RECOLLECTIONS

Experiencing classical enzymology in its prime

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I started my scientific education with one serious handicap: at an early age, I showed signs of having more talent in art than the average youngster. And, unlike what happens with the natural artistic ability of the vast majority of these budding artists, mine persisted beyond childhood. In fact, to my great surprise, on the strength of this talent, I was admitted to the prestigious specialized High School of Music and Art in New York.

Ironically, this ability was a significant handicap for my ultimate career in science because it shunted me into a milieu where I was exposed to four years of outstanding daily studio art instruction, but where I took hardly any science courses. Consequently, on graduation from high school, I knew very little about biology and chemistry. I was also uncertain and confused about what my next step should be, because the experience of four years of mingling with kids of exceptional talent convinced me that I was not in the same class. Had I persisted in trying to become a professional artist, I probably would have experienced first hand what has been said about most people facing this sad situation—that an insufficient talent is the cruelest of all temptations.

The turning point for me came, as I understand it has come to many others who ultimately choose science as a career, by my reading in my senior year in high school deKruif's *Microbe Hunters* and Sinclair Lewis' novel *Arrowsmith*. Reading these

books was a kind of epiphany. I knew immediately not only that I wanted to become a scientist, but that I wanted to do some kind of biomedical research. Parenthetically, I should add that my love of art has never abated and, to this day, continues to be a source of great pleasure as a hobby.

Although I didn't learn much science in high school, I did learn one lesson of enduring importance, especially as I began to take more academic courses. This lesson was about taking examinations at school. I learned that the outcome largely is determined not by what you do during the actual test, but by what you do in preparation for it.

In a similar way, there can be no doubt that one of the critical determinants for having a successful career as a scientist is the right kind of preparation, which in this case means working with outstanding scientists. The other determinant is luck!

In my own case, this preparation took place in three stages. My main interest in science at this time was still unfocused. It fluctuated between organic chemistry and biochemistry and came to rest, tentatively, somewhere between the two. Having learned that the University of Illinois had an outstanding chemistry department, and that tuition was not too high, I decided that was the place to go.

As an undergraduate at Illinois, and later when I was working for my Masters degree, I took most of the courses offered by both the organic and biochemistry divisions. To my dismay, however, I found that many of the chemistry courses emphasized what seemed to me to be useless facts, e.g., the exact conditions of temperature and pressure under which a synthetic reaction had to be carried out. These courses were so boring that I found my interest in chemistry fading. I even began to have doubts about my choice of chemistry as a career. Fortunately, these doubts were entirely dispelled when, in my senior year, I was lucky enough to take Professor W.C. Rose's course on Intermediary Metabolism. This introduction to what appeared to me to be the magical world of enzymes rekindled my flagging interest and helped focus my attention on the areas of chemistry that I found to be challenging.

Also in my senior year, I was able to gain additional experience in synthetic chemistry that was not bound by the strictures of a formal course. From time to time, a few students who appeared to be both serious and financially strapped were given the chance to work part-time for the Department of Chemistry synthesizing a variety of organic compounds that were needed

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Seymour Kaufman, Chief of the Laboratory of Neurochemistry, National Institute of Mental Health, was born in Brooklyn, New York, and received his B.S. degree in chemistry in 1945 and his M.S. degree in chemistry in 1946 from the University of Illinois. He earned his Ph.D. in biochemistry from Duke University, under the guidance of Professor Hans Neurath. After his Ph.D., he worked in Professor Severo Ochoa's department (Pharmacology) at New York University Medical School for a period of five years, first as a postdoctoral fellow, then as an Instructor and Assistant Professor. In 1954, he accepted a position at the National Institute of Mental Health, National Institutes of Health, in the Laboratory of General and Comparative Biochemistry. During the period from 1968 to 1971, he served as Acting Chief, Laboratory of Neurochemistry, National Institute of Mental Health; he was appointed Chief of the laboratory in 1971.

Dr. Kaufman was elected to the National Academy of Sciences and the American Academy of Arts and Sciences in 1987. He received the Hillebrand Prize in 1991.

by various faculty members for their research. The students were paid by the hour for each completed project. This was a great arrangement for everyone. The faculty member got needed compounds for very little money, and the student benefited, not only by getting valuable experience in the synthesis of a wide variety of compounds, but also by substantially supplementing what, in my case, was a meager bank account.

This expanded investment in organic chemistry paid many future dividends. First, unlike today, when most of the compounds that may be needed for research can be purchased, during the period I am talking about—40–50 years ago—almost every biochemical that we now take for granted, even L-amino acids, had to be isolated after chemical synthesis. In addition, although I didn't know it at the time, this training would prove to be invaluable when some years later, during the course of my research, I had to solve a challenging structural organic chemistry problem.

At Illinois, I also got my first taste of experimental research while working for my Masters degree in the laboratory of Dr. Carl Vestling in the Biochemistry Division. The enthusiasm for biochemistry of this gifted teacher and researcher was infectious and largely accounts for the fact that my initiation into research in the area of intermediary metabolism was a memorable experience which, despite the modesty of my accomplishment, led to my first publication in 1946—in the *Journal of Biological Chemistry*, at that. As most scientists will testify, it is difficult to top the thrill of opening a journal and seeing one's first paper in print.

My extracurricular experience with organic chemistry proved, unexpectedly, to be pivotal in my choice of where I would do my graduate work. Dr. Hans Neurath, in the Biochemistry Department at Duke University, was in the early stage of a research project aimed at studying the mechanism of action of the pancreatic proteases and, for this work, synthetic peptides that could serve as substrates for these enzymes were needed. He contacted the University of Illinois to try to find one or more Ph.D. candidates who might be interested in this subject and who were willing and able to take on this phase of the work. The two undergraduates who were recommended because of their extended experience with organic chemistry were myself and John Snoke. We joined a small group that Neurath had assembled, consisting of another graduate student, Elaine Elkins, and, serving the role of lieutenant, Dr. George Schwert. This proved to be a congenial group: John became one of my best friends and I married Elaine.

Neurath's long-term goal was the study of the properties of an enzyme-substrate complex. Prior to tackling what in those days loomed as daunting task, he proposed a preliminary study of the properties of a complex of the enzyme with a substrate analogue that was a potent inhibitor. This project, however, was aborted by a totally unexpected finding. Earlier, M. Bergmann and his coworkers had reported that chymotrypsin and trypsin could catalyze the hydrolysis of simple synthetic peptides and amides of the aromatic and basic amino acids, respectively. There seemed to be a reasonable chance that the esters of the corresponding substrate amides might be inhibitors of these enzymes. John and I prepared, among other esters, benzoyl-L-arginine methyl ester (BAME), as a potential inhibitor of trypsin, and benzoyl-L-tyrosine ethyl ester, as a potential inhibitor of chymotrypsin. George isolated pure crystalline trypsin and chymotrypsin.

Purely by chance, the first enzyme selected for study was trypsin. Because BAME had been synthesized as the hydrochloride, it had to be neutralized prior to testing it as a possible inhibitor. Accordingly, George proceeded to neutralize with alkali a solution of the benzoylarginine ester. We were both puzzled by what happened next. As alkali was added to the solution to bring it to neutrality, the needle on the pH meter rapidly drifted down indicating an acid pH. This drift happened again after another addition of alkali. Our first thought was that the pH meter was broken. Our second thought was that the ester was being hydrolyzed. This latter possibility, however, seemed unlikely because I knew that these esters were not that unstable. George then realized what was happening. Before we had started to neutralize the ester, he had neutralized the trypsin solution that was to be used in our experiment. And although he had carefully rinsed the glass electrode, a trace of the trypsin solution had remained and the residual amount of enzyme was catalyzing the hydrolysis of the arginine ester. We had accidentally discovered that trypsin can catalyze the rapid hydrolysis of ester analogues of its amide substrate. Very quickly, we showed that chymotrypsin and carboxypeptidase shared the ability. The original plan to study enzyme-inhibitor complexes was scrapped and we were each assigned the study of the esterase activity of one of the proteases for our thesis research. My assignment was chymotrypsin.

At the time when we stumbled upon the esterase activity of these proteases, it was a big deal. Enzymes were, after all, supposed to be specific. Now we know that the specificity of enzymes is relative. As I recall, if one uses enough chymotrypsin, it can even effect the splitting of a suitably placed carbon-carbon bond.

My three-plus years working in Hans' laboratory, including about eight months as a postdoctoral fellow, firmly consolidated my interest in enzymes, an interest that has never wavered. Under his tutelage, I was introduced to many aspects of physical biochemistry that turned out to be extremely useful in my later research. Of particular value was learning the principles that determine the solubility of proteins. As it turned out, I frequently had to face the problem of separating the individual components of a complex enzyme system and it was helpful to be able to go beyond a purely empirical approach to this kind of problem. From Hans, I also got a glimpse of the unique insights into enzyme mechanisms that thermodynamics and kinetics can provide. These insights also proved to be useful.

Based on my Ph.D. thesis research, I was offered a faculty position at the new Department of Biophysics at the University of Illinois. I was so dazzled and flattered by the offer that I almost made the mistake of accepting it. It would have been a mistake because at this stage, I was suffering from a serious case of tunnel vision. Research in enzymology meant research with proteolytic enzymes. It was all that I knew. Luckily, on the unanimous advice of my elders, I turned down this offer and, instead, accepted a postdoctoral position with Professor Severo Ochoa in the Department of Pharmacology at New York University (NYU) Medical School.

In this department, I was immediately plunged into a different, more dynamic world of enzymology—the fiercely competitive area of intermediary metabolism, specifically the enzymes of the citric acid cycle, glycolysis, fatty acid oxidation, the hydrogen-transport system, and CO₂ fixation. One got the feeling that every one of these important problems was ripe and that they would all be cracked within the next few years.

Fig. 1. Some members of Severo Ochoa's department (Pharmacology) at NYU Medical School in the early 1950s. From left to right: S. Ochoa, F. Lynen, D. Nachmansohn (colleague from Columbia University), S. Kaufman, A. deCampillo, J. Stern, and S. Ratner. (The seated woman is one of the technicians.)

Ochoa's department was larger than Neurath's, consisting of two junior faculty members (Seymour Korkes and Joe Stern) and two postdoctoral fellows (Giulio Cantoni and myself). In addition, there were two more senior members of the department, Sarah Ratner, already on her way to a distinguished, independent career, and Richard de Bodo, the only authentic pharmacologist. Ephriam Racker, from the Department of Microbiology at NYU, was a *de facto* member of the group. In addition, David Nachmansohn (from Columbia University), a colleague of Severo's since the time when they were both postdoctoral fellows in Meyerhof's laboratory in Germany, was a frequent visitor. The Nobel laureate Otto Loewi was an emeritus professor, but he occupied a cubby hole of an office located on another floor. There were no graduate students.

The department was organized along the lines of the great German universities; with few exceptions, everyone worked directly on Ochoa's research problems. Before coming to NYU, I had expected to find large teams working on a few different problems. That is not how it was. Rather, each fellow worked on a separate, but related project, most of them focused on enzymes that catalyze reactions of the citric acid cycle or those that feed into the cycle.

I had arrived at NYU worn out from the unrelenting pace involved in winding up my thesis research, writing the thesis, and finally defending it. In fact, I had been hoping that I might be able to catch my breath for a short period at NYU. I was, however, quickly disabused of such an idea. At the end of my first day in Ochoa's department, tired from the strain of coping with a totally unfamiliar setting, I was packing up some reprints for later reading when Korkes wandered by and asked what I was doing. I mentioned that I was leaving for home. He indicated that nobody left at this hour—it was 5:30 PM. Rather, this was the time when everyone—Severo and all the fellows—went down to the cafeteria for a cup of coffee and a snack, returning to the

laboratory to work until around 8:00 PM. Although the ostensible reason for this schedule was to avoid the awful evening rush-hour traffic, at least as compelling a reason was that it conformed with Severo's desire that all the fellows put in a maximum effort.

Initially, my research project consisted of the narrow task of separating lactic dehydrogenase from the partially purified preparations of malic enzyme. The contaminating dehydrogenase had blocked all attempts to determine whether pyruvate was an intermediate in the conversion of malate to lactate and CO_2 .

Because today's powerful protein fractionation techniques, such as ion exchange and sephadex chromatography, had not yet been developed, the armamentarium available for the purification of proteins was skimpy: fractionation with salt and organic solvents and adsorption to and elution from a variety of gels such as calcium phosphate and alumina C γ . I succeeded in effecting enough of a separation of the two enzymes to show that pyruvate is not a free intermediate in the reaction.

Despite this success and the technical challenges that this work presented, this problem never appealed to me. It had been well worked over by others before I was given a crack at it and by the time I started, it seemed stale. During one of Severo's frequent extended trips abroad, therefore, I conducted some preliminary experiments with the citric acid cycle enzyme responsible for the oxidative decarboxylation of α -ketoglutarate to succinate and CO_2 . In all previous unsuccessful attempts to isolate this enzyme, its activity in tissue extracts was assayed by following oxygen uptake. This assay demanded not only the solubilization of " α -ketoglutarate oxidase," but also the heroic job of solubilization of the whole hydrogen transport system. Instead, I decided to use an assay that was independent of the hydrogen transport system. It was based on an anaerobic dismutation reaction that had just been described by Hunter, in which the NAD^+ -dependent oxidative decarboxylation of α -ketoglutarate to CO_2

and succinate, catalyzed by α -ketoglutarate dehydrogenase, is coupled to the NADH-dependent reduction of a second molecule of α -ketoglutarate (in the presence of NH_3) to glutamate, a reaction catalyzed by glutamate dehydrogenase. The first time I tested an extract of pig heart fortified with a "kochsaft" (boiled tissue extract) to supply any coenzymes that might be needed in the reaction, I was astonished that the experiment worked; most of the added α -ketoglutarate had disappeared with robust formation of succinate and CO_2 . When Severo returned from his trip and learned about these results, he enthusiastically endorsed this research project.

Within two months of this initial experiment, it was possible to show that the oxidation of ketoglutarate was totally dependent on CoA and that one of the products was succinyl CoA, a compound that had not been described previously. Up to this point, the oxidation of ketoglutarate proved to be analogous to the recently reported oxidation of pyruvate to acetyl CoA. A key difference was that the oxidation of α -ketoglutarate was known to be coupled to phosphorylation (the so called "substrate level phosphorylation"). I was able to show that in the presence of ADP, the oxidation of α -ketoglutarate was coupled to the formation of ATP. Furthermore, the partially purified enzyme fraction that I was using also catalyzed the reverse reaction, where succinyl CoA is synthesized from succinate, ATP, and CoA. Because succinyl CoA reacts nonenzymatically with hydroxylamine to form the easily measured hydroxamic acid, this reverse reaction formed the basis for a convenient assay of the phosphorylation reaction that was independent of the oxidation of α -ketoglutarate. A further component of this system was also present in the heart fractions that I was using. This was an enzyme capable of hydrolyzing succinyl CoA, later designated succinyl CoA deacylase. Its activity competed with the phosphorylating enzyme (which we called the "P enzyme" until it was designated "succinic thiokinase"), and could therefore decrease the efficiency of ATP formation coupled to the oxidation of α -ketoglutarate.

We announced our results at the 1951 McCollum-Pratt Symposium on Phosphorus Metabolism. Presenting a lecture before this group of the leading scientists in this field, a group including, among others, the past and future Nobel Prize winners, O. Meyerhof, F. Lipmann, L. Leloir, E. Sutherland, A. Kornberg and, of course, Ochoa, was a heady experience for me. It was like being given a three day pass to Camelot.

After the meeting, when my feet once again touched the ground, we tried to assess where this project stood. It was clear that the system was a multicomponent one, consisting of at least three enzymes, α -ketoglutarate dehydrogenase, succinic thiokinase, and succinyl CoA deacylase. Subsequently, Green and his colleagues showed that with the heart system, GDP (or IDP), rather than ADP, was the primary phosphate receptor for the P enzyme and that another enzyme, nucleoside diphosphokinase, converts the resulting GTP (or ITP) to ATP; we and others showed that for the P enzyme from spinach and *Escherichia coli*, ADP was the primary acceptor.

It was also clear that given this level of complexity and the growing signs of intense competition from other laboratories, that I, working by myself, would be unable to remain competitive for long. Fortunately, just when my concerns over the future course of our research were mounting (and my ability to fall asleep was decreasing), Charles Gilvarg, a new postdoctoral associate in the laboratory, was assigned to work with me on the

α -ketoglutarate-P enzyme project. He proved to be an ideal colleague, extremely bright, with excellent training in both enzymology and bioorganic chemistry. Together, we succeeded in further purifying and characterizing all of the enzymes that I had first described at the phosphorus symposium. When this phase of the work was completed, I turned my attention to studies of the mechanism of the reaction catalyzed by the P enzyme and, working with another of Severo's several postdoctoral associates, S. Alivisatos, and using the P enzyme from spinach, we were able to show, among other things, that a phosphorylated enzyme is an intermediate in the reaction.

The time I spent working in Severo's laboratory, first as a postdoctoral fellow, and finally as an Assistant Professor, were memorable. During this period, many gaps in my earlier training were filled. Indeed, had they not been filled, they undoubtedly would have come back to haunt me later on. To mention only one of these, Severo was a virtuoso of coupled spectrophotometric assays of oxidative enzymes. Often, the reaction being studied would be coupled to as many as three or four other enzyme-catalyzed reactions until, at the end of the chain, an NAD- or NADP-dependent reaction in which the pyridine nucleotide was either oxidized or reduced could provide the basis for a continuous spectrophotometric assay of the activity of the enzyme in question. This experience with assays of this type would prove to be invaluable.

In addition to learning many useful specialized laboratory techniques that Severo had "inherited" from his mentors (e.g., Meyerhof)—such as the proper way to make a "kochsaft" and how to prepare an acetone powder of a tissue (which often could solubilize a protein)—there was a powerful synergy at work in the laboratory: Severo's growing reputation attracted a steady stream of scientists of renown such as F. Lynen, I. Gunsalus, T. Stadtman, and Bill Slater. Moreover, the caliber of the postdoctoral fellows who came to work with Severo was, in general, very high. Besides those already mentioned, there were people like Irnie Rose, Martin Flavin, and Marianne Grunberg-Manago, all of whom went on to achieve eminence. Marianne's work, in particular, contributed importantly to the research that later led to Severo's winning the Nobel Prize. These scientists enriched the experience of everyone in the department.

Also contributing to the rich experience was the scientific ambience of New York City. There were regularly scheduled meetings and evening lectures of the Harvey Society and, what was probably unique in its time, The Enzyme Club. These monthly get-togethers at the Enzyme Club featured a guest lecturer, usually a distinguished visitor from out of town or from abroad. The lecture was preceded by a cocktail hour and was presented while dinner was served. Faculty members from most of the local universities—NYU, Columbia University, City College, Cornell University Medical College, and Rockefeller attended, as well as a large contingent from Yale University. In addition to these formal meetings, there was a full schedule of daily scientific lectures at the local universities.

The discussion of this experience would be incomplete without some comment about some of Severo's personal traits. He shared with all of my other mentors great wisdom and intuition. In addition, he had some noteworthy characteristics which, although not unique, were more pronounced in him than in any other person whom I have met. He was fiercely competitive. Undoubtedly, this trait, combined with his attributes, contributed to his great success as a scientist. The downside of his competi-

tiveness was that he conveyed it to his postdoctoral fellows. As a result, of all the things that I experienced in Severo's laboratory, peace of mind was not one of them.

Closely related to Severo's competitiveness was his unusual ability to focus with great intensity—like a powerful burning glass—on his research. Always on the lookout for ways to eliminate distractions from his life (he had no children), he declared one day at lunch, for example, that he had decided to stop going to concerts and to give up reading the *New York Times* because these activities took too much time away from his work.

After spending almost five years in Severo's laboratory, I was anxious to begin my independent career. In fact, at this point I was beginning to wonder if all of these training years working as a postdoctoral associate were not, as Yeats said of life, an immense preparation for something that never happens.

In 1954, Giulio Cantoni, who had come to Severo's laboratory around the same time I had come, and who had since moved on to Western Reserve University, had recently accepted a position at the National Institute of Mental Health (NIMH) as head of the newly created Laboratory of Cellular Pharmacology. He offered me a staff position as an independent scientist in his laboratory.

When I arrived in Bethesda, I was dismayed to learn that the new laboratory existed only on paper and that it would be many months before construction would be completed. At first this delay struck me as an unqualified disaster. It proved to be, however, a fortunate development. It forced me to be deliberate in making one of the most important decisions faced by any young scientist—the choice of a first research project.

Although our laboratory was part of the basic research program at NIMH and the director of intramural research, Dr. S. Kety, had assured Giulio and myself that our research did not have to relate to the nervous system, I nonetheless tried to formulate a research problem that would have the features of a double-croctic, a variety of crossword puzzle where the solution in one dimension automatically leads to the solution in a second dimension. Adapted to my situation, that meant the selection of a basic problem that, if solved, would help advance our understanding of a clinical problem, ideally, one involving the brain.

From my years working on the α -ketoglutarate oxidizing system, I had developed and retained a keen interest in the mechanism of action of oxidative enzymes. After months of reading about oxidative enzymes, especially those that were not well understood, I decided to study the enzyme responsible for the conversion of phenylalanine to tyrosine. It certainly fit the criterion of being poorly understood. The mechanism of this oxidative

reaction was so obscure that it could not even be written as a simple balanced equation. The other criterion was also met because it was known that the inherited inability to convert phenylalanine to tyrosine was the cause of the disease phenylketonuria (PKU), a condition that leads to severe mental retardation.

Starting with extracts of rat liver, a tissue rich in this hydroxylation activity, I ultimately isolated from the extract four components that were essential for the conversion of phenylalanine to tyrosine, a nonprotein coenzyme and three enzymes. I identified the coenzyme as tetrahydrobiopterin (BH_4), a compound without any previously known metabolic function that had not been known to occur in mammalian tissues. One of the enzymes was the one that actually carries out the hydroxylation reaction, phenylalanine hydroxylase, whereas the other two proved to be ancillary enzymes necessary for the NADH-mediated regeneration of BH_4 which undergoes oxidation during the hydroxylation reaction. The discovery of BH_4 as the hydroxylation coenzyme (it is also an essential component of the tyrosine hydroxylase and tryptophan hydroxylase-hydroxylating systems, as well as of nitric oxide synthase) opened up a new field of enzymology.

As for the second dimension of the double-croctic puzzle, the successful fractionation of the hydroxylating system into its separate components was followed by our conclusive demonstration that the missing component in the classical form of PKU is phenylalanine hydroxylase. Some years later, after I had been appointed as the Chief of the Laboratory of Neurochemistry, we and others described less common, but often lethal variant forms of the disease, each traced to the lack of one of the other essential components of the hydroxylation system. Following this work, we helped develop methods of differential diagnosis and treatment of all of the variant forms of the disease (an effective therapy for classical PKU had been developed long before these variants had been discovered). The fact that more than 100 children with these lethal variant forms of PKU are alive today as a result of this basic research, and the consequent development by us and others of effective treatments, has been enormously rewarding.

I have mentioned how crucial adequate training is. Some prior experience in organic chemistry was critical to the successful determination, over a four- to five-year effort, of the structure of BH_4 . Also invaluable was my earlier experience in fractionating a complex multicomponent enzyme system into its separate parts.

I am well aware of how fortunate I was to have had the chance to experience the excitement of working in the area of classical enzymology before this term became a perjorative one.