R_f values of the product in Solvents A and B are 0·51 and 0·21 respectively. The molar ratio of acid-labile phosphorus to total phosphorus was 2·04. Treatment of the product with 1 N HCl at 100° for 15 min released 1 mole glucose per mole of H_2N-UDP-glucose (calculated on the basis of total phosphate). Spectral characteristics: $\lambda_{max}^{pH\ 1}$, 265 mμ; $\lambda_{max}^{pH\ 12}$, 285 mμ; A_{280}:A_{260} at pH 1, 0·61, at pH 12, 1·4.

Preparation of HO-dUTP. HO-dUTP was prepared from dUTP by a method similar to that used for the preparation of HO-UTP[9] except for the following changes to affect the purification of the product. The fractions eluted from DEAE-cellulose (HCO_3^-) column containing HO-dUTP were lyophilized and chromatographed on Whatman No. 3 MM paper with Solvent A for 40 hr. The product was separated completely from 5-bromodeoxyuridine-5′-triphosphate and dUTP. After elution from the paper and lyophilization, the dried product was dissolved in a minimum amount of water and was desalted on a column of Sephadex G-10. The solution containing HO-dUTP was lyophilized and kept at −20° until used. The product chromatographed on Whatman No. 3 MM paper with Solvent A migrated as a single ultraviolet-absorbing area which produced a purple color with ferric chloride.[16] Spectral characteristics: $\lambda_{max}^{pH\ 1}$, 282 mμ; $\lambda_{max}^{pH\ 12}$, 304 mμ; A_{280}:A_{260} at pH 1, 1·89, at pH 12, 0·9.

Assay of RNA polymerase. Incorporation of isotope from radioactive nucleoside triphosphates into an acid-insoluble product was used as a measure of RNA polymerase activity. The composition of the reaction mixture is described under each table and figure. Prior to use, the enzyme was diluted with a solution containing 2 mg of crystalline bovine plasma albumin per ml and 1 mM mercaptoethanol.[10] After incubation at 38°, the reaction was terminated by addition of 2 ml of 5% trichloroacetic acid at

0°. The acid-insoluble material was collected by filtration through membrane filters, washed and counted as described previously.[9] Data given in the figures and tables, except for those given in Table 1 and Fig. 1, were calculated after subtraction of blank values obtained when DNA or enzyme was omitted from the reaction mixture. Each table and figure represents data obtained from a single enzyme preparation. Specific activity[10] of the enzyme preparation varied from 700 to 1000, except for those used for Table 1 and Fig. 3, where the activity was 500 units/mg of protein.

Assay of DNA polymerase. The conversion of a ^{14}C-labeled deoxyribonucleoside triphosphate into an acid-insoluble product was used as a measure of DNA polymerase activity. The reaction mixtures were prepared as described by Zimmerman[17] and are indicated under each table and figure. Prior to use, the enzyme was diluted with a solution containing 1 mg of bovine serum albumin per ml containing 50 mM Tris, pH 7·5, and 10 mM mercaptoethanol.[17] After incubation at 37° for 30 min, the reaction was terminated by chilling and the addition of 2 ml of cold 5% trichloroacetic acid. Prior to addition of trichloroacetic acid, 0·1 ml of calf thymus DNA (0·5 mg/ml) was added as a carrier. The acid-insoluble material was collected by filtration through membrane filters, washed and counted as described previously.[9] The data given in figures and tables, except for those given in Fig. 5 and Tables 2 and 3, were calculated after subtraction of blank values obtained when DNA or enzyme was omitted from the reaction mixtures. An enzyme preparation having a specific activity of about 100 units[17] per mg was used. Each table and figure represents data from a single enzyme preparation.

Assay of UDP-glucose dehydrogenase. UDP-glucose dehydrogenase was assayed spectrophotometrically by measuring the rate of NAD^+ reduction as indicated by the increase in optical density at 340 mμ.[15] An enzyme preparation having a specific activity[18] of about 4000 units per mg of protein was used.

RESULTS AND DISCUSSION

Incorporation of H_2N-UTP into RNA. The extent to which H_2N-UTP replaced UTP in the synthesis of RNA by RNA polymerase is shown in Fig. 1 and Table 1. The substitution of H_2N-UTP for UTP in RNA synthesis was assessed from the incorporation of an accompanying natural substrate,[19] CTP-^{14}C or ATP-^{14}C. The data of Fig. 1 show that the incorporation of CMP-^{14}C into RNA was dependent on H_2N-UTP concentration and the maximum incorporation of CMP-^{14}C was achieved at approximately the same level of H_2N-UTP as that of UTP. It is apparent from the data of Table 1 that H_2N-UTP substituted specifically for UTP with an efficiency of about 40 per cent. The substrate activity of H_2N-UTP for RNA polymerase is consistent with the previous finding that H_2N-Urd is incorporated into RNA of Ehrlich ascites cells.[5]

Inhibition of UMP-^{14}C incorporation into RNA by H_2N-UTP. The inhibitory effect of H_2N-UTP on incorporation of UMP-^{14}C into RNA is shown in Fig. 2. The presence of an equimolar concentration of H_2N-UTP and UTP-^{14}C depressed incorporation of UMP-^{14}C into RNA by about 30 per cent. A maximum depression of about 60 per cent was produced by a 3-fold molar excess of H_2N-UTP. Conventional reciprocal plots of the data obtained in the absence and presence of H_2N-UTP suggested that the inhibition of RNA polymerase by the analog is competitive with respect to UTP (Fig. 3).

It has been shown that certain viruses grown in the presence of H_2N-Urd are hyper-

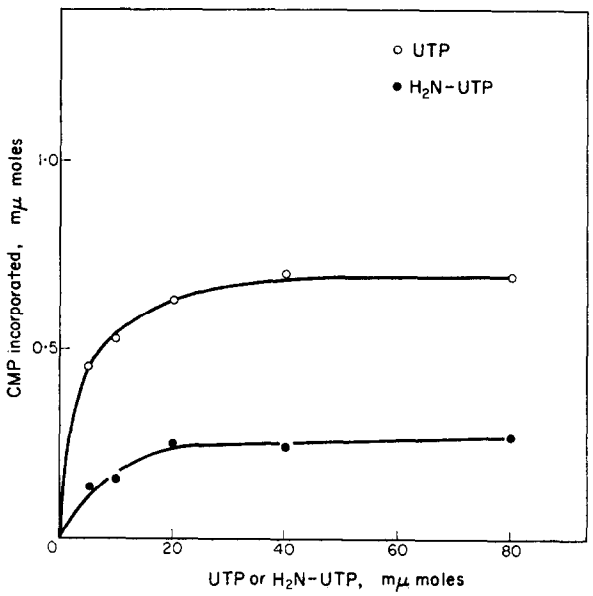

FIG. 1. Incorporation of CMP-^{14}C into RNA in the presence of various concentrations of H_2N-UTP and UTP. The reaction mixture, 0·5 ml, contained UTP or H_2N-UTP at concentrations which were varied as indicated; ATP, GTP and CTP-^{14}C (1×10^6 cpm/μmole) each at 80 μM; 4 mM $MgCl_2$; 2 mM $MnCl_2$; 2 mM mercaptoethanol; 2 mM spermidine-HCl; 50 mM Tris-HCl, pH 8·0; calf thymus DNA (equivalent to 0·90 absorbance unit at 260 mμ) and 1·2 μg enzyme. The incubation was carried out at 38° for 20 min. Isotope incorporated into acid-insoluble material was measured on Millipore filters as described in the text. The data were plotted after subtraction of blank value (0·02 mμmole) obtained by omission of H_2N-UTP and UTP.

TABLE 1. CAPACITY OF H_2N-UTP TO REPLACE UTP, CTP, ATP OR GTP IN RNA SYNTHESIS

Expt. no. and nucleoside triphosphate added*	Nucleotide incorporated (mμmoles)
1. CTP-^{14}C, ATP, GTP, UTP	4·67
2. As in expt. 1 minus DNA or enzyme	0·11
3. CTP-^{14}C, ATP, GTP, H_2N-UTP	1·85
4. As in expt. 3 minus H_2N-UTP	0·12
5. CTP-^{14}C, ATP, UTP, H_2N-UTP	0·20
6. As in expt. 5 minus H_2N-UTP	0·13
7. CTP-^{14}C, GTP, UTP, H_2N-UTP	0·37
8. As in expt. 7 minus H_2N-UTP	0·22
9. As in expt. 3 minus DNA or enzyme	0·07
10. As in expt. 3 plus RNAse	0·17
11. ATP-^{14}C, GTP, UTP, CTP	4·73
12. ATP-^{14}C, UTP, GTP, H_2N-UTP	0·36
13. As in expt. 12 minus H_2N-UTP	0·60
14. ATP-^{14}C, GTP, CTP, H_2N-UTP	1·95
15. As in expt. 14 minus H_2N-UTP	0·40

*Each incubation mixture, 0·5 ml, contained 80 μM of each of the following nucleoside triphosphates indicated to be present in each experiment: ATP, GTP, CTP, UTP, H_2N-UPT, CTP-^{14}C (1×10^6 cpm/μmole) and ATP-^{14}C ($1{\cdot}2 \times 10^6$ cpm/μmole). Other conditions were as described in Fig. 1, except that 10 μg enzyme was used.

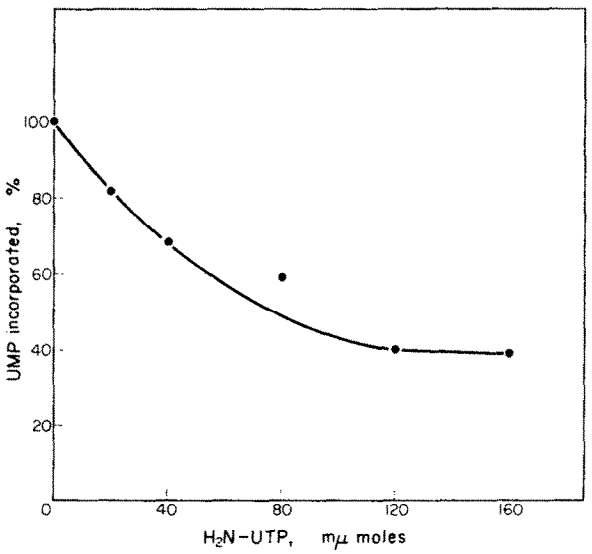

FIG. 2. Effect of H_2N-UTP on incorporation of UMP-^{14}C into RNA. The reaction mixtures were the same as described in Fig. 1 except that 80 μM each of UTP-^{14}C (1×10^6 cpm/μmole), ATP, GTP and CTP were present and the concentration of H_2N-UTP was varied as indicated. The incorporation of UMP-^{14}C into RNA in the absence of analog was 1·66 mμmoles, and this value was taken as 100 per cent incorporation.

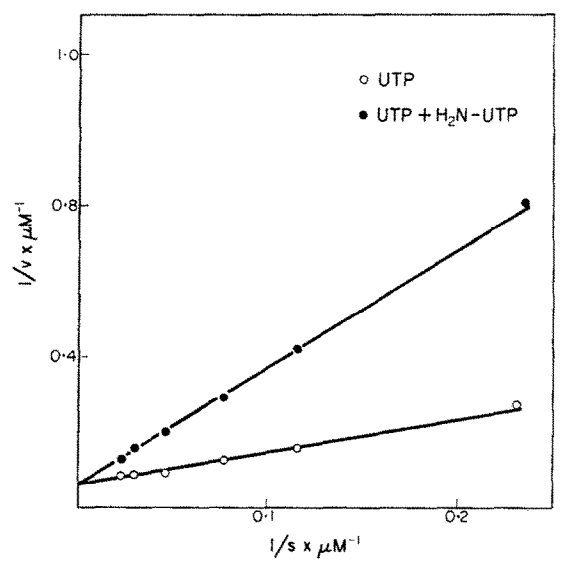

FIG. 3. Plots of the reciprocal of the rate of UMP-^{14}C incorporation (v) against the reciprocal of the molar concentration of UTP (s) in the presence and absence of H_2N-UTP. The reaction mixture, 0·5 ml, contained ATP, GTP and CTP, each at 80 μM, and 10 μg enzyme. The concentration of UTP-^{14}C (1×10^6 cpm/μmole) was varied as indicated and H_2N-UTP was present at 60μM concentration. Other conditions were as described in Fig. 1.

sensitized to nitrous acid.[20] This increased susceptibility to deaminating agent was considered to be due to substitution of this analog for uridine in RNA of those viruses. The present data show that H_2N-UTP substitutes for UTP to an extent of about 40 per cent in the RNA polymerase reaction. Although the incorporation of H_2N-UTP is substantial, it is apparent from the data of Fig. 2 that it does not inhibit RNA synthesis significantly. However, it is possible that high incorporation of H_2N-UTP into RNA may result in functional alterations which may contribute to the inhibitory action of H_2N-Urd.

Formation of H_2N-UDP-glucose and its effect on UDP-glucose dehydrogenase. The nucleoside triphosphate, H_2N-UTP was active as a substrate for UDP-glucose pyrophosphorylase of yeast. Conversion of H_2N-UTP into H_2N-UDP-glucose by this enzyme was about 50 per cent as compared to the conversion of UTP into UDP-glucose under similar conditions. The effectiveness of H_2N-UTP as substrate for UDP-glucose pyrophosphorylase is consistent with the previous findings that H_2N-Urd is converted to 5-aminouridine diphosphate sugar compounds in Ehrlich ascites cells.[5]

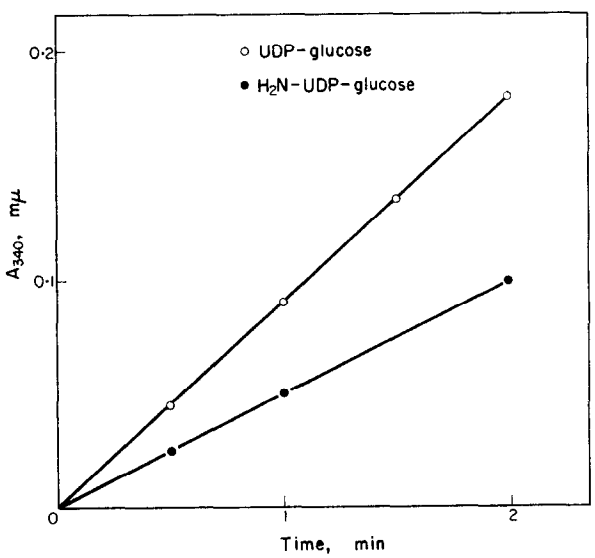

FIG. 4. Relative rates of reaction of UDP-glucose and H_2N-UDP-glucose with UDP-glucose dehydrogenase. The reaction mixture, 1·0 ml, contained 2·0 mM NAD^+, 50 mM Tris-acetate, pH 8·0, 0·21 mM UDP-glucose or H_2N-UDP-glucose and 90 units of enzyme.

Comparison of the initial rate of NAD^+ reduction by UDP-glucose dehydrogenase in the presence of H_2N-UDP-glucose or UDP-glucose showed that the analog was oxidized at a rate one-half that of UDP-glucose (Fig. 4).

Incorporation of HO-dUTP into DNA. The extent to which HO-dUTP replaced dTTP in the synthesis of DNA by DNA polymerase with salmon sperm DNA as template is shown in Table 2. The analog was about 37 per cent as efficient as dTTP. The substitution of HO-dUTP for dTTP in the DNA polymerase reaction was assessed from the incorporation of an accompanying radioactive natural substrate, dATP. The

TABLE 2. CAPACITY OF HO-dUTP TO REPLACE dTTP, dCTP, dATP OR dGTP IN DNA SYNTHESIS

Expt. no. and deoxyribonucleoside triphosphate added*	Nucleotide incorporated (mμmoles)
1. dATP-^{14}C, dCTP, dGTP, dTTP	0·55
2. dATP-^{14}C, dCTP, dTTP	0·02
3. As in expt. 2 plus HO-dUTP	0·03
4. dATP-^{14}C, dTTP, dGTP	0·04
5. As in expt. 4 plus HO-dUTP	0·07
6. dATP-^{14}C, dCTP, dGTP	0·04
7. As in expt. 6 plus HO-dUTP	0·23
8. dTTP-^{14}C, dCTP, dGTP, dATP	0·36
9. dTTP-^{14}C, dCTP, dGTP	0·02
10. As in expt. 9 plus HO-dUTP	0·01

*Each incubation mixture, 0·3 ml, contained 33·3 μM of each of the following deoxyribonucleoside triphosphates indicated to be present in each experiment: dGTP, dCTP, dTTP, dATP, HO-dUTP, dATP-^{14}C (9·7 × 10^5 cpm/μmole) and dTTP-^{14}C (7·3 × 10^5 cpm/μmole); 3·3 mM $MgCl_2$; 0·1 mM mercaptoethanol; 66·3 mM Tris-HCl, pH 7·4; 20 μg of salmon sperm DNA and 0·085 unit of enzyme. The incubation was carried out at 37° for 30 min. Isotope incorporated into acid-insoluble material was measured on Millipore filters as described in the text.

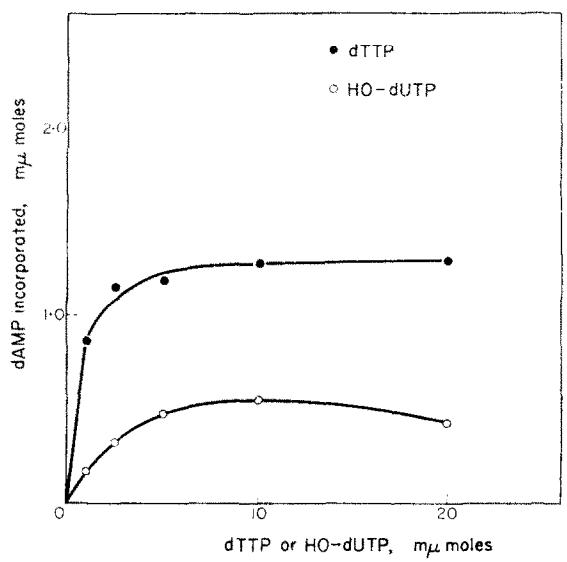

FIG. 5. Incorporation of dAMP-^{14}C into polynucleotide in the presence of various concentrations of HO-dUTP and dTTP. The reaction mixture, 0·3 ml, contained HO-dUTP or dTTP at concentrations which were varied as indicated; 33·3 μM dATP-^{14}C (9·7 × 10^5 cpm/μmole) and 0·031 absorbance unit at 260 mμ of dAT copolymer was used as template. Other conditions were the same as described in Table 2, except that dGTP and dCTP were omitted. The data were plotted after subtraction of blank value (0·01 mμmole) obtained by the omission of HO-dUTP and dTTP.

effectiveness of HO-dUTP as replacement for dTTP in the DNA polymerase reaction with poly dAT as template is shown in Fig. 5. The incorporation of dAMP-^{14}C was dependent on HO-dUTP concentration. Maximum incorporation of dAMP-^{14}C occurred at about the same concentration of HO-dUTP as that of dTTP.

The pK_a value of deoxythymidine is 10. It may be assumed that the pK_a value of HO-Urd (7·8) is not appreciably different from that of HO-dUrd. Thus, the decrease

TABLE 3. EFFECT OF pH ON THE ABILITY OF HO-dUTP TO SUBSTITUTE FOR dTTP IN THE DNA POLYMERASE REACTION

Deoxyribonucleoside triphosphate added*	dAMP incorporated† (%)				
	pH 7·5	pH 8·0	pH 8·5	pH 9·0	pH 9·5
dATP-^{14}C, dGTP, dCTP, dTTP	100 (0·25)	100 (0·27)	100 (0·19)	100 (0·17)	100 (0·06)
dATP-^{14}C, dGTP, dCTP, HO-dUTP	45	38	26	14	0

*Each incubation mixture, 0·3 ml, contained 16·6 μM dTTP or HO-dUTP; dGTP, dCTP and dATP-^{14}C (9·7 $\times$ 10^5 cpm/μmole) each at 33·3 μM; and 66·6 mM Tris-HCl at indicated pH values. Other conditions were as described in Table 3. The blank value obtained by omission of dTTP and HO-dUTP was subtracted from each value given in the table.
†Values in parentheses represent mμmoles of dAMP incorporated.

in the effectiveness of HO-dUTP as a substitute for dTTP with the increase in pH (Table 3) suggests that the undissociated form of the analog is the preferred substrate for DNA polymerase. A similar effect is observed when HO-UTP replaces UTP in the RNA polymerase raction.[9] These data follow the conclusion of Kahan and Hurwitz[19] that dissociated bases are not incorporated into polynucleotides by RNA polymerase, even though they possess hydrogen bonding groups at the same positions as the natural purine and pyrimidine they replace.

Inhibition by HO-dUTP of dAMP-^{14}C or dTMP-^{14}C incorporation into DNA. The inhibitory effect of HO-dUTP on incorporation of dATP-^{14}C or dTTP-^{14}C into DNA is shown in Fig. 6. The inhibitory effect of HO-dUTP on DNA synthesis was more

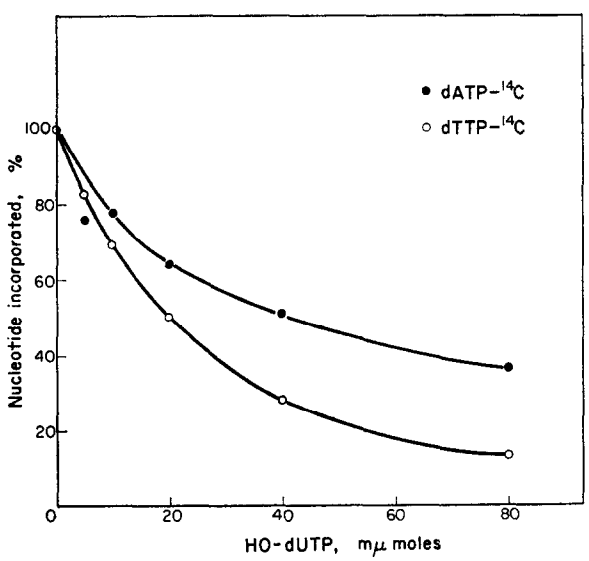

FIG. 6. Effect of HO-dUTP on the incorporation of dAMP-^{14}C and dTMP-^{14}C into DNA. The reaction mixtures were the same as described in Table 2, except that 33·3 μM each of dCTP, dGTP, dTTP and dATP-^{14}C (9·7 $\times$ 10^5 cpm/μmole) or 33·3 μM each of dCTP, dGTP, dATP and dTTP-^{14}C (7·3 $\times$ 10^5 cpm/μmole) were present. The concentration of HO-dUTP was varied as indicated. The incorporation of dAMP-^{14}C and dTMP-^{14}C into DNA in the absence of analog were 0·503 and 0·534 mμmoles, respectively, and these values were taken as 100% incorporation.

pronounced when dTTP was used as the labeled substrate. For example, at a molar ratio of HO-dUTP to dTTP-^{14}C of 4, the analog depressed the incorporation of dTMP-^{14}C into DNA by more than 70 per cent whereas the analog present in the same ratio produced only about 50 per cent inhibition of dAMP-^{14}C incorporation. This difference is consistent with the previous results, which show that HO-dUTP specifically substitutes for dTTP in the DNA polymerase reaction. Thus, incorporation of the analog into DNA would be expected to produce a corresponding decrease in dTTP-^{14}C incorporation.

A more detailed investigation of the nature of this inhibitory effect of HO-dUTP compared initial rates of dTMP incorporation in the presence of HO-dUTP at various concentrations of dTTP-^{14}C. Analysis of the data according to Lineweaver and Burk suggested that the analog acts as a competitive inhibitor of dTTP in the DNA polymerase reaction (Fig. 7).

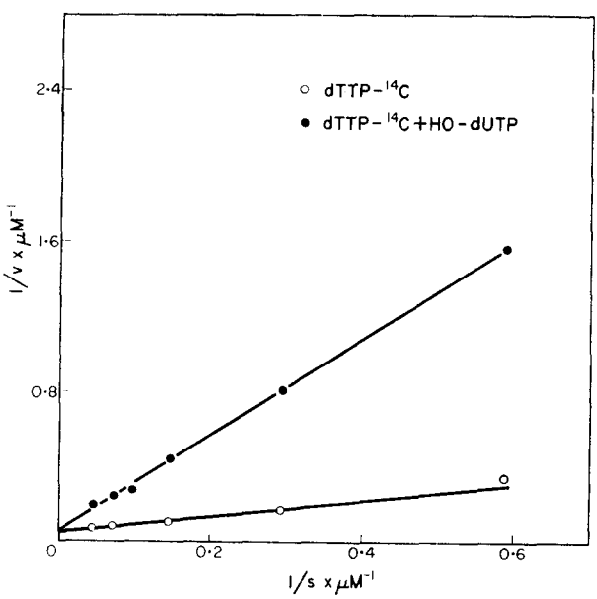

FIG. 7. Plots of the reciprocal of the rate of dTMP-^{14}C incorporation (v) against the reciprocal of the molar concentration of dTTP (s) in the presence and absence of HO-UTP. The reaction mixture, 0·3 ml, contained 33·3 μM dATP; 3·3 mM $MgCl_2$; 0·1 mM mercaptoethanol; 66·6 mM Tris-HCl, pH 7·4; 0·031 absorbance unit at 260 mμ of dAT copolymer and 0·17 unit of enzyme. The concentration of dTTP-^{14}C (7·3 × 10^5 cpm/μmole) was varied as indicated and HO-dUTP was present at 66·6 μM concentration. The rest of the conditions were as described in Table 2.

The observation that pH has a marked influence upon HO-dUTP incorporation into DNA, reflecting the low pK_a of HO-dUrd as compared to deoxythymidine, led to studies of the effect of pH on the inhibitory effect of HO-dUTP. The results showed that as pH values were increased the inhibitory effect of HO-dUTP on dTMP-^{14}C incorporation into DNA was decreased. For example, at pH 7·5, HO-dUTP decreased dTMP incorporation into DNA to about 50 per cent of the values obtained in the

absence of analog (Table 4). At pH 9·5, dTMP was incorporated to about 99 per cent of the control at the same molar ratio (2:1) of analog to dTTP-^{14}C.

A control experiment was conducted to determine the chemical stability of HO-dUTP at the pH values used in these studies. No change in absorptivity of light at 280 or 260 mμ occurred when a solution of HO-dUTP was maintained at room temperature in a buffer at pH 9·5 for 24 hr. Thus, the decreased effectiveness of HO-dUTP as substrate or inhibitor of DNA synthesis at higher pH values is not due to its degradation. From the data showing the extent to which HO-dUTP replaced dTTP in DNA synthesis (Table 2) and the amount to which dTTP incorporation into DNA is

TABLE 4. EFFECT OF pH ON THE INHIBITORY EFFECT OF HO-dUTP ON dTMP INCORPORATION

HO-dUTP* added (μM)	dTMP incorporated† (%)				
	pH 7·5	pH 8·0	pH 8·5	pH 9·0	pH 9·5
None	100 (0·33)	100 (0·31)	100 (0·21)	100 (0·17)	100 (0·10)
33·3	50	70	86	90	99

*Each incubation mixture contained 16·6 μM dTTP-^{14}C; dGTP, dCTP and dATP each at 33·3 μM; 66·6 mM Tris-HCl at indicated pH values. Other conditions were as described in Table 2.
†Values in parentheses represent mμmoles of dTMP incorporated.

depressed by this analog (Fig. 6), it appears likely that the inhibition of DNA synthesis by this analog is primarily due to its ability to act as an alternate substrate for dTTP. The effect of pH on the inhibitory effect of this analog in DNA synthesis is also explained by its action as an alternate substrate. This conclusion is supported by the data of Tables 3 and 4, which show that the inhibition is more pronounced at the optimum pH for the analog incorporation, and that the incorporation of HO-dUTP into DNA and its inhibitory effect on DNA synthesis both decrease progressively with the increase in pH values.

The behaviour of HO-dUTP in DNA polymerase reaction is distinctly different from that of HO-UTP in RNA polymerase reaction.[9] Unlike HO-dUTP, the inhibitory effect of HO-UTP on RNA synthesis cannot be explained by its ability to act as an alternate substrate for the ribonucleoside triphosphate which it replaces. Futhermore, the inhibitory effect of HO-UTP on RNA synthesis is more pronounced when the nucleotide exists primarily in the dissociated form.

Preliminary experiments showed that HO-dUrd is not phosphorylated to HO-dUTP by kinases from *E. coli* or Ehrlich ascites cells. Thus, the inhibitory effects of HO-dUrd cannot be explained by the effect of HO-dUTP as substrate of inhibitor of DNA synthesis in these cells.[3, 6] However, since there is a facile conversion of HO-Urd to its nucleotides in Ehrlich ascites cells, it is possible that the deoxyribonucleotide of the analog may be formed by the action of nucleotide reductase.

Acknowledgements—The authors are indebted to Mr. Carlos M. Medina for assistance and to Dr. Y. H. Huang for cooperation during the course of this work.

REFERENCES

1. M. ROBERTS and D. W. VISSER, *J. biol. Chem.* **194**, 695 (1952).
2. I. J. SLOTNICK, D. W. VISSER and S. C. RITTENBERG, *J. biol. Chem.* **203**, 647 (1953).
3. D. W. VISSER, in *Antimetabolites and Cancer* (Ed. C. P. RHOADS), p. 47, American Association for the Advancement of Science, Washington (1955).
4. D. W. VISSER, D. L. LAGERBORG and H. E. PEARSON, *Proc. Soc. exp. Biol. Med.* **79**, 571 (1952).
5. D. A. SMITH, P. ROY-BURMAN and D. W. VISSER, *Biochim. biophys. Acta* **119**, 221 (1966).
6. R. E. BELTZ and D. W. VISSER, *J. Am. chem. Soc.* **77**, 736 (1955).
7. H. E. PEARSON, D. L. LAGERBORG and D. W. VISSER, *Proc. Soc. exp. Biol. Med.* **93**, 61 (1956).
8. D. A. SMITH and D. W. VISSER, *J. biol. Chem.* **240**, 446 (1965).
9. S. ROY-BURMAN, P. ROY-BURMAN and D. W. VISSER, *J biol. Chem.* **241**, 781 (1966).
10. J. J. FURTH, J. HURWITZ and M. ANDERS, *J. biol. Chem.* **237**, 2611 (1962).
11. C. F. FOX, R. I. GUMPORT and S. B. WEISS, *J. biol. Chem.* **240**, 2101 (1965).
12. M. RABINOWITZ and I. H. GOLDBERG, *J. biol. Chem.* **238**, 1801 (1963).
13. J. L. STROMINGER, E. S. MAXWELL, J. AXELROD and H. M. KALCKAR, *J. biol. Chem.* **224**, 79 (1957).
14. S. N. CHAKRAVARTY and M. B. ROY, *Analyst, Lond.* **62**, 603 (1937).
15. P. ROY-BURMAN, S. ROY-BURMAN and D. W. VISSER, *J. biol. Chem.* **243**, 1692 (1968).
16. D. DAVIDSON and O. BAUDISCH, *J. biol. Chem.* **64**, 619 (1925).
17. B. K. ZIMMERMAN, *J. biol. Chem.* **241**, 2035 (1966).
18. N. D. GOLDBERG, J. L. DAHL and R. E. PARK, JR., *J. biol. Chem.* **238**, 3109 (1963).
19. F. M. KAHAN and J. HURWITZ, *J. biol. Chem.* **237**, 3778 (1962).
20. L. THIRY, *Virology* **28**, 543 (1966).