A Lifetime of Kinetics

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When Herb Tabor asked whether I would be willing to write a Reflections for the Journal of Biological Chemistry, I responded that I would be honored to do so. The majority of my early papers, after all, were published in the Journal, and I served for 10 years on the Editorial Board when 10 years was the maximum for such service. When Herb later asked whether I would take on another 5-year term, I reminded him of the agreed-to 10-year limit. For me, he said, they would make an exception. However I did not take the bait, and so this is my chance to make amends for turning him down. Fortunately, it will not take 5 years of work.

After graduating from Carleton College, I started graduate studies in the Department of Biochemistry at the University of Wisconsin. My advisor for that first year was Karl Paul Link, usually called K. P., who was a carbohydrate chemist. Link was both famous and eccentric: famous because he had discovered and isolated dicumarol as the active component of spoiled sweet clover hay that prevented blood from clotting. A synthetic dicumarol derivative became the active component of warfarin, which is still used today in both rats and people. In rats, it is a poison when eaten repeatedly. In people, warfarin (or Coumadin) is used to prevent blood clots. Link was eccentric for his willingness, almost eagerness, to argue with other faculty members and his rather unusual attire. In any case, after a few months of the biochemistry being taught at the time, I went to K. P. and said I was not really interested in nutrition (or carbohydrate chemistry) but rather in protein and enzyme structure. To my everlasting gratitude, Link contacted Robert (Bob) Alberty in the Chemistry Department and told him to accept me as a graduate student in chemistry. Bob felt compelled to acquiesce, as I later discovered, after being contacted by Professor Link, and so I joined and graduated from the Chemistry Department at the University of Wisconsin. Bob has written his own Reflections (1). At the time, one research project in the Alberty laboratory centered around enzyme kinetics and, in particular, the enzyme pig heart fumarase. I was assigned to that project. One could not buy the enzyme, of course, so trips to the meat packing company Oscar Mayer, located in Madison, were required to get pig hearts right off the line. Protein purification, as well as crystallization, was a hit-and-miss affair, but I was lucky enough to crystallize fumarase. I had left the purified protein dialyzing against 50% ammonium sulfate over a weekend. On Monday morning, there were the crystals, but I was disappointed to learn that when Vincent Massey spent a sabbatical in the lab, he had also crystallized the enzyme (2). Vince was a great colleague, and our studies on the pH dependence of fumarase kinetics were overlapping (3, 4). Bob Alberty was a wonderful mentor, supporting me through the rigors of chemistry and guiding me into the area of enzyme kinetics, which was to become my major interest for many years after leaving Wisconsin (Fig. 1).

The state of enzyme kinetics at that time (1951–1952) was not very well advanced. Certainly, the theory was there but essentially only for enzymes that involved a single substrate and single product. The rapid equilibrium derivation of the Michaelis-Menten equation was well known of course, and the steady-state derivations by Haldane had been published in 1930 (5), so the stage was set for more quantitative investigations. It is likely that World War II had delayed scientific progress on the development of enzyme kinetic theory.
I stopped work on fumarase after I left Wisconsin, but as I look back on the research I have done since my graduate school days, essentially all of it has been involved in kinetics of one sort or another. Some of these projects were undertaken after thoughtful consideration; others I just fell into. I am not sure which turned out better, but I remain convinced that kinetic processes, whether the rate of conformational changes within a macromolecule or of protein-protein interactions or of protein-ligand interactions or of cell-cell interactions, are what we need to study to figure out the process of life. After all, living systems do not exist at equilibrium. We can determine all the interactions that are present in the cell, but unless we know the time dependence of those interactions, we will never figure out how the cell works. It is a great challenge.

Bovine Liver Glutamate Dehydrogenase

In 1955, I moved to Washington University School of Medicine for a postdoctoral fellowship with Sidney Velick in the Biochemistry Department, chaired by the outstanding biochemist Carl Cori. During that time, Sid fundamentally left me alone, and I published three papers as the sole author (6–8), a rarity these days. After 2 years, Dr. Cori hired me and then asked what I was going to work on. Nowadays, it is the other way around. We expect faculty applicants to lay out in detail what they plan to do. Perhaps that is not such a great idea. In my case, I had searched the literature and thought I was interested in protein-protein interactions especially in an enzyme system. A perfect candidate appeared to be the bovine liver glutamate dehydrogenase (GDH). Other than the observation that it could use either NADH or NADPH as substrate (9), very little was known about the enzyme, and indeed, there were some early conflicting reports on the pH dependence and coenzyme specificity (10). H. J. Strecker in 1951 (11) and J. A. Olson and C. B. Anfinsen in 1952 (10) had, however, crystallized the enzyme from beef liver. Even by 1957, there were less than a dozen papers on the animal enzyme in the literature. And although Dr. Cori wondered why I did not want to work on his favorite enzyme, glycogen phosphorylase, he did not argue with me.

The choice of GDH turned out to be more fortuitous than I could have ever imagined. First, even in 1957, enzyme kinetics still dealt primarily with enzymes that involved only a single substrate. Indeed, most current text-
books do the same despite the fact that the great majority of enzymes use at least two, if not three or four, substrates. In the forward direction, GDH uses three: \( \alpha \)-ketoglutarate, ammonia, and either NADH or NADPH (called DPNH or TPNH in those days). I will mention later this unusual coenzyme specificity. With multiple substrates, enzymologists began to worry about whether the substrates were added sequentially or randomly to the enzyme. Deriving the equations, I found that the kinetic equations were different depending on how the three substrates were added and concluded that substrate addition for GDH was sequential (12). That conclusion has been challenged (13), probably correctly, but although important for characterization, I do not think this is an interesting issue any more. First, one can make very many complex kinetic mechanisms without much insight, but second, initial velocities do not define the in vivo situation in which both substrates and products exist.

My studies on GDH began in earnest in late 1957. After performing some initial experiments on the effect of cofactors on the sedimentation behavior (7, 14), I started the initial velocity studies. I decided to try to find some competitive inhibitors for the reaction. The most obvious choices were fragments of either NAD or NADP. On addition of ADP, however, the reaction went faster. That is not right. ADP should have been an inhibitor. The only way I felt that ADP could activate the reaction was if it were binding at a place other than the active site. That single observation started me on the path to look at the effect of a variety of purine nucleotides on enzymatic activity, and they were everywhere. Adenine nucleotides activated or inhibited depending on the conditions, guanine nucleotides inhibited, and the magnitude of the effects depended on whether one used NADH or NADPH as the substrate. Wow. This was incredible, and I published the results with adenine nucleotides in the Journal of Biological Chemistry in 1959 (15) and those with guanine nucleotides in 1962 (16). Jacques Monod must have noticed these papers. He had been invited to talk at Washington University, but his visit was very short: drive to the medical school, give a talk, and go back to the airport. He asked to see me, however, and as we walked to the parking lot, he invited me to spend a sabbatical in his laboratory at the Institut Pasteur. I immediately agreed. The sabbatical turned out to be quite short (just a few weeks) but a never to be forgotten experience. Jean-Pierre Changeux was working on threonine deaminase, reinvestigating the original finding (17) that the velocity of the reaction did not obey Michaelis-Menten kinetics but rather showed a sigmoidal dependence on substrate concentration. My first response to Jean-Pierre was that he was working with an impure enzyme and adding excess pyridoxal phosphate and why was he surprised, but I spent several weeks and used lots of paper writing out kinetic derivations involving multiple binding sites. I was all wrong, of course. This was the beginning of the concept of allosteric enzymes and the eventual and brilliant Monod, Wyman, and Changeux (MWC) theory of allosteric enzymes (18). Buzz Baldwin was in the Monod lab while the theory was being developed, and I do not think he ever got sufficient credit for his contribution (19).

After our initial studies, GDH became a rather popular enzyme to study. Some of this was because it seemed to be affected by a large number of compounds including sterols and also used a variety of substrates instead of glutamate. Gordon Tomkins was a leader in this field of hormone action. A brilliant biochemist, Gordon trumpeted the importance of GDH before his life was cut short after surgery at the age of 49.

I suppose GDH never became the model for an allosteric enzyme as did the later (20) example of aspartic transcarbamylase because the dependence of the initial velocity on substrate concentration followed Michaelis-Menten behavior rather than sigmoidal behavior. However, GDH is certainly an allosteric enzyme in the real sense of the word because ligands binding distant from the active site affect activity. At the same time as the MWC model was published, Dan Koshland and co-workers (21) published their theory of allosteric behavior (the KNF model) more along the lines that I had been thinking about. Meanwhile, as I continued to explore GDH kinetics, it was clear that NADH at high concentrations showed strong inhibition of the activity, whereas NADPH did not. Indeed, part of the reason for ADP activation was that it relieved NADH inhibition. While exploring the NADH inhibition, a student in the lab, Dave Bates, noticed and pointed out to me that the inhibition appeared to be time-dependent. That seemingly trivial observation got me thinking about two issues: if the velocity is time-dependent, then what is an initial velocity, and how could we analyze such behavior. The first of these issues led to the concept of hysteresis in enzymatic reactions, whereas the second led to the development of KinSim, our kinetic simulation program. Hysteresis, from the Greek, means to lag behind. I used it in the sense that the change in activity lagged behind the initial formation of the enzyme-ligand complex (22) and thus reflected a time-dependent conformational change. In the original 1970 paper I wrote on hysteresis, I presented an equation for this behavior: 

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P_t = v_f - (1/k')(v_f - v_0)(1 - e^{-kt})
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which represents product formation (assuming no significant substrate depletion) as
a function of time, where $v_0$ and $v_f$ are the initial and final velocities, respectively, and $k'$ is the apparent rate constant for the time-dependent conformational change. This equation has frequently been used by others (e.g. Ref. 23).

The hysteresis concept also seemed related to the issue of an apparent slow binding step, meaning that the apparent association rate constant for ligand binding might be well below that expected for a simple diffusion process. After the observation of inhibition of GDH by NADH, we noticed this behavior several years later in our study of AMP deaminase with the transition state analog (TSA) inhibitor deoxycoformycin (24). Curiously, a number of TSA inhibitors of various enzymes seem to show this behavior. Because TSA inhibitors usually bind with extremely high affinity, the question was how one interprets this slow binding. I interpret such data to mean that the TSA inhibitor initially binds rather poorly to the enzyme surface, followed by a slow conformational change that results in tight binding. And what does that mean? That the enzyme active site is not complementary to the transition state, as had been proposed, but rather to the substrate itself.

The original 1970 paper on hysteresis also contained a discussion of what slow processes might mean for metabolic pathways. Indeed, the title of the paper, “Kinetic Aspects of Regulation of Metabolic Processes: The Hysteretic Enzyme Concept,” clearly defined that I was thinking about how slow processes affected metabolism. We are nowhere near figuring this out, and until we do, we will not know how cells really work. I do consider this to be one of my most important contributions, and it was certainly novel for its time.

**KinSim**

The second issue raised by the finding of time-dependent processes was how to analyze them. Derivation of equations to describe the full time course of a reaction, especially multisubstrate reactions, was and still is horrendous. The equations are complex with numerous terms. As enzymologists know, no single equation can describe both the pre-steady-state and steady-state reactions because the first order differential equation has no explicit solution. Students coming into the lab in the early 1970s were just beginning to have experience in computer programming, and I was convinced that the only way to solve the problem was numerical integration of the differential equations. No such convenient program was available, so a progression of students attempted to write a program, in Fortran, that would be user friendly. Dave Bates undertook the problem and succeeded in writing a program for the only mainframe computer we had in 1973, a PDP-12/40 (25). The program was used to analyze the full time course of the GDH reaction (26). Bruce Barshop, an M.D./Ph.D. student, next undertook the problem as part of his Ph.D. work to make it more user friendly, and in collaboration with a computer expert, Rich Wrenn, he succeeded. The resulting program, KinSim for Kinetic Simulation, was published in 1983 (27) using the newer mainframe VAX computer. KinSim and its later cousin, FitSim (28), developed by Chris Zimmerle, were revelations. KinSim was a simple to use program that could describe any reaction under any set of conditions and compare the real data with the simulated data. It has been remarkably successful, and although many other programs have been developed over the intervening years, KinSim is still being used and has been ported from the mainframe VAX to desktop computers. That still amazes me because the code is at least 25 years old. One important aspect of KinSim and FitSim was that I provided the source code free of charge. Anyone could take it and change it if they wanted, and they have. It seemed to me at the time that because my research was supported by the National Institutes of Health (NIH), this was the right thing to do. Clearly, these days not everyone agrees with that position, but I have no regrets.

The usefulness of KinSim is as a tool to help one think about things that may be complicated. One of the important uses is to analyze the full time course of a reaction rather than just the initial velocity. There is, after all, a lot more kinetic information in the full time course. This has been vividly illustrated in the analysis of the kinetic mechanism of the *Escherichia coli* dihydrofolate reductase, which has been examined in my lab (29) and by Fierke et al. (30). This enzyme has a complex kinetic mechanism involving substrate-product complexes in such a way that there is almost never any free enzyme produced during the reaction. I suspect that may be true for many enzyme systems, but initial velocity studies will not reveal it. The use of the full time course for analysis of kinetic behavior is still not as fully exploited as it should be in my opinion.

GDH had another interesting property, which was that it polymerized. What the function of that polymerization might be is not clear, but Roberta Colman (my first post-doctoral student, who went on to do many excellent chemical modification studies using nucleotide analogs) showed that it was affected by purine nucleotides (31). Polymerization was to become a stronger interest in other later projects.

**The GDH Structure**

I stopped working with GDH because I could not crystallize it, and I felt that unless I knew the structure, there was little more I could find out. Now, the structure of the
bovine liver enzyme has been solved by Tom Smith and co-workers (32, 33), and it is a wonderful structure to behold. We had known that the protein was a hexamer, a dimer of trimers. What Smith found was that each subunit had an antenna and that the antennas from the trimers wound around each other. Furthermore, the antennas contained the binding sites for the allosteric effectors. That was particularly gratifying for us because we had done a large number of chemical modifications to identify the amino acid residues related to the allosteric properties (34–37), and there was excellent agreement between our studies and the final structure (33). One needs to remember that at the time we carried out the chemical modification studies, site-directed mutagenesis was not available. In those days, chemical modification was the only option, but not a very satisfying one.

**And One More Thing**

Mammalian GDH may be the only dehydrogenase that uses NADH and NADPH almost equally well. Even the prokaryotic enzymes use either one or the other but not both, and two different enzymes with different coenzyme specificity may exist in the same organism. Additionally, the prokaryotic enzymes do not show the same kind of allosteric behavior with respect to nucleotides as do the mammalian enzymes. Clearly, evolution had something in mind, but it is not clear what. Barry Goldin and I proposed that when using NAD or NADH, the enzyme functioned in a catabolic role, whereas when using NADP or NADPH, it functioned in an anabolic role, e.g. in the synthesis of fatty acids (38). We speculated that the single enzyme may perform very different functions in different tissues without the necessity of having two or more different enzymes. This issue has not been solved even today and remains a great problem for those interested in metabolomics.

**Life after GDH**

I have focused this paper on the early work with GDH and where it led, as befits a Reflections article. Many other projects have been subjects of our work, including kinetics of other regulatory enzymes and actin polymerization. Some 20 years ago, I became interested in a problem that had not been solved: the mechanism of protein folding. That involved incorporating fluorine-labeled amino acids into the protein and devising a method of doing stopped-flow $^{19}$F NMR (39, 40). Much has happened over the last 20 years, and we are getting closer to the answers. Recently, I undertook another yet unsolved problem: the mechanism of aggregation of proteins related to neurodegenerative diseases, the so-called intrinsically disordered proteins.

**Epilogue**

Life has been punctuated by periods of great good luck. I have learned to expect the unexpected or at least not to ignore it. I have been fortunate enough to meet and interact with many fantastic scientists. I have also been fortunate to have great students, many of whom I have not mentioned here because I was reflecting back on those early years. They all have taught me, and I apologize for not mentioning them.

As I approach my 80th year, I do so with undiminished enthusiasm for science. I am troubled, however, by what has happened to the NIH grant system. Not long ago, the success rate for competitive investigator-initiated grants was at least 30%. In the past several years, it has fallen dramatically for such grants and is lower now than I can ever remember in my almost 50 years of obtaining NIH funding support. This is bad for all scientists, especially young investigators, and for science in general. It will take years to recover. And that is shameful.

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**REFERENCES**