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Studies of N^5, N^{10}-methylene tetrahydrofolate reductase from porcine kidney and mouse L1210-induced tumor tissues, purification and interaction with antifolates

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STUDIES OF N^5,N^{10}-METHYLENE TETRAHYDROFOLATE REDUCTASE FROM PORCINE KIDNEY AND MOUSE L1210-INDUCED TUMOR TISSUES: PURIFICATION AND INTERACTION WITH ANTIFOLATES

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This thesis, by David W. Jayme, is accepted in its present form by the Graduate Section of Biochemistry, Department of Chemistry of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.

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INTRODUCTION

Cancer Chemotherapy

"Cancer is one of the oldest diseases affecting mankind, and the efforts at treatment have been recorded as long ago as the use of arsenic pastes in ancient Egypt. Today, cancer is second only to cardiovascular disease as the major cause of death in the United States. Each year an estimated 650,000 new cases are diagnosed and over one million known patients continue treatment. The major difficulties in mounting a rapid scientific assault on cancer are that it encompasses more than 100 clinically distinct diseases and is inextricably linked to fundamental life processes that still are not completely understood"(1).

In the attempt to "cure" cancer, i.e. provide a normal life expectancy for the treated cancer patient, clinical treatment has evolved to the single or combined utilization of three modalities: surgery, radiotherapy, and chemotherapy. While the prognosis for a panecea is neither imminent nor, perhaps, feasible, recent scientific advances both in the early diagnosis of malignancy and its subsequent treatment have fostered a "humble optimism" among clinicians and researchers.

While surgical therapy may well be the only means by which certain neoplastic diseases may be cured, many patients as a result

of prolonged incubation prior to diagnosis have surpassed the range of surgical care. A prominent investigator in cancer chemotherapy recently stated, using the carcinoma of the colon as an example, that "over 10 percent...are unresectable at the time of operation, and an additional 25 percent have visible metastases which cannot be resected"(2). He further suggested factors in addition to the extent of metastatic involvement of the disease which limit surgical approaches, such as the general condition of the patient, interference with vital organ function, and cosmetic considerations. Nevertheless, while surgery may not provide a cure in cases where the malignancy has been disseminated, the removal of large tumor masses and other surgical procedures have been demonstrated to increase the effectiveness of supplementary radiotherapy and chemotherapy.

Although radiotherapy is the preferential modality for the cure of certain radiosensitive malignancies, its general role is in palliative therapy and as an adjuvant to surgery or chemotherapy. Adjuvant radiotherapy has been demonstrated to facilitate the surgical removal of certain tumors by initiating a reduction in tumor size prior to surgery. Palliative radiotherapy has been successfully combined with systemic chemotherapy in the cure of patients with childhood solid tumors. As with surgery, radiotherapy is able to cure only with early detection and diagnosis: once the malignancy has spread to other parts of the body, such localized modes of therapy, while able to eradicate the primary locus of neoplasia, cannot control metastasis.

The objectives of cancer chemotherapy are two-fold: initially, to produce a complete remission, i.e. to eliminate all clinical

evidence of the disease; and secondly, to maintain remission as long as possible. Bodey and Frei (2) estimate that remission induction therapy should produce about a 90-99% reduction in malignant cells, a goal which has been achieved in several types of neoplastic diseases, both localized and systemic.

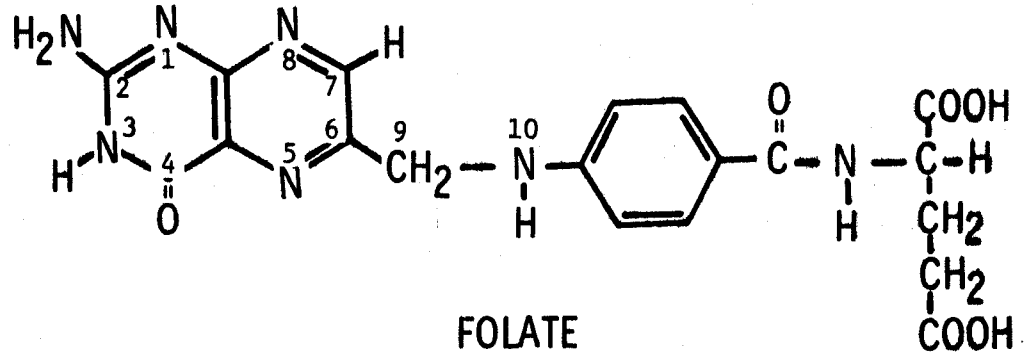
Chemotherapeutic agents vary widely with regard to their source, mode of action, effectiveness and toxicity, and consequently must be subjected to a variety of screening processes prior to their use in clinical practice. Essentially, the diversity of antitumor agents may be classified into five major categories: alkylating agents, antimetabolites, plant alkaloids, antibiotics, and endocrine agents. Much could be said concerning each of these categories of drugs and their mechanism of action and major toxicity, but such an analysis exceeds the scope of this work. Rather, it is more pertinent to this discussion to focus on the area of antimetabolites, in particular those compounds, natural and synthetic, which are antagonistic to enzymes involved in folate metabolism.

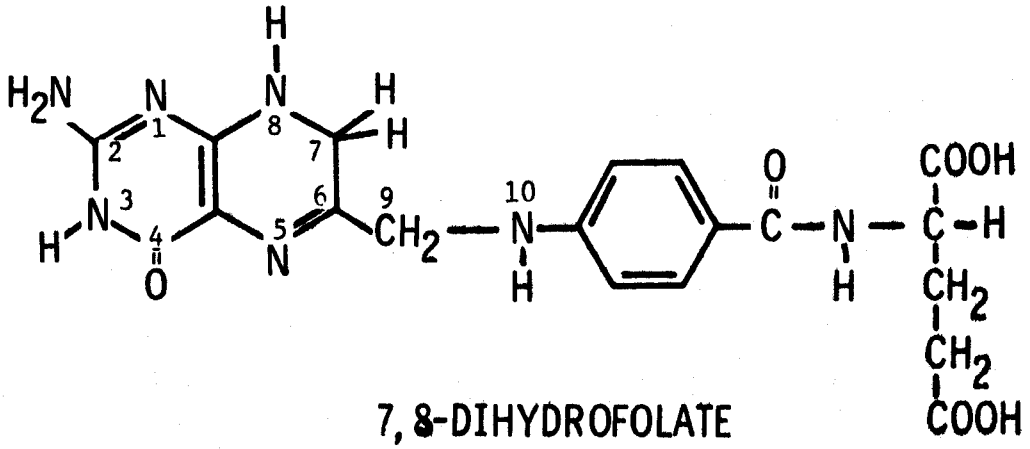
Folic acid (pteroylglutamic acid) has been demonstrated to function principally in the transfer of one carbon units of varying levels of oxidation, primarily as derivatives of tetrahydrofolate. The known folate-dependent interconversions of one-carbon units are summarized in Figure 2. These reactions may be classified as follows:

Condensation

$$HCOO^- + FH_4 \rightleftarrows N^5\text{-formyl } FH_4$$

$$HCHO + FH_4 \rightleftarrows N^5,N^{10}\text{-methylene } FH_4$$





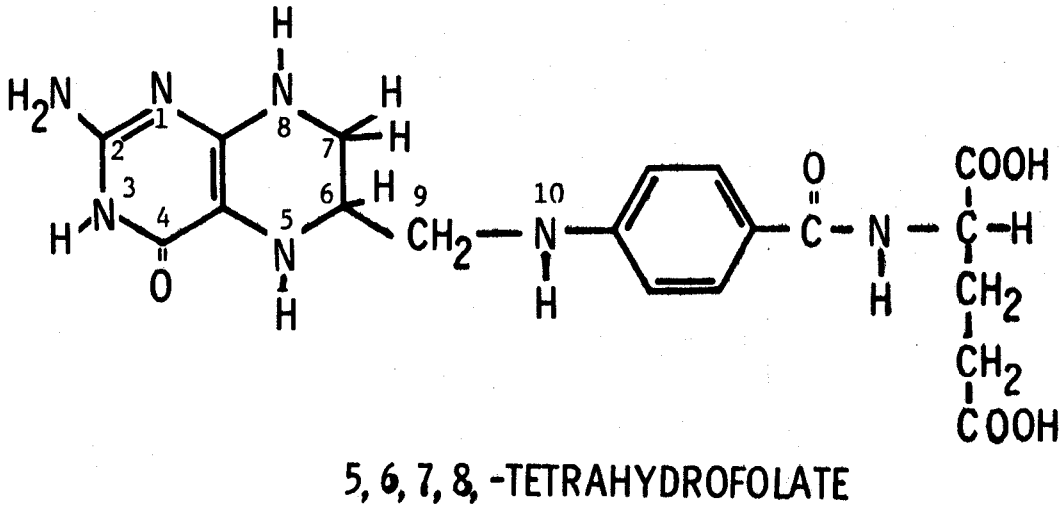
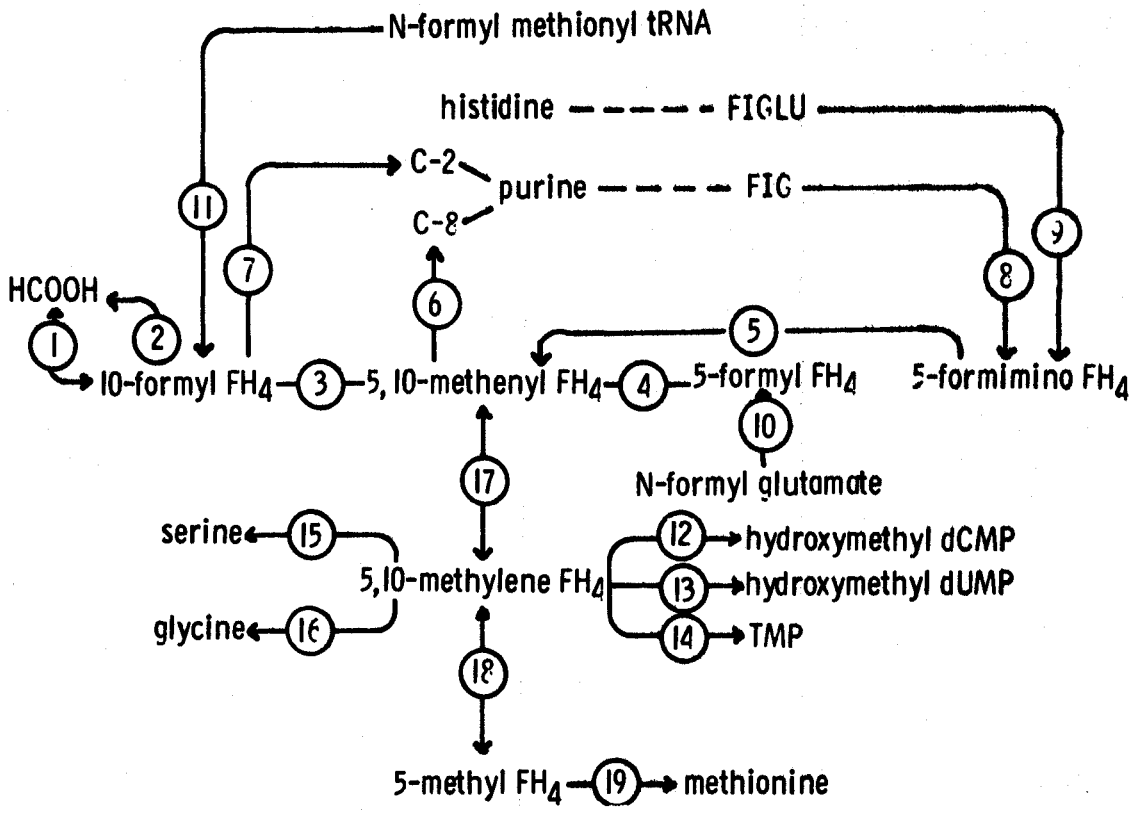


Fig. 1. The Structure of Folate and Its Reduced Forms



Arrows indicate reversibility or irreversibility of reactions. Numbers refer to the following enzymes: 1, 10-formyltetrahydrofolate synthetase; 2, 10-formyltetrahydrofolate deacylase; 3, 5, 10-methenyltetrahydrofolate cyclohydrolase; 4, 5-formyltetrahydrofolate cyclodehydrase; 5, 5-formiminotetrahydrofolate cyclodeaminase; 6, glycinamide ribonucleotide transformylase; 7, 5-amino-4-imidazole carboxamide ribonucleotide transformylase; 8, formiminoglycine formiminotransferase; 9, formiminoglutamate formiminotransferase; 10, N-formylglutamate transformylase; 11, methionyl-tRNA transformylase; 12, deoxycytidylate hydroxymethyltransferase; 13, deoxyuridylate hydroxymethyltransferase; 14, thymidylate synthetase; 15, serine hydroxymethyltransferase; 16, enzyme system responsible for oxidative decarboxylation of glycine; 17, 5, 10-methylenetetrahydrofolate dehydrogenase; 18, 5, 10-methylenetetrahydrofolate reductase; and 19, 5-methyltetrahydrofolate-homocysteine transmethylase.

Fig. 2. Tetrahydrofolate-Mediated Transfer Reactions Involving One-Carbon Groups

Group Transfer

$$\text{L-serine} + FH_4 \rightleftarrows \text{glycine} + N^5,N^{10}\text{-methylene } FH_4 + H_2O$$

Oxidative Decarboxylation

$$\text{glycine} + FH_4 + NAD^{\oplus} \rightleftarrows N^5,N^{10}\text{-methylene } FH_4 + NH_4^{\oplus} + NADH + CO_2$$

Catabolism of Purine and Histidine

$$\text{Purine ring} \rightarrow \rightarrow \rightarrow 5\text{-formimino } FH_4$$

$$\text{Histidine} \rightarrow \rightarrow \rightarrow 5\text{-formimino } FH_4$$

The two major reactions involved in the interconversion of these tetrahydrofolate derivatives to levels of higher or lower oxidation are catalyzed by two important branch-point enzymes: N^5,N^{10}-methylene tetrahydrofolate reductase and N^5,N^{10}-methylene tetrahydrofolate dehydrogenase.

$$N^5,N^{10}\text{-methylene } FH_4 + NADPH + H^{\oplus} \underset{\text{Reductase}}{\rightleftarrows} N^5\text{-methyl } FH_4 + NADP^+$$

$$N^5,N^{10}\text{-methylene } FH_4 + NADP^{\oplus} \underset{\text{Dehydrogenase}}{\rightleftarrows} N^5,N^{10}\text{-methenyl } FH_4 + NADPH + H^{\oplus}$$

Depending on the oxidation level of the folate coenzyme, the tetrahydrofolate derivative may then be utilized to donate one-carbon units into three known biosynthetic pathways in mammalian systems:

1. N^5-methyl FH_4 may serve as the source of the methyl group which is added to homocysteine to form methionine in the vitamin B_{12}-dependent methionine synthetase reaction. Methionine is later

S-adenosylated for methylation reactions involving the formation of a number of biogenic amines.

2. N^5,N^{10}-methylene FH_4 may be utilized directly as the source of methyl groups in several reactions of pyrimidine nucleotide biosynthesis, in particular, the thymidylate synthetase reaction which involves the folate dependent methylation of deoxyuridylate to yield thymidylate.

$$N^5,N^{10}\text{-methylene } FH_4 + dUMP \rightleftarrows dTMP + FH_2$$

3. Two transformylase reactions have been shown to be involved in purine ring biosynthesis, in each case catalyzing the donation of one-carbon units at the formyl level.

$$\text{glycinamide ribotide} + N^5,N^{10}\text{-methenyl } FH_4 \rightleftarrows \text{formylglycinamide ribotide} + FH_4$$

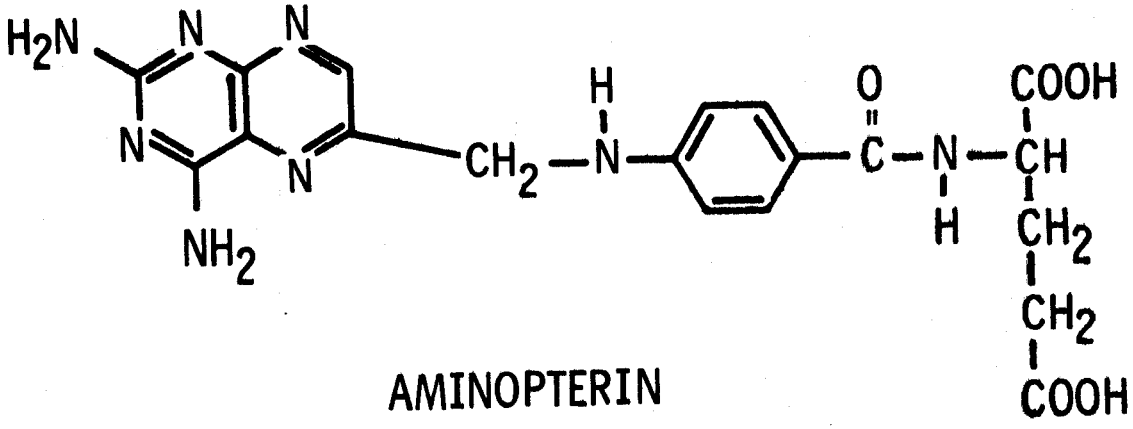
$$\text{5-amino-4-imidazole carboxamide ribotide} + \text{10-formyl } FH_4 \rightleftarrows \text{5-Formamido-4-imidazole carboxamide ribotide} + FH_4$$

Particularly as a result of their direct participation in nucleotide biosynthesis, the reactions involved in folate metabolism have long been investigated by cancer researchers. The rationale is simple and straightforward: cancer may be characterized by apparently unregulated mitotic activity and cellular proliferation, which would require a continuous, elevated rate of production of genetic material. Inhibition of the synthesis of nucleic acid precursors would thus reduce the rate of cellular proliferation.

It was suggested by Friedkin (3) that inhibition of thymidylate synthetase by an antimetabolite would produce a form of "thymine-less death" of neoplastic tissue. It was later discovered that a more efficient enzyme block of thymidylate synthesis could be applied to dihydrofolate reductase (4). Substrate analogs such as 5-fluorouracil, 6-mercaptopurine and 6-thioguanine showed some promise in the direct inhibition of thymidylate synthetase. However, aminopterin and its methylated derivative, methotrexate, which are stoichiometric inhibitors of the dihydrofolate reductase reaction (see Figure 4), were more effective. While the action of such antifolate drugs was non-specific, in that it affected both normal and tumor cells, the therapy was quantitatively effective in checking the rapid proliferation of the malignant cells.

While antifolates, such as methotrexate and aminopterin, have been effective in cancer chemotherapy, particularly in the maintenance of remission with minimal toxicity once complete remission has been induced by vincristin or prednisone administration, it has become apparent that the dihydrofolate reductase block exhibits some weaknesses. Although the inhibitor constant for methotrexate combination with dihydrofolate reductase is in the range of 10^{-8} to 10^{-9}M, which constitutes stoichiometric inhibition, there has been evidence upon prolonged drug administration of the induction of an enhanced dihydrofolate reductase level.

Consequently, investigators began to consider other folate-dependent enzymes as targets for chemotherapeutic drugs, but initially the outlook was dismal. While several enzymes in key positions in the



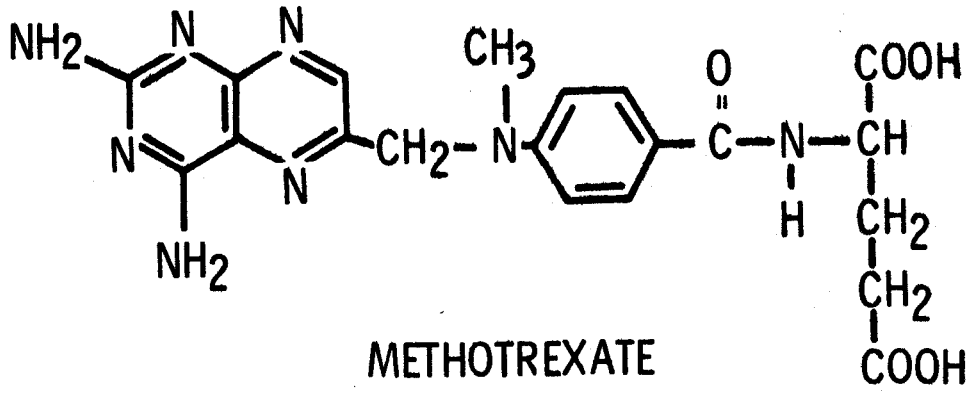


Fig. 3. The Structure of the Two Classical Antifolates, Aminopterin and Methotrexate

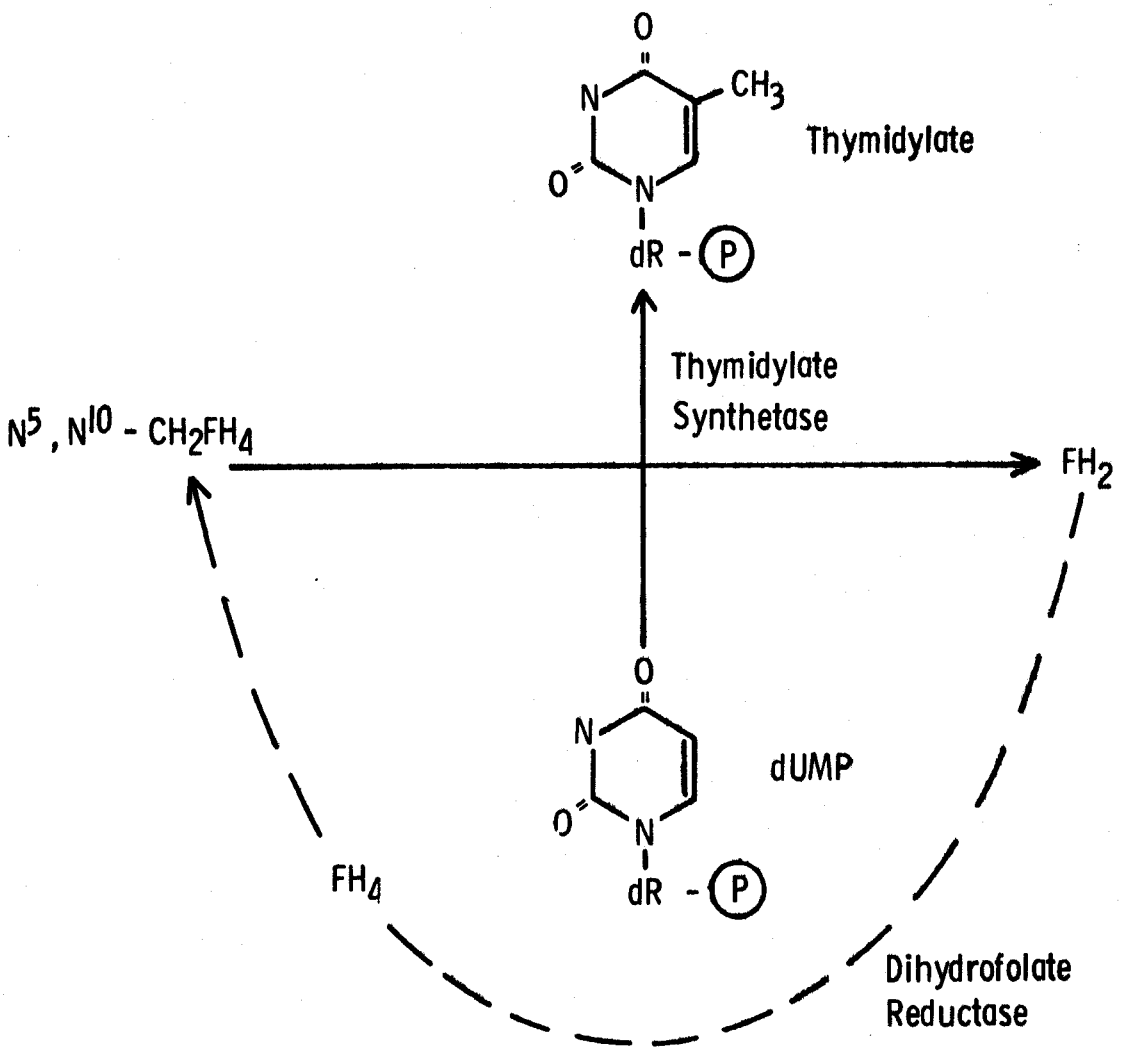


Fig. 4. Schematic Diagram of the Reactions Catalyzed by Thymidylate Synthetase--Dihydrofolate Reductase

biosynthesis of DNA precursors could be inhibited by antifolate compounds, in order to achieve the required circulating inhibitor concentration to reduce enzyme activity, the patient would have to receive lethal dosages of the chemotherapeutic agent. However, a recent clinical publication (5) describing a combination therapy successful in the treatment of osteogenic sarcoma which required the administration of near lethal doses of methotrexate, followed by rescue with citrovorum factor (5-formyl tetrahydrofolate) reported circulating inhibitor levels in the range of 10^{-3} to 10^{-4}M. This discovery generated considerable enthusiasm in our laboratory, as such relatively high levels of antifolates have been demonstrated to inhibit a number of folate-dependent enzymes. In response to a contract assigned to Dr. John H. Mangum by the National Cancer Institute of the National Institutes of Health, we are attempting the purification of five key enzymes involved in folate metabolism. My project has been concerned with the purification of N^5,N^{10}-methylene tetrahydrofolate reductase from porcine kidney and from mouse L1210-induced solid tumor tissue. My objective has been to purify the enzyme from each tissue to a sufficient extent as to be free of other folate-dependent enzyme contaminants, and to compare the "normal" enzyme with the "tumor" enzyme, with a particular emphasis on its kinetic properties and inhibition by potential chemotherapeutic drugs.

ENZYME PURIFICATION

Historical Aspects

Methylene tetrahydrofolate reductase activity was first reported in 1959 by Donaldson and Keresztesy, when they observed that incubation of a hot aqueous extract of horse liver with liver homogenates resulted in a very significant increase in citrovorum factor activity (6,7). It was then postulated and later verified (8) that prefolic A, in crude form, was converted to N^5-formyl tetrahydrofolate (citrovorum factor) by two separate enzymatic reactions. The first reaction converted prefolic A to tetrahydrofolate in the presence of a suitable electron acceptor by the action of a flavin adenine dinucleotide-linked enzyme system. Then, in the presence of a transformylating enzyme and suitable substrate (e.g. formyl-L-glutamate), tetrahydrofolate is formylated to N^5-formyl tetrahydrofolate. When it was observed (9) that formaldehyde was released in the oxidation of prefolic A to tetrahydrofolate, Buchanan (10) suggested that prefolic A and the newly discovered intermediate of methionine biosynthesis (11) might be the same compound. Donaldson and Keresztesy subsequently reported (8) that the artificial oxidation of prefolic A by an electron-accepting dye involved the enzyme-catalyzed formation of an N^5,N^{10}-methylene bridge compound which then hydrolyzed to yield tetrahydrofolic acid and formaldehyde. In addition, they presented evidence

for the physiological forward reaction of this FAD-dependent enzyme which catalyzed the synthesis of prefolic A from tetrahydrofolate and formaldehyde (or methanol) in the presence of flavin adenine dinucleotide and reduced diphosphopyridine nucleotide.

Although there were several studies of methylene tetrahydrofolate reductase activity, using relatively crude bacterial and liver preparations (8,12-14), Katzen and Buchanan (15) succeeded in purifying the enzyme from an auxotrophic mutant of *Escherichia coli* (113-3). By incubating this strain of bacterium in methionine and vitamin B_{12}-deficient medium, a derepressed condition favoring a high methylene-tetrahydrofolate reductase activity, Katzen and Buchanan were able to cultivate bacterial cells with a 20-fold increase in specific activity over repressed controls. Following disruption of the cells, DNAase treatment and centrifugation, the crude extract was subjected to protamine sulfate fractionation and ammonium sulfate fractionation, followed by column chromatography on DEAE-cellulose, which resulted in an enzyme preparation which was purified 104-fold above the derepressed crude extract.

Methylene tetrahydrofolate reductase has been isolated and partially purified from several mammalian tissues, including equine liver (8), porcine liver (16,17), rat liver (16), and bovine brain (18). Of particular value, both in the suggestion of a general purification format, which we have modified, and the availability of significant kinetic data useful for comparison, was the paper of Kutzbach and Stokstad (16).

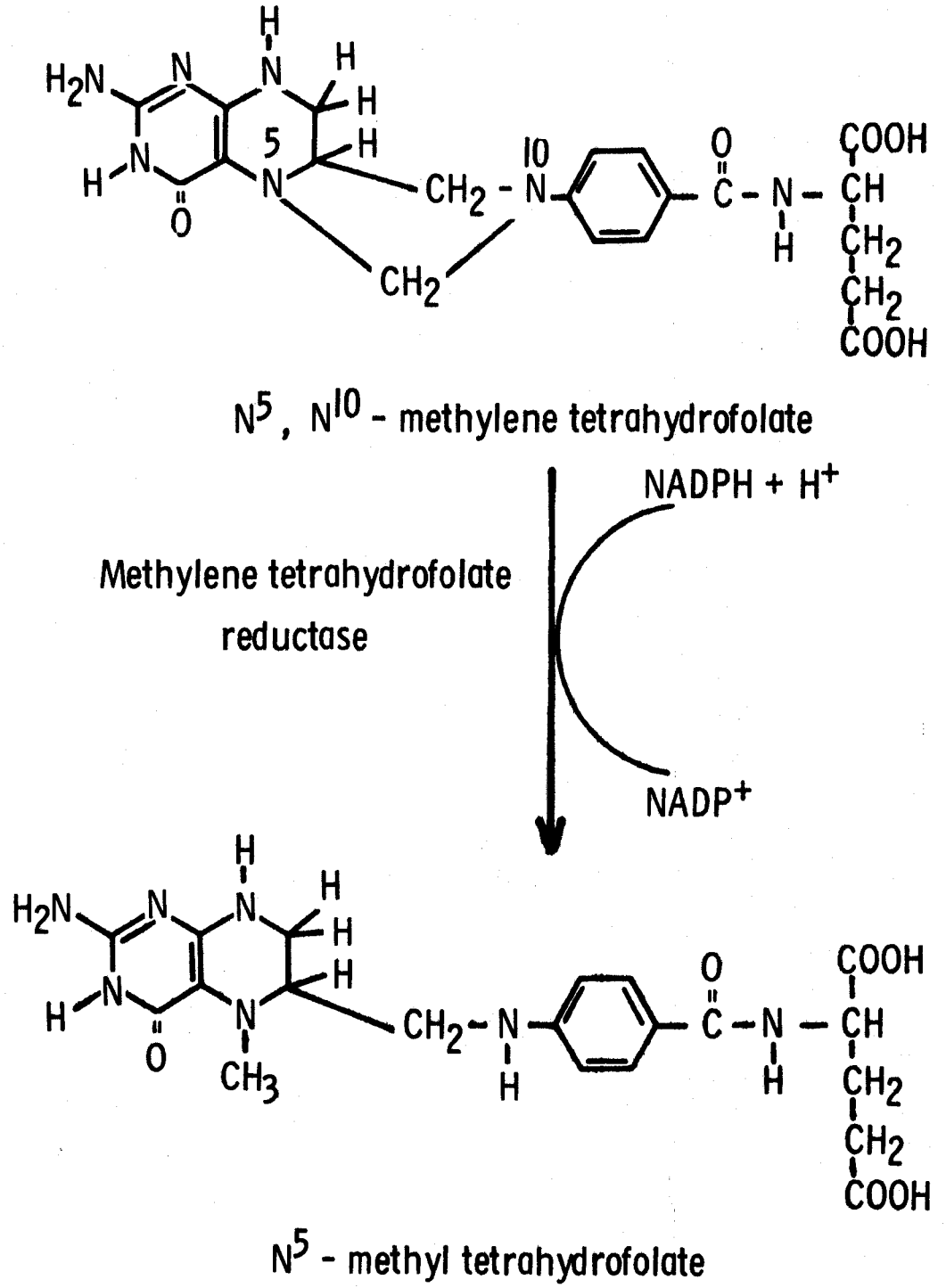


Fig. 5. The Reduction of N^5,N^{10}-methylene Tetrahydrofolate to N^5-methyl Tetrahydrofolate Catalyzed by Methylene Tetrahydrofolate Reductase

Materials and Methods

The chemicals used in this study were obtained from the following sources: FAD, NADPH, and folic acid - Sigma Chemical Co.; [C^{14}]-formaldehyde - New England Nuclear Corp.; Menadione - Calbiochem; Dimedon - J. T. Baker Chemical Co. Other reagents were commercial products of analytical grade.

DE-52, a preswollen, microgranular diethylaminoethyl(DEAE)-cellulose preparation obtained from Whatman Biochemicals, Ltd. and DEAE-sephadex A-50 from Pharmacia Fine Chemicals, Inc. were used for column chromatographic separations.

Tetrahydrofolate was prepared by the catalytic hydrogenation of folate according to the method of O'Dell *et al.* (19) as modified by Hatefi *et al.* (20). The radioactive substrate for the assay in the reverse direction, [C^{14}]-N^5-methyl tetrahydrofolate, was prepared according to the method of Blair and Saunders (21) as adapted from Keresztesy and Donaldson (22). One millicurie of [C^{14}]-formaldehyde was diluted with cold formaldehyde and combined with tetrahydrofolate in the reaction vessel in an approximate molar ratio of 1:1. The reaction mixture was flushed with nitrogen prior to the addition of the tetrahydrofolate and nitrogen was bubbled continuously throughout the course of reaction to prevent air oxidation. The condensation product, the N^5,N^{10}-methylene bridge compound, was then reduced with potassium borohydride (200mg KBH_4/100mg FH_4) to N^5-methyl tetrahydrofolate, in which the methyl group bears the radioactive label. The reaction mixture was then loaded onto a DEAE-cellulose column

(3cm x 10cm) which had been washed and equilibrated with 1 liter of 0.133 M ammonium acetate in 0.01 M mercaptoethanol. The N^5-methyl tetrahydrofolate was separated from unreacted tetrahydrofolate and other folate contaminants by elution from a linear gradient generated by 400 ml each of 0.133 M and 0.400 M ammonium acetate in 0.01 M mercaptoethanol. The purity of the product was determined by scanning the UV spectra using a Cary 118 spectrophotometer (λ_{max} = 290 nm and λmin ≈ 245 nm), and the combined fractions were lyophilized to remove the volatile salt and concentrate the product. The lyophilized material was resuspended in water and stored at -40°C.

Protein concentration was determined by the Biuret method (23) in the early steps of purification. Column fractions were monitored by measuring absorbance at 280 nm in a Beckman DU Spectrophotometer. The Folin method according to Lowry, et al. (24) as modified by Ross and Schatz (25) was used to determine the concentration of protein at the final step of purification.

During purification, methylene tetrahydrofolate reductase was routinely assayed in the reverse direction using menadione as the electron acceptor and [C^{14}]-N^5methyl tetrahydrofolate as the substrate (8,16). With the highly purified enzyme, a spectrophotometric assay in the forward direction as well as a quantitization of the menadione reductase activity of the enzyme have been successfully employed (16) and reported to coincide with the artificial assay method used in this study.

The reaction volume for the incubation was 0.5 ml, containing 100 µmoles of ascrobate, 0.5 µmoles of FAD, approximately 350 nmoles

of menadione (added after preincubation), 250 nmoles of [C^{14}]-N^5-methyl tetrahydrofolate (2000 cpm/nmole), and an aliquot of enzyme solution. The mixture was pre-incubated at 37°C for five minutes and menadione (0.1 ml of a saturated solution in water at room temperature) was added with mild agitation to start the reaction. Assay tubes were incubated at 37°C for 20 minutes and the reaction halted by the addition of 0.3 ml dimedon reagent [3 mg/ml dimedon in 1 M acetate buffer (pH 4.5)] with mixing and by heating the tubes in a boiling water bath for five minutes to denature the enzyme. After cooling the assay tubes in ice, the isotopically labeled formaldehyde-dimedon condensation product was extracted into 3 ml of toluene by vigorous agitation using a Vortex mixer for 20 seconds. The toluene layer was separated from the aqueous phase by centrifugation in a clinical, desk-top centrifuge, and 1.0 ml of the upper toluene phase was withdrawn, mixed with 10 ml of Aquasol (NEN) scintillation cocktail and counted in a Packard TRI-CARB liquid scintillation spectrometer.

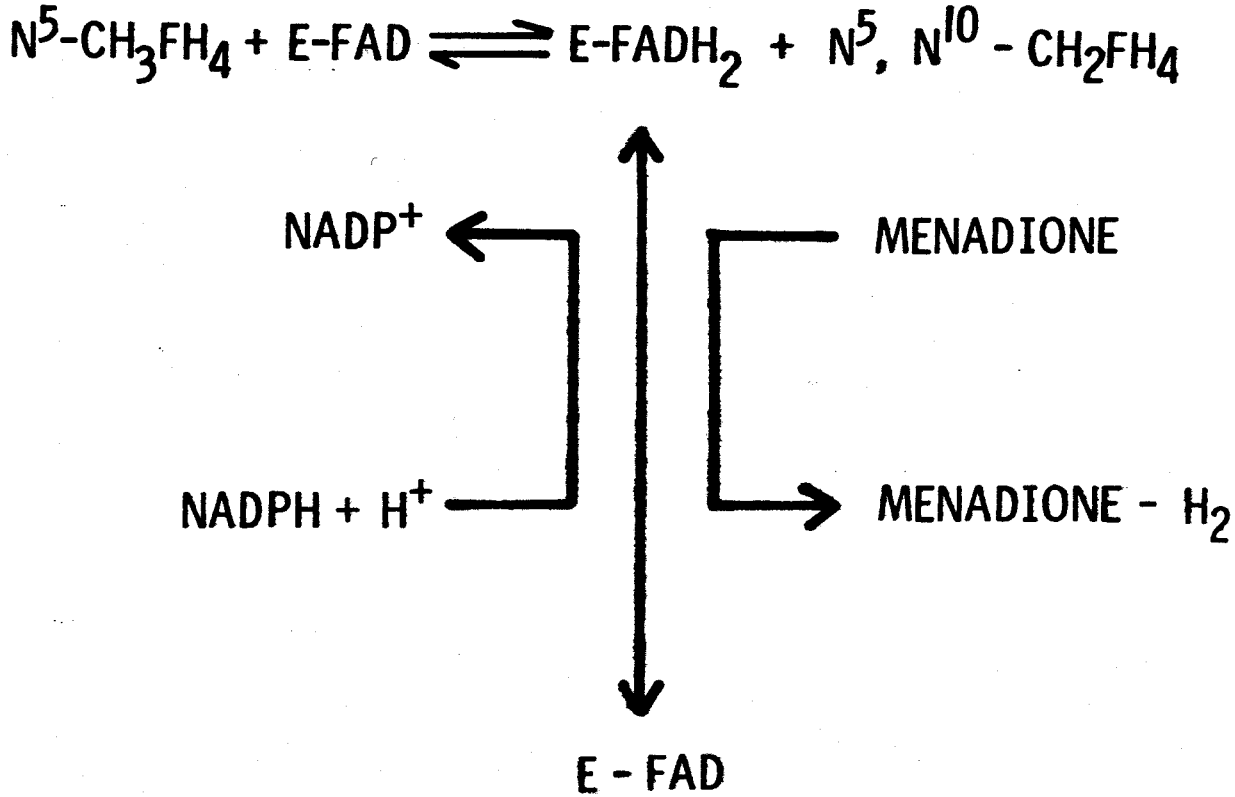


Fig. 6. Reaction Utilized for the Assay of Methylene Tetrahydrofolate Reductase

Purification Procedure

Identical procedures were employed in the purification of N^5,N^{10}-methylene tetrahydrofolate reductase from pig kidney and from mouse L1210-induced tumor tissue. Fresh pig kidney was obtained "hot-off the hog", as it were, from a local slaughterhouse, frozen immediately in dry ice and kept at -40°C. Mouse tumor tissue was obtained by subcutaneously inoculating the flanks of laboratory mice with a leukemic strain obtained from a host ascites tumor. Seven days following inoculation, the solid tumors (about 1/2 g each) were excised and stored at -80°C. The procedure for purification described below is for a preparation of approximately 500 g of tissue. All steps in the purification were performed at 0-4°C, unless otherwise indicated.

1. Extraction: 500 g of tissue were partially thawed, and homogenized with 1.2 liters of 0.05 M potassium phosphate buffer (pH 7.2) in a Waring blendor for 3 minutes. The crude homogenate was filtered through several layers of cheesecloth, the filtrate adjusted to pH 6.0 with 4 M acetic acid, and centrifuged in a Sorvall RC2-B refrigerated centrifuge at 27,000 x g for 30 minutes.

2. Fractionation with $(NH_4)_2SO_4$: Solid $(NH_4)_2SO_4$ was added with constant stirring to the supernatant to effect 25% saturation [144 g $(NH_4)_2SO_4$/liter of enzyme solution]. The ammonium sulfate-protein solution was allowed to equilibrate with constant stirring for 15 minutes and then centrifuged at 27,000 x g for 35 minutes. Solid $(NH_4)_2SO_4$ was similarly added to the 25% supernatant [125 g $(NH_4)_2SO_4$/liter of solution] to achieve 45% of saturation. This solution

was stirred and centrifuged as before. The resulting pellet (representing a 25-45% cut) was homogenized in a Potter-Elvehjem glass homogenizer, resuspended in cold distilled water and brought to a total volume of 300 ml.

3. Acid precipitation: The resuspended ammonium sulfate fraction was adjusted to pH 4.5 by the dropwise addition of 4 M acetic acid and allowed to stir for 10 minutes, followed by centrifugation at 35,000 x g for 10 minutes. The pellet was resuspended in 100 ml of 0.05 M potassium phosphate buffer (pH 7.2), pH adjusted to 7.2 with 1 M NH_4OH, and the preparation allowed to stand overnight. The suspension was then centrifuged at 35,000 x g for 15 minutes to yield a clear, yellow supernatant. The precipitate was discarded.

4. Acetone Fractionation: Sufficient 1 M potassium phosphate buffer (pH 7.2) was added to the clear, yellow supernatant to bring the phosphate concentration to approximately 0.1 M, and the enzyme solution was chilled in an ice-salt mixture to 0°C. One-half volume of acetone (-25°C) was added rapidly with stirring and the turbid solution was centrifuged (-15°C) at 35,000 x g for 10 minutes. The supernatant was decanted into a pre-chilled beaker and an additional one-half volume of acetone added rapidly with stirring, followed by centrifugation (-15°C) at 35,000 x g for 10 minutes. The supernatant was discarded and the fairly solid, white pellet was thoroughly aspirated, then resuspended in a minimum volume (about 40 ml) of 0.05 M potassium phosphate buffer (pH 7.2). The suspension was aspirated again, allowed to stand for 60 minutes, and centrifuged at 35,000 x g for 15 minutes.

The clear, yellow supernatant was carefully withdrawn and samples were assayed for reductase activity. As we found the acetone fractionation to be less predictable than other steps in the purification procedure, we deemed it wise to delay the combination of simultaneously run preparations until the completion of the acetone step and verification of reductase activity. In the case of the pig kidney preparation, the active fractions from four processings were combined at this stage of purification.

5. DEAE-cellulose chromatography: A 3 cm x 25 cm column was prepared from DEAE-cellulose DE-52 (Whatman) and equilibrated with 0.05 M potassium phosphate buffer (pH 7.2) until the effluent registered a pH of 7.2. The combined acetone fractions in the case of the pig kidney preparation and a single preparation in the case of the tumor tissue were loaded onto the column and washed with 2 liters of 0.15 M potassium phosphate buffer (pH 7.2). The enzyme apparently had a high affinity for the positively-charged matrix, as a sharp, yellow band was readily visible near the top of the column. The protein was then eluted from a linear ionic gradient of 250 ml each of 0.4 M and 0.6 M potassium phosphate buffer (pH 7.2) and collected in 5 ml fractions at a flow rate of 60 ml/hour. The protein was monitored by measuring the absorbance at 280 nm and assayed for reductase activity.

6. DEAE-sephadex chromatography: A shorter column (3 cm x 12 cm) of DEAE-sephadex A-50 was prepared and equilibrated with 0.15 M KCl. The active fractions from the DE-52 column were combined and diluted 1:3 with water to lower the ionic strength and permit

adsorption onto the column. The adsorbed protein was washed with 2 liters of 0.2 M KCl and eluted from a linear ionic gradient of 300 ml each of 0.4 M and 0.6 M KCl. The active fractions were monitored and assayed as described previously and the combined fractions were made 0.002 M in mercaptoethanol.

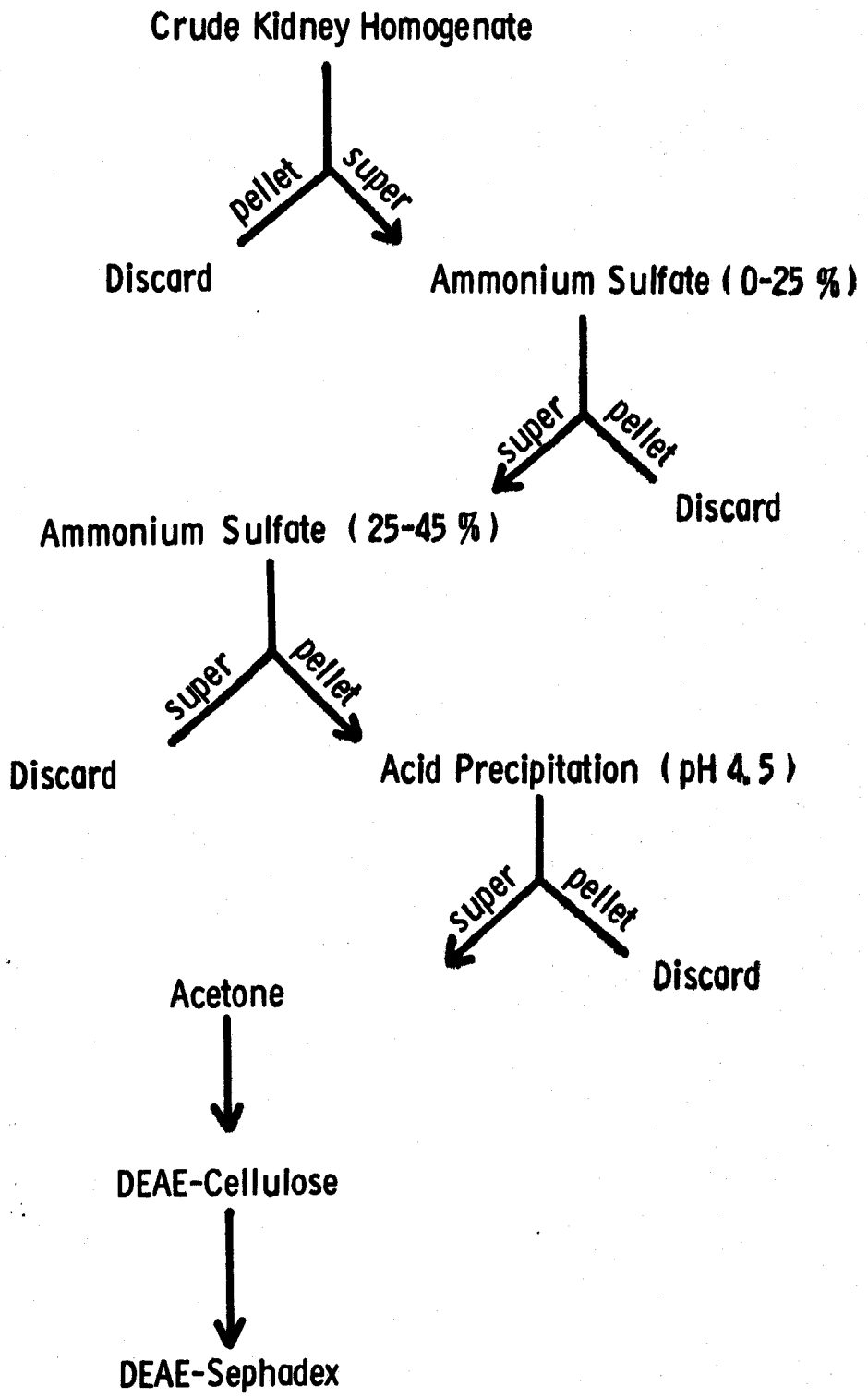


Fig. 7. Flowchart of the Purification of Methylene Tetrahydrofolate Reductase

Results and Discussion

The results for the purification of methylene tetrahydrofolate reductase from porcine kidney are summarized in Table I. We would estimate the enzyme to be of approximately equal purity with the reported purification from rat liver (specific activity = 108 nmoles/min/mg protein) of Kutzbach and Stokstad (16). The higher fold purification reported for our preparation reflects the difference in enzyme levels in the two tissues. While we do not attempt to report a degree of purity, it would appear from the sharpness and symmetry of the column elution pattern (Figure 8) that there is a significant correlation between reductase activity and protein levels, as also illustrated by the superimposable protein and activity peaks. The specific activity remained relatively constant in all fractions.

The purification summary for methylene tetrahydrofolate reductase isolated from mouse L1210-induced tumor tissue is displayed in Table II. As is evident, we chose to purify the tumor enzyme only through the first ion exchange column. It is interesting to note that the kidney enzyme and the tumor enzyme behaved identically throughout the purification process, even to the extent of displaying similar column elution profiles. The similarity in the two enzymes was also reflected in the constant ratio of specific activities at each stage of purification.

The first three steps in our purification scheme were modified from that of Kutzbach and Stokstad (16), the only major alteration being in the ammonium sulfate fraction, where we found only negligible

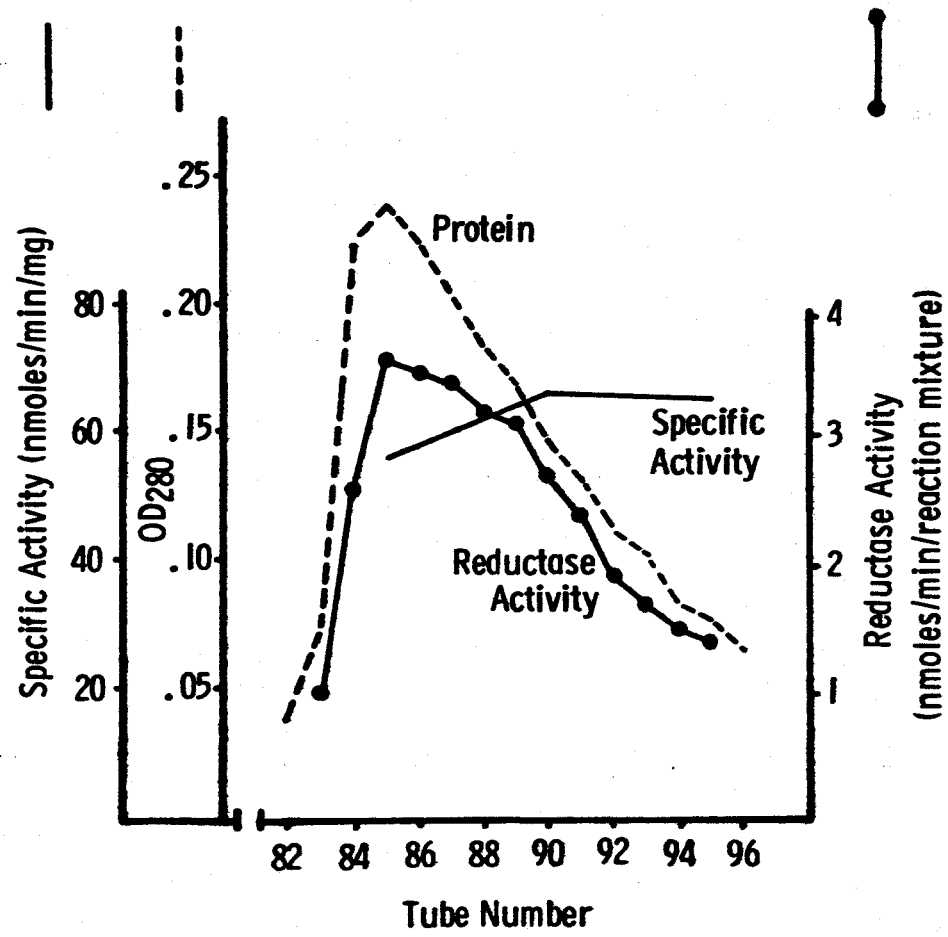


Fig. 8. Elution Profile of Methylene Tetrahydrofolate Reductase from DEAE-Sephadex Column

Table I

Purification of Methylene Tetrahydrofolate Reductase from Porcine Kidney

Purification Step	Total Protein (mgs)	Total Activity (Units)*	Specific Acitivty (Units/mg)	% Recovery	Fold
Crude Homogenate	483,000	63,000	0.1	100	----
Supernatant (pH 6.0)	109,000	55,900	0.5	89	5
$(NH_4)_2SO_4$ (25-45%)	28,560	45,100	1.6	72	16
Acid Precipitation (pH 4.5)	4,130	38,000	9.2	61	92
Acetone Fractionation	1,092	15,000	13.5	24	135
DEAE-Cellulose	150	8,800	58.3	14	583
DEAE-Sephadex	20	2,120	105.0	3	1050

*expressed in nmoles/min

Table II

Purification of Methylene Tetrahydrofolate Reductase
from Mouse L1210-Induced Tumor Tissue

Purification Step	Total Protein (mgs)	Total Activity (Units)*	Specific Activity (Units/mg)	% Recovery	Fold
Crude Homogenate	20,400	816	0.04	100	----
Supernatant (pH 6.0)	3,065	613	0.20	75	5
$(NH_4)_2SO_4$ (25-45%)	840	496	0.59	61	15
Acid Precipitation (pH 4.5)	164	336	2.05	41	51
Acetone Fractionation	23	142	6.24	17	156
DEAE-Cellulose	8	124	15.48	15	387

*expressed in nmoles/min

reductase activity in the 0-25% fraction. Thus, we were able to achieve a significantly higher degree of purification by retaining the more active 25-45% cut. Some attempts have been made, both in this laboratory (26) and elsewhere (17), at using a protamine sulfate step prior to ammonium sulfate fractionation. While a reasonably good fold purification was achieved, we rejected this method as being too costly in terms of loss of total activity.

The acetone step was, to my knowledge, the first reported use of precipitation by organic solvents in the purification of methylene tetrahydrofolate reductase from mammalian tissue. Considerable effort was expended in the optimization of conditions for this differential solvent precipitation, particularly in correlating the phosphate concentration with the volume of acetone to be added for maximum reductase yield. Care also needed to be exercised in lowering the temperature of the enzyme solution to minimize loss of enzyme activity as a result of freezing and adherence to the walls of the reaction vessel. Despite the difficulties involved in standardizing this step, although it accounted for only a two-fold increase in specific activity above the previous step, it succeeded in solving two problems which had plagued earlier investigators: 1) it lowered the protein concentration sufficiently as to facilitate subsequent chromatographic purification; and 2) it purified the reductase fraction of contaminating methionine synthetase and serine transhydroxymethylase activities, a significant prerequisite for the kinetic and inhibition studies which will be discussed in the next chapter.

It was determined by initial batch elution studies that at neutral pH methylene tetrahydrofolate reductase was bound tightly to the diethylaminoethyl group. We found that a column of DEAE-cellulose DE-52 would concentrate both the kidney and tumor enzymes into a single band near the top of the column, detectable due to the tightly-bound flavin prosthetic group. Several protein contaminants appeared to be removed by the wash with buffer of low ionic strength and the active enzyme was eluted in a single, relatively narrow peak by the increasing ionic gradient. As previously mentioned, both enzymes were eluted with an approximately equal volume and ionic strength of potassium phosphate buffer.

DEAE-sephadex A-50 chromatography was an attempt to combine the property of ion exchange associated with the diethylaminoethyl functional group with the molecular sieve of the sephadex beads. While this step did result in a two-fold purification above the DEAE-cellulose fractions, we anticipate that the enzyme will be purified to a greater degree of homogeneity by affinity chromatography (27-30).

Some additional observations pertaining to the purified methylene tetrahydrofolate reductase are appropriate at this point in the discussion. It is evident that, in the sequence of purification, the enzyme has retained a considerable amount of its initial activity despite relatively drastic procedures, such as the acid precipitation at pH 4.5 and the acetone fractionation. This would appear to indicate that the flavin moiety associated with the enzyme is tightly bound to the apoenzyme. An "Achilles heel" on the otherwise extremely stable enzyme appears to be a free sulfhydryl group associated with the

catalytic activity. The purified reductase was observed to lose activity rapidly with refrigeration; however, the enzyme retained approximately 95% activity after storage for one month at 0-4°C when made 0.002 M in mercaptoethanol, a reagent commonly utilized to prevent air oxidation of sulfhydryl groups. Supporting evidence for this hypothesis is provided in the report of almost complete inhibition of reductase activity by p-hydroxymercuribenzoate (17), an enzyme inhibitor which is postulated to react with free sulfhydryl groups.

ENZYME CHARACTERIZATION

Kinetic Properties

The kinetics of N^5,N^{10} methylene tetrahydrofolate reductase from both kidney and tumor tissue have been studied. Both enzymes exhibit characteristic Michaelis-Menten saturation kinetics with respect to the reverse assay technique employed, using menadione as the electron acceptor and [C^{14}]-N^5-methyl tetrahydrofolate as the variable substrate. The linear Lineweaver-Burk double reciprocal plots were utilized as a criterion for determination of the maximum velocity (V_{max}) and the Michaelis constant (Km), as well as for the classification of the types of inhibition (31-33). The type of inhibition has been classified according to Cleland (32): competitive inhibition alters the slope of the double reciprocal plot without affecting the intercept; non-competitive inhibition alters both the slope and the intercept, with a resultant convergence point to the left of the ordinate axis. Competition is defined to be linear when replot of the slope vs. inhibitor concentration yields a straight line. On the basis of these kinetics data, we have reported that the kidney enzyme exhibits a Km of 2.0×10^{-4} M and a V_{max} of 67 nmoles/min/mg protein; whereas the tumor enzyme has a Km of 2.4×10^{-4} M and a V_{max} of 29 nmoles/min/mg portein. Thus, to the extent that each enzyme has been purified, it would appear that the "normal" enzyme has a slightly

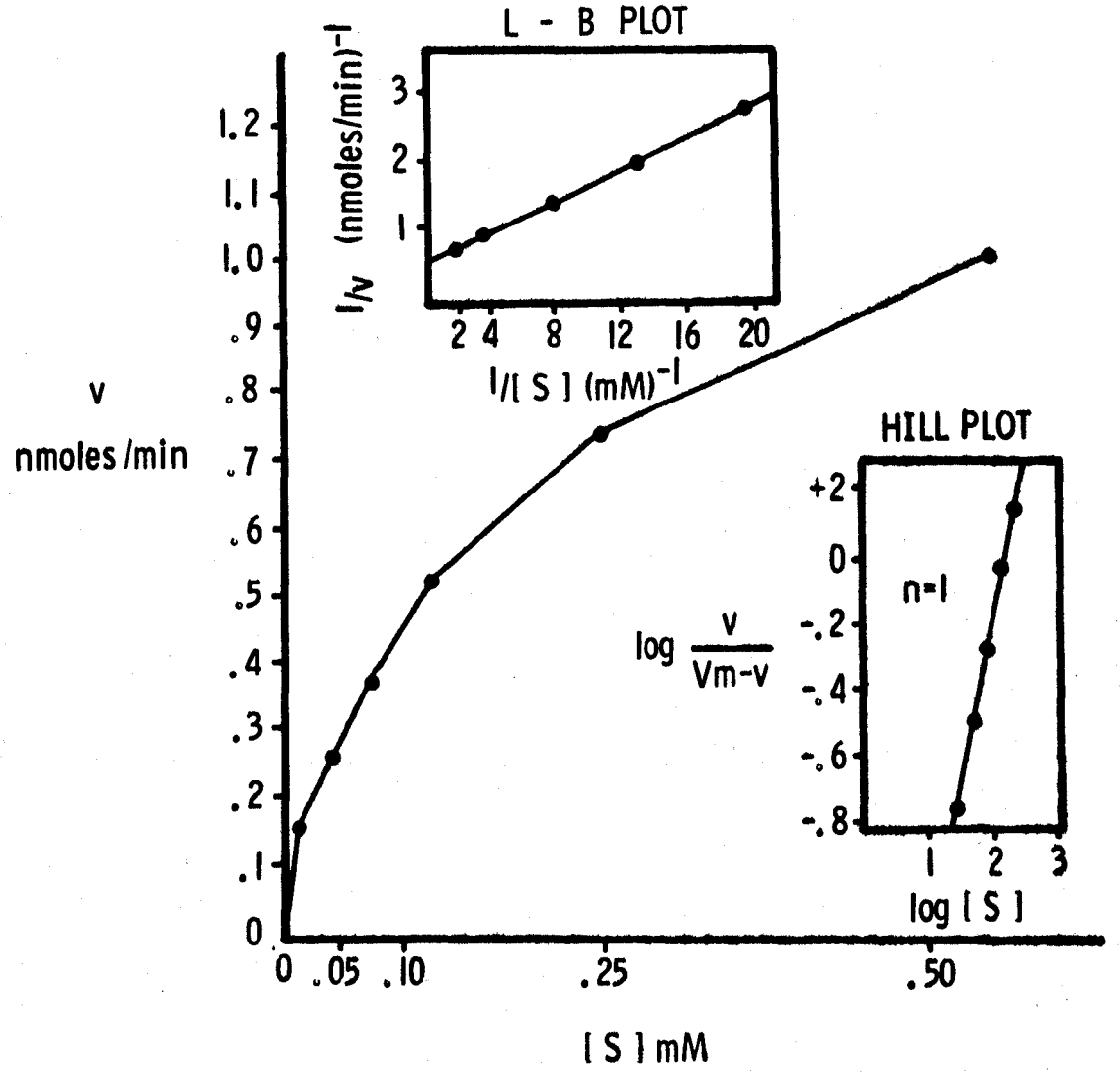


Fig. 9. Saturation of Kidney Methylene Tetrahydrofolate Reductase by N^5-methyl Tetrahydrofolate

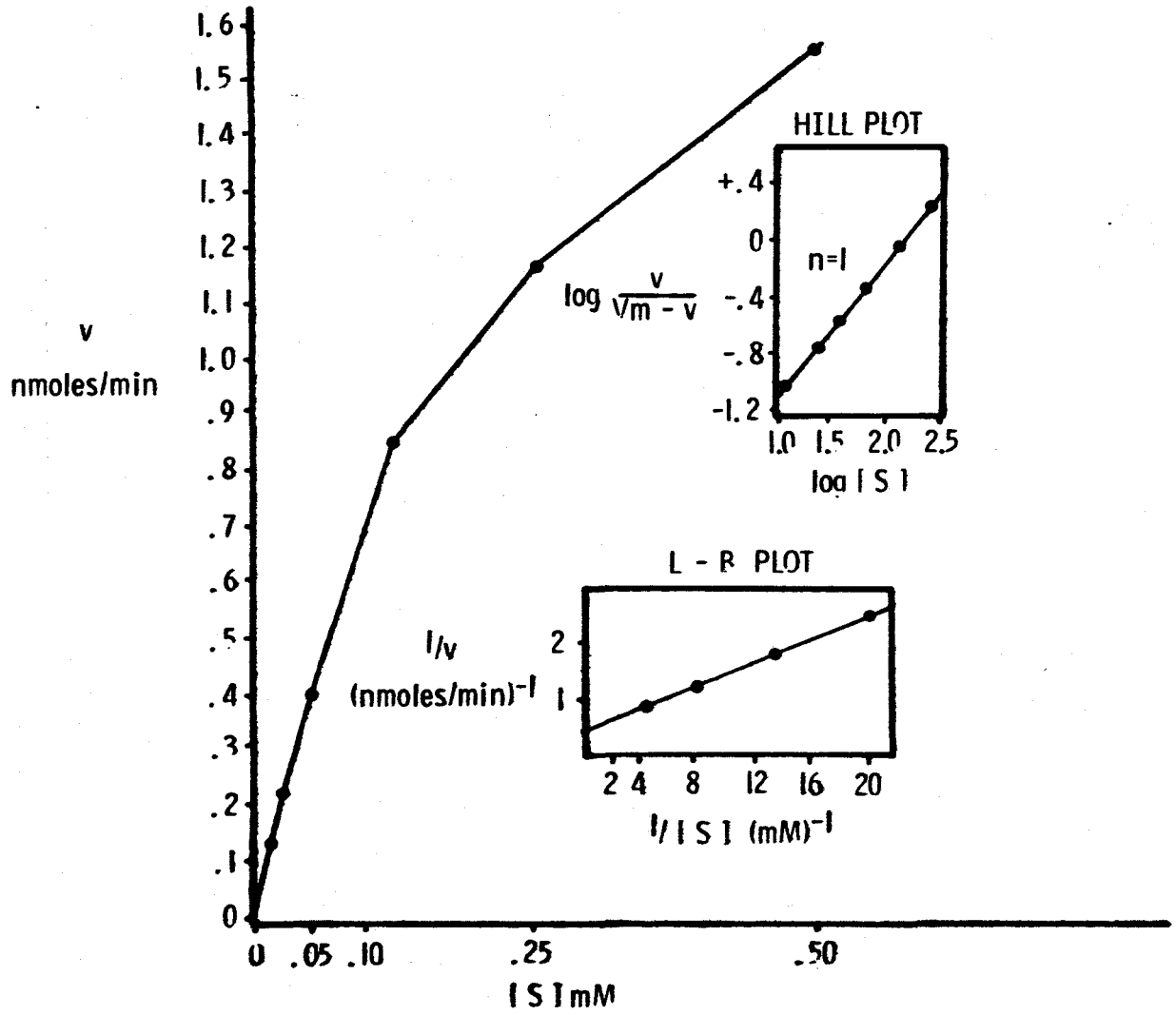


Fig. 10. Saturation of Tumor Methylene Tetrahydrofolate Reductase by N^5-methyl Tetrahydrofolate

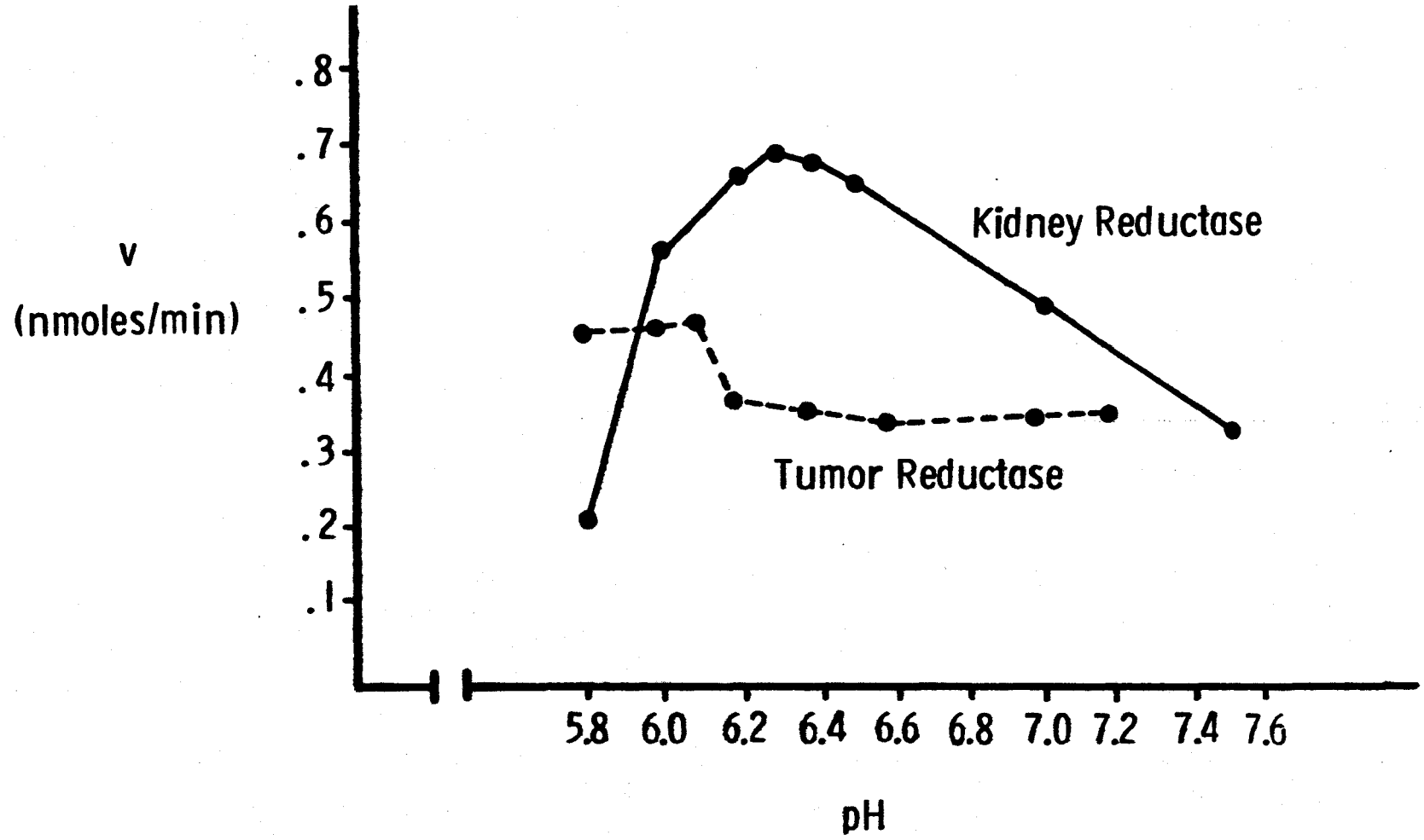


Fig. 11. Comparison of pH Optima for Purified Preparations of Methylene Tetrahydrofolate Reductase from Kidney and Tumor Tissue

greater affinity for N^5-methyl tetrahydrofolate and a considerably higher maximum velocity. Exactly what the physiological significance of this observation might be cannot readily be determined until a more extensive investigation of the regulatory controls involved in one-carbon metabolism is attempted.

The Hill plot (31), a kinetic tool for determination of the number of interacting substrate sites per molecule of enzyme, is linear for both enzymes and yields an "n" value of 1. Both enzymes exhibit fairly broad pH optima for assay in the reverse direction with an optimum velocity at pH 6.3 for the kidney enzyme and a slightly more acidic pH optimum of 6.1 for the tumor enzyme.

Inhibition Studies

Overview

As mentioned in the introductory chapter, a primary justification for this research effort was the screening of a number of natural and synthetic compounds for methylene tetrahydrofolate reductase inhibition. By consolidating data regarding the biochemistry and toxicology of such potential chemotherapeutic drugs, the National Cancer Institute hopes to eliminate the trivial compounds so that available resources can be more judiciously applied to the clinical trial of a few promising anti-cancer agents. To this end, we have investigated a number of compounds: some furnished by the National Cancer Institute, others by cooperating laboratories, still others that we have synthesized in our laboratory. These potential chemotherapeutic drugs, generally either alkylating agents or antifolates, have been routinely

screened at various concentrations for inhibition of methylene tetrahydrofolate reductase activity by the previously described artificial assay technique. The inhibitor constant (K_i) for each of these compounds was determined by the method of Dixon (31).

Synthesis of N^5-Alkyl Tetrahydrofolate and N^5-Alkyl Tetrahydrohomofolate Derivatives

For the purpose of analyzing the effect of the higher N^5-alkyl homologs of N^5-methyl tetrahydrofolate on methylene tetrahydrofolate reductase activity, as well as other enzymes involved in folate-dependent, one-carbon metabolism, we have synthesized the following compounds: N^5-ethyl tetrahydrofolate, N^5-propyl tetrahydrofolate, N^5-butyl tetrahydrofolate, N^5-methyl tetrahydrohomofolate, [C^{14}]-N^5-methyl tetrahydrofolate, [C^{14}]-N^5-ethyl tetrahydrofolate, and [C^{14}]-N^5-ethyl tetrahydrohomofolate. Both tetrahydrofolate and tetrahydrohomofolate were routinely prepared by the platinum oxide catalyzed reduction of folate (homofolate) in glacial acetic acid under hydrogen gas pressure according to the method of O'dell (19) as modified by Hatefi (20). The N^5-alkyl analogs were then formed by the addition of the appropriate aldehyde, yielding the N^5,N^{10}-bridged condensation product, which was subsequently reduced by KBH_4 (21) and purified from folate contaminants by ion exchange chromatography, as previously described in the methods section. The purity of the product was determined spectrophotometrically. Typical spectra are illustrated in Figure 12.

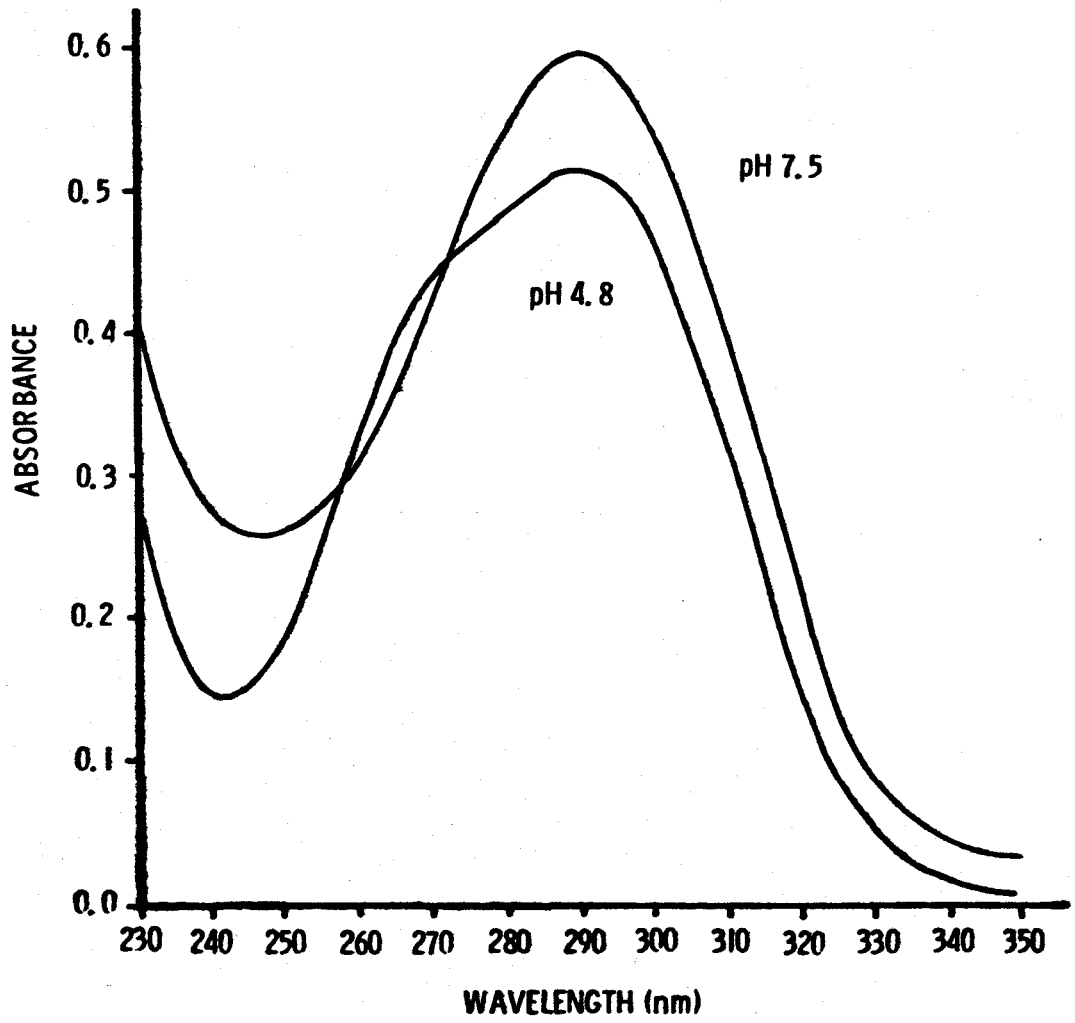


Fig. 12. Typical Spectrophotometric Scan of Isolated N^5-alkyl Tetrahydrofolate

Inhibition of Kidney Reductase by N^5-Alkyl Tetrahydrofolate Derivatives

Studies of inhibition of the kidney enzyme by N^5-ethyl tetrahydrofolate, N^5-propyl tetrahydrofolate and N^5-butyl tetrahydrofolate have indicated that these compounds are competitive inhibitors. K_i values have been determined to be 6.0×10^{-4} M, 4.0×10^{-4} M and 2.3×10^{-4} M, respectively, with replots of these data illustrating linear competition. We had anticipated, based on the relative steric hindrance of the higher homologs, that the order of inhibition would be: N^5-ethyl > N^5-propyl > N^5-butyl tetrahydrofolate. As the sequence of these compounds was reversed from what we had predicted, we postulated that the effective inhibitor concentration was diminishing during the course of reaction, the most likely explanation being a turnover of labile ethyl groups with a reduced turnover rate in the higher homologs. Preliminary results indicated that methylene tetrahydrofolate reductase will catalyze the oxidation of [C^{14}]-N^5-ethyl tetrahydrofolate to the [C^{14}]-N^5,N^{10}-ethylidene tetrahydrofolate adduct; however, further studies will be required to confirm this observation. N^5-methyl tetrahydrohomofolate was shown to inhibit the kidney reductase non-competitively (K_i = 1.7×10^{-4} M) and preliminary studies with [C^{14}]-N^5-methyl tetrahydrohomofolate have shown no evidence of turnover.

Reductase Inhibition by Alkylating Agents

The alkylating agents examined for reductase inhibition were solely those of the Baker antifolate variety. These compounds,

Table III

Inhibition of Methylene Tetrahydrofolate Reductase by N^5-Alkyl Tetrahydrofolate and N^5-Alkyl Tetrahydrohomofolate Derivatives

Compound	Type of Inhibition	Slope Replot	K_i
N^5-ethyl tetrahydrofolate	Competitive	Linear	6.0×10^{-4} M
N^5-propyl tetrahydrofolate	Competitive	Linear	4.0×10^{-4} M
N^5-butyl tetrahydrofolate	Competitive	Linear	2.3×10^{-4} M
N^5-methyl tetrahydrohomofolate	Non-competitive	---	1.7×10^{-4} M

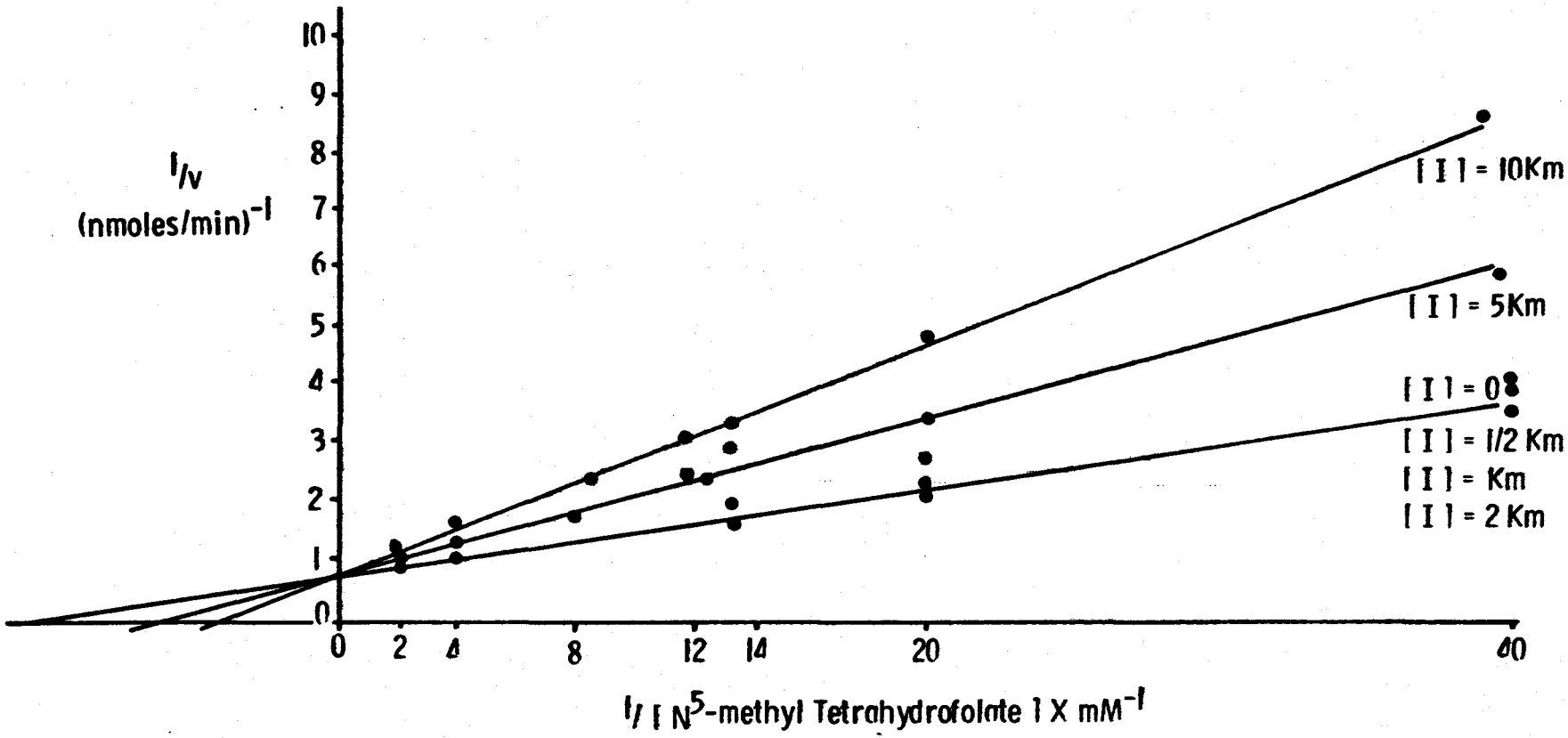


Fig. 13. Typical Lineweaver-Burk Plot Illustrating Competitive Inhibition of Methylene Tetrahydrofolate Reductase Activity by N^5-alkyl Tetrahydrofolate Derivatives

structurally similar to natural folate substrates, contain a sulfonyl fluoride functional group, which is presumed to alkylate the enzyme at a locus which would inhibit enzyme activity. Preliminary screening studies with Baker antifolates showed them to inhibit reductase activity only negligibly at physiologically significant concentrations. Upon the suggestion that our pre-incubation time of five minutes might be insufficient to permit the alkylation reaction, time course assays were performed with pre-incubation times of 5, 20, 35, 65, and 125 minutes. While this type of assay revealed some valuable information regarding the inhibition of another enzyme currently being studied in this laboratory, serine transhydroxymethylase, the time of pre-incubation had no significant effect on methylene tetrahydrofolate reductase inhibition by Baker antifolates.

Reductase Inhibition by Folate Analogs

K_i values were determined for reductase inhibition by seven folate analogs. The results for the inhibition of the kidney and tumor enzymes are summarized in Table IV.

Time course screens were performed on each of the above compounds with pre-incubation times of 5 minutes and 125 minutes, with no significant change in the degree of enzyme inhibition. A general observation is that the tumor enzyme appears more resistant to inhibition by folate analogs than the kidney enzyme. Whether this is of any physiological significance or merely a function of the difference in enzyme sources can only be determined by the comparison of normal tissue with a less artificial cancer model. Of significance is the

observation that, under the condition of this assay, methotrexate, a drug used clinically in the treatment of a number of solid and systemic cancers, appears to be less effective at inhibiting methylene tetrahydrofolate reductase activity than several other folate compounds. This is particularly apparent with the kidney enzyme, where aminopterin and leukovorin exhibit approximately 10-fold higher inhibition and the 2',5'-dichloro-derivative of methotrexate improves the inhibition by 100-fold relative to the methotrexate standard. Since these compounds may not inhibit dihydrofolate reductase as effectively as methotrexate, should they be determined to have an inhibitory effect on other folate dependent enzymes, such as methionine synthetase or serine transhydroxymethylase, it would suggest their possible implementation in clinical trials in combination therapy with methotrexate to maintain remission even with elevated levels of dihydrofolate reductase.

Another application of these antifolate compounds, if not as pleasing to one's humanitarian senses, at least more practically imminent, is its possible utilization in affinity chromatography for further enzyme purification. Similar compounds are readily covalently-linked to a cyanogen bromide activated carbohydrate (e.g. agarose) support medium, and this technique is currently under investigation in this laboratory.

Table IV

K_i Values of Selected Inhibitors of Methylene Tetrahydrofolate Reductase

Compound	Type of Inhibition	Kidney Enzyme	Tumor Enzyme
Methotrexate	Non-Competitive	2.3×10^{-4} M	5.4×10^{-4} M
Aminopterin	Non-Competitive	1.8×10^{-5} M	3.4×10^{-4} M
Dichloromethotrexate	Non-Competitive	3.8×10^{-6} M	8.2×10^{-5} M
Leukovorin	Non-Competitive	1.7×10^{-5} M	1.9×10^{-4} M
Homofolate	Non-Competitive	Not determined	5.1×10^{-4} M
Tetrahydrofolate	Non-Competitive	1.6×10^{-4} M	2.3×10^{-4} M
N^5-methyl Tetrahydrohomofolate	Non-Competitive	1.7×10^{-4} M	1.9×10^{-4} M

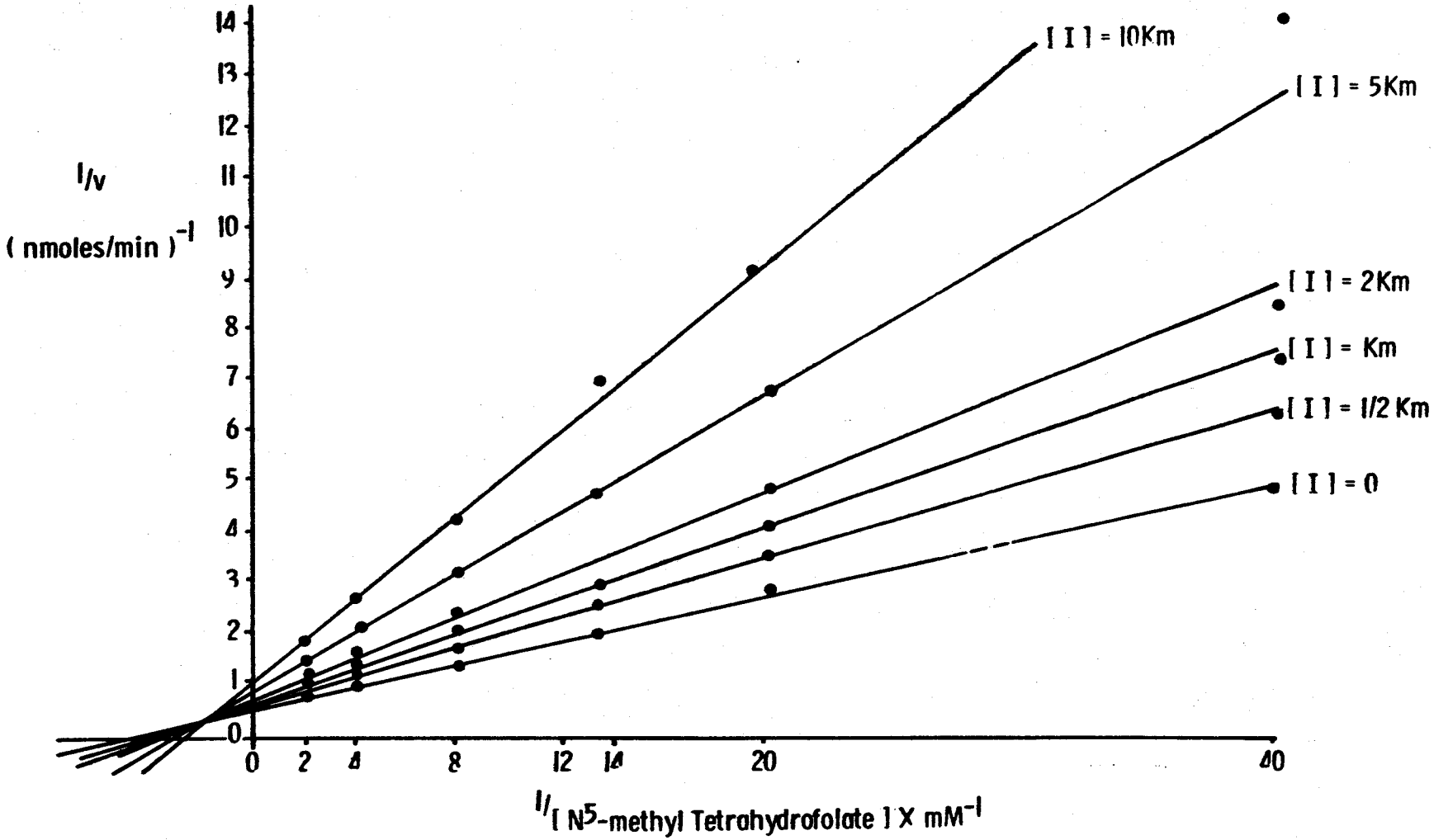


Fig. 14. Typical Lineweaver-Burk Plot Illustrating Non-Competitive Inhibition of Methylene Tetrahydrofolate Reductase Activity by Folate Analogs

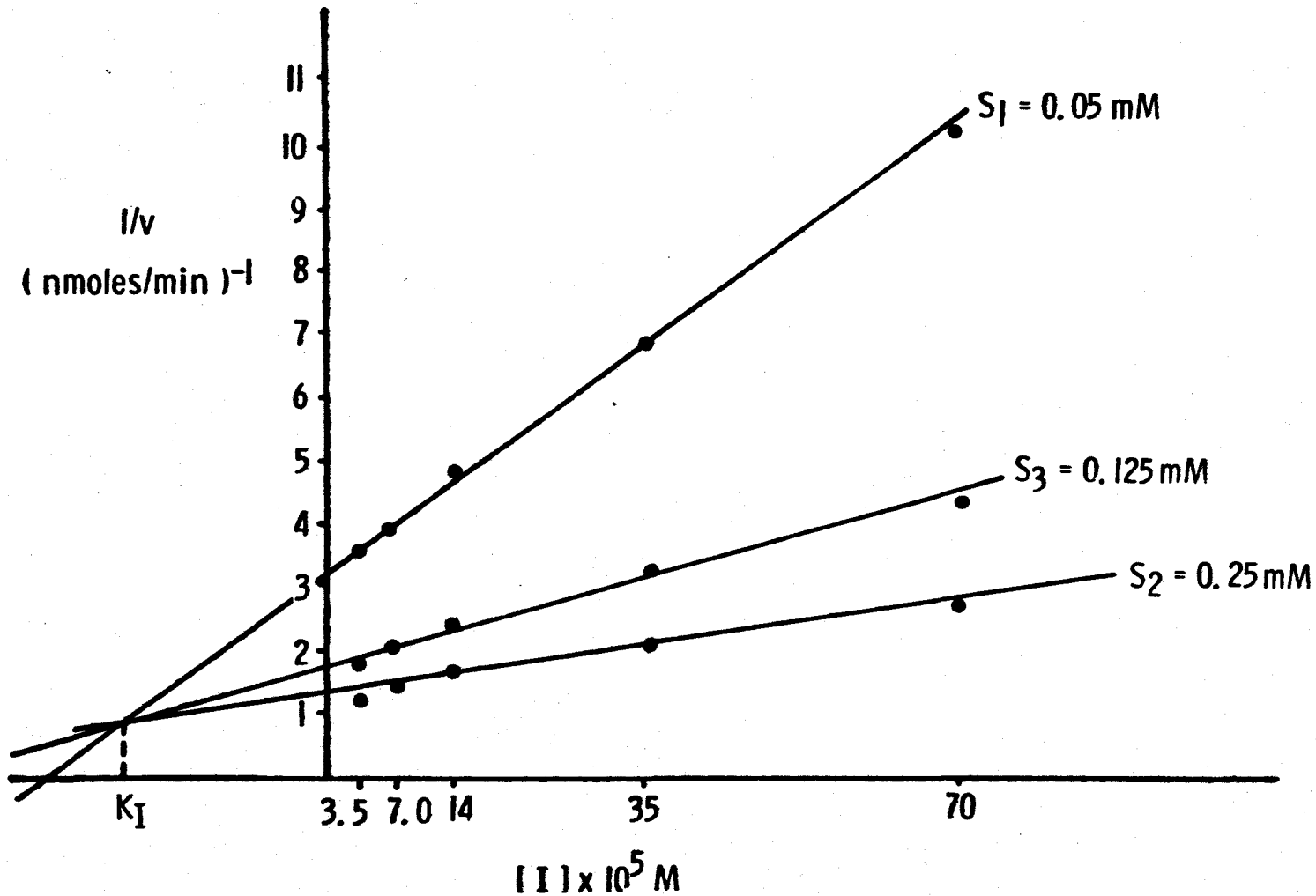


Fig. 15. Dixon Plot for the Determination of K_i Values

SUMMARY

Methylene tetrahydrofolate reductase has been partially purified 1000-fold from porcine kidney and 400-fold from mouse L1210-induced tumor tissue by similar procedures. While the two enzymes behaved similarly throughout purification, kinetic characterization and inhibition studies have revealed subtle differences between the "normal" and tumor enzymes which, upon further investigation, might prove valuable in the specific inhibition of malignancy. These differences include a slight variation in pH-activity profile, general enzyme stability in the purified state, substrate affinity, and resistance to enzyme inhibition by folate analogs.

Several compounds have been demonstrated to inhibit methylene tetrahydrofolate reductase activity at physiological levels, which suggests their possible implementation, pending the outcome of toxicology trials, in cancer chemotherapy in combination with methotrexate. It is anticipated that, through the use of these inhibitors in affinity chromatography, methylene tetrahydrofolate reductase will shortly be purified to a greater degree of homogeneity.

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STUDIES OF N^5,N^{10}-METHYLENE TETRAHYDROFOLATE REDUCTASE FROM PORCINE KIDNEY AND MOUSE L1210-INDUCED TUMOR TISSUE: PURIFICATION AND INTERACTION WITH ANTIFOLATES

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M. S. Degree, December 1975

ABSTRACT

Methylene tetrahydrofolate reductase has been purified 1000-fold from porcine kidney and 400-fold from mouse L1210-induced tumor tissue by classical methods. The enzyme preparations have been demonstrated to be essentially free of contaminating methionine synthetase and serine transhydroxymethylase activity.

Studies of the kinetic properties of the kidney and tumor enzymes, with respect to the reverse reaction using N^5-methyl tetrahydrofolate as the variable substrate, have indicated K_m values of 2.0 and 2.4 x 10^{-4} M, respectively. Inhibition of this key branch point enzyme in folate metabolism by a number of antimetabolites indicates that several of these antifolate compounds exhibit enzyme inhibition superior to that of methotrexate, a drug extensively utilized in the maintenance of remission in cancer chemotherapy.

COMMITTEE APPROVAL: