Allen, J. D., & Thoma, J. A. (1978) *Biochemistry 17* (preceding paper in this issue).
Bardsley, W. G., & Childs, R. E. (1975) *Biochem. J. 149,* 313.
Chipman, D. M. (1971) *Biochemistry 10,* 1714.
Chipman, D. M., Pollock, J. J., & Sharon, N. (1968) *J. Biol. Chem. 243,* 487.
Cleland, W. W. (1967) *Adv. Enzymol. 29,* 1-32.
Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2,* 1-65.
Cleland, W. W. (1975) *Biochemistry 14,* 3220.
Dahlquist, F. W., Rand-Meir, T., & Raftery, M. A. (1969) *Biochemistry 8,* 4214.
Huber, R. E., & Thompson, D. J. (1973) *Biochemistry 12,* 4011.
Jarabak, R., & Westley, J. (1974) *Biochemistry 13,* 3237.
Jermyn, M. A. (1962a) *Aust. J. Biol. Sci. 15,* 233.
Jermyn, M. A. (1962b) *Aust. J. Biol. Sci. 15,* 248.
King, E. L., & Altman, C. (1956) *J. Phys. Chem. 60,* 1375.
Lee, H., & Wilson, I. B. (1971) *Biochim. Biophys. Acta 242,* 519.
Legler, G. (1968) *Biochim. Biophys. Acta 151,* 728.
McLean, C., Werner, D. A., & Aminoff, D. (1973) *Anal. Biochem. 55,* 72.
Nakamura, L. K. (1970) *Can. J. Biochem. 48,* 1260.
Robyt, J. F., & French, D. (1970) *J. Biol. Chem. 245,* 3917.
Thoma, J. A., Spradlin, J. E., & Dygert, S. (1971) *Enzymes, 3rd Ed. 5,* 115-189.
Umezurike, G. M. (1975) *Biochim. Biophys. Acta 397,* 164.
Voet, J. G., & Abeles, R. H. (1970) *J. Biol. Chem. 245,* 1020.

Enzymatic Preparation of the 5′-Triphosphates of 2′-Deoxytubercidin, 2′-Deoxytoyocamycin, and 2′-Deoxyformycin and the Allosteric Effects of These Nucleotides on Ribonucleotide Reductase†

Shirley A. Brinkley, Arthur Lewis, Walter J. Critz, Linda L. Witt, Leroy B. Townsend, and Raymond L. Blakley*

ABSTRACT: Analogues of 2′-deoxyadenosine are potentially of interest as chemotherapeutic agents since the corresponding 5′-triphosphates are expected to mimic the action of dATP in causing allosteric inhibition of mammalian ribonucleotide reductase, but to be metabolically more stable than dATP. A synthetic route to analogues of dATP via preparative reduction of some ATP analogues is described, which could be used for large scale preparation of the dATP analogues and the corresponding 2′-deoxyribonucleosides. The naturally occurring nucleosides tubercidin, toyocamycin, and formycin have been converted to the corresponding 5′-triphosphates by published procedures and purified by ion-exchange chromatography. Tubercidin triphosphate has been reduced to 2′-deoxytubercidin 5′-triphosphate (dTuTP) enzymatically in the presence of the ribonucleotide reductase from *Lactobacillus leichmannii* with about 90% yield. After purification by chromatography on an organoborate column and by ion-exchange chromatography on DEAE-cellulose the recovery of dTuTP was about 76% with 9% recovery of unreacted tubercidin triphosphate. In the absence of allosteric activators, toyocamycin triphosphate (ToTP) and formycin triphosphate (FTP) are not reduced by the *L. leichmannii* reductase, although FTP binds to the catalytic site as shown by its linear competitive inhibition of the reduction of ATP, GTP, and UTP with inhibition constants in the range 0.3 to 0.6 mM. However, in the presence of 1 mM dGTP, FTP is reduced at about the same rate as ATP, and ToTP at about half this rate. With FTP as substrate the true K_m for dGTP is 0.029 ± 0.011 mM and the kinetically determined dissociation constant 0.106 ± 0.033 mM. In the presence of 0.5 mM dGTP the yield of dFTP from the organoborate column averaged 49% with recovery of 39% of the original FTP. dGTP was largely separated from dFTP on this column. dTuTP, dFTP, and dToTP are all able to mimic dATP as activators of CTP reduction by the *L. leichmannii* reductase. The kinetically determined dissociation constants for the enzyme-activator complexes and for the enzyme-CTP-activator complexes indicate that dTuTP, dToTP, and dFTP all bind to the allosteric site with about the same affinity as dATP.

A number of considerations suggest that ribonucleotide reductase is the rate-limiting enzyme in DNA biosynthesis. The enzyme is present at very low levels in vertebrate tissues, but activity rapidly increases when the tissue commences proliferation. Thus Larsson (1969) and King & Lancker (1969) have shown that a short period after partial hepatectomy of rats there is a rapid increase in reductase activity in the regenerating liver that progresses for 20-30 h. Other rapidly proliferating cells such as Yaba poxvirus tumor (Gordon & Fiel, 1969), leukemic mouse spleen (Fujioka & Silber, 1970), and Ehrlich ascites tumor (Cory & Whitford, 1972) show a similar correlation between cell growth and ribonucleotide reductase activity. There is also a strict parallelism between the measured levels of ribonucleotide reductase and the fraction of L-cells that are synthesizing DNA (Turner et al., 1968). An even more impressive report is that of Elford et al. (1970) who studied the level of ribonucleotide reductase activity in a series of minimum deviation hepatomas. They

† From the Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52242, and the Department of Biopharmaceutical Sciences, College of Pharmacy, University of Utah, Salt Lake City, Utah 84112. *Received December 22, 1977.* This research was supported by United States Public Health Service Research Grant CA 16254 and Research Contracts N01-CM-43806 and CM-77142 from the Division of Cancer Treatment NCI, National Institutes of Health, Department of Health Education and Welfare.

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found a remarkable correlation between the growth rate of the tumors, as measured by their mean generation time, and the level of reductase activity. The reductase activity spanned a 200-fold range whereas two other enzymes of the DNA biosynthetic pathway increased to a much smaller extent. Thymidylate synthetase increased only by about 20-fold and thymidine kinase by 40-fold. The level of ribonucleotide reductase activity therefore correlates much more closely with the rate of DNA synthesis than does the level of other enzymes such as thymidine kinase (Cory & Whitford, 1972; Elford et al., 1970).

Although this evidence is admittedly not conclusive for the rate-limiting function of ribonucleotide reductase, it is so strongly suggestive that this enzyme should be further investigated as a suitable target for chemotherapeutic agents. An enzyme which is at all times low in activity compared with others in the pathway and which correlates so closely with the rate of DNA synthesis and cellular proliferation is an ideal target for inhibitors designed to slow the entire pathway.

Certain known chemotherapeutic agents in fact appear to act by inhibiting ribonucleotide reductase. Hydroxyurea, which has antileukemic activity in both mice and humans (Young & Hodas, 1964; Frenkel et al., 1964), has been shown by in vitro studies to inhibit DNA synthesis but not synthesis of RNA or protein, and its inhibitory effect on DNA synthesis has been shown to be caused by inhibition of ribonucleotide reductase (Frenkel et al., 1964; Young et al., 1967). Evidence has also been obtained that another series of chemotherapeutic agents, the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones, also act by inhibiting ribonucleotide reductase (Moore et al., 1971; Agrawal et al., 1972; Booth et al., 1974).

The nature of another type of suitable inhibitor for ribonucleotide reductase is suggested by what is known of physiological regulators of this enzyme. In work with partially purified mammalian ribonucleotide reductase from various sources (Larsson, 1969; Fujioka & Silber, 1970; Moore & Hurlbert, 1966; Moore, 1967) as well as in cell-free extracts (Gordon & Fiel, 1969), dATP has been shown to inhibit the reduction of one or more ribonucleotide substrates. Evidence that this regulatory mechanism operates in vivo is provided by the fact that deoxyadenosine is a potent inhibitor of the incorporation of precursors into DNA (Langer & Klenow, 1960; Maley & Maley, 1961). The other inhibitor of mammalian ribonucleotide reductase is dTTP (Larsson, 1969; Fujioka & Silber, 1970; Moore & Hurlbert, 1966) and again the corresponding nucleoside, thymidine, is a powerful inhibitor in vivo (Hakala & Taylor, 1959; Morris & Fischer, 1960; Bjursell & Reichard, 1973). In this case the effect of dTTP is to inhibit rather specifically the reduction of CDP with a consequent drop in the pool of dCTP (Morris & Fischer, 1960; Whittle, 1966).

Many adenosine-like nucleoside antibiotics have been synthesized and several have been isolated from natural sources. However, the corresponding 2′-deoxy derivatives and their phosphates have not been extensively studied, primarily because of the difficulty encountered in their chemical synthesis. Robins & Muhs (1976) have published a six-step synthetic route for converting tubercidin into 2′-deoxytubercidin in a 27% yield. However, at two steps, this requires extensive purification from the side-products which are formed in large amounts. 2′-Deoxyformycin has similarly been prepared from formycin (Jain et al., 1973; Robins et al., 1973) but in very low yield since the 3′-deoxy derivative is the major product in these procedures. More recently, a good yield of 2′-deoxytoyocamycin and 2′-deoxysangivamycin has been obtained from chemical manipulations of the parent ribonucleosides

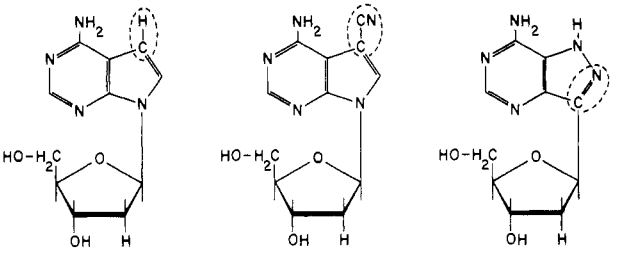

FIGURE 1: Structures of deoxytubercidin, deoxytoyocamycin, and deoxyformycin. The part of the structure that differs from deoxyadenosine is enclosed by the dashed line.

(Maruyama, T., Roti Roti, L. W., and Townsend, L. B., manuscript in preparation).

The possibility of an enzymatic route to deoxyadenosine and analogues is suggested by the report of Suhadolnick et al. (1968) who showed that tubercidin triphosphate is a substrate for the ribonucleotide reductase of *Lactobacillus leichmannii* and that toyocamycin triphosphate is also a substrate in the presence of dGTP. We report studies designed to show the feasibility of such a synthetic route. An important feature of the method is that it employs the bacterial reductase to reduce ATP analogues to dATP analogues on a preparative scale. Dephosphorylation of these nucleotides provides the nucleosides shown in Figure 1.

Experimental Procedure

Materials

The adenosine analogues, tubercidin, toyocamycin and formycin were obtained from commercial sources. Ribonucleotide reductase was prepared from *L. leichmanni* according to the procedure of Hoffman & Blakley (1975) through the second ammonium sulfate precipitation. The enzyme was stored at 5 °C under nitrogen in 0.1 M sodium citrate buffer, pH 5.0, containing 0.02% sodium azide. The enzyme used for preparative reduction of ATP analogues had a specific activity of 0.5 to 1.0 unit (μmol of ATP reduced per min) per mg of protein. Enzyme used in kinetic studies had a specific activity of 2 units per mg and was prepared by the procedure of Singh et al. (1977). The enzyme was assayed colorimetrically as described elsewhere (Blakley, 1978) with ATP as substrate.

Methods

Phosphorylation of Ribonucleoside Analogues. Tubercidin 5′-phosphate and formycin 5′-phosphate were prepared from the unblocked nucleosides by the method of Yoshikawa et al. (1967) and isolated as the free phosphates according to the procedure used for AMP by Imai et al. (1969). Although toyocamycin phosphate can be prepared by direct phosphorylation of the unblocked nucleoside, it was found that improved yields could be obtained by using the 2′,3′-*O*-isopropylidine derivative, with removal of the blocking group subsequently. The monophosphates were checked for purity by thin-layer chromatography on Silic AR-7GF plates (Mallinckrodt) with 0.1 M ammonium chloride–acetonitrile (3:7 or 2:9, v/v) as solvent.

Triphosphates (TuTP,[1] ToTP, and FTP) were prepared from the monophosphates via their morpholidate by reaction

[1] Abbreviations used: TuTP, FTP, and ToTP, the 5′-triphosphates of tubercidin, formycin, and toyocamycin, respectively; dTuTP, dFTP, and dToTP, the 2′-deoxy 5′-triphosphates of tubercidin, formycin, and toyocamycin, respectively.

TABLE I: Effect of Experimental Conditions on Yield of dATP Analogues.[a]

Substrate		Enzyme:substrate ratio (units/μmol)	dGTP concn (mM)	Product recovery (%)		Substrate recovery (%)
Identity	Amount (μmol)			After dihydroxyborylcellulose	After DEAE-cellulose	
TuTP	3	0.067	0			
TuTP	16	0.067	0	87	77	4
TuTP	3	0.083	0	94		
TuTP	6	*b*	0	87	84	12
TuTP	10	0.042	0	88		
TuTP	400	0.083	0	85		
TuTP	120	0.083	0	94	78	6
TuTP	360	0.083	0	88	65	12
TuTP	420	0.10	0	86	84	
FTP	30	0.13	0.05	42		46
FTP	10	0.38	0.05	52		
FTP	3	0.2	0.15	38	32	37
FTP	3	0.2	0.50	46		55
FTP	25	0.5	1.0	49	30	19
FTP	187	1.0	1.0	67		
ToTP	24	0.1	1.0	40	34	
ToTP	65	0.5	1.0	73	70	

[a] For other details, see the Experimental Procedure section. [b] Sepharose-bound ribonucleotide reductase was used in this instance. [c] Purification on Sephadex G-10 was used instead of DEAE-cellulose.

with bis(tri-*n*-butylammonium) pyrophosphate as described by Moffatt & Khorana (1971). The optimum reaction time was found to be 3.5 h. The triphosphates were separated by chromatography on DEAE-cellulose (HCO_3^-) using a linear gradient of 0.05 to 0.5 N triethylammonium bicarbonate (pH 7.5) and converted to their sodium salts as described by Hoard & Ott (1965). The triphosphates were homogeneous as judged by paper chromatography (descending on Whatman No. 1, with isobutyric acid–1 M ammonium hydroxide–0.1 M EDTA, 25:15:0.4, as solvent) and high voltage electrophoresis (Whatman 3 MM paper in 1 M ammonium formate buffer, pH 3.8, 2000 V).

Diphosphates (TuDP, FDP, and ToDP) were prepared and isolated, in an identical manner, by reaction of the morpholidates with tri-*n*-butyl phosphate. The diphosphates were similarly checked for chromatographic and electrophoretic homogeneity. FDP was used as its triethylammonium salt, whereas ToDP and TuDP were converted to their sodium salts.

Enzymatic Reduction of ATP Analogues. The reaction mixture contained 1 M sodium acetate, 50 mM potassium phosphate, 25 mM dihydrolipoate, 0.8 mM EDTA, 8 μM adenosylcobalamin, *L. leichmannii* ribonucleotide reductase, and 2 mM analogue. The mixture was adjusted to pH 7.3. Adenosylacobalamin was added last in a dim light and from this point light was excluded from the reaction mixture. A typical reaction mixture contained 400 μmol of TuTP and 33 units of ribonucleotide reductase in a volume of 200 mL (Table I). For reduction of ToTP or FTP, dGTP (0.05 to 1 mM) was also present. Reductions were carried out at 37 °C under a nitrogen atmosphere for 1.5 h.

Dihydroxyboryl-Subsituted Cellulose. N-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose was synthesized according to the procedure of Weith et al. (1970). Since the aminoethyl substitution was 0.35 mequiv per g of cellulose instead of 1 mequiv/g, 2.8 times more aminoethylcellulose was used for the synthesis; i.e., 72 g (25 mequiv) of aminoethylcellulose were reacted with 5.9 g of dihydroxyborylphenylsuccinamic acid. The degree of substitution of the boryl derivative, as calculated by the method of Weith et al. (1977), was 0.12 mmol per g of aminoethylcellulose.

The ammonia wash which contained most of the unreacted *N*-(*m*-dihydroxyborylphenyl)succinamic acid was stored at −100 °C until used for recovery of the boryl derivative. Ammonia was removed by concentration of the solution on a rotary evaporator to 0.25 of the original volume. The concentration was then diluted fivefold with water, the pH adjusted to 4.1 with HCl, and the solution applied to a column of Dowex 50 (H^+ form, 2.2 × 29 cm for 500 mL of solution). The boryl derivative was washed through the column with water, and fractions absorbing at 260 nm were pooled and evaporated to dryness under reduced pressure. The white residue was crystallized from water: mp 173–174 °C. The recovered material had ϵ_{260} 4800 in 0.2 M ammonia and λ_{max} = 243 nm, ϵ_{max} = 10 800 in 1 M potassium phosphate pH 7.3; recovery: approximately 50% of the unreacted material.

Determination of the Reduction of ATP Analogues. Since the analogues of dATP did not react with the diphenylamine reagent normally used for determining reduction rates, the reduction of these ATP analogues cannot be followed by the normal colorimetric assay. The course of the reaction can be followed by removing samples of the reaction mixture at intervals and passing them through dihydroxyboryl-substituted cellulose columns. The amount of deoxyribonucleotide formed can then be estimated from the absorbance at the appropriate wavelength of material passing unretarded through the column (see also Figure 2). This is similar to the method of Moore et al. (1974). When this method was applied to the reduction of ToTP or FTP, the separation on the column of dGTP from the deoxyribonucleotide product was sufficient to prevent significant interference by dGTP, provided the concentration of dGTP in the reaction was not greater than 0.5 mM. The recovery of total nucleotides from the column was between 85 and 94% for TuTP reduction and about 40–73% for reduction of FTP or ToTP.

Isolation of Deoxyribonucleotides. dATP analogues were isolated from their respective reaction mixtures using the dihydroxyborylphenylsuccinamylaminoethylcellulose column previously equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 0.02% sodium azide, at 2–4 °C. Approximately 200 mL of reaction mixture containing 400 μmol of nucleotide was applied directly to a 4.5 × 70 cm col-

TABLE II: Chromatographic Data for Ribonucleotides and Deoxyribonucleotides.[a]

	R_f	
	Solvent A	Solvent B
ATP	0.38	0.92
dATP	0.51	0.92
TuTP	0.02	0.90
dTuTP	0.37	0.91
FTP	0.27	0.94
FDP	0.37	
dFTP	0.41	0.90
ToTP	0.38	0.95
dToTP	0.51	0.92
dGTP	0.27	0.97

[a] Chromatography was performed on thin-layer cellulose in two solvent systems: (A) isobutyric acid:concentrated NH_4OH:H_2O (66:1:33 v/v); and (B) 5% Na_2HPO_4 saturated with isoamyl alcohol.

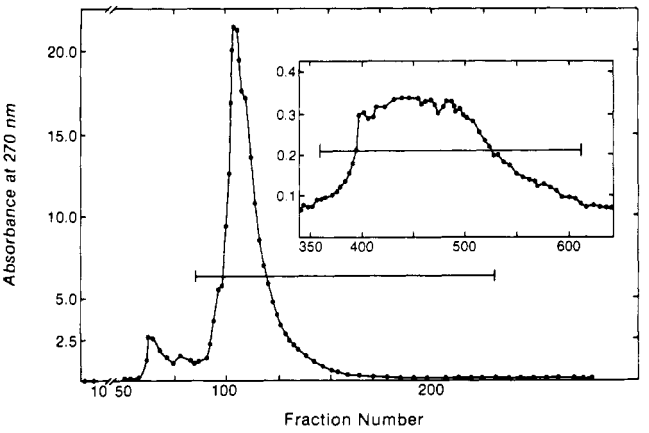

FIGURE 2: Separation of dTuTP from other components in the reaction mixture. The reaction mixture, prepared as described in the Methods Section and initially containing 400 μmol of TuTP in 200 mL, was incubated for 1.5 h at 37 °C and then applied to the dihydroxylboryl-substituted cellulose colum (4.5 × 70 cm) in the cold. Elution was carried out with 50 mM potassium phosphate buffer, pH 7.5, containing 0.02% sodium azide. The elute was collected in 10.4-mL fractions and monitored at 270 nm. TLC showed that dTuTP was present in the major peak with maximum in fraction 105, whereas unreacted TuTP appeared in the small broad peak with maximum at fraction 444. The two small early peaks contained other components of the reaction mixture. Note that in the insert the abscissa scale is contracted and the ordinate expanded.

umn and elution performed with the same buffer at 2–4 °C. The effluent was collected in 10-mL fractions and monitored at the appropriate wavelength until all absorbing material had been eluted. Fractions containing the deoxynucleotide were pooled and diluted fivefold with water. The relevant spectrophotometric data for the nucleosides, which were assumed to apply to ribonucleotides and deoxyribonucleotides also, are as follows: tubercidin, λ_{max} 270 nm, ϵ 12.1 × 10^3; toyocamycin, λ_{max} 277 nm, ϵ 14.3 × 10^3; formycin, λ_{max} 295 nm, ϵ 10.8 × 10^3 (Suhadolnik, 1970).

DEAE-Cellulose Chromatography. The deoxyribonucleotides were further purified and desalted on a DEAE-cellulose column previously equilibrated with 50 mM triethylammonium bicarbonate buffer, pH 7.5, at 2–4 °C. The diluted fractions from the previous column (~120 μmol of nucleotide) were applied to the DEAE-cellulose column (1.2 × 39 cm) at 4 °C and the column washed overnight with 2 to 3 L of water at 4 °C. The nucleotides were eluted with a linear gradient prepared by starting with 300 mL of water in the mixing chamber and 300 mL of 0.5 M triethylammonium bicarbonate buffer, pH 7.5, in the reservoir. In the case of dToTP and dFTP the vessels each contained 800 mL. The effluent was collected in 8-mL fractions and monitored at the absorbance maximum of the nucleotide, and also at 253 nm if dGTP was present. Fractions containing the deoxyribonucleotide were pooled and freeze-dried. The residue was partially dissolved in ethanol (95% v/v) and evaporated to dryness under reduced pressure to remove triethylammonium bicarbonate. This was repeated three or four times. Finally, the residue was dissolved in water and freeze-dried twice more. The final residue was stored at −100 °C.

Thin-Layer Chromatography. The analogues were chromatographed on thin-layer cellulose containing fluorescent indicator (Eastman Kodak) in either solvent A, isobutyric acid:NH_4OH:H_2O (66:1:33 v/v), or solvent B, 5% Na_2HPO_4 saturated with isoamyl alcohol. R_f values are listed in Table II.

Phosphate Determinations. These were carried out according to the procedure of Ames & Dubin (1960) with the 100 °C incubation time reduced to 7 min.

Determination of Kinetic Parameters. For determination of inhibition constants or activation constants, the spectrophotometric assay was used (Vitols et al., 1967; Blakley, 1978). Reaction mixtures contained 0.2 M potassium phosphate buffer, pH 7.5, 4 mM EDTA, 0.2 mM NADPH, 20 μM adenosylcobalamin, 7 μM thioredoxin, 0.06 unit of thioredoxin reductase, 0.05 unit of ribonucleotide reductase, and various concentrations of ribonucleotide substrate and activating or inhibitory nucleotides.

Kinetic parameters for activation of CTP reduction by dATP or its analogues were obtained by fitting data to the equation:

$$v = \frac{VA}{K\left(\frac{1 + I/K_{IN}}{1 + I/K_{ID}}\right) + A}$$

Fitting was accomplished with the use of a computer program provided by Dr. W. W. Cleland. V and v are the maximum and measured velocity, respectively; A is the concentration of CTP, I is the concentration of dATP or its analogue, K is the Michaelis constant for CTP in absence of activator, K_{IN} is the dissociation constant for the enzyme–activator complex, and K_{ID} for the enzyme–CTP–activator complex. A graphical method (Segal, 1975) was used to obtain initial estimates of dissociation constants and the Michaelis constant, or to obtain approximate values when the data were not good enough for the computer program to work.

In the case of dGTP activation of FTP reduction, since no reduction occurred unless dGTP was also present, the data were fitted to the equation for a bi-sequential reaction:

$$v = \frac{VAB}{K_{iA}K_B + K_AB + K_BA + AB}$$

Fitting was accomplished by the use of a computer program provided by Dr. W. W. Cleland (1963). V and v have the same meanings as previously, A and B are the concentrations of dGTP and FTP respectively, K_{iA} is the dissociation constant for the dGTP–enzyme complex, K_A is the Michaelis constant, and K_B is the Michaelis constant for FTP.

Data for inhibition of the reduction of ATP, GTP, or UTP were fitted to the equation for linear competitive inhibition by the use of Cleland's computer program (Cleland, 1963). Apparent Michaelis constants were determined by the use of the hyper program of Cleland (1963).

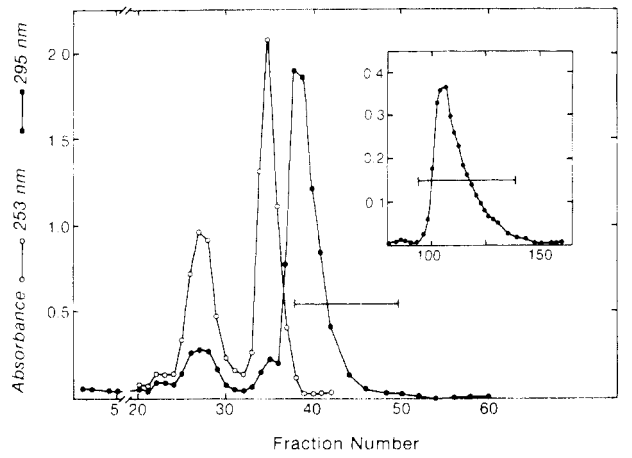

FIGURE 3: Partial separation of dFTP from other components of the reaction mixture. The reaction mixture, prepared as described in the Method Section, initially contained μmol of FTP and 0.6 unit of reductase. After incubation for 1.5 h, the reaction mixture was applied to the dihydroxyboryl-substituted cellulose column (0.9 × 87 cm) and eluted with 50 mM potassium phosphate buffer, pH 7.5, containing 0.02% sodium azide. The effluent was collected in 2-mL fractions and the absorbance monitored at 295 nm and 253 nm, the absorbance maxima for dFTP and dGTP, respectively. Absorbance at 253 nm in the region of dGTP and dFTP overlap was corrected for the contribution by dFTP which was calculated from the absorbance at 295 nm and a ratio of absorbance at 253 nm to absorbance at 295 nm of 0.32 for dFTP. Note that in the insert the abscissa scale is contracted and the ordinate expanded.

Results

Enzymatic Reduction of TuTP. The ribonucleotide reductase from *L. leichmannii* can readily be purified on a large scale to a specific activity suitable for use in the preparative reduction of ribonucleotides (Singh et al., 1977). Since this reductase has substrate specificity for triphosphates (Blakley, 1966), the adenosine analogues tubercidin, toyocamycin, and formycin were converted to the 5′-triphosphates by established procedures, as described in the Methods section, and tested as substrates for the reductase.

TuTP was readily reduced under usual assay conditions, and in the spectrophotometric assay the apparent K_m was 0.55 ± 0.10 mM (cf. for ATP, K_m = 0.80 ± 0.11 mM) and the maximum velocity was 92% that for ATP. No activator was required for reduction of TuTP, but dGTP produced an activation similar to that seen for ATP. When the reaction was allowed to proceed under the conditions specified for dTuTP preparation in the Methods section, extensive formation of dTuTP occurred if the amount of enzyme present (in units of activity) was 0.08 times the amount of TuTP (in μmol; Table I). A study of the time course of the reduction showed that 98% reduction occurred in 1.5 h.

Reduction of ToTP and FTP. Neither of these nucleotides served as substrates in the absence of activators. Nevertheless, they bind to the catalytic site since they are inhibitors of the reduction of other substrates. FTP was found to be a linear competitive inhibitor of the reduction of ATP, GTP, and UTP and K_i values of 0.93, 0.67, and 3.4 mM were obtained in the presence of these respective substrates. FTP did not, however, cause inhibition of CTP reduction. Since dFTP is an activator of CTP reduction (see below) and since ribonucleoside triphosphates can bind weakly at the regulatory site (Blakley, 1978), it may be assumed that FTP combines at the regulatory site and causes some activation of CTP reduction. This activation would offset the competitive inhibition caused by FTP combination at the catalytic site, resulting in the observed absence of any net effect. No activation effects would be anticipated for other substrates, since dATP specifically activates only CTP reduction (Vitols et al., 1967) and analogues presumably do likewise.

In the presence of dGTP, both ToTP and FTP can be reduced by the *L. leichmannii* reductase. The kinetics of the activation of FTP reduction by dGTP indicated that the Michaelis constant for FTP is 0.13 ± 0.03 mM, the Michaelis constant for dGTP is 0.029 ± 0.011 mM, and the dissociation constant for the enzyme-dGTP complex is 0.106 ± 0.033 mM. ToTP and FTP are reduced in the presence of dGTP at rates comparable to that for ATP reduction. Thus in the presence of 1 mM dGTP the relative rates of reduction of the nucleotides ATP, FTP, and ToTP, each at a concentration of 0.4 mM, were 1.0, 0.91, and 0.50.

Reduction of ToTP and FTP was not as complete as in the case of TuTP (Table I). Thus reduction of FTP by reductase (0.2 unit per μmol of FTP) in the presence of 0.5 mM dGTP was only 46% complete in 1.5 h. This may be due to displacement of dGTP from the activator site by dFTP or dToTP. The yield can be improved a little by a larger amount of enzyme (Table I).

Isolation of Deoxyribonucleotide Analogues. Dihydroxyboryl-substituted cellulose columns not only provided a suitable means of following the progress of the reduction (see Methods) but were also the primary means for the isolation of the dATP analogue products. As illustrated in Figure 2 the dATP analogues separated well from unreacted ribonucleoside triphosphate and were largely separated from other components of the reaction mixture, provided the sample load was appropriate. In the case of ToTP and FTP reaction mixtures, the dGTP was also largely separated from the product. Thus in Figure 3, 1.8% of the nucleotide in the pooled fractions was dGTP. The fractions containing the dATP analogue were pooled for further purification by chromatography on DEAE-cellulose as described in the Methods section.

After lyophilization, the deoxynucleotide products were pure as determined by thin-layer chromatography in two solvent systems, by absorption spectra and a comparison of absorbance with hydrolyzable phosphate. Ratios of total phosphorus to nucleoside (as determined by absorbance) for the various nucleotides were as follows: TuTP, 3.16; dTuTP, 3.03; ToTP, 4.30; dToTP, 3.04; FTP, 3.02; and dFTP, 3.38. The high value in the case of ToTP was presumably due to inorganic phosphate or pyrophosphate that had been incompletely removed, despite the purification procedures.

The yield of pure deoxynucleotide (based on ribonucleotide in the reaction mixture) was about 76% in the case of dTuTP and about 30% for dFTP and dToTP (Table I).

Activation of CTP Reduction by dTuTP, dToTP, and dFTP. Since the ultimate interest in these analogues stems from the possibility that they may be able to mimic dATP as negative effectors of mammalian ribonucleotide reductase, it was of interest, as a preliminary step, to determine whether they have the same effect as dATP in the regulation of the reductase of *L. leichmannii*. Like dATP they all proved to be activators for CTP reduction, and affected only the apparent Michaelis constant for CTP and not the maximum velocity over the range of CTP concentrations used (0.02 to 0.71 mM). The decrease in slope of the double-reciprocal plot ($1/v$ vs. $1/[CTP]$) due to the presence of the activator was hyperbolically related to activator concentration. The dissociation constants obtained for the activator complexes by fitting of the kinetic data to the appropriate rate equation are given in Table III. None of the dATP analogues at a concentration of 1 mM caused any activation of the reduction of 1 mM GTP, ATP, or UTP.

TABLE III: Kinetic Constants for the Activation by dATP and Its Analogues of CTP Reduction in the Presence of the Ribonucleotide Reductase of *L. leichmanii*.[a]

Nucleotide activator	K_{IN} (mM)	K_{ID} (mM)	αK_m[a]
dATP	0.116 ± 0.079	0.014 ± 0.006	0.039
dTuTP	0.057	0.011	0.062
dToTP	0.40 ± 0.28	0.013 ± 0.004	0.011
dFTP	0.057	0.015	0.084

[a] K_{IN} is the dissociation constant for the enzyme–activator complex, and K_{ID} the dissociation constant for the enzyme–CTP–activator complex. [b] αK_m is the Micahelis constant for CTP in the presence of saturating activator. K_m for CTP in absence of activator was 0.32 mM.

Discussion

The data presented above clearly indicate the convenience and economic feasibility of using the *L. leichmannii* ribonucleotide reductase for reducing TuTP, ToTP, or FTP to the corresponding 2′-deoxynucleotides. Our results indicate excellent conversion of TuTP and reasonable conversion of the other two analogues in reaction mixtures containing about 0.1 unit of reductase activity per μmol of nucleotide. A single commerical fermentation of *L. leichmannii* on the 500-L scale can produce 1000 g of cell paste, from which 1270 units of enzyme can be purified to the stage of the second ammonium sulfate fractionation (Singh et al., 1977). This enzyme, even if used only once, would therefore reduce about 13 mmol of TuTP or about 6 mmol of ToTP or FTP. However, it may be possible to increase considerably the yield from a given amount of enzyme by adding additional substrates (and perhaps coenzyme) at intervals, during a more prolonged incubation period. Alternatively, enzyme might be separated from other constituents of the reaction mixture on Sephadex G-25 and used again. Finally, the behavior of enzyme insolubilized by attachment to Sepharose 4B has been explored and preliminary results indicate that insolubilized enzyme can be used a number of times. By the use of such procedures the yield of deoxyribonucleotide produced with enzyme from a 500-L fermentation should be increased to at least 50 mmol or about 29 g.

The absolute requirement for dGTP as an activator for the reduction of FTP and particularly of ToTP is surprising in view of the ready reduction of TuTP and ATP in the absence of dGTP (although the latter is a nonessential activator for ATP and TuTP). Why the additional CN group prevents ToTP reduction unless activator is present is unclear. dGTP does not seem to be necessary for binding of FTP to the catalytic site to occur since, in absence of dGTP, FTP is a linear competitive inhibitor of the reduction of ATP, GTP, and UTP. Further, the K_i for this inhibition (~1 mM) is about the same as the K_m for ATP and CTP and not a great deal higher than the K_m for FTP in the presence of dGTP (0.13 mM). Thus the effect of dGTP on the binding of FTP and ToTP does not explain the absolute requirement for the activator and an effect on the catalytic process itself must be assumed.

Many of the effects produced by deoxyribonucleotide activators, on reactions catalyzed by the *L. leichmannii* reductase (e.g., isotope exchange, radical intermediate formation, coenzyme degradation), can be explained by the increased binding of adenosylcobalamin which activators cause (Singh et al., 1977). However, this cannot be the only effect of activators, since each deoxyribonucleotide is quite specific in activating the reduction of a single substrate (Vitols et al., 1967). The general effect on coenzyme binding and the specific effects on substrate reduction are apparently both mediated by binding of activator at a single regulatory site, and binding causes the enzyme to undergo a considerable conformational transition since the sedimentation constant of the protein decreases significantly (Singh et al., 1977). The absolute requirement for binding of dGTP at the regulatory site in the case of ToTP and FTP reduction is a striking demonstration of a specific activation effect. This requirement for dGTP is unlikely to be related to enhancement of coenzyme binding by the latter, since activation of FTP reduction is still required at relatively high coenzyme concentrations (12 μM). Presumably binding of dGTP at the regulatory site specifies a conformational transition which is *essential* for the proper orientation of catalytic groups at the active site with respect to bound FTP or ToTP and optimizes their orientation with respect to bound ATP or TuTP.

The activating effects of dTuTP, dToTP, and dFTP with *L. leichmannii* ribonucleotide reductase suggest that these analogues not only bind just as well as dATP (i.e., with similar association constants) to the regulatory site, but that the decrease in the apparent Michaelis constant for CTP which they produce is similar to that brought about by dATP (Table III). It is clear, therefore, that the analogues substitute effectively for dATP at the regulatory site of this enzyme. Whether they would bind as well to the allosteric site of mammalian ribonucleotide reductase is, of course, uncertain, since there are great differences between the *Lactobacillus* reductase and the mammalian enzyme. Nevertheless, the ability of the analogues to substitute for dATP at the active site of the *Lactobacillus* enzyme indicates that the stereochemical differences do not a priori exclude the possibility of binding to the regulatory site of other ribonucleotide reductases.

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References

Agrawal, K. C., Booth, B. A., Moore, E. C., & Sartorelli, A. C. (1972) *J. Med. Chem. 15,* 1154–1158.

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235,* 769–775.

Bjursell, G., & Reichard, P. (1973) *J. Biol. Chem. 248,* 3904–3909.

Blakley, R. L. (1966) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 25,* 1633–1638.

Blakley, R. L. (1978) *Methods Enzymol.* (in press).

Booth, B. A., Agrawal, K. C., Moore, E. C., & Sartorelli, A. C. (1974) *Cancer Res. 34,* 1308–1314.

Cleland, W. W. (1963) *Nature (London) 198,* 463–465.

Cory, J. G., & Whitford, T. W., Jr. (1972) *Cancer Res. 23,* 1301–1306.

Elford, H. L., Freese, M., Passamani, E., & Morris, H. P. (1970) *J. Biol. Chem. 245,* 5228–5233.

Frenkel, E. P., Skinner, W. N., & Smiley, J. E. (1964) *Cancer Chemother. Rep. 40,* 19–22.

Fujioka, S., & Silber, R. (1970) *J. Biol. Chem. 245,* 1688–1693.

Gordon, H. L., & Fiel, R. J. (1969) *Cancer Res. 29,* 1350–1355.

Hakala, M. T., & Taylor, E. (1959) *J. Biol. Chem. 234,* 126–128.

Hoard, D. E., & Ott, D. G. (1965) *J. Am. Chem. Soc. 87,*

1785–1788.
Hoffmann, P. J., & Blakley, R. L. (1975) *Biochemistry 14,* 4804–4812.
Imai, K., Fujii, S., Takanohashi, K., Furukawa, Y., Masuda, T., & Honjo, M. (1969) *J. Org. Chem. 34,* 1547–1550.
Jain, T. C., Russel, A. F., & Moffatt, J. G. (1973) *J. Org. Chem. 38,* 3179–3186.
King, C. D., & Lancker, J. L. V. (1969) *Arch. Biochem. Biophys. 129,* 603–608.
Langer, L., & Klenow, H. (1960) *Biochim. Biophys. Acta 37,* 33–37.
Larsson, A. (1969) *Eur. J. Biochem. 11,* 113–121.
Maley, G. F., & Maley, F. (1961) *J. Biol. Chem. 235,* 2964–2967.
Moffat, J. G., & Khorana, H. G. (1971) *J. Am. Chem. Soc. 83,* 649–658.
Moore, E. C. (1967) *Methods Enzymol. 12A,* 155–164.
Moore, E. C., & Hurlbert, R. B. (1966) *J. Biol. Chem. 214,* 4802–4809.
Moore, E. C., Booth, B. A., & Sartorelli, A. C. (1971) *Cancer Res. 31,* 235–238.
Moore, E. C., Peterson, D., Yang, L. Y., Yeung, C. Y., & Neff, N. F. (1974) *Biochemistry 13,* 2904–2907.
Morris, N. R., & Fischer, G. A. (1960) *Biochim. Biophys. Acta 42,* 183–184.
Robins, M. J., & Muhs, W. H. (1976) *J. Chem. Soc., Chem. Commun.* 269.
Robins, M. J., McCarthy, J. R., Jr., Jones, R. A. & Mengel, R. (1973) *Can. J. Chem. 51,* 1313–1321.
Segal, I. H. (1975) *Enzyme Kinetics,* p 227 ff, Wiley, New York, N.Y.
Singh, D., Tamao, Y., & Blakley, R. L. (1977) *Adv. Enzyme Regul. 15,* 81–100.
Suhadolnik, R. J. (1970) *Nucleoside Antibiotics,* pp 298–389, Wiley, New York, N.Y.
Suhadolnik, R. J., Finkel, S. I., & Chassy, B. M. (1968) *J. Biol. Chem. 243,* 3532–3537.
Turner, M. K., Abrams, R., & Liebeman, I. (1968) *J. Biol. Chem. 243,* 3725–3728.
Vitols, E., Brownson, C., Gardiner, M., & Blakley, R. L. (1967) *J. Biol. Chem. 242,* 3035–3041.
Weith, H. L., Wiebers, J. L., & Gilham, P. J. (1970) *Biochemistry 9,* 4396–4401.
Whittle, E. D. (1966) *Biochim. Biophys. Acta 114,* 44–60.
Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50,* 5065–5068.
Young, C. W., & Hodas, S. (1964) *Science 146,* 1172–1174.
Young, C. W., Schochetman, G., & Karnofsky, D. A. (1967) *Cancer Res. 27,* 526–534.

Kinetics of Ligand Binding to Dihydrofolate Reductase: Binary Complex Formation with NADPH and Coenzyme Analogues†

Susan M. J. Dunn, John G. Batchelor,‡ and Rodney W. King*

ABSTRACT: The reaction between dihydrofolate reductase from *L. casei* MTX/R and NADPH or its analogues has been investigated by stopped-flow fluorescence techniques. The formation of the enzyme–coenzyme complex is characterized by quenching of the near ultraviolet fluorescence of the enzyme and enhancement of the fluorescence of the NADPH chromophore. These spectral changes take place in two phases: the faster characterized by an apparent bimolecular rate constant near 10^7 M^{-1} s^{-1}, the slower by a unimolecular rate constant whose value is dependent on pH and varies from 0.06 to 0.012 s^{-1}. The relative amplitudes of the fast and slow phases are also pH dependent, the fast change dominating at low pH. There is no direct coupling of the fast and slow reactions, since the slow phase appears in the reaction trace only after the fast phase has been saturated. These results suggest that the enzyme exists in at least two interconvertible forms whose relative proportions are pH dependent. NADPH appears to bind rapidly and exclusively to one of these forms, the slow phase probably being a reflection of the interconversion of the forms followed by rapid coenzyme binding. The fast association rate is dependent on the viscosity of the medium and has an enthalpy of activation of only 2.5 kcal mol^{-1}, suggesting that the association is diffusion controlled. The binding of deamino-NADPH and etheno-NADPH follows the same kinetic pattern as that of NADPH; however, the ligands 3-acetylpyridine-NADPH, thionicotinamide-NADPH, and $NADP^+$ exhibit a third phase of relatively small amplitude and intermediate rate in the association reaction. It is proposed that in these cases the coenzyme analogues can bind to either form of the enzyme and that the intermediate rate reflects the interconversion of the binary complexes.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). The enzyme is therefore necessary for the formation of THF and its derivatives which are essential cofactors in the important metabolic reactions involving transfer of one-carbon units. In conjunction with thymidylate synthetase, the enzyme is essential for thymidylate biosynthesis and hence for DNA synthesis.

The enzyme has been the subject of extensive study (reviewed by Blakley, 1969) not only because of its importance in intermediary metabolism but also because it is the target

† From the National Institute for Medical Research, London NW7 1AA, Great Britain. *Received November 3, 1977; revised manuscript received March 3, 1978.* S.M.J.D. holds an MRC research studentship.
‡ Present address: Medical Computing Unit, Charing Cross Hospital, London W6 8RF, Great Britain.

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