Title
Part I: Novel alkyl alkanethiolsulfonate sulfhydryl reagents. Part II: Isotope probes of the mechanism of thymidylate synthetase

Author
Bruice, Thomas Wayne

Publication Date
1980

Peer reviewed|Thesis/dissertation

Part I. NOVEL ALKYL ALKANETHIOLSULFONATE SULFHYDRYL REAGENTS

Part II. ISOTOPE PROBES OF THE MECHANISM OF THYMIDYLATE SYNTHETASE

by

THOMAS WAYNE BRUICE

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date University Librarian

Degree Conferred: SEP 24 1980

ACKNOWLEDGMENTS

With most sincere and grateful thanks to Dan Santi and George Kenyon for their expert guidance during my graduate work. Equally appreciated has been their constant enthusiasm and patience; this despite perhaps just a touch of a certain well-meaning, but it is possible also an exasperating, stubbornness on my part. I shall always value their association during these studies, and I hope forever their friendship.

I wish also to thank the members of Dr. Santi's and Dr. Kenyon's research groups for their assistance, especially Al Pogolotti for ever generously sharing his experimental expertise and knowledge, and Kathleen Ramos for her help in preparing this manuscript.

Finally, I should like to thank my father, Thomas Charles Bruice, for his undying support and advice and for being the finest example I know of that which I strive to be.

I wish to acknowledge the National Institutes of Health for Training Grant T32 CA 09270 for T.W.B., Grant AM 17323 awarded to G.L.K., and Grant CA 14394 awarded to D.V.S. for support of this research.

VITA

April 24, 1952-- born-- Los Angeles, California.

1974-- B.A. in Chemistry with Honors from Revelle College of the University of California, San Diego.

1974-1975--Teaching Assistant, Department of Biochemistry and Biophysics, University of California, San Francisco.

1976-1980--Research Assistant, Department of Biochemistry and Biophysics, University of California, San Francisco.

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PART II. ISOTOPE PROBES OF THE MECHANISM OF THYMIDYLATE SYNTHETASE

by

THOMAS W. BRUICE

PART I

A simple, general scheme for the synthesis of sulfhydryl-specific alkyl alkanethiolsulfonate ($RSSO_2R'$) reagents has been developed. The reagents aminoethyl methanethiolsulfonate and benzyl methanethiolsulfonate were used to modify stoichiometrically, selectively and reversibly the sulfhydryl groups of the ethyl ester and p-nitroanilide derivatives of N-α-acetyl-L-cysteine, glutathione and the A chain of bovine insulin under mild conditions. The modification products possess the cysteinyl mixed-disulfide side-chains ($>CyS_{\beta}-SR$), β-S-(β-aminoethanethiol)- and β-S-(benzylthiol)-, respectively, which structurally resemble the side-chains of lysine and phenylalanine, respectively.

For both trypsin-catalyzed hydrolyses of the β-S-(β-aminoethanethiol)-cysteine analogs and α-chymotrypsin-catalyzed hydrolyses of the β-S-(benzylthiol)-cysteine analogs it is surprising that although the specificity constants, k_{cat}/K_m, for the analog esters compare favorably with those for analogously derivatized and identically assayed specific reference amino acid esters, specificity constants for the analog amides compare much less favorably with those for reference amino acid amides. Further, no detectable enzyme-catalyzed hydrolysis of the amide bonds at the sites of modified cysteine residues in the A

chain of insulin was observed. Evidence is presented which suggests that the transition-state free energies of acylation are very large for the modification products, to the extent that there is a change in rate-determining step for the modified cysteine ester substrates from deacylation to acylation. This is the first report of a change in rate-determining step for serine protease amino acid substrates possessing side-chains of equal or greater length than those of the preferred amino acid substrates.

PART II

The secondary α-tritium kinetic isotope effects for dissociation of the covalent, ternary complexes formed with thymidylate (dTMP) synthetase, [2-^{14}C,6-^{3}H]-5-fluoro-2'-deoxyuridylate ([2-^{14}C,6-^{3}H]FdUMP) and (6R)-5,10-methylene-5,6,7,8-tetrahydropteroylpoly-L-glutamates ((6R)-CH_2-H_4Pte-L-Glu_n) are the same (k_H/k_T = 1.229, n=1; 1.211, n=5) at 25°C, within experimental error, as the isotope effect measured at equilibrium (K_T/K_H = 1.240, n=1). The magnitudes of the isotope effects provides direct proof that the complex formed is a stable 5,6-dihydropyrimidine analog of a steady-state intermediate recently demonstrated to form in reactions catalyzed by this enzyme. Dissociation of complexes formed using dTMP synthetase, FdUMP and (6R), L-[^{3}H]CH_2-[2-^{14}C]H_4folate (n=1) was accompanied by a very small secondary α-tritium kinetic isotope effect (k_H/k_T = 1.004-1.033). It is concluded that all covalent bond making/breaking steps involving either FdUMP or the cofactor are pre-equilibrium steps prior to the rate-determining step in the direction of dissociation. The rate-determining step is localized at the binding of ligands and/or at a subsequent conformational change of the enzyme and cofactor in the direction of association.

Using an isotope-trapping procedure, noncovalent, binary complexes of dTMP synthetase and either [6-^{3}H]FdUMP or (6R),L-CH_2-[6-^{3}H]H_4folate

were shown to partition identically between irreversible dissociation of radioactivity and "trapping" of ca. 0.8 mol of high specific radioactivity, covalent, ternary complex/mol of enzyme dimer. Thus, the order of binding to the native enzyme is fully random. It also may be deduced that the rate-determining step occurs after initial formation of noncovalent, ternary complexes. Equilibrium dissociation constants for the noncovalent, binary complexes were obtained: K_d^{FdUMP} = 21 μM and $K_d^{CH_2\text{-}H_4folate}$ = 44 μM.

There was a 3.3-fold decrease in the half-life at 25°C for exchange of [2-^{14}C]FdUMP from 1.0 μM covalent [2-^{14}C]FdUMP—(6R),L-CH_2-[6-^{3}H]H_4folate—dTMP synthetase complexes as [(6R),L-CH_2-H_4folate] was raised from 0.1 to 1.0 mM holding [FdUMP] at 0.1 mM; no other concentration dependencies of rates of exchange of FdUMP or cofactor were observed. Kinetic analysis reveals that the binding order under the conditions of isotope exchange experiments is completely ordered with FdUMP binding before CH_2-H_4folate.

A minimal model for association/dissociation of covalent complexes is proposed: upon the random order, noncovalent binding of both FdUMP and CH_2-H_4folate to one subunit of the native form of the enzyme there is a rate-limiting, ligand induced conformational change of the enzyme which results in continued binding to the other subunit being ordered; binding to the two subunits, then, is overall sequential.

PART I

NOVEL ALKYL ALKANETHIOLSULFONATE SULFHYDRYL REAGENTS

INTRODUCTION

Sulfhydryl groups of cysteinyl residues of peptides and proteins are generally the most reactive of all amino acid side-chain functionalities under normal physiological conditions. They may be readily alkylated, acylated, arylated, and oxidized, and will form complexes with many heavy-metal ions.

Several reviews on sulfhydryl reagents (Fontana & Scoffone, 1969, 1972; Jocelyn, 1972; Friedman, 1973; Torchinskii, 1974; Kenyon & Bruice, 1977) have appeared in recent years. And, since the publication of "Chemical Modification of Proteins" by Means and Feeney in 1971, in which approximately forty types of sulfhydryl reagents were described and extensively referenced, there has been burgeoning interest in the development of new such reagents.

Traditional sulfhydryl reagents deliver groups that nearly always may be categorized as follows: blocking and labeling groups, reporter groups, cross-linking groups, and affinity labeling groups (Kenyon & Bruice, 1977). Most of these earlier sulfhydryl reagents deliver groups that fall in the category of blocking and labeling groups, which may be either reversible[1] or irreversible. In general, these

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[1]The reactions of many sulfhydryl blocking groups may be reversed. However, in many instances the reversals are extremely slow processes and/or require harsh conditions destructive to protein structure. By easily reversible, it is meant removable in a reasonably short period of time under conditions that preserve the native structure of a polypeptide or protein; any reagent that cannot be thus removed is defined as irreversible.

groups are designed to be structurally and chemically relatively innocuous so that when covalently bound they act merely to block the activity of, or to titrate or label (sometimes isotopically) any number of sulfhydryl groups.

Reagents which deliver cross-linking groups possess one functionality that reacts initially and selectively with sulfhydryls and then another functionality that reacts (often under altered conditions) with another nearby group, which may or may not be another sulfhydryl. This second reaction is sometimes, but not always, selective. Older reagents in this class have almost exclusively been structurally symmetrical and used to generate cross-linking bridges between two nearby sulfhydryl groups.

There is a scarcity of reagents which deliver reporter or affinity labeling groups and still react satisfactorily and are of any general practical utility. Although increased effort has been expended in the 1970's toward the design of suitable sulfhydryl-specific reagents for these purposes, there is room for much further work.

The reporter groups generally serve to provide chemical and physical information about the environment of the sulfhydryl group that is labeled (and sometimes to quantitate such groups), with or without a wide variety of post-labeling perturbations. They exhibit easily detectable physical or chemical changes that are exquisitely sensitive to variable aspects of their microenvironment, such as polarity and hydrophobicity. These groups most often fall into one of three subclasses: chromophoric groups, fluorescent probes and stable nitroxide radical

spin labels.

Sulfhydryl-specific affinity labeling groups are designed to be structurally analogous to some ligand that normally shows specificity in association to a protein-binding site containing at least one sulfhydryl. Once the affinity labeling group is bound noncovalently, a reactive moiety is designed to be so positioned in approximation to the binding site sulfhydryl that it can react with it to anchor the affinity label covalently. Thus selectivity in modification of only binding site sulfhydryls is achieved. These reagents may be designed to accomplish further purposes once they are thus secured, but their outstanding features are those just described.

Sulfhydryl reagents may be placed into yet another general class, that of simple oxidizing agents. Prior to 1971 a number of such reagents had been used and seemed to fulfill their task satisfactorily. Very little new interest in designing new reagents in this class has been generated.

If one were to list the properties desirable for "ideal" sulfhydryl reagents, they likely would be something on the order of: 1) Variability of the type of group delivered and hence versatility of application, 2) independence of reaction with the sulfhydryl group on the nature of the group delivered, 3) reaction conditions that are mild and nondestructive to proteins, 4) rapidity of reaction, 5) high selectivity of reaction with only cysteinyl sulfhydryls of proteins, 6) quantitative conversion to the desired modification product without large excesses of reagent, and 7) rapid reversibility of reaction under conditions

nondestructive to protein structure. Unfortunately, few if any of the existing sulfhydryl reagents fulfill all of the above criteria; most have serious drawbacks of one sort or another which restrict their practical use (Kenyon & Bruice, 1977). Therefore, there still exists an important need to create new sulfhydryl reagents which more fully satisfy these criteria.

These studies describe a continuing effort to demonstrate this intent of purpose with a type of sulfhydryl-specific reagents known as alkyl alkanethiolsulfonates. These are conceptually and chemically a distinct class of sulfhydryl reagents; as a result, we think they offer far more potential than any other developed to date.

CHAPTER 1

ALKYL ALKANETHIOLSULFONATE SULFHYDRYL REAGENTS: BACKGROUND

The fundamental reaction of alkyl alkanethiolsulfonate sulfhydryl-specific reagents with protein sulfhydryls is shown in Figure 1. As far as has been investigated, it appears to proceed with fulfillment of the seven criteria that are desirable of protein sulfhydryl reagents that have been discussed in the Introduction. This argues for consideration of these reagents separately as a chemical class, rather than by grouping of individual alkyl alkanethiolsulfonates according to function as can be done with other sulfhydryl reagents (Kenyon & Bruice, 1977). However, the potential of these reagents has merely been implied, and demonstration of the criteria in question for a wide variety of these reagents for a wide variety of specific biochemical purposes has yet to be done.

Alkyl alkanethiolsulfonates have been used previously for the chemical synthesis of mixed disulfides[2] of simple organic compounds by Boldyrev et al. (1954, 1961, 1966) and Dunbar and Rogers (1966). A closely related aryl arenethiolsulfonate was used by Field and Giles (1971) to block a sulfhydryl group of creatine kinase, but the conclusion was drawn that this reagent "shows no advantage over Ellman's reagent."

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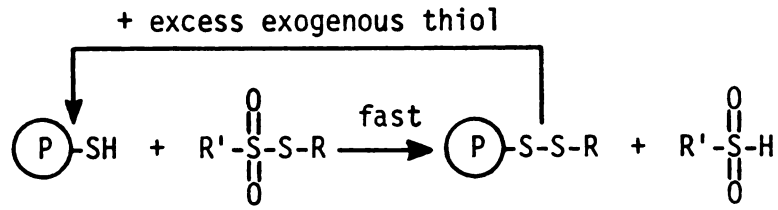
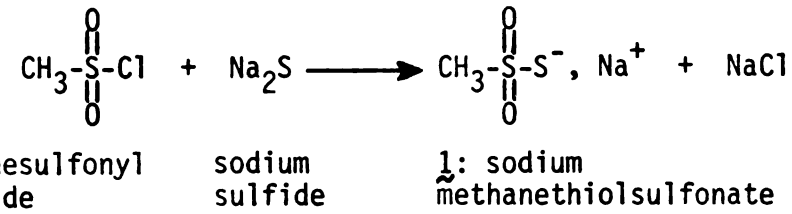
[2]Mixed-disulfides also may be conveniently synthesized using alkoxycarbonylalkyl disulfides (Brois et al., 1970; Smith et al., 1975) or by using N-(alkanethio)phthalimides (Harpp & Back, 1971).

The simplest and thus far most widely used reagent in this class is methyl methanethiolsulfonate (Smith & Kenyon, 1974; Smith et al., 1975; Figure 1), which delivers the small, uncharged, non-hydrogen-bonding CH_3S-group. In addition, since this group is a portion of the side-chain moiety of methionine, perturbations of a protein as a result of its introduction are generally as slight as those observed with any other sulfhydryl reagent.

Methyl methanethiolsulfonate (MMTS) has been used to titrate two enzymes, papain and glyceraldehyde-3-phosphate dehydrogenase, that had been previously shown, using a variety of methods, to conatain active site sulfhydryls essential for their catalytic activity (Smith et al., 1975). Complete and rapid inhibition was observed and 1.0 mol of CH_3S-group was incorporated per mole of papain. The clean stoichiometry of CH_3S- incorporation paralleling loss of sulfhydryl-dependent activity confirms the absolute selectivity of MMTS for active and/or accessible sulfhydryl groups in preference to all other reactive protein groups. When rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was similarly titrated with MMTS, two thiol groups per subunit were found to be modified, one much more rapidly than the other. Complete inactivation concurrent with blocking of the more reactive thiol was observed.

In contrast to these expected results, the behavior of creatine kinase with MMTS was anomalous (Smith & Kenyon, 1974; Smith et al., 1975). It had been previously suggested, after titrations with reagents such as iodoacetamide of what appeared to be one unique, active-site sulfhydryl per subunit of this enzyme, that this thiol was required for





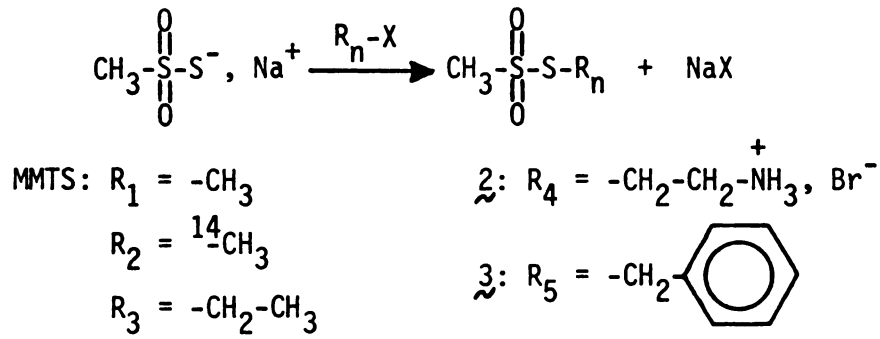


FIGURE 1: Reaction of alkyl alkanethiolsulfonates with protein sulfhydryl groups and synthesis of alkyl methanethiolsulfonate reagents. (P)-SH denotes a protein sulfhydryl group. R_1-R_3 groups shown are those of reagents made prior to the instigation of these studies.

activity. When 1.0 equivalent of CH_3S- was incorporated per subunit, however, approximately 20% residual activity remained. The presence of this CH_3S- group afforded complete protection against further inactivation by normally inhibitory iodoacetamide.

These results are consistent with the conclusion that the iodoacetamide-sensitive active-site sulfhydryl group is not essential for enzymic activity. Preliminary evidence based on both EPR and kinetic studies indicates that blocking of this sulfhydryl residue with CH_3S- interferes with nucleotide binding, not with binding of the guanidino substrates (Markham et al., 1975).

Further evidence for relatively small protein structure perturbations upon treatment with MMTS was provided in a study of the blocking of the sulfhydryl groups of Escherichia coli succinic thiokinase (Nishimura et al., 1975). At a point where approximately 4 mol of CH_3S- were incorporated per mole of enzyme, 80% of thiokinase activity was lost, but no loss of antigenicity (as measured by microcomplement fixation) was observed. In contrast, losses of both thiokinase activity and antigenicity were observed upon similar treatment with 5,5'-dithiobis-(2-nitrobenzoic acid), ethylmercurithiosalicylate, and p-hydroxymercuribenzoate.

The behavior of MMTS as a sulfhydryl titrant of yeast alcohol dehydrogenase is also unusual (Klinman, 1975a). The kinetics of inactivation by excess reagent are biphasic; 35% of activity is lost in a burst, corresponding to incorporation of one CH_3S- group per subunit, followed by a slower first-order loss of greater than 98% of the

activity concurrent with incorporation of a second equivalent of CH_3S-. No such biphasic kinetic behavior is observed with similar titrations of the affinity label, styrene oxide (Klinman, 1975b).

MMTS has also been used for the potentiometric determination of inonizations at the active-site of papain (Lewis et al., 1976), to block apparent intramolecular disulfide interchange in the same enzyme (Fink & Angelides, 1976), to titrate active-site sulfhydryls of beef heart isocitrate dehydrogenase (Levy & Villafranca, 1977), and to synthesize the mixed methyldisulfide of CoA (Currier & Mautner, 1976a,b). This CoA derivative is a potent inhibitor of choline acetyltransferase (squid ganglia).

MMTS and several other alkyl methanethiolsulfonates have been prepared from the common precursor sodium methanethiolsulfonate by simple S_N2 displacement reactions as shown in Figure 1 (Smith & Kenyon, 1974; Smith et al., 1975; Kenyon & Bruice, 1977). It is hoped that a wide variety of new alkyl methanethiolsulfonates of possible biochemical interest should be easily synthetically accessible. For example, it is likely that most of the sulfhydryl-specific reagents discussed in a recent review (Kenyon & Bruice, 1977), the vast majority of which are irreversible S-alkylating reagents, may be chemically transformed to their corresponding alkyl methanethiolsulfonate in a one-step S-alkylation using sodium methanethiolsulfonate. This should improve both the rapidity and selectivity of reaction of the resulting reagent with protein sulfhydryls and remove the usual requirement of excesses of reagent needed to afford complete modification. The resulting blocking

groups could then also be readily removed, if desired.

The goals of this part of this thesis are first to ascertain whether alkyl methanethiolsulfonates with R groups that are larger and more complex than methyl or ethyl can be easily made from sodium methanethiolsulfonate. Second, will they then still react with sulfhydryls with fulfillment of the criteria presented earlier? Third, can they thus be used to deliver RS- groups for highly specific biochemical purposes?

CHAPTER 2

SYNTHESIS OF ALKYL METHANETHIOLSULFONATE REAGENTS AND THEIR UTILIZATION FOR THE CHEMICAL MODIFICATION OF CYSTEINYL SULFHYDRYL GROUPS[3]

ABSTRACT

A simple, general scheme for the synthesis of sulfhydryl-specific alkyl alkanethiolsulfonate ($RSSO_2R'$) reagents has been developed. To provide a demonstration of the largely untapped potential of these reagents, two new ones, aminoethyl methanethiolsulfonate (2) and benzyl methanethiolsulfonate (3) were used to modify reversibly, stoichiometrically and selectively the sulfhydryl groups of the ethyl ester (4) and p-nitroanilide (7) derivatives of N-α-acetyl-L-cysteine, glutathione and the A chain of bovine insulin under mild conditions. The corresponding β-S-(β-aminoethanethiol)- and β-S-(benzylthiol)- derivatives of cysteine (8-11) and of the peptides (12-15) were afforded. These have mixed-disulfide side-chains structurally similar to those of lysine and phenylalanine, respectively; the significance of these structural similarities is elucidated in the accompanying chapter (3).

There long has been an interest in the chemical modification of sulfhydryl groups of cysteinyl residues of peptides and proteins, both because they generally are the most reactive of all amino acid side-chain moieties under normal physiological conditions, and because they often play important functional roles. Although many reagents for this purpose presently are known, most have significant limits to their usefulness (Kenyon & Bruice, 1977; Means & Feeney, 1971). Therefore, we have pursued the design of a new class of versatile sulfhydryl reagents, the alkyl alkanethiolsulfonates.

The first application of an alkyl alkanethiolsulfonate for biochemical purposes was the rapid, stoichiometric, selective, and reversible modification under mild conditions of accessible cysteinyl sulfhydryl groups (-SH) of several enzymes with methyl methanethiolsulfonate, reported in 1975 by Smith, Maggio and Kenyon (Figure 1). Methyl methanethiolsulfonate since has proven very useful in a number of biochemical investigations, which we recently have reviewed (Kenyon & Bruice, 1977).

Simple $RSSO_2R'$ reagents made to date can be used only to effect blocking of a reactive -SH group (Smith et al., 1975; Currier & Mautner,

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[3]Preliminary reports of portions of this work have appeared: Bruice et al. (1976) and Kenyon & Bruice (1977).

1977). However, the apparent independence of these reactions on the nature of both the R and R' moieties suggested to us 1) the possibility of devising a general synthetic scheme to provide reagents with the same R' group, and 2) that these reagents could be used to deliver "customized" RS-groups for highly specialized biochemical purposes.

To demonstrate this potential, the primary concerns of this chapter have been the design, synthesis, and properties of both some new alkyl methanethiolsulfonate reagents, and the products of their reactions with cysteine-containing model compounds and peptides. It was expected that certain reaction products would fulfill the specialized function of being substrates for trypsin and α-chymotrypsin by virtue of the structural analogies of their side-chains with those of lysine or phenylalanine, respectively; this is the subject of the following chapter 3.

EXPERIMENTAL PROCEDURES

Materials. Materials used were of the highest purity commercially available. β-bromoethylamine hydrobromide and benzyl bromide were from Aldrich, N-α-acetyl-L-cysteine and glutathione (reduced) were from Sigma, dihydrolipoamide glass beads were from Pierce, L-cystine-bis-p-nitroanilide was from Nutritional Biochemicals and spectrograde Me_2SO was from Matheson, Coleman and Bell. Silica gel (60-200 mesh) was purchased from Baker, 0.1 mm cellulose glass TLC plates (MN 300) were from Brinkman, and micropolyamide sheets were from Schleicher and Schuell. Tetra-S-sulfonated A chain of insulin (bovine pancreas; anhydrous

powder) was a gift from Dr. F. H. Carpenter (Department of Biochemistry, University of California, Berkeley).

Methods. Infrared spectra were recorded using a Beckman 4 spectrometer. Ultraviolet and visible spectra and absorbance measurements were taken on either Gilford 6040 or 2000 recorders equipped with Beckman DU or DUR monochrometers, respectively, or on a Cary 118-C recording spectrophotometer. Proton nuclear magnetic resonance spectra were obtained using either a Varian A-60A or a Perkin-Elmer R12-B, 60-MHz spectrometers. Low resolution chemical ionization mass spectra and high-resolution, accurate mass measurements were determined on a modified AEI MS 902 mass spectrometer using isobutane as reagent gas. Melting points are uncorrected unless otherwise indicated. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. DTNB[4,5] (Ellman's

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[4]Abbreviations used: BAEE, N-α-benzoyl-L-arginine ethyl ester; BTEE, N-α-benzoyl-L-tyrosine ethyl ester; BApNA, N-α-benzoyl-DL-arginine-p-nitroanilide; APpNA, N-α-benzoyl-DL-phenylalanine-p-nitroanilide; AEBCME, β-S-(β-aminoethyl)-N-α-benzoyl-L-cysteine methyl ester·HCl; BLME, N-α-benzoyl-L-lysine methyl ester·p-tosylate; AEBCA, β-S-(β-aminoethyl)-N-α-benzoyl-L-cysteine amide; BAA, N-α-benzoyl-L-arginine amide; AECTPE, β-S-(β-aminoethyl)-N-α-toluene-p-sulfonyl-L-cysteine n-propyl ester; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); NEM, N-ethylmaleimide; TPCK, L-1-p-tosylamide-2-phenylethylchloromethyl ketone; TLCK, N-α-p-tosyl-L-lysine-chloromethyl ketone; NMM, N-methyl morpholine; DTT, dithiothreitol; CNT, 3-carboxylato-4-nitrothiophenolate; TLC, thin layer chromatography; TLE, thin layer electrophoresis; DSS, sodium 2,2-dimethyl-2-silapentone-5-sulfonate; EDTA, ethylenediaminetetraacetic acid; λ, wavelength; S_o, initial substrate concentration; E_o, initial enzyme concentration.

[5]Since numerous values for the CNT ion extinction coefficient have been reported, we have used DTNB assays semi-quantitatively only. Experimental errors for NEM titrations were typically $\leq \pm 5\%$.

reagent) assays were performed by the method of Robyt et al. (1971) and NEM assays using the methods of Cleland (1964). TLC analyses (silica gel) were performed as described in the Appendix to Part I.

Chemical Syntheses

Sodium Methanethiolsulfonate (1) was prepared by the method of Kenyon & Bruice (1977). The hygroscopic product gave the previously unreported NMR spectrum (D_2O; DSS standard): δ 3.36 (3H, s). Anal. calcd. for $CH_3O_2S_2Na$: C, 8.95; H, 2.25; S, 47.81. Found: C, 8.80; H, 2.40; S, 47.70. A similar procedure for the synthesis of 1 was reported recently (Currier & Mautner, 1977).

Aminoethyl Methanethiolsulfonate Hydrobromide (2). β-Bromoethylamine·HBr (13.38 g, 65.3 mmol) and 1 (8.76 g, 65.3 mmol) in 125 mL of absolute ethanol were heated at reflux for 5 hr. The reaction mixture was cooled to 0° C, filtered, concentrated cooled and filtered again to remove NaBr co-product. The combined NaBr sediments were washed with several mL of ice-cold absolute ethanol which were combined with the ethanolic solution of 2 (significant losses of product occurred during these steps owing to the similar solubilities of the bromide salts of both co-porducts). Solvent was removed under high vacuum at room temperature. The resulting light yellow, opaque oil (Johnston & Gallagher, 1961) was kept at 4°C for about 12 hr, and was warmed to room temperature to give oily crystals. Recrystallization from absolute ethanol yielded 7.7 g (50%) of a light beige powder, mp 109-111° C. Anal. calcd. for $C_3H_{10}NO_2S_2Br$: C, 15.23; H, 4.27; N, 5.93; S, 27.16;

Br, 33.84. Found: C, 15.01; H, 4.09; N, 6.05; S, 26.88; Br, 34.10. The NMR spectrum (D_2O; DSS standard) showed a broad multiplet at δ 3.45-3.73.

Benzyl Methanethiolsulfonate (3) was prepared by a method analogous to that used for 2, employing dry benzyl bromide (10.20 g, 59.6 mmol) and 1 (8.00 g, 59.6 mmol). One-half in. long, colorless crystals were obtained after two recrystallizations from ethanol to remove all traces of the oily, moderately ethanol-soluble benzyl bromide. The final yield was 6.58 g (55%), mp 40-42.5° C. Anal. calcd. for $C_8H_{10}O_2S_2$: C, 47.50; H, 4.98; S, 31.70. Found: C, 47.64; H, 5.09; S, 31.42. The NMR spectrum ($CDCl_3$) showed peaks at δ 2.94 (3H,s) 4.38 (2H,s), and 7.38 (5H,s).

N-α-Acetyl-L-Cysteine Ethyl Ester (4). Anhydrous conditions and minimal exposure to air were observed at all steps. Hydrogen chloride gas, dried by passage through a conc. H_2SO_4 trap, was bubbled for 60 min through a 25 mL stirred ethanolic solution of N-α-acetyl-L-cysteine (1.00 g, 6.13 mmol) at a rate so adjusted as to maintain the temperature ≤ 65° C. Ethanol·HCl was removed at reduced pressure, as were three following washes (25-50 mL each) with $CHCl_3$, to give a co- colorless, clear oil. This was either dried and stored in vacuo, or was triturated with Et_2O (75-100 mL), cooled in a dry ice-acetone bath, stored 1-5 days at 0-4° C, and slowly brought to boiling (50-55° C) at which point a crystalline cake formed that was washed by filtration twice with ice-cold ether. Recrystallization was achieved by cooling to 0° C followed by gradual warming to room temperature of a saturated

solution in ether. Typical yields were 0.82 g (70%) of snow white product (mp 46-48° C dec). Anal. calcd. for $C_7H_{13}NO_3S$: C, 43.96; H, 6.85; N, 7.32; S, 16.77. Found: C, 43.8; H, 6.9; N, 7.1; S, 16.6. The NMR spectrum ($CDCl_3$) showed peaks at δ 1.32 (3 H, t, J = 7 Hz), 2.07 (3 H,s), 3.06 (2H, d of d, J = 9 Hz, J = 4 Hz) 4.31 (2H, q, J = 7 Hz), 4.88 (1 H, br m , 7.20 (1H, br m), and 9.40 (1H, br m). Product 4 (either crystals or oil) was fully reduced when titrated with DTNB, but was subject to hydrolysis and oxidation upon TLC.

L-Cystine-bis-p-nitroanilide Dihydrochloride (5) was prepared both under anhydrous conditions and in indirect light. Impure L-cystine-bis-p-nitroanilide (2.25 g, ∿ 4.69 mmol) was dissolved in a minimal volume (∿ 300 mL) of stirred CH_3OH at reflux, added dropwise over 60 min. Insoluble residue was removed from the cooled solution by filtration. HCl gas, dried as above for 4, was passed (≥ 4 mL/min) through the stirred solution for 60 min, and product was isolated as a flocculent white precipitate upon dropwise addition with vigorous stirring of ≥ two equal volumes (∿ 700-800 mL) of Et_2O. It was washed with ice-cold methanol:ether (1:1) by filtration and dried to yield 1.49 g (66%) of 5, mp 221.3 - 222.6° C dec (corrected). Anal. calcd. for $C_{18}H_{22}N_6O_6S_2Cl_2$: C, 39.06; H, 4.01; N, 15.19. Found: C, 38.71; H, 4.23; N, 14.89.

N-α,N'-α'-Diacetyl-L-cystine-bis-p-nitroanilide (6): In a minimal volume (∿ 126 mL) of anhydrous pyridine containing 9.76 mmol of freshly distilled triethylamine was dissolved 4.88 mmol (2.70 g) of 5. Dry acetic anhydride (10.7 mmol, 1.10 g) was added dropwise (over

∿ 2 min) to the stirred solution at 0° C. Solvent was removed after 5 min, the light colored oil dissolved in 160 mL of $CHCl_3$, washed twice and successively with 80 mL each of 10% acetic acid, H_2O, 10% $NaHCO_3$, and H_2O, and then dried over anhydrous $MgSO_4$. $CHCl_3$ was evaporated and the remaining oil was added dropwise to 500 mL of vigorously stirred absolute ethanol at reflux. Two crops of off-white, flocculent precipitate that formed upon slow cooling to 0° C were collected and washed with ice-cold ethanol. Total yield was 2.41 g (88%), mp 248.3-249° C dec. Anal. calcd. for $C_{22}H_{24}N_6O_8S_2$: C, 46.80; H, 4.29; N, 14.89; S, 11.36. Found: C, 46.90; H, 4.51; N, 14.66; S, 11.54. The IR spectrum (Nujol mull) showed an intense band at 1750 cm^{-1} that was not present in the spectrum of L-cystine-bis-p-nitroanilide. An NMR spectrum (Me_2SO-d_6, 2 drops D_2O) exhibited peaks at δ 1.96 (6H, s), 3.17 (4H, br m), 4.75 (2 H, br m), and 8.00 (8H, d of d, J = 22 Hz, J = 9 Hz).

N-α-Acetyl-L-cysteine-p-nitroanilide (7) was prepared *in situ* immediately prior to its use. DTT_{red} (Cleland, 1964) was dried *in vacuo*, then weighed to make a stock solution (∿ 0.65 M) of ∿100 mg DTT/1.0 mL (≤ ±1% error) of dry N_2-saturated absolute ethanol; the solution was stored for short periods at 0-4° C under N_2. The reducing eqs/mL of this solution also was determined experimentally both by DTNB and NEM titrations. Then, 1.02 eqs (2% molar excess) of DTT_{red} were added volumetrically to a solution (6.83 mM) of carefully weighed 6 (0.39 g, 0.68 mmol) in 100 mL of dry, N_2-saturated Me_2SO.

Model Alkanethiolation Reactions

N-α-Acetyl-L-cysteine Ethyl Ester with Aminoethyl Methanethiolsulfonate Hydrobromide. The reaction was performed under anhydrous conditions with minimal exposure to air. A 25 mL, N_2-saturated ethanolic solution of 4 (oil; ∿0.400 g, ∿2.09 mmol) was made and the concentration (∿0.84 mM) was checked independently (as was the stability of 4) by DTNB titrations, and pH-stat titrations of acid produced upon total ester hydrolysis (pH 10.0-10.5). Any of 1.00, 1.05 or 1.20 (2:4) molar equivalents of 2 were added at 0-23° C, and the approximate rate and the extent of reaction were monitored by DTNB titrations and TLC analysis. A 4.0 mL sample of the post-reaction mixture was concentrated to ≤ 0.5 mL and passed through a silica gel column (1.8 x 98 cm, all Teflon connections) with anhydrous $CHCl_3$: ethanol (2:3). Column eluants were monitored by ninhydrin assays. Completely resolved fractions containing β-S-(β-aminoethanethiol)-N-α-acetyl-L-cysteine ethyl ester (8) were pooled, concentrated, and aliquots were used to obtain both a chemical ionization mass spectrum and an accurate mass measurement of the parent ion by high resolution mass spectrometry. Anal. calcd. for $C_9H_{18}N_2O_3S_2$ (free base): peak mass, 267.0837. Found: peak mass, 267.0866 ± 0.003.

N-α-Acetyl-L-cysteine Ethyl Ester with Benzyl Methanethiolsulfonate. The reaction was performed in 10 mL of ethanol similarly to the preparation of 8 to yield β-S-(benzylthiol)-N-α-acetyl-L-cysteine ethyl ester (9). The silica gel column (1.8 x 52 cm) solvent system was ethanol:

$CHCl_3$ (2:3) and column eluants were monitored spectrophotometrically at 249 nm. Inch long, clear, colorless needles of pure 9 were obtained from pooled, supersaturated column eluant of the product peak. Anal. calcd. for $C_{14}H_{19}NO_3S_2$: C, 53.65; H, 6.11; N, 4.47; peak mass, 314.0885. Found, C, 53.36; H, 6.07; N, 4.34; peak mass, 314.0875 ± 0.003.

N-α-Acetyl-L-cysteine-p-nitroanilide with Aminoethyl Methanethiolsulfonate hydrobromide. To a 50 mL stirred, N_2-saturated Me_2SO (or absolute ethanol) solution of *in situ* generated 7 was added either 1.05 or 1.10 (2:7) molar eqs. of 2, and both the approximate rate and the extent of reaction were monitored by DTNB titrations and TLC. Solvent was removed, and the residual clear, light orange-yellow oil was dissolved in dry, N_2-saturated, "spectrograde" Me_2SO to give a 15.00 mL final volume stock solution of β-S-(β-aminoethanethiol)-N-α-acetyl-L-cysteine-p-nitroanilide hydrobromide (10). The product was characterized further spectrophotometrically (see Figure 4 of following chapter).

N-α-Acetyl-L-cysteine-p-nitroanilide with Benzyl Methanethiolsulfonate. The reaction was performed analogously to the preparatiion of 10; to the other 50 mL solution of 7 was added benzyl methanethiolsulfonate (3). The product, β-S-(benzylthiol)-N-α-acetyl-L-cysteine-p-nitroanilide (11), was a clear, light yellow oil after removal of solvent.

Alkanethiolations of Glutathione (GSH) were performed using either i) a 1.25 molar ratio ($RSSO_2CH_3$:GSH): 1.0 mM GSH, 1.25 mM 2 or 3; 0.1 M $CaCl_2$, pH 7.6 (pH-stat) or 0.1 M triethylammonium bicarbonate buffer, pH 8.0; 30-37° C, 30-45 min incubation under argon in

tightly sealed Reacti-Vials with Mini-Nert caps (Pierce Chem. Co.), or ii) a 2.00 molar ratio ($RSSO_2CH_3$:GSH): 23.0 mM GSH, 46.0 mM 2 or 3; 0.1 M triethylammonium bicarbonate buffer, pH 8.0; with incubations as before. Reactions were monitored by DTNB assays. The modification products were characterized on paper chromatography and cellulose TLC (see Peptide Mapping below).

Alkanethiolations of A Chain of Insulin. Aliquots of a 0.38 mM (assuming 0.3 g H_2O/g anhydrous protein; concentration verified by spectrophotometric determination of tyrosinate residues {2 per A chain}; Edelhoch, 1967) stock solution of tetra-S-sulfonated A chain of insulin in deoxygenated 0.1 M triethylammonium bicarbonate buffer, pH 8.0, under argon in Reacti-Vials with Mini-Nert caps, were treated with dihydrolipoamide glass beads (0.22 sulfhydryl meq/g) in a 10:1 eqs ratio (DHL: S-sulfonated cysteine residue) for 30 min, 37° C, with constant mixing. Reductive cleavage was monitored to completion with DTNB assays. The solution was transferred to a new Reacti-Vial, 1.0 to 2.0:1.0 eqs of reagent (2 or 3: cysteinyl residue) were added (to ≥ 1.52 mM), the solution was incubated under argon, 37° C, 30 min, and the alkanethiolation reactions were monitored by DTNB assays. Products were characterized by TLC and TLE (see Peptide Mapping below).

Peptide Mapping

Characterizations of alkanethiolation products were performed by any of the following: cellulose TLC [∿2 nmol/application; n-butanol: glacial acetic acid: H_2O (2:1:1), pH 2-3; t-butanol:methyl ethyl ketone:

H_2O:NH_4OH (4:3:2:1), pH 9.5-10; pyridine:glacial acetic acid:H_2O (25:1:224), pH 6.5], paper chromatography [Wattman 1 MM, 30 cm, ascending mode, pH 2-3 TLC solvent system (co-chromatographed glycine standards for digests of modified GSH only)], cellulose TLC [$\sim$ 2 nmol/application, 700 V (constant), 9-15 mAmp, 15-60 min, pyridine:glacial acetic acid:H_2O (100:4:11), pH 6.5] (Dawson et al., 1974), and by 2-dimensional peptide mapping [20 x 20 cm cellulose glass plates, TLC pH 2-3, TLE pH 6.5]. Visualization was made with sprays of fluorescamine [0.03% in AR acetone after triethylamine:acetone (1:100)], ninhydrin [0.3% ethanolic solution] and o-phthalaldehyde [for glycine (Dawson et al., 1974)].

RESULTS

Chemical Syntheses and Model Reactions

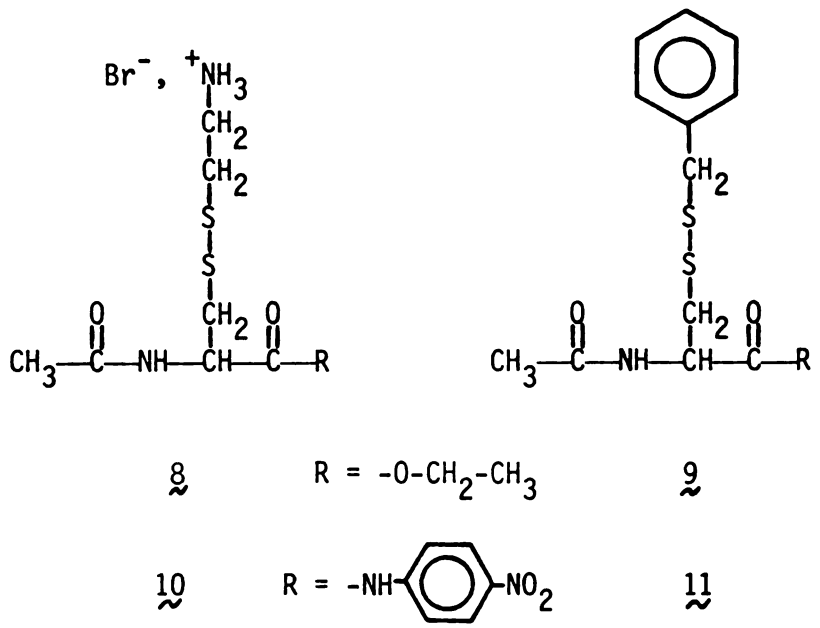
Syntheses of new, sulfhydryl-specific, alkyl alkanethiolsulfonate reagents, 2 and 3, utilized parallel, nearly quantitative (prior to purifications) two-step reaction sequences through a common intermediate, 1. Derivatives of α-L-cysteine, 4,5,6, and 7 were synthesized using well-established types of reactions, and analytically pure 4 and 7 obtained could be stored without decomposition.

Reactions of N-α-acetyl-L-cysteine ethyl ester (4) with alkyl methanethiolsulfonates, 2 or 3, proceeded quantitatively using 0 to 20% molar excesses of reagents. Therefore, post-reaction ethanolic solutions contained only methanesulfinic acid as a co-product, in some cases excesses of the alkanethiolating reagents, and either 8 or 9 (Figure 2). TLC and DTNB assays indicated stoichiometric product formation within

several minutes, and thus could be used to characterize conveniently the alkanethiolation reactions of 2 and 3 with cysteinyl sulfhydryl groups.

Reactions of N-α-acetyl-L-cysteine-p-nitroanilide (7) with 2 and 3 in 5 or 10% molar excess in Me_2SO were stoichiometric and rapid, as indicated by TLC and DTNB analyses. For use in enzyme kinetic studies (see following chapter) 10 and 11 were made in situ using only a 5% molar excess of reagents. Thus, post-reaction Me_2SO solutions contained only stoichiometric amounts of 10 or 11 (Figure 2), methanesulfinic acid, DTT_{ox}, and $\leq$ 2 to 5% of both innocuous mixed-disulfides, formed from DTT and reagents, and excess 2 or 3. Unfortunately, neither 10 nor 11 could be easily isolated and, since they were not volatile enough for mass spectral analyses, they were further characterized spectrophotometrically (see Figure 4 of following chapter). The mixed-disulfide products, 8, 9, 10 and 11, could be kept under specified conditions without detectable decomposition.

Reduced glutathione and the A chain of bovine insulin were treated with 1 to 2 sulfhydryl equivalents of 2 or 3 in buffered, aqueous solutions under mild conditions. In all cases, the reactions were rapid, stoichiometric and selective for cysteinyl sulfhydryl groups, indicated by DTNB (complete loss of free sulfhydryl groups) and various paper, TLC, TLE and combined TLC-TLE analyses (complete disappearance of unmodified peptide spots and appearance of new, single spots).



8 R = -O-CH2-CH3 9

10 R = -NH-(C6H4)-NO2 11

FIGURE 2: Products of model alkanethiolation reactions of reagents 2 and 3 with L-cysteine derivatives 4 and 7.

DISCUSSION

Synthesis of Alkyl Methanethiolsulfonate Reagents. In alkanethiolation reactions an alkanesulfinic acid ($R'SO_2H$) is released (Figure 1). The nature of the R'-moiety of the $RSSO_2R'$ reagent is of no consequence, therefore, unless it adversely affects the stability and/or reactivity with thiols. Accordingly, a major goal of ours has been to devise a general scheme for the practical synthesis of new reagents, all of which would possess a simple methyl group as the R' moiety.

The results of this and previous work (Smith et al., 1975) indicate that virtually any new $RSSO_2CH_3$ reagent should be obtainable in high yield in a simple, one-step S-alkylation of the appropriate alkyl halide (or a functionality otherwise susceptible to nucleophilic substitution by thiolate anion) with sodium methanethiolsulfonate. In these reactions care should be taken to eliminate water, as its prolonged presence might be expected to lead to hydrolysis of the $RSSO_2CH_3$ product, yielding methanesulfonic acid and the corresponding alkanethiol (Pappas, 1977). However, this is not a consideration in subsequent rapid alkanethiolations, even of cysteinyl sulfhydryl groups of polypeptides in dilute, aqueous solutions.

Modification of Cysteinyl Sulfhydryl Groups with Alkyl Methanethiolsulfonates should be performed in dilute solutions of either anhydrous organic or buffered aqueous and aqueous-organic solvents to avoid both methanesulfinic acid-catalyzed (CH_3SO_2H; pK_a 2.28, 25° C; Wudl et al., 1967) disproportionation (Kice & Ekman, 1975) and hydrolysis (Pappas, 1977) of the mixed-disulfide products. Whereas $CH_3SO_2^-$ is fairly stable

at room temperature, CH_3SO_2H is subject to rapid decomposition, especially in the presence of O_2 and H_2O, to yield an incompletely characterized mixture of low molecular weight, volatile products (Wudl et al., 1967). We have shown, however, that at least under the mild conditions used in this work, the stability of the mixed-disulfide bond of the alkanethiolation products is not adversely affected by the continued presence of any of these side-products. Thus, the desired cysteinyl mixed-disulfide products apparently can be generated in situ and used for many purposes without further purification.

Chemistry of Reactions of Alkyl Alkanethiolsulfonates with Sulfhydryl Groups. The results of this study both confirm and extend those of previous studies (Smith et al., 1975; Kenyon & Bruice, 1977; Currier & Mautner, 1977) and indicate that alkanethiolation reactions of $RSSO_2R'$ reagents with -SH groups proceed: (i) independent of the nature of both the R' and most importantly the R groups (presumably within steric limitations), (ii) under mild conditions, nondestructive to proteins, (iii) with high selectivity for cysteinyl sulfhydryls, (iv) extremely rapidly, (v) quantitatively to complete conversion to the mixed-disulfide without large excesses of reagents, and (vi) reversibly to regenerate free sulfhydryl groups upon addition of large molar excesses of thiols (see chapter 3). These highly desirable characteristics endow this class of reagents with unusual versatility for potential exploitation.

We can account qualitatively for the observed facility of this nucleophilic substitution reaction. As shown in Figure 3, nucleophilic attack by relatively "soft" thiol or thiolate groups of cysteine (16)

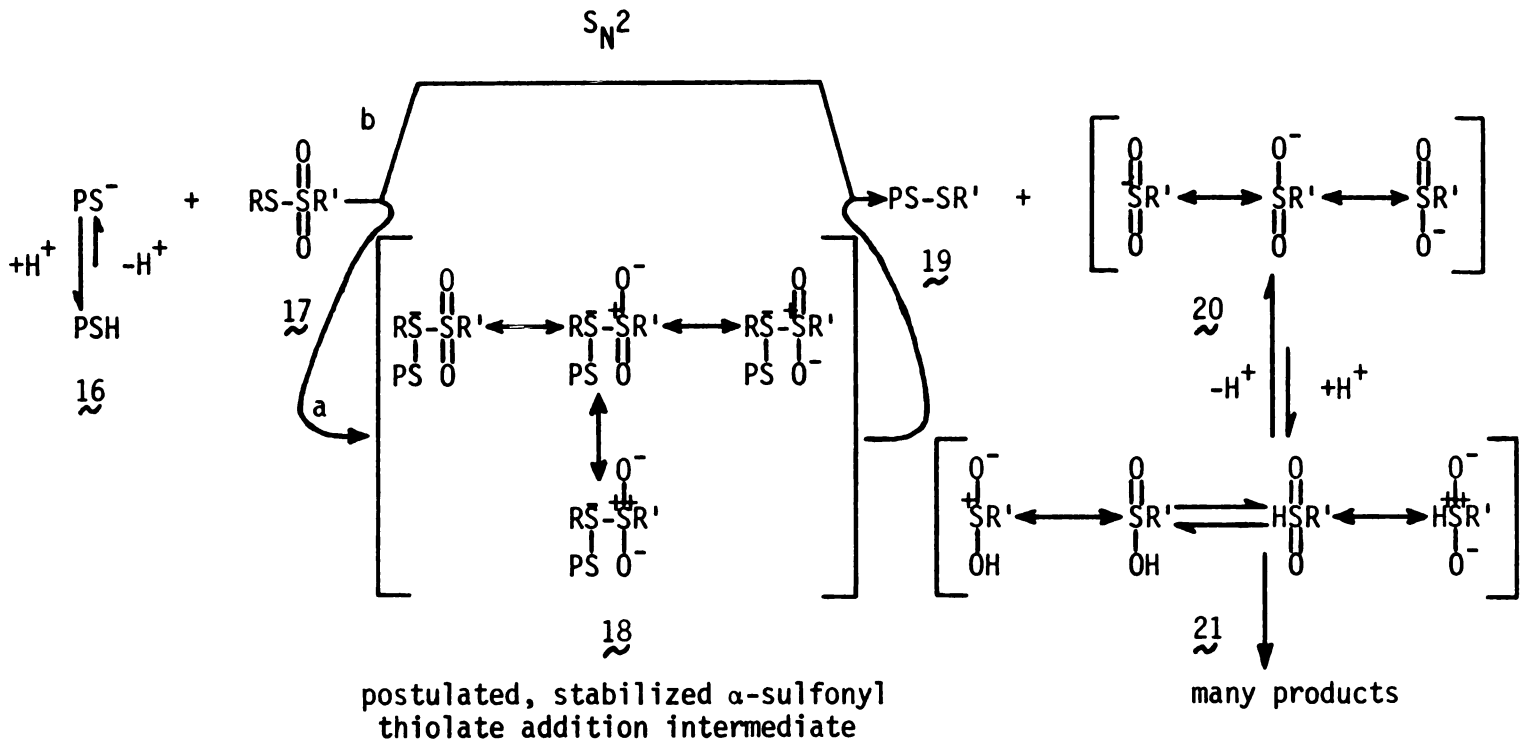


FIGURE 3: Plausible reaction mechanisms of alkyl alkanethiolsulfonates with sulfhydryl groups. PSH denotes a compound possessing an accessible, reactive sulfhydryl group, such as the amino acid cysteine, alone, or in peptides and proteins.

on the "soft" sulfide sulfur in $RSSO_2R'$ (17) to form a new sulfur-sulfur bond (18 or 19), should proceed readily with early charge transfer. The reaction may proceed (path a) through the formation of a resonance-stabilized addition intermediate (18) of the type that generally has been implicated in nucleophilic substitution at S-S bonds (as in $RS-SO_2R'$). Of all common anions, the thiolate ion (16) is most reactive in effecting facile nucleophilic scission of S-S bonds. Regardless of mechanism, scission of the $S-SO_2$ bond of the reagent should be rapid, but especially so in an S_N2 mechanism (path b), because it is polarized (Pappas, 1977). And, the alkanesulfinate (20) then released is an excellent leaving group because of reasonance stabilization (Hendrickson et al., 1970). Subsequent rapid decomposition of 21 (observed for $R' < C_{12}$) formed in buffered aqueous or aqueous-organic solvents into many products (> 20 where $R' = -CH_3$; Wudl et al., 1967) also would provide a large driving force for reaction.

Modification of cysteinyl sulfhydryl groups with the reagents of this study, 2 and 3, yields unusual amino acid moieties with unique mixed-disulfide side-chains. The structures of these have purposefully been designed to be analogous with respect to the functional groups but different in other respects to lysine and phenylalanine. The significances of these similarities and differences in terms of kinetic behavior as substrates for either trypsin or α-chymotrypsin are examined in the accompanying chapter. This will provide a demonstration of a highly specialized biochemical application of new $RSSO_2CH_3$ reagents that are both more complex and more easily synthetically accessible than any other $RSSO_2R'$ reagents studied to date.

CHAPTER 3

APPLICATION OF NOVEL ALKYL METHANETHIOLSULFONATE REAGENTS FOR THE GENERATION OF KINETICALLY UNUSUAL SUBSTRATES FOR TRYPSIN AND α-CHYMOTRYPSIN BY MODIFICATION OF L-CYSTEINE[3]

ABSTRACT

N-α-Acetyl-L-cysteine ethyl ester (4) and its mixed-disulfide derivatives generated in the preceeding chapter (8 and 9) undergo anomalously rapid specific acid- and specific base-catalyzed ester hydrolyses; this is not observed for either N-α-benzoyl-L-arginine ethyl ester or N-α-benzoyl-L-tyrosine ethyl ester. Kinetics of trypsin-catalyzed hydrolyses of the β-S-(β-aminoethanethiol)-analogs (8 and 10) and of α-chymotrypsin-catalyzed hydrolyses of the β-S-(benzylthiol)-analogs (9 and 11) were compared to those for analogously derivatized and identically assayed, specific reference amino acid substrates. For both enzymes it is surprising that the specificity constants, k_{cat}/K_m, for analog esters (8 and 9) compare favorably with those for reference esters, whereas those for analog amides (10 and 11) compare much less favorably with those for reference amides. Further, no detectable enzyme-catalyzed hydrosysis of the amide bonds at the sites of modified cysteine residues in the A chain of insulin was observed. Evidence is presented which suggests that the transition-state free energies of acylation are very large for 8-15, to the extent that there is a change in rate-determining step for the analog ester substrates 8 and 9 from deacylation, rate-determining for the reference ester substrates, to acylation. This is the first report of such a change in rate-determining step for serine protease amino acid substrates possessing side-chains of equal or greater length than those of the preferred amino acid substrates. However, these conclusions are entirely consistent with certain recent mechanistically-based predictions (Bachovchin and Roberts, 1978).

In the accompanying chapter we reported first that new alkyl methanethiolsulfonates ($RSSO_2CH_3$) with bulky charged or aromatic R groups are synthetically as easily accessible as earlier reagents with methyl or ethyl R groups *via* S-alkylation reactions with sodium methanethiolsulfonate. Second, we showed that the presence of these R groups had no interfering effects on the facile reactions of the new reagents, **2** and **3**, with the sulfhydryl groups of cysteine in amino acid derivatives and in the peptides glutathione and the A chain of bovine insulin. In this chapter we describe a specialized biochemical application of these reagents; the generation of new substrates for trypsin and α-chymotrypsin by modification of L-cysteine residues.

In addition to the likelihood that they would be substrates, we thought it probable that the modification products also would be of interest because of the consequences to substrate behavior of having both large and disulfide-containing side-chains. It was known, for example, that lysine derivatives are better substrates for trypsin than the corresponding homo-lysine derivative possessing a larger side-chain (Kitagawa & Izumiya, 1959; Izumiya *et al.*, 1959), and that one sulfur heteroatom in the side-chains of aminoethylated derivatives of L-cysteine results in substrates that are significantly less kinetically specific for

trypsin than analogously derivatized L-lysine for which the side-chain is isosteric (Raftery & Cole, 1963; Cole, 1967).

Indeed, even though the substrate specificities for these enzymes have been studied exhaustively, we have found in this study that some alkanethiolated cysteine derivatives which are substrates exhibit kinetics implicating certain changes in rate-determining step which have not been observed before. These results are fully consistent with predictions which recently have been generated from a formulation of the mechanism of serine proteases which obviates the requirement of a charge-relay (Bachovchin & Roberts, 1978).

EXPERIMENTAL PROCEDURES

Materials and Methods. These were the same as reported in the preceding chapter and include the reagents 2 and 3, and modification products 8-15 synthesized therein. In addition, trypsin used was: Type I 1,200 BAEE[4] units/mg at 25° C, Sigma, or A grade, 3,700 National Formulary units/mg at 25° C, Calbiochem. α-Chymotrypsin used was Type II, 43 International units/mg at 25° C, Sigma.

Computer analyses were performed on a Hewlett-Packard Model 9821A. Static measurements of pH and pH*[6] (Hui Bon Hoa & Douzou, 1973; Maurel et al., 1975; Fink, 1976) were made using Radiometer PHM 26 meters and glass electrodes, and kinetics were carried out on a Radiometer TTT 2 pH-stat using NaOH solutions standardized against reference solutions

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[6] pH* is the experimentally observed apparent protonic activity in an aqueous-organic solvent. Values of pH* were used to give rough estimations of true protonic activities and, more importantly, to indicate any changes in protonic activities during kinetic assays.

of potassium acid phthalate.

Kinetic Assays of Trypsin- and α-Chymotrypsin-Catalyzed Hydrolysis Reactions

Ethyl Ester Hydrolyses. A pH-stat holding 2.0 mL solutions of the desired substrate concentrations and 75.0 mM $CaCl_2$-0.75 mM EDTA at 30 ± 0.5° C with endpoint pH's (±0.05) of 7.60 for tryptic and 7.80 for chymotryptic assays was used. Initial rates of hydrolyses were followed to < 5% completion, under conditions of $S_o >> E_o$ and $K_m >> E_o$. Values for S_o (8 and 9) were varied over at least a 10-fold range about K_m. For each substrate and E_o at least one assay was followed to completion. Kinetic assays of reference substrates, BAEE and BTEE, were run only under the conditions of $S_o >> K_m >> E_o$. Kinetic experiments were repeated at least twice.

Changes in specific activities of trypsin and α-chymotrypsin in the presence of increasing concentrations of Me_2SO were determined spectrophotometrically using specific amino acid ester substrates under conditions of $S_o >> K_m >> E_o$.

p-Nitroanilide hydrolyses. In solutions of ∿ 1.0 absorbance unit made as for assays below the largest value for the difference in absorbances for p-nitroaniline (recrystallized from water, mp 147.7-149.5° C) and for each p-nitroanilide substrate was seen at 389 nm (Figure 4): p-nitroaniline ε_{389} = 15,562, BApNA ε_{389} = 485, APpNA ε_{389} = 485, 10 ε_{389} = 780, 11 ε_{389} = 494 (ε = molar extinction coefficient).

Kinetic assays (Erlanger et al., 1961; Fink, 1974; Fink & Wildi,

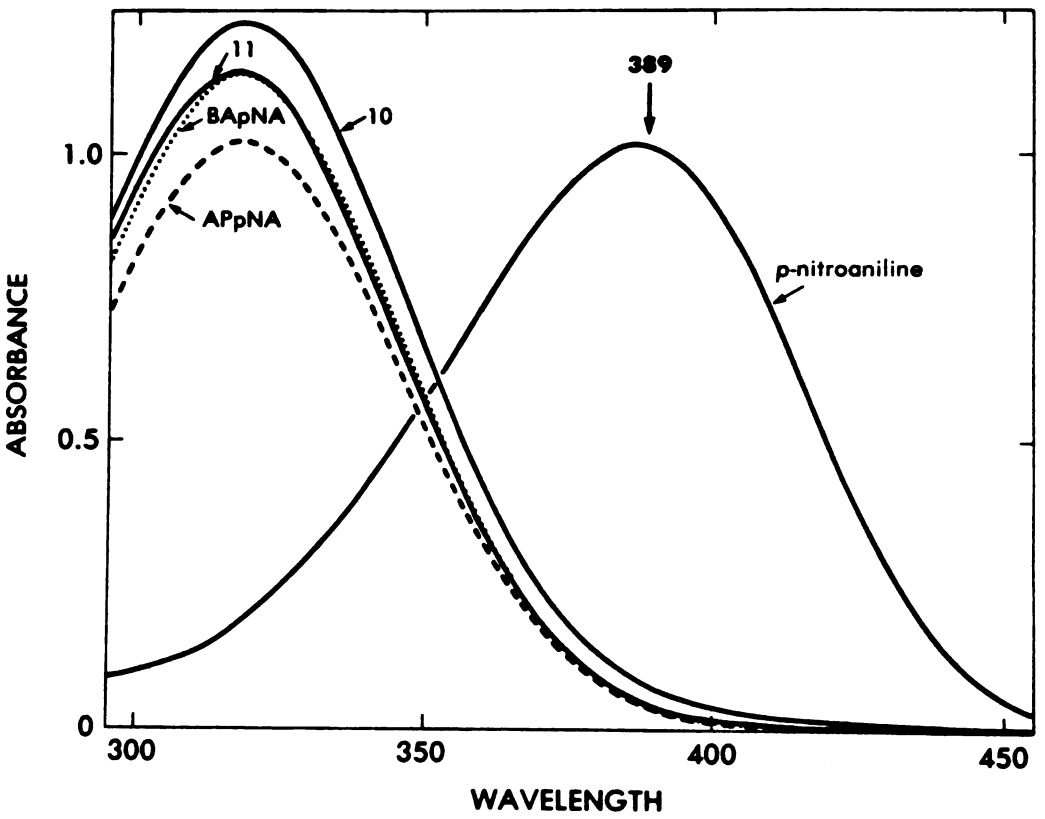

FIGURE 4: Ultraviolet--visible absorbance spectra of reference and analog p-nitroanilide substrates and of the common product of hydrolysis, p-nitroaniline. Spectra and extinction coefficients were obtained as described in text.

1974; Fink, 1976) performed with 1.500 mL total volume solutions, maintained at 10.0 ± 0.8° C in the thermostated cell component of a Cary 118-C, containing 33.3% Me_2SO (v/v), 40.0 m$\underline{M}$ NMM-HCl, and 50.0 m$\underline{M}$ $CaCl_2$, at pH* 7.89 ± 0.03 followed the rate of differential absorbance changes at 389 nm after addition of enzyme. Because of the limited substrate solubilities and very slow observed rates, kinetic reactions were followed in the region between pseudo-first-order [$\underline{K}_m \leq (8.4 - 57.7)\underline{S}_o$] and initial velocity [$\underline{S}_o \leq (0.4\text{-}1.4)\underline{K}_m$] conditions, and where possible with $\underline{S}_o >> \underline{E}_o$, but always with $\underline{S}_o \geq 2 \cdot \underline{E}_o$. Even in the latter case, the calculated extent of reaction was always < 5%. For each substrate and $\underline{E}_o$ at least one assay was followed for ≥ 5% total calculated reaction. Kinetic experiments were repeated at least twice.

Enzyme Digests and Peptide Mapping

Solutions of *in situ* alkanethiolated glutathione and the A chain of insulin, pH 8.0, were incubated in the sealed Reacti-Vials at 37° C, in the dark, 24 to 52 hr, with 1:3.8 to 100 [$\underline{E}_o$: modified peptide (W/W)] trypsin or α-chymotrypsin (6×10^{-7} to 8×10^{-5} $\underline{M}$). Aliquots were removed periodically and assayed spectrophotometrically by the method of Mitz and Schleuter (1958). Following incubations, enzyme activity was verified by assaying aliquots of the digests by the pH-stat method using saturating concentrations of BAEE (for trypsin) or BTEE (for α-chymotrypsin). Enzyme digests were lyophilized, treated with performic acid ($P\text{-}S\text{-}S\text{-}R \xrightarrow[-RSO_3^-]{+HCO_3H} P\text{-}SO_3^-$) by the method of Hirs (1967), lyophilyzed three times (2 water washes) and re-dissolved in doubly-distilled water

(to ∿ 0.5 nmol/μL). Control experiments were performed identically except for the absence of the enzyme digestion or alkanethiolation steps.

Characterizations of enzyme digestion products were performed by the paper chromatography, TLC, TLE and combined TLC-TLE methods reported in the preceding chapter for alkanethiolation product analyses. In addition, digests also were subjected to N-terminal analysis by the semi-micro (1/10th scale; 1 mmol/application to a 5 x 5 cm micropolyamide sheet) dansyl-chloride method of Woods and Wang (1967).

RESULTS

Experiments with Ethyl Ester Substrate Analogs. The enzyme-catalyzed rate of ester hydrolysis was calculated as the difference between pH-stat determined initial rates in the absence (if significant) and then in the presence of enzyme (Ingles & Knowles, 1966). For *in situ* assayed **8** and **9** rates were monitored after rapid neutralization of methanesulfinic acid. The rates of lyate species-catalyzed hydrolysis of BAEE and BTEE were immeasurable under assay conditions over >> v_i periods.

Rates of hydrolyses in the absence of enzymes were surprisingly rapid and approximately the same (within an order of magnitude) for identical concentrations at **4**, **8** and **9** at pH's of 7.6 or 7.8. The presence of either $CaCl_2$ or EDTA did not significantly influence the rates of hydrolyses. At a given pH (7.0-8.0), initial rates were linearly dependent on the concentration (over a > 10-fold range) of **8** or **9**, and were identical

when the substrates were either generated in situ or purified by column chromatography. At high pH (12.0) the hydrolytic reactions followed first-order kinetics for > 4 half-lives; equivalents of standardized base consumed upon completion were equal ($\sim\pm5\%$) to the initial concentration of derivative.

The dependence of log k_{obsd} on pH for hydrolysis of 9 (Figure 5) only was determined because, unlike either 4 or 8, it has no ionizable groups, but at 5.2 mM required for detection of rates oiling-out was a problem. However, log k_{obsd} varied linearly with slopes of plus or minus 1.0 as the pH was raised (from 8.5 to 12.0) or lowered (from 5.5 to 2.5), respectively. At pH 3.0 the rate constant for specific acid catalysis (k_H = 3.2 x 10^2 M^{-1} min^{-1}) and at pH 11.0 the rate constant for specific base catalysis (k_{OH} = 2.2 x 10^2 M^{-1} min^{-1} were calculated as shown below (-log K_w = 13.833 at 30° C).

$$k_{obsd} = k_H a_H + k_{OH} \frac{K_w}{a_H}$$

At pH 7.7, neglecting the possibility of spontaneous-catalyzed hydrolysis, a minimal value for -log k_{obsd} of 3.8 min^{-1} [$k_{obsd} \geq 1.6 \times 10^{-4}$ min^{-1} << k_{cat} = 1.8 x 10^2 min^{-1} (Table I)] was calculated.

Under conditions of $K_m \geq\geq S_o >> E_o$ or $K_m >> E_o >> S_o$ (for 8 with trypsin and 9 with α-chymotrypsin) initial rates were linear and directly proportional to the concentration (varied over a ≥ 10-fold range) of the limiting species, and proportional to S_o under conditions of $K_m >> S_o \geq E_o$. Also, hydrolysis curves for equivalents of base titrated to

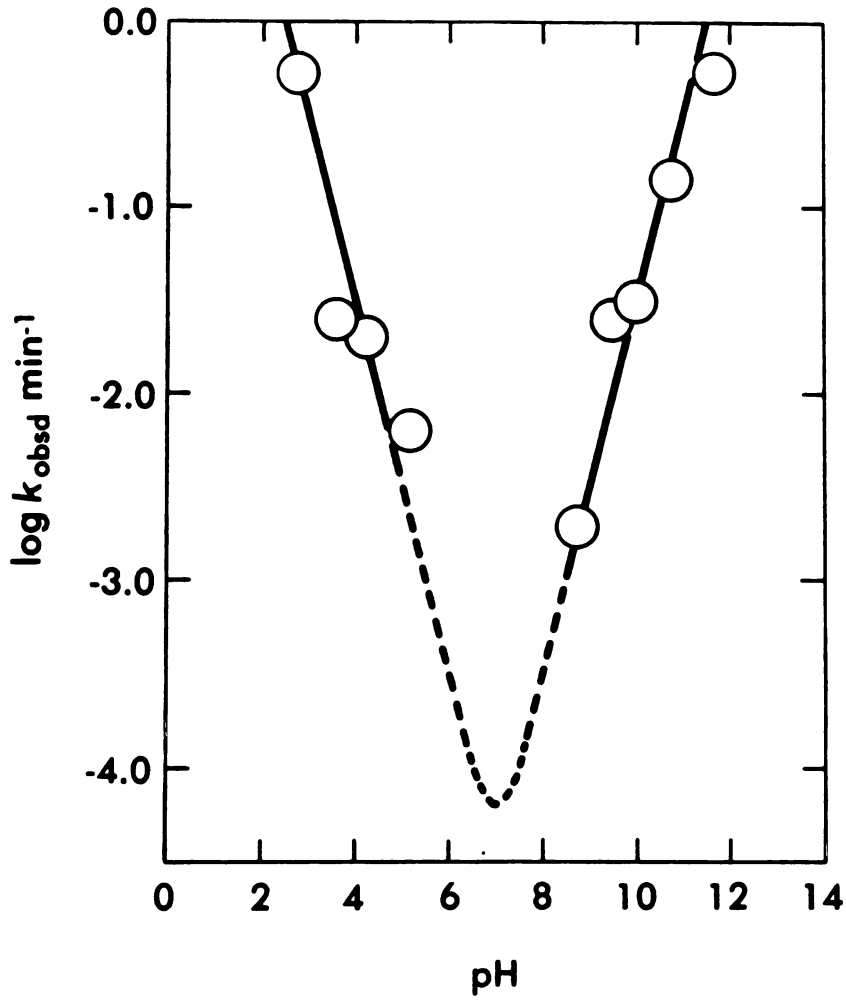

FIGURE 5: Preliminary pH vs. log rate profile for the nonenzymatic hydrolysis of 9, demonstrating specific acid and specific base catalysis of hydrolysis. Profile was determined as described in text.

completion (using $E_o \geq 5.0 \times 10^{-6}$ M) vs. time were typical for serine protease-catalyzed reactions. Michaelis-Menten kinetic parameters are shown in Table I.

Control experiments were performed under conditions of $S_o >> K_m >> E_o$, where appropriate. Over >> v_i periods of time no significant rates were observed (i) with enzymes alone at high concentrations ($\geq 10^{-5}$ M) (Ingles & Knowles, 1966), (ii) in assays of enzymes with 4, (iii) in assays of trypsin with 9 or α-chymotrypsin with 8 (even with $E_o \geq 5.0 \times 10^{-6}$ M) and (iv) in assay solutions containing only 2 or 3. Rates of enzyme-catalyzed hydrolyses of BAEE or BTEE were the same in the absence or presence of large molar excesses of 2 or 3. Rates were identical for 8 and 9 either generated in situ or column purified (minus excess 2 or 3 and methanesulfinic acid). Large molar excesses of β-mercaptoethanol had no effect on v_i's for BAEE or BTEE, but abolished the rates for 8 and 9; for $S_o > [\beta\text{-mercaptoethanol}]_o$, rates immediately decreased proportionally to the amount of exogenous thiol added.

Product analysis for the assumed enzyme-catalyzed hydrolysis was performed as follows. Solvent was removed from the pool of column-purified 8 (≥200 mg; limited solubility of 9 prevented an analogous analysis) and an NMR spectrum (D_2O; DSS standard; taken immediately to avoid significant hydrolysis) of ≥100 mg showed major peaks at δ 1.27 (methyl, t, J = 7 Hz), 2.07 (methyl, s), 3.70 (methylene, q, J = 7 Hz), 4.30 (methylene, t, J = 6 Hz). After addition of 4 drops of absolute ethanol, new methyl triplet (δ 1.17) and methylene quartet (δ 3.63) peaks appeared shifted 7 Hz upfield from the analogous peaks of the

ethyl ester of 8, showing that these latter peaks were not due to solvent. Another ∿100 mg of oil was incubated in a 22 mM assay solution at pH 7.6 (pH-stat), 30° C, for 45 min with 2 x 10^{-6} M trypsin [<1/100, E_o/8 (w/w)]. Solvent was removed, and the sample alternately was washed with D_2O and dried *in vacuo* (2 times). Its NMR spectrum (as before) was superimposable on that for 8 from controls incubated in the absence of enzyme, except for the complete absence of ethyl ester peaks at δ1.27 (methyl, t) and 3.70 (methylene, q).

Experiments with p-Nitroanilide Substrate Analogs. In aqueous-organic assay solutions of 1/3 (v/v) Me_2SO, the p-nitroanilide substrates had minimally satisfactory solubilities on the order of 1 mM at 10° C; the enzymes still retained substantial relative specific activities (trypsin 131% and α-chymotrypsin 55%, *vs.* 100% specific activity as defined in the absence of Me_2SO) and were stable to denaturation.

Only the p-nitroanilide moieties of analytically pure reference substrates and of *in situ* generated analog substrates absorbed in the 500-300 nm range. Increases in absorbance at 389 nm during enzyme-catalyzed hydrolyses were due solely to release of free p-nitroaniline.

Initial rates of tryptic hydrolysis of 10 and α-chymotryptic hydrolysis of 11 were linear and directly proportional to the concentration (varied over a ≥ 10-fold range) of the limiting species under pseudo-first-order conditions of $K_m \geq\geq S_o >> E_o$ or $K_m >> E_o >> S_o$ and proportional to S_o when $K_m >> S_o > E_o$. Michaelis-Menten kinetic parameters are shown in Table I.

Wherever appropriate, control experiments were performed under the most

rapid velocity conditions possible ($\underline{S}_o >> \underline{E}_o$). In substrate assay solutions lacking enzyme, no significant lyate species-catalyzed p-nitroanilide hydrolysis was observed even at 30° C for >42 hrs. Enzyme activities did not diminish noticeably over >> v_i periods of time. Neither DTT_{ox} alone nor equimolar amounts of both DTT_{red} and 2 or 3 added in large molar excess over $\underline{S}_o$ had any effects on the rates of trypsin- or α-chymotrypsin-catalyzed hydrolyses of BApNA or APpNA, respectively. A 100-fold excess over $\underline{S}_o$ of β-mercaptoethanol had no immediate effect on initial velocities with BApNA or APpNA, but with 10 or 11 there were immediate, irreversible cessations of rates. When trypsin or α-chymotrypsin were pre-incubated at 10° C for 30-120 min in the presence of a 10-100 molar excess of the active site specific alkylating inhibitors, TLCK or TPCK, respectively, no rates were observed in later assays of the reference or analog substrates.

For 10 and 11 obtaining product analyses was made difficult by the slow enzyme-catalyzed rates which precluded following of reactions to completion. Spectra taken during the reactions, however, showed the appearance of free p-nitroaniline concomitant with disappearance of the substrate, to extents dependent on both $\underline{E}_o$ and the time periods of the reactions.

Experiments with Alkanethiolated Peptides. No detectable trypsin-catalyzed hydrolyses of aminoethanethiolated or α-chymotrypsin-catalyzed hydrolyses of benzylthiolated glutathione (12 and 13, respectively) or the A chain of bovine insulin (14 and 15, respectively) under a variety

of conditions of concentrations, ratios of enzyme to modified peptide and incubation times, were observed using several methods of analyses. These included a direct spectrophotometric technique (Mitz & Schlueter, 1958), a dansyl chloride N-terminal analysis and various systems of paper chromatography and cellulose TLC and TLE. All methods unambiguously gave parallel results for both alkanethiolated and unmodified peptide digests which were incubated for prolonged periods (>2 days, 37° C, optimal pH); afterwards, significant enzyme activities were demonstrated independently. Thus, we can conclude that the modified residues of the peptides in question (12-15) are not sites of cleavage, or at best are extremely poor sites of action for either trypsin or α-chymotrypsin.

DISCUSSION

Rates of Nonenzymatic Hydrolyses of Cysteine Ethyl Ester Derivatives 4, 8 and 9 surprisingly were observed to be at least two orders of magnitude larger than those for BTEE and BAEE (immeasurable over a period of several hours) assayed identically; similarly rapid rates also have been reported for cysteine methyl ester (Wataya et al., 1976). They therefore likely were not due to metal ion catalysis (Hay & Morris, 1976), especially since the presence or absence of either EDTA or Ca^{2+} ions were without significant effect. Our preliminary studies have shown that the anomalous pH-stat rates are indeed a result of non-enzymatic hydrolysis that minimally is subject (at least for 9) to specific-acid and specific-base catalysis (Figure 5). However, these studies

gave no other mechanistic information. Yet, we may now say that it is unlikely that differences of this magnitude in non-enzymatic rates can be attributed solely to polar, steric, or resonance effects operative only on the cysteine derivatives (De Tar & Tenpas, 1976). It is not obvious, then, why these rapid rates are observed, and so it is apparent that more definitive answers will require a mechanistic study outside the scope and interests of this work.

However, such an intriguing observation prompts us to speculate briefly that at least at neutral pH the β- or γ-sulfur atoms may be acting as intramolecular nucleophilic catalysts in the hydrolysis of 4, 8 and 9; catalysis of hydrolysis of certain lysine derivatives by the ε-amino group has been observed (Hay & Morris, 1976). Although this has not been reported previously to our knowledge, both free sulfhydryl- and mono-sulfide sulfurs are good nucleophiles (Lindley, 1956; Hupe & Jencks, 1977; Ando, 1977), and in some systems effective anchimeric assistance has been observed (Livant & Martin, 1976; Heller et al., 1977; Burighel et al., 1971); disulfides are implicated here as good nucleophiles towards a carbonyl center. Further, in the tetrahedral intermediates at the α-carbonyl carbon that would be expected, proper orbital orientation of the sulfur (at least for 4) and carbonyl oxygen heteroatoms bonded to this carbon may exist to provide a powerful stereoelectronic driving force for expelling the leaving group, especially since the oxygen atom would be negatively charged (Deslongchamps, 1976). At extremes of pH, either specific-acid or specific-base catalysis of hydrolysis may predominate over intramolecular nucleophilic catalysis (at

least for 9).

Structure of the Mixed-Disulfide Side-Chains of Alkanethiolated Cysteine. Both the β-S-(β-aminoethanethiol)- and β-S-(benzylthiol)-side-chains are longer than the corresponding side-chains of Lys or Phe, which they are structurally identical to at their distal ends. However, evidence existed suggesting that the steric tolerances of the substrate side-chain binding sites of the respective enzymes (Kossiakoff et al., 1977; Scofield et al., 1977; Brot & Bender, 1969; Hansch & Coats, 1970) would be sufficient to accomodate their binding.

As depicted in Figure 6, the covalent radius of a sulfide sulfur atom is no larger than that of a methylene unit. Also, the C-C and S-S and S-C bond lengths, and the $-CH_2-CH_2-CH_2$ and $-CH_2-S-S-$ bond angles are similar for the juxtaposed side-chains. For unstrained CCSSCC structural units in which the disulfide bond is β-methylene substituted and both R_α and R'_α are so small as not to interact sterically, there will exist a wide range of conformations about the C-S bond. And, the C-S-S bond angles will vary with changes in the dihedral angle, χ(CS-SC), within the approximate range shown in Figure 6. Most importantly, at ambient temperatures values for χ of $\sim\pm$ 90-98° are only slightly preferred; χ rotational free energy barriers may be estimated to range from ~7.0-9.0 kcal/mol (Cotton & Wilkinson, 1972; Van Wart et al., 1976; Mattice, 1977; Fraser et al., 1971), and thus at least near rotational isomeric equilibrium should exist.

From these considerations at least 8, 9, 10 and 11, and probably 14 and 15, could reasonably be expected to be substrates for trypsin and

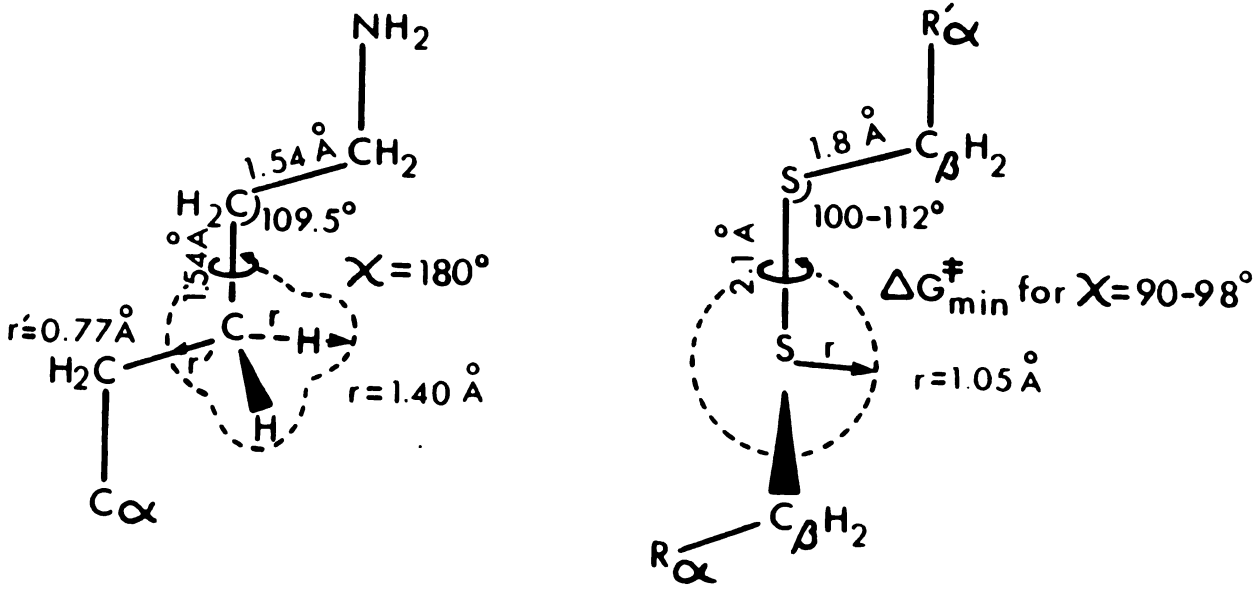

FIGURE 6: Schematic representation of mixed-disulfide side-chains of β-S-(alkanethiol)-cysteine compared to the side-chain of lysine. Notation and parameters were obtained from Pappas (1977); Kice & Ekman (1975); Van Wart et al., (1976); and Cotton & Wilkinson (1972).

α-chymotrypsin. However, as mentioned earlier, the kinetic consequences of having either abnormally large or mono-sulfide containing side-chains alone are non-trivial. It would not be unexpected if the unusual combination of the former and di-sulfide containing side-chains were to result in interesting substrate behavior.

Alkyl Methanethiolsulfonate Modified Cysteine Esters and Amides as Substrates for Trypsin or α-Chymotrypsin. In a preliminary study (Bruice et al., 1976) we found that both the ethyl esters 8 and 9 were good substrates for trypsin and α-chymotrypsin, respectively, confirming our expectations. We thus were encouraged to determine "relative specificity constants", $(k_{cat}/K_m)_{analog}/(k_{cat}/K_m)_{reference}$, (Brot & Bender, 1969; Bender & Kezdy, 1965). These are sensitive only to structural differences in the side-chains of acyl α-L-amino acid substrate analogs compared to highly specific reference acyl α-L-amino acid substrates[7] with the same

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[7] Convenient syntheses for the reference substrates, APpNA and BApNA, yield racemates. Since it is known that the binding constants (K_n^D) for the D-isomers, which are at best extremely poor substrates (Ingles & Knowles, 1967; Jencks, 1969), are less than or equal to those (K_p^L) for the L-isomers, the maximal possible deviations of the observed maximum velocities (V_{obsd}; and hence k_{cat}) and binding constants (K_{obsd}) from the true values (V_p^L and K_p^L) for the L-isomers will be as shown below (assuming $K_p^L = K_n^D$; Jencks, 1969). As can be seen, the errors introduced are not large, and only differences >> 2-fold in the K_m and k_{cat} values for 10 or 11 vs. BApNA or APpNA, respectively, are considered significant.

$$V_{obsd} = V_p^L \frac{K_p^L}{K_p^L + K_n^D} = \tfrac{1}{2} V_p^L$$

$$K_{obsd} = K_p^L + K_n^D = 2K_p^L$$

LEGEND TO TABLE I

[a]Kinetic parameters (and standard errors) were obtained by computer curve fitting of the data points to the Michaelis-Menten equation; values for E_o used were determined from mg crystalline enzyme/mL and are subject to errors equivalent to the magnitude of the discrepancy (usually 10-25%; Bender *et al*, 1967) between these values and the true concentration of active sites in solution. Values for k_{cat} were calculated assuming molecular weights of 23,800 for trypsin and of 21,600 for α-chymotrypsin. Parameters for ethyl ester substrates were obtained by pH-stat assay described in text: E_o stock solutions (10^{-3} M HCl)-trypsin 1.00-1.05 mg/mL, α-chymotrypsin 1.00-1.02 mg/mL; S_o stock solutions (stored 0-4°C) - BTEE 1.1 x 10^{-3} M (50% methanol W/W), BAEE 2.6 x 10^{-3} M (H_2O or 0.1 M $CaCl_2$), 8 6.7-8.4 x 10^{-2} M and 9 7.2-20 x 10^{-2} M (8 and 9 in absolute ethanol dried over 3Å molecular seives). Parameters for p-nitroanilide substrates were obtained by spectrophotometric assay described in text, where the actual $\Delta A_{obsd} = A_{\text{p-nitroaniline released}} - A_{\text{substrate-p-nitroanilide consumed}}$. Knowing values for ε_{389} for the substrates and p-nitroaniline, ΔA_{obsd} could be calculated for complete hydrolysis of a 1 M solution of substrate to give a 1 M p-nitroaniline solution. Correction factors were then calculated as $A_{\text{p-nitroaniline released}}/\Delta A_{obsd}$ and were: BApNA 1.032, APpNA 1.032, 10 1.053, 11 1.033. Finally; true $A_{\text{p-nitroaniline released}} = \Delta A_{obsd} \times$ correction factor. E_o stock solutions (10^{-3} M HCl) - trypsin 9.1 mg/mL, α-chymotrypsin 9.2 mg/mL; S_o stock solutions {all at near maximal concentrations in N_2-saturated, spectrograde Me_2SO, stored under N_2 at 0-4°C (frozen) in the dark} - APpNA 3.9 x 10^{-2} M, BApNA 3.0 x 10^{-2} M, 10 4.4 x 10^{-2} M, 11 4.4 x 10^{-3} M, [b]Apparent specificity constant. [c]Apparent relative specificity constant. [d]from Bender *et al.*, 1961. [e]from Schwert & Eisenberg, 1949; pH 7.8, 30 C. [f]from Elmore *et al.*, 1967; pH 8.0, 25°C (standard deviations given). [g]from Wang & Carpenter, 1968; pH 8.0, 30°C.

TABLE I: Kinetic Data for Trypsin- and α-Chymotrypsin-Catalyzed Hydrolyses of Reference and Analog Substrates[a]

Substrate	$K_m \times 10^4$ (M)	$k_{cat} \times 10^2$ (sec^{-1})	k_{cat}/K_m[b] $\times 10^{-1}$ ($sec^{-1}\ M^{-1}$)	$\frac{(k_{cat}/K_m)_{analog}}{(k_{cat}/K_m)_{reference}}$[c] $\times 10^2$
		α-Chymotrypsin		
9	7.3 ± 1.5	300 ± 0.8	410 ± 65	1.8 ± 0.19
BTEE	1.2[d]	2,700 ± 170	22,000 ± 1,400	
11	6.8 ± 0.93	0.0013 ± 0.00011	0.0019 ± 0.00017	0.17 ± 0.015
APpNA	1.8 ± 0.0067	0.20 ± 0.00072	1.1 ± 0.0085	
		Trypsin		
8	8.3 ± 1.1	780 ± 24	940 ± 96	3.4 ± 0.25
BAEE	0.80[e]	2,200 ± 64	27,000 ± 80	
AEBCME[f]	0.94 ± 0.063	2,100 ± 400	22,000	22
BLME[f]	0.17 ± 0.004	1,700 ± 10	99,000	
10	12 ± 0.39	0.0099 ± 0.000095	0.0081 ± 0.00018	0.020 ± 0.00012
BApNA	15 ± 2.2	59 ± 7.9	40 ± 0.63	
AEBCA[g]	43	33	7.7	6.8
BAA[g]	25	280	110	

leaving group, when assayed identically. These constants are largely independent of the nature of the N-acyl moieties (Brot & Bender, 1969; Bender & Kézdy, 1965; Bender et al., 1964; Ingles and Knowles, 1967, 1968).

In contrast to the results of previous studies (Brot & Bender, 1969; Bender & Kézdy, 1965) the relative specificity constants of Table I indicate that for the same analog acyl amino acids the ester substrates, 8 and 9, are apparently much more specific than the very poor amide substrates, 10 and 11. In view of the latter it is not surprising that no detectable enzyme-catalyzed cleavage of peptide bonds at sites of alkanethiolation of the A chain of bovine insulin for 14 and 15 was observed. This is especially true since the assay methods were less sensitive and the peptide structure may be expected to adversely affect both the interactions with the enzyme and the range of freely allowed conformations of the mixed-disulfide side-chains. Similar arguments may apply for the products of modification of glutathione, 12 and 13, but in this case the presence of an abnormal γ-Glu residue preceding the Cys residue may alone account for the failure to detect enzymatic cleavage (Ingles & Knowles, 1968).

We may assume that these apparent specificity differences between ester and amide substrate analogs are not a result of changes in reaction pathway (Fink, 1974), since the mechanistic characteristics of reactions catalyzed by either trypsin or α-chymotrypsin are identical for both the most and least specific substrates (Bender & Kézdy, 1965).[8] They then can be attributed predominately to differences in

values of k_{cat} rather than K_m; (k_{cat} analog ester/k_{cat} reference ester) > (k_{cat} analog amide/k_{cat} reference amide), whereas (K_m analog ester/K_m reference ester) ≃ (K_m analog amide/K_m reference amide) (Table I). The binding constants (K_s) for these juxtaposed substrates are probably similar also, since it is unlikely that highly different K_s values could be mutually offset in every case by compensatory differences in rate terms to give the similar K_m values observed in Table I. These observations and interpretations are consistent with the knowledge that large differences in kinetic specificities of substrates for serine proteases usually reflect overriding variations in catalytic vs. binding ability (Jencks, 1969; Bender et al., 1964; Ingles & Knowles, 1967; Jencks, 1966).

For the serine proteases k_{cat} generally relates to the single catalytic step with the highest transition-state free energy, $\Delta G^{\ddagger}$ (as $\Delta G^{\ddagger}_{highest}$ increases, k_{cat} decreases; Bender & Kézdy, 1965; Bender et al., 1964; Zerner & Bender, 1964). Thus, using the k_{cat} values of Table I, the most plausible relationships of $\Delta G^{\ddagger}$ values of individual catalytic steps for reference and analog substrates can be determined (Figure 7). The large upward perturbation (vertical arrow in Figure 7) from $\Delta G^{\ddagger}_{acyl.}$ for BApNA and APpNA to $\Delta G^{\ddagger}_{acyl.}$ for 10 and 11 reflects the large differences in k_{cat} values between these analog and reference

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[8] It is noteworthy that concentrations of Me_2SO even greater than the 33.3% employed here in all assays of p-nitroanilide substrates only, alter neither the kinetic schemes or mechanisms of action of either trypsin or α-chymotrypsin (Fink, 1974; Fink & Wildi, 1974; Fink, 1976).

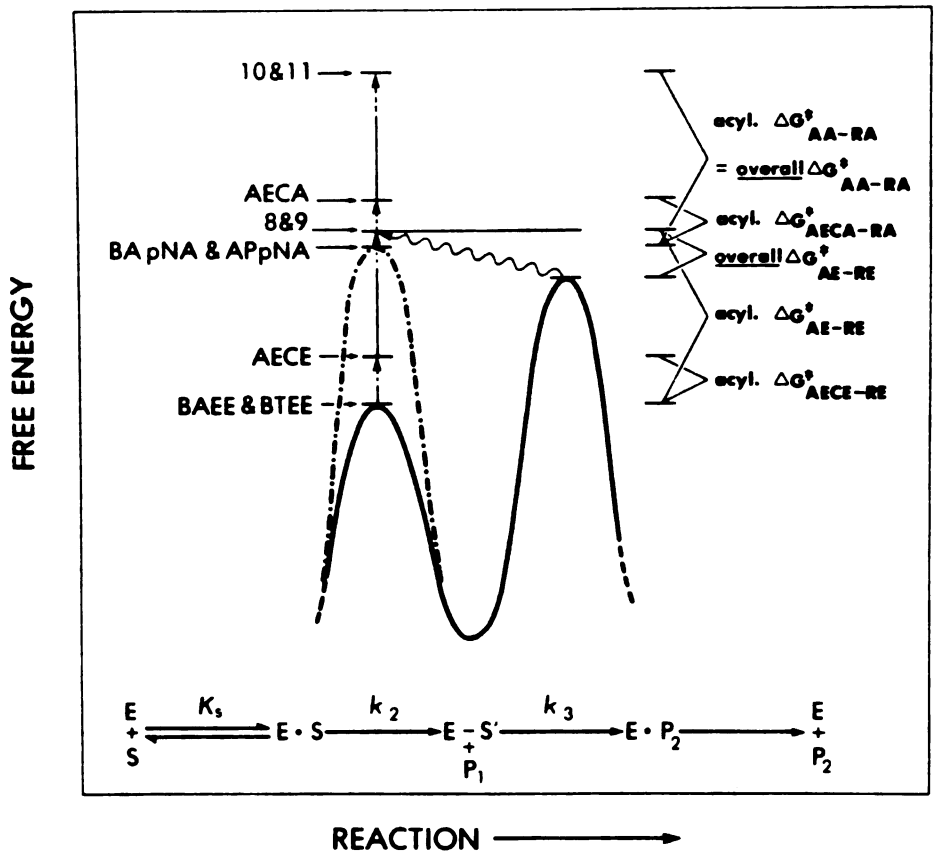

FIGURE 7: Reaction coordinate - free energy diagram showing proposed relationships of transition-state free energies of individual catalytic steps for the trypsin- and α-chymotrypsin-catalyzed hydrolyses of reference and cysteinyl β-S-derivatized analog substrates. The standard free energy values have been arbitrarily chosen on an undefined scale since they have not been quantitatively determined. Shown below is the general equation for reactions normally catalyzed by serine proteases. Tetrahedral intermediates in both acylation (with rate constant k_2) and deacylation (with rate constant k_3) have not been depicted; their omission does not affect the overall conclusions (Bender et al., 1964). AECE (kinetic data from Elmore et al., 1967) and AECA (kinetic data from Wang & Carpenter, 1968) = β-S-(β-aminoethyl)-N-α-acyl-L-cysteine esters and amides, respectively. AA and AE = analog amides (10, 11) and esters (8, 9), respectively. RA and RE = reference amides (BApNA, APpNA) and esters (BAEE, BTEE), respectively. —— = free energy profile for highly specific reference esters and —·—·— = free energy profile for highly specific reference amides. ⟨∿ is used to show that there is a change in rate-determining-step for 8 and 9 from deacylation (the rate-determining-step for reference esters, as shown) to acylation (the rate-determining-step for highly specific reference amides, as shown). See text for an explanation for the significance of the vertical arrows.

amides. The magnitude of these perturbations, however, must be equivalent for both esters and amides of the same modified acyl cysteine moieties; an equal size arrow depicts the difference in $\Delta G^{\ddagger}_{acyl.}$ for BAEE and BTEE vs. $\Delta G^{\ddagger}_{acyl.}$ for 8 and 9. In other words, 8 and 9 are equally as nonspecific as 10 and 11. This means that the relative specificity constants for the former are both misleading and meaningless. This is expected if the k_{cat} values for 8 and 9 are kinetically nonequivalent to those for highly specific ester substrates; for the latter it is known that $k_{cat} \simeq k_3$ (Bender et al., 1964). A large increase in $\Delta G^{\ddagger}_{acyl.}$ for 8 and 9 vs. for reference esters could result in a change in rate-determining step for the former from deacylation to acylation, $k_{cat} \simeq k_2$, such that k_2 for 8 and 9 is not much different than k_3 for BAEE and BTEE (as depicted in Figure 7). This interpretation is consistent with the results of the Eadie plot (Figure 8) for the tryptic hydrolysis of 8. It shows a curve of apparent substrate inhibition at high concentrations characteristically seen for amide substrates, and not a curve of presumed substrate activation (Ingles & Knowles, 1966) at high concentrations characteristically seen with less nonspecific ester substrates (Wang & Carpenter, 1968). Unfortunately, a more rigorously quantitative analysis is not possible without prior determination of the individual binding and kinetic constants for the analog substrates (Bender & Kezdy, 1965; Ingles & Knowles, 1967, 1968); this is not expected to be a simple task for these ethyl ester analog substrates with k_{cat} values of the magnitude observed (Hunkapillar et al., 1976).

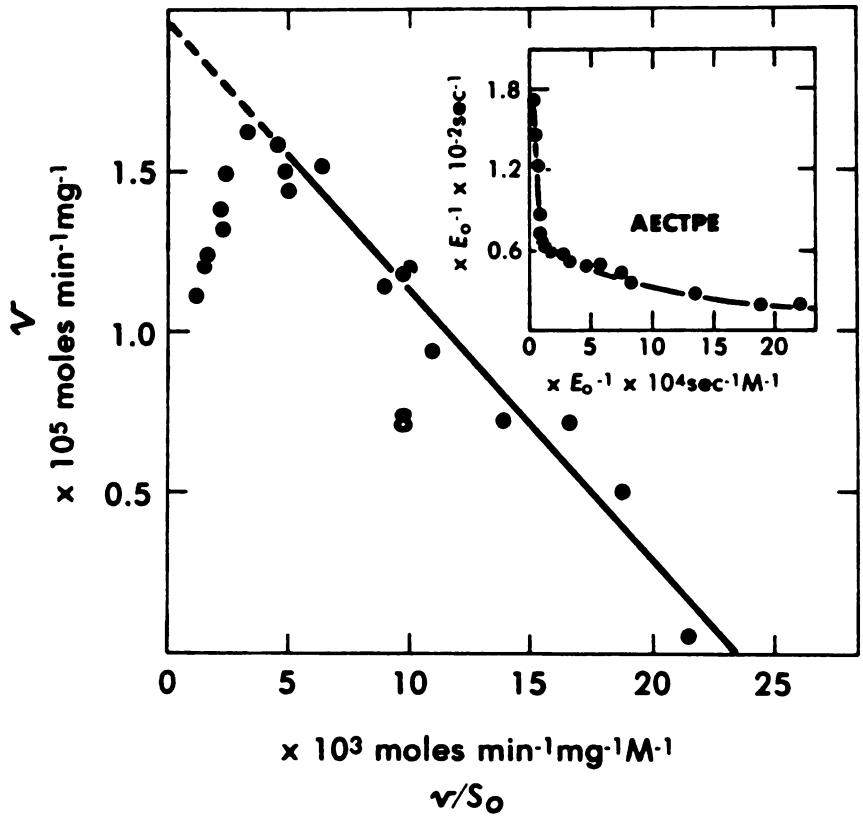

FIGURE 8: Comparison of Eadie plots for trypsin-catalyzed hyrolyses of β-S-(β-aminoethanethiol)- (8; pH-stat assay as in text) and β-S-(β-aminoethyl)- (inset; pH 8.0, 25°C, from Elmore et al., 1967) derivatized N-α-acyl-L-cysteine ester nonspecific substrates. AECTPE = β-S-(β-aminoethyl)-N-α-toluene-p-sulfonyl-L-cysteine n-propyl ester.

Rate-determining acylation has been observed previously only for a few non-specific ester substrates with very small side-chains (Jencks, 1969; Zerner & Bender, 1964), because of which they are lacking a defined three-point locus, a postulated requirement for specificity (Ingles & Knowles, 1967, 1968). This is the first report, to our knowledge, of acyl amino acid ester substrates with large side-chains for which acylation appears to be rate-determining. Both the existence of such substrates and mechanistic rationale for their kinetic behavior are implied in a recent formulation of the mechanism of serine proteases which does not invoke the enigmatic charge-relay system. Herein it was predicted that certain substrates--we propose 8-11 and perhaps 14 and 15--could exist which would be so constituted as to produce such stabilized tetrahedral intermediates in acylation that they should accumulate; their formation would be faster than their decomposition to the serine ester plus leaving group (E-S' + P; Fig. 7) which would be overall rate-determining (Bachovchin & Roberts, 1978). Hence, there would be a change in rate-determining-step for esters of this type (8 and 9) from the deacylation step that is rate-determining for other ester substrates to acylation. It is reasonable to assume that the combination of abnormally large and disulfide-containing side-chains creates severe constraints on enzyme-substrate conformations during acylation (Fink & Wildi, 1974; Schultz et al., 1977), such that those which presumably are important in the initial stabilization of tetrahedral intermediates in acylation are allowed, but the ones that facilitate their subsequent decomposition are hindered (Deslongchamps, 1975;

Hunkapiller et al., 1976; Bachovchin & Roberts, 1978; Lehn & Wipff, 1980).

APPENDIX

THIN LAYER CHROMATOGRAPHY OF SYNTHETIC PRODUCTS OF CHAPTER 2

LEGEND TO TABLE II

[a]Silica gel TLC sheets with fluorescent indicator (Eastman 6060) were used. Visualization of TLC was achieved always by fluorescence quenching and/or reaction with I_2 vapor and, where appropriate, also by sprays of ninhydrin (0.3% in ethanol), DTNB (standard assay stock solution) and 6N KOH. All chromatographic mixed solvent systems are given in ratios by volume: A, ethanol:ligroin:$CHCl_3$ (2:1:2); B, ethanol:$CHCl_3$ (2:3); C, ethanol:ethyl acetate (1:4); D, $CHCl_3$:ethanol (3:7); E, benzene (neat); F, toluene (neat); G, $CHCl_3$:ethanol (2:3); H, CH_3CN: CH_3OH:10% acetic acid (2:2:1); I, ethyl acetate:acetone (8:1); J, ethanol:ligroin:$CHCl_3$(2:2:1); K, ethanol:ethyl acetate (1:4); L, ethanol:$CHCl_3$ (9:1); M, CH_3OH:H_2O (9:1); N, acetone:CH_3OH:H_2O (2:2:1). A single spot with the indicated R_f was obtained for all compounds shown and + denotes reaction and - no reaction with the indicated reagent.

TABLE II: Results of TLC and Chemical Analyses of Products[a]

Product	R_f	Solvent system	DTNB	Ninhydrin	KOH
2 ~	0.34	A		+	
	0.41	B		+	
	0.23	C		+	
3 ~	0.79	D			
	0.64	E			
	0.61	F			
5 ~	0.46	G	-	+	+
	0.76	H	-	+	+
	0.32	I	-	+	+
6 ~	0.81	G	-	-	+
	0.85	H	-	-	+
	0.61	I	-	-	+
7 ~	0.80	G	-	+	+
	0.81	H	-	+	+
	0.57	I	-	+	+
8 ~	0.48	J		+	
	0.51	B		+	
	0.16	K		+	
9 ~	0.81	L			
	0.90	B			
10 ~	0.69	M		+	+
	0.77	N		+	+
11 ~	0.86	G			+
	0.88	H			+
	0.61	I			+
	0.28	E			+

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PART II

ISOTOPE PROBES OF THE MECHANISM OF THYMIDYLATE SYNTHETASE

INTRODUCTION

Thymidylate synthetase (EC 2.1.1.45) catalyzes the conversion of 2'-deoxyuridylate (dUMP) to 2'-deoxythymidylate (dTMP) by reductive methylation using (6R)-5,10-methylene-5,6,7,8-tetrahydropteroyloligo-L-glutamates (CH_2-H_4PteGlu$_n$, where n = ca. 3-7 depending on the organism) as the source both of one-carbon units and hydride equivalents; 7,8-dihydropteroyloligoglutamates (H_2PteGlu$_n$) are formed concomitantly (Figure 1). This reaction is unique in its mode of utilization of CH_2-H_4PteGlu$_n$; with other enzymic reactions employing reduced folate cofactors for carbon transfer, H_4PteGlu$_n$ act as one-carbon unit carriers and are regenerated unchanged (Friedkin, 1973). The monoglutamate form of the cofactor, CH_2-H_4folate, may be substituted in the reaction *in vitro*, and since most of the current understanding of the enzymic reaction comes from work using this cofactor it is customary to refer to it in discussions of the mechanism. Since there is a limited amount of CH_2-H_4PteGlu$_n$ within a cell, continuous synthesis of dTMP is maintained *via* sequential reduction and hydroxymethyl transfer reactions upon the H_2PteGlu$_n$ formed in the dTMP synthetase reaction. Thus, there are three enzyme-catalyzed reactions which function in concert for the continuous production of dTMP and regeneration of CH_2-H_4folate, and they may be referred to as the "dTMP synthesis cycle" (Figure 2; Huennekens *et al.*, 1963).

Thymidylate synthetase is the sole *de novo* source of dTMP and any interruption of the "dTMP synthesis cycle" results in a loss of cell (Cohen, 1971) or whole organism viability (Friedkin, 1973). This occurs in a manner closely analogous to the characteristic mode of "thymineless

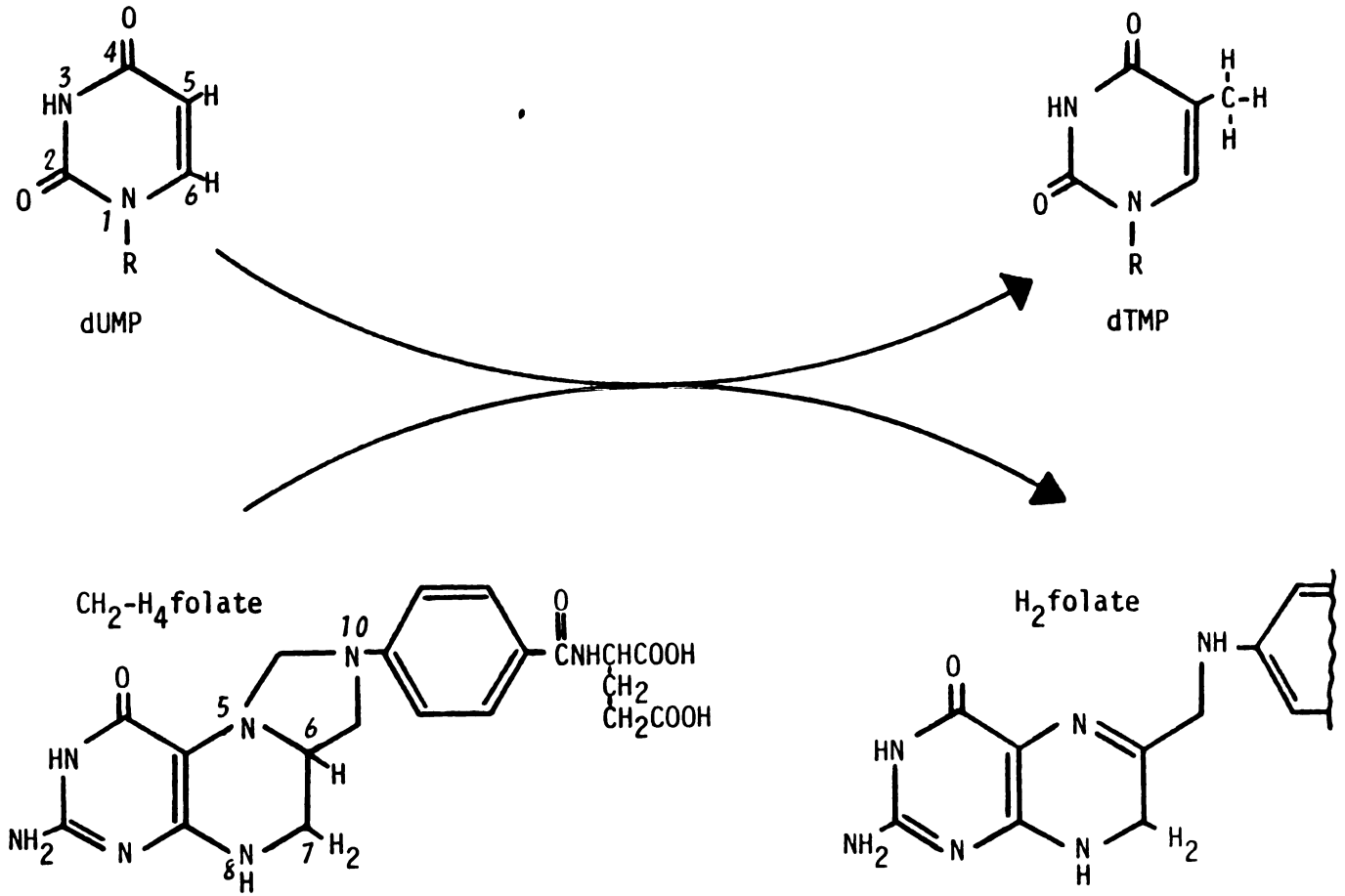

FIGURE 1: Thymidylate synthetase catalyzed conversion of dUMP to dTMP using CH_2-H_4folate as cofactor. R = 5'-phospho-2'-deoxy-β-ribofuranose.

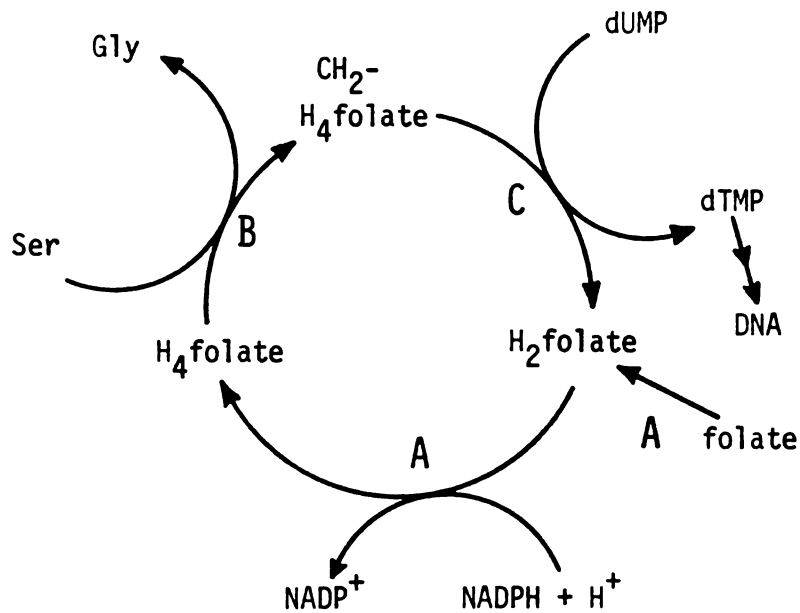

FIGURE 2: dTMP synthesis cycle. Enzymes involved are (A) dihydrofolate reductase, (B) serine hydroxymethyl transferase, and (C) thymidylate synthetase.

death" (Cohen, 1971) of bacteria when deprived of essential thymine precursor (Garrett, 1979). Salvage pathways for degradation products are insufficient to promote cell proliferation or even to maintain cell viability upon continued absence of de novo dTMP generation (Friedkin, 1973). It is not surprising, therefore, that enzymes of the dTMP synthetase cycle are important targets of cytotoxic agents used in cancer chemotherapy. As examples, 5-fluoro-2'-deoxyuridylate (FdUMP), a metabolite of 5-fluorouracil, is a potent inhibitor of thymidylate synthetase and directly inhibits the formation of dTMP, and inhibitors of dihydrofolate reductase such as trimethoprim and methotrexate prevent regeneration of H_4folate from H_2folate and deplete the cell of CH_2-H_4-folate necessary for dTMP synthesis.

The second part of this thesis is concerned with several features of the mechanism of thymidylate synthetase. Much, if not most of what is now known of the mechanism of this enzyme (for a review see Pogolotti & Santi, 1977) comes from diverse studies of interactions with nucleotide and/or cofactor analogs and inhibitors; foremost among these has been FdUMP. The studies reported here combine the approaches of using FdUMP and isotopic probes to ferret out salient aspects of the enzyme mechanism which would not easily be obtained from the normal catalyzed reaction using other more traditional enzymological methods. The isotope probes employed are 1) secondary α-hydrogen isotope effects, 2) isotope exchange, and 3) isotope-trapping or partitioning. The utilization of such methods to aid in the elucidation of enzyme mechanisms began in the early 1960's and is ever burgeoning and increasingly

important for this purpose; excellent reviews on isotopic probes in enzyme studies recently have been published (Purich, 1980). However, most applications of these tools have been for enzyme catalyzed reactions, and thus we have had to modify the methodology where appropriate for use in investigating the non-catalytic (i.e., there is no turnover of enzyme), enzyme-facilitated partial reaction with FdUMP. The simplicity of this partial reaction and its complete analogy (as far as is known) to the first part of the normal reaction with dUMP suggested it would be well suited for isotope probe analyses. Although the goal of these studies is a better understanding of the exact enzymatic mechanism of thymidylate synthetase, it reasonably can be expected that the isotope probe approaches and methodology delineated here can be applied to other enzyme systems (Bruice et al., 1980). The knowledge gained also may facilitate the design of new, clinically more efficacious drugs and/or drug regimens (Santi, 1980).

CHAPTER 1

ON THE MECHANISM OF THYMIDYLATE SYNTHETASE: BACKGROUND

It was recognized some time ago that a minimal mechanism for thymidylate synthetase must involve at least two steps: transfer of the one-carbon unit of the cofactor to the pyrimidine base with subsequent reduction to the 5-methyl moiety (Friedkin, 1959; Figure 3). That the cofactor serves as the reductant has been shown in a number of laboratories by experiments demonstrating that tritium of CH_2-[6-^{3}H]H_4-folate is transferred to the methyl group of dTMP (Pastore & Friedkin, 1962; Blakley et al., 1963; Lorenson et al., 1967). Later, Lomax and Greenberg (1967) observed that in the presence of CH_2-H_4folate the E. coli enzyme catalyzes exchange of the hydrogen at the 5-position of dUMP for protons of solvent. This reaction represents an electrophilic substitution and, in all likelihood, is mechanistically related to the first step of the overall reaction.

Fundamental to understanding the mechanism of an enzyme is a thorough knowledge of organic reaction mechanisms of chemical counterparts. Fairly comprehensive model studies have been performed in which chemical analogies have been constructed for the two basic steps of the enzymic reaction. In summary, model reactions of both electrophilic substitution at the 5-position of uracil derivatives (viz. alkylation by CH_2-H_4folate and 5-H exchange; Kalman, 1971; Hayatsu, 1976; Pogolotti

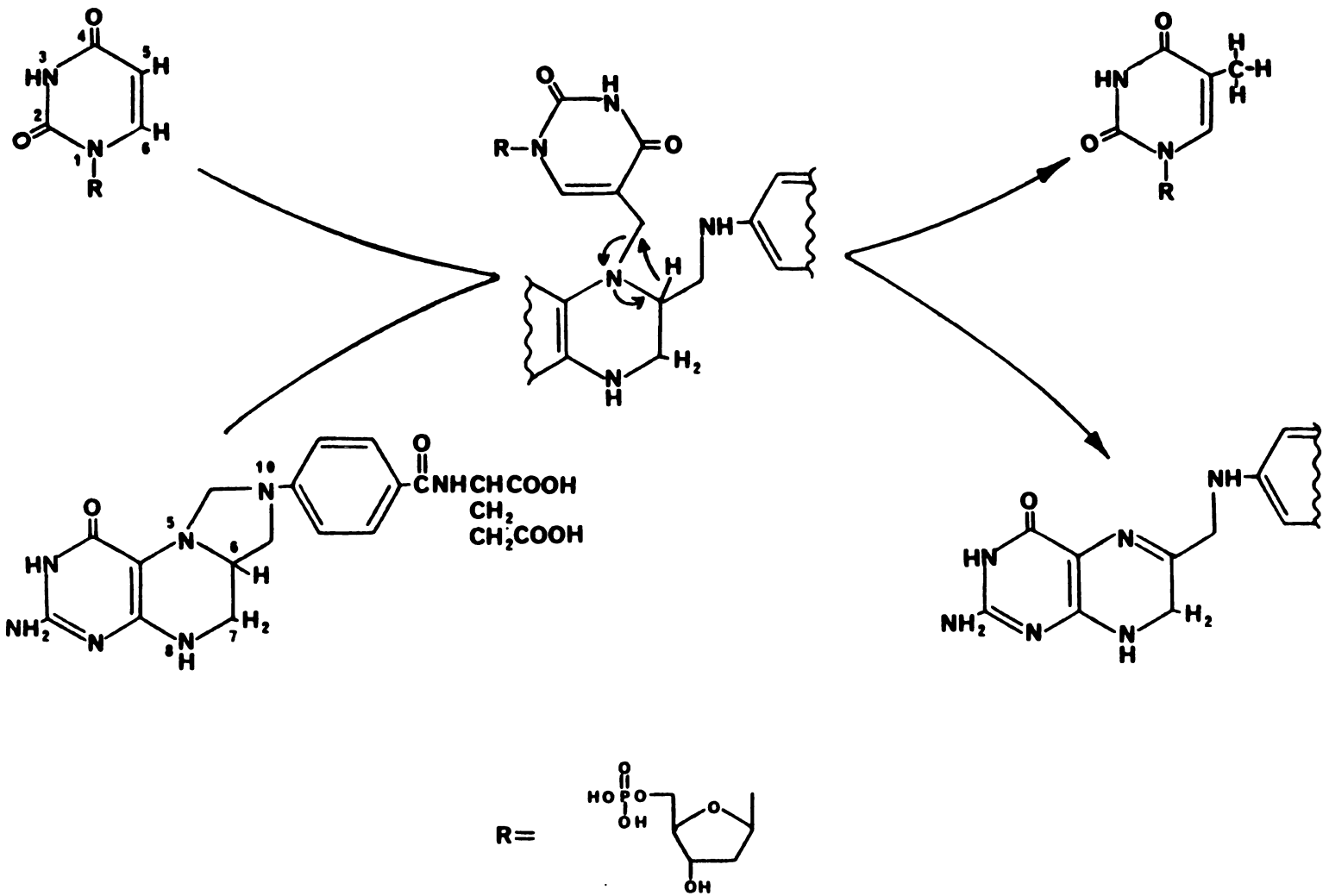

FIGURE 3: Minimal mechanism for dTMP synthetase proposed by Friedkin in 1959.

& Santi, 1977) and nucleophilic substution at the 5-carbon of thyminyl derivatives (viz. hydride transfer from C-6 of H_4folate to the incipient methyl group of dTMP; Pogolotti & Santi, 1974) are greatly facilitated by addition of a nucleophile to the 6-position of the pyrimidine heterocycle.

The conclusions of these model studies were extrapolated to propose the first chemically reasonable minimal mechanism for the thymidylate synthetase reaction, which is depicted in Figure 4 (Pogolotti & Santi, 1974). The reaction is initiated by attack of an enzyme nucleophile at the 6-position of the pyrimidine heterocycle to give the reactive enolate $\underset{\sim}{1}$. Subsequent electrophilic substitution of the reactive iminium ion form of the cofactor at the activated 5-position of the substrate gives a covalent, ternary structure $\underset{\sim}{2}$ in which the 5,6-bond is saturated. Abstraction of the 5-H yields the enolate $\underset{\sim}{3}$ which is poised for a β-elimination to produce the highly reactive exocyclic methylene intermediate $\underset{\sim}{4}$ and H_4folate, bound to the enzyme non-covalently in close proximity. Intermolecular hydride transfer from the C-6 of H_4folate to $\underset{\sim}{4}$, followed by β-elimination yields dTMP, H_2folate and native enzyme.

Of course, the validity of the conclusions derived from chemical studies depends on the demonstration that intermediates and conversions proposed do, in fact, occur in the enzymatic reaction. Most of the work confirming the salient aspects of the mechanism depicted in Figure 4 has been relatively recent, subsequent to the finding that the methotrexate resistant strains of Lactobacillus casei are rich sources of a stable enzyme by virtue of gene amplification (Crusberg

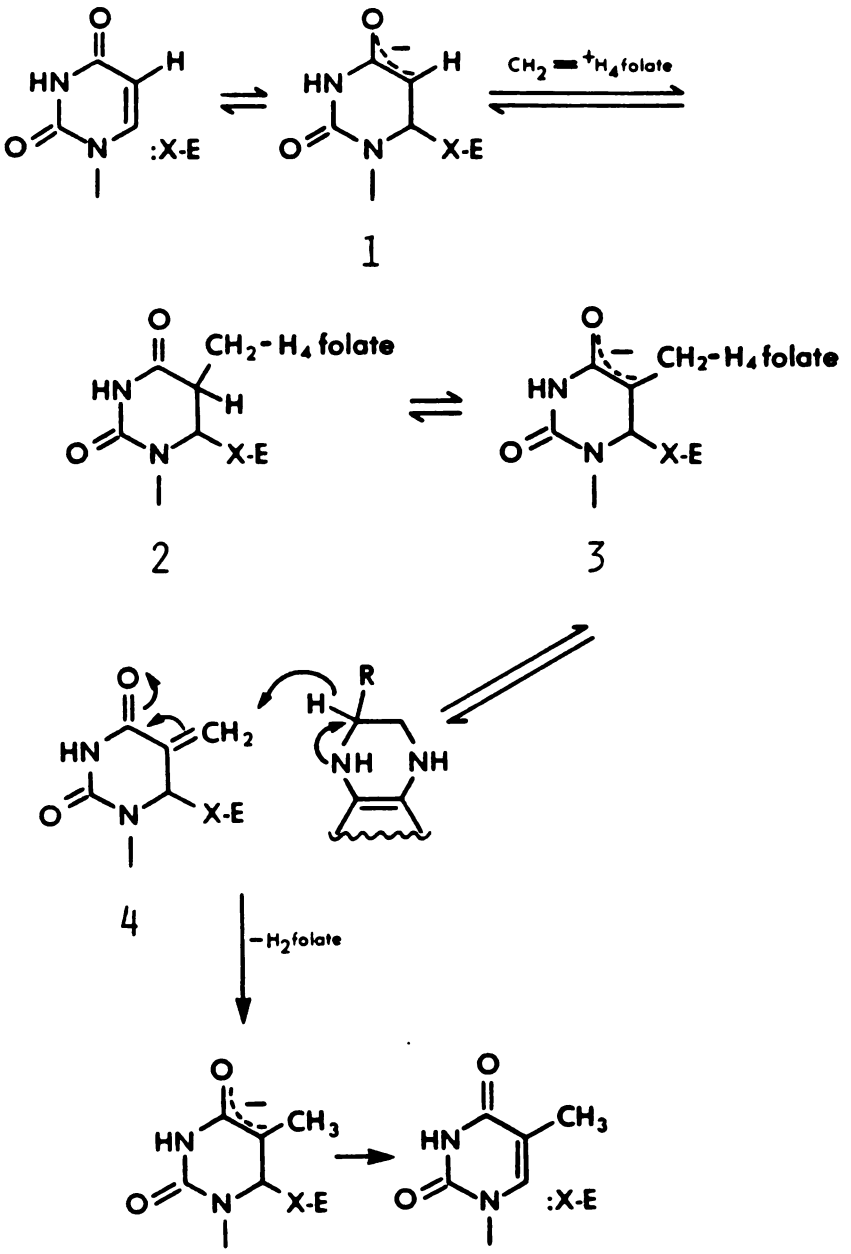

FIGURE 4: Mechanism proposed for the thymidylate synthetase reaction based on model studies.

et al., 1970; Dunlap et al., 1971). This enzyme can be readily purified. It also has been crystallized to afford homogeneous enzyme (Koeppe et al., 1975; Wataya & Santi, 1977a). It has a MW of 70,000 and consists of two 35,000 MW subunits which have identical primary structures (Maley et al., 1979; Bellisario et al., 1979). The enzyme exists in at least two interconvertible forms: the oxidized form (E_O) is catalytically inactive but can be converted to the active form (E_R) upon treatment with thiols, presumably via reduction of disulfides to provide essential sulfhydryl group(s); there are four sulfhydryls/dimer in the E_R form.

Direct enzymic support for major aspects of the originally proposed mechanism of thymidylate synthetase was first obtained from studies of its interaction with FdUMP. It has been known for some time that FdUMP is a potent inhibitor of thymidylate synthetase (Heidelberger et al., 1960; Blakley, 1969), but the nature and mechanism of inhibition was a topic of considerable controversy. In 1972 it was demonstrated (Santi & McHenry, 1972) that the dimeric enzyme from L. casei interacts with FdUMP in a reaction that is dependent on the presence of CH_2-H_4folate. That a covalent complex likely was formed was first deduced from indirect evidence using the native enzyme (Santi & McHenry, 1972; Santi et al., 1974b; Langenbach et al., 1972; Danenberg et al., 1974), and from what was known regarding model chemical systems.

Several lines of evidence later conclusively demonstrated that a reversible covalent bond is formed between FdUMP and the enzyme within

the complex. (1) From studies of the relative rates of association and dissociation of FdUMP, the dissociation constant of the nucleotide in the ternary complex was calculated to be ca. 10^{-12} to -13 M (Santi et al., 1974b). (2) The enzyme—FdUMP—CH_2-H_4folate complex can be treated with a number of protein denaturants (urea, guanidine hydrochloride, etc.) without apparent dissociation of protein-bound ligands (Danenberg et al., 1974; Santi & McHenry, 1972; Santi et al., 1974b). With few exceptions, such treatment is sufficient to disrupt noncovalent interactions between low molecular weight ligands and their protein receptors. (3) Although denaturation of the enzyme yields stable protein-bound ligands, ligands bound to the native complex slowly dissociate unchanged (Lam et al., 1976; Santi et al., 1974b). Thus, dissociation of the stable complex requires catalytic integrity of the enzyme. (4) Upon formation of the ternary complex there is a decrease of absorbance at 269 nm which corresponds to stoichiometric loss of the pyrimidine chromophore of FdUMP (Danenberg et al., 1974; Santi et al., 1974b). This result strongly suggests that the 5,6-double bond of the pyrimidine has become saturated when FdUMP is bound in the complex. (5) In a preliminary study, dissociation of [6-^{3}H]FdUMP from the complex was reported to be accompanied by a secondary α-tritium kinetic isotope effect (k_H/k_T) of 1.23 (Santi et al., 1974b). This would correspond to a deuterium isotope effect of 1.15 and demonstrates that the 6-carbon of the heterocycle undergoes sp^3 to sp^2 rehybridization during this reaction, as required if the 5,6-bond of FdUMP is saturated in the

complex. (6) Proteolytic digestion of the complex yields an active site peptide that is covalently bound to FdUMP and CH_2-H_4folate (Sommer & Santi, 1974; Pogolotti et al., 1976). The ^{19}F nuclear magnetic resonance spectrum of the fragment is consistent with the C-F bond being flanked by CH and CH_2 groups (James et al., 1976).

From this evidence, the structure of the enzyme—FdUMP—CH_2-H_4folate complex currently is believed to be as depicted in Figure 5. As shown, a nucleophile of the enzyme is covalently bound to the 6-position of FdUMP and the 5-position of FdUMP is covalently bound to the methylene group of the cofactor. The nucleophile which attacks the 6-position of FdUMP is most likely the thiol of Cys, although Thr is still a possibility (Pogolotti et al., 1976; Bellisario et al., 1979; Maley et al., 1979).

The mechanism of inhibition of FdUMP is now understood in some detail. Comparing structure 2 of Figure 4 with Figure 5, it can be seen that FdUMP enters into conversions until it forms an intermediate analogous to 2 (Figure 4) in the normal enzyme reaction, excepting that a fluorine rather than hydrogen is at the 5-position. The subsequent conversion of 2 to 3 involves proton abstraction which, of course, is not possible with the fluorinated intermediate. Thus, FdUMP behaves as a quasi-substrate and accumulates as a stable analog of a covalently-bound steady-state intermediate of the normal enzymic reaction. Because of the stability and spectral properties of the active site peptide possessing FdUMP and CH_2-H_4folate (Sommer & Santi, 1974; Pogolotti et al., 1976), it was concluded that the cofactor was attached via its

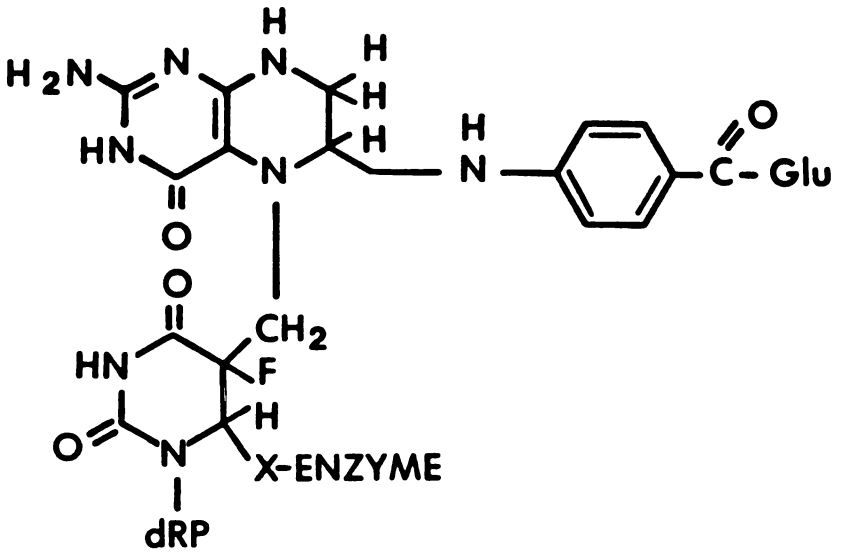

FIGURE 5: Structure of the covalent FdUMP—CH_2-H_4folate—dTMP synthetase complex. dRP = 5'-phospho-2'-deoxy-β-ribofuranose.

5-nitrogen. This disqualifies free formaldehyde as an intermediate in the reaction and implicates the 5-iminium cation (5-CH_2=$^+H_4$folate) as the reactive intermediate (Kallen & Jencks, 1966); this is in complete accord with recent studies by Tatum et al., (1977) who demonstrated that the methyl group of dTMP is formed with essentially complete retention of stereochemistry using pro-chiral CH_2-H_4folate. In addition, preliminary ^{19}F-nmr studies of the peptide (James et al., 1976) indicate that the enzyme and cofactor add across the 5,6-C=C of FdUMP in a trans-fashion.

In a more recent ^{19}F-nmr refinement (Byrd et al., 1978) it was deduced that in the complex formed with the native enzyme, the enzyme nucleophile at C-6 and the methylene group at C-5 of the pyrimidine are in a trans-diaxial structure, but that upon denaturation there is a subtle conformational change of the heterocycle to a trans-diequatorial conformation; presumably the latter is true also for the peptide complex. Likely, these stereochemical features of the FdUMP complex relate to the analogous study-state intermediate for the enzymatic reaction and, if so, the stereochemistry of many intermediates of this reaction may be deduced.

Much also is now known of other aspects of the interaction of FdUMP, CH_2-H_4folate and thymidylate synthetase. Equilibrium dialysis studies (Galivan et al., 1976) have shown that FdUMP forms a reversible, noncovalent binary complex with thymidylate synthetase with a K_d value of ca. 10^{-5}M; no binding of CH_2-H_4folate in the absence of a nucleotide was observed. These results and studies of heat stability

(Lorenson et al., 1967), initial velocity, product inhibition and competitive inhibition kinetics (Lorenson et al., 1967; Dolnick & Cheng, 1977; Daron & Aull, 1978; Danenberg & Danenberg, 1978) and kinetics of ternary complex breakdown (Danenberg & Danenberg, 1978) generally have been interpreted as evidence for an ordered, sequential binding; nucleotide binds first while cofactor obligatorily binds second. The validity of the results of at least some of these studies is in question. Upon binding of both ligands, a reversible, noncovalent ternary complex is formed in which the K_d of FdUMP has been estimated to be ca. 10^{-8}M (Wataya et al., 1977). It may be surmised from available data that one or more intermediates are formed subsequent to this complex. One of these must be a result of conversion of CH_2-H_4folate to the 5-iminium ion 5-CH_2=$^+H_4$folate (Tatum et al., 1977; Pogolotti et al., 1976). Also, there is substantial evidence (Sharma & Kisliuk, 1973; Danenberg et al., 1974; Galivan et al., 1975; Donato et al., 1976; Santi et al., 1976a; Kisliuk et al., 1976; Dolnick & Cheng, 1977, 1978; Lockshin & Danenberg, 1980) for the existence of a large conformational change either concomitant with or immediately after formation of the noncovalent complex and prior to the first covalent bond forming steps.

The final product which accumulates has both FdUMP and CH_2-H_4folate covalently bound to the enzyme. The dissociation constant of FdUMP from the complex has been estimated by kinetic experiments to be 10^{-12} to 10^{-13}M. Although for convenience the covalent complex is often referred to as a ternary complex, it clearly has been established that two equiva-

lents of FdUMP and CH_2-H_4folate can bind per 70,000 MW dimer (Galivan et al., 1974; Santi et al., 1974b). Both the K_d and rate of dissociation for FdUMP in these complexes are reported to be identical for both subunits (Santi et al., 1974b; Danenberg & Danenberg, 1978, 1979). At less than saturating concentrations of FdUMP and/or CH_2-H_4folate, species have been detected and isolated wherein there is only one equivalent of both ligands per dimer (Danenberg et al., 1974; Donato et al., 1976).

Interestingly, equilibrium dialysis studies (Galivan et al., 1976) have shown that in Tris-HCl FdUMP binds to two sites with the same affinity in the absence of folates and with added methotrexate or (6S)-CH_2-H_4folate, but binds with a high and low affinity site in either Tris-HCl in the presence of H_2folate or in phosphate buffer in the presence of H_2folate or (6S)-CH_2-H_4folate. In phosphate buffer in the absence of folates only one FdUMP binding site was detected. In either Tris-HCl or phosphate buffer only one site was found for dUMP and dTMP and binding was tighter in the former. Methotrexate and H_2folate showed single site binding in the presence of dUMP. In other studies one dUMP binding site also was shown by circular dichroism (Leary et al., 1975), but a second, weaker binding site was shown calorimetrically (Beaudette, et al., 1977). Binding to a single site in the absence of nucleotides has been found for CH_2-H_4PteGlu$_4$ by equilibrium dialysis (Galivan et al., 1976b) and to at least one site for (6S)-CH_2-H_4folate by fluorescence quenching (Kisliuk et al., 1976) and is implied for pteroylpolyglutamates from protection afforded against proteolytic

inactivations (Galivan et al., 1977). The existence of two independent and nonidentical sites for various mono- and polyglutamate cofactors and analogs in the presence of dUMP has been inferred from kinetic inhibition studies (Dolnick & Cheng, 1978; Cheng et al., 1979).

The conclusions of these binding studies, though not entirely consistent, do seem to point towards a functional nonequivalence of the chemically identical subunits in the native, dimeric enzyme. Other data, some apparently in support of and some in conflict with this interpretation, comes mainly from sulfhydryl modification studies. Consistent with most FdUMP binding studies, 1.4-1.8 mol of N-ethylmaleimide, iodoacetamide or p-chloromercuribenzoate/mol dimeric enzyme (Plese & Dunlap, 1977) and 1.5-1.6 mol of methyl methanethiolsulfonate/mol dimer (Lewis et al., 1978) were incorporated concomitant with complete loss of activity and ability to form covalent complexes. However, it also has been reported that treatment of native enzyme with iodoacetamide, N-ethylmaleimide (NEM) 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 5-HgdUMP and other classical sulfhydryl group alkylating, oxidizing and complex forming reagents modifies only one essential cysteine of the four cysteines in the enzyme; complete losses both of catalytic activity and of FdUMP and dUMP binding ability were observed (Danenberg et al., 1974; Dunlap et al., 1971; Galivan et al., 1977; Leary et al., 1975). Further evidence that it is one, unique cysteine that is modified comes from nucleotide protection experiments (Galivan et al., 1977; Leary et al., 1975) and thiol reagent competition experiments (Galivan et al., 1977). Also, complete enzyme inactivation has been

observed upon release of only 1 mol of carboxy-terminus Val/mol dimer (Aull et al., 1974).

Further examination of the relationship between thiol modification and enzyme activity revealed that after iodoacetamide inactivation ca. one-half of the native enzyme activity could be restored upon addition of excess mercaptoethanol (Leary et al., 1975); this suggests a reactivation of the remaining unmodified subunit. In the most recent and informative study (Danenberg & Danenberg, 1979) of this kind the following was reported: treatment of native enzyme with NEM or DTNB gave modification of only one of the four cysteines with complete loss of activity and FdUMP binding ability. However, in the presence of excess CH_2-H_4folate and 1.0 mol FdUMP/mol dimer there was still exactly one sulfhydryl group available for modification. The extent of modification decreased to less than one proportional to an increase in FdUMP of greater than 1 mol/mol dimer. When an excess of FdUMP and CH_2-H_4folate were used, complete protection from modification was achieved. Integration of these results with many of those cited here has led to the proposal (Danenberg & Danenberg, 1979) of a ligand-induced sequential model for subunit interactions; only one site is accessible on the free enzyme until the binding of both ligands, nucleotide first and CH_2-H_4folate second, cause a conformational change that opens the second, internally identical site.

Other studies have elucidated, elaborated and confirmed key features of the mechanism of thymidylate synthetase. Recently, it has been demonstrated that L. casei thymidylate synthetase catalyzes the exchange

of the 5-H of dUMP for protons of water in the absence of CH_2-H_4folate; the cofactor does not affect V_{max} of the reaction, but K_m is decreased by about 10-fold (Pogolotti et al., 1979). This is the first direct evidence that nucleophilic attack at the 6-position of dUMP to give a 5,6-dihydropyrimidine intermediate (2 in Figure 4) is a requisite feature of a reaction catalyzed by the enzyme.

Thymidylate synthetase also catalyzes the dehalogenation of BrdUMP and IdUMP. The details of the mechanism of this reaction recently have been clarified (Garrett et al., 1979). The reaction does not require CH_2-H_4folate, but stoichiometrically consumes two equivalents of thiol. This is exactly analogous to extensively studied chemical mechanisms of dehalogenation of 5-Br- and 5-I-uracil (see reviews by Hayatsu, 1976; Sander, 1977) for which it is well established that the reactions involve nucleophilic attack at the 6-position and an oxidation (e.g. $2RSH \longrightarrow (RS)_2$). Further support for this mechanism comes from secondary isotope effect studies using [2-^{14}C,6-^{3}H]pyrimidines. The cysteine-mediated dehalogenation of [2-^{14}C,6-^{3}H]BrdUrd, which is known to proceed via initial thiol attack at C-6 (Wataya et al., 1973; Sedor et al., 1974), shows k_T/k_H=1.17 (Wataya & Santi, 1977b). Likewise, the thymidylate synthetase catalyzed dehalogenation of [2-^{14}C,6-^{3}H]BrdUMP shows k_T/k_H=1.26 (Garrett et al., 1979); this verifies the existence of a transient 5,6-dihydropyrimidine intermediate, analogous to the structure proposed for the FdUMP ternary complex, in a reaction catalyzed by the enzyme.

Considerable efforts have been devoted to the development of inhibitors of dTMP synthetase, an area which periodically has been reviewed (Friedkin, 1973; Baker, 1967; Santi, 1980). Cofactor analogs which have shown the most potent inhibitory properties include H_4-homofolate, methotrexate, 5-CHO-pteroylpentaglutamate, 5,8-deazafolate and 10-CH_3-5,8-deazafolate. Analogs of dUMP have been extensively investigated (Santi et al., 1979; Santi, 1980) and a limited QSAR analysis has been performed (Wataya et al., 1977). Among the potent nucleotide inhibitors that have been developed are AraFUMP, FdUMP, 5-trifluoromethyl-dUMP and vinylogous derivatives, 5-nitro-dUMP, 5-mercapto-dUMP and 5-formyl-dUMP. Analogs of dUMP are known which are good (a) reversible inhibitors, (b) irreversible inhibitors, and (c) quasisubstrate or "suicide substrates" for dTMP synthetase. Enzymatic and model reactions for several of these have provided further evidence supporting the involvement of nucleophilic attack at the 6-position of the pyrimidine heterocycle in reactions of the enzyme.

The goal of obtaining a better understanding of the mechanism of thymidylate synthetase is approached in this part of this thesis via a more extensive investigation of the mechanism of interaction of the enzyme with cofactor and FdUMP. Various isotope probes are utilized. Chapter 2 deals with attempts to locate the rate-determining step of the partial reaction and to order the sequence of other key steps in relation to it. Chapter 3 is concerned with a further elaboration of these aspects and with ascertaining the binding order of the ligands and hence with the identification of productive, reversible intermedi-

ates on the pathway from native enzyme to enzyme to which two equivalents each of FdUMP and CH_2-H_4folate are covalently bound.

In Chapter 4 a minimal mechanism for the interaction of dTMP synthetase with CH_2-H_4folate and FdUMP is proposed. This model can accomodate the results of the isotope probe studies of this thesis, is consistent with currently accepted features of the enzyme mechanism and can resolve much of the confusion and controversy surrounding incompletely understood aspects that have been cited in this chapter.

CHAPTER 2

SECONDARY α-HYDROGEN ISOTOPE EFFECT PROBES OF THE MECHANISM OF REACTION OF THYMIDYLATE SYNTHETASE WITH 5-FLUORO-2'-DEOXYURIDYLATE AND (6R)-5,10-METHYLENE-5,6,7,8-TETRAHYDROPTEROYL-MONO- AND PENTA-L-GLUTAMATE

ABSTRACT

Thymidylate (dTMP) synthetase facilitates a partial reaction with 5-fluoro-2'-deoxyuridylate (FdUMP) and 5,10-methylene-5,6,7,8-tetrahydropteroylpolyglutamates (CH_2-H_4PteGlu$_n$; where n is ca. 1-7) which parallels the normal enzymic reaction with 2'-deoxyuridylate to the point where a stable, covalent, ternary complex analog of a steady-state intermediate on the normal reaction pathway accumulates. The dissociation of the complex formed with [2-^{14}C,6-^{3}H]FdUMP and CH_2-H_4folate (n=1) or CH_2-H_4PteGlu$_5$ has been found to proceed with a substantial secondary α-tritium kinetic isotope effect (k_H/k_T = 1.229 and 1.211, respectively), but the rate with the latter was some 6-fold slower; no isotope effect was observed upon formation of the complex. The equilibrium isotope effect for the same complexes was identical (K_T/K_H = 1.240) within experimental error. The magnitudes of these isotope effects provides direct proof that the complex formed is a 5,6-dihydropyrimidine, analogous to what recently has been shown for reactions catalyzed by the enzyme. Comparison of this isotope effect data also leads to the conclusion that the last covalent bond change, the cleavage of the bond between the enzyme nucleophile and FdUMP, occurs as a pre-equilibrium step prior to the rate-determining step in the direction of dissociation . The lack of a significant secondary α-tritium kinetic isotope effect (k_H/k_T = 1.004-1.033) for dissociation of complexes formed with [^{3}H]CH_2-[2-^{14}C]H_4folate leads us to conclude that the expected large isotope effects for conversions of the sp^3 one-carbon unit in the complex to an sp^2 iminium ion and of the iminium ion to sp^3 CH_2-H_4folate cancel as both steps are fully pre-equilibrium. A minimal model is presented which places the rate-determining step at binding/release of ligands and/or at a subsequent conformational change of the enzyme and the cofactor in the association direction; this change also may occur upon binding/release of ligands and is suggested to result in conversion of CH_2-H_4folate to 5-$CH_2{=}^{+}H_4$folate, the reactive species in the reaction.

In the presence of an appropriate reduced folate cofactor, dTMP[1] synthetase facilitates a reversible partial reaction with the quasi-substrate inhibitor FdUMP as shown in Figure 6. Considerable data (Santi et al., 1974a,b; Danenberg et al., 1974; Santi et al., 1976 a,b; Pogolotti & Santi, 1974, 1977) suggests that the steps of this reaction parallel those with dUMP to the point where a stable, covalent analog of a steady-state intermediate on the normal catalytic reaction pathway accumulates. In fact, studies of this interaction have been a principle source of information pertaining to the mechanism of this enzyme. In continuing this line of investigation, here is described the results of a secondary α-hydrogen isotope effect analysis of the reaction of FdUMP, the mono- and penta-Glu forms of the cofactor and dTMP synthetase from Lactobacillus casei. The observation of large secondary α-tritium

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[1]Abbreviations used are as follows: HPLC, high performance liquid chromatography; NMM, N-methylmorpholine; dUMP, 2'-deoxyuridylate; dTMP, 2'-deoxythymidylate; FdUrd, 5-fluoro-2'-deoxyuridine; FdUMP, 5-fluoro-2'-deoxyuridylate; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; CH_2-H_4folate, 5,10-methylene-H_4folate; CH_2-$H_4PteGlu_5$, 5,10-methylene-5,6,7,8-tetrahydropteroyl-pentaglutamate. The glutamate residue(s) of all folates have the L (S) configuration. The stereochemistry at C-6 of H_4folates utilizes the R,S-designation now that the absolute stereochemistry at this carbon is known (Fontecilla-Camps et al., 1979); the naturally occurring (+) isomer of CH_2-H_4folate is designated as 6R.

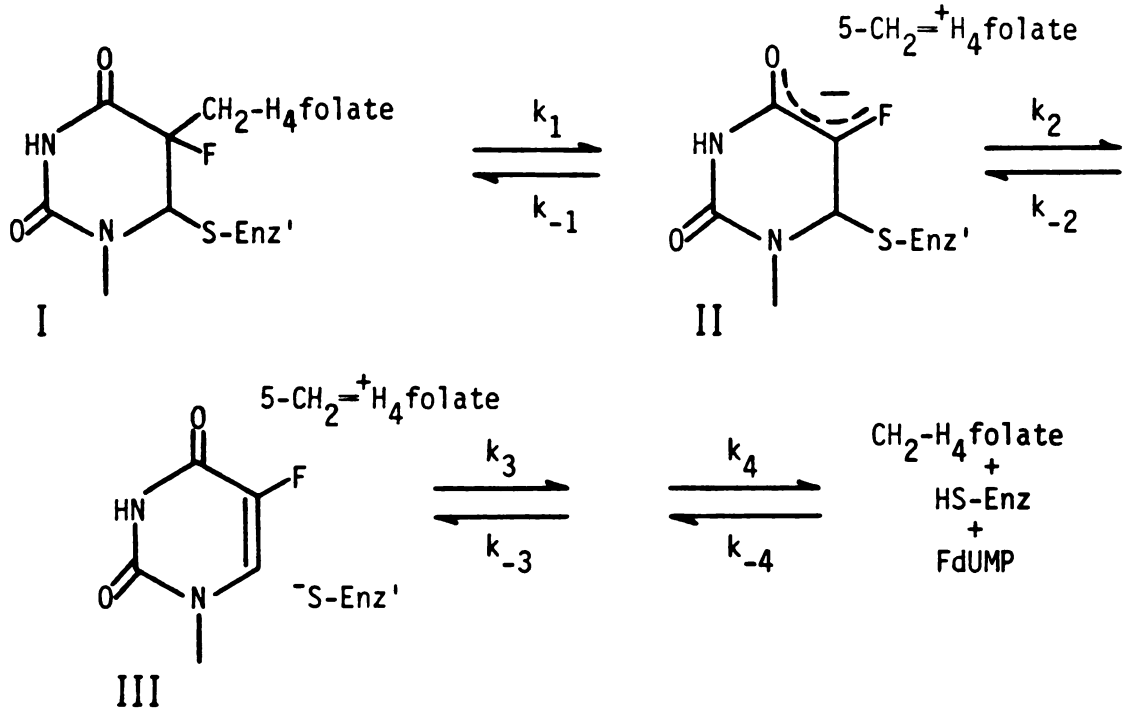

FIGURE 6: Proposed minimal mechanism for dissociation/association of the covalent FdUMP—CH_2-H_4folate—dTMP synthetase complex.

kinetic[2] and equilibrium isotope effects provides conclusive evidence that the complex formed is a 5,6-dihydropyrimidine; attack of an enzyme nucleophile at the C-6 position of the pyrimidine heterocycle serves to "activate" the neighboring C-5 position for subsequent electrophilic attack by the reactive form of the cofactor. These salient mechanistic aspects long have been proposed for extensively studied chemical counterparts (Pogolotti & Santi, 1977) and recently also have been demonstrated for several reactions catalyzed by dTMP synthetase (Pogolotti et al.,1979; Garrett et al., 1979; Bruice et al., 1980). In addition, from examination of the various isotope effects observed, the rate-determining step of the partial reaction has been partially localized and the positions of steps involving carbon rehybridizing, covalent bond changes of FdUMP and CH_2-H_4PteGlu$_{1-5}$ in relation to this step have been assigned.

EXPERIMENTAL PROCEDURES

Materials. dTMP synthetase from methotrexate-resistant Lactobacillus casei (Crusberg et al., 1970) was purified by a modification (Wataya and Santi, 1977a) of the method of Galivan et al. (1975). The preparation catalyzed the formation of 4 μmol dTMP/min/mg as determined spectrophotometrically (Wahba & Friedkin, 1962) using conditions previously described (Santi & Sakai, 1971). The concentration of FdUMP

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[2]The first observation of a significant secondary α-hydrogen isotope effect in a dTMP synthetase reaction was reported by Santi and coworkers (Santi et al., 1974b) using [6-^{3}H,2-^{14}C]FdUMP.

binding sites was calculated from the equivalence point determined upon spectrophotometric titration of the enzyme with FdUMP in the presence of CH_2-H_4folate as previously described (Garrett et al., 1979). Using $\varepsilon_{278} = 1.07 \times 10^5$ M^{-1} cm^{-1} (Santi et al.,1974b), and a molecular weight of 70,000 for the enzyme, the preparations used here were calculated to possess 80 to 90% of the theoretical FdUMP binding sites. Purified H_2folate reductase from Escherichia coli RT500 (120 units/mg) was a gift from Dr. David P. Baccanari (Burroughs Wellcome Co.) and was assayed by a reported method (Baccanari et al., 1975). G-6-P dehydrogenase from Leuconostoc mesenteroides (695 NAD units/mg) was purchased from Worthington and was assayed by the method described in their technical bulletin. H_2folate was prepared by the method of Futterman (1957). (6R,S),L-H_4folate was prepared by reduction of folate (Hatefi et al., 1960) and (6R)-CH_2-H_4Pte-L-Glu_5 was a gift from Professor Jesse Rabinowitz (University of California, Berkeley). FdUrd was obtained from P. L. Biochemicals and was converted to FdUMP using Escherichia coli dThd kinase as previously described (Wataya and Santi, 1977a); the latter was purified by chromatography on DEAE-cellulose (Wataya and Santi, 1977a) and Aminex A-27 (Garrett et al., 1979). Concentrations of FdUMP were determined using $\varepsilon_{269}^{pH\ 2} = 8950$. N-Methylmorpholine (NMM) was Pierce Chem. Co. Sequanal grade. All other reagents were of the highest purity commercially available. Doubly-distilled, charcoal and millipore-filtered water was used throughout. The standard NMM buffer contained 50 mM NMM·HCl (pH 7.4), 25 mM $MgCl_2$, 1 mM EDTA, 6.5 mM H_2CO and 75 mM 2-mercaptoethanol.

[2-^{14}C]Folate (55 mCi/mmol) was obtained from Amersham. [^{3}H]H_2CO (85 mCi/mmol) was obtained from New England Nuclear. [2-^{14}C]FdUrd (52 mCi/mmol) and [6-^{3}H]FdUrd (15 Ci/mmol) were obtained from Moravek Biochemicals. Radioactive FdUMP was prepared and purified as described for the unlabeled nucleotide.

High Pressure Liquid Chromatography. The purity of nucleotides and folate derivatives used in this work was verified by HPLC analysis shortly before their use. Reverse phase HPLC was performed at ambient temperature using a Lichrosorb C_{18} column (4.6 x 250 mm). System A contained 5 mM $(n\text{-}Bu)_4N^+$ phosphate (pH 7.5) and 30% (V/V) MeOH; system B contained 0.1 M potassium phosphate (pH 6.0) and 5% (V/V) acetonitrile.

Preparation of Labeled and Unlabeled CH_2-H_4Folates. (6R),L-CH_2-H_4folate was prepared by a modification of the method of Pastore and Friedkin (1962). The reaction mixture (4.8 ml) contained 300 mM Tris·HCl (pH 7.2), 21 mM H_2folate, 42 mM H_2CO, 160 mM 2-mercaptoethanol, 61 mM G-6-P, 2.3 mM NADPH, 21 units/ml G-6-P dehydrogenase and 6.4 units/ml H_2folate reductase; the reaction was initiated with H_2folate reductase. After 30 min at ambient temperature, under argon and protected from light, there was no further increase in A_{340}; the A_{294}/A_{340} had changed from ca. 3 to 18. The reaction was cooled to 0°, quenched by adjusting the pH to 9.5 with NaOH or 10 vols of 0.4 M $Et_3N^+H\cdot HCO_3^-$ (pH 9.5) and was lyophilized. The residue was redissolved in a minimal volume (ca. 0.5 ml) of 0.4 M $Et_3N^+H\cdot HCO_3^-$ (pH 9.5), 20 mM 2-mercaptoethanol and 1 mM H_2CO, and insolubles were removed by centrifugation.

The solution was applied to a Biogel P2 column (1.4 x 73 cm; 200-400 mesh) previously equilibrated with 20 mM $Et_3N^+H \cdot HCO_3^-$ (pH 9.5), 20 mM 2-mercaptoethanol and 1 mM H_2CO. The column was eluted with the same buffer at 15 ml/hr, at 4°, protected from light and 2 ml fractions were collected. Fractions 17 to 23 contained (6R),L-CH_2-H_4folate as assayed with dTMP synthetase; these were pooled, lyophilized and dissolved in 20 mM $Et_3N^+H \cdot HCO_3^-$ (pH 9.5), 20 mM 2-mercaptoethanol and a 2-fold excess of H_2CO. Typical overall yields were 80-95% of (6R),L-CH_2-H_4folate of 95% purity as determined by A_{294}, HPLC (system A, RV = 8.2 ml)and dTMP synthetase assay. Solutions could be stored under argon at -80° for at least one year without decomposition.

[2-^{14}C]H_2Folate was prepared by a modification of previously reported procedures (Futterman, 1957; Coward et al., 1974). A solution (0.45 ml) containing 4 mM [2-^{14}C]folate (21.3 mCi/mmol), 0.2 M 2-mercaptoethanol, 0.2 M potassium phosphate (pH 7.5) and 0.4 M $Na_2S_2O_4$ was incubated for 30 minutes under argon at ambient temperature, protected from light. To this was added 3.5 ml of a solution containing 0.5 M $Et_3N^+H \cdot HCO_3^-$ (pH 7.0) and 50 mM 2-mercaptoethanol. The solution was applied to a DEAE-Sephadex column (9 x 40 mm; 40-120 μ) pre-equilibrated with the aforementioned buffer at 4°. The column was eluted at 4° with a 24 ml linear gradient (0.5 to 1.2 M) of $Et_3N^+H \cdot HCO_3^-$ (pH 7.0) containing 50 mM 2-mercaptoethanol; the flow rate was 0.1 ml/min and the product eluted at ca. 0.9 M salt. Fractions containing the product were combined, lyophilized, and re-dissolved in 0.5 ml of 0.5 M tris·HCl (pH 7.2) and 0.25 M 2-mercaptoethanol. The overall recovery

of [2-^{14}C]H_2folate was 77%, which was determined to be 97% pure by HPLC (system A, RV = 7.8 ml) and enzymic reduction using H_2folate reductase.

(6R),L-[^{3}H]CH_2-[2-^{14}C]H_4folate was prepared as previously described for unlabeled cofactor with the following modifications: (a) The reaction was performed in a total volume of 0.8 ml and the final concentration of [2-^{14}C]H_2folate was 3.8 mM and that of [^{3}H]H_2CO (85 mCi/mmol) was 14.8 mM. (b) Purification was performed on a 1.0 x 27 cm Biogel P2 column at a flow rate of 0.3 ml/min, and H_2CO was omitted from the equilibrating and eluting buffer. The reaction proceeded to at least 97% completion as determined spectrophotometrically, and a 93% recovery of ^{14}C dpm's was obtained from the pooled, product-containing fractions of the Biogel column. The cofactor was determined to be at least 95% radiochemically pure by HPLC analysis (system A, RV = 8.2 ml; system B, RV = 28 ml), and the ternary complex formed with FdUMP and dTMP synthetase and isolated by gel filtration had a $^3H/^{14}C$ = 5.000. The final solution of the cofactor contained approximately a 4-fold excess of free [^{3}H]H_2CO because of some co-elution in the Biogel chromatography. The pooled fractions were lyophilized, re-dissolved in 0.80 ml of 20 mM $Et_3N^+H\cdot HCO_3^-$ (pH 9.5) and 20 mM 2-mercaptoethanol, and stored under argon at -80°.

Formation and Isolation of Ternary Complexes. FdUMP—CH_2-H_4folate—dTMP synthetase complexes were formed under conditions specified in the Results section. Filter assays of the complex were performed using Bac-T-flex nitrocellulose filters (0.45 micron; 2.4 cm; Schleicher and Schuell) as previously described (Santi et al., 1974a) except that

filters were pre-soaked and washed in 75 mM K phosphate (pH 7.4); filtration efficiencies were typically ca. 90%. Separations of the complex from free ligands by Sephadex G-25 filtration were performed at 4° using 75 mM K phosphate (pH 7.4) and 10 mM 2-mercaptoethanol for equilibration and elution. In all chromatographic separations of double-labeled isotopes, the entire radioactive peak was pooled prior to counting to avoid isotopic separation.

Determination of Secondary α-Hydrogen Isotope Effects. Double-labeled [^{3}H,^{14}C]FdUMP—CH_2-H_4folate—enzyme complexes were separated from free radioactive ligands by adsorption to nitrocellulose filters or by gel filtration as described elsewhere. The ^{3}H and ^{14}C radioactivities were obtained by counting samples in 10 ml Aqueous Counting Scintillant (Amersham) 3 to 10 times, collecting a minimum of 2 x 10^5 total counts of ^{14}C, and dpm for each isotope were calculated with appropriate corrections for ^{14}C spillover by the external standard ratio method. Standard errors (SE) for determination of $^{3}H/^{14}C$ ratios and ^{14}C dpm were maximally 0.25% and 0.5%, respectively. Isotope effects accompanying the dissociation of the FdUMP—CH_2-H_4folate—enzyme complex were calculated from the isotopic ratios remaining in the reactant complex as described by Melander (1960). These also were calculated as the ratio of the first-order rate constants for dissociation of ^{3}H and ^{1}H (^{14}C) as previously described (Santi et al., 1974b). Both methods gave essentially identical results. For equilibrium isotope effects, the complex was separated from unbound ligands after equilibrium was reached, and the equilibrium isotope effect was calculated as

the ratio of $^{3}H/^{14}C$ of the complex to $^{3}H/^{14}C$ of the unbound ligands.

Miscellaneous. Uv spectra were obtained on a Cary 118 spectrophotometer and radioactivity was monitored on an Isocap 300 liquid scintillation counter. DPM calculations were performed by the external standard ratio method and were aided by a tape-fed Hewlett Packard 11255 A computer. HPLC was performed using a Hewlett-Packard 1084b instrument.

RESULTS

Kinetic Isotope Effect upon Dissociation of the [2-^{14}C,6-^{3}H]-FdUMP—CH_2-H_4PteGlu$_{1-5}$—dTMP Synthetase Complex. To a solution (3.4 ml) of the ternary complex was added unlabeled FdUMP (1.1 ml) to give a final solution (4.5 ml) containing in the standard NMM buffer, 0.45 μM dTMP synthetase, 51 μM (6R),L-CH_2-H_4folate, 2.3 μM [2-^{14}C,6-^{3}H]-FdUMP (49.7 mCi ^{14}C/mmol; $^{3}H/^{14}C$ = 4.549), and 340 μM FdUMP. The mixture was kept at 25.0° under nitrogen, protected from light. Triplicate aliquots were removed at intervals up to ca. 35 hr and the ternary complex was isolated by adsorption on nitrocellulose filters; the aliquots filtered were progressivley larger (20-500 μl) with time to obtain sufficient and constant dpm in the isolated complex as the reaction proceeded. The triplicate samples were counted, showing standard errors for $^{3}H/^{14}C$ within 0.8% of the mean. From these, the dissociation of the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4folate—dTMP synthetase complex was shown to be first order with k_H = 0.12 hr^{-1}. Fig. 7 shows the data from which k_H/k_T was calculated to be 1.229 ± 0.009 (n = 12). Similar

FIGURE 7: Secondary α-hydrogen isotope effect upon dissociation of the [2-^{14},6-^{3}H]FdUMP—CH_2-H_4folate—dTMP synthetase complex. The data points represent the $^{3}H/^{14}C$ ratio of [2-^{14}C,6-^{3}H]FdUMP bound in the complex isolated over the course of the reaction. The solid line depicts the theoretical isotopic ratios (Melander, 1960) for a reaction with k_H/k_T = 1.229. Experimental details are described in the text.

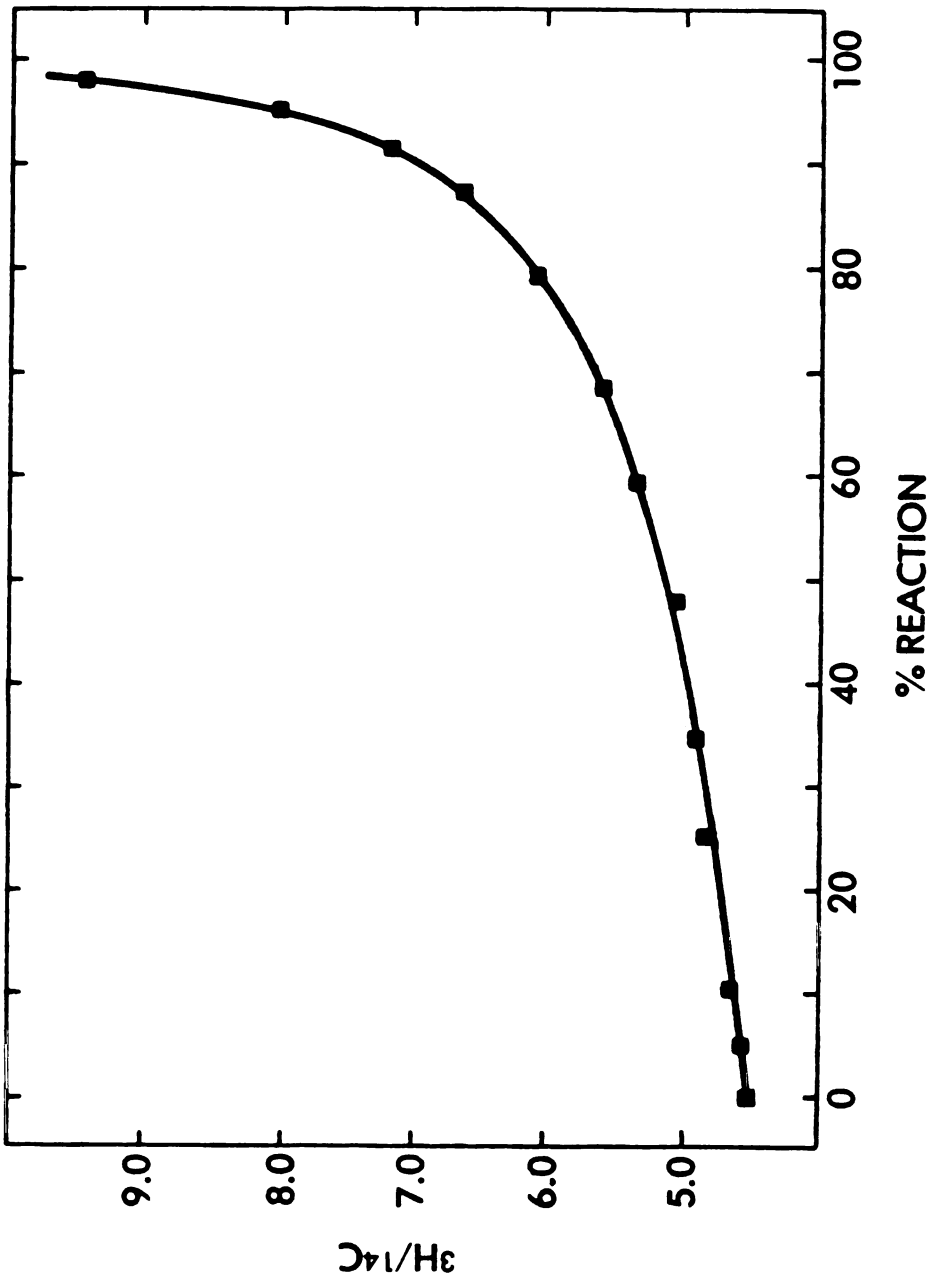

3H/14C
9.0
8.0
7.0
6.0
5.0
0
20
40
60
80
100
% REACTION

FIGURE 8: Secondary α-hydrogen isotope effect upon dissociation of the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4PteGlu$_5$—dTMP synthetase complex. The data points represent the ^{3}H/^{14}C ratio of [2-^{14}C,6-^{3}H]-FdUMP bound in the complex isolated over the course of the reaction. The solid line depicts the theoretical isotopic ratios (Melander, 1960) for a reaction with k_H/k_T = 1.211. Experimental details are described in the text.

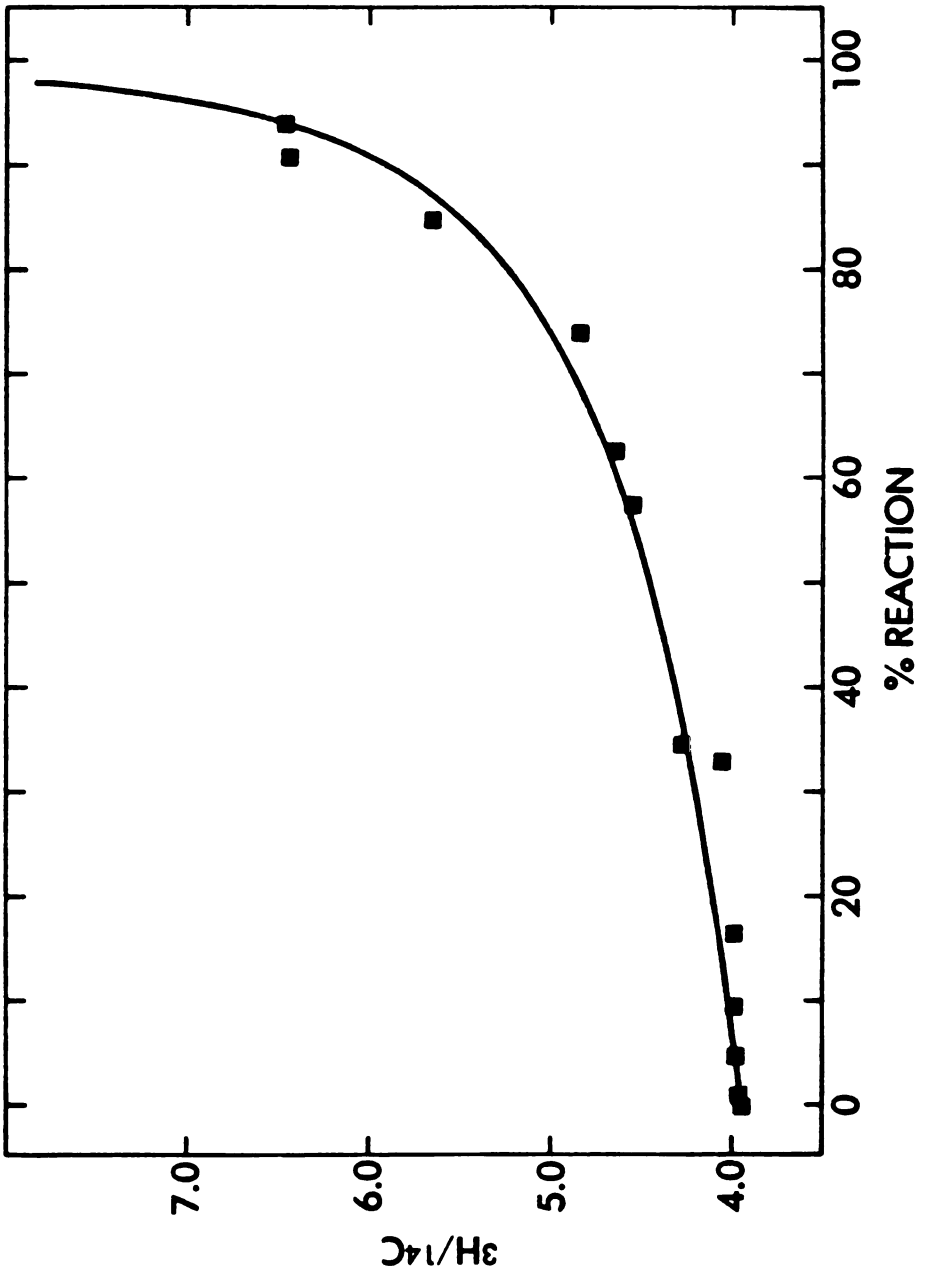

7.0
6.0
5.0
4.0
3H/14C
0
20
40
60
80
100
% REACTION

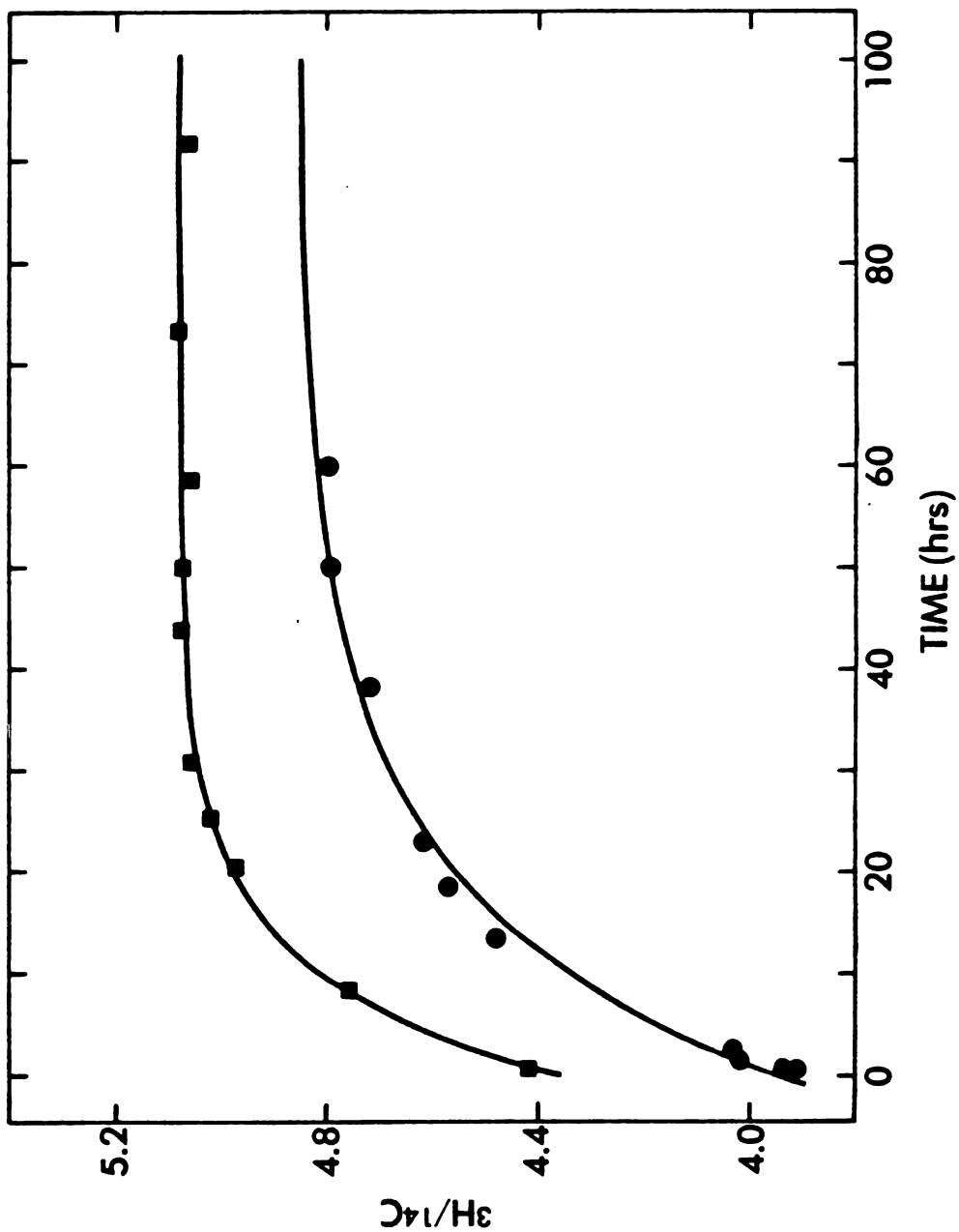
FIGURE 9: Secondary α-hydrogen equilibrium isotope effect for the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4folate—dTMP synthetase complex. The data points represent the $^{3}H/^{14}C$ ratio of complexes made with 7.5 μM [2-^{14}C,6-^{3}H]FdUMP (■) or 340 μM [2-^{14}C,6-^{3}H]FdUMP (●) which were isolated over the course of isotopic approach to equilibrium. The solid lines depict the theoretical isotopic ratios at any time $(^{3}H/^{14}C)_t$ calculated according to the equation:

$$(^{3}H/^{14}C)_t = (^{3}H/^{14}C)_o + [(^{3}H/^{14}C)_\infty - (^{3}H/^{14}C)_o] - [(^{3}H/^{14}C)_\infty - (^{3}H/^{14}C)_o]e^{-kt},$$

where $(^{3}H/^{14}C)_o$ and $(^{3}H/^{14}C)_\infty$ are the initial ratio prior to initiation of equilibration and the final ratio at equilibrium, respectively. $(^{3}H/^{14}C)_o$ was determined experimentally as described in the text. $(^{3}H/^{14}C)_\infty$ and k were determined by a computer-aided, linear least squares fit of the data points to the equation for isotopic approach to equilibrium (Jencks, 1969):

$$-\ln\left[\frac{(^{3}H/^{14}C)_\infty - (^{3}H/^{14}C)_t}{(^{3}H/^{14}C)_\infty}\right] = kt.$$

Experimental details are described in the text.


5.2
4.8
4.4
4.0
3H/14C
0
20
40
60
80
100
TIME (hrs)

experiments using variable amounts of unlabeled FdUMP (10 to 100-fold excess over labeled FdUMP) demonstrated that both the rate constant for dissociation and the kinetic isotope effect were independent of the concentration of the free ligand.

The kinetic isotope effect upon dissociation of complexes made using (6R)-CH_2-H_4Pte-L-Glu_5 was determined identically as described above, except that aliquots were removed from the final mixture up to ca. 140 hr. Dissociation of the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4PteGlu_5—dTMP synthetase complex was shown to be first order with k_H = 2.02 x 10^{-2} hr^{-1}. Fig. 8 shows the data from which the kinetic isotope effect was calculated to be 1.211 ± 0.027 (n = 12).

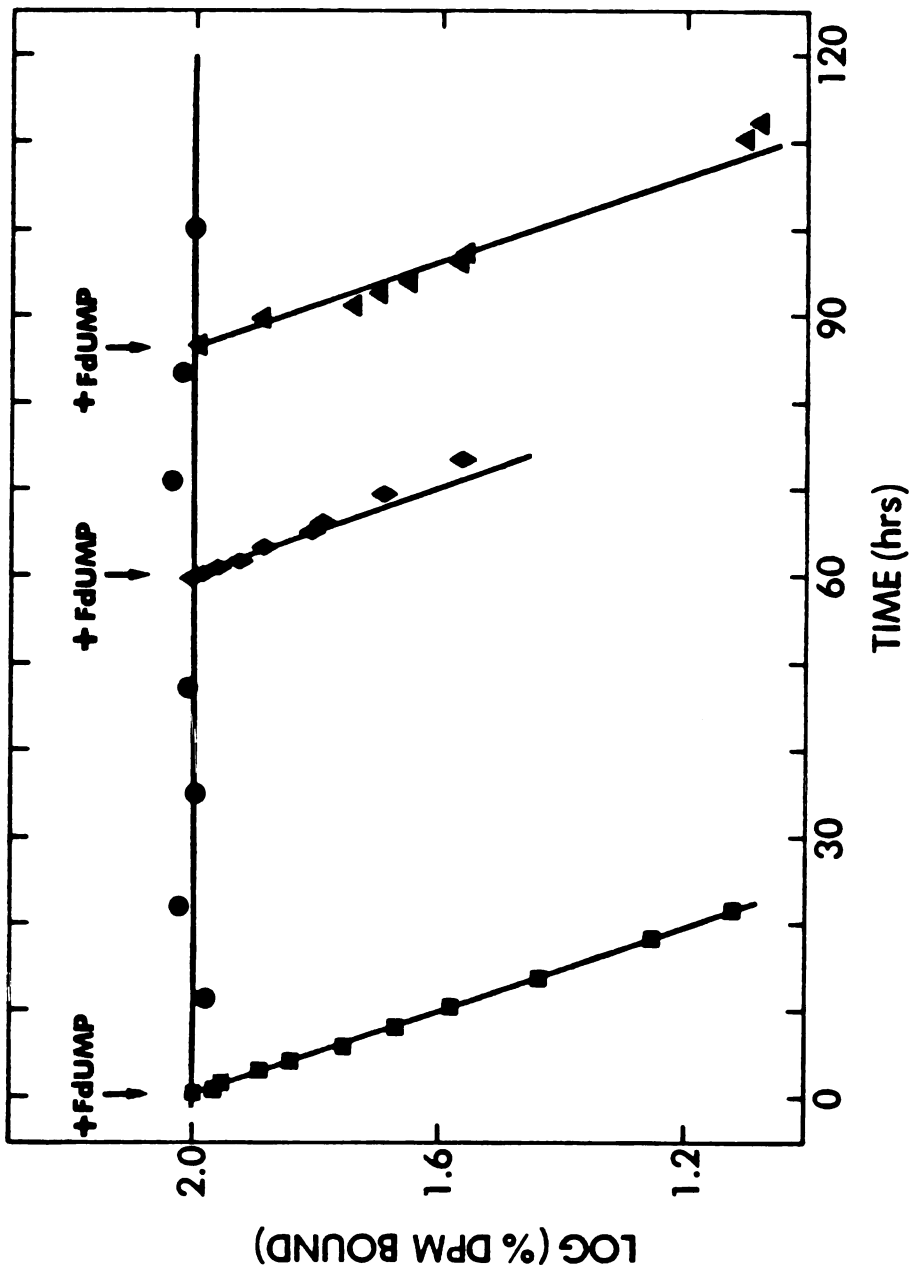
Equilibrium Isotope Effect for the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4-folate—dTMP Synthetase Complex. The ternary complex (700 µl) was prepared in the standard NMM buffer using 0.17 mM (6R),L-CH_2-H_4folate, 1.5 µM dTMP synthetase and 7.5 µM [2-^{14}C,6-^{3}H]FdUMP (49.7 mCi ^{14}C/mmol; $^{3}H/^{14}C$ = 4.419) and was kept under nitrogen at 25.0°, protected from light. CH_2-H_4folate was omitted in controls. Nitrocellulose filtration of 20 µl aliquots demonstrated that at t = 0, the $^{3}H/^{14}C$ of the complex was identical to that of the FdUMP used, but it gradually increased with time until isotopic equilibrium was reached at 55-60 hrs (Figure 9). There was no further change up to 90 hr. The change in the $^{3}H/^{14}C$ of the complex was first-order with k = 0.10 hr^{-1} and a calculated flux of 2.1 x 10^{-7} M hr^{-1} (Jencks, 1969). Unlabeled FdUMP (100-fold molar excess over labeled FdUMP) was added to 200 µl aliquots of solutions identical to the above, except that [6-^{3}H]FdUMP (253

mCi/mmol) was substituted for [2-^{14}C,6-^{3}H]FdUMP, at 0.5, 60 and 85 hr, and the rate of dissociation of labeled complex was determined over ca. 1.5 half-lives as previously described (Fig. 10). The facts that the amount of complex was unchanged over this period, and that the first-order rate constants for dissociation throughout this period were identical ($k = 2.0 \times 10^{-3}$ min^{-1}) indicated that the complex was not modified upon prolonged incubation.

To determine the equilibrium isotope effect, three separate solutions (800 µl) of the complex were prepared as described above and were kept under nitrogen at 25.0°, protected from light, for 60-65 hr. Three 200 µl aliquots of each solution were passed through a Sephadex G-25 column (1.0 x 29 cm), equilibrated and eluted with 75 mM potassium phosphate-10 mM 2-mercaptoethanol (pH 7.4) at 4°, and ca. 0.8 ml fractions were collected. The fractions containing bound and free [2-^{14}C,6-^{3}H]FdUMP were individually pooled and triplicate aliquots of each were counted. Samples of the free [2-^{14}C,6-^{3}H]FdUMP also were subjected to HPLC analysis (System A) and were demonstrated not to have undergone appreciable decomposition during the period of incubation and analysis; 96% of the radioactive material was FdUMP (RV = 8.0 ml) and 4% was FdUrd (RV = 4.4 ml). Not considering the impurity, the equilibrium isotope effect calculated from these data gave K_T/K_H = 1.240 ± 0.001 (n = 9); assuming that all 4% FdUrd was present at the outset of the experiment, a lower limit of K_T/K_H was calculated to be 1.228 ± 0.001.

The secondary equilibrium isotope effect also was determined for

FIGURE 10: Demonstration that [6-^{3}H]FdUMP—CH_2-H_4folate—dTMP synthetase complexes are not modified upon prolonged incubation under conditions used to determine equilibrium isotope effects. Data points depict the amount of [6-^{3}H]FdUMP bound in the complex isolated over time in the absence (●) and in the presence of exogenous, unlabeled FdUMP added in 100-fold molar excess over [6-^{3}H]FdUMP at 0.5 (■), 60 (◆) and 85 hr (▲). First-order rate constants for dissociation of [6-^{3}H]-FdUMP were calculated from the solid lines: $k = 2.0 \times 10^{-3}$ min^{-1} for ■, ◆, and ▲.


+FdUMP
+FdUMP
+FdUMP
2.0
1.6
1.2
LOG (% DPM BOUND)
0
30
60
90
120
TIME (hrs)

complexes formed in solutions (300 µl) containing 51 µM (6R),L-CH_2-H_4folate, 1.5 µM dTMP synthetase and 0.34 mM [2-^{14}C,6-^{3}H]FdUMP (49.7 mCi ^{14}C/mmol; $^{3}H/^{14}C$ = 3.912) in standard NMM buffer; controls omitted CH_2-H_4folate. The reaction was incubated as before and was followed by nitrocellulose filtration of 20 µl aliquots at selected intervals up to 60 hrs (Fig. 9). It was first-order with k = 5.5 x 10^{-2} hr^{-1} and R = 1.6 x 10^{-7} M hr^{-1}. Because of the large molar excess of labeled FdUMP over enzyme sites, the $^{3}H/^{14}C$ of the unbound FdUMP pool does not change significantly with time; the equilibrium isotope effect was calculated simply as the ratio of the $^{3}H/^{14}C$ of the complex at equilibrium to the $^{3}H/^{14}C$ of the [2-^{14}C,6-^{3}H]FdUMP used, which was the same as the $^{3}H/^{14}C$ of the initially formed complex: K_T/K_H = 1.223 ± 0.014 (n = 10).

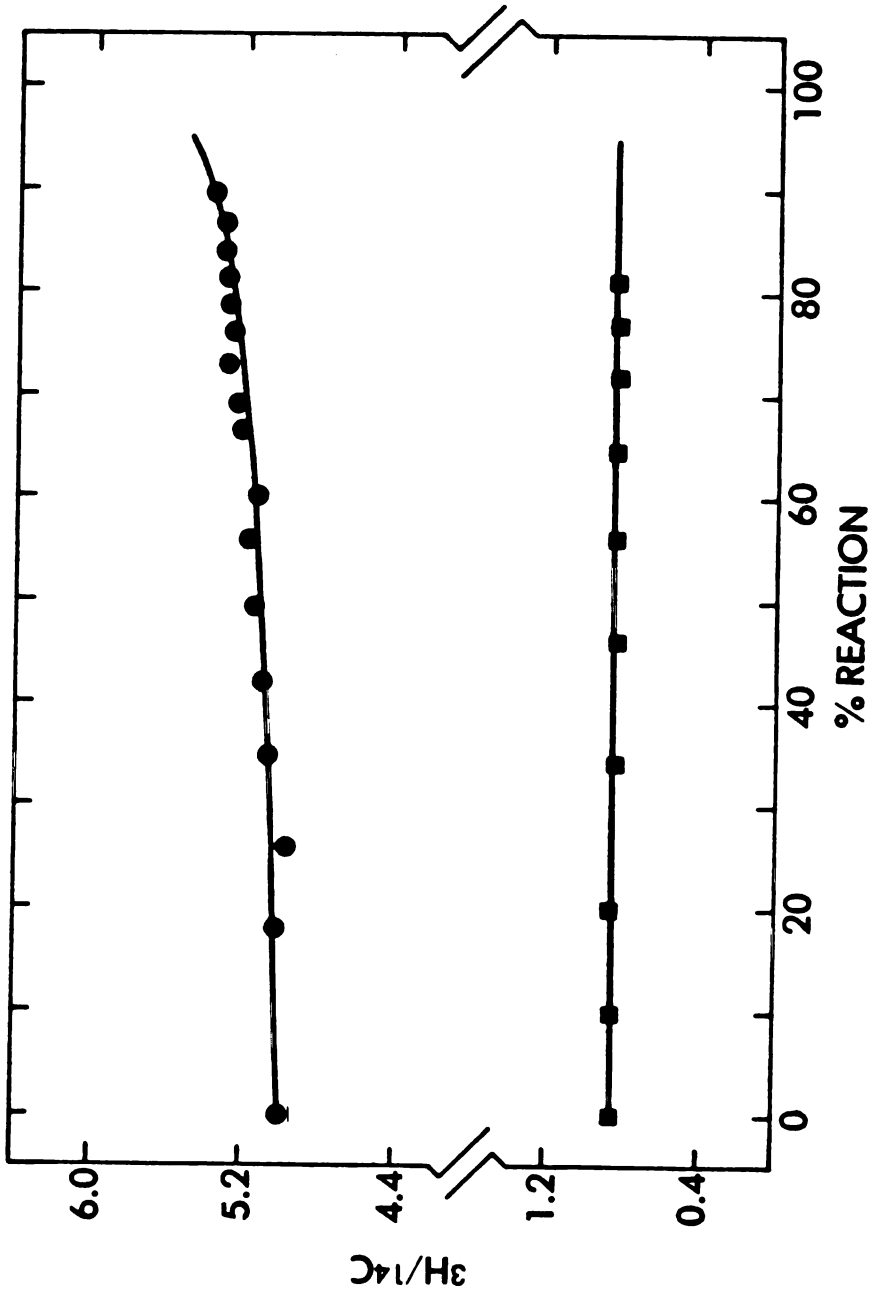
Kinetic Isotope Effect upon Dissociation of the FdUMP—[^{3}H]CH_2-[2-^{14}C]H_4Folate—dTMP Synthetase Complex. The complex was formed in a solution (2.5 ml) containing in the standard NMM buffer, 19 µM dTMP synthetase, 2.1 mM FdUMP and 94 µM (6R),L-[^{3}H]CH_2-[2-^{14}C]H_4folate (1.1 x 10^{7} ^{14}C dpm). After 30 min at 4°, under argon and protected from light, the complex was isolated by gel filtration through a Sephadex G-25 column (1.8 x 57 cm), equilibrated and eluted with NMM buffer minus formaldehyde at 4° (gel filtration was necessary to remove free [^{3}H]H_2CO which interfered with the nitrocellulose filter assay). Under identical conditions, <1.5% of the total ^{14}C dpm recovered was eluted in the macromolecular peak when [2-^{14}C]-H_2folate was used in place of the double-labeled cofactor. Triplicate solutions (7.8 ml each) were prepared which contained 1.2 µM of the isolated complex (3 x 10^{6} ^{14}C dpm), 48 µM FdUMP, and 340 µM (6R),L-CH_2-H_4folate in the standard NMM buffer, and were incubated at 25.0°, under argon and protected from light; controls omitted the cofactor. Duplicate

aliquots (30-520 µl) containing ca. 3000 bound ^{14}C dpm were removed at intervals up to ca. 35 hr and the ternary complex was isolated on nitrocellulose filters; controls lacking FdUMP showed negligible adsorption of radioactivity (0.4%) providing free $[^{3}H]H_2CO$ was removed as described above. The duplicate aliquots of each of the three experiments (total of 6 determinations per time point) were counted, each showing standard errors of $^{3}H/^{14}C$ within 0.68% of the mean. The dissociation of the radioactive ligands was first-order for at least 6 half-lives with $k_H = 0.14\ hr^{-1}$. The $^{3}H/^{14}C$ ratio of the complex remaining did not change appreciably during the period when over 85% of the labeled cofactor had dissociated (Fig. 11) and k_H/k_T was calculated to be 1.033 ± 0.004 (n = 16). A value of k_H/k_T = 1.004 ± 0.013 (n = 9) was obtained (Fig. 11) for experiments performed identically except that complexes were formed using (6R,S),L-$[^{3}H]CH_2$-$[2-^{14}C]H_4$folate and were pre-incubated at ambient temperature for >12 hr prior to gel filtration (to assure complex formation at all enzyme active sites with the 6R isomer only). In the absence of added unlabeled cofactor no dissociation of the radioactive complex was observed after as long as 35 hr.

DISCUSSION

Figure 6 depicts what we currently believe to be the most reasonable minimal mechanism for dissociation/association of the covalent FdUMP—CH_2-H_4folate—dTMP synthetase complex. The first step in dissociation of the complex ($\underset{\sim}{I}$), k_1, involves cleavage of the linkage

FIGURE 11: Secondary α-hydrogen isotope effect upon dissociation of the FdUMP—[^{3}H]CH_2-[2-^{14}C]H_4folate—dTMP synthetase complex. The data points represent the $^{3}H/^{14}C$ ratio of complexes made with (6R),L-[^{3}H]CH_2-[2-^{14}C]H_4folate (●) or (6R,S),L-[^{3}H]CH_2-[2-^{14}C]H_4folate (■) which were isolated over the course of the reaction. The solid lines depict the theoretical isotopic ratios (Melander, 1960) for reactions with k_H/k_T = 1.033 (●) and k_H/k_T = 1.004 (■).


6.0
5.2
4.4
1.2
0.4
0
20
40
60
80
100
% REACTION
3H/14C

between the 5-position of the bound FdUMP and the cofactor to form the covalently bound enolate II (or equivalent enol) and the 5-iminium ion form of the cofactor, $CH_2=^{+}H_4$folate; this step involves sp^3 to sp^2 rehybridization of the one-carbon unit of the bound cofactor. While the sp^2 hybridized iminium ion intermediate has not been directly demonstrated, its existence is strongly supported by chemical rationale, model studies (Kallen & Jencks, 1966; Benkovic & Bullard, 1973; Benkovic, 1978), and recent stereochemical studies on the chirality of the one-carbon transfer in the dTMP synthetase reaction (Tatum et al., 1977; Benkovic, 1980). The second step in dissociation, k_2, involves β-elimination of the enzyme nucleophile of II to provide non-covalently bound FdUMP; it is here where sp^2 rehybridization of the 6-carbon of FdUMP occurs. The step k_3/k_{-3} represents a conformational change of the enzyme, and perhaps of the cofactor, which is induced upon formation of non-covalent, reversible, ternary complexes (III).

Evidence that a substantial conformational change of dTMP synthetase occurs upon formation of the ternary complex with FdUMP and CH_2-H_4folate recently has been obtained from hydrodynamic studies (Lockshin and Danenberg, 1980) and provides direct support for previous proposals derived from spectral studies of analogous non-covalent and covalent complexes. The covalent FdUMP—CH_2-H_4folate—dTMP synthetase complex has a unique uv spectrum with maxima above 330 nm ($\Delta\varepsilon_{330-335}$ = 17,700 M^{-1} cm^{-1} for difference spectra omitting FdUMP in reference cell; ε_{375} = 7,070 M^{-1} cm^{-1}) which are not found in the protein or unbound ligands (Sharma & Kisliuk, 1973; Danenberg et al., 1974; Santi et al., 1976a;

Donato et al., 1976). Because denaturation of the complex does not disrupt covalent bonds but results in a loss of high wave-length absorbance, it was surmised that the spectral properties of the complex were a result of electronic perturbation of the chromophores of the cofactor bound in the ternary complex. Circular dichroism and fluorescence spectral studies indicated that the environment of tryptophan residues of the enzyme also are altered in the ternary complex (Galivan et al., 1975; Donato et al., 1976; Kisliuk et al., 1976), and it is reasonable to suggest that the pteridine and/or p-aminobenzoylglutamate moieties of the cofactor strongly interact with the indole ring of tryptophan residues within the ternary complex. These spectral properties cannot be attributed to covalent bond formation of the bound ligands, since they also are observed when folate analogs which do not involve covalent bond formation are used to form ternary complexes (Galivan et al., 1975; Kisliuk et al., 1976; Santi et al., 1976a). Since these spectral changes are not prevalent in binary complexes, we conclude that the conformational changes (-Enz to -Enz') leading to perturbations of the chromophore occur either upon formation (k_{-4}) of the initial non-covalent ternary complex or, as depicted in Figure 6, immediately thereafter (k_{-3}).

In this study, secondary α-hydrogen isotope effects have been used to clarify relationships of covalent bond changes at the 6-position of FdUMP and at the one-carbon unit of CH_2-$H_4PteGlu_{1-5}$ with rate-limiting processes in the dissociation and formation of the FdUMP—CH_2-$H_4PteGlu_{1-5}$—dTMP synthetase complex. The use and interpretation of

secondary α-hydrogen isotope effects have been extensively reviewed (Melander, 1960; Collins & Bowman, 1970; Kirsch, 1977), and only a brief summary of kinetic and equilibrium secondary isotope effects as they apply to interpretation of this study is provided here. Isotopic substitution of a non-reactive, non-exchangeable hydrogen attached to a carbon atom which undergoes a covalent bond change may alter the equilibrium or rate of a reaction. The maximal isotope effect is manifested at equilibrium and may be estimated by the use of fractionation factors derived from simple models of the two relevant states (Hartshorn & Shiner, 1972; Buddenbaum & Shiner, 1977). For the sp^3 to sp^2 conversion of the C-6 of $\underset{\sim}{I}$ to FdUMP, a maximal α-tritium isotope effect of 30% is calculated, with isotopic enrichment in FdUMP. The overall conversion of the $C^5\text{-}CH_2(T)\text{-}N^{10}$ of $\underset{\sim}{I}$ to the $N^5\text{-}CH_2(T)\text{-}N^{10}$ of $CH_2\text{-}H_4$folate involves the intermediacy of $CH_2{=}^{+}H_4$folate and is calculated similarly to proceed with an equilibrium α-tritium isotope effect of only 5%, with isotopic enrichment in $CH_2\text{-}H_4$folate[3]. While

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[3]Calculated values are from W. W. Cleland, Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, in a personal communication. They are derived from a combination of experimental values for equilibrium isotope effects and values calculated from fractionation factors, many of which recently have been published (W. W. Cleland, 1980). The behavior of S in isotope effect systems is too poorly known to place too much import on the value of 1.30 for the equilibrium effect in going from $\underset{\sim}{I}$ to $\underset{\sim}{III}$. The fractionation factor used for $CH_2(D)SH$ relative to $CH_3(D)$ is 1.067 and the value obtained from back-calculation from the experimentally observed isotope effect of 1.24 is 1.03 (after correction for a tritium vs. deuterium effect). The differences in these fraction factors (1.067 vs. 1.03) could well be a result of geometric constraints in the active site of the enzyme. Significant changes in fractionation factors concomitant with transition-state compression as a mechanism of enzymic catalysis appears to be operative in O to S transmethylation reactions catalyzed by catechol O-methyltransferase (Gray et al., 1979; Hegazi et al., 1979).

no simple model is available to estimate a fractionation factor for $CH_2=^{+}H_4$folate, it would certainly be larger than for $-CH_2=CH_2-$, and α-tritium isotope effects greater than 13% would be expected in conversions between $CH_2=^{+}H_4$folate and $\underset{\sim}{I}$ or CH_2-H_4folate.

A few idealized generalizations may be made regarding kinetic secondary α-hydrogen isotope effects, which are sufficient for interpretation of the results described here. Unlike equilibrium isotope effects, the magnitude of observed secondary kinetic isotope effects depends on where in the reaction the relevant covalent bond change occurs with regard to rate-limiting processes. If the covalent bond change is pre-rate-determining, that is, a rapid equilibrium step, isotopic equilibrium will have occurred before the rate-determining step occurs, hence before completion of the monitored reaction. There will be full isotopic enrichment or depletion of the intermediate participating in the rate-limiting step, and a maximal kinetic α-hydrogen isotope effect will be observed; it will be equal to the equilibirum isotope effect. Conversely, if the relevant covalent bond change is after the rate-determining step, the isotopic substitution will have no effect on this step or the observed rate, and no kinetic isotope effect will be detected. From the above, it follows that a secondary kinetic isotope effect which results from a pre-rate-deterimining covalent bond change in one direction of a reaction would not be manifested in the kinetics of the reverse reaction where the covalent bond change is post-rate-

determining. The interpretation of kinetic α-secondary isotope effects is more complex when the covalent bond change occurs at the rate-determining step. In this case, if the transition state structure is intermediate between that of the reactant and product, the interpretation is relatively straightforward: the kinetic isotope effect will not be maximal (i.e., it will be smaller than the equilibrium isotope effect) and it will be observed regardless of the direction in which the reaction kinetics are studied. The caveats in relating secondary isotope effects to rate-limiting processes lie in situations where the relevant covalent bond changes occur at the rate-determining step and the transition state structure closely resembles the reactant or product. In general, the closer the structure of the transition state resembles the reactants or products, the more the kinetic isotope effect will resemble what has been described for covalent bond changes which are post- or pre-rate-determining, respectively. In the extreme cases, a reaction in which the transition state structure closely resembles the reactant will show no isotope effect, and one in which the transition state closely resembles the product will show a maximal kinetic isotope effect; to our knowledge, this situation is rare and we will argue that secondary kinetic isotope effects which are equal in magnitude to secondary equilibrium isotope effects--at least in this study--represent covalent bond changes before the rate-determining step.

Dissociation of [6-^{3}H]FdUMP from the FdUMP—CH_2-H_4folate—dTMP synthetase complex is sufficiently slow (k_H = 0.12 hr^{-1}) to permit direct measurement of the $^{3}H/^{14}C$ remaining in the complex as the first-

order reaction proceeds. In agreement with a previously reported result (Santi et al., 1974b), dissociation of [2-^{14}C,6-^{3}H]FdUMP from the covalent complex $\underset{\sim}{I}$ proceeded with k_H/k_T = 1.29 (Table 1). When [2-^{14}C,6-^{3}H]FdUMP was combined with CH_2-H_4folate and the enzyme, the $^{3}H/^{14}C$ of the complex formed was identical initially to that of the ligand used, that is, there was no kinetic isotope effect upon formation of the complex; ^{3}H enrichment of the complex occurred with a first-order approach to isotopic equilibrium. After reaching isotopic equilibrium, comparison of the $^{3}H/^{14}C$ of the bound and free [2-^{14}C,6-^{3}H]-FdUMP showed that the equilibrium isotope effect (K_H/K_T= 1.240) was identical, within experimental error (Table 1), to the kinetic isotope effect observed upon dissociation. Similarly, the kinetic isotope effect was identical for complexes formed using either CH_2-H_4PteGlu$_1$ or-Glu$_5$, a naturally occuring cofactor, and both equilibrium and kinetic measurements were invariant with respect to FdUMP concentration (Table 1). It is satisfying that the values for these isotope effects are in general accord with the 30% value calculated earlier from fractionation factors, and exact agreement is not expected since the latter are derived from imperfect models for bound and free FdUMP.[3]

The observation of a deuterium isotope effect of greater than 10%, equivalent to a tritium isotope effect of 15% (Swain et al., 1958), generally is accepted to provide conclusive evidence for carbon re-hybridization at or before the rate-limiting step in a reaction. Thus, the magnitude of the secondary normal kinetic and equilibrium isotope effects reported here for the dTMP synthetase facilitated reactions of

TABLE 1: Secondary α-Tritium Isotope Effects for the Reactions of FdUMP—dTMP Synthetase—Cofactor Complexes.

Ternary Complex[a]	Reaction	Isotope Effect[b]
[2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4folate—Enz	dissociation	k_H/k_T = 1.229 ± 0.009 (n=12)
[2-^{14}C,6-^{3}H]FdUMP—CH_2-$H_4$$PteGlu_5$—Enz	dissociation	k_H/k_T = 1.211 ± 0.027 (n=12)
[2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4folate—Enz	equilibration	K_T/K_H = 1.240 ± 0.001 (n=9)[c] = 1.223 ± 0.014 (n=10)[d]
FdUMP—[^{3}H]CH_2-[2-^{14}C]H_4folate—Enz	dissociation	k_H/k_T = 1.033 ± 0.004 (n=16) = 1.004 ± 0.013 (n=9)[e]

[a] Cofactors used to make complexes have (6R),L- stereochemistry unless otherwise indicated.
[b] Isotope effects were determined as described in the text at 25.0°, pH 7.4.
[c] [2-^{14}C,6-^{3}H]FdUMP = 7.5 μM. [d] [2-^{14}C,6-^{3}H]FdUMP = 340 μM. [e] Complex formed using (6R,S),L-[^{3}H]CH_2-[2-^{14}C]H_4folate.

FdUMP—CH_2-H_4PteGlu$_{1-5}$—dTMP synthetase complexes convincingly demonstrates the occurrence of sp^3 to sp^2 rehybridization at the 6-carbon of the pyrimidine heterocycle at or before the rate-determining step of the reaction in the direction of dissociation. This confirms the existence of a 5,6-dihydropyrimidine intermediate in this reaction pathway. These results are in complete accord with and verify important aspects of the mechanism originally proposed (Santi & Brewer, 1968; Pogolotti & Santi, 1974, 1977) and recently shown to hold for reactions catalyzed by this enzyme: exchange of the 5-H of dUMP for protons of water (Pogolotti et al., 1979) and dehalogenation of BrdUMP and IdUMP (Garrett et al., 1979). This agreement reasonably should establish the validity of presuming further interpretations of the isotope effect results with the non-catalytic FdUMP partial reaction to apply to reactions catalyzed by dTMP synthetase.

The most likely interpretation for the equivalence of the kinetic and equilibrium isotope effect results in this study is that in dissociation of the complex (I), cleavage of the covalent bond between the enzyme nucleophile and FdUMP (k_2) is fully pre-rate-determining. For the general reasons discussed earlier, the alternate interpretation that bond cleavage is the rate-limiting step with C-6 of the pyrimidine heterocycle being completely sp^2 hybridized in a very late transition-state is unlikely. This will become even more apparent from arguments presented below. Regardless, these experiments rule out k_1 as the rate-determining step.

The complex formed with [^{3}H]CH_2-[2-^{14}C]H_4folate showed essentially

no secondary kinetic isotope effect upon dissociation; 0.4-3.3% (Table 1). This suggests that the sp^3 to sp^2 rehybridization of the one-carbon unit at k_1 is largely cancelled by the inverse isotope effect at k_3 or k_4 which occurs upon conversion of the iminium ion, 5-CH_2=$^+$$H_4$-folate, to the sp^3 hybridized 5,10-CH_2-H_4folate. By juxtaposition with the studies with FdUMP, conversion of the 5,6-dihydropyrimidine I to give the iminium ion at II must be pre-rate-determining in dissociation of the complex; that is, the conversion must occur prior to k_2, since k_{-2} is required in the association direction to activate C-5 for attack by the iminium ion. Thus, the subsequent conversion of the iminium ion to the sp^3 hybridized 5,10-CH_2-H_4folate must also occur before the rate-determining step, or at the rate-determining step with the one-carbon unit being nearly completely sp^3 rehybridized in a late transition state. This must be true in order for there to be cancellation of the isotope effects of each conversion which are expected to be large (>13%) from fractionation factors. That the results show a 0.4-3.3% normal (k_H/k_T) kinetic isotope effect *vs.* a calculated 5% inverse (k_T/k_H) effect is not inconsistent with these assignments of positions of rehybridization steps relative to the rate-determining step. A relatively small difference of 5-8% can be accounted for by any of experimental error (obviously $\geq$ 2.9%), inaccuracies in fractionation factor calculations from incompletely analogous model reactions, and possible geometric perturbations enforced by the enzyme.

As previously argued, the earliest step in the dissociation of

the complex which might be rate-determining is k_2, i.e., cleavage of the enzyme-FdUMP covalent bond. If this were the rate-determining step, then from the above, both the C-6 of FdUMP and the one-carbon unit of the cofactor would have to be rehybridized concurrently at this step in late transition states with the C-6 of FdUMP completely sp^2 rehybridized and the one-carbon unit of the cofactor completely sp^3 rehybridized. As the occurrence of either transition-state structure above is rare and there is no logical reason at this time to believe these processes should be coupled in this manner, it can be considered highly unlikely that k_2 is the rate-determining step.

From what thus far has been discussed, we conclude that the rate-determining step in the dissociation of the FdUMP—CH_2-H_4folate—dTMP synthetase complex is after cleavage of the covalent bond linking the enzyme to FdUMP (k_2). That is, the rate-limiting step(s) must occur at k_3/k_{-3}, the conformational change, and/or at k_4/k_{-4}, the release/binding of ligands. In accord with this conclusion, dissociation of [2-^{14}C,6-^{3}H]-FdUMP from the covalent FdUMP—CH_2-H_4PteGlu$_5$—enzyme complex proceeds with an isotope effect essentially identical to that observed with the complex formed with CH_2-H_4folate (Table 1), but some six-fold slower. As the ca. 23% isotope effect is maximal for this reaction (with the reasonable assumption that additional polyglutamates do not affect the reactive moieties), we again may conclude that cleavage of the enzyme-FdUMP covalent bond in this complex either is pre-equilibrium or is rate-determining with a very late transition-state in which sp^2 rehybridization of the 6-C of FdUMP essentially is complete. For reasons

just discussed, the latter is considered unlikely; the difference in rates of dissociation of FdUMP from complexes formed with CH_2-H_4PteGlu$_5$ versus CH_2-H_4PteGlu must reflect rate-determining step(s) subsequent to k_2. This is in agreement with a number of studies (Friedkin et al., 1974; Galivan & Maley, 1976b, Galivan et al., 1977; Dolnick & Cheng, 1978; Kisliuk & Gaumont, 1979) which have demonstrated that pteroyl-polyglutamates bing much tighter to dTMP stnthetase than do pteroyl-monoglutamates; i.e., ternary complexes formed with the former are more stable and dissociate more slowly.

The isotope effect data presented in this study do not permit an unequivocal assignment of the temporal position where the interconversion of the iminium ion, 5-CH_2=$^+H_4$folate, and 5,10-CH_2-H_4folate occurs, at k_2/k_{-2}, k_3/k_{-3} or k_4/k_{-4}, but as discussed it argues strongly against k_2/k_{-2}. However, consideration of chemistry involved, and aforementioned observations provoke an interesting speculation on this aspect. As described previously, various spectral measurements suggest that the pteridine and/or the p-aminobenzoylglutamate functions of CH_2-H_4folate are perturbed upon formation of non-covalent, rapidly reversible FdUMP·CH_2-H_4folate·dTMP synthetase complexes. We suggest that these perturbations result from favorable interactions of these moieties of the cofactor with the enzyme which results in a "substrate-induced strain" of the labile 5-membered, methylene-bridged ring and as a result converts it to the 5-iminium ion; concomitant would be an enzyme conformational change. This may occur either in a concerted fashion upon binding of the second ligand to form the non-covalent,

ternary complex, k_{-4}/k_4, or immediately thereafter as a separate step, k_{-3}/k_3.

Concluding Remarks. There is good reason to believe that nucleophilic attack at the 6-position of the uracil or cytosine heterocycle may be a common mechanistic feature utilized by many enzymes to enhance the reactivity at various sites of the heterocycle (Santi et al., 1978). From what is known thus far, this is almost certainly correct for a variety of enzyme-catalyzed electrophilic substitution reactions occurring at the 5-position of these heterocycles. These would include the dUMP- and dCMP-hydroxymethylases, the pyrimidine methylases of nucleic acids, pseudo-uridylate synthetase, and a large number of yet uncharacterized enzymes that alkylate the 5-position of minor bases found in tRNA. Indeed, this lab recently has demonstrated that dUMP hydroxymethylase from SP01-infected Bacillus subtilis proceeds by this mechanism (Kunitani & Santi, 1980). In addition, compared to the unaltered bases, 5,6-dihydrouracil and 5,6-dihydrocytosine derivatives are chemically much more reactive both toward nucleophilic substitution at the 4-position of the heterocycle and towards glycosidic bond cleavage (Santi et al., 1978). It is, therefore, not unreasonable to suggest that at least some of the fifty or more enzymes that catalyze such reactions might also operate via nucleophilic attack at the 6-position of the heterocycle to achieve saturation of the 5,6-double bond. The use of secondary α-hydrogen isotope effects provides a simple tool that may be useful in ascertaining whether these hypotheses are correct. Although dTMP synthetase has been used as a paradigm in this and a recently

reported study (Garrett et al., 1979; Bruice et al., 1980), the general procedures should be useful in detecting dihydropyrimidine intermediates formed in other chemical and enzymic reactions, providing rehybridization of carbon occurs before or at the rate-determining step.

CHAPTER 3

ISOTOPE-TRAPPING AND ISOTOPE EXCHANGE PROBES OF THE INTERACTION OF THYMIDYLATE SYNTHETASE WITH 5-FLUORO-2'-DEOXYURIDYLATE AND 5,10-METHYLENE-5,6,7,8-TETRAHYDROFOLATE[4]

ABSTRACT

Using a modification of the isotope-trapping method (Rose, 1980), the formation of *productive*, noncovalent binary complexes with thymidylate (dTMP) synthetase and [6-^{3}H]5-fluoro-2'-deoxyuridylate ([6-^{3}H]-FdUMP) or (6*R*),*L*-5,10-methylene-[6-^{3}H]5,6,7,8-tetrahydrofolate ((6*R*),*L*-CH_2-[6-^{3}H]H_4folate) was demonstrated; when added to solutions containing large molar excesses of unlabeled FdUMP and CH_2-H_4folate there was identical partitioning under optimal conditions of both binary complexes between irreversible dissociation of radioactivity and maximal "trapping" of *ca.* 0.8 mol of high specific radioactivity covalent ternary complex/mol of enzyme dimer. These results show that the order of binding to the native enzyme is fully random. Also, with the concentrations of ligands used the overall rate-determining step in formation of covalent complexes may be deduced to occur after initial formation of noncovalent ternary complexes. Equilibrium dissociation constants for the noncovalent, binary complexes were obtained using this technique: K_d^{FdUMP} = 21 μ*M* and $K_d^{CH_2\text{-}H_4folate}$ = 44 μ*M*. The concentrations of the unlabeled ligands which give one-half the maximum amount of trapping of binary complexes in the final covalent ternary complexes, $K_{\frac{1}{2}}$, also were determined; for trapping of enzyme·[6-^{3}H]FdUMP by (6*R*),*L*-CH_2-H_4folate $K_{\frac{1}{2}}^{CH_2\text{-}H_4folate}$ = 150 μ*M*, similarly $K_{\frac{1}{2}}^{FdUMP}$ = 18 μ*M*. The extent of partitioning to covalent ternary complexes was greatly inhibited (*ca.* 90%) for enzyme·(6*R*),*L*-CH_2-[6-^{3}H]H_4folate complexes and was inhibited to a lesser extent for enzyme·[6-^{3}H]FdUMP complexes by high concentrations (m*M*) of unlabeled CH_2-H_4folate. Binding of CH_2-H_4folate to a second site to form nonproductive complexes is offered as one likely explanation. Rates of isotope exchange at 25° C of varying concentrations of unlabeled FdUMP and (6*R*),*L*-CH_2-H_4folate for labeled ligands in 1.0 μ*M* covalent [2-^{14}C]FdUMP—(6*R*),*L*-CH_2-[6-^{3}H]H_4folate—dTMP synthetase complexes were measured and in all cases were first-order over > 2 half-lives. There was a 3.3-fold decrease in the half-life for exchange of

[6-^{3}H]FdUMP as the [CH_2-H_4folate] was raised from 0.1 to 1.0 m$\underline{M}$ holding the [FdUMP] at 0.1 m$\underline{M}$. A kinetic analysis reveals that the binding order under the conditions of these experiments is completely ordered with FdUMP binding before CH_2-H_4folate in agreement with results of recently reported, similar experiments (Danenberg & Danenberg, 1978). An hypothesis is presented which accounts for the different binding order results of the isotope-trapping and isotope exchange experiments while satisfying microscopic reversibility.

In the preceding chapter, the magnitude of secondary α-hydrogen isotope effects accompanying the interaction of dTMP[1] synthetase with FdUMP and CH_2-H_4folate provides conclusive evidence that this partial reaction proceeds *via* nucleophilic attack at the 6-position of the heterocycle and results in formation of a 5,6-dihydropyrimidine covalent complex. The formation of transient 5,6-dihydropyrimidine intermediates recently has been demonstrated in reactions *catalyzed* by dTMP synthetase (Pogolotti *et al.*, 1979; Garrett *et al.*, 1979). Thus, these results corroborate previous evidence (see Pogolotti & Santi, 1977) that the steps of the partial reaction with the inhibitor are analogous to those of the normal reaction with dUMP to the point of formation of covalent complexes.

Considerable confusion and controversy remains concerning non-catalytic features of the enzyme mechanism such as the binding stoichiometry, binding order, and whether the subunits are functionally identical and independent (see Galivan *et al.*, 1976a; Danenberg & Danenberg,

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[4]Certain portions of this work were performed previously in this laboratory, but have not been published. Equilibrium dialysis of binding of FdUMP to dTMP synthetase was performed by Dr. Norman Gravitz and some of the isotope-trapping experiments were performed preliminarily by Dr. Yasuko Tomozawa.

1978; 1979, and references cited therein). Encouraged by our earlier success with secondary isotope effect analyses, here we describe the application of additional isotope probes to the FdUMP partial reaction as a most effective means to address such questions.

Primarily, we have employed a modification of the isotope-trapping method (Krishnaswamy et al., 1962; Rose et al., 1974; for a recent review see Rose, 1980) which can provide information relating both to kinetic and equilibrium aspects of bisubstrate reactions. In particular, the ability to ascertain the existence of a productive binary complex and higher order of association complexes in rapid equilibrium with such a complex is the outstanding feature of the isotope-trapping method which distinguishes it from approaches such as steady-state kinetics and equilibrium dialysis.

Recently, Danenberg and Danenberg (1978) studied isotope exchange (for recent reviews, see Boyer, 1978; Purich, 1980) of a single ligand ([^{3}H]FdUMP or [^{14}C]CH_2-H_4folate) in covalent FdUMP——CH_2-H_4folate—dTMP synthetase complexes and concluded that association and dissociation of nucleotide and cofactor are fully ordered. As our preliminary isotope-trapping results gave evidence for a random contribution to binding, we have looked at similar but more informative simultaneous isotope exchange of [^{14}C]FdUMP and [^{3}H]CH_2-H_4folate in covalent complexes.

The isotope-trapping and exchange techniques used here are shown to be highly complementary. Information obtained includes evidence for functional differences of native and fully complexed enzyme, the order of ligand association/dissociation, identification and partial

kinetic characterization of productive binding intermediates and pathways, evidence suggestive of the existence, nature and kinetic significance of non-productive complexes, and a further localization of the rate-determining step(s).

In the following chapter, integration of results of the isotope effect, trapping and exchange studies reported in this work allows a formulation of a minimal model of the formation/breakdown of covalent FdUMP—CH_2-H_4folate—dTMP synthetase complexes which is consistent with current understanding of the mechanism of dTMP synthetase.

EXPERIMENTAL PROCEDURES

Materials and Methods. Wherever appropriate, materials described in the previous chapter were used. These included dTMP synthetase, H_2folate reductase, G-6-P dehydrogenase, H_2folate, (6R,S),L-H_4folate, (6R),L-H_4folate, (6R),L-CH_2-H_4folate, enzymatically synthesized FdUMP and [2-^{14}C]FdUMP, and N-methyl morpholine (NMM). Hexokinase from yeast (500 units/mg) was purchased from Sigma Chemical Co. and was assayed by the method of Darrow and Colowick (1962). D-[1-^{3}H]-glucose (18.0 Ci/mmol) was obtained from New England Nuclear. (6R,S),L-CH_2-[6-^{3}H]H_4folate was prepared as previously described (Santi et al., 1976a). ATP, D-G-6-P, β-NAD, β-NADP and FdUMP were from Sigma and D-glucose was from Mallinckrodt. Cellulose dialysis membranes were from Union Carbide, and equilibrium dialysis was performed in micro-dialysis cells produced by Peter Hoeffer Co.

Reverse phase HPLC was performed at ambient temperature with

1.0 ml/min flow rates; nucleotides were monitored at 254 nm and folate derivatives at 280 nm. System A contained 5 mM $(n\text{-}Bu)_4N^+$ phosphate (pH 7.5) and 30% (V/V) MeOH and system B contained 0.1 M potassium phosphate (pH 6.0) and 5% (V/V) acetonitrile and were used with a Lichrosorb C_{18} column (4.6 x 250 mm). System C contained 0.1 M potassium phosphate (pH 6.0) and 1% (V/V) acetonitrile and was used with an Altex Ultrasphere column (4.6 x 250 mm). Determinations of radioactivity, the standard NMM buffer used, formation and isolation on nitrocellulose filters of covalent, ternary complexes, and miscellaneous procedures were as described in the preceding chapter. One unit of enzyme activity refers to conversion of one μmol of substrate/min under specified assay conditions.

Preparation of (6R),L-CH_2-[6-^{3}H]H_4folate. A modification of the method of Pastore and Friedkin (1962) was used. The initial reaction mixture (4.95 ml) contained in order of addition: 520 mM Tris·HCl (pH 7.2), 156 mM 2-mercaptoethanol, 12 mM $MgCl_2$, 20 mM H_2folate, 41 mM H_2CO, 2.0 mM D-[1-^{3}H]glucose (316 mCi/mmol), 4.1 mM ATP, 2.2 mM β-NADP, 9.62 units/ml hexokinase, 20.2 units/ml G-6-P dehydrogenase, and 6.1 units/ml H_2folate reductase. Reaction was initiated with H_2folate reductase; there was no further decrease in A_{340} after 35 min. G-6-P (290 μmol in 200 μl) was then added to a final concentration of 56 mM. Further decrease in A_{340} was nearly complete in 15 more min, but the reaction was allowed to proceed for 95 min total. The overall decrease in A_{340} indicated ca. 100% product formation, and HPLC analysis in System A showed 97% of total radioactivity associated with the product

peak. The reaction was cooled to 0° C, quenched with 10 vols (50 ml) of 0.4 M $Et_3N^+H \cdot HCO_3^-$ (pH 9.5) and was lyophilized. The residue was redissolved in a minimal vol. (ca. 3.3 ml) of 0.4 M $Et_3N^+H \cdot HCO_3^-$ (pH 9.5), 20 mM 2-mercaptoethanol and 1 mM H_2CO and insolubles were removed by centrifugation. The solution was applied to a Biogel P-2 column (1.5 x 75 cm; 200-400 mesh) previously equilibrated and then eluted with 20 mM $Et_3N^+H \cdot HCO_3^-$ (pH 9.5), 20 mM 2-mercaptoethanol and 1 mM H_2CO at a flow rate of 16 ml/hr at 4° C, protected from light. 2 ml fractions were collected and fractions 31 to 43 were pooled. These corresponded to the center of the peak containing (6R),L-CH_2-[6-^{3}H]H_4folate as assayed by radioactivity and with dTMP synthetase by the method of Santi and Sakai (1971); 56% of ^{3}H dpm in the reaction were recovered. The cofactor was determined to be at least 99% radiochemically pure by HPLC analysis (System A, RV = 8.2 ml; system B, RV = 28 ml) and was 96% pure by spectrophotometric assays with dTMP synthetase; specific radioactivity was 39 mCi/mmol. The pooled fractions were lyophilized, re-dissolved in 3.9 ml of 20 mM $Et_3N^+H \cdot HCO_3^-$ (pH 9.5), 20 mM 2-mercaptoethanol and 30 mM H_2CO to a concentration of 12 mM of (6R),L-CH_2-[6-^{3}H]H_4folate, and stored under argon at -80° C.

Isotope Trapping: Partitioning of Noncovalent, Binary Complexes. Pulse solutions (Rose, 1980) containing dTMP synthetase and [6-^{3}H]FdUMP or CH_2-[6-^{3}H]H_4folate, and chase solutions containing FdUMP and CH_2-H_4folate in large molar excess over the labeled ligand, were both made in standard NMM buffer and equilibrated 10 to 30 min at ambient temperature. Aliquots of 50 µl of pulse solutions were injected into 450 µl

of chase solutions in 1.5 ml polypropylene tubes and rapid mixing under a stream of argon was achieved with a micro stir bar (2 x 7 mm) and magnetic stirrer. Reaction mixtures were sealed under argon, kept at ambient temperature 10 to 30 min and placed on ice prior to assaying. Within 1 hr the radioactivity trapped in covalent, ternary complexes in 200 to 400 µl aliquots of the reaction mixture was determined by adsorption to nitrocellulose filters followed by liquid scintillation counting; total radioactivity was determined by counting 20 to 40 µl of the reaction mixture. After mixing pulse and chase solutions the trapped dpm reached a constant value in < 1-2 min, and remained constant over at least 60 min at 4 ° C. Total filter-bound dpm were corrected both for background dpm of controls omitting the second ligand in the chase required for complex formation, < 0.13% of dpm applied/filter and < 3% of dpm bound/filter, and for filtration efficiency (Santi et al., 1974a), typically ca. 90%; they were not corrected for binding of low specific activity ligands of controls where enzyme was added to solutions of pre-mixed labeled and unlabeled ligands, since the dpm bound were ≤ background dpm.

Where indicated, binding curves were constructed by computerized, non-linear least-squares curve fitting to the appropriate binding equation. Estimated K_d values were used to calculate concentrations of unbound ligands in pulse solutions which were used to obtain improved estimates of K_d values by curve fitting. This procedure was reiterated until the error in consecutively determined K_d values was maximally 1%.

The concentrations of total enzyme binding sites for each series of pulse solutions was determined: an excess of the labeled ligand and the unlabeled second ligand required for ternary complex formation were added to the solution of enzyme and radioactivity of complexes formed was determined after adsorption to nitrocellulose filters.

Isotope Exchange: Kinetics of Dissociation of Covalent, Ternary Complexes. To identical solutions of the double-labeled [2-^{14}C]FdUMP—CH_2-[6-^{3}H]H_4folate—dTMP synthetase complex were added variable amounts of unlabeled FdUMP and (6R),L-CH_2-H_4folate. Final solutions (1.2 ml) contained in the standard NMM buffer, 1.0 μM [2-^{14}C]FdUMP (52 mCi/mmol), 1.0 μM (6R),L-CH_2-[6-^{3}H]H_4folate (37 mCi/mmol), 0.5 μM dTMP synthetase, 450 μM K phosphate from the enzyme stock solution buffer and either 0.1 mM (6R),L-CH_2-H_4folate and 0, 0.1, 0.3, 1.0 or 3.0 mM FdUMP, or 0.1 mM FdUMP and 0, 0.1, 0.3, 1.0 or 3.0 mM (6R),L-CH_2-H_4folate. Control solutions omitted either [2-^{14}C]FdUMP or (6R),L-CH_2-[6-^{3}H]H_4folate and also FdUMP and (6R),L-CH_2-H_4folate. The mixtures were kept at 25.0° , under argon, protected from light. Aliquots of 100 μl (ca. 8.3 x 10^3 ^{3}H dpm) were removed at intervals up to ca. 14 hr and the ternary complexes were isolated by adsorption on nitrocellulose filters and then counted.

Experiments also were performed identically to those described except that potassium phosphate was either present at 50 mM (pH 7.2) or was completely removed by gel-filtration of stock enzyme (in 60 mM K phosphate (pH 6.0) and 1 mM EDTA) on Sephadex G-25 with standard NMM eluting buffer.

Equilibrium Dialysis. Cellulose membranes were boiled for 10 min in 5% Na_2CO_3 solution containing 50 mM EDTA, washed well in distilled water and stored at 5° C in 10% aqueous ethanol. Solutions (100 µl) of protein and [2-^{14}C]FdUMP (24.5 mCi/mmol) were added to opposite sides of the membrane to give final concentrations of 7.5 µM dTMP synthetase and from 1 to 300 µM FdUMP in standard NMM buffer; controls omitted enzyme. Dialysis was performed at ambient temperature and equilibrium was achieved in ca. 3 hr. Duplicate 40 µl aliquots were removed from each side of the membrane after 3-4 hr and the radioactivity in each was determined. The amount of dTMP synthetase-bound FdUMP was determined from the difference in dpm between the two sides of the membrane. The concentration of free FdUMP was calculated as the difference between the total radioactivity and the bound radioactivity on the protein side of the membrane and was corrected for a 4% [2-^{14}C]FdUrd (RV = 4.0 ml) impurity in the [2-^{14}C]FdUMP used as determined by HPLC (system A).

RESULTS

Isotope-Trapping: Partitioning of Noncovalent, Binary FdUMP· or CH_2-H_4folate·dTMP Synthetase Complexes. The dependence of the amount of high specific radioactivity complex formed on the concentration of [6-^{3}H]FdUMP in the pulse is shown in Figure 12. The pulse solution (50 µl) contained 6.2 to 120 µM [6-^{3}H]FdUMP (16 mCi/mmol) and 8.0 µM dTMP synthetase. The chase solution (450 µl) contained 660 µM unlabeled FdUMP, ca. 50 to 1000 times that in the pulse solution, and 830 µM (6R),L-CH_2-H_4folate. The dissociation constant for the noncovalent,

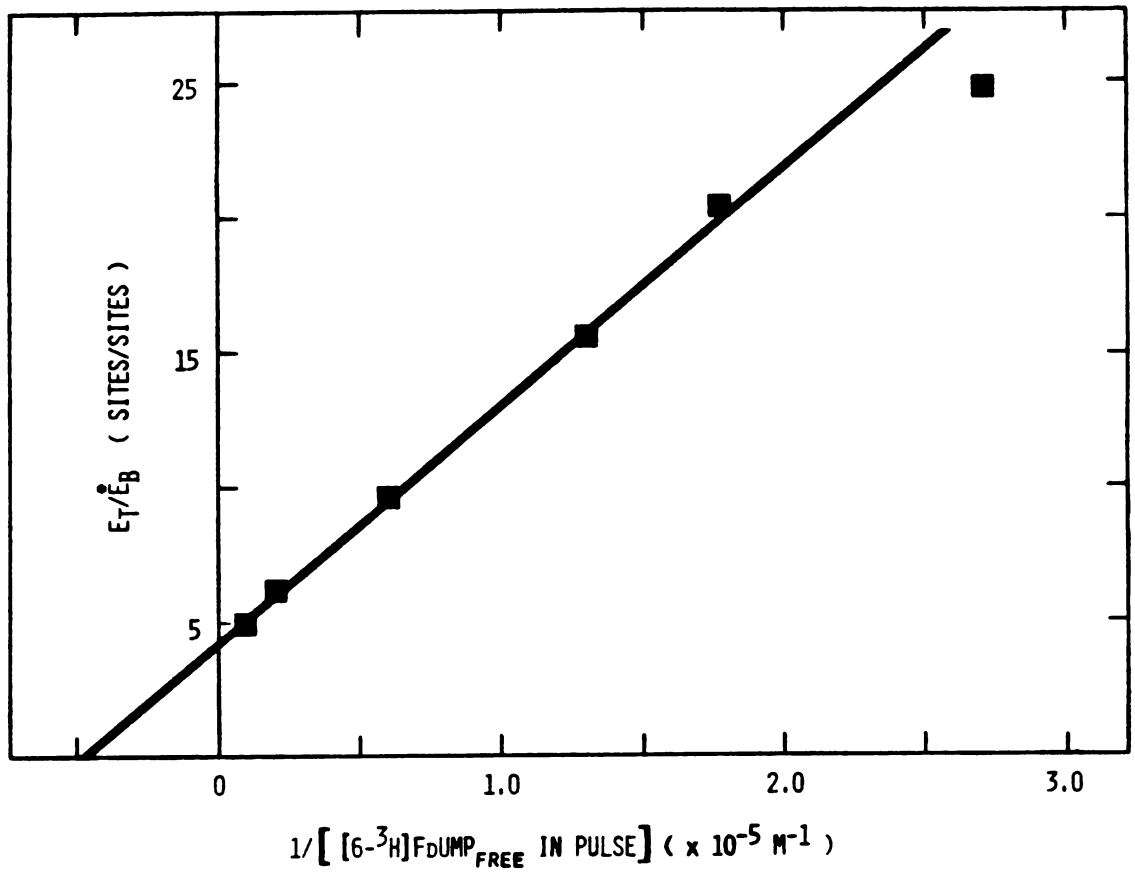


FIGURE 12: The dependence of isotope-trapping of radioactive, covalent complexes on the concentration of [6-^{3}H]FdUMP in pulse solutions. Pulse solutions contained 8.0 μM dTMP synthetase and 6.2-120 μM [6-^{3}H]FdUMP (16 mCi/mmol). Chase solutions contained 660 μM FdUMP and 830 μM (6R),L-CH_2-H_4folate. The data points represent the ratio of the concentrations of total available binding sites for formation of ternary complexes, E_T, to that of nitrocellulose filter-bound, high specific radioactivity, covalent ternary complexes, E_B^*, that are trapped as a function of the calculated concentration of unbound [6-^{3}H]FdUMP in pulse solutions. The solid line depicts the computer generated best fit of the data points to the double-reciprocal form of a single ligand binding equation (Segel, 1975). Experimental details are described in the text.

binary enzyme·FdUMP complex, K_d^{FdUMP}, calculated from the abcissa intercept is 21 μM. In two experiments performed identically except that (6R,S),L-CH_2-H_4folate was used in the chase, values for K_d^{FdUMP} of 16 and 25 μM were obtained. The ordinate intercept of Figure 12 shows that for an extrapolated infinite concentration of [6-^{3}H]FdUMP in the pulse for this experiment, the fraction of total enzyme binding sites trapped in covalent complexes is 0.24. For the experiments using (6R,S),L-CH_2-H_4folate in the chase values of 0.24 and 0.30 were obtained.

In Figure 13 is shown the effect of varying the concentration of (6R),L-CH_2-H_4folate in the chase from 83 to 830 μM on the amount of tritiated complex formed. The pulse solution contained 47 μM [6-^{3}H]FdUMP (16 mCi/mmol) and 7.5 μM dTMP synthetase, and there was 2.2 mM FdUMP in the chase. From the abcissa intercept is obtained the value of 0.15 mM for $K_{\frac{1}{2}}^{CH_2-H_4folate}$, the concentration of (6R),L-CH_2-H_4folate in the final pulse-chase solution required for half-maximal trapping of binary enzyme·FdUMP complexes (Rose, 1980). A similar experiment performed using (6R,S),L-CH_2-H_4folate in the chase yielded a value for $K_{\frac{1}{2}}^{CH_2-H_4folate}$ of 0.31 mM. From the ordinate intercept of Figure 13 it is seen that at an extrapolated infinite concentration of (6R),L-CH_2-H_4folate in the chase for this experiment, the expected fraction of total enzyme binding sites trapped in covalent complexes is 0.27. Using the K_d^{FdUMP} value of 21 μM, the concentration of the enzyme·FdUMP complex in the pulse for the experiment of Figure 13 is calculated to be 4.8 μM. The maximum fraction of total enzyme binding sites trapped in covalent complexes that can be expected at extrapolated infinite concentrations of [6-^{3}H]FdUMP in the

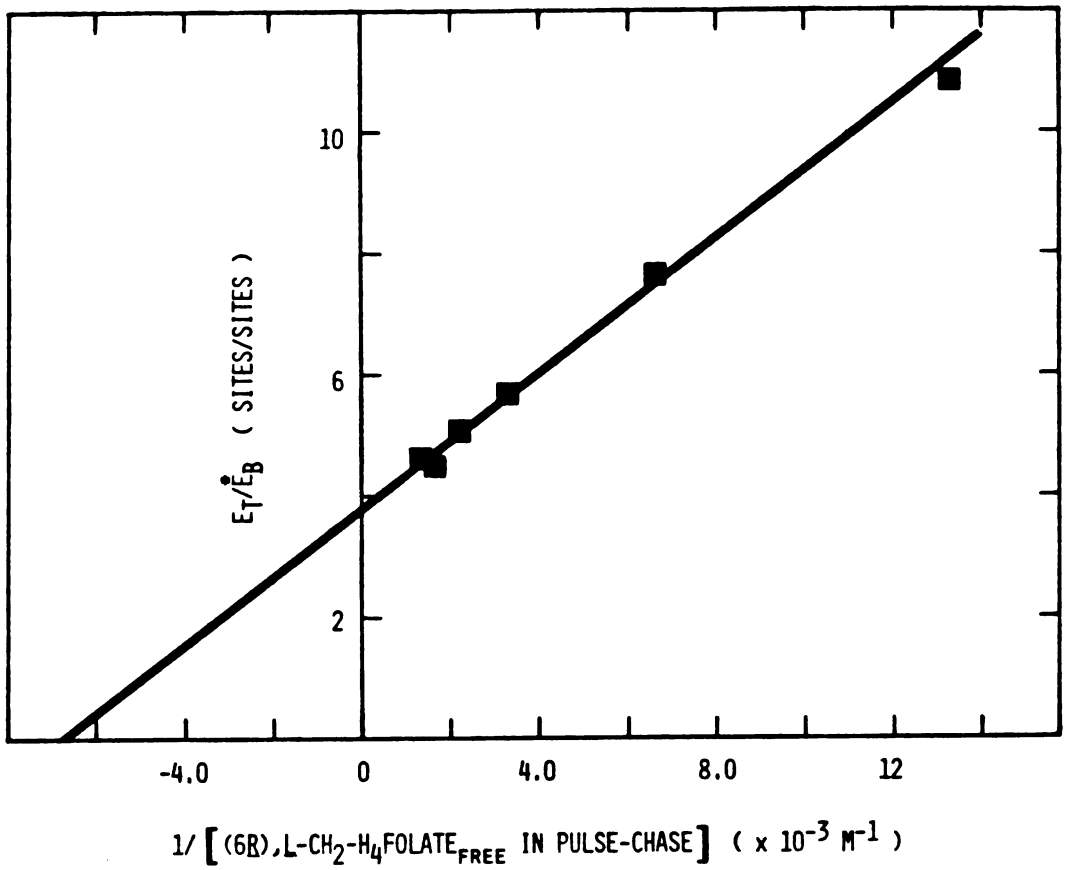


FIGURE 13: The dependence of isotope-trapping of covalent complexes containing [6-^{3}H]FdUMP on the concentration of (6R),L-CH_2-H_4folate in chase solutions. Pulse solutions contained 7.5 μM dTMP synthetase and 47 μM [6-^{3}H]FdUMP (16 mCi/mmol). Chase solutions contained 2.2 mM FdUMP and 83-830 μM (6R),L-CH_2-H_4folate. The data points represent the ratio of the concentrations of total available binding sites for formation of ternary complexes, E_T, to that of nitrocellulose filter-bound, high specific radioactivity, covalent ternary complexes, E_B^*, that are trapped as a function of the concentration of unbound (6R),L-CH_2-H_4folate in the final reaction mixtures. The solid line depicts the computer generated best fit of the data points to the double-reciprocal form of a single ligand binding equation (Segel, 1975); this equation uses values for the unbound concentration of the variable ligand, but in this experiment the total and free concentrations are essentially identical (±<1%). Experimental details are described in the text.

pulse and of (6R),L-CH_2-H_4folate in the chase, or the partition coefficient, is calculated as (15.0 μM enzyme sites in the pulse/9.6 μM sites as enzyme·FdUMP complex in the pulse) x 0.27 and is 0.42.

The dependence of the amount of complex formed with [6-^{3}H]FdUMP 157 mCi/mmol) on the concentration of unlabeled FdUMP in the chase of from 0.069 to 6.9 mM, is depicted in Figure 14; the broken line represents the theoretical curve if the effect were solely one of binding of diluted specific activity [6-^{3}H]FdUMP in the pulse-chase solution in the case of no trapping due to complete dissociation of the high specific activity [6-^{3}H]FdUMP. In this experiment there was 24 μM enzyme and 450 μM [6-^{3}H]FdUMP in the pulse and 0.34 or 8.0 mM (6R,S),L-CH_2-H_4folate in the chase. The extent to which the experimental curves are displaced upwards from the theoretical curve represents the amount of trapping at the indicated unlabeled FdUMP concentration. It is noted that the other experiments described above used concentrations of from 0.66 to 2.2 mM of FdUMP in chase solutions.

In Figure 15 is shown the dependence of the amount of tritiated complex formed on the concentration of (6R),L-CH_2-[6-^{3}H]H_4folate (39 mCi/mmol) in the pulse solution. The pulse (50 μl) contained 7.4 to 370 μM [^{3}H]cofactor and 11 μM dTMP synthetase. The chase solution (450 μl) contained 14 mM of unlabeled (6R,S),L-CH_2-H_4folate, ca. 170 to 8,400 times more of the (6R)-isomer as in the pulse solution, and 3.8 mM FdUMP. The $K_d^{CH_2\text{-}H_4\text{folate}}$ value obtained from this curve is 44 μM. In a similar experiment using 4.0 to 280 μM (6R,S),L-CH_2-[6-^{3}H]H_4folate (163 mCi/mmol) and 7.3 μM dTMP synthetase in the pulse, and 2.4 mM

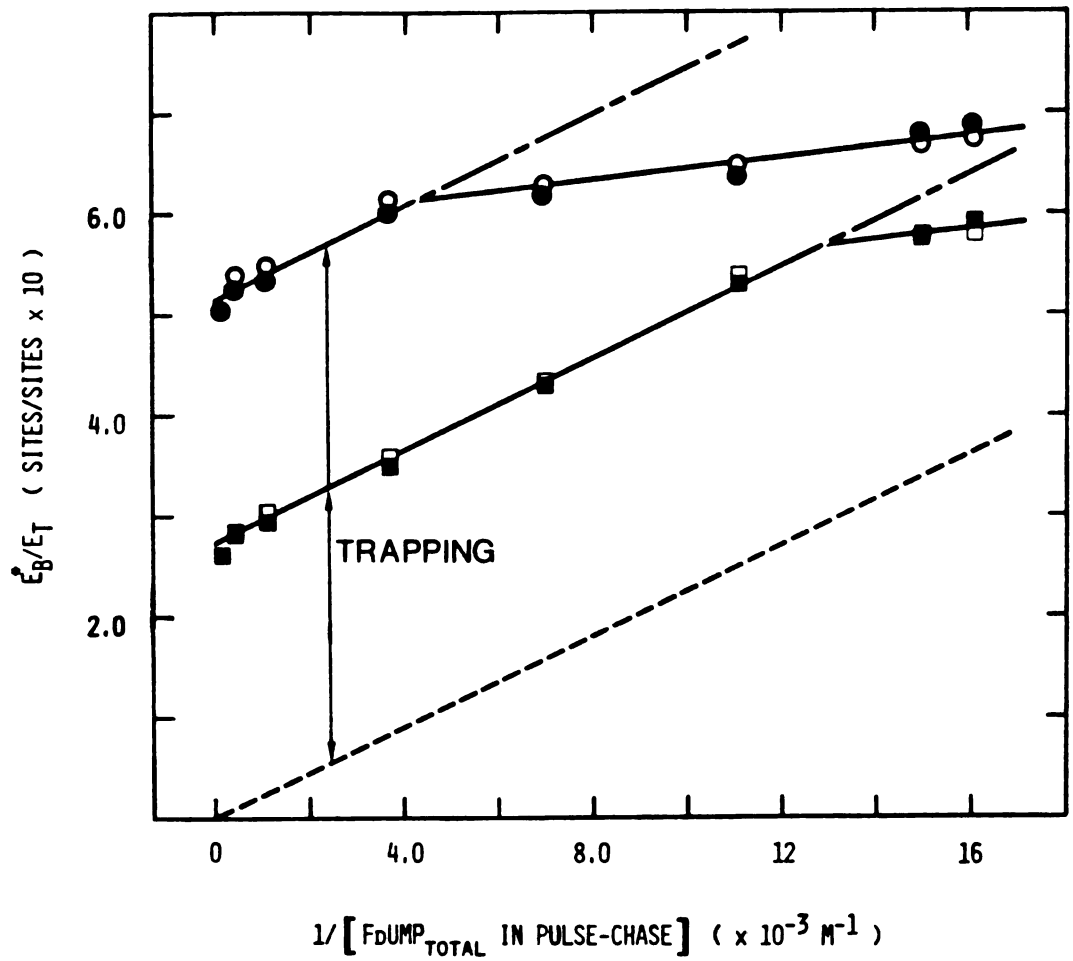


FIGURE 14: The dependence of isotope-trapping of covalent complexes containing [6-^{3}H]FdUMP on the concentration of FdUMP in chase solutions at two different concentrations of (6R,S),L-CH_2-H_4folate in chase solutions. Pulse solutions contained 24 μM dTMP synthetase and 450 μM [6-^{3}H]FdUMP (157 mCi/mmol). Chase solutions contained 0.069-6.9 mM of enzymatically synthesized (○,□) or chemically made (●,■) FdUMP and 0.34 mM (□,■) or 8.0 mM (○,●) (6R,S),L-CH_2-H_4folate. The data points represent the ratio of the concentrations of nitrocellulose filter-bound, high specific radioactivity, covalent ternary complexes, E^*_B, that are trapped as a function of the total concentration of FdUMP in the final reaction mixture, to that of total available binding sites for formation of ternary complexes, E_T. The solid lines depict the visually determined best fit of the data points to straight lines. The dashed line through the origin represents the calculated ratio of low specific radioactivity E^*_B/E_T expected as an artifact of incomplete dilution of free [6-^{3}H]FdUMP with FdUMP as described in the text. The vertical arrows depict the extent of trapping of only high specific radioactivity E^*_B. Experimental details are described in the text.

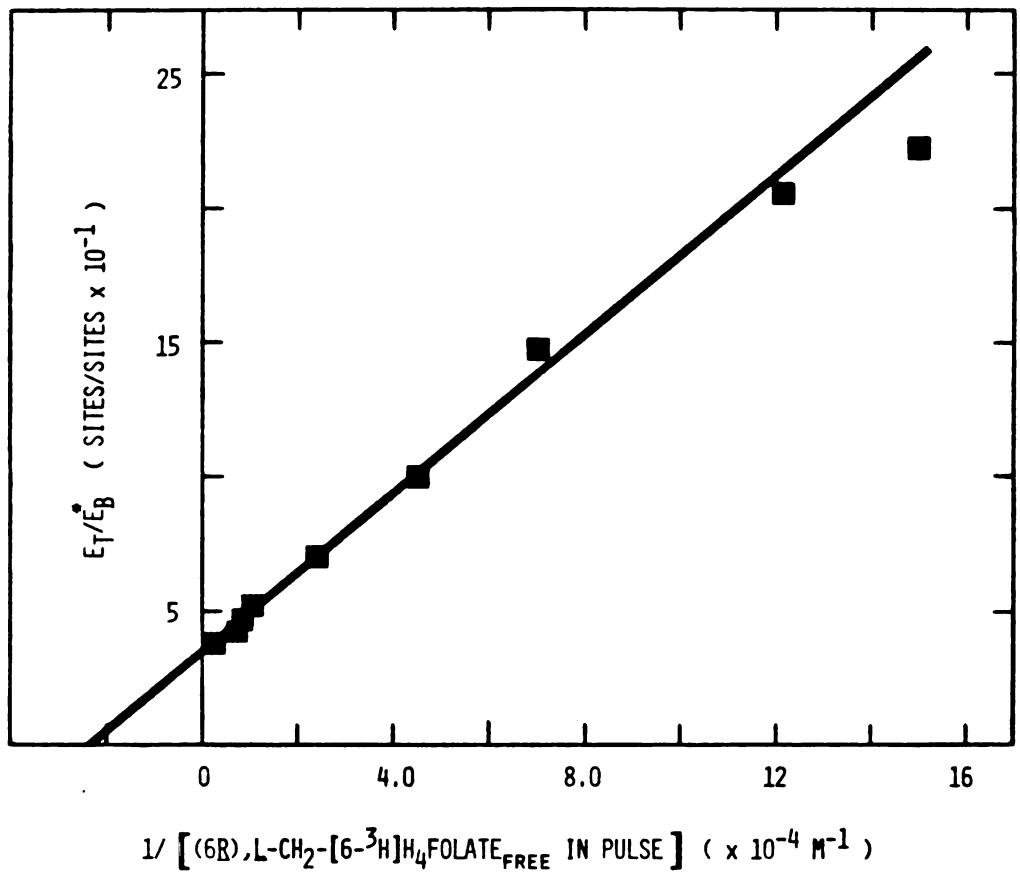


FIGURE 15: The dependence of isotope-trapping of radioactive, covalent complexes on the concentration of (6R),L-CH_2-[6-^{3}H]H_4folate in pulse solutions. Pulse solutions contained 11 μM dTMP synthetase and 7.4-370 μM (6R),L-CH_2-[6-^{3}H]H_4folate (39 mCi/mmol). Chase solutions contained 14 mM (6R,S),L-CH_2-H_4folate and 3.8 mM FdUMP. The data points represent the ratio of the concentration of total available binding sites for formation of ternary complexes, E_T, to that of nitrocellulose filter-bound, high specific radioactivity, covalent ternary complexes, E_B^*, that are trapped as a function of the calculated concentration of unbound (6R),L-CH_2-[6-^{3}H]H_4folate in pulse solutions. The solid line depicts the computer generated best fit of the data points to the double-reciprocal form of a single ligand binding equation (Segel, 1975). Experimental details are described in the text.

(6R,S),L-CH_2-H_4folate and 1.4 mM FdUMP in the chase, a $K_d^{CH_2-H_4folate}$ value of 40 μM was obtained. For an extrapolated infinite concentration of (6R),L-CH_2-[6-^{3}H]H_4folate in the pulse for the experiment of Figure 15, the calculated fraction of binding sites trapped in covalent complexes is 0.030; for the experiment using (6R,S),L-CH_2-[6-^{3}H]H_4folate in the pulse and a lower concentration of (6R,S),L-CH_2-H_4folate in the chase, the analogous value is 0.22. As shown in Figure 17 below, under conditions identical to those of Figures 15 and 16 here, the extent of trapping observed is the same using [^{3}H](6R)- or [^{3}H](6R,S)-cofactor in the pulse, but is affected by the concentration of (6R,S)-cofactor in the chase.

Figure 16 shows the effect of varying the concentration of FdUMP in the chase from 3.0 to 300 μM on the amount of [^{3}H]cofactor trapped in the complex; the chase also contained 14 mM (6R,S),L-CH_2-H_4folate. The pulse contained 390 μM (6R),L-CH_2-[6-^{3}H]H_4folate (39 mCi/mmol) and 11 μM dTMP synthetase. The $K_{\frac{1}{2}}^{FdUMP}$ value for trapping of noncovalent enzyme·[^{3}H]cofactor complexes by FdUMP is 18 μM. A value of 20 μM was obtained from an experiment using 290 μM (6R,S),L-CH_2-[6-^{3}H]H_4folate (163 mCi/mmol) and 7.2 μM dTMP synthetase in the pulse and 1.3 to 130 μM FdUMP and 2.6 mM (6R,S),L-CH_2-H_4folate in the chase. At an extrapolated infinite concentration of FdUMP in the chase for the experiment of Figure 16, the calculated fraction of binding sites trapped in covalent complexes is 0.021; for the experiment using racemic [^{3}H]cofactor in the pulse and a lower concentration of cofactor in the chase the value is 0.18. Using the $K_d^{CH_2-H_4folate}$ value of 44 μM, the concentration of the enzyme·(6R),L-

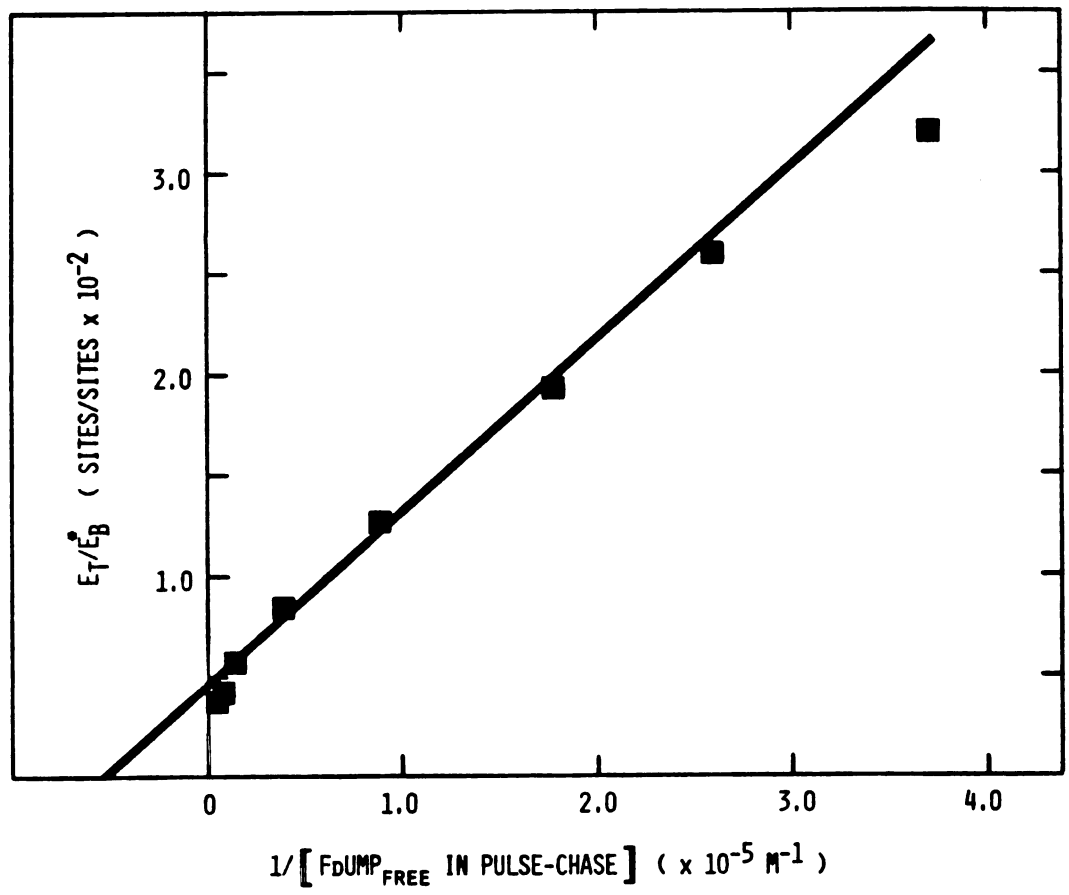
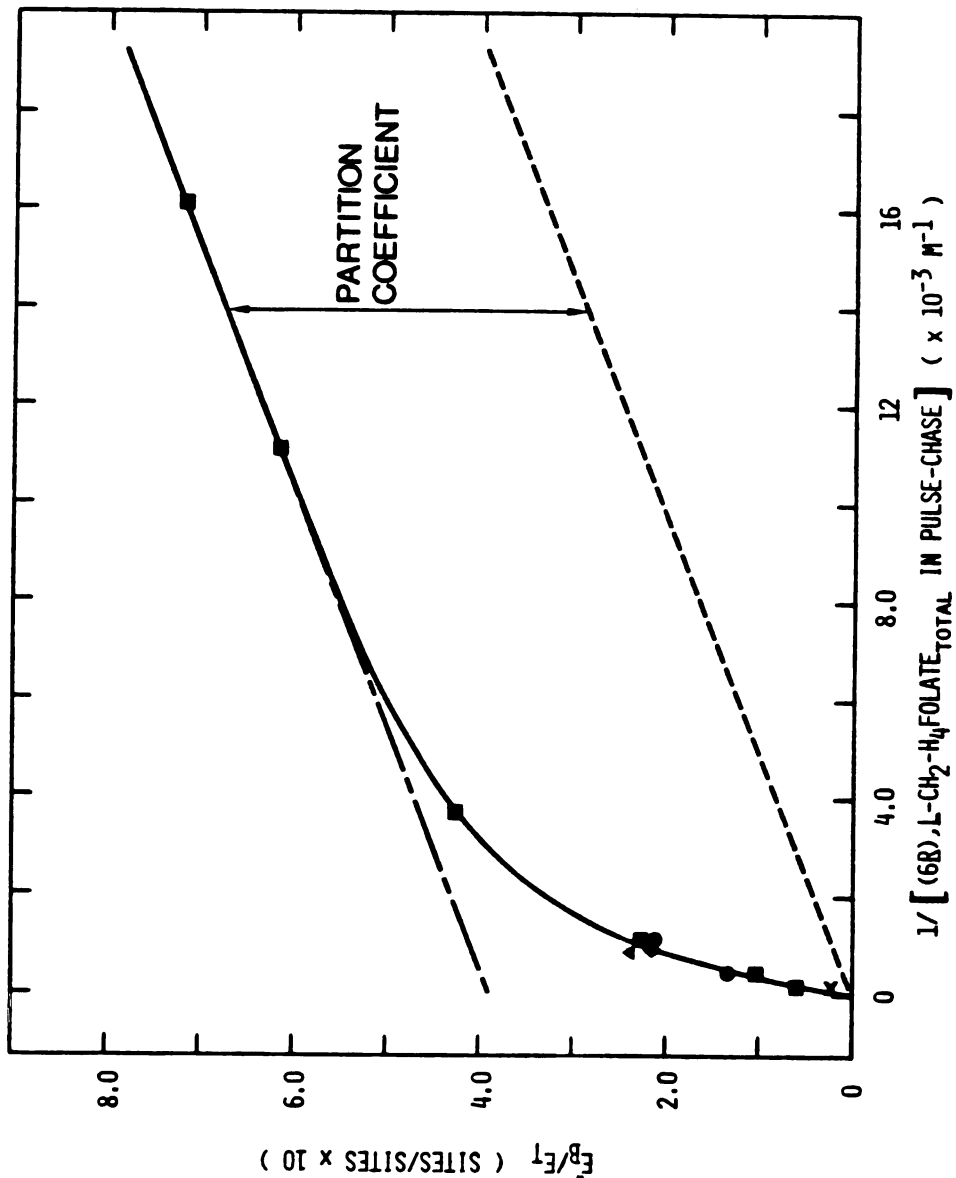


FIGURE 16: The dependence of isotope-trapping of covalent complexes containing (6R),L-CH_2-[6-^{3}H]H_4folate on the concentration of FdUMP in chase solutions. Pulse solutions contained 11 μM dTMP synthetase and 390 μM (6R),L-CH_2[6-^{3}H]H_4folate (39 mCi/mmol). Chase solutions contained 14 mM (6R,S),L-CH_2-H_4folate and 3.0-300 μM FdUMP. The data points represent the ratio of the concentrations of total available binding sites for formation of ternary complexes, E_T, to that of nitrocellulose filter-bound, high specific radioactivity, covalent ternary complexes, E_B^*, that are trapped as a function of the concentration of unbound FdUMP in the final reaction solution. The solid line is a visual best fit of the data points. A computer generated best fit of the data points to the double-reciprocal form of a single ligand binding equation (Segel, 1975) yielded a biphasic curve because of the heavy weighting of the two points at highest [FdUMP] in the reaction; for further possible significance of these two points see Appendix 3. In this experiment the total and free concentrations of the variable ligand, FdUMP, are essentially identical (±<1%). Experimental details are described in the text.

CH_2-[6-^{3}H]H_4folate complex in the pulse for the experiment of Figure 16 is calculated to be 10 μM, assuming two binding sites for cofactor/enzyme dimer. The maximum fraction of total enzyme binding sites trapped in covalent complexes that is expected as the concentrations of (6R),L-CH_2-[6-^{3}H]H_4folate in the pulse and of FdUMP in the chase approach infinity is calculated as (22 μM enzyme sites in the pulse/20 μM sites as enzyme·cofactor complexes in the pulse) x 0.021 and is 0.024. For the experiment employing racemic [^{3}H]cofactor in the pulse and a lower concentration of cofactor in the chase, the value is 0.24.

The dependence of the amount of covalent complex formed with (6R),L-CH_2-[6-^{3}H]H_4folate on the concentration from 0.14 to 14 mM of unlabeled (6R,S),L-CH_2-H_4folate in the chase is shown in Figure 17; the broken line depicts the theoretical effect of specific radioactivity dilution as described for the experiment using [^{3}H]FdUMP shown in Figure 14. The pulse contained 11 μM enzyme and 400 μM (6R),L-CH_2-[6-^{3}H]H_4folate (39 mCi/mmol), and the chase also contained 3.8 mM FdUMP. Inspection of Figures 15 and 16 shows that 400 μM [^{3}H]cofactor in the pulse and 3.8 mM FdUMP in the chase, respectively, are very near the concentrations required to give maximum trapping of labeled covalent complexes. And, the extent of displacement of the experimental curve from the curve for radioactivity dilution (Figure 17) represents the amount of trapping. Thus, at low concentrations of cofactor in the chase, where the slope of the experimental curve is as expected, a partition coefficient value of 0.39 is calculated. As the concentration of cofactor in the chase increases, a point is reached where the extent of trapping decreases. The value

FIGURE 17: The dependence of isotope-trapping of covalent complexes containing (6R),L-CH_2-[6-^{3}H]H_4folate on the concentration of CH_2-H_4folate in chase solutions. Shown are: experimental points (■) for pulse solutions containing 11 μM dTMP synthetase and 400 μM (6R),L-CH_2-[6-^{3}H]H_4folate (39 mCi/mmol) and chase solutions containing 3.8 mM FdUMP and 0.14-14 mM chemically made (6R,S),L-CH_2-H_4folate; experimental points (●) for pulse solutions containing 6.8 μM dTMP synthetase and 310 μM (6R),L-CH_2-[6-^{3}H]H_4folate (39 mCi/mmol) and chase solutions containing 3.8 mM FdUMP and 1.0 or 2.5 mM enzymatically synthesized (6R),L-CH_2-H_4folate; the point (◆) obtained from extrapolation as the concentration of labeled cofactor approaches infinity for the experiment where the pulse solutions contained 7.3 μM dTMP synthetase and 4.0-280 μM (6R,S),L-CH_2-[6-^{3}H]H_4folate (163 mCi/mmol) and chase solutions contained 1.4 mM FdUMP and 2.4 mM (6R,S),L-CH_2-H_4folate; the point (▲) corresponding to the partition coefficient (see text) for the experiment where the pulse solutions contained 7.2 μM dTMP synthetase and 290 μM (6R,S),L-CH_2-[6-^{3}H]H_4folate (163 mCi/mmol) and the chase solutions contained 1.3-130 μM FdUMP and 2.6 mM (6R,S),L-CH_2-H_4folate; and the point (✖) corresponding to the partition coefficient of Figure 16. The data points represent the ratio of the concentrations of nitrocellulose filter-bound, high specific radioactivity, covalent ternary complexes, E_B^*, that are trapped as a function of the total concentration of the (6R),L-CH_2-H_4folate isomer in the final reaction solution, to that of total available binding sites for formation of ternary complexes, E_T. The solid line depicts the visually determined best fit of the data points to a curve. The dashed line through the origin represents the calculated ratio of low specific radioactivity E_B^*/E_T expected as an artifact of incomplete dilution of free CH_2-[6-^{3}H]H_4folate with CH_2-H_4folate as described in the text. The vertical arrow depicts the extent of trapping of only high specific radioactivity E_B^* and is approximately equal to the partition coefficient at low concentrations of CH_2-H_4folate where the two lines are parallel. Experimental details are described in the text.


PARTITION
COEFFICIENT
E_B^*/E_T (SITES/SITE x 10)
8.0
6.0
4.0
2.0
0
0
4.0
8.0
12
16
$1/[(6R),L\text{-}CH_2\text{-}H_4FOLATE_{TOTAL}$ IN PULSE-CHASE$]$ (x 10^{-3} M^{-1})

for maximum trapping (0.22) obtained in the previously described experiment varying (6R,S),L-CH_2-[6-^{3}H]H_4folate in the pulse, the partition coefficient (0.24) from the experiment using this same cofactor in the pulse and varying the concentration of FdUMP in the chase, and the partition coefficient (0.024) obtained from the experiment of Figure 16, all fall on the descending portion of this curve.

In chase solutions, substitution of commercially obtained FdUMP (84% pure, HPLC system C, RV = 5.1 ml), and (6R,S),L-CH_2-H_4folate (ca. 90% pure as assayed with dTMP synthetase; Santi & Sakai, 1971), for purified compounds prepared enzymatically in this lab, had no pronounced effects on the results (see Figures 14 and 17); concentrations of FdUMP and CH_2-H_4folate reported in the above experiments are corrected for purity. In pulse solutions containing [^{3}H]FdUMP, the presence or absence of 6.5 mM H_2CO did not affect the results, viz. possible hydroxymethylation of the enzyme; H_2CO was required to stabilize [^{3}H]CH_2-H_4folate in pulse solutions.

Isotope Exchange: Kinetics of Dissociation of Covalent, Ternary FdUMP—CH_2-H_4folate—dTMP Synthetase Complexes. The dissociation of the [2-^{14}C]FdUMP—(6R),L-CH_2-[6-^{3}H]H_4folate—dTMP synthetase complexes in all cases was shown to be first-order. When the concentrations of unlabeled cofactor were held constant and those of unlabeled FdUMP were varied, the rates of dissociation of labeled FdUMP were the same, $k = 1.09 \pm 0.02$(S.E.) $\times 10^{-1}$ hr^{-1} (Figure 18, A), as were those for labeled cofactor, $k = 1.04 \pm 0.02 \times 10^{-1}$ hr^{-1} (Figure 18, B); when unlabeled FdUMP was omitted there was no dissociation of [2-^{14}C]FdUMP (Figure 18, A). When the concentrations of unlabeled FdUMP were held constant and those

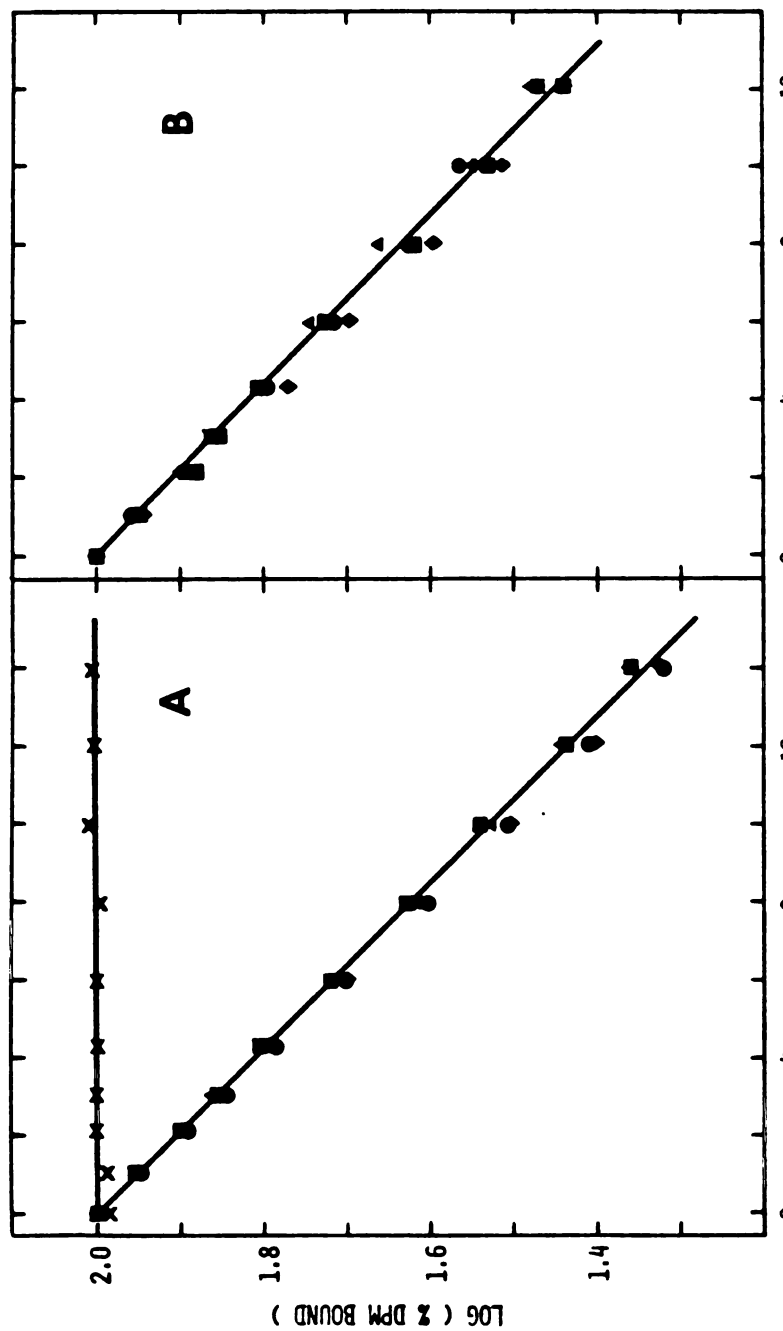


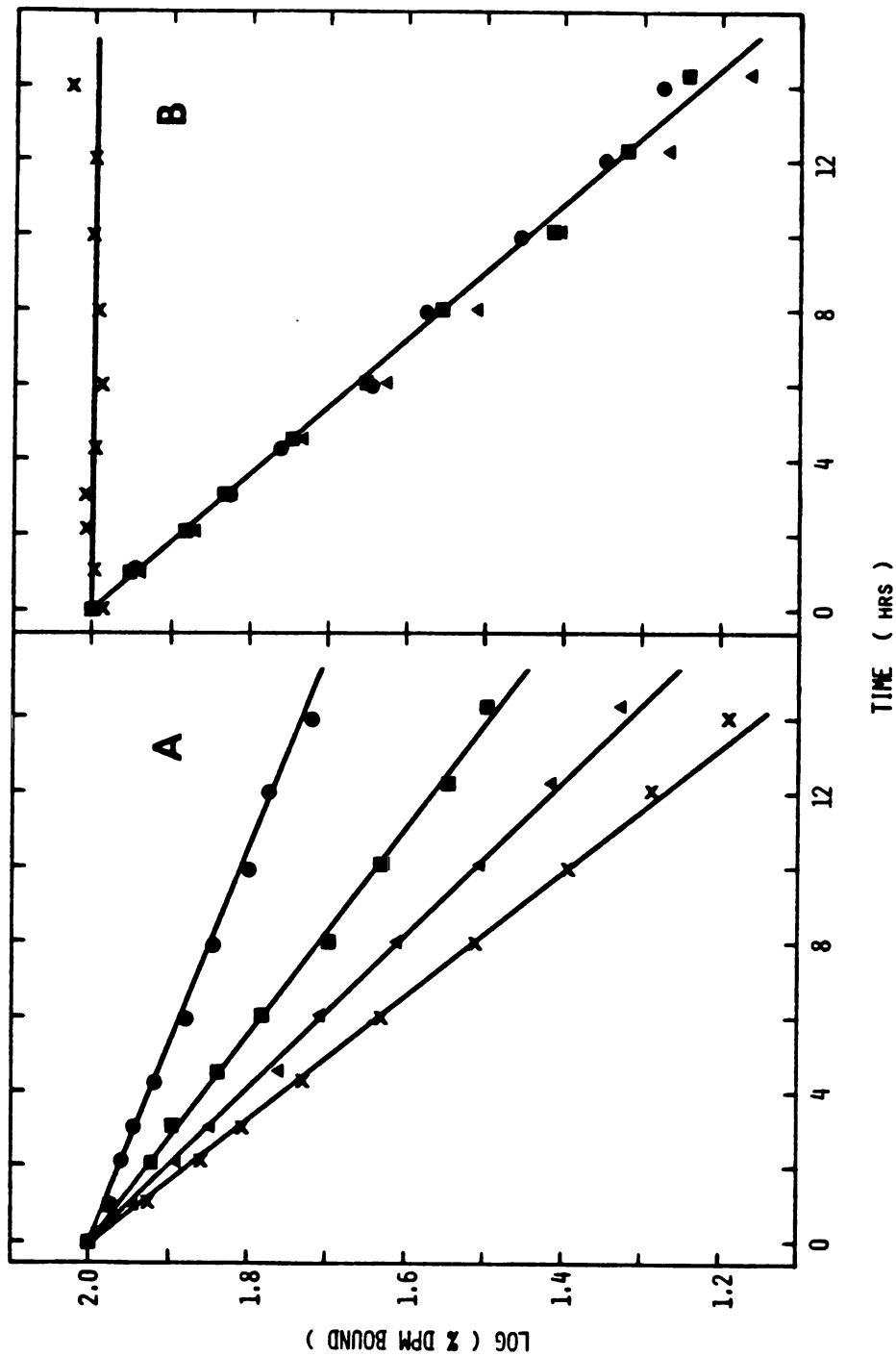
FIGURE 18: The dependence of the rates of isotope exchange of ligands in covalent [2-^{14}C]FdUMP—(6R),L-CH_2-[6-^{3}H]H_4folate—dTMP synthetase complexes on the concentration of FdUMP. The data points represent the [2-^{14}C]FdUMP, **A**, and the (6R),L-CH_2-[6-^{3}H]H_4folate, **B**, bound in the complex isolated over the course of the reaction. The reaction mixtures contained 1.0 μM double-labeled complex, 1.0 μM (6R),L-CH_2-H_4folate and 0 (✕), 0.1 (▲), 0.3 (■), 1.0 (●), and 3.0 mM (◆) FdUMP. Experimental details are described in the text.

of unlabeled cofactor were varied, the rates of dissociation of labeled cofactor were invariant, $k = 1.28 \pm 0.04 \times 10^{-1}$ hr^{-1} (Figure 19, B); when unlabeled cofactor was omitted there was no dissociation of (6R),L-CH_2-[6-^{3}H]H_4folate (Figure 19, B). However, for [2-^{14}C]FdUMP dissociation $k = 1.34$, 1.08, 0.841 and 0.455×10^{-1} hr^{-1} at 0, 0.1, 0.3 and 1.0 mM unlabeled cofactor (Figure 19, A); aberrant kinetics (not shown) were observed for the solution containing 3.0 mM cofactor in which there was a significant pH change, ca. 1 pH unit, and are not considered further. These results were unaffected by potassium phosphate over the range of 0 to 50 mM (plots not shown).

Equilibrium Dialysis: Binding of FdUMP to dTMP Synthetase. From a double-reciprocal plot (not shown) for binding of [2-^{14}C]FdUMP to dTMP synthetase a dissociation constant was calculated, $K_d = 20$ μM, and at an extrapolated infinite concentration binding of 2 mol FdUMP/mol enzyme dimer was indicated. The Scatchard plot (not shown) of the binding data was linear, showing that the affinity of FdUMP for both sites was the same.

Although equilibrium was reached in 3 hr, HPLC analysis (system A) of a 40 μl aliquot from the side containing protein showed no degradation of [2-^{14}C]FdUMP (RV = 7.5 ml) even after > 5 hr. In controls omitting [2-^{14}C]FdUMP, enzyme activity remained essentially unchanged as determined by the equivalence point of FdUMP titration of active sites performed spectrophotometrically (Garrett et al., 1979).

FIGURE 19: The dependence of the rates of isotope exchange of ligands in covalent [2-^{14}C]FdUMP—(6R),L-CH_2-[6-^{3}H]H_4folate—dTMP synthetase complexes on the concentration of (6R),L-CH_2-H_4folate. The data points represent the[2-^{14}C]FdUMP, **A** , and the (6R),L-CH_2-[6-^{3}H]H_4folate, **B** , bound in the complex isolated over the course of the reaction. The reaction mixtures contained 1.0 μM double-labeled covalent complex, 1.0 μM FdUMP and 0 (✕), 0.1 (▲), 0.3 (■), and 1.0 (●) (6R),L-CH_2-H_4folate. Experimental details are described in the text.


A
B
LOG (% DPM BOUND)
2.0
1.8
1.6
1.4
1.2
0
4
8
12
TIME (HRS)

DISCUSSION

The isotope-trapping method described by Rose (1980) for enzyme-catalyzed reactions was adapted here for the formation of covalent FdUMP—CH_2-H_4folate—dTMP synthetase complexes, as depicted in Figure 20. The "pulse" solution contains pre-equilibrated enzyme E and one of the two ligands of high specific radioactivity, $\overset{*}{A}$. It is diluted ten-fold with rapid mixing into a "chase" solution containing the unlabeled form of this ligand, A, and the second ligand, B, such that both are in large molar excess over $\overset{*}{A}$ in the final reaction mixture.

The partitioning of any noncovalent, binary $E\overset{*}{A}$ complex pre-formed in the pulse between free $\overset{*}{A}$ and final covalent, ternary complex, $\underline{\acute{E}\overset{*}{A}B}$, when it is added to the chase will depend on its flux along alternative routes given in the minimal scheme (Figure 20). The steps with rate constants k_2 and k_7 are written as irreversible because of the presence of excess unlabeled A in the final reaction mixture. That is, this large pool of unlabeled material serves to dilute the specific activity of any $\overset{*}{A}$ that dissociates from noncovalent complexes, such that high specific radioactivity covalent complexes ($\underline{\acute{E}\overset{*}{A}B}$) can form only from intermediates in which $\overset{*}{A}$ has remained bound. The step(s) with rate constant k_5 also is written as irreversible because the rate of dissociation of $\underline{\acute{E}\overset{*}{A}B}$ complexes under the experimental conditions is negligible. The amount of $E\overset{*}{A}$ complex that becomes "trapped" in the $\underline{\acute{E}\overset{*}{A}B}$ complex, then, is determined by scintillation counting of the covalent complex after adsorption to nitrocellulose filters.

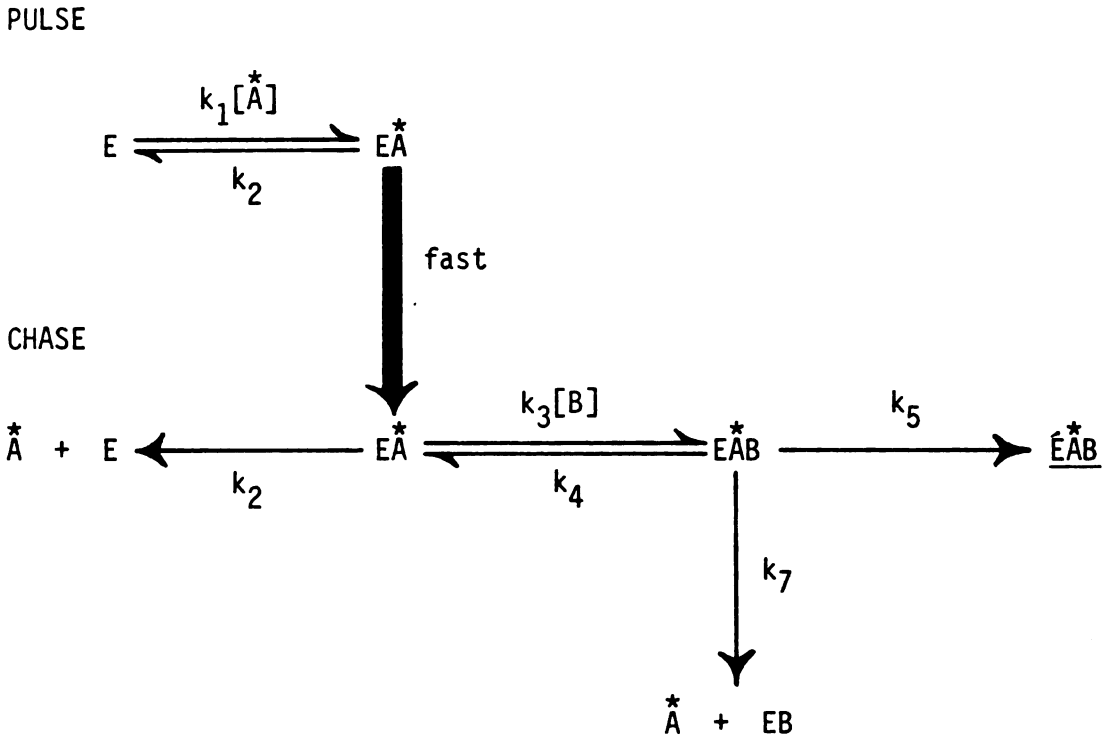


FIGURE 20: Isotope-trapping scheme for the formation of covalent FdUMP—CH_2-H_4folate—dTMP synthetase complexes. Nomenclature used: E, dTMP synthetase; $\overset{*}{A}$, high specific radioactivity ligand (either [6-^{3}H]FdUMP or CH_2-[6-^{3}H]H_4folate); A, unlabeled ligand; B, the other unlabeled ligand; $\underline{\overset{*}{E}AB}$, high specific radioactivity covalent, ternary complex. The solid arrow depicts rapid mixing of Pulse and Chase solutions. For simplicity, the interactions of a monomeric enzyme are shown, but these will be the same for each of the chemically identical subunits of dTMP synthetase (Maley *et al.*, 1979; Bellisario *et al.*, 1979) if they also function identically and independently. E denotes a conformational form of the enzyme distinct from the native form; see Figure 6 and explanation in text of chapter 2.

It is imperative to know the amount of $\overset{*}{E}A$ complex in the pulse to fully exploit the isotope-trapping method (Rose, 1980). When $[\overset{*}{A}]$ is varied and concentrations of all other components are held constant the partitioning of $\overset{*}{E}A$ is invariant, and changes in the amount of covalent $\underline{\acute{E}AB}$ complex formed are a function of the amount of $\overset{*}{E}A$ in the pulse. Thus, by varying the concentration of [6-^{3}H]FdUMP in the pulse a value for the dissociation constant, K_d^{FdUMP}, of 21 μM for the E·[6-^{3}H]-FdUMP complex is obtained from Figure 12. This value agrees closely with that of 20 μM determined by equilibrium dialysis both in this study under identical conditions and previously in 50 mM Tris-HCl (pH 7.1) and 20 mM $MgCl_2$ (Galivan et al., 1976a). The dissociation constant for FdUMP in the initially formed, noncovalent $\overset{*}{E}AB$ complex, $K_d^{'FdUMP}$, has been estimated from kinetic data (Wataya et al., 1977) to be ca. 10^{-8} M. Since this $K_d^{'FdUMP}$ is ca. 10^3-fold less than K_d^{FdUMP}, binding of the first ligand (A) facilitates binding of the second ligand (B).

When [B] is raised and concentrations of all other components are fixed, more $\overset{*}{E}AB$ is formed due to increased trapping of $\overset{*}{E}A$ by B (Figure 20). If $[\overset{*}{A}]$ approaches infinity in the pulse, all enzyme binding sites for $\overset{*}{A}$ are introduced into the chase as $\overset{*}{E}A$. And, as [B] approaches infinity also, all $\overset{*}{E}A$ is irreversibly converted to $\overset{*}{E}AB$ which will then form $\underline{\acute{E}AB}$ and/or lose $\overset{*}{A}$ irreversibly (Figure 20). Under these extrapolated conditions when all enzyme initially is converted to noncovalent E·[6-^{3}H]FdUMP·(6R),L-CH_2-H_4folate complexes, only a fraction, 0.42, partitions to give final $\underline{\acute{E}AB}$ complex.[5] The [B] that causes half this

maximum amount of trapping is defined as $K_{\frac{1}{2}}$, which is analogous to K_m in steady-state kinetics (Rose, 1980). For trapping of E·[6-^{3}H]FdUMP by CH_2-H_4folate a value for $K_{\frac{1}{2}}^{CH_2\text{-}H_4folate}$ of 0.15 m$\underline{M}$ is obtained from Figure 13.

There is a caveat in the interpretation of the value of 0.42 for the maximum fraction of trapping. In the determination of total enzyme binding sites in the presence of molar excesses of [6-^{3}H]FdUMP and CH_2-H_4folate, two mol covalent complex/mol dimer are formed (Santi et al., 1974b; Galivan et al., 1974), but this is no assurance that in the absence of CH_2-H_4folate functional E·[6-^{3}H]FdUMP complexes on both subunits are formed in the pulse. However, under identical conditions two mol of [2-^{14}C]FdUMP were bound with equal affinity for every mol of enzyme dimer as determined by equilibrium dialysis, suggesting this may be the case. Regardless, the partition coefficient of 0.42 represents maximally 0.84 mol of covalent complex/mol dimer, and even if productive $E\overset{*}{A}$ complexes are formed on only one subunit, there is still incomplete trapping.

One explanation for this is that some $\overset{*}{A}$ dissociates from $E\overset{*}{A}B$ (Figure 20). Simultaneously or alternatively, there may be formation of non-productive complexes along another pathway(s) not depicted in the

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[5]Values for K_d, $K_{\frac{1}{2}}$ and partitioning for these experiments are essentially the same using either (6$\underline{R}$),$\underline{L}$- or (6$\underline{R}$,$\underline{S}$),$\underline{L}$-CH_2-H_4folate as the trapping ligand (B) in chase solutions. This is expected since the 6$\underline{S}$ isomer binds only very poorly to dTMP synthetase even in the presence of dUMP or FdUMP (Galivan et al., 1976a), and the 6$\underline{R}$ isomer always was in large molar excess over noncovalent E·[6-^{3}H]FdUMP complexes.

minimal scheme (Rose, 1980). Non-productive complex formation could be the same for both subunits of dTMP synthetase if they are functionally identical, or could occur on one subunit if they are functionally non-identical. If some $\overset{*}{A}$ does dissociate from $E\overset{*}{A}B$, then the fact that some $\overset{*}{A}$ also is trapped in $\underline{\acute{E}\overset{*}{A}B}$ argues that k_7 and k_5 must be of similar magnitude (Rose, 1980). However, the dissociation step with rate constant k_7 would not be allowed in an ordered binding scheme where B obligatorily dissociates from $E\overset{*}{A}B$ prior to $\overset{*}{A}$, as has been reported (Danenberg & Danenberg, 1978).

Similar interpretations are made from experiments in which $\overset{*}{A}$ was CH_2-[6-^{3}H]H_4folate and B was FdUMP (Figure 20). Using either [^{3}H]6R- or [^{3}H]6R,S-cofactor, double-reciprocal plots showing the dependence of the extent of trapping on the concentration of the unbound [^{3}H]6R-isomer in the pulse were linear and values for $K_d^{CH_2\text{-}H_4\text{folate}}$ of 44 μM (Figure 15) and 40 μM (plot not shown), respectively, were obtained. If the 6S-isomer binds to dTMP synthetase (Kisliuk et al., 1976) the K_d must be << 40 μM and essentially only E·(6R),L-CH_2-[6-^{3}H]H_4folate complexes form in equilibrated pulse solutions. Further supported by the finding of Galivan et al. (1976b) that (6R),L-CH_2-H_4PteGlu$_4$ binds to dTMP synthetase with a $K_d \approx 20$ μM, these isotope-trapping results argue against an earlier conclusion of these workers (Galivan et al., 1976a) that (6R),L-CH_2-H_4PteGlu$_1$ does not bind; why binding was not detected is unclear since the $K_d^{CH_2\text{-}H_4\text{folate}}$ value of 44 μM obtained here is only ca. two times greater than the dissociation constant for FdUMP binding which was detected by the equilibrium dialysis method used by these workers.

When [FdUMP] in the chase is varied, values for $K_{\frac{1}{2}}^{FdUMP}$ of 18 and 20 μM are obtained using constant concentrations in the pulse of (6R),L- (Figure 16) and (6R,S),L-CH_2-[6-^{3}H]H_4folate, respectively. This is further evidence that the same E·(6R),L-CH_2-[6-^{3}H]-H_4folate·FdUMP complex partitions in the final reaction mixtures. These values for $K_{\frac{1}{2}}^{FdUMP}$ are of the same order of magnitude as those for $K_{\frac{1}{2}}^{CH_2-H_4folate}$.

The experiments depicted in Figures 15 and 16 were performed using the [^{3}H](6R)-isomer in the pulse and high concentrations (14 mM) of (6R,S),L-CH_2-H_4folate in the chase. Under these conditions, when both [$\overset{*}{A}$] and [B] were increased without limit a calculated partition coefficient of only 0.024 was obtained.[6] However, in parallel experiments using the [^{3}H](6R,S)-racemate in the pulse and lower concentrations of (6R,S),L-CH_2-H_4folate in the chase (2.4-2.6 mM) a partition coefficient of 0.24 was obtained.

The partition coefficient values for both sets of experiments above fall on the curve (Figure 17) for the experiment where a constant concentration of (6R),L-CH_2-[6-^{3}H]H_4folate is in the pulse and [(6R,S),L-CH_2-H_4folate] in the chase is varied holding concentrations of all other components constant; i.e., the values are dependent on the concentration of cofactor in the chase. Differences in these values are not a result,

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[6] That the trapping value of 0.030 at very high [B] is larger than the calculated value of 0.024 as [B] approaches infinity may be ascribed to a reasonably expected degree of error in such low trapping values for the experiments of Figures 15 and 16.

therefore, of using either [^{3}H](6R)- or [^{3}H](6R,S)-cofactor in the pulse, consistent with previous arguments that the presence of the 6S-isomer in the pulse has little effect on $K_d^{CH_2\text{-}H_4\text{folate}}$ and $K_{\frac{1}{2}}^{FdUMP}$ values. When (6R),L-CH_2-H_4folate is used in the chase for the experiments of Figure 17 the results are the same. Comparisons of these experimental points and calculated partition coefficient values on one curve (Figure 17) are valid since the concentrations of [^{3}H](6R)-cofactor and FdUMP in the experiment are close to those for maximum trapping (see Figures 15 and 16, respectively). Because of these high [$\overset{*}{A}$] and [B] we may obtain an estimate for the true partition coefficient from this experimental curve. It is the value of 0.39 for the extent of trapping of high specific radioactivity $\underline{E\overset{*}{A}B}$ complexes which is observed at low [A] where apparent inhibition of trapping is absent (Figure 17). The possibilities for incomplete trapping discussed as when $\overset{*}{A}$ is [6-^{3}H]-FdUMP apply here also.

The diminished trapping observed at high [cofactor] in the chase may be due to one of two possibilities.[7] First, nonproductive complexes with cofactor may form (Rose, 1980). Cofactor could bind at a non-catalytic site to effect allosteric inhibition, but no allosteric effects for dTMP synthetase have been seen to date. More likely, then, would be a competition between CH_2-H_4folate and FdUMP for binding to the same site, such that at high [CH_2-H_4folate] predominantly nonpro-

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[7]Possible hysteretic effects cannot be entirely discounted at this time, but linearity of the double-reciprocal plots of previously described Figures argue against the presence of a mixing artifact (Rose, 1980).

ductive complexes are formed. Second, the relatively large size (MW 457) and hence low diffusion coefficient of CH_2-H_4folate may effectively block trapping of the E·[^{3}H]cofactor complex by FdUMP (Rose et al., 1974; Rose, 1980).

Support for the hypothesis that the extent of trapping may be dependent on competing effects of CH_2-H_4folate and FdUMP in chase solutions is shown in Figure 14. The primary effect of increasing [CH_2-H_4folate] (B) is to increase the trapping of E·[6-^{3}H]FdUMP complexes. At high [FdUMP] (A) the extent of trapping of high specific radioactivity $\underline{\acute{E}\overset{*}{A}B}$ complexes[8] is independent of [A]. However, the curves appear to be biphasic and the [A] at which the break points occur are dependent on [B]; as [B] is increased, higher [A] is needed to overcome the secondary effect of B of apparent inhibition of trapping.

Demonstration of formation of productive $E\overset{*}{A}$ complexes when $\overset{*}{A}$ is either [6-^{3}H]FdUMP or CH_2-[6-^{3}H]H_4folate is direct proof for a random binding order contribution in formation of $\underline{\acute{E}\overset{*}{A}B}$ complexes (Rose, 1980). It is apparent that shifts in equilibria effected by variations in the concentrations of FdUMP and CH_2-H_4folate may affect the extent of trapping observed (Rose, 1980), and hence the degree of preference for binding one ligand or the other. However, equivalent values were obtained for maximal trapping of either $E\overset{*}{A}$ complex of ca. 0.8 mol of covalent

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[8]The trapping value at high [FdUMP] and [CH_2-H_4folate] of ca. 0.5, as determined in Figure 14, is slightly larger than the partition coefficient of 0.42 obtained from the results of Figures 12 and 13. This difference may be attributed to normal experimental error and is not considered significant.

complex/mol of enzyme dimer. Thus, the equilibria that occur under optimal conditions of isotope-trapping are such that the allowed mode of binding to the native enzyme is random (Himoe, 1976).

In addition, for experimentally used high [$\overset{*}{A}$] and [B] trapping values were achieved which are very nearly the same as the maximum trapping values calculated as [$\overset{*}{A}$] and [B] approach infinity. Since this maximum value is only ca. 40% of the available sites, it may be concluded that the rate-determining step in formation of covalent complexes under the conditions of the studies reported here occurs after formation of noncovalent ternary complexes, i.e., $k_5 < k_7$ in Figure 20.

In contrast to the random binding observed here, previous studies have been interpreted as evidence for wholly ordered binding; nucleotide binds first and cofactor second. Danenberg and Danenberg (1978) have obtained the best evidence for this to date using an isotope exchange technique (Boyer, 1978; Purich, 1980). However, conditions used were substantially different from those used in this laboratory.

A modification of the method of Danenberg and Danenberg (1978) has been employed here under conditions identical to those of the isotope-trapping experiments and avoids most of the pitfalls of binding and steady-state kinetic methods. We have determined the dependence of the rates of exchange of labeled for unlabeled ligands in [2-^{14}C]FdUMP—(6R),L-CH_2-[6-^{3}H]H_4folate—dTMP synthetase complexes on the concentrations of unlabeled FdUMP and CH_2-H_4folate. Exchange of ligands in complexes containing either [^{3}H]FdUMP or [^{3}H]CH_2-H_4folate upon variation of only the complimentary unlabeled ligand was the approach used pre-

viously by Danenberg and Danenberg (1978). The results of the experiments performed here are shown in Figures 18 and 19.

For all experiments isotopic exchange was first-order for > two half-lives in accordance with theory (Jencks, 1969). Figure 18 shows that the rates of exchange of [2-^{14}C]FdUMP and (6R),L-CH_2-[6-^{3}H]H_4folate are unaffected in the presence of (6R),L-CH_2-H_4folate as [FdUMP] is varied. In the analogous experiment where [FdUMP] is constant and [(6R),L-CH_2-H_4folate] is varied, the rates of exchange of (6R),L-CH_2-[6-^{3}H]H_4-folate[9] are unaffected (Figure 19, B), but those of [2-^{14}C]FdUMP are (Figure 19, A). The $t_{\frac{1}{2}}$ values from Figure 19, A, are a linear function of [CH_2-H_4folate] and decrease 3.3-fold as the cofactor concentration is raised from 0 to 1.0 mM (plot not shown). These results suggest that dissociation is at least partially ordered, where FdUMP leaves second and hence is suppressed from doing so at high concentrations of CH_2-H_4folate (Danenberg & Danenberg, 1978).

Table 2 shows the equations for first-order rate constants for exchange of $\overset{*}{A}$ and $\overset{*}{B}$ ([2-^{14}C]FdUMP and (6R),L-CH_2-[6-^{3}H]H_4folate, respectively) from covalent ternary complexes by the extremes of mechanism that could apply under the conditions of the experiments performed here. For the completely random mechanism only, the rate equations for exchange of $\overset{*}{A}$ and $\overset{*}{B}$ are of identical form, as required for a symmetrical

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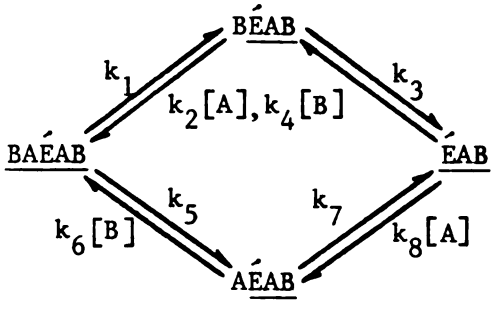
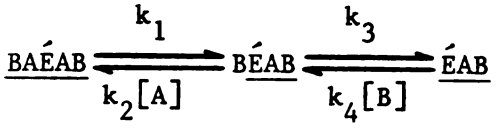
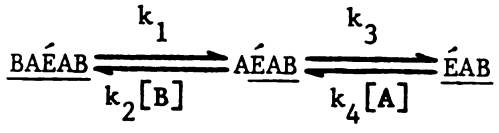
[9]The half-lives for dissociation of (6R),L-CH_2-[6-^{3}H]H_4folate, although unaffected by varying [FdUMP] or [(6R),L-CH_2-H_4folate], are 5.43 ± 0.178 hr and 6.66 ± 0.122 hr in Figures 18, B and 19, B, respectively. These differences are not considered meaningful and are ascribed to deterministic errors between series of experiments.

LEGEND TO TABLE 2

[a]First-order rate constants are derived by the partition analysis method of Cleland (1975). Dissociation from only one of two identical subunits is considered for simplicity, and depicted are the equilibria that may exist in the isotope exchange experiments in the presence of large molar excesses of A and B; exchange of radioactivity in one subunit is accounted for as shown. All radioactivity can be exchanged by a subsequent dissociation from the second subunit after the first binds unlabeled A and B; in each mechanism considered the equations for k for exchange from the second subunit will be of identical form as shown for exchange from the first subunit. The existence of noncovalent ternary complexes as the first species formed in dissociation has been omitted, but the interpretations remain unchanged. $\underline{\acute{E}AB}$ denotes covalent complex at one subunit. Ligands on the right of the enzyme $\acute{E}$ depict binding at one subunit and those on the left binding to the other subunit. $\acute{E}$ denotes a conformational form of the enzyme distinct from the native form; see Figure 6 and explanation in text of chapter 2. $\overset{*}{A}$ and $\overset{*}{B}$ denote high specific radioactivity [2-^{14}C]FdUMP and (6$\underline{R}$),$\underline{L}$-CH_2-[6-^{3}H]H_4folate, respectively.

TABLE 2: First-Order Rate Constants, k, for Exchange of Ligands from Covalent Ternary Complexes.[a]

Mechanism	k	
	$\overset{*}{A}$ Exchange	$\overset{*}{B}$ Exchange
Random: 	$k_1 + k_5 \frac{k_7}{k_7 + k_6[B]}$	$k_5 + k_1 \frac{k_3}{k_3 + k_2[A]}$
Ordered, A Dissociates First: 	k_1	$k_1 \frac{k_3}{k_3 + k_2[A]}$
Ordered, B Dissociates First: 	$k_1 \frac{k_3}{k_3 + k_2[B]}$	k_1

system. Rates of exchange of $\overset{*}{A}$ and $\overset{*}{B}$ are inversely dependent on the concentrations of B and A, respectively, but not on A and B; at infinite concentrations of B or A, respectively, there will be less than full suppression of dissociation because of a second term in each rate equation which is concentration-independent. For both ordered mechanisms, when $\overset{*}{A}$ and $\overset{*}{B}$ dissociate last, infinite concentrations of B or A, respectively, will completely suppress dissociation, but changing A or B, respectively will have no effect. When $\overset{*}{A}$ or $\overset{*}{B}$ dissociate first the rates for both are unaffected by the concentrations of either B or A. From inspection, the data of Figures 18 and 19 appears to be consistent with a fully ordered mechanism where B dissociates first. In fact, a best fit plot of k vs. 1/[cofactor] for the data of Figure 19, A (not shown) is hyperbolic and extrapolates to a zero rate at infinite cofactor concentration; the absence of a detectable, finite rate of dissociation of [2-^{14}C]FdUMP at this concentration indicates there is no contribution to dissociation of a cofactor concentration independent term for the random mechanism. A plot of 1/k vs. [B] for the data of Figure 19, A is linear (not shown) and from the ordinate intercept is obtained a value for k_1 of 1.32×10^{-1} hr^{-1} which is identical to the dissociation rate constant of 1.34×10^{-1} hr^{-1} for the curve of Figure 19, A, where CH_2-H_4folate is omitted. These are the results expected from the reciprocal of the rate equation for exchange of $\overset{*}{A}$ in the ordered mechanism where B is released first (Table 2): $1/k = 1/k_1 + k_2[B]/k_1k_3$.

This fully ordered mechanism also was implicated by Danenberg and Danenberg (1978). These authors argued that binding to native enzyme also must be ordered with nucleotide first and cofactor second, hence, kinetically significant, productive enzyme·CH_2-H_4folate complexes could not exist. This conclusion is in contrast to the fully random binding obtained using the isotope-trapping method. In order to satisfy microscopic reversibility, this requires the existence of functionally nonidentical native and fully bound (i.e., A and B bound at the active site of both subunits) enzyme and that there be separation into two partial reactions of the equilibria that occur under the conditions of isotope-trapping vs. isotope exchange. In other words, association/dissociation of A and B to the native form of the enzyme is random, but to the bound form of the enzyme is ordered; only binding intermediates containing the native form of the enzyme are present under the conditions of isotope-trapping, and for isotope exchange only intermediates containing the complexed form of the enzyme are present.

CHAPTER 4

A MODEL FOR THE INTERACTION OF THYMIDYLATE SYNTHETASE WITH 5-FLUORO-2'-DEOXYURIDYLATE AND 5,10-METHYLENE-5,6,7,8-TETRAHYDROFOLATE

Any model for the interaction of dTMP synthetase with FdUMP and CH_2-H_4folate must accomodate the results of the isotope probe studies of this thesis and must be consistent with generally accepted features of the mechanism of the enzyme (Pogolotti & Santi, 1977). It also must address certain aspects of the mechanism for which credible data have been obtained in support of both sides of controversial interpretations (Danenberg & Danenberg, 1978, 1979; Galivan et al., 1976a). Among these are questions concerning the stoichiometry and affinity of binding of folate derivatives and pyrimidine nucleotides, whether subunits in the native enzyme function identically, and whether the native enzyme and enzyme to which nucleotide and cofactor are bound are functionally identical.

A hypothetical mechanism for the association/dissociation of covalent FdUMP—CH_2-H_4folate—dTMP synthetase complexes has been constructed which can answer these questions. It features a generalized treatment for the binding of ligands to the subunits of the native form of the enzyme, such that incorporation of future results may be allowed. A more limited model has been presented recently by Danenberg and Danenberg (1979), but it fails to account for some notable observations. The important distinctions between proposals of these authors and the model proposed here are noted. First the model is presented and then it is supported. Direct experimental evidence for salient aspects are described, followed by a corroborating discussion of how the model accounts for pertinent results of other experiments and how it resolves the aforementioned controversial issues. Evidence also is presented

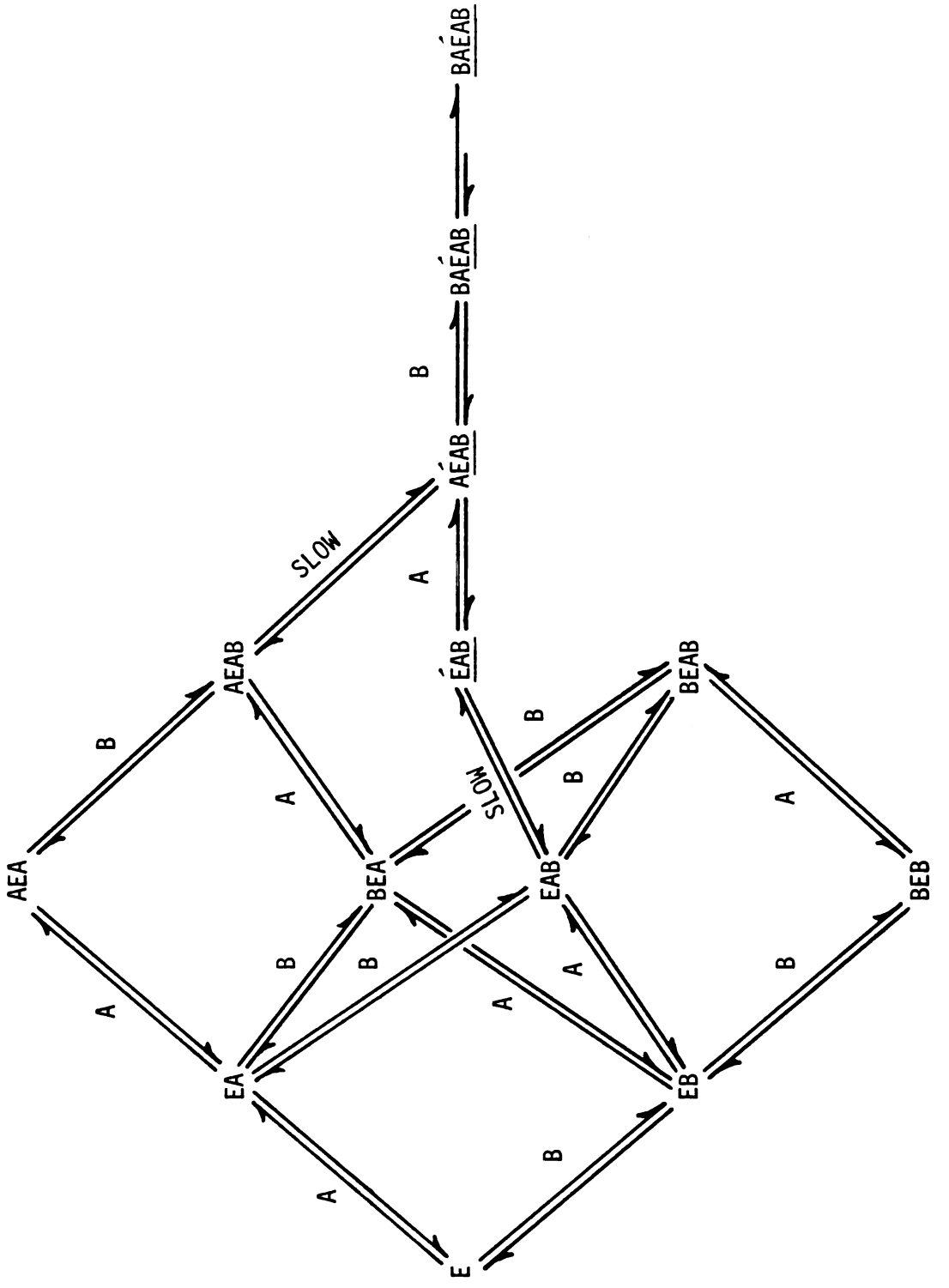
in support of a prominent prediction of the model.

PRESENTATION OF THE MODEL

Figure 21 shows the postulated minimal mechanism for the association/dissociation of covalent FdUMP—CH_2-H_4folate—dTMP synthetase complexes. The central feature of this model is a ligand induced conformational change of the dimeric enzyme upon noncovalent binding of both nucleotide (A) and cofactor (B) to one of the subunits. As a consequence, the native form of the enzyme (E), which binds A and B in a random fashion, is converted to a form ($\acute{E}$) for which continued binding to the other subunit is fully ordered. Thus, it is proposed that there is overall sequential binding of the two subunits of dTMP synthetase (Koshland, 1970).

The model is presented in Figure 21 in a general form that can account for the binding of any nucleotide and/or cofactor. As shown, it is presumed that the active sites of both subunits of the native enzyme are sterically accessible for binding of nucleotide and cofactor. However, at least under certain conditions, binding of some ligands to one subunit may be sterically and/or otherwise more restricted than to the other subunit. This is dealt with in the model simply by making the equilibrium binding constants for specified intermediates unfavorable with respect to those for other intermediates as described later. Therefore, the model can accomodate the binding of ligands with from equal to widely disparate affinities for the two

FIGURE 21: Proposed minimal mechanism for the interaction of dTMP synthetase with FdUMP and (6R),L-CH_2-H_4folate. Nomenclature used: E, native form of dTMP synthetase; ^{2}A, ^{4}nucleotide; B, cofactor; É, conformationally and functionally altered form of the enzyme distinct from the native form; ligands, A and/or B, shown to the left vs. the right of E or É denote binding to different subunits of the dimeric enzyme; underlines denote the presence of covalent bonds between nucleotide and cofactor and the indicated subunit(s) of dTMP synthetase.


BAÉAB
BAÉAB
AÉAB
AÉAB
ÉAB
ÉAB
AEAB
AEAB
BEAB
BEAB
EAB
EAB
BEA
BEA
AEA
AEA
EA
EA
EB
EB
BEB
BEB
E
E
SLOW
SLOW
A
B

subunits. Following is a description of the progression of events that are proposed to occur in the formation of covalent complexes with both subunits of the enzyme.

Initially, A and B can bind in a random order to either subunit of E until both are noncovalently bound to one subunit. At this point, the other subunit may be empty (EAB) or A (AEAB) or B (BEAB) may be noncovalently bound to it as a consequence of the unrestricted mode of binding. For the EAB and AEAB intermediates only, there is a conformational change of E to É upon or immediately after the binding of A and B at one site; BEAB is a nonproductive complex. The conformational change most likely is the overall rate-determining step, but if it occurs before the rate-determining step the model will still be consistent with experimental results. This ligand induced conformational change is a prerequisite to formation of covalent bonds. Attack at the 6-position of A by the active site nucleophile of the subunit to which A and B are bound is the first covalent bond forming step. It may occur at, but more likely occurs after the overall rate-limiting step. Formation of a covalent ternary complex with one subunit, (A)ÉAB, may occur independently of subsequent binding to the other subunit. For the now conformationally altered enzyme, É, continued binding to the remaining, unbound subunit is fully ordered with A binding before B. The position of equilibrium greatly favors binding to this subunit of the É form of the enzyme even if binding was very weak or undetected to this same subunit when in the E form. Finally, formation of covalent bonds after binding of A and B to the second subunit results in enzyme

with both subunits covalently complexed, BA$\acute{E}$AB. The subunits of this $\acute{E}$ form of the enzyme function identically; for example, the affinity of binding of A or B is the same for both subunits.

The reason why the conformational change and covalent bond formation should be allowed for AEAB is because the same productive A$\acute{E}$AB complex will result as from binding of A to $\acute{E}$AB. However, if a conformational change and formation of covalent bonds were allowed for BEAB, a B$\acute{E}$AB species would result which would be in violation of the order of binding to the second subunit. It thus is assumed that the presence of B alone in the second subunit of the E form of the enzyme prevents the conformation change.

JUSTIFICATION FOR THE MODEL

Binding of Nucleotides or Cofactors to the Native Enzyme. The question whether the subunits in the native enzyme are functionally identical or nonidentical has been a subject of controversy (for previous discussions, see: Galivan et al., 1976a; Danenberg & Danenberg, 1978, 1979; Chapter 1, Ph.D. thesis). Although the subunits have the same primary structure (Maley et al., 1979; Bellisario et al., 1979), in certain binding and modification experiments higher order structural/functional differences are indicated, which will be discussed later, and it has been suggested by Galivan et al. (1976a) that there is only one active subunit/dimer. The recent model of Danenberg & Danenberg (1979) is a refined version of this idea, wherein it is proposed that

there is only one sterically accessible active site/dimer prior to binding of both nucleotide and cofactor which opens up the other site.

A pertinent point in this regard is that two binding sites/mol of enzyme for FdUMP (Galivan et al., 1976a; this study), dUMP (Beaudette et al., 1977) and 5-nitro-dUMP (Matsuda et al., 1978) in the absence of folate derivatives have been demonstrated. This argues that both subunits in the native enzyme must be accessible for binding, though not necessarily to the same degree. Any steric restrictions that may exist cannot be severe, at least for the nucleotide binding site, because FdUMP binds to both subunits with equal affinity in the absence of cofactor in NMM (this study) or Tris (Galivan et al., 1976a); there may be a greater effect on the binding of larger ligands such as folate derivatives. Therefore, other explanations for the observed functional nonequivalencies of the subunits in addition to a possible steric contribution must be proposed.

Nonequivalent binding of some ligands to the subunits, for any number of possible reasons, may always be expressed in terms of shifts in the position of binding equilibria for one subunit relative to the other; under certain experimental conditions if $k_{AEA \longrightarrow EA} >> [A]k_{EA \longrightarrow AEA}$, for example, the amount of AEA present at equilibrium may not be easily detectable, as when A is dUMP (Galivan et al., 1976a). Similarly, the reported binding of CH_2-H_4PteGlu$_4$ (Galivan et al., 1976b) but not of CH_2-H_4folate (Galivan et al., 1976a), which we have shown in the previous chapter does bind to E, may be resolved in like manner.

Ligand Induced Conformational Change. There is considerable spectral evidence for a conformational change involving both dTMP synthetase and CH_2-H_4folate in the formation of ternary complexes with FdUMP; a summary was presented in a previous chapter (2). As discussed, these spectral changes occur either upon or immediately after noncovalent binding of one mol (Sharma & Kisliuk, 1975) of FdUMP and CH_2-H_4folate/mol of enzyme, and before the first covalent bond forming steps. Studies of the hydrodynamic properties of bound and free enzyme (Lockshin & Danenberg, 1980) and of ligand protection against proteolysis (Galivan et al., 1977b) have shown that conformational changes are appreciable and significantly alter the properties of the enzyme. Thus, a ligand induced conformational change upon binding of nucleotide and cofactor to one subunit is compelling as the mechanism whereby the native enzyme may be converted to a functionally different form of the enzyme.

Relationship of Conformational Change, Rate-Determining and Covalent Bond Forming Steps. It is proposed here that the first covalent bond forming step occurs in the first subunit to bind both A and B subsequent to the major conformational change. From the results of the secondary α-hydrogen isotope effect experiments of chapter 2 it can be concluded that the rate-determining step in formation of BAEAB almost certainly occurs before, but no later than this first covalent bond forming step, (A)EAB $\longrightarrow$ (A)ÉAB. Only in this situation do the carbon rehybridization steps of covalent complex formation with both subunits in the sequential binding mechanism come after the rate-

determining step in the direction of association (Figure 21). As discussed in chapter 3, the results of isotope-trapping experiments suggest that the rate-determining step occurs after formation of noncovalent ternary complexes at experimentally used high concentrations of A and B. Therefore, both the conformational change and the rate-determining step occur <u>after</u> formation of the first noncovalent ternary complex and <u>prior</u> to the first covalent bond forming step. The only other probable step that likely may occur within these limits is the conversion of CH_2-H_4folate to the 5-iminium ion, 5-CH_2=$^+H_4$folate, which as discussed in chapter 2 may be surmised to be concurrent with the conformational change of the enzyme. Therefore, in the absence of a persuasive reason for invoking other steps besides the conformational change of the enzyme and cofactor within these limits, it is reasonable to assume the conformational change is the step that <u>is</u> rate-limiting[10].

<u>Isotope Exchange</u>. In the isotope exchange experiments of chapter 3, [A] and [B] are high, $\geq$ 0.1 m<u>M</u>. Therefore, in comparison to the rate of binding of these unlabeled ligands to <u>ÉAB</u> and A<u>ÉAB</u> intermediates formed by dissociation of labeled ligands, the rate-limiting conformational change step on either allowed pathway, <u>ÉAB</u> $\longrightarrow$ EAB or A<u>ÉAB</u> $\longrightarrow$ AEAB, will be very slow; partitioning of these intermediates

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[10]Should some other step that is rate-determining, such as ionization of an active site group, occur within the limits of noncovalent complex formation and formation of covalent bonds, then a conformational change that occurs after noncovalent complex formation but <u>before</u> the rate-limiting step also would satisfy the arguments that follow.

will greatly favor formation of BAÉAB, K_d = ca. $10^{-11 \text{ to } -13}$ M (Santi et al., 1974b), and >99.99% of enzyme binding sites will be fully bound at all times. Thus, as shown in Figure 21 the ligand exchange equilibria involves only species which contain the É form of the enzyme. The first-order loss of labeled ligand(s) over >2-6 half-lives observed for these double ligand (chapter 3) and earlier single ligand (Santi et al., 1974b; Danenberg & Danenberg, 1978; this thesis, chapter 2) exchange experiments suggests that a single class of binding sites is monitored; that is, the subunits of É are functionally identical and independent. In other words, exchange of ligands in both subunits proceeds with the same first-order rate constants (Figures 18 and 19; Table 2) in the fully ordered manner previously described.

Isotope-Trapping. In pulse solutions of isotope-trapping experiments there is equilibrium binding of either nucleotide or cofactor and the form of the enzyme (E) that is present prior to the conformational change induced only in the presence of both ligands. It is the trapping of complexes present in the pulse that provides evidence of the binding order. Significant trapping of high specific radioactivity covalent complexes when $\overset{*}{A}$ of Figure 20 is either cofactor or nucleotide confirms that both ligands can bind to E to form productive complexes in the pulse. Thus, random binding to native dTMP synthetase is indicated as shown in Figure 21.

Trapping of the first fully bound subunit occurs because the conformational change, with rate constant $\simeq k_5$ in Figure 20, is not much slower than dissociation of noncovalently bound $\overset{*}{A}$ from $E\overset{*}{A}B$, with rate

constant k_7 in Figure 20. Depending on the position of equilibrium, there may be trapping of nucleotides ($\overset{*}{A}$ of Figure 21) which bind to both subunits, like FdUMP, on the second subunit as well. As previously discussed, in the presence of high [$\overset{*}{A}$] and [B] partitioning of $\overset{*}{A}E\underline{AB}$ will be greatly towards $\underline{B\overset{*}{A}E\overset{*}{A}B}$. The formation of covalent bonds may be the essentially irreversible step for trapping of $\overset{*}{A}$ at the second subunit because of the relatively slow rate of ligand exchange from $\underline{B\overset{*}{A}E\overset{*}{A}B}$ as demonstrated in the isotope exchange experiments. However, maximum trapping of $\overset{*}{A}$ gives only ca. 0.8 mol of covalent complex/mol of dimer, and if the positions of equilibrium for complex formation for the two subunits are substantially different then all complex may be formed on only one subunit/dimer.

The model shown in Figure 21 is not fully symmetrical; i.e., AEAB is a productive complex whereas BEAB is a nonproductive complex. There is no reason, a priori then, to expect that the values for maximum trapping should be the same for isotope-trapping experiments when the pulse solution contains $Fd\overset{*}{U}MP$ as for when it contains $CH_2\text{-}H_4\overset{*}{f}olate$. The fact that identical values are observed under optimal conditions can be accomodated by this model, however; it may be presumed that the equilibrium and rate constants for the various steps depicted are such that the fluxes through productive pathways are the same for both types of experiments.

Nonproductive Complexes with Cofactor. A salient consequence of the asymmetry of the model is that cofactor at high concentrations should trap those intermediates to which it can noncovalently bind to

both subunits. It thus will divert them from productive pathways to form BEAB complexes which are nonproductive for reasons presented earlier. In accord with this proposal are the observations of substrate inhibition in steady-state kinetics at high concentrations of cofactor, >ca. 1 mM (Danenberg & Danenberg, 1978) and of noncompetitive kinetics with a number of reduced folated derivatives (Danenberg et al., 1974; Slavik & Zakrzewski, 1967; Dolnick & Cheng, 1978; Kisliuk & Gaumont, 1979; Cheng et al., 1979). From these, nonequivalent binding of cofactor to a second site has been proposed. The minimal mechanism presented here predicts that for many binding intermediates the competition between A and B for further binding should determine the extent of their partitioning to covalent complexes vs. to nonproductive BEAB complexes. As suggested in chapter 3, just such an explanation would account for the non-linearity of the plots of Figures 14 and 17 and for the low values for trapping of covalent complexes obtained from Figures 15 and 16. The results of all of the isotope-trapping experiments performed in this work, in fact, are qualitatively consistent with the model of Figure 21 (see Appendix 3 for further explanations).

Modification Studies. Further support for this model is derived from the fact that it can satisfactorily account for many incompletely understood results of previous studies. Several laboratories have reported that treatment of native TMP synthetase with a variety of sulfhydryl reagents results in incorporation of only one mol of reagent/mol of dimer with concomitant complete loss of FdUMP binding

ability and catalytic activity (see Galivan et al., 1976a and Danenberg & Danenberg, 1979 and references cited therein)[11]; similar results were obtained upon release of one mol of carboxy terminal valine/mol of native enzyme (Aull et al., 1974a). The model in Figure 21 suggests that such modification of one subunit could abolish activity of the other subunit by inhibiting the ligand induced conformational change of both subunits requisite for covalent complex formation or catalysis. Modification of only one subunit with sulfhydryl reagents may be due to either a reagent induced conformational change which results in a sterically blocked second subunit or one subunit in the native enzyme may be inaccessible to the reagents used. Finally, these interpretations are consistent with the titration of complete loss of activity upon binding of two mol of FdUMP and CH_2-H_4folate/mol of enzyme (Santi et al., 1974; Galivan et al., 1974) which clearly demonstrates the existence of two active sites/dimer in the presence of molar excesses of nucleotide and cofactor.

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[11] It should be noted that sulfhydryl group modification reactions are as much dependent on the nature of the reagent and the conditions used as on the characteristics of the protein (Kenyon & Bruice, 1977). The reagents used in the studies cited are large enough that at least some could be excluded from a more sterically restricted second subunit due to size alone whereas certain ligands, such as FdUMP, may not be. Unfavorable interactive forces may repel other reagents. And, under certain conditions, methyl methanethiolsulfonate (Lewis et al., 1978), N-ethylmaleimide, p-chloromercuribenzoate and iodoacetamide (Plese and Dunlap, 1977) have been reported to react with ca. two sulfhydryl groups/dimer. Therefore, caution should be exercised in the interpretation of these results of sulfhydryl group modification studies. In light of other evidence discussed here it probably is incorrect to conclude that one of the subunits of the enzyme is completely inaccessible to binding and/or catalytically inactive as has been proposed (Galivan et al., 1976a; Danenberg & Danenberg, 1979).

Treatment of covalent complexes formed using either 0.5 or 1.0 mol of FdUMP/mol of enzyme and excess CH_2-H_4folate with sulfhydryl reagents results in incorporation of one mol of reagent/mol enzyme for both kinds of complexes (Danenberg & Danenberg, 1979). Only when >1.0 mol of FdUMP/mol of enzyme is used is accordingly less than one mol of reagent/mol of enzyme incorporated. Further, the rates of reaction of 5,5'-dithio-bis-(2-nitrobenzoic acid) and N-ethylmaleimide with 1:1:1 (A:B:dimer) complexes is ca. two-fold faster than with native enzyme. These results indicate that complexes formed with less than 2 mol of FdUMP/mol of enzyme contain a different form of the enzyme, $\acute{E}$ in Figure 21, in which the active site of the subunit not initially bound is more accessible and contains a more reactive sulfhydryl group than in the native enzyme. Modification of this sulfhydryl group does not inactivate the initially bound subunit of the enzyme, but it appears to substitute for ligands bound at this second subunit; the rate of ligand exchange in the one complexed subunit is the same as for fully complexed enzyme (Danenberg & Danenberg, 1979). This indicates there are the same exchange equilibria for both subunits involving only $\acute{E}$ species in these experiments.

Exchange vs. Dissociation. An earlier observation that the rate of dissociation of 2:2:1 (A:B:dimer) complexes is faster than that of 1:1:1 complexes (Aull et al., 1974b) was interpreted by Danenberg & Danenberg (1979) as indicating the two complexed subunits are functionally nonidentical, contrary to the results of isotope exchange experiments of chapter 3. However, rates of dissociation of these complexes

in the absence of excess A and B should not be confused with rates of ligand exchange in complexes in the presence of excess A and B. The rates of ligand exchange for both subunits are the same because only species containing $\acute{E}$ are formed; i.e., results of these experiments truly are indicative of the nature of the subunits in the complexed form of the enzyme. In the dissociation experiments above, the same intermediates containing $\acute{E}$ that are formed in isotope exchange are involved in the dissociation of the first complexed subunit of the 2:2:1 complex, BA$\acute{E}$AB to $\acute{E}$AB in Figure 21. But in the sequential binding model, dissociation of 1:1:1 complexes, $\acute{E}$AB in Figure 21, is initiated by conversion of $\acute{E}$AB to EAB, which includes the rate-determining step of the overall dissociation reaction and results in the formation of an intermediate containing the E form of the enzyme. Thus, the overall rate of dissociation of BA$\acute{E}$AB is not expected to be the same as that of $\acute{E}$AB, and further, these experiments cannot be interpreted as reflecting solely the nature of the subunits in the complexed form of the enzyme.

Binding of Folate Derivatives in the Presence of Nucleotides. Only single site binding of certain folate derivatives, even in the presence of pyrimidine nucleotides (Galivan et al., 1976a), has been reported. The high concentrations (mM) of CH_2-H_4folate required to effect inhibition of steady-state kinetics and of isotope-trapping of covalent complexes suggests it binds weakly to the initially noncomplexed subunit. That is, the diminished trapping observed only at high concentrations of CH_2-H_4folate in Figure 17 has been attributed here to

binding of B to EAB to form the nonproductive BEAB complex. Folate derivatives bind more weakly than CH_2-H_4folate to the more strongly binding subunit of the native enzyme (Galivan et al., 1976a; Santi et al., 1974b) and so binding to the other subunit may not have been detected. In these binding studies, combinations of CH_2-H_4folate and a nucleotide other than dUMP or of dUMP and a folate derivative other than CH_2-H_4folate were used, for which covalent bonds are not formed. The precisely defined geometry and enzyme-ligand interactions implicit for the proposed conformational change probably are lacking, such that generation of a more accessible/tigher binding second subunit in the $\acute{E}$ form of the enzyme cannot occur.

Effects of Phosphate. Both the stoichiometry and affinity of binding to dTMP synthetase of FdUMP alone or in the presence of folate derivatives other than CH_2-H_4folate are significantly affected by as little as 5 mM phosphate (Galivan et al., 1976a). From the arguments just presented, all the binding species that may be formed in these studies should contain only the E form of the enzyme. In contrast, neither the stoichiometry or affinity of binding of FdUMP in the presence of CH_2-H_4folate is affected by phosphate (Galivan et al., 1976a), and $\acute{B}A\acute{E}AB$ complexes are formed. Further, the results of the isotope exchange experiments of chapter 3 are the same in 0 or 50 mM potassium phosphate. In these experiments, only intermediates containing the $\acute{E}$ form of the enzyme are formed. Juxtaposition of the results of these experiments alone cannot provide for definitive conclusions. However, the results are consistent with what is expected based on the

model of Figure 21 if it is presumed that the presence of phosphate significantly affects the binding of FdUMP to the E form of the enzyme but has no detectable affect on the binding of FdUMP to the $\acute{E}$ form of the enzyme.

CONCLUSIONS

In summary, the minimal model proposed in this chapter (Figure 21) is in complete accord with current understanding of the interaction of dTMP synthetase with FdUMP and CH_2-H_4folate. And, since the salient features of this model have at least some experimental support, it appears to provide a credible description of the mechanism of the interaction. This model also reasonably may be expected to describe the normal reaction with dUMP to the point where a steady-state 5,6-dihydropyrimidine intermediate (Pogolotti et al., 1979, Garrett et al., 1979) is formed because of the aforementioned analogies of established features of the partial reaction with those of the first part of the catalyzed reaction. Many important aspects of the mechanism of dTMP synthetase remain unanswered, and it is hoped that the model presented here may be useful in directing more reasoned and focused efforts in needed areas.

The proposed mechanism is most unusal (Purich, 1980) in that after random binding of nucleotide and cofactor to one subunit, subsequent binding to the other subunit is obligatorily ordered. Thus, either isotope-trapping or isotope exchange methods employed in the absence

of the other easily could have led to the misinterpretations of wholely random or ordered (Danenberg & Danenberg, 1978) binding, respectively, in formation of fully complexed enzyme. There is no reason, a priori, why such functional differences should not exist for native and complexed forms of other multimeric enzymes (Segel, 1975). Although dTMP synthetase is used as a paradigm in the studies described in this thesis, the approach of using a combination of isotopic probes should be useful in elucidating the mode of ligand association/dissociation and other important aspects of the mechanisms of these enzymes.

APPENDICES

APPENDIX 1

REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY OF COMPOUNDS OF INTEREST

LEGEND TO TABLE 3

[a]HPLC was performed using a Hewlett-Packard 1084b instrument at ambient temperature with 1.0 ml/min flow rates; pyrimidine derivatives were monitored at 254 nm and folate derivatives at 280 nm. Injections were made of ca. 10 to 100 µl containing ca. 0.02 to 0.1 µmol of the specified compounds. [b]Volume of eluate from sample injection to peak concentration. [c]Altex column: 4.6 x 250 mm. [d]5 mM $(n\text{-}Bu)_4N^+$ phosphate (pH 7.5) and 30% (V/V) MeOH. [e]0.1 M potassium phosphate (pH 6.0) and 5% (V/V) acetonitrile. [f]0.1 M potassium phosphate (pH 6.0) and 1% (V/V) acetonitrile. [g]Unidentified nucleotide impurity (ca. 15%) in commercially obtained (Sigma) FdUMP that can be separated only in this system. [h]Common contaminants from the conversion of FdUrd to FdUMP catalyzed by Escherichia coli dThd Kinase, even after purification on diethylaminoethyl cellulose (Wataya & Santi, 1977). [i]Partial decomposition observed; this system is sueful for qualitative identification only of reduced folates. [j]N-(p-aminobenzoyl)-L-glutamic acid; a major decomposition product of reduced folates. [k]Unidentified decomposition product of CH_2-H_4folate. [l]Present as an antioxidant in mM concentrations of all solutions of reduced folates. [m]The product of oxidation of 2-mercaptoethanol.

TABLE 3: Separation of Pyrimidine Derivatives and Folate Derivatives by Reverse Phase High Pressure Liquid Chromatography[a]

Compound	Retention Volume (ml)[b]		
	Lichrosorb C_{18}[c]		Ultrasphere[c]
	Buffer A[d]	Buffer B[e]	Buffer C[f]
dUrd	3.4		
dUMP	4.6		
FUra	3.4		ca. 30
FdUrd	4.0		20
FdUMP	7.5		5.1
unknown[g]			6.1
AMP[h]	5.7		
ADP[h]	11		
ATP[h]	>15		
folic acid	14	22	
H_2folate	7.8	21	
CH_2-H_4folate	8.2	28[i]	
PABG[j]	6.7	3.8	
unknown[k]	21		
2-mercaptoethanol[l]	3.7	5.0	
2,2'-dihydroxydiethyl-disulfide[l,m]	4.6	12	

APPENDIX 2

SECONDARY α-HYDROGEN KINETIC ISOTOPE EFFECT UPON DISSOCIATION OF THE [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4FOLATE—dTMP SYNTHETASE COMPLEX AT 10°C.

In preliminary studies by McHenry (1974), biphasic Arrhenius plots with break points occurring at ca. 12-15°C were obtained both for second-order rate constants for association of covalent FdUMP—CH_2-H_4folate—dTMP synthetase complexes and for kinetically estimated dissociation constants for binding of CH_2-H_4folate to dTMP synthetase. The interpretation of this work is unclear at present, and the curvature in these plots simply may be artifactual. If not, the plots are diagnostic of different activation energies above and below the transition temperature for the processes being studied, indicating a change in mechanism at this temperature. There are numerous possible explanations for this: a change in conformation of the enzyme, a change in rate-limiting step, a change in the interaction between subunits, an effect of temperature on substrate or cofactor, etc. (Cornish-Bowden, 1979). Obviously, some of these may be expected to alter the transition-state structure or the position with respect to the rate-limiting step of isotopically sensitive covalent bond changing steps. Thus, the possibility of observing significantly different secondary α-hydrogen isotope effects for the association/dissociation of covalent complexes at temperatures above vs. below the break point is implied. Any differences found could provide insight into the nature of the temperature dependent change in mechanism. These arguments and the fact that isotope effects on enzyme mechanisms as a function of temperature rarely have been studied (Northrop, 1977), have prompted the preliminary experiment reported here.

EXPERIMENTAL

Materials and experimental procedures used were the same as described in Chapter 2.

RESULTS

Kinetic Isotope Effect upon Dissociation of the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4Folate—dTMP Synthetase Complex at 10°C. This isotope effect was determined identically to that for the same reaction at 25°C reported in Chapter 2 except that aliquots of the reaction mix were removed at intervals up to ca. 300 hr. Dissociation of the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4folate—dTMP synthetase complex was found to be first-order with $k_H = 1.3 \times 10^{-4}$ min^{-1} ($t_{1/2}$ = 90.1 hr) as shown in Figure 22. The isotope effect was calculated as the ratio of the first-order rate constants for dissociation of ^{3}H and ^{1}H (^{14}C) determined directly from Figure 22: $k_H/k_T = 1.295$. It is noted that standard errors in this detemination are not available from this graphical method, but this is of no consequence in the ensuing discussion.

DISCUSSION

Maximal secondary α-hydrogen isotope effects are manifested at equilibrium and may be estimated from fractionation factors derived from simple model reactions (Hartshorn & Shiner, 1972; Buddenbaum &

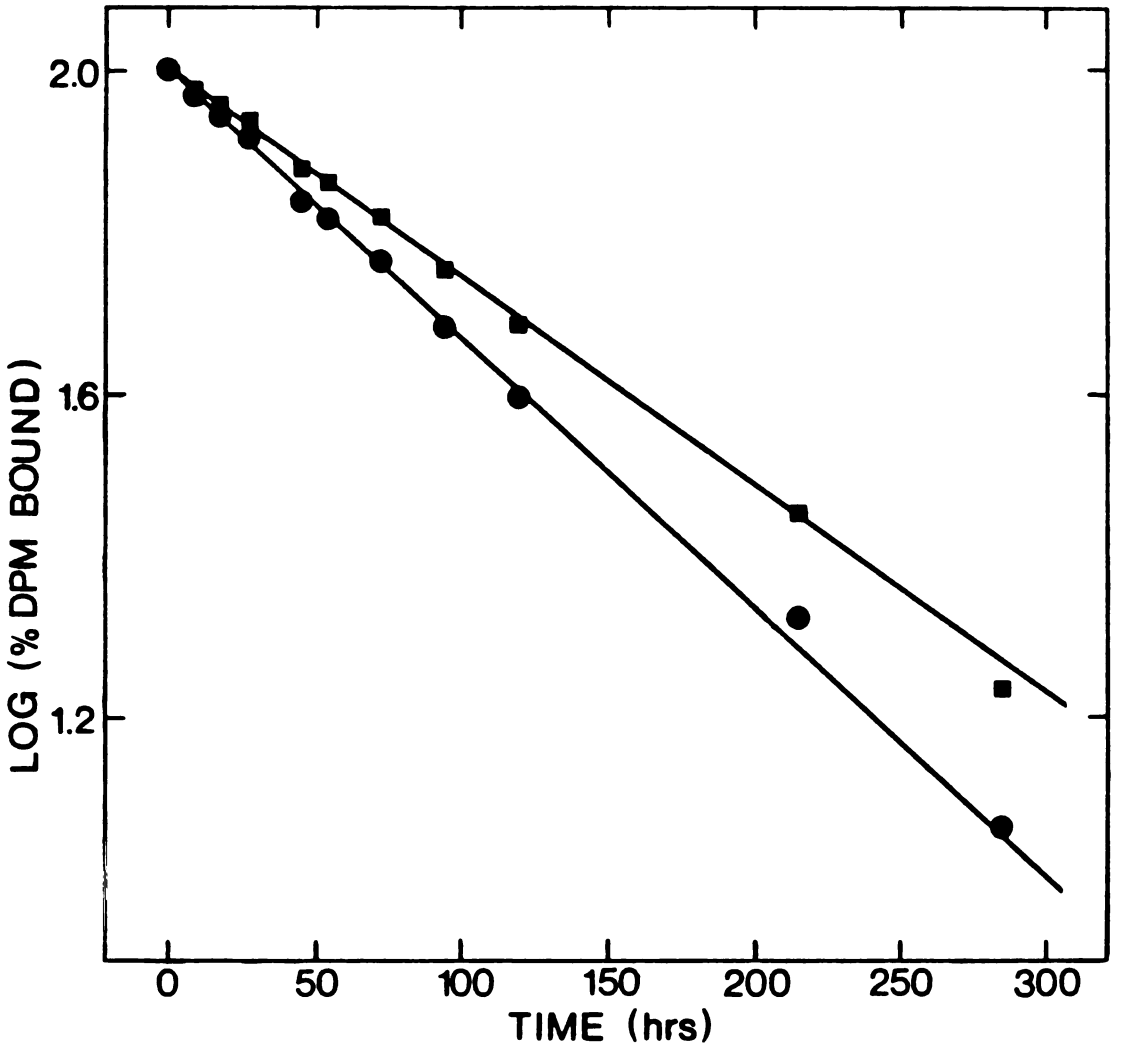

FIGURE 22: Secondary α-hydrogen isotope effect upon dissociation of the [2-^{14}C,6-^{3}H]FdUMP—CH_2-H_4folate—dTMP synthetase complex at 10° C. The data points represent the [2-^{14}C]- (●) and [6-^{3}H]FdUMP (■) bound in the complex isolated over the course of the reaction. Experimental details are described in the text.

Shiner, 1977). Thus, the equilibrium isotope effects (EIE) are dependent only on the structures of the products and reactants and the enzyme acts merely as a catalyst to enhance the rate of approach to equilibrium. Unlike equilibrium isotope effects, the magnitude of observed secondary kinetic isotope effects (KIE) depends on several factors and are not necessarily expected to be maximal. However, the fact that the measured secondary α-hydrogen KIE for dissociation of [2-^{14}C,6-^{3}H]FdUMP——CH_2H_4folate——dTMP synthetase complexes is the same as the EIE shows the kinetically measured effect is maximal at 25°C and relates to a full pre-equilibrium step (Chapter 2).

It is known that intrinsic isotope effects change with temperature--they decrease with increasing temperature--but, within the temperature range wherein enzymes maintain their native integrity the changes are small and may be considered insignificant (Northrop, 1977). Thus, a significant difference in magnitudes of isotope effects measured at 25°C and at < 15°C would be proof of a change in mechanism. Obviously, to ensure the best chances of seeing any differences isotope effects well below 15°C should be measured. However, as the temperature is lowered the rates of interactions of dTMP synthetase with FdUMP and CH_2-H_4folate become slow, to the extent that the necessary meaurements can be made practicably only as low as ca. 10°C. Although this temperature is not much below the transition temperature seen by McHenry, a preliminary isotope effect experiment at 10°C was deemed justified in light of the significance of the possible results.

The rigorous approach would be to measure both equilibrium and kinetic isotope effects at 10°C, but the time to reach equilibrium at this temperature is prohibitively long; artifacts introduced by enzyme denaturation, CH_2-H_4folate decomposition, etc. reasonably could be expected. However, since the KIE=EIE at 25°C using [2-^{14}C,6-^{3}H]FdUMP and the equilibrium isotope effect is not expected to be greatly different at 10°C, in practice only the KIE at 10°C need be measured, at least initially; only if $KIE_{10°} << KIE_{25°}$ would the results have significance for a temperature dependent change in mechanism. Further, satisfactory estimates for the KIE at 10°C if it were essentially temperature independent, i.e., still a full pre-equilibrium effect, can be calculated in two ways:

I) From Melander (1960): $E_{aT} - E_{aH} = RT^2[d\ln(k_T/k_H)/dT]$; not a useful eq. in this form. However, from the Arrhenius eq.:

$$k_T = A_T e^{-E_{aH}/RT} \quad \text{and} \quad k_H = A_H e^{-E_{aT}/RT}$$

Substituting for k_T/k_H and reducing: $E_{aT} - E_{aH} = RT\ln(k_T/k_H)$; a useful, integrated form of the Melander eq.

Solving for the data at 25°C: $E_{aT} - E_{aH} = 122.097$

The $KIE_{10°}$ is now calculated: $k_T/k_H = 0.805$ or $k_H/k_T = 1.243$

II) a semi-empirical equation derived by Hogg *et al.* (1980) is:

$$(k_H/k_T)_{T°} = e^{[2981n(k_H/k_T)_{298}/T]}$$

substituting for $(k_H/k_T)_{298} = 1.229$: $(k_H/k_T)_{283} = 1.243$

Identical values for $KIE_{10^o}^{calc.}$ of 1.243 are obtained from either method. The $KIE_{10^o}^{obsd.}$ is 1.295 and $KIE_{10^o}^{obsd.} - KIE_{10^o}^{calc.}$ is only 0.052. The difference is <u>not</u> large and data of enormous quality would be required to make a definitive statement since it is common for small deterministic errors of this magnitude in different experiments to far outweigh in importance the statistically determined standard errors obtained for a particular experiment (Kirsch, 1977). For example, in chapter 2 KIE values of 1.004 and 1.033 were obtained in different experiments for the dissociation of FdUMP—[^{3}H]CH_2-[2-^{14}C]H_4folate—dTMP synthetase complexes even though the standard errors for each value were much less than the difference between the values. At this point it was decided that the difficulties in performing secondary isotope effect experiments for the interaction of dTMP synthetase with FdUMP and CH_2-H_4folate at low temperatures far outweighed the now diminished probability of obtaining consequential results. However, it should not be concluded in general that studies of the temperature dependence of isotope effects on enzyme reactions cannot yield useful information.

APPENDIX 3

EXPLANATIONS FOR RESULTS OF INDIVIDUAL ISOTOPE-TRAPPING EXPERIMENTS ACCORDING TO THE MODEL OF FIGURE 21.

A general statement may be made concerning the interpretation of the results of these experiments. The model depicted in Figure 21 remains largely undefined in terms of quantitative kinetic analysis. And, the absolute and relative concentrations of components used in the experiments performed here are of limited range for the sake of practicality. Coupled with the complexity of interactions of even this minimal mechanism, these facts render it impossible at the present time to make all but the most basic qualitative conclusions.

Figure 17: In this experiment [$\overset{*}{B}$] is fixed and a constant [$\overset{*}{EB}$] will be present in the reaction mixture, either introduced from the pulse or formed from dissociation of $\overset{*}{B}E\overset{*}{B}$. At low [B] the higher [A] will trap essentially all $\overset{*}{EB}$ to form productive $EA\overset{*}{B}$ or $AEA\overset{*}{B}$ complexes. As [B] is increased $\overset{*}{EB}$ will partition between trapping by A or B, and at very high [B] mostly $BEA\overset{*}{B}$ complexes will be formed and trapping of covalent complexes will be low. It should be noted that if some $\overset{*}{B}E\overset{*}{B}$ partitions to $\overset{*}{B}EA\overset{*}{B}$, even at low [A], then the estimated partition coefficient obtained from Figure 17 at these concentrations may be lower than the trapping value that would be obtained with lower [$\overset{*}{B}$]; i.e. where $\overset{*}{EB}$ rather than $\overset{*}{B}E\overset{*}{B}$ is the major species in the pulse.

Figure 14: This experiment is the complement to that above; with a fixed, high [$\overset{*}{A}$] the predominate species in the pulse will be $\overset{*}{A}E\overset{*}{A}$. This intermediate can partition in the forward direction only to give productive complexes to an extent dependent on the rate of trapping by B; at higher [B] the major effect is increased trapping of covalent complexes. However, at higher [B] more of whatever $E\overset{*}{A}$ is

present will partition to give nonproductive complexes. This partitioning is dependent on competition between B and A for trapping of $E\overset{*}{A}$ and so formation of nonproductive $BE\overset{*}{A}\overset{*}{B}$ can be overcome at very high [A].

Figure 15: Here [B] is high and most $(\overset{*}{B})E\overset{*}{B}$ should partition to $(\overset{*}{B})E\overset{*}{A}\overset{*}{B}$ giving low values for trapping of covalent complexes. Since [A] and [B] are fixed the partitioning between trapping by A and B is constant and as $[\overset{*}{B}]$ is varied there is a direct effect on the amount of $E\overset{*}{B}$ which partitions to covalent complexes; the linear plot that is observed is expected.

Figure 16: Here [B] also is high and low values for trapping of covalent complexes are expected analogous to the above experiment. The [B] is in fact greater than the highest [A] used. From Figure 17 above it is seen that significant competition for trapping of $E\overset{*}{B}$ by A and B is not apparent until $[A] \approx [B]$. Thus, this effect is not expected to be pronounced under the conditions of this experiment; the expected downward deviation from linearity that would be expected for such an effect in a double-reciprocal plot may just be beginning to be seen for the two points at highest [A] in Figure 16.

Figure 13: In this experiment a high $[\overset{*}{A}]$ is used and so the major species in the pulse should be $\overset{*}{A}E\overset{*}{A}$, as for Figure 14, and the extent of trapping of covalent complexes should increase as [B] is raised. At the same time any $E\overset{*}{A}$ present will be trapped to a larger extent in nonproductive complexes; development of non-linearity in a double-reciprocal plot such as that of Figure 13 would be expected under certain conditions.

That the plot of Figure 13 appears linear is easily explained as a consequence of a small amount of $E\overset{*}{A}$ being formed and/or a small effect of partitioning of the $E\overset{*}{A}$ present to give nonproductive complexes over the only ca. 10-fold range of [B] used in this experiment. It should be noted that in the extrapolation to infinite [B] the value for maximum trapping obtained from the y-intercept will be less the amount of $E\overset{*}{A}$ that partitions to nonproductive complexes.

Figure 12: For this experiment a constant ratio of partitioning of $E\overset{*}{A}$ between fixed [A] and [B] is expected. As $[\overset{*}{A}]$ is raised the amount of $E\overset{*}{A}$ relative to $\overset{*}{A}E\overset{*}{A}$ in the pulse decreases and the result is both increased formation of productive complexes and decreased formation of nonproductive complexes. Whether the net result should be a linear or non-linear double reciprocal plot cannot be predicted, but if it is the latter, arguments similar to those proposed above for Figure 13 can be made to account for the linear plot obtained.

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