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NEVER A DULL ENZYME

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GROWING UP WITHOUT SCIENCE (1918–1942)

The current generation of scientists may be suprised to know that I had no formal research training. I was well started in a career of clinical medicine until World War II placed me in the National Institutes of Health (NIH) where I soon became an eager investigator of rat nutrition. Three years later, in 1945, I responded to the lure of enzymes and have remained faithful to them ever since.

Science was unknown in my family and circle of friends. Once, in 1947,

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when I was in the biochemistry department of Washington University in St. Louis, working under the guidance of Carl and Gerty Cori, Gerty told me that Carl had collected beetles and butterflies in his youth, and then asked: "Arthur, what did you collect?" "Matchbook covers," was my sheepish response. What else? They were the dominant flora in the Brooklyn streets where I played and in the subways where my father often risked being trampled when he stooped to add one more to my collection.

My early education in grade school and Abraham Lincoln High School in Brooklyn was distinguished only by "skipping" a few grades and finishing three years ahead of schedule. I recall nothing inspirational from teachers or courses except encouragement to get good marks. I remember the glow of my chemistry teacher when I received a grade of 100 in New York State Regents examination. It was the first time, in more than twenty years of teaching, a student of his had gotten a perfect grade. Once when I boasted about this to my wife, Sylvy, she remarked that she too had gotten 100, not only in chemistry, but also in algebra and geometry.

I chose the cachet of City College in uptown Manhattan over nearby Brooklyn College, even though commuting from Bath Beach (near Coney Island) meant three hours a day in crowded subways. Competition among a large body of bright and highly motivated students was fierce in all subjects. I carried over my high school interest in chemistry, but the prospects for employment in college teaching or industry were dismal. For lack of graduate studies or research laboratories at City College then, these possibilities barely existed. At age 19 in 1937, with a Bachelor of Science degree, and no jobs to be had in the depths of the Great Depression, I welcomed the haven that medical school would provide for four more years.

Throughout college I worked evenings, weekends, and school holidays as a salesman in men's furnishings stores. This left little time for study or sleep and none for leisure. With these earnings, a New York State Regents Scholarship of $100 a year, no college tuition, and frugal living, I saved enough to see myself through the first half of medical school at the University of Rochester.

I enjoyed medical school and the training to become a doctor. Among my courses, biochemistry seemed rather dull. The descriptive emphasis on the constituents of tissues, blood, and urine reflected biochemistry in the United States in the 1930s. The dynamism of cellular energy exchanges and macromolecules was still unknown, and the importance of enzymes had not penetrated my course or textbook. By contrast, anatomy and physiology presented integrated and awesome structures and functions. The aberrations presented in pathology and bacteriology were absorbing, as were the responsibilities to diagnose and treat patients during the clinical years.

Did I as a medical student consider a career in research? Not really. I

expected to practice internal medicine, preferably in an academic setting; the idea of spending a significant fraction of my future days in the laboratory had no appeal. The medical school of the University of Rochester granted some students fellowships to take a year out for research. I had hoped but failed to get such an award from any of the departments. In those years, ethnic and religious barriers were formidable, even within the enlightened circle of academic science.

I did some research on my own, which grew out of curiosity about jaundice. I had noticed a slightly yellow discoloration of the whites of my eyes, and found that my blood bilirubin level was elevated and my tolerance to injected bilirubin reduced. I made similar measurements on as many medical students and patients as I could. I collected samples at odd moments and did the analyses on a borrowed bench, late at night and on weekends. The report I published (1) called attention to the frequent occurrence of high bilirubin levels and reduced capacity to eliminate bilirubin, now recognized as signs of the benign familial trait called Gilbert's Disease.

Looking back, I realized that I enjoyed collecting data. I kept on collecting bilirubin measurements during my internship year and started setting up to do more analyses in the small sickbay of a Navy ship soon after I joined it. A lucky consequence was that the publication of my student work on jaundice attracted attention and led to my transfer from sea duty to do research at the NIH, a rare assignment at that time.

JOINING THE VITAMIN HUNTERS (1942–1945)

The Nutrition Laboratory at NIH to which I was assigned in the fall of 1942 as a commissioned officer in the U. S. Public Health Service had been started by Joseph Goldberger (1874–1929). He was among the first to recognize that a vitamin deficiency can cause an epidemic disease, and in tracking the missing vitamin in the diets of pellagra patients, he emerged as one of the greatest of the vitamin hunters. W. H. (Henry) Sebrell, whom he had trained, was now chief of the laboratory and my senior boss. The laboratory had moved in 1938 from downtown Washington to suburban Bethesda, Maryland, but some of Goldberger's animal caretakers, kitchen staff, and diet notebooks, as well as his aura were still around.

My initial project as a nutritionist was to find out why rats fed a purified ("synthetic") diet containing a sulfa drug developed a severe blood disorder in a few weeks and died. A stock animal ration or inclusion of a yeast or liver supplement in the purified diet was effective in preventing and curing the disease (2). After other vitamin hunters (3) with the use of a microbial assay had succeeded in isolating folic acid and made it available to us, we could

show that an induced deficiency of this vitamin was responsible for the sulfa drug effect.

It seemed clear that sulfa drugs, as analogues of para-aminobenzoic acid (PABA), a component of folic acid, were preventing bacteria from synthesizing this essential constituent and thus preventing their growth. We also knew that many animals rely on their intestinal bacteria for an adequate supply of folic acid and other vitamins, including vitamin K. I was therefore puzzled by a report (4) that PABA prevented the sulfa drug from producing a vitamin K deficiency even when given by injection. This result was taken to mean that the sulfa drug was not exerting its toxic effect on the intestinal bacteria, but somewhere else in the body.

I repeated the experiments with PABA injections and sulfa drugs, and developed a method to measure the amounts of vitamin K and PABA in the intestinal contents and feces. Ample quantities of vitamin K were produced by the intestinal bacteria of rats on a purified diet and this production was eliminated by sulfa drugs. As for rats injected with PABA, high levels of this substance accumulated in the intestinal contents, amounts sufficient to offset the action of the sulfa drug taken in the diet. These findings were reported in my first contribution to the *Journal of Biological Chemistry* (5), one of a very few biochemical papers from the NIH.

With the isolation of folic acid, it was apparent that virtually all the vitamins had been discovered. But we did not understand what most of the vitamins did in the body. How was folic acid serving in the growth of blood cells? What clues did the structure of folic acid offer to understanding its precise metabolic function? Could this understanding explain why sulfa drugs kill bacteria but not animal cells?

The answers to these questions, as well as to similar questions about the functions of the other vitamins, would be answered in the next two decades by enzymology. Just as the microbe hunters, who led the way in the first two decades of this century, were succeeded in the 1920s and 1930s by the vitamin hunters, so the latter would be overrun in the next two decades by the enzyme hunters.

I had come to nutrition in its twilight, decades late for the excitement and adventures of the early vitamin hunters who had solved the riddles of diseases that had plagued the world for centuries. My envy of their exploits impelled me to search for a new frontier. The discoveries of each of the vitamins—nicotinic acid, riboflavin, and thiamine in intermediary metabolism, and folic acid in nucleotide biosynthesis—became part of my heritage as I went on to learn about their biochemical functions. The rush to biochemistry depopulated the ranks of nutrition. How tragic that diet remains to this day as controversial as politics and the science of nutrition is in disarray.

FROM RATS TO ENZYMES (1945–1947)

By 1945, with the war over, I had become bored with feeding rats variations of purified diets. I was excited reading for the first time about enzymes, coenzymes, and ATP, in papers by Otto Warburg, Otto Meyerhof, Carl Cori, Herman Kalckar, and Fritz Lipmann. I had learned nothing about these things or people in medical school. While at NIH, I was startled and fascinated by a seminar in which Edward Tatum described his and George Beadle's work with *Neurospora* mutants and their one gene-one enzyme hypothesis. I knew even less about genetics than about biochemistry.

Fortunately, I was able to persuade Dr. Sebrell to let me quit my nutritional work and go to a laboratory where I could learn about ATP and enzymes. Immediately, I apprenticed myself to Bernard Horecker, a friend at NIH, who had been studying effects of DDT on cockroaches and was returning to the subject of his doctoral dissertation, the cytochromes of cellular respiration. Bernie introduced me to succinoxidase, cytochrome c, and the Beckman Model DU spectrometer. The unsolved problem of oxidative phosphorylation seemed to me to be the most important thing to do in biochemistry.

While still in uniform, I spent the year 1946 with Severo Ochoa at New York University Medical School; it was one of the happiest and most exhilarating in my life. Never had my learning curve been so sharply exponential and sustained. And in the few waking hours outside the laboratory, Sylvy and I discovered the theater, music, and museums that are the heartthrob of New York. Despite my being a native of Brooklyn and having attended City College in uptown Manhattan, and despite Sylvy's many visits from Rochester where she grew up and studied biochemistry, we were strangers to the city.

My mission from Ochoa was to purify heart muscle aconitase. This was my first solo stab at enzyme purification. We expected to resolve the activity into two enzymes to account for the successive subtraction and readdition of a water molecule that converts citric to isocitric acid. Despite repeated failure (aconitase proved to be one enzyme), this immersion in enzymology was intoxicating. Aside from the fascination of seeing an enzyme in action, the pace of the experimental work was breathtaking. By coupling aconitase action to isocitrate dehydrogenase, spectrophotometric assays could be performed in a few minutes, and many ideas could be tested and discarded in the course of a day. Late evenings were occupied preparing a series of protocols for the following day. What a contrast with the tedious pace of nutritional experiments on rats.

In my work on aconitase, I learned the philosophy and practice of enzyme purification. To attain the goal of a pure protein, the notebook record of an

enzyme purification should withstand the scrutiny of an auditor or bank examiner. Not that I ever regarded the enterprise as a business or banking operation. Rather, it often seemed like the ascent of an uncharted mountain: the logistics resembled supplying successively higher base camps; protein fatalities and confusing contaminants resembled the adventure of unexpected storms and hardships. Gratifying views along the way fed the anticipation of what would be seen from the top. The ultimate reward of a pure enzyme was tantamount to the unobstructed and commanding view from the summit. Beyond the grand vista and thrill of being there first, there was no need for descent, but rather the prospect of ascending even more inviting mountains, each with the promise of even grander views.

I was luckier in my second attempt at enzyme purification when I joined Ochoa and Alan Mehler, his first graduate student, in purifying the liver malic enzyme, the enzyme that converts malic to lactic acid (6). Mehler was already on the scene when I arrived in Ochoa's lab and became my indefatigable and devoted tutor. Having always been the youngest in my class, it was a shock to find that I was so far behind someone four years my junior.

To let me pursue my training and the problem of aerobic phosphorylation, the NIH extended my stay with Ochoa to a full year, and allowed me another six months in the laboratory of Carl and Gerty Cori at the Washington University Medical School in St. Louis. Right after the war, the Cori laboratory was the mecca of enzymology. There I joined a young Swedish visitor, Olov Lindberg, who was investigating a striking observation made six years earlier by Ochoa when he worked in the Cori laboratory. Liver particles metabolizing pyruvic and related acids produced inorganic pyrophosphate (PP), a compound previously unknown as a cellular constituent. We began by ruling out the possibility that PP was released from an unstable form of ATP, but then found little else to guide us.

Later, while trying to enhance the levels of respiration and coupled aerobic phosphorylation by kidney particles, we observed a strong stimulation by NAD, and discovered that the effect could be traced to AMP generated by its hydrolysis.

$$\underset{\text{(NAD)}}{\text{Nicotinamide-ribose-P-P-ribose-adenine}} + H_2O \rightarrow \underset{\text{(NicRP)}}{\text{Nicotinamide-ribose-P}} + \text{AMP} \qquad 1.$$

The AMP produced by NAD cleavage stimulated the reaction because it served as an acceptor of inorganic phosphate to form ATP. This mundane result marked the end of my search for the source of ATP in aerobic phosphorylation. The search had been doomed from the start because I was committed to finding discrete soluble enzymes that linked the synthesis of

ATP to respiration. As the late Albert Lehninger recognized a few years later, these enzymes are firmly embedded in mitochondria.

MY ROOKIE YEAR (1948)

Nineteen forty-eight, the year I set up my own biochemistry lab, was a great year for me. When I returned to the NIH, my former laboratory space in the Nutrition Division (in Building 4) was occupied. Just about then, one of the frequent organizational convulsions in the Industrial Hygiene Division (in Building 2) threatened Bernie Horecker and Leon Heppel, a close friend and medical school classmate, with a transfer to Cincinnati. Fortunately, Henry Sebrell agreed to let me start an Enzyme Section that would include the three of us in a few laboratory rooms in Building 3. (Considering the present mammoth size of the NIH, covering 300 acres and employing 13,000 people, it is hard to believe that in 1947 there were only six small buildings and that the research emphasis was still on infectious disease, dominated by a small corps of commissioned medical officers.)

I continued the work on the rabbit kidney enzyme that Lindberg and I had discovered in St. Louis and established that it cleaves NAD at the pyrophosphate linkage (7). However, the enzyme was firmly attached to tissue particles and there was little hope of obtaining it in pure form. At the suggestion of the late Sidney Colowick and Oliver Lowry, I looked for and found a similar enzyme activity in potatoes, from which it could readily be extracted in a free, soluble form (8).

The purified enzyme cleaved not only NAD, but all nucleotides with a pyrophosphate bond. I called the enzyme *nucleotide pyrophosphatase*. By using the enzyme to cleave NADP, I could show that the position of the extra phosphate, then unknown, was part of the AMP moiety on carbon 2 of the ribose. Best of all, having isolated NicRP from NAD cleavage, I wondered whether it might serve in the synthesis of NAD. It did! Enzymes purified from yeast and liver condensed NicRP and ATP to produce not only NAD, but PP as well, the first clue to the origin of PP after years of speculation. The reaction was readily reversible and could support a vigorous exchange of PP with ATP (9).

$$\underset{}{\text{NicRP}} + \underset{(\text{ATP})}{\text{PPPRA}} \rightleftarrows \underset{}{\text{NicRPPRA}} + \underset{(\text{NAD})}{\text{PP}} \qquad 2.$$

This mechanism immediately led us to the discovery of the enzyme that synthesizes flavin adenine dinucleotide (FAD) from riboflavin phosphate and ATP (10). In the ensuing years, the mechanism of nucleotidyl transfer from a

nucleoside triphosphate for the biosynthesis of coenzymes was discovered again and again in the biosynthesis of proteins, lipids, carbohydrates, and nucleic acids. A variety of phosphoric, carboxylic, and sulfuric acids (XO^-) accept a nucleotidyl group from a nucleoside triphosphate (PPPRN) to generate an activated form of XO^- with the release of PP.

$$XO^- + PPPRN \rightleftarrows XO-PRN + PP \xrightarrow{} 2\ Pi \qquad 3.$$

Hydrolysis of PP by a strong and ubiquitous inorganic pyrophosphatase drives these reversible condensations toward biosynthesis (11).

What a wondrous enzyme, the humble potato pyrophosphatase! It helped solve an aspect of NADP structure, set up the discovery of coenzyme biosynthesis, and with it a major theme in biochemistry, and then led me on to the enzymes that assemble DNA, genes, and chromosomes.

OROTIC ACID IS ON THE MAIN TRACK (1953–1955)

In 1955, two years after the historic Watson and Crick reports (12) of the double helix and its implications for replication, I found an enzyme that synthesizes DNA chains from simple building blocks. Based on this chronology, it is commonly assumed that the Watson-Crick discovery spurred me to search for the enzymes of replication. But that is not the way it happened. In 1953, DNA was far from the center of my interests. The significance of the double helix did not intrude on my work until 1956, when the enzyme that assembles the nucleotide building blocks into a DNA chain was already in hand.

My interest in the replication of DNA, the focus of my research for the past 33 years, developed primarily from a fascination with enzymes. Having found an enzyme that incorporates a nucleotide into a coenzyme, I began, around 1950, to wonder about enzymes that might assemble the many nucleotides that make up the chains of nucleic acids, particularly RNA. But first we had to know the building blocks of the nucleic acids. It was not at all obvious in 1950 what they might be. Was the backbone assembled first and were the bases attached later? Was each link added to the chain as a single nucleotide? If so, was the phosphate in each component nucleotide initially attached to carbon number three or five, or to either one randomly or in a cyclic form to both?

In anticipating what the building block might be, I was influenced by what I had learned from the biosynthesis of coenzymes. I also felt that in searching for the form of the nucleotide that might serve as a building block for RNA and DNA, it would help to know how a nucleotide itself is built from simpler

molecules, and thus what its nascent form might be. Inasmuch as Jack Buchanan and Bob Greenberg were already pursuing purine biosynthesis, I decided to go after the pyrimidines.

During a brief interlude, I acted on the hunch that biosynthesis of the phosphodiester bond, accessible in phospholipids, might offer a model for building the backbone of nucleic acids. In exploratory experiments with [^{32}P]-α-glycerophosphate and [^{14}C]-phosphoryl choline, I could find no evidence for their condensation to form the diester (glycerophosphoryl choline), but I did stumble on the formation of phosphatidic acid and phosphatidyl choline in the cell-free extract (13). I worked out the enzymatic synthesis of phosphatidic acid (14), the key precursor of phospholipids, but still was eager to get away from greasy molecules and return to pyrimidines and the aqueous phase. In the future, I would not rely on intuition about model systems, but would head toward an objective directly.

Osamu Hayaishi came as a postdoctoral fellow in 1950 experienced in the use of soil bacterial enrichment cultures. Among the huge variety of species in soil, at least one can be found that will respond to virtually every natural organic compound and use it as a source of carbon and energy. Believing too that reversibility of metabolic pathways might provide clues to biosynthesis, we examined the breakdown of uracil and thymine in extracts of bacteria isolated from soil by aerobic enrichment on these pyrimidines. Uracil and thymine were converted to the corresponding barbiturates, not at all promising as biosynthetic precursors (15). But the next year, during my first visit to California, H. A. Barker helped me find an anaerobe in San Francisco Bay mud that consumed orotic acid. Back at NIH, with the participation of Irving Lieberman, who had been a student of Barker, studies of this organism identified as metabolic products dihydroorotic acid and carbamyl aspartate (16), which later proved to be intermediates in the biosynthesis of orotic acid.

Orotic acid was known from intact cell studies to be a precursor of nucleic acid pyrimidines, but it was uncertain whether it was on the main track or connected to it by a spur. With orotic acid tagged in its carboxyl group, the release of CO_2 to form uracil might lead us to the enzyme that took orotic "up" to nucleic acid. CO_2 release by extracts from yeast or liver was terribly feeble, yet showed a tantalizing requirement for ATP and ribose 5P. One happy day, instead of using extracts of either yeast or liver, I combined them. The reaction was explosive, hundreds of times greater than before, one of those rare moments in a scientific lifetime.

The enzyme abundant in liver extracts transferred a PP group from ATP to carbon 1 of ribose 5P to produce the novel phosphoribosyl pyrophosphate (PRPP) (17), later recognized as the key precursor of purine nucleotides, histidine, tryptophan, and NAD. The enzyme in yeast extracts (actually two

enzymes) formed orotidine 5P, which then was decarboxylated to UMP (18), the direct precursor of all the nucleic acid pyrimidines (Figure 1).

The transfer of pyrophosphate to ribose 5P entails an attack on the middle phosphate of ATP, as Gobind Khorana showed during one of his whirlwind and productive visits to my lab (19). (Other examples of this unusual reaction are the synthesis of thiamine PP, and guanosine tetraphosphate.) PRPP synthetase remains one of my favorite enzymes. As I wrote in the 1975 Festschrift for Ochoa (*Reflections on Biochemistry*, Pergamon Press): "Most of us anticipated that ribosyl activation for nucleotide biosynthesis would use the same device of phosphorylation, so well known for glucose. But the novelty of pyrophosphorylation used by this enzyme (coupled with elimination of inorganic pyrophosphate upon subsequent condensations) established my unalloyed awe for the ingenuity and fitness of an enzyme."

Knowing that PRPP enables a free pyrimidine (orotic acid) to be converted directly to a nucleotide, we sought and found enzymes that used PRPP to convert free purines (adenine, hypoxanthine, guanine) directly to nucleotides (20). Yet, I also knew from Buchanan's and Greenberg's studies (21, 22) that a purine ring is assembled from the very outset attached to ribose phosphate (later shown to be derived from PRPP). These facts, coupled with the knowledge that nucleotides can be formed from nucleosides by kinases, made it clear to me that cells have alternate pathways to the biosynthesis of nucleotides: salvage of preformed bases and nucleosides, and de novo routes from smaller molecules (e.g. sugar phosphates, amino acids, ammonia, one-carbon units). We have since realized that the role of salvage pathways can be as vital as the de novo pathways even under normal conditions when the de novo routes are not blocked by mutation, drugs, disease, or excessive traffic (23).

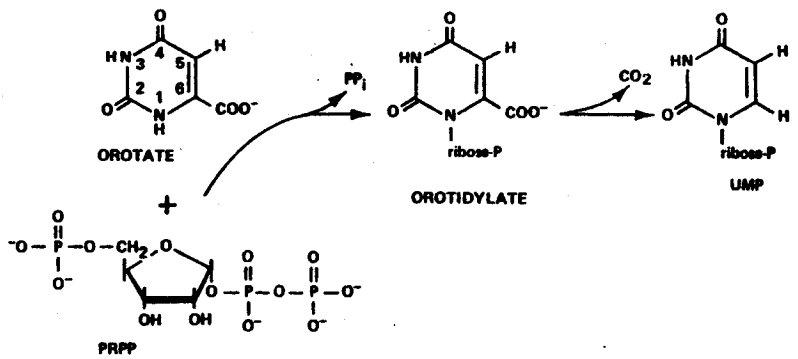

Figure 1 Condensation of orotic acid with PRPP produces the nucelotide, orotidylate (orotidine 5P), which upon decarboxylation generates uridine 5P (UMP).

DISCOVERY OF DNA POLYMERASE (1955–1959)

Having learned how the likely nucleotide building blocks of nucleic acids are synthesized and activated in cells, it seemed natural that in 1954 I would look for the enzymes that assemble them into RNA and DNA. Such an attempt might have been considered by some as audacious. Synthesis of starch and fat, once regarded as impossible outside the living cell, had been achieved with enzymes in the test tube. But, the monotonous array of sugar units in starch or the acetic acid units in fat was a far cry from the assembly of DNA, thousands of times larger and genetically precise.

Yet, I was only following the classical biochemical traditions practiced by my teachers. It always seemed to me that a biochemist devoted to enzymes could, if persistent, reconstitute any metabolic event in the test tube as well as the cell does it. In fact better! Without the constraints under which an intact cell must operate, the biochemist can manipulate the concentrations of substrates and enzymes and arrange the medium around them to favor the reaction of his choice.

I have adhered to the rule that all chemical reactions in the cell proceed through the catalysis and control of enzymes. Once, in a seminar on the enzymes that degrade orotic acid (16), I realized that my audience in the Washington University chemistry department was drifting away. In a last-ditch attempt to gain their attention, I pronounced loudly that every chemical event in the cell depends on the action of an enzyme. At that point, Joseph Kennedy, the brilliant young chairman, awoke: "Do you mean to tell us that something as simple as the hydration of carbon dioxide (to form bicarbonate) needs an enzyme?" The Lord had delivered him into my hands. "Yes, Joe, cells have an enzyme, called carbonic anhydrase. It enhances the rate of that reaction more than a million-fold."

By 1954, the rapidly growing *Escherichia coli* cell had become a favored object of biochemical and genetic studies, and for me had replaced yeast and animal tissues as the preferred source of enzymes. To explore the synthesis of RNA, Uri Littauer, a postdoctoral fellow, and I prepared [^{14}C-adenine]-ATP and maintained it as ATP with a regenerating system. Upon incubation with an *E. coli* extract, a small but significant amount of the radioactivity was incorporated into an acid-insoluble form, presumably RNA, and we proceeded eagerly to purify the activity responsible.

I also pursued the synthesis of DNA. Here, I had the invaluable help of Morris Friedkin, who had synthesized ^{14}C-thymidine and was studying its uptake into the DNA of rabbit bone marrow or onion root tip cells. Disinclined to work with cell-free extracts, he generously saved the spent reaction fluid from which I recovered radioactive thymidine to use in trials with extracts of *E. coli*.