

# SELECTIVE INHIBITORS OF DIHYDROFOLATE REDUCTASE

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by

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My interest in nucleic acids, their constituents and metabolism can be traced to the discovery of adenosine triphosphate in muscle by Fiske and Subbarow (1). I had entered Harvard University as a candidate for a Ph.D. degree in 1928 and transferred to Harvard Medical School in 1929. Otto Folin, then head of the Department of Biological Chemistry, designated my first predoctoral year to be spent with Cyrus J. Fiske, then involved in momentous discoveries of phosphocreatine and the labile phosphorus compounds of muscle and other tissues. I was soon immersed in the discovery of analytical tools with which to follow the metabolism of adenosine triphosphate (2).

Other lines of thought coalesced with this interest when I joined Burroughs Wellcome Co. in 1942 as the sole member of the Biochemistry Department. Meantime, the antimetabolite principle had been expressed by Woods (3) and Fildes (4). I saw the opportunity to explore nucleic acid biosynthesis in a new and revealing way by employing synthetic analogues of the purine and pyrimidine bases in a system utilizing these heterocyclic compounds for biosynthesis.

I was able to interest Elvira Falco who was then an assistant in the company's Bacteriology Department. Together we worked out a system using *Lactobacillus casei*, which would grow either with a then-unknown "L-casei" growth factor or with a mixture of thymine and a purine (Fig. 1) (5). This system quickly gave us encouraging results. In a simple screening test for antibacterial activity, analogues were found to inhibit strongly not only the *L. casei* system but pathogenic bacteria as well. We had added toxicity testing in growing rats and other biological screening procedures and were becoming more and more excited by the results.

By 1947, six or seven of us were pursuing this work, and the feeling in the group was, "Now we have the chemotherapeutic agents; we need only to find the diseases in which they will be active." At that point I made two arrangements for collaborative studies, one with Sloan Kettering Institute for antitumor testing using sarcoma 180 in mice, and another with outside

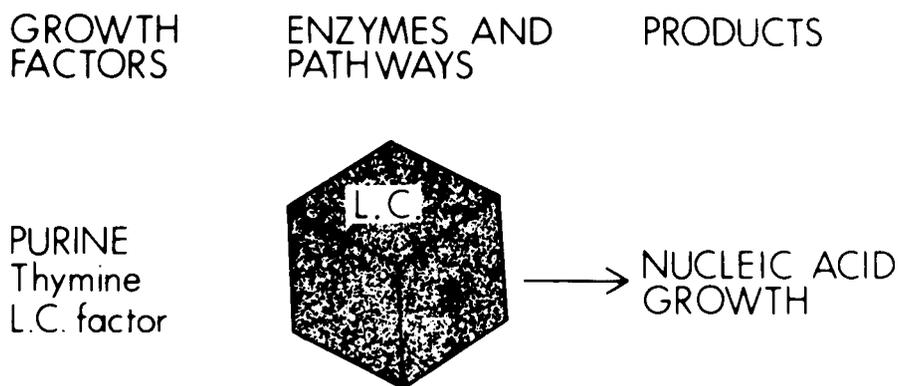


Figure 1. The use of *Lactobacillus casei* as a black box of enzymes and metabolic pathways concerned with nucleic acid metabolism (9).

laboratories for expansion of antibacterial and antimalarial testing. The antimalarial testing was included through the insight of Peter B. Russell, also a member of our research group. Russell noted the resemblance of a 5-phenyl-2,4-diaminopyrimidine to a hypothetical conformation of the antimalarial proguanil. It turned out later that this theory was prescient; the dihydrotriazine is the active metabolite of proguanil (6).

The next year, two leads developed almost simultaneously. Falco began a series of selective inhibitors of dihydrofolate reductase with the synthesis of 2,4-diamino-5-phenoxy pyrimidine, and Gertrude Elion synthesized 2,6-diaminopurine (7). The latter was among the first four compounds we submitted to Sloan Kettering Institute. It was found to be active in the S-180 test, was taken into clinical trial by Joseph H. Burchenal and gave at least one spectacular remission (8). This was sufficient to establish cancer chemotherapy as a continuing primary goal of our group. The purine analogue story, which has been a major theme in Gertrude Elion's career, is the topic of her Nobel address.

The main theme of this essay is the continuing topic of selective inhibitors of dihydrofolate reductase (DHFR). Biochemical knowledge of the role of folates was developing concomitantly. Figure 2 shows that dihydrofolate is synthesized *de novo* in prokaryotes (microorganisms) while the higher species of eukaryotes (host) must have the vitamin preformed. I will pick up the story of selective inhibitors of dihydrofolate reductase *in medias res* and carry it forward to current exciting developments.

A short review of our line of research was presented in a symposium honoring Sir Henry Wellcome (9). Papers in *Advances in Enzymology* (10), *Advances in Enzyme Regulation* (11), and *Enzyme Inhibitors as Drugs* (12) tell the story of selectivity among 2,4-diaminopyrimidines from its first recognition before 1950 to its confirmation. Proof was developed through inhibitor analysis sequencing, conformations, induced mitogenesis, computer assisted conformation studies, and new syntheses based on this type of information.

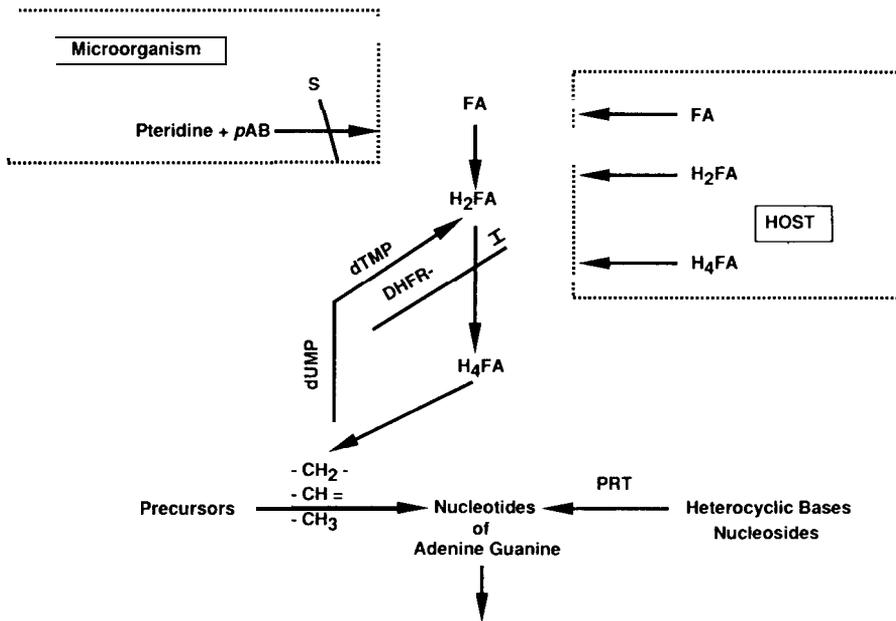


Figure 2. Folate metabolism. Upper left shows biosynthesis in prokaryotes; upper right shows the uptake of preformed vitamin in mammals. The subsequent utilization for the biosynthesis of nucleic acid components is shown below. The importance of dihydrofolate reductase (DHFR) and the selectivity of its inhibitors takes a central place. Folic acid (FA); dihydrofolic acid (H<sub>2</sub>FA); tetrahydrofolate (H<sub>4</sub>FA); thymidylate (dTMP); deoxyuridylate (dUMP); p-aminobenzoic acid (pAB); phosphoribosyl transferase (PRT) (10).

The earliest proof of the mechanism of action of these compounds is illustrated in Fig. 3 (13). The growth of *Streptococcus faecium* was easily inhibited by a diaminopyrimidine when the growth was induced by folic acid, but it took 500 to 1000 times as much inhibitor when folinic acid (tetrahydrofolate) was supplied. This was correctly interpreted as inhibition of the yet-unknown enzyme responsible for the reduction of folate to tetrahydrofolate.

By 1950, we had concluded that we were dealing with selective inhibitors of dihydrofolate reductase. From this work it appeared probable that details of fine structure in dihydrofolic reductase vary from species to species and that a given inhibitor may exhibit considerable selectivity as a result of looser or tighter binding to the corresponding enzyme of host or parasite, respectively (14). The full structural analogue of MTX shows little selectivity, while pyrimethamine is highly active against the malarial enzyme and trimethoprim against a bacterial enzyme. Neither has notable toxicity against the rat liver enzyme.

Isolation and characterization of this enzyme led first to inhibitor analysis (Table 1). This documented unequivocally the different structures of representative dihydrofolate reductases from different sources. This was soon followed by amino acid analyses and sequence determinations.

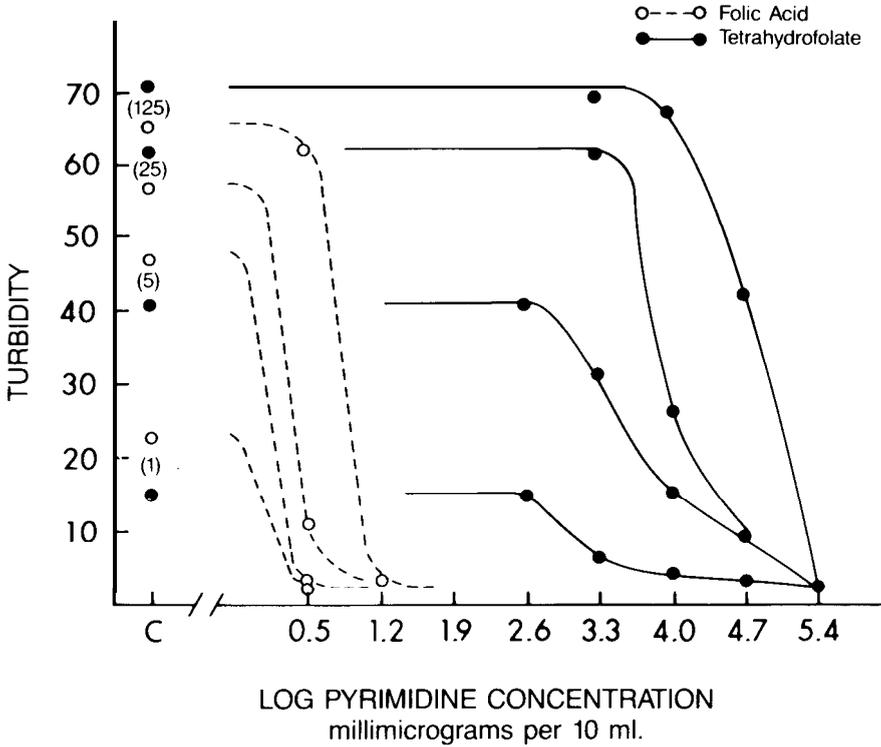


Figure 3. The concentration of pyrimethamine required to inhibit growth of *Streptococcus faecium* depends on whether folic acid (○) or a form of tetrahydrofolate (●) is used in the medium (13).

Table I. The concentration (I.C.<sub>50</sub>) of methotrexate (MTX) pyrimethamine (Pyr) or trimethoprim (Tmp) required to inhibit the DHFK derived from *E. coli*, rat liver and *P. berghei* (9).

**INHIBITOR ANALYSIS**  
I.C.<sub>50</sub> × 10<sup>8</sup>

Enzyme	<i>E. Coli</i>	Rat Liver	<i>P. Berghei</i>
Compound			
MTX	0.1	0.2	0.07
Pyr	250	70	0.05
Tmp	0.5	3 × 10 <sup>3</sup>	7.0

Concomitantly developing knowledge of the roles of folate derivations in biosyntheses and metabolism gave a fuller appreciation of the significance of inhibition of dihydrofolate reductase (Figs. 4, 5). The activity of the enzyme is necessary to produce tetrahydrofolate initially and to recycle it after its reoxidation, molecule for molecule, in the formation of thymidylate from deoxyuridylate.

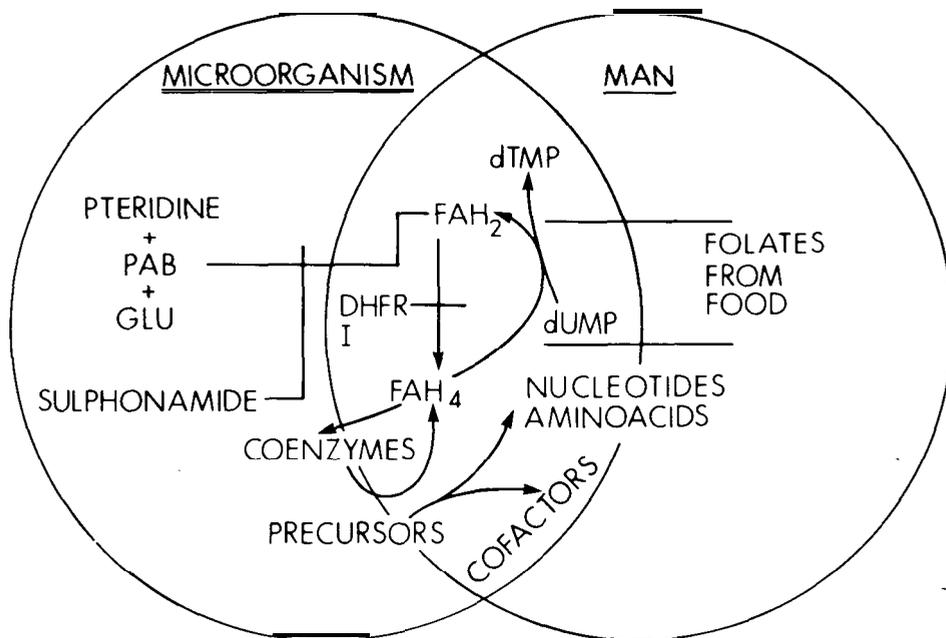


Figure 4. The metabolic reactions catalyzed by derivatives of tetrahydrofolate (FAH4): thymidylate (dTMP); deoxyuridylate (dUMP); *p*-aminobenzoic acid (PAB); glutamate (GLU) (12).

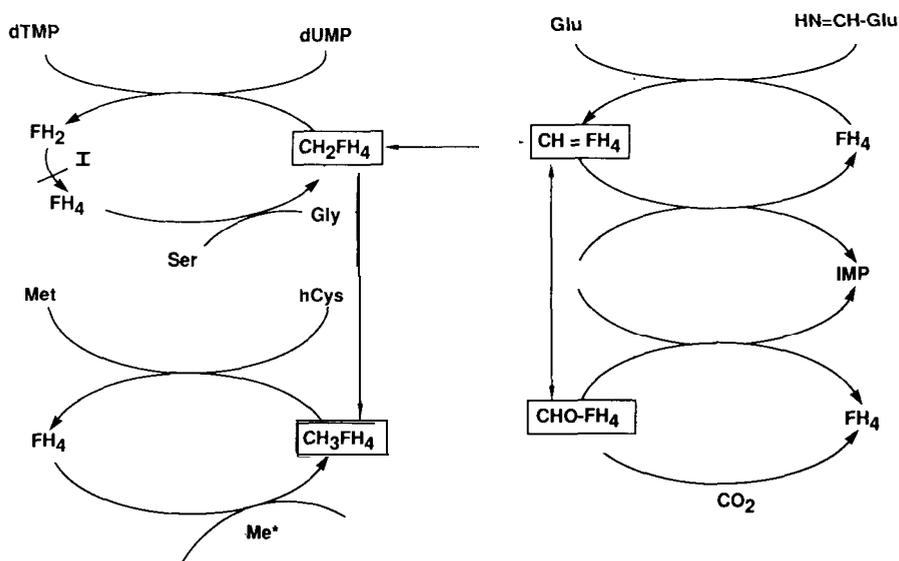


Figure 5. Details of reactions of specific folate-containing cofactors. Counter clockwise, formyl, methanyl, methylene, and methyl tetrahydrofolates (FH4). Serine (Ser); methionine (Met); inosinate (IMP). See Table 1 and Fig. 4 for other abbreviations (12).

## DIHYDROFOLATE REDUCTASE

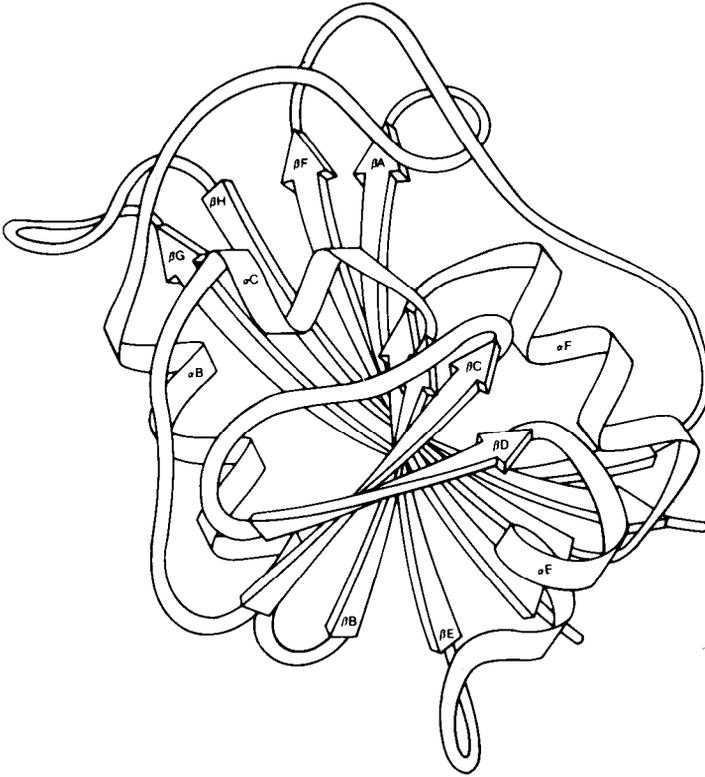


Figure 6. A graphic illustration of the configuration of the dihydrofolate reductase molecule (16).

Amino acid sequence determinations brought out a full appreciation of the high variability of the basic constituents of this enzyme in a range of species (15). Between the enzymes of two bacterial species there is only 30 percent homology, and between prokaryotes and eukaryotes only 30 percent. It is only the higher species that exhibit as much as 90 percent homology. Hitchings and Roth found 16 identities between the enzymes from *Escherichia coli* and those from the mouse tumor L1210 (12). They predicted correctly that study of a wider range of enzymes would reduce the number of identities. If one takes into account enzymes not in the mainstream, e.g. those from protozoa plasmids, the number is even smaller.

The mainstream enzymes (from bacterial and mammalian sources) have similar conformations. That published by Richardson (16) may be regarded as the type (Fig. 6). Although mammalian enzymes are larger than this bacterial enzyme by some 3000 daltons, the extra residues exist as loops that do not greatly alter the main conformation. On the other hand, the enzymes from other types of organism can be so different as to raise doubts about whether they are intrinsically dihydrofolate reductase, or whether their activity in that field is secondary to some other unknown function, e.g., Matthews (17). Figure 7 (18) shows some possible origins of Type II DHFR.

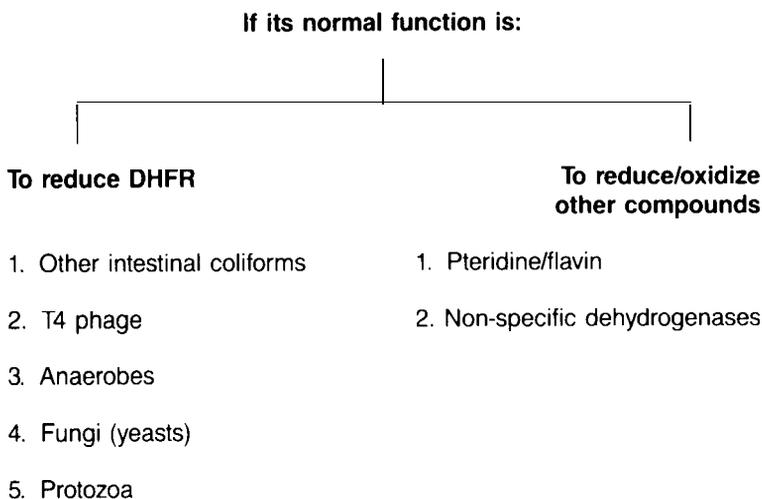


Figure 7. Possible origins of untypical dihydrofolate reductase (18)

It is pertinent at this point to refer to some of the applications of selective inhibitors of dihydrofolate reductase. A major application is, of course, the establishment of co-trimoxazole as an antibacterial of major importance. Its creation derived from the knowledge that in combining trimethoprim with sulfamethoxazole, one was creating a sequential blockade of a major biosynthetic pathway in a bacterium or in other prokaryotes. The minimal effects on the host reflect the absence in eukaryotes of the reactions leading to the biosynthesis of dihydrofolate such as occurs in prokaryotic cells.

The strong potentiation that occurs is indicated by the data of Table 2 (19), which show that minimum inhibitory concentrations of one component may be reduced as much as 10- or 20-fold when the second component is also present. Moreover, the combination may be effective against organisms that would not be inhibited by the individual drugs, e.g., *Klebsiella sp.* and *Streptococcus* Group C, where the individual inhibitory concentrations would be borderline or unattainable.

One further illustration of the possible utility of biochemically related inhibitors is given in Fig. 8 (20), where the addition of 8-azaguanine to an already potentiative combination of diaveridine (B.W. 21OU49) with sulfadiazine enhances the potency. Such biochemically orientated triple combinations have not been used in a major way, but their potential remains exploitable.

I should like to focus now on the uses of individual inhibitors of the enzyme dihydrofolate reductase.

Chronologically first, and perhaps still first in importance in cancer chemotherapy, is methotrexate from the Lederle Laboratories. Methotrexate had assumed a role in the therapy of acute leukemia as early as 1948 (21) and is still of major interest today. As shown in Table 1, however, it is

Table 2. Effect on minimal inhibitory concentration (MIC) on combining one part of trimethoprim with 20 parts sulfamethoxazole.

Organism	MIC ( $\mu\text{g/ml}$ )			
	Sulfamethoxazole		Trimethoprim	
	Alone	Mixture	Alone	Mixture
<i>Streptococcus pyogenes</i>	>100	1	1	0.05
<i>Streptococcus pneumoniae</i>	30	2	2	0.1
<i>Staphylococcus aureus</i>	3	0.3	1	0.015
<i>Haemophilus influenzae</i>	10	0.3	1	0.015
<i>Bordetella pertussis</i>	50	4	3	0.2
<i>Klebsiella pneumoniae</i>	>100	4	1	0.2
<i>Klebsiella aerogenes</i>	>100	4	1	0.2
<i>Escherichia coli</i>	3	1	0.3	0.05
<i>Salmonella typhimurium</i>	10	1	0.3	0.05
<i>Shigella sonnei</i>	10	1	0.3	0.05
<i>Proteus vulgaris</i>	30	3	3	0.15

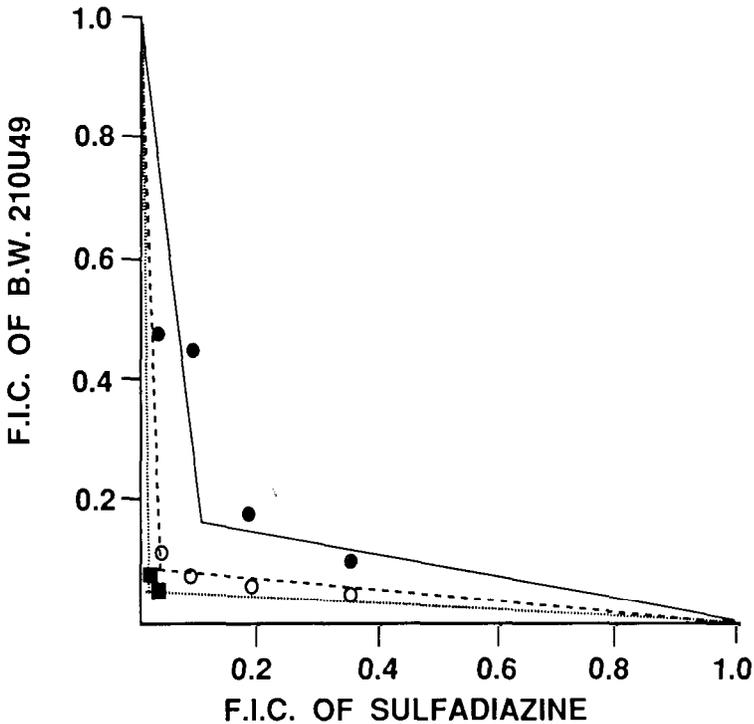


Figure 8. Fractional inhibitory concentrations of B.W. 210U49 and sulfadiazine required for 50 percent inhibition of *P. vulgaris* in the presence and absence of 8-azaguanine: ●—●, no 8-azaguanine; ○—○, 0.0 1  $\mu\text{g/ml}$ ; ■—■, 10  $\mu\text{g/ml}$ . (20).

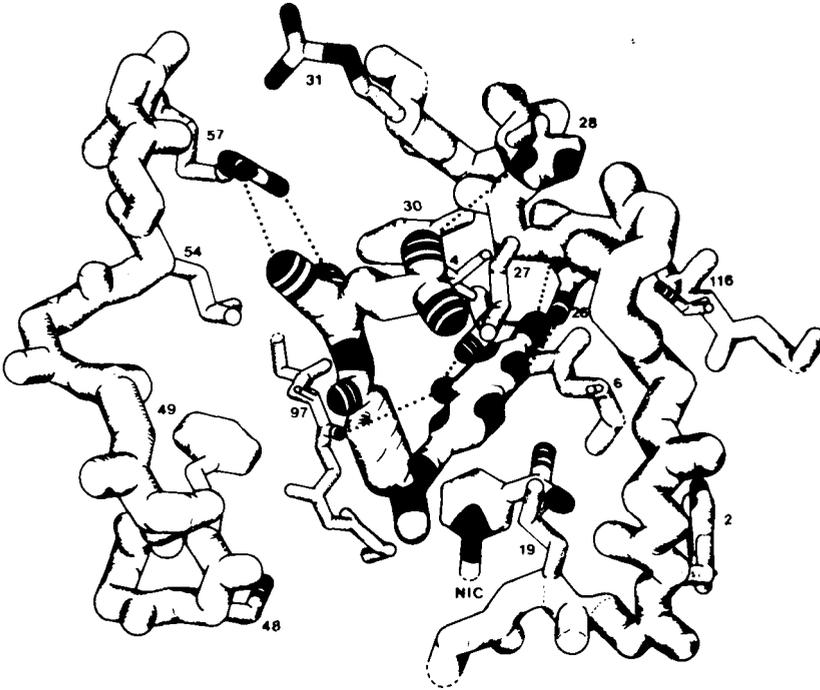


Figure 9. Schematic illustration of the active site of *L. casei* dihydrofolate reductase showing the binding of methotrexate and nicotinamide adenine dinucleotide phosphate (NADPH) (22).

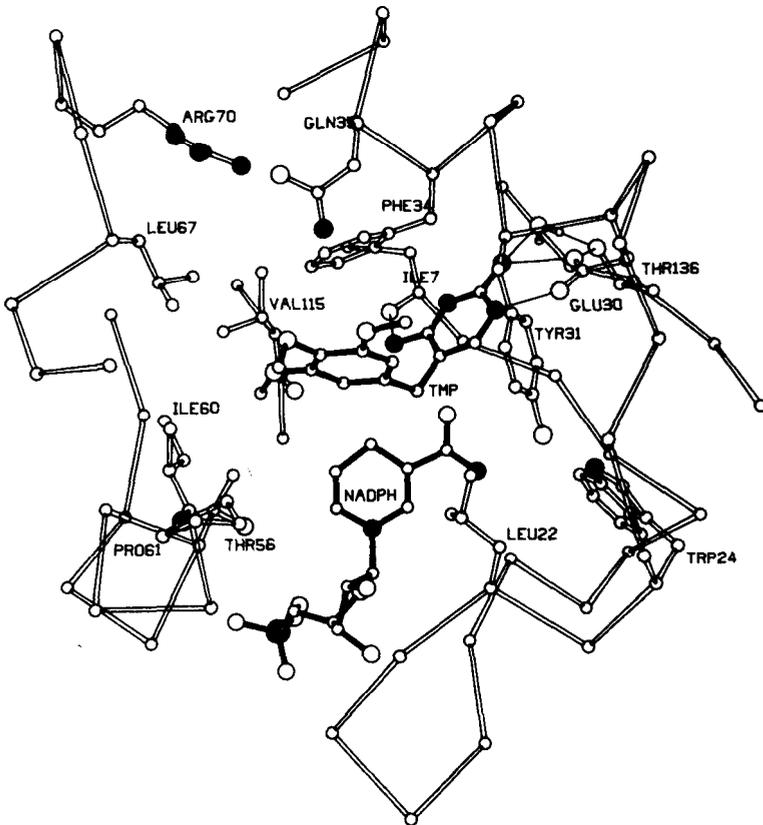


Figure 10. Schematic illustration of the cofactor in chicken liver DHFR showing the binding of trimethoprim (TMP) and NADPH (23).

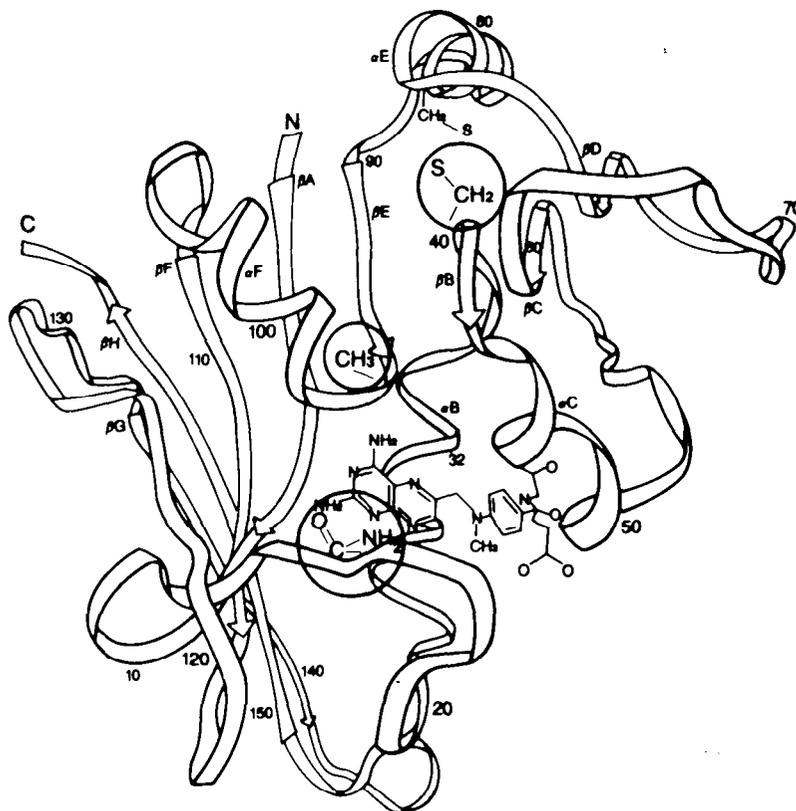


Figure 11. *E. coli* dihydrofolate reductase with induced specific mutations (24).

relatively unselective. Its therapeutic utility depends mainly on differences in uptake, glutamylation, cellular excretion, cellular metabolic balance, and other factors.

Whereas methotrexate is a close structural analogue of folate, our own antifols were significantly different and highly selective. These facts will be brought out by examination of details of structure and active centers of several enzymes.

For example, it may be illustrated by contrasting the fine structure of the complex of methotrexate and cofactor in the *L. casei* DHFR (Fig. 9) (22) with that of trimethoprim and cofactor in the chicken liver enzyme (Fig. 10) (23). It is obvious that the methotrexate molecule is much more space-filling than trimethoprim. Such contrasts are observable in all situations where the small molecule inhibitors are observed, and the contrasts in the active centers of the enzymes are consistent with the selectivities of the small molecule-inhibitors.

There have been a number of studies of the functions of specific amino acid residues. One of the most cogent was reported by Kraut and coworkers (24) in a paper that combined several experiments. In this study of the *E. coli* enzyme, the replacement of aspartate by asparagine reduced activity to

**Table 3.** Comparison of binding constants and kinetic values of polyglutamates in enzymatic reactions in folate metabolism. Thymidylate synthase (T.S.); AICAR transformylase (AICAR TF); GAR transformylase (GAR TF) (25). Number of glutamate residues (n).

Enzyme	n	n/1	
		$K_m$	$V_{max}$
DHFR	5	1	—
serine HOCH <sub>2</sub> - transferase	5	0.0025	—
5, 10-CH <sub>2</sub> -H <sub>4</sub> F dehydrogenase	3	<1	1.7
10-CHO-H <sub>4</sub> F synthetase	3	0.01	0.5
10-CHO-H <sub>4</sub> F synthetase	5	0.001	0.25
5, 10 CH-H <sub>4</sub> F cyclohydrolase	5	0.5	1.5
T.S.	3	0.2	—
T.S.	5	0.014	3
AICAR-TF	3	0.01	1
GAR-TF	3	0.8	5

0.1% of that of the unaltered enzyme, showing the importance of the ionizable carboxyl group (Fig. 11). The substitution of alanyl for glycyl, next up, completely inactivated the enzyme, probably by distorting its conformation. However, substitution of SH for OH (cysteine for serine) had little effect. This type of experiment may provide the background for the synthesis of new and useful inhibitors.

An aspect of folate metabolism that appeared relatively late is the identification of the high molecular weight derivatives of folic acid as polyglutamates. Since the chain length of most of these exceeds that of the longest form that can be transported into the cell efficiently, most cells contain a pteroylpolyglutamate synthetase. The number of glutamyl residues varies with time and with cell type. The immense complexities of this situation are only beginning to appear. Nevertheless, it is interesting and instructive to compare the binding constants and kinetic values of polyglutamates in some of the enzymatic reactions involved in folate metabolism (Table 3) (25).

**Table 4.** The effect of pentaglutamylation on the binding of methotrexate (MTX) to specific tetrahydrofolate-utilizing synthetic enzymes (26).

Enzyme	Folate Cofactor	K <sub>i</sub> (μM)		MTX
		MTX	MTX-Glu <sub>5</sub>	MTX-Glu <sub>5</sub>
Thymidylate Synthase	5,10-CH <sub>2</sub> -H <sub>4</sub> PteGlu <sub>1</sub>	13.0	0.047	277
	5,10-CH <sub>2</sub> -H <sub>4</sub> PteGlu <sub>5</sub>	14.3	0.056	254
AICAR Transformylase	10-CHO-H <sub>4</sub> PteGlu <sub>1</sub>	143.9	0.057	2508
	10-CHO-H <sub>4</sub> PteGlu <sub>5</sub>	40.0	5.89	6.8
GAR Transformylase	10-CHO-H <sub>4</sub> PteGlu <sub>1</sub>	80	2.5	32
	10-CHO-H <sub>4</sub> PteGlu <sub>5</sub>	84	22.0	4

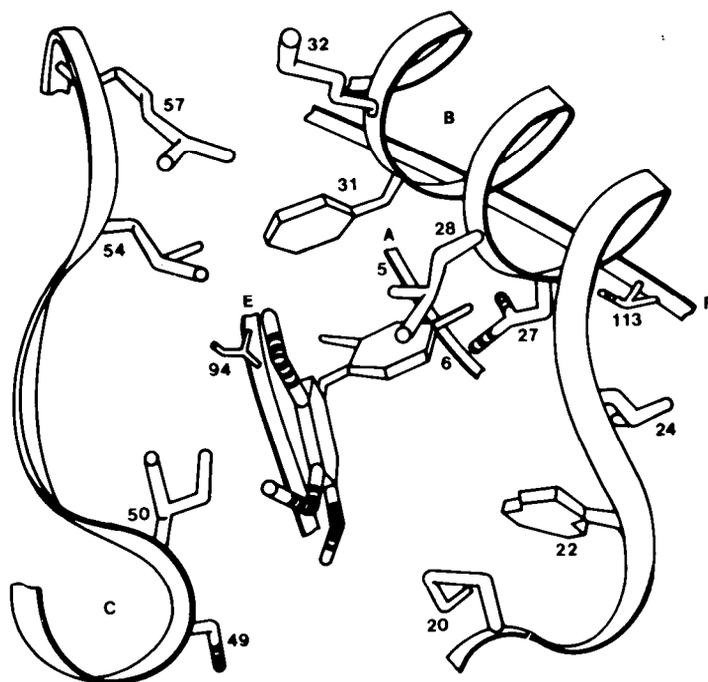


Figure 12. Fit of trimethoprim (center) in the DHFR of *E. coli* (27).

The inhibitor methotrexate is subject to polyglutamylation as well as the tetrahydrofolate. In many cases the ratio of folate polyglutamate to inhibitor polyglutamate is not markedly changed, but it is predictable that among the vast number of possibilities some of the anomalies and some of the therapeutic indices will find solid explanations. A few of the effects of glutamylation on specific biosynthetic enzymes are shown in Table 4 (26).

I wish to turn now to the intimate details of the structure of specific enzymes and their exploitation in the synthesis of more active inhibitors. Figure 12 (27) shows the fit of trimethoprim in the dihydrofolate reductase of *E. coli*. In the upper left (residue 57), one can see the guanidino group of arginine. This was exploited by Lee Kuyper who replaced the *m*-methoxyl of trimethoprim with a series of carboxyalkoxy radicals. The optimum length proved to be 5 carbon atoms, and the product, the carboxyl amyloxy derivation, bound the enzyme some 50 times more tightly than did trimethoprim (28). In the same vein, Barbara Roth synthesized bromoacetoxyphenoxy analogues of trimethoprim and found that the derivative bound to the histidine-22 residue of the enzyme with a covalent linkage (29). These examples may be regarded as probes of a vast and exciting future.

Resistance to antifolates in multiple aspects appeared soon after work on these inhibitors began. Among various expressions of resistance (Fig. 13) was a loss of the cellular transport mechanism, which has stimulated the investigation of lipophilic inhibitors that do not depend on this mechanism

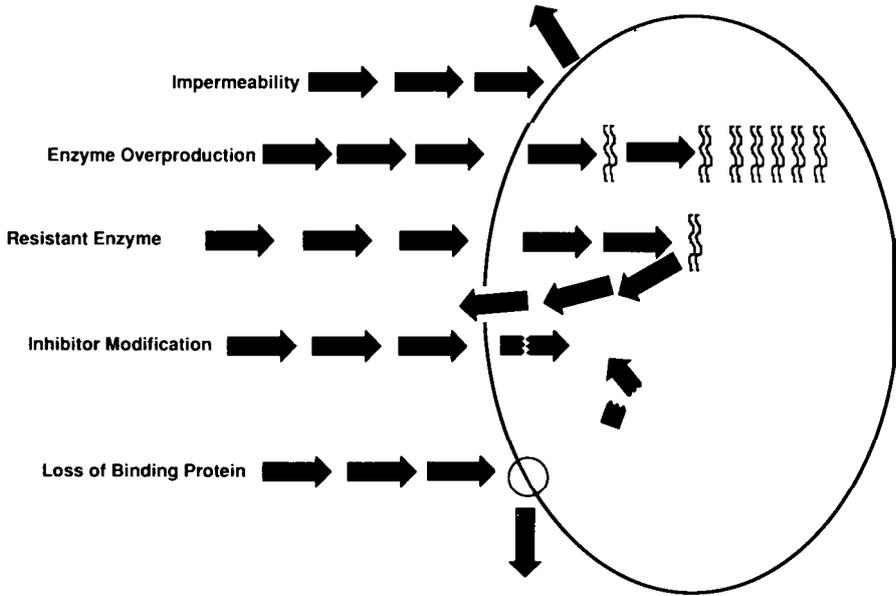
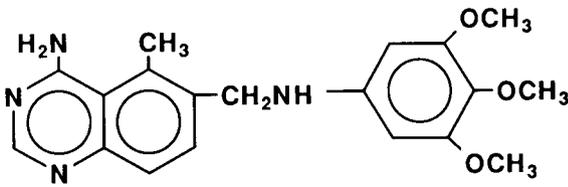


Figure 13. Mechanisms of resistance to inhibitors of dihydrofolate reductase (18).

### TRIMETREXATE



### PIRITREXIM

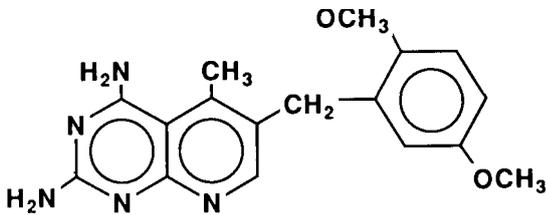


Figure 14. Chemical structures of trimetrexate and piritrexim, two potentially useful drugs.

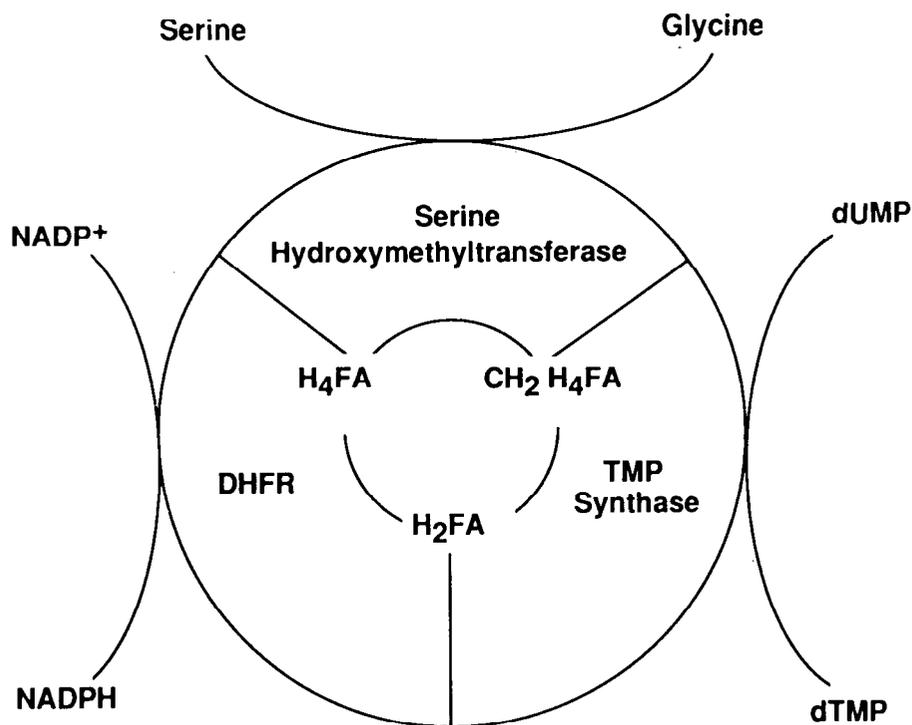


Figure 15. The thymidylate synthetic cycle. Methylene tetrahydrofolate (CH<sub>2</sub> H<sub>4</sub>FA); Nicotinamide adenine dinucleotide phosphate (NADPH).

for transport into the cell. At present the two potentially useful drugs are trimetrexate and piritrexim (Fig. 14). Exploitation of these inhibitors is in its infancy, but each has exhibited interesting properties of its own. The effects of trimetrexate and methotrexate are compared in Table 5 (30).

Table 5. Efficacy of trimetrexate and methotrexate against transplantable rodent tumors and in xenografts (30).

	Tumor System	Trimetrexate	MTX
Murine	L1210 leukemia	+ +	+ +
	P388 leukemia	+ +	+ +
	B16 melanoma	+ +	- -
	Lewis lung carcinoma	- -	- -
	Colon 26	+ +	- -
	Colon 38	+	- -
	CD8F1 mammary	+ +	+
Human xenograft	Mammary MX-1	- -	- -
	Lung LX-1	- -	- -
	Colon CX-1	- -	- -

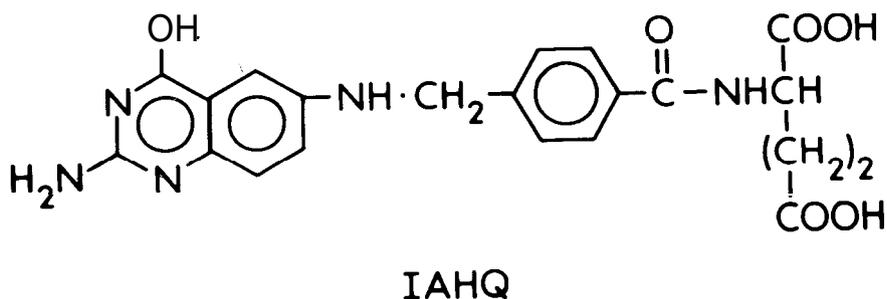
**Table 6.** Effect of the piritrexim-sulfadiazine combination in treatment of murine toxoplasmosis. Sulfadiazine (S) was administered orally by gavage at concentrations of 4, 40, and 400 mg/kg per day, and piritrexim (P) was administered ip at a concentration of 20 mg/kg per day (32).

Treatment (concentration)*	Percent cumulative mortality: day						
	3	6	9	12	15	18	20
None	0	0	100	—	—	—	—
S (4)	0	20	60	100	—	—	—
P + S (4)	0	20	40	60	80	80	80
S (40)	0	20	40	100	—	—	—
P + S (40)	20	20	20	40	80	80	80
S (400)	0	0	80	100	—	—	—
P + S (400)	0	0	0	0	0	0	0

Both trimetrexate and piritrexim have shown interesting activities against infectious prokaryotic organisms. The combination of trimetrexate and leucovorin has been used successfully to treat pneumocystic pneumonia in AIDS patients (31). Table 6 shows the effect of combining piritrexim and sulfadiazine to treat murine toxoplasmosis (32).

Finally, to be mentioned is a selective inhibitor of thymidylate synthase (Fig. 15). The first of these is 5,8-dideazaisopteroylglutamate (IAHQ) (Fig. 16). IAHQ and specific inhibitors of other biosynthetic reactions involving tetrahydrofolate represent the beginning of a biochemically orientated improved cancer chemotherapy (33).

I am incredibly blessed to have been involved for well over four decades in a field that continues to become more exciting with every passing year. Figure 17 (34) charts the history of increasing knowledge concerning dihydrofolate reductase, beginning with our work in the 1940s. Those early, untargeted studies led to the development of useful drugs for a wide variety of diseases (Fig. 18) and has justified our belief that this approach to drug discovery is more fruitful than narrow targeting. In 1988, this line of research continues to generate useful new compounds.



**Figure 16.** Chemical structure of IAHQ(5,8-dideazaisopteroylglutamate), a selective inhibitor of thymidylate synthase.

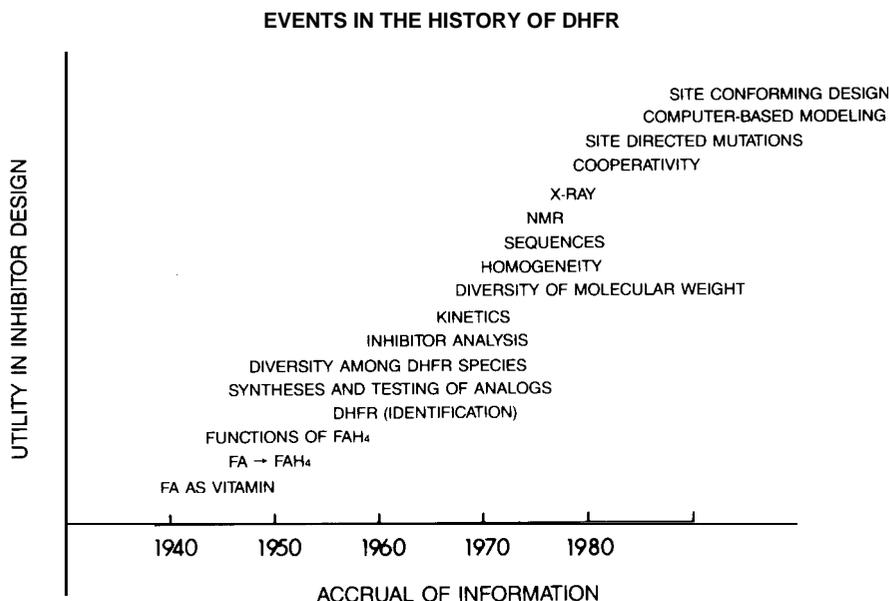


Figure 17. Accrual of information about dihydrofolate reductase, beginning with untargeted studies in the 1940s and continuing in the 1980s with discovery of new useful compounds and methods of drugs design (34)

<b>BASE ANALOGS</b>	
<u>Antileukemic</u>	<u>Immunosuppressant</u>
2,6-Diaminopurine	—————→
6-Thioguanine	—————→
6-Mercaptopurine	—————→ Azathioprine
<u>Hypouric</u>	<u>Antiprotozoal</u>
Allopurinol	—————→ Allopurinol Riboside
<b>DHFR INHIBITORS</b>	
Pyrimethamine	Antimalarial
Trimethoprim	Antibacterial
Piritrexim	Antitumor
<b>SUGAR ANALOGS</b>	
Antiherpetic	
Acyclovir	(acyclic guanosine)
Anti AIDS	
3-Azidothymidine (zidovudine)	

Figure 18. Drug therapies that have emerged from dihydrofolate reductase inhibitors.

I should like to close with a quotation from one of my own papers:

"To the biochemical chemotherapist, it is not only a matter of faith, but an obvious fact, that every cell type must have a characteristic biochemical pattern, and therefore be susceptible to attack at some locus or *loci* critical for its survival and replication." (35).

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