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RECOLLECTIONS

In search of dihydrofolate reductase

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Dihydrofolate reductase is a fascinating enzyme. Nearly four decades after it was discovered in the late 1950s, the enzyme continues to command the attention of scientists in a variety of disciplines. Biochemists interested in protein structure and function appreciate the monomeric form and low molecular weight of dihydrofolate reductase (ca. 20 kDa)—properties that make it an ideal subject for X-ray crystallography, NMR spectroscopy, kinetic measurements, and site-directed mutagenesis. Molecular biologists use the enzyme as a selectable genetic marker and as a model for gene amplification. Oncologists take advantage of the identification of dihydrofolate reductase as the target for Methotrexate to design optimal regimens for use of the drug in cancer chemotherapy. Information about structural features of the enzyme that account for the tight binding of Methotrexate and alterations in structure or amount of the enzyme that are responsible for drug resistance provides guidance to pharmacologists, as Hitchings (1989) pointed out in his Nobel Lecture, for the design of anti-folates directed against tumors, pathogenic bacteria, and malaria parasites. Each year, the large number of literature citations attest to the continued popularity of dihydrofolate reductase. It is truly an "enzyme for all seasons."

The properties of dihydrofolate reductases from various sources have been documented in several comprehensive reviews (Gready, 1980; Blakely, 1984; Freisheim & Matthews, 1984). Less well-known, however, are the events that stimulated the search for, and discovery of, the enzyme. This quest was conducted by groups with somewhat different objectives and experimental approaches. The story of how these investigators converged upon dihydrofolate reductase, apart from possible historical interest, may serve to remind us that, in science as in business, large rewards can accrue from small investments.

Folate-dependent enzymes (1950–1960)

The search for dihydrofolate reductase was preceded by the recognition of folate-dependent enzymes (pteroproteins) that occurred in the 1950s—a period that defined the field. It was also, to borrow a phrase from Hans Neurath (1994), a "Golden Era" for enzymology in general. The broad outlines of various metabolic pathways had been established by experiments with labeled compounds, augmented by some inspired guesses, and attention was turning toward the assay, isolation, and characterization of enzymes that catalyzed the individual steps in these pathways. Activities barely discernable in tissue or cell extracts were being transformed by hard labor and persistence into visible amounts of purified, highly active proteins. This accomplishment may be difficult for today's investigators to appreciate, because enzymes are now produced in bulk by cloning and overexpression techniques, and Nature's originals can be reconstructed to suit the needs of the investigation.

The Enzyme Game was open to everyone. Starting materials were tissues available from laboratory animals and local abattoirs or from cells provided by accommodating bacteria. The necessary reagents (often homemade or, when all else failed, purchased from Sigma) were relatively few and simple. Equipment needs also were modest—minimum essentials were little more than a blender, centrifuge, pH meter, ice bucket, constant-temperature water bath, and Beckman DU spectrophotometer; regrettably, gel electrophoresis for assessing the progress of protein purification had not yet been introduced. Although lightly armed, the investigators were remarkably successful in prying

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Frank M. Huennekens received his Ph.D. in chemistry from the University of California Berkeley in 1948. After postdoctoral training with David Green in the Institute for Enzyme Research at the University of Wisconsin, he joined the Department of Biochemistry at the University of Washington and rose to the rank of Professor. In 1962, he became the founding Chairman and Member of the Department of Biochemistry at Scripps Clinic and Research Foundation in La Jolla, and he was appointed Adjunct Professor in the Departments of Chemistry and Biology at the University of California San Diego. Since 1992, he has been a Member Emeritus of The Scripps Research Institute, and he served recently as Visiting Professor in the Department of Biological Chemistry at the University of California Davis.

Dr. Huennekens has been a member of the Editorial Boards of various journals, including *Biochemistry*, *Journal of Biological Chemistry*, and *Cancer Research*, and on Advisory Boards and Councils of the National Cancer Institute and the American Cancer Society. Currently, he is a member of an Advisory Board to the Burroughs Wellcome Fund. His research program was supported continuously for more than 30 years by ACS and NCI. In 1985, he received one of the first Outstanding Investigator grants from NCI. Honors include the Paul-Lewis Award in Enzyme Chemistry from the American Chemical Society, the F. Gowland Hopkins medal from the International Pteridine Symposium, the C. Chester Stock Award from Memorial Sloan Kettering Cancer Center, and membership in the American Academy of Arts and Sciences. In 1989, he was the honoree at a symposium titled, "Critical Issues in Cancer Chemotherapy."

open Nature's box of biocatalysts. Many of the vast array of enzymes displayed on Metabolic Pathway charts that decorate office and laboratory walls were discovered during the 1950s. In retrospect, it was a period of adventure—there was no shortage of intriguing problems. Progress was easy to follow: results were published in a limited number of journals and presented at the Federation meetings in Atlantic City. Research grants, thanks to the largesse of the Federal government, were abundant, and all was right with the world.

Critical information about folate-dependent enzymes emerged during this decade. The stage had been set by four seminal observations from earlier nutritional, metabolic, and chemical studies. (1) Administration of [14C]formate to animals resulted in labeling of the 2- and 8-carbon atoms of purines, the methyl of thymine, the hydroxymethyl of serine, and the methyl of methionine. The term "one-carbon metabolism" was used to describe this interrelationship. (2) The B-vitamin pteroylglutamic acid (Fig. 1), subsequently given the more euphonious name "folic acid," was isolated from plants, liver, and yeast, and shown to be an essential growth factor for animals. A deficiency of folic acid compromised the labeling described above, thereby linking the vitamin to one-carbon metabolism. (3) Another folate compound, folinic acid (also known as "leukovorin" or "Citrovorum factor"), was also isolated from natural sources and shown to be the 5-formyl derivative of tetrahydrofolic acid (Fig. 1). Because it was known that B-vitamins are converted to coenzymes that have suitable structures to "carry" mobile metabolic groups (reviewed by Huennekens, 1956), a comparison of folic acid and folinic acid suggested that the coenzyme form of the vitamin was tetrahydrofolic acid and the mobile group was a formyl fragment. (4) The structures of folic acid and folinic acid were elucidated, and both compounds were synthesized. During the course of this work, conducted by groups at Lederle, Lilly, Parke Davis, and University of Texas, dihydrofolic acid, tetrahydrofolic acid, and the 5- and 10-formyl and 5,10-methenyl derivatives of tetrahydrofolic acid were prepared. Enzymologists are forever grateful to the chemists for having made these compounds readily available.

It should be noted parenthetically that folinic acid, the most stable of the reduced folates and used, therefore, as a nutrient in cell cultures and as a "rescue agent" in cancer therapy with high-dose levels of Methotrexate, has been much abused by the

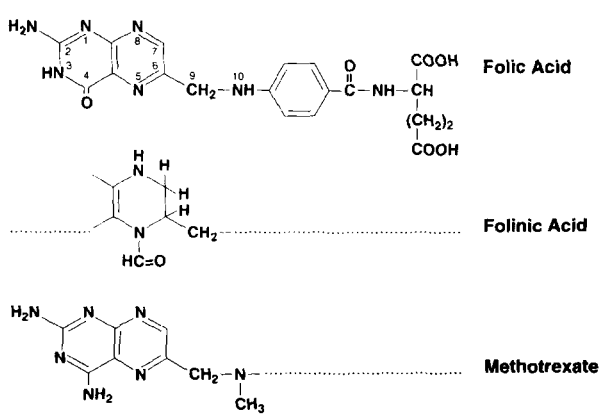

Fig. 1. Folic acid, folinic acid, and Methotrexate. Note that most naturally occurring folate compounds are γ-polyglutamates.

persistent argument that it is an artifact, created from other folate compounds during isolation procedures. However, the fact that it can be synthesized enzymatically from *N*-formylglutamate and converted enzymatically to methenyltetrahydrofolate (see below), argues strongly for its occurrence as a natural product.

Based upon the above observations and equipped, fortunately, with key compounds, investigators began to search for folate-dependent enzymes involved in one-carbon metabolism. It must be remembered, given the experimental constraints of that period, that large amounts of enzymes were not available, and emphasis was placed, therefore, upon their purification only to the stage where distracting activities had been eliminated, which allowed substrates and products to be identified and mechanisms to be studied. Characterization of the physical properties of the enzymes had to be deferred until more purified preparations were available.

For convenience, let us now fast-forward to the end of the 1950s in order to appreciate, via Figure 2, what was accomplished during the preceding decade. Most of the principal reactions catalyzed by folate-dependent enzymes, and the one-carbon (C1)-coenzyme complexes involved in these reactions, had been identified. Highlights of this progress were recorded by the traditional (sometimes single-page) Communications, many of which appeared in the *Journal of the American Chemical Society*.

The picture in Figure 2 was constructed piece-by-piece, much as a jigsaw puzzle is assembled. The role of folates in purine synthesis was one of the first problems to be investigated. Because formate was known to provide two of the carbon atoms in the purine ring, reaction of "activated" formate with two adjacent amino groups of putative precursors was a logical process for achieving ring closure. Working with extracts of avian liver, G.R. Greenberg found that folinate, along with ATP, stimulated the final step in purine synthesis, viz., conversion of 5-aminoimidazole-4-carboxamide ribonucleotide to inosinic acid. It was shown subsequently that the transformylase, which delivers the formyl group from the coenzyme to the purine skeleton, actually utilizes not the 5-formyl, but the 10-formyl derivative of tetrahydrofolate. This apparent contradiction was resolved independently by G.R. Greenberg and D.M. Greenberg, who demonstrated that 5-formyltetrahydrofolate is converted, via an ATP-dependent cyclodehydrase, to the 5,10-methenyl derivative and that the latter opens selectively to produce 10-formyltetrahydrofolate (Fig. 2). 5-Formiminotetrahydrofolate, identified by work from the laboratories of Tabor, Rabinowitz, Waelsch, and Silverman as an intermediate in the degradation of purines and histidine, is also a precursor of 5,10-methenyltetrahydrofolate; in this instance, the reaction, catalyzed by cyclodeaminase, involves the loss of ammonia rather than water, and ATP is not required.

The ring-opening reaction catalyzed by cyclohydrolase is reversible and, at neutral pH, the equilibrium favors the 10-formyl derivative; even in the absence of the enzyme, the reaction proceeds at a reasonable rate at neutral pH. This facile interchange between two of the formyl–coenzyme complexes hampered identification of the actual reactant in each of the two steps in purine synthesis and also in the oxido-reduction reaction that interconverts the formyl and methylene C1 units while attached to the coenzyme (see below).

The mechanism by which formate is transformed into a formyl derivative of tetrahydrofolate, and hence into purines, also received attention. Because ATP was required for this process,

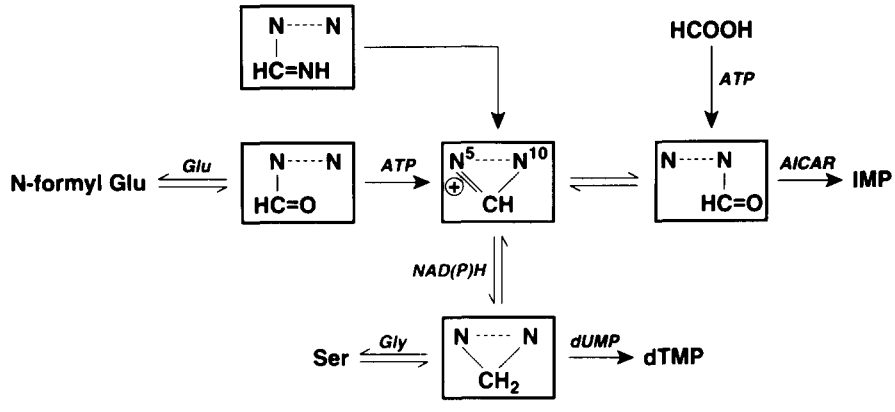

Fig. 2. Folate-dependent reactions in one-carbon metabolism. Status of the field 1950-1960. N-----N represents the 5- and 10 nitrogen atoms of tetrahydrofolate. AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide. Note that 10-formyltetrahydrofolate was shown later to be the precursor also of carbon atom 8 of purines via a reaction in which it formylates glycinamide ribonucleotide.

it seemed likely (by analogy with the "activation" of acetate to form acetyl CoA) that a similar reaction was operative. This hypothesis was verified by G.R. Greenberg, who demonstrated that addition of formate, tetrahydrofolate, and ATP to avian liver extracts resulted in the production of 10-formyltetrahydrofolate.

Another fertile area of investigation centered about the interconversion of serine to glycine and formaldehyde, catalyzed by serine hydroxymethyltransferase. This reaction is important because serine, arising from glucose, is the principal source of C1 units for the reactions shown in Figure 2. The research groups of Blakely, Sakami, D.M. Greenberg, and Kisliuk demonstrated that pyridoxal phosphate and tetrahydrofolate were cofactors for this reaction. The latter requirement suggested that "active formaldehyde" or "active hydroxymethyl," rather than free HCHO, might be the actual reactant in serine synthesis or breakdown. Chemical considerations led to the proposal that the activated species is the 5,10-methylene derivative of tetrahydrofolate. As shown subsequently, *nonenzymatic* interaction of HCHO with the favorably positioned N-5 and N-10 of tetrahydrofolate to form a stable five-membered ring is rapid and complete at neutral pH.

Identification of 5,10-methylenetetrahydrofolate as a C1-coenzyme complex provided an important clue to the mechanism of thymidylate synthesis. Although the reaction formally involved the methylation of deoxyuridylate, the identity of the methyl donor was unknown. 5-Methyltetrahydrofolate had not yet been discovered, and it was difficult to envision how a formyl or methylene derivative of tetrahydrofolate could function as a methylating agent. To answer this question, Friedkin and D.M. Greenberg proposed independently that, in the thymidylate synthase-catalyzed reaction, methylene-tetrahydrofolate was indeed the C1 donor, and that the requisite reducing power was provided by the tetrahydrofolate moiety of the complex. This was a novel concept: in all previously known reactions involving the folate coenzyme, the latter had served as an inert carrier of the C1 unit. Later, Friedkin suggested an ingenious mechanism for the reaction, namely that the methylene group becomes unhinged from the 10-position of the coenzyme and forms a covalent bond with the 5-position of deoxyuridylate. Cleavage of this intermediate, via a hydride ion from C-6 of the coenzyme, then leaves a methyl group on the pyrimidine and the coenzyme at the *dihydro* stage.

Throughout the above studies, various investigators had noted that some link existed between C1 units at the formyl and methylene level. Pyridine nucleotides appeared to mediate this interconversion, but it was not clear whether NAD or NADP was involved and whether the C1 units were free or attached to the folate coenzyme.

To summarize, by the end of the decade in 1960, the general pattern of one-carbon metabolism mediated by the folate coenzyme, tetrahydrofolate, was defined by the integrated network shown in Figure 2. Studies on the synthesis of purines, serine, and thymidylate had revealed that both formyl and methylene groups could be carried by the coenzyme. This fulfilled the prophesy made by Welch and Nichol (1952), who suggested that folic acid played a role in the metabolism of C1 units at these two oxidation levels. Four different formate-coenzyme complexes and one methylene-coenzyme complex had been identified. In the early 1960s, another complex, 5-methyltetrahydrofolate, and the NADPH-linked enzyme responsible for its production from the methylene derivative, were discovered by the laboratories of Keresztesy and Buchanan, and Buchanan then demonstrated that 5-methyltetrahydrofolate was the methyl donor in methionine synthesis; these reactions are omitted from Figure 2.

Introduction of our laboratory to folate-dependent enzymes

My interest in biochemistry resulted from a series of fortuitous events. As an undergraduate in the Chemistry Department at the University of California Berkeley (1940-1943), I was completely unaware of the Biochemistry Department located at the opposite end of the campus. After serving as an officer in the Marine Corps in the Pacific Theater, I returned to Berkeley in 1946 as a graduate student and joined the research group of Melvin Calvin. He, along with Glenn Seaborg and Kenneth Pitzer, were the rising young stars in the department. The legendary Professor G.N. Lewis, who still presided majestically over the Tuesday evening seminars, had left as his legacy a department that subjected students to a heavy load of physical chemistry and

thermodynamics. Over the years, however, I have come to appreciate the benefits of having been exposed to the quantitation and rigor of those subjects. My Ph.D. thesis research consisted of a study of the photochemical properties of metalloporphyrins and chlorins, the results of which, we hoped, would provide some clue to the mechanism of the light-trapping reaction in photosynthesis. To my surprise, the biochemist D.M. Greenberg (whom I came to know later as a friendly competitor) was appointed to my Advisory Committee. Except for asking me about "high-energy phosphate compounds" on my oral examination, he mercifully did not expose my ignorance of biochemistry.

In addition to appreciating Calvin's laissez faire attitude with respect to directing my research, I was tremendously impressed by the range of his eclectic interests which, at that time, were beginning to extend into the biological sciences. Near the completion of my thesis work, Calvin organized an informal study group (which I attended, largely out of curiosity but with growing interest) to discuss David Green's small but powerful book titled, *Biological Oxidations*. By curious coincidence, Calvin and Green happened to meet shortly thereafter (on an airplane flight, as I recall). David mentioned that he had just become Director of the newly created Institute for Enzyme Research at the University of Wisconsin and that he was looking for a postdoctoral fellow with a strong background in chemistry. I was recommended, offered the position, and accepted. Arriving in Madison in the summer of 1948, I plunged into the arcane world of enzymes. David, already well-established as a leader in the purification and characterization of soluble enzymes, had just embarked upon the more difficult task of working with particulate enzymes, later shown to be localized in mitochondria. After some studies in collaboration with Henry Mahler on malate dehydrogenase and propionate oxidation in particulate preparations, I turned to flavin nucleotides and, with Rao Sanadi, developed a procedure for the purification of FAD.

The three years that I spent at the Enzyme Institute opened the door to the exciting world of biochemistry and shaped my future career. My appreciation of David Green as a scientist, mentor, and friend is recorded in a memorial (Huennekens, 1984).

In 1952, I was most pleased to accept an invitation from Hans Neurath, who had just moved from Duke University to become Chairman of the Department of Biochemistry at the University of Washington, to join the new department as an Assistant Professor. My arrival in Seattle coincided with that of Walt Dandliker, a colleague in Calvin's group who later became one of the first members of our Biochemistry Department at Scripps Clinic and Research Foundation (now The Scripps Research Institute). It was a young group—all of the faculty, except for Hans, were Assistant Professors. The continued sprightliness of the department, even at the conclusion of the 1950s, is evident from the group photograph of its members that was used by Hans to illustrate his article for the Recollections series (Neurath, 1994). As a sign of the times, all of the gentlemen wore ties and were clean-shaven. My own favorite picture of that era (Fig. 3), taken in 1962, shows four of the Department Staff (assembled, as I recall, to attend an address by President John Kennedy) posing solemnly in academic attire against a backdrop of the UW Medical School.

Unknown to us at the time, the department was a cradle of future Nobel Laureates (Ed Krebs, Eddy Fischer, and Marty Rodbell) and several department chairmen. Those of us in the latter category, including Don Hanahan, learned much from Hans' crisp and efficient administrative style. He also insisted upon high standards in research and teaching. The latter was a formidable challenge for me, because I had never taken any formal courses in biochemistry. Whatever success I may have had over the years in teaching biochemistry to medical students is due, most probably, to the fact that I learned the subject from the viewpoint of a chemist.

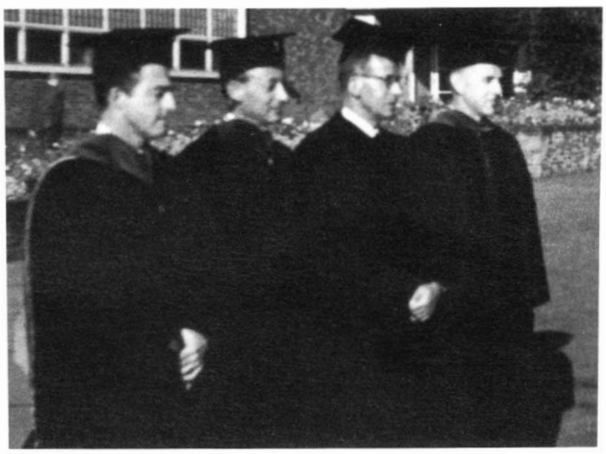

Fig. 3. Staff members, Department of Biochemistry, University of Washington. Left to right: Frank Huennekens, Edmond Fischer, Hans Neurath, and Edwin Krebs.

By the mid-1950s, I was settled comfortably in the department and had begun to assemble a small research group. Our program focused initially on flavin nucleotides and flavoproteins. Gordon Kilgour, one of the first graduate students, developed a simple but convenient chemical synthesis of FAD via the carbodiimide-promoted condensation of FMN and AMP. Our interest in nucleotides was stimulated by the opportunity of exchange visits with Gobind Khorana and his colleagues in nearby Vancouver.

The program in our laboratory soon expanded to include a collaborative effort on erythrocyte enzymes and erythrocyte preservation with Beverly Gabrio and Clement Finch in the Department of Medicine. Those studies were my introduction to the applicability of biochemistry to medical research. I was particularly intrigued by the potential of chemotherapy, as exemplified by the promising results with Methotrexate in the treatment of childhood leukemia, but I was puzzled (in my naivete as a chemist/biochemist) about the failure of the drug to retain its efficacy in the recurrent treatment of patients. This prompted some reading about folates, which appeared to hold the key to understanding how the drug functioned. The folate field was just opening up, and it seemed that there might be an opportunity for us to use our previous experience with flavins to make a contribution to this related field. More important, the possibility of combining the intellectual pleasure derived from doing basic research with results that might be beneficial to terminally ill children was a powerful incentive.

The point man for our program was Youssef (Joe) Hatefi, a graduate student, who later became a postdoctoral fellow with David Green (where he participated in the discovery of Coenzyme Q and developed the procedure for separation of Complexes I–IV of the mitochondrial electron transport system). In

1963, I was pleased to welcome him as one of the first staff members of our Biochemistry Department at Scripps, where he continues to be an international authority in the field of mitochondrial enzymes and oxidative phosphorylation. For the initial foray into folates, we elected to study the cofactor requirements of serine hydroxymethyltransferase in a soluble beef liver system. Joe, along with Lorel Kay (a research assistant, who had worked previously with Calvin), developed an assay system in which serine scission was coupled to oxidation of the C1 unit to the formate level. Confirming the results of previous investigators, they found that the reaction required pyridoxal phosphate, tetrahydrofolate, and NADP. Oxygen uptake, resulting from oxidation of NADPH by endogenous enzymes in the preparation, allowed the primary reaction to be monitored continuously. This study focused our attention upon interconversion of the C1-folate coenzyme units at the formaldehyde and formate levels. Mary Osborn (now Professor of Microbiology at the University of Connecticut) then joined the laboratory as a graduate student and began a fruitful collaboration with Joe Hatefi. They partially purified and characterized the NADP-linked methylenetetrahydrofolate dehydrogenase, folinate cyclodehydrase, and a system responsible for deacylation of 10-formyltetrahydrofolate. The cyclodehydrase, now termed 5,10-methenyltetrahydrofolate synthetase, remained relatively dormant until 1984, when it was purified to homogeneity from *Lactobacillus casei* by Ched Grimshaw, a postdoctoral fellow in our laboratory, and from rabbit liver by Schirch.

After Joe's departure for Madison, Mary continued the assault on folate-dependent enzymes. From two lines of evidence, she established that 5,10-methenyltetrahydrofolate, rather than either of the other formyl derivatives, was the participant in the dehydrogenase-catalyzed reaction. First, when a purified preparation of the enzyme in which cyclohydrolase had been removed was used, oxidation of 5,10-methylenetetrahydrofolate produced 5,10-methenyltetrahydrofolate, which was identified by its absorbance spectrum. Second, only the 5,10-methenyl derivative served as a substrate for the reaction in the reductive direction (measured by disappearance of NADPH). After the fact, it was evident that 5,10-methenyltetrahydrofolate with its reactive $\oplus N{=}C-$ structure, rather than the more inert 5- or 10-formyl derivatives, was the obvious substrate for reduction by a hydride ion from the pyridine nucleotide NADPH. During the course of this work, she also demonstrated that 5,10-methenyltetrahydrofolate was readily reduced by borohydride (a few minutes at room temperature sufficed) to the methylene compound; ironically, if more vigorous conditions had been used, we might have discovered 5-methyltetrahydrofolate, which can be prepared by treatment of methylenetetrahydrofolate with borohydride for longer times and at higher temperatures. This study concluded with a detailed investigation, in collaboration with Preston Talbert, that provided additional chemical and enzymatic evidence to establish the structure of "active formaldehyde" as 5,10-methylenetetrahydrofolate.

Mary Osborn finished her productive career in the laboratory by collaborating with Helen Whiteley in the Department of Microbiology on the purification, properties, and mechanism of action of the formate-activating enzyme from *Micrococcus aerogenes*. (In a concurrent study by Rabinowitz, the counterpart from *Clostridium cylindrosporum* was the first of the folate-dependent enzymes to be crystallized.) Mary also coauthored two of the first reviews on folate coenzymes and one-carbon metabolism, one in *Science* (Huennekens et al., 1958) and the other in *Advances in Enzymology* (Huennekens & Osborn, 1959).

The discovery of dihydrofolate reductase

By the mid-1950s, tetrahydrofolate had been established as the coenzyme for C1 groups involved in purine, serine, and thymidylate synthesis and in the activation of formate. The stage was set, therefore, to address the problem of how the coenzyme was generated from the vitamin. It was not entirely a journey into the wilderness, because some guideposts had been erected by earlier investigators whose work had touched indirectly upon this problem. Their studies, using cells or cell-free preparations from bacterial or mammalian sources, had focused upon the conversion of folate to folinate. This was a convenient system to examine, because the reaction product was a stable compound that could be measured in minute amounts by the sensitive *Leuconostoc citrovorum* growth assay; it is likely, however, that the assay also measured other reduced folates. It was generally believed, as indicated, for example, by Nichol and Welch (1950), that folinate synthesis involved the reduction of folate (probably in two steps with dihydrofolate as the intermediate), followed by the conversion of tetrahydrofolate to the 5-formyl derivative via an undefined route.

Some evidence had been obtained that NADH or NADPH provided the reducing power for the folate → trahydrofolate conversion, but this situation was not clarified until 1957–1958. The initial breakthrough, as is often the case, resulted from a study with a somewhat tangential objective. While investigating the "inactivation" of folic acid by tissue extracts, Silverman and Keresztesy observed that the process was stimulated by NADH and NADPH, from which they deduced that folate was reduced enzymatically to tetrahydrofolate and that the latter underwent nonenzymatic degradation. One of the products was identified as *p*-aminobenzoylglutamate, whose ability to be diazotized and converted to a colored product was utilized as an assay for the overall reaction. Pursuing this lead, Futterman, a colleague of the these investigators, fractionated a chicken liver extract and obtained a preparation that catalyzed the reduction of both folate and dihydrofolate to tetrahydrofolate (Futterman, 1957). The latter product accumulated and could be measured by the above colorimetric reaction, or it could be converted to folinate upon addition of *N*-formylglutamate and a partially purified transformylase. Folate was reduced only by NADPH, whereas dihydrofolate was reduced either by NADPH or NADH. These results led to the erroneous conclusion that separate enzymes were involved in the reduction of the two substrates. After his seminal contributions, Sidney Futterman apparently left the folate field. I regret that I never had the opportunity to meet him and express my admiration for his accomplishments.

More or less concurrent with Futterman's report, Zakrzewski and Nichol (1958), also working with a chicken liver preparation termed "folate hydrogenase" and using the colorimetric assay for tetrahydrofolate, found that NADPH was more effective than NADH in the reduction of folate.

The initial contributions of our laboratory to the dihydrofolate reductase story have been recorded previously (Huennekens, 1963). While pursuing the folate-dependent enzymes, we had

made routine use of tetrahydrofolate prepared by catalytic hydrogenation of folate, but the question kept arising, "How is the coenzyme synthesized enzymatically?" Mary Osborn, who had finished her Ph.D. thesis and was looking for a short project prior to beginning postdoctoral work with Bernard Horecker at New York University, thought that the problem was attractive and suitable for the time limitation. Recognizing from preliminary experiments with chicken liver extracts that dihydrofolate and NADPH were clearly the preferable substrates for the enzyme, Mary developed a continuous spectrophotometric assay (decrease in absorbance at 340 mμ) for the reaction. This assay, which allowed large numbers of fractions to be evaluated rapidly, greatly facilitated purification of the enzyme (Osborn & Huennekens, 1958).

Use of this preparation, from which troublesome contaminants had been removed, in conjunction with an NADPH-generating system (G-6-P, G-6-P dehydrogenase, and NADP) that minimized the concentration of the pyridine nucleotide, allowed the conversion of dihydrofolate to tetrahydrofolate to be demonstrated spectrophotometrically. Formation of the tetrahydrofolate was also verified by coupling dihydrofolate reductase to serine hydroxymethyltransferase, methylenetetrahydrofolate dehydrogenase, and cyclohydrolase, and observing the appearance of 10-formyltetrahydrofolate.

Although reduction of dihydrofolate went virtually to completion, the reaction could be reversed to a small but measurable degree by admixing high concentrations of NADP and tetrahydrofolate with the enzyme. Several years later, another colleague, Chris Mathews (now Professor of Biochemistry at Oregon State University and co-author of an excellent text in biochemistry), found that the equilibrium constant at pH 7.0 was ca. 6×10^4 for the reduction of dihydrofolate to tetrahydrofolate.

Further studies from our laboratory and elsewhere made it clear that a single enzyme was responsible for the reduction of dihydrofolate and, at a much slower rate, folate (Fig. 4). NADPH was the reductant, although weak activity could be observed with NADH. The pronounced preference for dihydrofolate as the substrate indicates that the major function of dihydrofolate reductase is regeneration of tetrahydrofolate produced in the reaction catalyzed by thymidylate synthase during cell replication, and that synthesis of the tetrahydrofolate from the vitamin, a less frequent need, is a secondary function.

In view of the substrate specificity of the enzyme, we suggested that it should be called "dihydrofolate reductase," rather than "folate reductase." The latter name, however, persisted in the literature for several years.

The enzymatic studies also provided information about the structure of dihydrofolate. This compound, prepared by chemical reduction of folate, was expected to have either the 5,6- or 7,8-dihydro structure. (A 5,8-dihydro structure, later shown to be the form of the substrate for dihydropteridine reductase, was not considered at that time.) 5,6-Dihydrofolate would have an asymmetric center at C-6, whereas the 7,8-form would have no chirality in the pyrazine portion. Because tetrahydrofolate produced via dihydrofolate reductase reacted completely when used as the substrate in other enzymatic reactions, the precursor dihydrofolate must be the 7,8-tautomer. This structure, which had been predicted by O'Dell from indirect evidence, was confirmed later by the NMR studies of Friedkin and Jardetzky. Subsequent work by Chris Mathews led to the use of dihydrofolate reductase as a convenient tool for preparation of the physiologically active form of tetrahydrofolate.

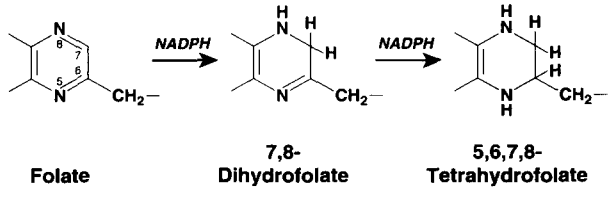

Fig. 4. Reactions catalyzed by dihydrofolate reductase. The pyrazine moiety of the folate structure is shown.

The availability of dihydrofolate reductase made it possible to investigate the mechanism by which folate and dihydrofolate are reduced by NADPH. The concept that reduced pyridine nucleotides provided hydride ions was relatively new at that time and, although such a mechanism could be visualized readily for the reduction of pyruvate or acetaldehyde by NADH, there was little precedent for its applicability to heterocyclic rings. Hydride ion-reduction of dihydrofolate seemed reasonable, because the semi-isolated N5-C6 double bond would be susceptible to protonation of the nitrogen, followed by attack on the cationic carbon by the hydride ion. However, a similar mechanism for the fully aromatic ring in folate was (and still is) difficult to envision.

Dihydrofolate reductase: Target for Methotrexate

The requirement of folic acid for the growth of mammals, and especially for the development of blood cells, stimulated the pharmaceutical industry to undertake the preparation of analogues that might have anti-growth activity. Aminopterin, the 2,4-diamino analogue of the vitamin, which was synthesized in 1947 by the Lederle group, met this expectation. The lethal nature of the 2,4-diamino structure was also exploited by Hitchings and Elion, who had found that 2,4-diaminopyrimidines were highly toxic to bacterial and mammalian cells, and that this effect could be reversed by folinic acid. The first clinical trial with aminopterin, conducted by Farber, produced dramatic remissions in children with acute lymphocytic leukemia. This desirable outcome, unfortunately, was not permanent, and further doses of the drug had progressively diminishing effects. Nevertheless, these results encouraged the synthesis of additional folic acid analogues. From the multitude of compounds prepared, amethopterin or Methotrexate, the 2,4-diamino-10-methyl analogue, became the agent of choice, and it continues to be used extensively in cancer chemotherapy. The wealth of information that has been obtained about the mode of action of Methotrexate, and the mechanisms by which tumor cells develop resistance to it, has made this agent a paradigm for drug development (Bertino, 1993; Huennekens, 1994).

Following the demonstration of the clinical potential of Methotrexate, attention became focused upon the identification of its intracellular target. Because folinic acid was able to reverse the cellular toxicity of Methotrexate, it was inferred that the drug might be inhibiting the folate → tetrahydrofolate conversion. This supposition became reality in 1957 and 1958 when three groups approached the problem at the enzymatic level. Futterman, using the partially purified preparation from chicken liver that had been developed for examining the reduction of folate and dihydrofolate, found that aminopterin and Methotrexate

(at nanomolar concentrations) inhibited both processes (Futterman, 1957). Zakrzewski and Nichol (1958) reported similar observations using folate as the substrate. In our laboratory, Mary Osborn and Melvin Freeman, a medical student (now a prominent ophthalmologist), using the preparation specific for dihydrofolate and NADPH and the spectrophotometric assay system that was amenable to kinetic measurements, determined the inhibitor constants for interaction of Methotrexate and aminopterin with the enzyme (Osborn et al., 1958). Subsequently, claims have been made for other enzymes as putative targets for Methotrexate, but it has become generally accepted that dihydrofolate reductase is the principal, and probably the only meaningful, target for the drug, and that its effect on cell replication is due largely to indirect inhibition of thymidylate synthesis.

Following these observations, my colleagues in our other laboratory at the King County Blood Bank in downtown Seattle (a troublesome commute for me from the Biochemistry Department in the Medical School) extended the work to leukemic leukocytes. Through the cooperation of Clement Finch and several other hematologists, we were able to obtain blood samples from a number of patients with various types of leukemia. Joe Bertino (now American Cancer Society Professor at Sloan-Kettering), who has continued to make major contributions to both clinical and basic aspects of folate-dependent enzymes and antifolates, in collaboration with Bob Silber (now Professor of Medicine at Duke University) and Beverly Gabrio, found that cells from leukemia patients contained measurable levels of dihydrofolate reductase and that it, too, was extremely sensitive to Methotrexate; the enzyme could not be detected in normal leukocytes (Bertino et al., 1960). Shortly thereafter, an important observation was made independently by Fischer and by Nichol and his colleagues, viz., that when cultured tumor cells were treated with sublethal amounts of Methotrexate, the level of the enzyme increased. Similarly, Friedkin and Goldin observed similar results when mice implanted with L1210 cells were treated with the drug. Our group made a detailed study of a patient with acute myelogenous leukemia undergoing treatment with Methotrexate and charted the progressively increasing level of dihydrofolate reductase that followed each course of therapy. Treatment of patients with other forms of leukemia produced the same kind of results, but levels of the enzyme were unchanged when other chemotherapeutic agents were employed (Bertino et al., 1962). An increase in the level of dihydrofolate reductase, resulting from treatment with Methotrexate, was the first of several mechanisms of resistance to this drug to be discovered.

Subsequently, we and others utilized this "induction" phenomenon to develop cell lines with increased levels of dihydrofolate reductase in order to make it available in quantity for purification procedures. Methotrexate-induced increase in level of the enzyme also provided Schimke with a model system that revealed the mechanism of gene amplification. Fluorescein-Methotrexate, developed by John Whiteley in our laboratory, enabled Schimke to label individual cells with elevated levels of the enzyme and allow them to be isolated by a cell-sorter.

Subsequent developments with dihydrofolate reductase

It is well beyond the scope of this article to continue with a detailed account of the explosion of work on dihydrofolate reductase after 1960. Various groups obtained highly purified preparations of the enzyme from a number of sources (reviewed by Gready, 1980; Blakely, 1984; Freisheim & Matthews, 1984). In our laboratory, the chicken liver enzyme was isolated by Chris Mathews, and the counterpart from a Methotrexate-resistant subline of *L. casei* was obtained by postdoctoral fellows Bruce Dunlap, Nigel Harding, Larry Gunderson, Jim Freisheim, and Fritz Otting. Purification of dihydrofolate reductases was greatly facilitated by the use of drug-resistant cells that overproduced the enzyme and by the introduction of affinity chromatography using immobilized Methotrexate.

The availability of pure dihydrofolate reductases opened the door to detailed structural studies. Amino acid sequences appeared – among the the first was the sequence of the *L. casei* enzyme obtained by Jim Freisheim at the University of Cincinnati. NMR spectroscopy measurements by Blakely and by Roberts provided considerable information about the interaction of specific amino acid residues with substrates and inhibitors. The 3-dimensional structure of the *L. casei* and *Escherichia coli* enzymes, obtained by Kraut and Matthews using X-ray crystallography, revealed the full beauty of the enzyme and helped to answer some previously puzzling questions, such as why Methotrexate is bound much more strongly than the folate substrates. Kinetic measurements by Morrison, Appleman and Blakely, and Benkovic produced a detailed picture of the catalytic mechanism. And finally, cloning and site-directed mutagenesis procedures have allowed the enzyme and mutant constructs to be produced in quantities far beyond even the most vivid imagination of investigators in the 1950s.

After 1960, although our interests began to shift from folate-dependent enzymes to B_{12}-dependent enzymes and to the transport of folate compounds, we made some contributions on multiple forms of dihydrofolate reductase, activation of the enzyme by various agents, and the formation of electrophoretically stable binary and ternary complexes of the enzyme with substrates and inhibitors; the latter work provided an experimental tool for the NMR and crystallographic studies. Most recently, we have introduced Methotrexate-α-peptides (e.g., MTX-Phe) as prodrugs that can be activated by carboxypeptidase/monoclonal antibody conjugates, thereby creating the possibility of treating tumors selectively with high local concentrations of the parent drug (Vitols et al., 1995).

The lessons learned

The Dihydrofolate Reductase story illustrates an important lesson in scientific research: a project that may appear limited in scope can expand to become a large program. Joining the crowd and working on trendy or "cutting-edge" projects is tempting, but young investigators should not overlook rewards that may result from pursuing, as Philip Handler once counseled, "whatever seems amusing." My colleagues and I followed this guideline, and we were not disappointed.

I am deeply grateful to the talented group of postdoctoral associates, graduate students, medical students, and research assistants, who have been responsible not only for producing the experimental results from our laboratory, but also for sharing in the formulation of the research plans. Some of these colleagues, who have been mentioned in this article, are portrayed in a picture (Fig. 5) of the speakers, chairpersons, and organizers of the symposium titled, "Critical Issues in Cancer Chemotherapy" (Weber, 1989).

Fig. 5. Speakers, chairpersons, and organizers of the Symposium on Critical Issues in Chemotherapy, held at The Scripps Research Institute in 1989. The lectures were published as Volume 29 of *Advances in Enzyme Regulation*, edited by George Weber. Bottom row (left to right): Edward Reich, Karin Vitols, Joseph Bertino, George Weber, Frank Huennekens, Bruce Chabner, George Hitchings, Kunio Yagi. Middle row: Bruce Dunlap, John Galivan, Dietrich Niethammer, Mary Osborn, Kenneth Harrap, Joseph Cory, Youssef Hatefi. Top row: John Whiteley, Frank Maley, Robert Jackson, David Matthews, Robert Silber, Roland Robins, Gary Henderson, James Freisheim. Photograph reproduced through the courtesy of Dr. George Weber and Pergamon Press.

On occasion, I have been asked what one should look for in selecting prospective coworkers. Aptitude for research is obviously important. To slightly paraphrase Edward Wilson, the Harvard entomologist who spoke to this point, "For the inept hunter, the woods are always empty" (Wilson, 1994). But equally important are the passion to do research and the imagination to plan research strategy. There is also an undefined factor—Richard Willstatter used to ask prospective co-workers "Haben Sie gluck?," and David Green expressed a similar thought, "There are winners and losers in research; look for the winners."

The setting for research is also important. Each of my career changes has involved a new and challenging enterprise, where I have been privileged to be associated with individuals noted for their collegiality and shared purpose. Research flourishes in that kind of atmosphere. I would urge young investigators, in seeking their first independent position, to give serious consideration to outposts where opportunities exist, not only to create new centers of research, but also to chart the course and guide the ship.

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