The Effect of Raney Nickel on the Covalent Thymidylate Synthetase–5-Fluoro-2′-deoxyuridylate–5,10-Methylenetetrahydrofolate Complex†

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ABSTRACT: Raney nickel (Ni(H)) catalyzes a specific reductive cleavage of carbon–sulfur bonds and, therefore, can be used to determine whether compounds are covalently bound to proteins through a sulfide linkage. When the covalent thymidylate synthetase–[^{3}H]5-fluoro-2′-deoxyuridylic acid–[^{14}C]-5,10-CH_2H_4-folate complex (Langenbach et al. (1972a), *Biochem. Biophys. Res. Commun. 48,* 1565) was denatured and then shaken with Ni(H) at 25 °C, both isotopes were rapidly cleaved from the protein, with identical reaction halftimes of less than 10 min. The liberated radioactivity was filterable through nitro-cellulose filters and comigrated with small molecules on Sephadex G-25. Both labels migrated identically upon paper chromatography. A [^{3}H]5-fluoro-2′-deoxyuridylic acid–[^{35}S]thymidylate synthetase complex was formed with enzyme isolated from *Lactobacillus casei* grown in the presence of [^{35}S]cysteine. This complex, upon Ni(H) treatment, released both tritium and sulfur-35 at identical rates. Control experiments on amino acids showed that only the sulfur-containing amino acids are degraded by Ni(H). Cysteine was rapidly converted to alanine and methionine to α-aminobutyric acid. 5-Carboxymethylcysteine and 5-uracilylcysteine, simple models for the ternary enzyme–5-fluoro-2′-deoxyuridylic acid–5,10-CH_2H_4-folate complex, were converted to alanine at the same rate that 5-fluoro-2′-deoxyuridylic acid (FdUrd-5′-P) was cleaved from the enzyme. Native ribonuclease, which has a tightly coiled structure, was not affected by the reagent, but carboxymethylated ribonuclease was desulfurized. Amino acid analysis of Ni(H)-treated thymidylate synthetase showed that cysteine was the only amino acid degraded. Gel electrophoresis of the proteins after exposure to Ni(H) showed no breakage of polypeptide chains. These results support a sulfide linkage between FdUrd-5′-P and thymidylate synthetase in the covalent complex.

The important anticancer drugs FUra[1] and FdUrd are effective as a result of conversion in vivo to the nucleotide (FdUrd-5′-P), which is a powerful inhibitor of thymidylate synthetase (methylenetetrahydrofolate:dUrd-5′-P-C-methyltransferase, EC 2.1.1.b) (Cohen et al., 1958; Heidelberger et al., 1960; Hartmann and Heidelberger, 1961; Reyes and Heidelberger, 1965). Considerable effort has been devoted in this laboratory to a study of the strong interaction between FdUrd-5′-P and thymidylate synthetase, not only in the hope of discovering principles which could be applied to the design of other drugs, but also as an aid in understanding the mechanism of this important enzyme.

Thymidylate synthetase, FdUrd-5′-P, and the cofactor, 5,10-CH_2H_4-folate have been shown to form a stable covalent complex (Langenbach et al., 1972a; Danenberg et al., 1974; Santi and McHenry, 1972). All of the evidence indicates that FdUrd-5′-P behaves as a "K_{cat}" inhibitor (Rando, 1974) or quasi-substrate, initially forming a reversible complex that becomes covalently bound through a partial catalytic conversion carried out by the enzyme. It had been postulated that one of the first steps in the catalytic sequence is the addition of a nucleophile to the 5,6 double bond of dUrd-5′-P (Santi and Brewer, 1968; Kalman, 1971; Santi and Brewer, 1973). Because of the close analogy between the reactions of dUrd-5′-P and FdUrd-5′-P with thymidylate synthetase, it is likely that the same nucleophile that performs the initial catalytic step also covalently binds FdUrd-5′-P to the enzyme. Considerable evidence has accumulated that the nucleophilic catalytic species is the sulfhydryl group of a cysteine residue (Dunlap et al., 1971; Kalman, 1971; Kalman, 1972; Wataya and Hayatsu, 1972). However, recently threonine or histidine has been proposed as the candidate nucleophilic species (Sommer and Santi, 1974).

Raney nickel (Ni(H)) causes a reductive cleavage of carbon–sulfur bonds in organic compounds, usually resulting in the formation of a new carbon–hydrogen bond. Since Ni(H) has been used successfully in structure determinations which depend on the selective excision of sulfur (Fieser and Fieser, 1967), this reagent could also be used as a diagnostic test for sulfide linkages between a protein and covalently bound molecules. In this report, experiments are presented in which we studied the effects of Ni(H) on amino acids and a protein model (ribonuclease) and then applied the reagent to the covalent thymidylate synthetase–FdUrd-5′-P–5,10-CH_2H_4-folate complex.

Materials and Methods

An amethopterin-resistant strain of *Lactobacillus casei* was kindly provided by Dr. F. M. Huennekens and Dr. R. B. Dunlap and was grown and purified according to the procedure of Dunlap et al. (1971) with the slight changes described previously (Danenberg, et al., 1974). The enzyme used in these experiments was homogeneous by sodium do-

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[1] Abbreviations used are: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUrd-5′-P, 5-fluoro-2′-deoxyuridylic acid; dUrd-5′-P, 2′-deoxyuridylic acid; 5,10-CH_2H_4-folate, 5,10-methylenetetrahydrofolic acid; Ni(H), activated Raney nickel.

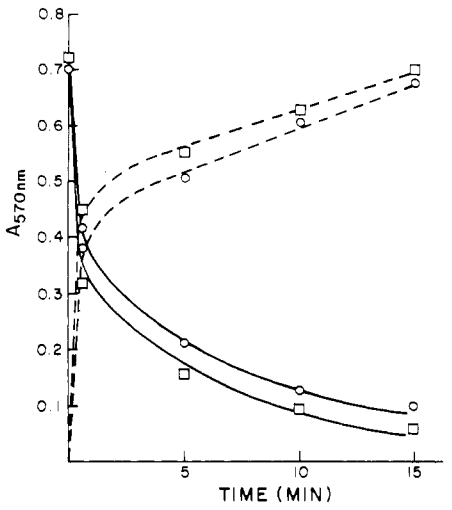

FIGURE 1: Desulfuration of 5-carboxymethylcysteine and 5-uracilylcysteine with Ni(H). The amino acids were treated with Ni(H) as described in Materials and Methods, and equal aliquots were removed at intervals from the reaction mixture. Separation of alanine from 5-carboxymethylcysteine or 5-uracilylcysteine was accomplished by thin-layer chromatography on cellulose plates, eluted with 1-butanol–acetic acid–water, 6:2:2. Areas corresponding to the amino acids were located using standards, removed, and analyzed for amino acid content with ninhydrin. The graph shows the disappearance of 5-carboxymethylcysteine (— □ —) and 5-uracilylcysteine (— ○ —), and the corresponding appearance of alanine (- - - □ - - -, - - - ○ - - -).

Table I: An Equimolar Mixture of Amino Acids Treated with Ni(H).

	Before	After Ni(H)
Lys	1.00	1.06
His	1.00	0.92
Arg	1.00	0.90
Thr	1.00	1.05
Ser	1.00	0.89
Pro	1.00	1.10
Gly	1.00	1.07
Ala	1.00	2.10
Cys	1.00	0.00
Val	1.00	1.12
Met	1.00	0.22
Ile	1.00	1.07
Leu	1.00	1.06
α-Amino butyric acid	0.00	0.70

decyl sulfate–gel electrophoresis. [6-^{3}H]FdUrd (6.7 Ci/mmol) was obtained from New England Nuclear Corp. Amino acids and bovine pancreas ribonuclease were obtained from Sigma Chemical Co. The Raney nickel catalyst (No. 28) was a product of W. R. Grace and Co.

Amino acid analyses were carried out on a Beckman Model 120C amino acid analyzer, except the amino acid analysis in Table IV, which was done at AAA Laboratory, Seattle, Washington. Oxidation of protein samples was carried out by the method of Moore (1963). Reduction of ribonuclease and carboxymethylation with iodoacetic acid were done according to Crestfield et al. (1963a). Protein concentrations were determined by the method of Lowry et al. (1951). 1- and 3-carboxymethylhistidines were synthesized by the method of Crestfield et al. (1963a), and 5-uracilylcysteine by the method of Wataya et al. (1973). Gel electrophoresis was carried out as previously described (Langenbach et al., 1972a).

Preparation of [6-^{3}H]FdUrd-5′-P. [6-^{3}H]FdUrd (0.037 mg; specific activity 6.7 Ci/mmol) was diluted with 0.37 mg of nonradioactive FdUrd to a specific activity of 0.6 Ci/mmol and then phosphorylated according to the procedure of Yoshikawa et al. (1967). When phosphorylation was complete, as determined by disappearance of FdUrd upon thin-layer chromatography (chloroform–methanol, 4:1 on silica gel), the reaction mixture was adjusted to pH 8.0 with 0.1 N NaOH, and the triethyl phosphate was extracted with ether from the aqueous solution. The solution was reduced in vacuo to a small volume and streaked onto a sheet of Whatman No. 40 paper (46 × 57 cm). The sheet was eluted with 1-butanol–acetic acid–water, 5:2:3. In this system FdUrd-5′-P has an R_f of 0.4. The band corresponding to [6-^{3}H]FdUrd-5′-P was cut out, and the nucleotide was removed from the paper with water. The yield was 75% based on the initial amount of radioactivity. The product was in all respects identical with authentic samples of FdUrd-5′-P. Recently, Chiu and Dunlap (1974) reported a similar procedure for synthesis of FdUrd-5′-P.

Treatment of Thymidylate Synthetase Complexes with Ni(H). The purified enzyme (0.1 mg) was incubated with excess FdUrd-5′-P and 5,10-CH_2H_4-folate and denatured as previously described (Danenberg et al., 1974). The denatured enzyme–FdUrd-5′-P–5,10-CH_2H_4-folate complex was freed of thiols by successive ultrafiltrations in an Amicon PM-10 ultrafiltration apparatus using 0.1 M potassium phosphate buffer, pH 7.5. The volume was adjusted to 1.0 ml, and 30 mg of Ni(H) was added as a slurry in distilled water. The mixture was stirred vigorously by taping the test tube to a Vortex stirrer. Aliquots were removed at intervals, centrifuged to remove particles of the catalyst, and filtered through nitro-cellulose filters according to the procedure developed by Yarus and Berg (1970) and Santi et al. (1974). The filters were dried under an infrared lamp and counted in a toluene–PPO–POPOP solution.

Treatment of Ribonuclease and Amino Acids with Ni(H). The amino acid (10 μmol) or pancreatic ribonuclease (2.0 mg), either in the native form or as the carboxymethylated protein, was dissolved in 1.0 ml of distilled water. Ni(H) (60 mg) was then added and the solution stirred on a Vortex mixer. For samples to be analyzed on the amino acid analyzer, the catalyst was removed by centrifugation, and the protein solution was passed through a small column (0.65 × 5 cm) of Chelex-100 chelating resin to remove metal ions. The column was washed with 10 ml of 1 N ammonium hydroxide, which was then evaporated to dryness.

Recovery of Adsorbed Radioactivity from the Ni(H). After the enzyme–[^{3}H]FdUrd-5′-P–^{14}C-labeled cofactor complex had been shaken with the Ni(H), the catalyst was removed by centrifugation and washed several times with distilled water. To the catalyst was then added 1.0 ml of a solution of 10^{-3} M FdUrd-5′-P and 10^{-3} M 5,10-CH_2H_4-folate, containing 1 mg of bovine serum albumin. This mixture was shaken at room temperature for 18 h. The metal particles were again removed by centrifugation.

Isolation of [^{35}S]Thymidylate Synthetase. The medium for growing *Lactobacillus casei* (*L. casei*) was made up according to Crusberg et al. (1970). After 6 l. of medium had been inoculated with methotrexate-resistant *L. casei,* 0.5 mCi (10 μmol) of [^{35}S]cysteine was added. The mixture was incubated at 37 °C until the bacteria reached log phase. The cells were then removed by centrifugation, resulting in a yield of 20 g of packed cells (wet weight). Isola-

Table II: The Effect of Ni(H) on the Sulfur-Containing Amino Acids of Carboxymethylated Ribonuclease.

	Ribonuclease Lit.[a]	Ni(H)-Treated Ribonuclease	Ni(H)-Treated CM-Ribonuclease
Cys	8.1[b]	6.7	0
Met	3.8	4.0	0
Val	8.7	7.9	8.0
Ala	11.9	11.0	16.9

[a] Crestfield et al. (1963b). [b] Number of amino acid residues per molecule of enzyme.

tion of the [^{35}S]thymidylate synthetase was carried out by the method described above. From this quantity of cells, 2.1 mg of purified enzyme was obtained (specific activity 2.7 units/mg). The incorporation of [^{35}S]cysteine into thymidylate synthetase was 3.2×10^4 dpm per mg of protein.

Results

The Effect of Ni(H) on Model Systems. a. Amino Acids. A number of amino acids were treated with Ni(H) to determine the conditions required for desulfuration, as well as to measure rates of reaction and degree of specificity. 5-Carboxymethylcysteine and 5-uracilylcysteine, which can be considered as simple models for the enzyme-FdUrd-5′-P-cofactor complex in which the linkage is a sulfide bond, were exposed to the metal in aqueous solution at room temperature, and aliquots were removed at short intervals for chromatographic analysis. The disappearance of the sulfur-containing amino acid and the generation of alanine were monitored (Figure 1). It is apparent that both 5-carboxymethylcysteine and 5-uracilylcysteine are almost completely converted to alanine under these very mild conditions in less than 10 min. Both compounds have similar rates of desulfuration, although the 5-uracilylcysteine reacted slightly faster. The reactivity of cysteine to Ni(H) was similar to that of carboxymethylcysteine, whereas cysteine reacted about twofold more slowly, as estimated by TLC.

The rationale behind the experiments presented in this paper is that in amino acid and protein systems, Ni(H) will be completely specific for cleavage of carbon–sulfur bonds; i.e., cysteine derivatives and methionine will be degraded, but other amino acids will be unaffected. The studies described in this section were done in order to verify the specificity of the reagent. A standard mixture of amino acids was treated with Ni(H) under the conditions described in Materials and Methods. Upon analysis, the ratios of all amino acids were found to remain the same except those of cysteine, which was quantitatively converted to alanine, and methionine, which was partially converted to α-aminobutyric acid (Table I). It is significant to note that the carbon–oxygen bonds of threonine, *N,O*-diacetylthreonine, and serine, in contrast to the carbon–sulfur bond of cysteine and methionine, were completely unaffected by this reagent. Similarly, no cleavage of the carbon–nitrogen bonds of 1- and 3-carboxymethylhistidines could be detected under these conditions. Threonine and histidine have been proposed as candidate nucleophiles through which FdUrd-5′-P becomes covalently bound to thymidylate synthetase (Sommer and Santi, 1974).

b. Ribonuclease was chosen as a model for a study of the effects of Ni(H) on proteins because its high cysteine content would facilitate the amino acid analysis. We wished to determine whether proteins could be effectively desulfurized and also to study the effects of the reagent on the polypeptide chain of a protein. Ribonuclease was treated with Ni(H) for varying lengths of time from 30 min to 3 h under the conditions described in Materials and Methods and then subjected to acid hydrolysis and amino acid analysis. Sodium dodecyl sulfate–gel electrophoresis of the protein before hydrolysis showed no discernible cleavage of the polypeptide chain upon Ni(H) treatment. Amino acid analysis showed that neither the cysteine nor methionine content of native ribonuclease was appreciably decreased after exposure to the reagent for as long as 3 h (Table II). Thus, it appears that the disulfide bridges of this enzyme are not sterically accessible to the metal particles in the heterogeneous suspension. To unravel the native structure of ribonuclease, and prevent refolding, ribonuclease was reduced with thiols and carboxymethylated (Crestfield et al., 1963a). After the protein had been modified in this manner, Ni(H) treatment for 1 h and acid hydrolysis of the protein gave an amino acid mixture in which no 5-carboxymethylcysteine or methionine were detected (Table II). Again, sodium dodecyl sulfate–gel electrophoresis showed that no cleavage of the peptide chain of the carboxymethylated protein had occurred under these conditions. From these results it is apparent that Ni(H) is not only specific for cleavage of carbon–sulfur bonds, but also is a reagent selective for accessible sulfhydryl groups on proteins.

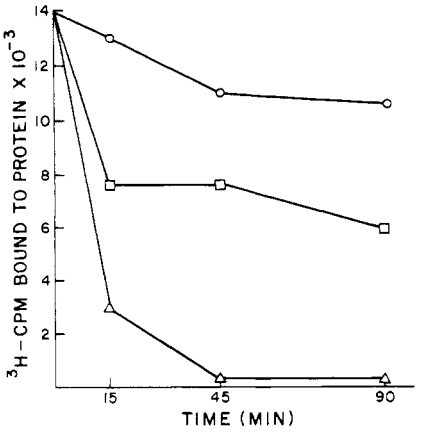

FIGURE 2: Nitro-cellulose filtration of thymidylate synthetase-[^{3}H]FdUrd-5′-P-cofactor treated with Ni(H). Aliquots (0.1 ml) were removed at intervals and filtered through nitro-cellulose filters as described in Materials and Methods. At the same time, aliquots were removed and analyzed for protein content. The counts are corrected for adsorption of ~30% of the protein to the catalyst. (— O —) Control, with no Ni(H) added; (— □ —) 30 mg of Ni(H) plus 0.1 mmol of β-mercaptoethanol after 5 min; (— △ —) 30 mg of Ni(H).

Effect of Ni(H) on the Thymidylate Synthetase-FdUrd-5′-P-5,10-CH_2H_4-folate Complex. If FdUrd-5′-P is covalently bound to thymidylate synthetase via a sulfide linkage, we expected that Ni(H) would be able to free the nucleotide from the protein. The enzyme-[^{3}H]FdUrd-5′-P-cofactor complex was allowed to form and then was denatured with sodium dodecyl sulfate as previously described (Danenberg et al. 1974). When the denatured complex was shaken with Ni(H) under the standard conditions described in Materials and Methods, a rapid removal of [^{3}H]FdUrd-5′-P from the protein was observed (Figure 2). In 15 min, there was an 80% loss of radioactivity as determined by the nitro-cellulose filtration method and, in 45 min, all the [^{3}H]FdUrd-5′-P had been cleaved from the protein. As evidence that this phenomenon is due to a specific interaction of the catalyst with carbon–sulfur bonds, we found that addition of β-

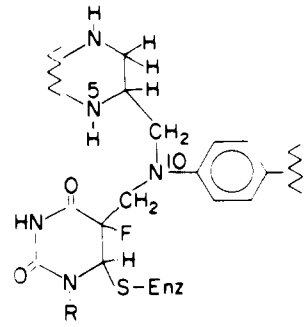

FIGURE 3: Proposed structure for the covalent thymidylate synthetase-FdUrd-5′-P-5,10-CH_2H_4-folate complex, where R = 5-phospho-2′-deoxyribosyl.

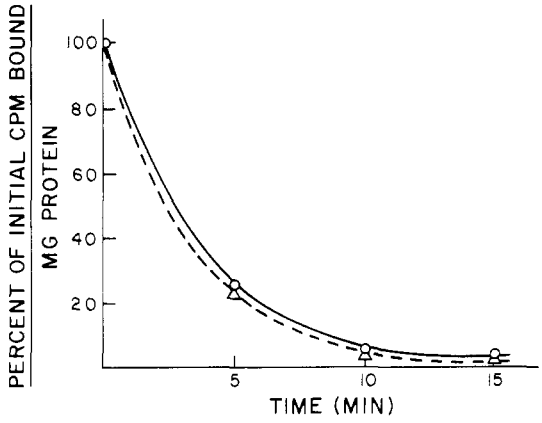

FIGURE 4: Treatment of the double-labeled thymidylate synthetase-[^{3}H]FdUrd-5′-P-[^{14}C]-5,10-CH_2H_4-folate complex with Ni(H). The procedure was the same as that described under Figure 2. (— o —) Tritium; (— Δ —) ^{14}C.

mercaptoethanol in large excess over the enzyme complex immediately quenched the cleavage reaction (Figure 2).

In the structure that we previously proposed (Langenbach et al., 1972a) for the ternary thymidylate synthetase-FdUrd-5′-P-5,10-CH_2H_4-folate complex, the nucleotide and cofactor are connected to each other by a methylene bridge formed from the reactive 5,10-methylene group of the cofactor (Figure 3). Therefore, rupture of the bond holding FdUrd-5′-P to the enzyme should result in simultaneous release of both the nucleotide and 5,10-CH_2H_4-folate. On the other hand, if the reaction proceeds through a methylated enzyme intermediate, the cofactor should remain with the protein. To differentiate between these possibilities and thereby test the correctness of the proposed structure, Ni(H) treatment was applied to the double-labeled enzyme-[^{3}H]FdUrd-5′-P-^{14}C-labeled cofactor complex. A rapid and simultaneous cleavage of both isotopes from the protein was observed (Figure 4), in which 80% of the radioactivity was liberated in 5 min, providing evidence that both moieties are attached to the protein through the same sulfide bond.

If covalently bound FdUrd-5′-P can be separated from the protein by a reagent that specifically cleaves carbon-sulfur bonds, there should also be a parallel loss of the sulfur through which the nucleotide is bound. To test this prediction, we grew *L. casei* in the presence of [^{35}S]cysteine, and isolated [^{35}S]thymidylate synthetase. The [^{3}H]FdUrd-5′-P-cofactor-[^{35}S]enzyme complex was then treated with Ni(H). A time course of the cleavage reaction (Table III) shows that both tritium and sulfur-35 were removed from the protein at identical rates.

Table III: Ni(H) Treatment of the [^{3}H]FdUrd-5′-P-Cofactor-^{35}S-Labeled Enzyme Complex.[a]

Time (min)	Fraction of [^{3}H]FdUMP Bound	Fraction of S-35 Remaining
0	1.0	1.0
5	0.35	0.34
10	0.044	0.044
15	0.0235	0.029
25	0.020	0.01

[a] [^{35}S]Thymidylate synthetase (0.25 unit) was incubated with excess [^{3}H]FdUrd-5′-P and 5,10-CH_2H_4-folate for 15 min in 50 mM Tris, pH 7.5, with 10 mM β-mercaptoethanol in a total volume of 1.0 ml. Then 2 mg of sodium dodecyl sulfate was added, and the mixture was heated in a steam bath for 3 min to denature the enzyme. The solution was placed on a Sephadex G-25 column and the [^{3}H]FdUrd-5′-P-[^{35}S] enzyme complex isolated. The complex was treated with 30 mg of Ni(H), and aliquots were removed at the intervals indicated. Measurements of [^{3}H]FdUrd-5′-P binding were performed as described in Materials and Methods. The ratios in the table are corrected for the amount of protein in the sample.

Recovery of the Released Radioactivity. Only 10% of the [^{3}H]FdUrd-5′-P and ^{14}C-labeled cofactor freed from the protein by the Ni(H) treatment was found in the filtrate after nitro-cellulose filtration. Most of the released radioactivity remained adsorbed to the catalyst. In order to desorb this material, the metal particles were removed by centrifugation and shaken in a solution containing FdUrd-5′-P, cofactor, and bovine serum albumin. In this manner, about 50% of the radioactivity that had originally been associated with the protein was recovered. This material was found to be completely filterable through nitro-cellulose filters which had a lower molecular weight limit of 10 000. Sephadex G-25 analysis (Figure 5) showed that the desorbed radioactive material had a molecular weight of less than 1000, and eluted together with small molecules such as mononucleotides.

The desorbed material was further analyzed by paper chromatography. Both isotopes were found to have the same R_f values (0.50), intermediate between those of FdUrd-5′-P (0.70) and 5,10-CH_2H_4-folate (0.35), indicating that both the [^{3}H]FdUrd-5′-P and ^{14}C-labeled cofactor moieties comprise portions of a single compound. This is consistent with the proposed structure for the complex since we would expect that, even if the bond between the FdUrd-5′-P moiety and the enzyme were cleaved, the nucleotide and cofactor would still remain attached via the methylene bridge.

Amino Acid Analysis of the Modified Protein. Amino acid analyses were performed on acid hydrolysates of the FdUrd-5′-P-5,10-CH_2H_4-folate-thymidylate synthetase complex treated with Ni(H) for 60 min and a sample of the native enzyme. The results are compared in Table IV. The cysteine content of the Ni(H)-treated complex was 64% lower than that of the native enzyme, corresponding to a loss of 1.3 cysteine residues per subunit. There was a corresponding increase in the alanine content of the Ni(H)-treated enzyme, as expected from the reaction of Ni(H) with cysteine. The ratios of the remaining amino acids were not appreciably different in the two samples. These analyses confirm the specificity of Ni(H) for sulfur-containing amino acids.

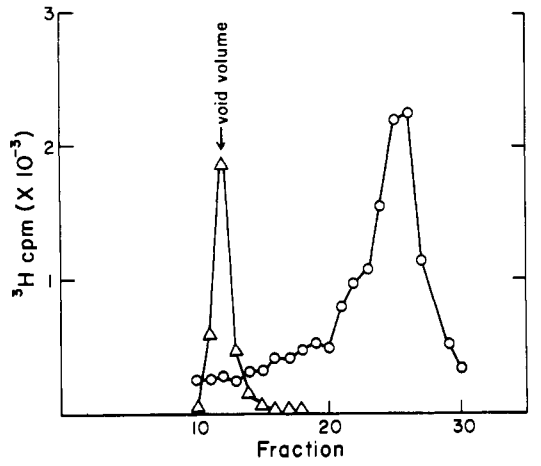

FIGURE 5: Sephadex G-25 chromatography of the radioactive material cleaved from the enzyme-[^{3}H]FdUrd-5′-P-cofactor complex by Ni(H), and subsequently desorbed from the metal: (— Δ —) enzyme-[^{3}H]FdUrd-5′-P complex before Ni(H) treatment; (— O —) radioactivity recovered from the catalyst as described in Materials and Methods, corresponding to molecules under a molecular weight of 1000.

Effect of Ni(H) on Enzyme Activity. Treatment of native thymidylate synthetase with Ni(H) resulted in a rapid loss of enzyme activity (Table V). The decrease in catalytic activity of the enzyme correlated well with the rate of degradation of cysteine residues, as measured by loss of sulfur-35 from the protein. The Ni(H)-treated enzyme was incubated with [^{3}H]FdUrd-5′-P and cofactor, but no binding of the nucleotide was detected.

Gel Electrophoresis. To determine whether Ni(H) can cleave the polypeptide chain of thymidylate synthetase, sodium dodecyl sulfate-gel electrophoresis of the enzyme-[^{3}H]FdUrd-5′-P-cofactor complex was performed before and after Ni(H) treatment. The gels were scanned for protein content and then sliced and counted. Enzyme from which 90% of the radioactivity had been removed by the nickel migrated identically with the untreated complex.

Table IV: The Effect of Ni(H) on the Amino Acid Composition of Thymidylate Synthetase.

	Native Enzyme[a]	Ni(H)-Treated Enzyme	Ratio
Ala	18.0	19.8	1.10
Arg	9.8	9.7	0.99
Asp	31.5	30.6	0.97
Cys	2.1	0.74	0.36
Glu	24.5	23.3	0.95
Gly	16.3	16.7	1.04
Ile	12.0	11.9	0.99
Leu	29.1	28.7	0.98
Lys	15.4	16.2	1.06
Met	5.2	4.6	0.89
Phe	15.5	15.4	0.99
Pro	14.7	15.3	1.04
Ser	11.3	12.0	1.06
Thr	12.6	12.8	1.01
Tyr	11.7	11.2	0.97
Val	12.0	12.7	0.99

[a] Residues per subunit.

Table V: The Effect of Ni(H) on the Enzymatic Activity and Cysteine Content of Thymidylate Synthetase.[a]

Time (min)	Fraction of Enzymatic Activity Remaining	Fraction of Sulfur-35 Remaining
0	1.0	1.0
5	0.30	0.39
10	0.06	0.20
30	0.0	0.08

[a] [^{35}S]Thymidylate synthethase was passed through a Sephadex G-25 column to remove thiols. The enzyme (0.10 unit) was then shaken with Ni(H) (30 mg). Aliquots were removed at the times indicated and assayed for enzymatic activity, protein content, and sulfur-35 content. The values in the table have been corrected for the amount of protein in the aliquot.

Discussion

The discovery by Lomax and Greenberg (1967) that thymidylate synthetase catalyzes an exchange between water and the C-5 proton of dUrd-5′-P has been important in determining the course of subsequent work on the reaction mechanism of this enzyme. It has been suggested, based on studies with model systems (Santi and Brewer, 1968; Kalman, 1971; Santi and Brewer, 1973), that this exchange occurs as a result of addition of nucleophile to the 5,6 double bond of the pyrimidine portion of dUrd-5′-P. We have postulated that the presence of the fluorine atom at the 5-position of FdUrd-5′-P prevents elimination of this nucleophilic species with the result that the fluorinated nucleotide remains covalently bound to the enzyme (Langenbach et al., 1972a).

An important question in elucidating the mechanism of thymidylate synthetase is the identity of the nucleophile through which FdUrd-5′-P binds. Then, if the analogy between the stable enzyme-FdUrd-5′-P-cofactor complex and the transition state of the enzymic reaction is correct (Danenberg et al., 1974), this is also the group that participates in the initial catalytic step.

There is considerable evidence that the nucleophilic catalyst in question is the sulfhydryl group of a cysteine residue. Glutathione and bisulfite have been found to be the most effective in promoting exchange of the C-5 proton of uridine, analogously to the enzymatic reaction with dUrd-5′-P (Kalman, 1971; Wataya and Hayatsu, 1972). Other nucleophiles such as hydroxyl groups require high temperature conditions and extreme pH's, as well as favorable molecular proximity (Santi and Brewer, 1973). Dunlap et al. (1971) have demonstrated that the catalytic activity of the enzyme is destroyed when one sulfhydryl group per enzyme molecule has reacted with *p*-chloromercuribenzoate. Since both substrates, dUrd-5′-P and 5,10-CH_2H_4-folate, protect the enzyme against inactivation by sulfhydryl reagents (Langenbach et al., 1972b), the essential sulfhydryl group is most likely within the active site. Treatment of the enzyme with hydroxyethyl disulfide prevents covalent binding of FdUrd-5′-P, but the enzyme still retains the ability to bind the nucleotide reversibly (Kalman and Im, 1973). Showdomycin (3-β-D-ribofuranosylmaleimide) 5′-phosphate, which is both a sulfhydryl reagent and an analogue of dUrd-5′-P, inactivates thymidylate synthetase much faster than the nucleoside analogue, showdomycin (Kalman, 1972). Since addition of a phosphate group would confer substrate-like specificity to the reagent, this result suggests that a sulfhydryl group in the active site is alkylated. The enzyme is protected against this reagent by dUrd-5′-P. The data cited above strongly indicate that a sulfhydryl group is intimately involved in catalyzing the reaction as well as in the covalent binding of FdUrd-5′-P.

Recently, however, data have been published which suggest that FdUrd-5′-P may not be covalently bound to a cysteine residue of the enzyme (McHenry and Santi, 1974). It was found that there was little difference in the uptake of sulfhydryl reagents between the free enzyme and the enzyme–FdUrd-5′-P complex. Furthermore, when a small peptide containing FdUrd-5′-P was hydrolyzed in strong acid, no cysteine was found in the hydrolysate (Sommer and Santi, 1974).

To resolve the controversy surrounding the role of the catalytically essential sulfhydryl group of thymidylate synthetase, we sought an analytical method that would clearly show whether FdUrd-5′-P and the enzyme were bound to each other by a sulfide linkage. Ni(H) appeared to be the ideal reagent for this purpose since it has the unique property of specifically excising sulfur atoms from organic molecules (Pettit and Van Tamelen, 1962). Indeed, when we treated amino acids with this reagent, there was no cleavage of the carbon–oxygen or carbon–nitrogen bonds of serine, threonine, *N,O*-diacetylthreonine, or carboxymethylhistidine. The only reaction observed was degradation of cysteine and methionine to produce alanine and α-aminobutyric acid, respectively. Thus, if FdUrd-5′-P were bound to the enzyme through an ether linkage (resulting from addition of threonine or serine to the 5,6 double bond) or through an amino linkage (resulting from addition of histidine, lysine, or arginine) as postulated (Sommer and Santi, 1974), the nucleotide should remain bound to the protein in the presence of Ni(H). On the contrary, we have observed that Ni(H) causes a rapid breakdown of the enzyme–FdUrd-5′-P–cofactor complex with the release of both the nucleotide and cofactor moieties from the protein. The basis for this conclusion is the following. (a) The nucleotide–cofactor portion of the complex is liberated as a small fragment that readily passes through nitro-cellulose filters and co-migrates with molecules of molecular weight less than 1000 on Sephadex G-25. Thus, we can preclude the possibility that the observed loss of radioactivity upon nitro-cellulose filtration is actually removal of the enzyme–FdUrd-5′-P–cofactor from solution by a preferential and tight adsorption to the catalyst. In that case, the radioactivity recovered from the nickel would still be of high molecular weight. (b) The cleavage of the ligands from the protein depends upon a specific interaction of the catalyst with sulfur atoms. When excess sulfhydryl groups are added to the reaction mixture, the cleavage reaction is rapidly quenched with no further release of radioactive ligands from the protein. (c) [^{3}H]FdUrd-5′-P and sulfur-35 are cleaved from the protein at the same rate. This is a necessary condition if a sulfide bond exists between the nucleotide and the enzyme. (d) Decomposition of the ternary complex by harsh reaction conditions is also ruled out. The desulfuration reaction occurs rapidly under very mild conditions; the reduction of cysteine to alanine has a halftime of 5 min when stirred with Raney nickel at room temperature. Chapeville et al. (1962) employed similar conditions in their classic experiment in which cysteinyl-tRNA^{Cys} was treated with Raney nickel. The resulting alanyl-tRNA^{Cys} that was obtained retained full biochemical activity, showing that tRNA determines the specificity of amino acid transfer. We have shown that the disulfide bridges of native ribonuclease are not affected by Raney nickel, but only when ribonuclease is denatured by reduction and carboxymethylation does desulfuration occur. From these observations and the gel electrophoresis results, it can be concluded that the structural integrity of macromolecules is not significantly affected by adsorption to the catalyst.

In view of the demonstrated specificity of the Raney nickel catalyzed desulfuration reaction, the results presented here provide strong evidence in favor of a sulfide linkage between FdUrd-5′-P and thymidylate synthetase. By analogy, therefore, a sulfhydryl group is the nucleophilic species that initiates the enzymic reaction.

We believe that Raney nickel has potential as a reagent useful to protein chemistry in general. It could be used, for example, to demonstrate whether a nonspecific reagent such as iodoacetic acid has alkylated sulfhydryl residues in a protein, in addition to its use in determining whether ligands become bound to protein by sulfhydryl addition. During the course of this work, Corbett and Nettesheim (1974) reported that Raney nickel cleaves covalently bound carcinogens from proteins, and they considered this as evidence that the hydrocarbon metabolites are bound through cysteine or homocysteine.

Acknowledgment

We are deeply indebted to Dr. Charles B. Kasper and Mrs. Marilynn Sikes for performing the amino acid analyses described in Tables I and II, and to Dr. Richard G. Moran for growing the sulfur-35-labeled *L. casei*.

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Specificity of Thrombin: Evidence for Selectivity in Acylation Rather Than Binding for *p*-Nitrophenyl α-Amino-*p*-toluate†

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ABSTRACT: The lysyl ester analogue *p*-nitrophenyl α-amino-*p*-toluate hydrobromide was synthesized, and its reactions with thrombin, trypsin, and plasmin were studied by stopped-flow and conventional methods. Kinetic parameters were compared with those determined for the arginyl ester analogue, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride, with these enzymes. By following nitrophenol release or proflavin absorption changes in the stopped-flow spectrophotometer, the constants K_s (enzyme-substrate binding), k_2 (acylation), and k_3 (deacylation) were determined. The major findings were: (1) K_s values were similar regardless of the substrate or the enzyme; (2) k_3 was approximately the same for the reaction of the lysyl ester analogue with any enzyme; (3) k_2 for the lysyl ester analogue was 1100 times greater with trypsin than with thrombin; and (4) k_2 with thrombin was 60 times greater for the arginyl than for the lysyl ester analogue. The results suggest that the limited cleavage of lysyl bonds by thrombin is due in part to restricted acylation rather than substrate binding. The active site of thrombin, compared with that of trypsin, appears to have a more stringent requirement for the spatial relationship between the cationic group and the bond cleaved in substrates.

Crucial to thrombin's key role in blood coagulation is its restricted proteolytic specificity. Thrombin hydrolyzes only four arginyl-glycine bonds in its main physiological substrate, fibrinogen. It cleaves only two such bonds in activating factor XIII (Schwartz et al., 1973; Takagi and Doolittle, 1974). Thrombin also shows a marked preference for cleavage of arginyl rather than lysyl small-molecule substrates. In contrast, plasmin, a homologous protease, preferentially cleaves lysyl substrates, while trypsin hydrolyzes both arginyl and lysyl bonds with little discrimination (Weinstein and Doolittle, 1972).

Studies with synthetic peptide substrates and defined fibrinogen fragments (Hageman and Scheraga, 1974) have indicated that the high specificity of thrombin for protein substrates resides both in the active site and in secondary binding sites outside, but close to, the active center. The structural requirements for active site binding and reaction can be determined by investigations into the kinetic behavior of small-molecule substrate analogues of arginine and lysine.

In a previous study (Fasco and Fenton, 1973), the effect of N_β substituents on the hydrolysis of *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (NPGB)[1] by trypsin, thrombin, and plasmin was examined. The results of that

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[1] Abbreviations used are: PMS-trypsin, phenylmethylsulfonyl-trypsin; CU, Michigan Department of Health casein unit; NIH unit, National Institutes of Health clotting unit; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; NPMT, *p*-nitrophenyl α-amino-*p*-toluate hydrobromide; K_s, enzyme-substrate binding constant; k_2, acylation constant; k_3, deacylation constant.