Moments of Discovery: My Favorite Experiments

Even before completing my undergraduate stay at Penn State University, I had decided to get a Ph.D. in biochemistry to help me get a good job in the pharmaceutical or food technology industry. However, sometime during my last year I came across a series of papers from the Biochemistry Department at Western Reserve University in Cleveland, Ohio, an institution I had not previously considered as a place to do graduate work. Those reports described the use of radioisotopes to track metabolic reactions in vivo and in vitro. Reading further, one name, Harland Wood, dominated the list of authors (Fig. 1). Soon after World War II ended, Wood had been recruited to the Western Reserve University medical school from the University of Minnesota to resurrect a virtually moribund biochemistry department. Intrigued by the new technology, I decided to explore this new approach. By 1948 when I arrived as a graduate student, Wood's carefully chosen group of younger faculty constituted one of the top departments in the country.

Because of an unfortunate miscommunication, I found that I had inadvertently applied to and been accepted into the remnant of the defunct biochemistry department. Although keenly disappointed, I tried to make the best of it by teaming up with two former members of the department, Leonard Skeggs and Jack Leonard, to develop a new kind of artificial kidney. During the ensuing 2 years, I became pretty adept at surgical removal of dog kidneys and keeping the animals alive by dialysis. Recognizing my frustration during one of the biochemistry graduate courses I was obliged to take, Wood asked if I was interested in joining his department and completing my thesis research there. It was a break that changed my life. During the next 2 years, I completed my thesis research on the mechanism by which one-carbon compounds, formate, formaldehyde, and methanol, are converted to the methyl group of methionine. Although Warwick Sakami, one of the young professors in the department, was my nominal adviser, it was Wood who provided the inspiration that set the tone of my career in research.

Wood's scientific exploits as a graduate student and postdoctoral fellow were legendary among members of the department. While working for his Ph.D. at Iowa State University, he made the startling discovery that heterotrophic organisms (those that live on complex carbon compounds for their source of energy and biosynthetic needs) incorporated carbon dioxide into their cellular substituents. This property, previously believed to exist only in photosynthetic plants and autotrophic microbes, was met with considerable disbelief. Characteristically, Wood was not about to let the doubts persist and he turned to isotopes to prove his point. After a brief fling with radioactive carbon-11, whose incredibly short half-life made it difficult to use, he chose the stable carbon-13 isotope to trace the metabolic utilization of carbon dioxide. Because an enriched source of carbon-13 was not readily available in the late 1930s and early 1940s, he proceeded to prepare some himself. He built a water-cooled thermal diffusion column in a five-story abandoned elevator shaft that enabled him to separate sufficient quantities of carbon-13 for his experiments. However, he also needed a way to measure the abundance of carbon-13 in the metabolic products. Getting advice from Alfred Nier, a physicist at the
university, he built a mass spectrophotometer from scratch. This display of dogged self-sufficiency reflects his early experiences on the family's Iowa farm and the remarkable work ethic he and his brothers and sisters acquired from their parents and the community in which they were raised.

Wood's devotion to research and to those who shared his commitment to science showed through his outwardly gruff manner. “Hanging out” with the graduate students during the many late evenings when he lingered in the laboratory before heading home were the times I treasured most. His unremitting honesty and forthrightness in the way he practiced science provided the model we all tried to emulate. Wood's response to criticism showed through during a visit from the legendary Hans Krebs. Some years before, Wood had concluded that citric acid could not be one of the intermediates in the Krebs cycle for metabolizing dicarboxylic acids. He based his conclusion on the finding of an unanticipated isotope distribution in the glucose portion of liver glycogen and in intermediates of the cycle after feeding a variety of $^{14}$C-labeled metabolic precursors. He concluded that citric acid, a chemically symmetric molecule, could not be an obligatory intermediate in the cycle. For a period, Krebs' original formulation of the cycle stood amended with cis-aconitic acid replacing citric acid. Krebs' lecture during that visit provided a wholly novel way to explain how citric acid could be metabolized in an asymmetric way, thereby accounting for Wood's findings. Alexander Ogston had pointed out that citric acid was asymmetric with respect to its chirality and that the enzyme aconitase could bind and metabolize citric acid in an asymmetric manner. Entirely free from any apparent pique, defensiveness, or embarrassment, Wood was forthright in acknowledging Krebs' ingenious explanation and admitted that in this instance the isotope result had been misleading, and he stressed the necessity of understanding the enzyme mechanisms. The magnanimity and generosity of his praise and the unself-conscious manner in which he responded brought home to me his often repeated admonition that criticism focused on the science was not meant to diminish one as a person. It was important for me to keep that in mind when he vigorously contested what I thought were clever interpretations or speculations. David A. Goldthwait and Richard W. Hanson captured the essence of the man in their National Academy of Sciences Biographical Memoir: “...as a man without pretensions, whose opinions and decisions were always based on principles and not on personal factors, a man whose mind was open to new ideas and concepts, a man who by his example and encouragement got the best out of his associates, and a man who, once he made up his mind, would drive straight toward his goal. In him one felt the warmth, strength and integrity that made him unique” (1).

Much to the benefit of the students and faculty, Wood's reputation enticed many of the world's leading biochemists to visit and present their latest findings. Besides Krebs, the ones I recall as being most influential were Carl Cori, Severo Ochoa, Fritz Lipmann, Feodor Lynen,
Albert Lehninger, Herman Kalckar, and Arthur Kornberg. The latter two made a special impression. Aside from the fact that I and most others could barely understand Kalckar’s heavily Danish-inflected English, his almost childish, joyous personality made his still novel use of spectrophotometry for studying nucleotide enzymology seem all the more exciting. Arthur Kornberg was one of the rising “stars” in enzymology but equally notable for me at the time was that he and I graduated from the same Brooklyn High School, Abraham Lincoln, albeit about 10 years apart.

As I was finishing the research for my thesis, I decided that it would be important for me to get more intensive training in enzymology. Aware of that decision, Wood arranged for me to have a postdoctoral position with Carl and Gerty Cori at Washington University. Much to his chagrin, I told him that I preferred not to live in St. Louis because of its vestiges of racial segregation and notoriously torrid summers; perhaps apocryphal, foreign consular officials were said to receive a “tropical pay bonus” during their assignment in St. Louis. Turning down what was possibly a career-making opportunity because of an aversion to living in St. Louis was in Wood’s mind shortsighted, if not foolish. Disappointing Wood in his aspirations for my future was painful, but in time my decision was forgiven, perhaps because he appreciated my independence. Having thought hard about what I would do instead, I decided to spend the first of two planned postdoctoral years working with Herman Kalckar in Copenhagen, Denmark and to spend the second year in Arthur Kornberg’s laboratory at the National Institutes of Health.

Herman Kalckar came to the United States in the late 1930s and was forced to remain throughout World War II. During the eight or so years he spent in the United States he was among the earliest to formulate the concept of high energy bonds as the form in which free energy was captured and stored during oxidative metabolism. Most people were captivated by his charmingly buoyant and fun-loving manner despite their inability to understand what he was saying. As I learned later, it was not just his Danish-like English that confused people, for even the Danes found him difficult to follow when he spoke his native language. Soon after the war ended and the situation in Denmark was near normal, Kalckar returned to Copenhagen and established an active laboratory at the university’s Institute of Cytophysiology (Fig. 2).

When I arrived in the fall of 1952, he had already assembled an interesting collection of fellows from Sweden, India, Italy, Scotland, Australia, and Canada. The international makeup of the laboratory made English the lingua franca for our scientific and social discourse. The locals in the laboratory were amused and tolerant as they listened to our futile promises to
refrain from speaking English in the laboratory; their references to Danish as a “throat disease” made us feel less inadequate. James D. Watson had been in the laboratory the year before I arrived to learn some nucleic acid chemistry, ostensibly on the advice of his graduate professor, Salvador Luria. His stay, however, was brief probably because of his outspoken disdain for biochemistry and his belief that Kalckar had little interest in genes or DNA. That assessment was probably a result of Kalckar’s inattention to the laboratory that year, for when I learned to translate Kalckar’s mutterings, it was apparent that his interests in biology were wide ranging and most often stimulating and provocative.

Although still not fully recovered from the Nazi occupation, the Danes were welcoming and extraordinarily hospitable, occasionally to our embarrassment. Living in Taarbaek, a small upscale fishing village bordering on the King’s private deer park on the outskirts of Copenhagen, was a welcome relief from 4 years in Cleveland and from my wife Millie’s 4 years of nursing at the university’s hospital. My daily commute to and from the institute and the bike ride home through the woods to our “villa” provided the quiet time for preparing and thinking about the experiments of the day. My American Cancer Society fellowship stipend ($3600 per year), which one of our Danish friends speculated might have been more than the King’s allowance, allowed us to live well and to sample the sights and culture of the Europe we had only read about.

Kalckar was a dreamer, often seeking novel explanations for paradoxical observations. One of these originated from a suggestion by Thomas Rosenberg, a physicist friend from the nearby Niels Bohr Institute. Responding to a proposal that insulin acted on the phosphorylation of glucose by hexokinase, they speculated that the hexokinase reaction occurred in two steps; the first product was a high energy glucose 6-metaphosphate that was then hydrated to form glucose 6-phosphate; insulin was presumed to stimulate the hydration step. Commonly, such musings formed the basis for discussions during afternoon tea. At one discussion, I outlined a way to test that possibility. Presuming that the first step was reversible and being aware that ITP was also a substrate for hexokinase, it seemed plausible to expect a glucose-dependent transfer of phosphate from ATP to IDP or from ITP to ADP. At the time, Wolfgang (Bill) Joklik, a postdoctoral fellow from Australia via a Ph.D. at Oxford, joined the laboratory and we agreed to test that idea. Because $^{32}$P-labeled ATP and ITP were unavailable commercially at the time, we made our own. A rabbit was injected with $^{32}$P-labeled phosphate and ATP was harvested from the skeletal muscles, an exercise Bill and I still chortle over when we recall how the rabbit nearly sabotaged our effort.

The experiment to test the Rosenberg-Kalckar hypothesis led to a serendipitous and more interesting result. The terminal phosphate of ATP was transferred to IDP and from ITP to ADP, but neither reaction was influenced by the presence of glucose. Clearly this finding was inconsistent with the hypothesis’ predicted key role for glucose. Following up on that result, Joklik and I discovered that the transphosphorylation activity was because of a previously unknown enzyme that uses ATP to phosphorylate the four ribo- and deoxyribonucleoside diphosphates to the respective triphosphates; we dubbed the enzyme nucleoside diphosphokinase or Nudiki for short (2). Although the new activity was first detected in vertebrate muscle, we purified it from yeast. Subsequently the enzyme was found to be widely distributed in pro- and eukaryotes, not surprisingly because it plays a critical role in generating the “building blocks” for RNA and DNA synthesis.

One of the papers that got lots of discussion at our tea times was a report from Fritz Lipmann, Feodor Lynen, and their respective collaborators Mary Ellen Jones and Helmut Hilz (3). That report alleged that the long standing puzzle of how eukaryotic organisms make acetyl-CoA had been solved. Lipmann’s and Lynen’s towering reputations and the novelty of their proposal were enough to capture my attention. At the time, the synthesis of acetyl-CoA in bacteria was known to occur via two separate enzymatic reactions. For aceto kinase,

$$\text{ATP} + \text{acetate} \leftrightarrow \text{acetyl phosphate} + \text{ADP}$$

and for phosphotransacetylase

$$\text{Acetyl phosphate} + \text{CoA} \leftrightarrow \text{acetyl-CoA} + \text{P}_i$$

By contrast, in yeast and animal cells, acetyl-CoA appeared to be made by a single enzyme (acetyl-CoA synthetase), seemingly in a single reaction.

$$\text{ATP} + \text{acetate} + \text{CoA} \leftrightarrow \text{acetyl-CoA} + \text{AMP} + \text{PP}_i$$
The authors' claim that the reaction proceeds in discrete steps involving successive enzyme-bound intermediates was particularly provocative.

\[
\begin{align*}
\text{ATP} + \text{enzyme} & \leftrightarrow \text{enzyme-adenylate} + \text{PP}_i \\
\text{Enzyme-adenylate} + \text{CoA} & \leftrightarrow \text{enzyme-CoA} + \text{AMP} \\
\text{Enzyme-CoA} + \text{acetate} & \leftrightarrow \text{acetyl-CoA} + \text{enzyme}
\end{align*}
\]

The evidence supporting their proposal relied on observations that the enzyme alone catalyzed an exchange of \(^{32}\text{P}\)-labeled \(\text{PP}_i\) with the pyrophosphoryl moiety of ATP, as well as an exchange of \(^{14}\text{C}\)-labeled acetate with the acetyl group of acetyl-CoA. Both of these findings were consistent with their formulation of three partial reactions and accounted for the energetics and stoichiometry of the overall conversions. Although enzyme-bound intermediates had previously been suspected as intermediates in certain protease reactions, to my knowledge none had been isolated and characterized. Were analogous enzyme-bound nucleotides formed with other nucleoside triphosphates? Could they be, I wondered, activated substrates for nucleic acid synthesis? As a start, it seemed worth trying to isolate the enzyme-AMP compound when I got to Kornberg's laboratory.

The voyage back to the United States was filled with regrets at leaving the idyllic stay in Denmark. However, there was also the excitement of a new laboratory and a chance to do something different. Ironically, although I had earlier turned down the opportunity to work at Washington University, Kornberg (Fig. 3) informed me about halfway through the year that he had decided to move from the National Institutes of Health to St. Louis to rebuild Washington University's department of microbiology. Later, I learned from him that of the several post-doctoral applicants he had accepted to join his laboratory I was the only one who agreed to the change in location.
It was a typical fall day in mid-November, 1953, when I arrived at Washington University Medical School to join Kornberg’s laboratory. Located at the top of an antiquated clinic building, the only way to reach the microbiology department was by passing down a long corridor lined with patients in various states of despair waiting their turn to see a doctor. The final ascent was via a somewhat “ancient” elevator, which, after the sliding gate was secured, inched and lurched its way upward to the fourth floor. On my arrival, the shabbiness of the surroundings was plainly evident. The main corridor’s bare bulb lighting, suspended from the unusually high ceilings characteristic of buildings of that vintage, could well have been installed when gas lights went out of fashion. It was hard to realize that that department had been where Sol Spiegelman and Al Hershey had helped usher in molecular biology and where some notable work in medical immunology had been done. However, Kornberg’s enthusiasm at my arrival, his excitement about those who would follow, and the prospects for the soon to be completed renovations lessened the importance of the shabby surroundings. Kornberg’s escorted tour of the laboratories that had been newly done over, including the one I was to work in, assured me that I had made the right choice for the second year of my postdoctoral fellowship. After settling on a place to live, I was ready to get to the laboratory.

In our meeting to discuss what I would work on, I told Kornberg of my keenness on trying to isolate the enzyme-AMP compound proposed by the Lipmann-Lynen team. He was decidedly cool to my suggestion, expressing considerable skepticism of their claim. Indeed, he believed that the ATP-PPi exchange reactions offered to support the model could be explained in other ways. For example, the reversible reactions leading to the synthesis of the coenzymes nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) (4, 5) result in the production of PPi and, therefore, even trace amounts of NAD or FAD in their enzyme preparation could account for the observed ATP-PPi exchange. Nevertheless, despite Kornberg’s reservations, I was “unleashed” to explore my hunch with only the admonition to purify the enzyme before testing whether their observations and predictions held up.

Within a relatively short time, using the formation of acetyl-CoA as the assay, I had a substantially purified enzyme. Much to my dismay neither of the two kinds of exchange reactions were detectable. Setting about to determine what was needed to reconstitute the ATP-PPi exchange, I confirmed that with all the reactants present there was a robust exchange of labeled PPi with ATP but none when acetate and CoA were omitted. Surprisingly, acetate alone was able to completely restore the enzyme’s ability to promote the exchange of ATP with PPi. I inferred that ATP reacted with acetate to produce acetyl adenylate, a nucleotidyl analogue of acetyl phosphate, with the concomitant formation of PPi (6). It was also plausible that like acetyl phosphate, acetyl adenylate could serve as the acetyl donor to CoA. However, my attempts to isolate the putative acetyl adenylate from the reaction failed.

Seeking to verify my conjecture, I decided to synthesize acetyl adenylate chemically. Being a novel compound, I contacted David Lipkin, a specialist in phosphate chemistry on the Washington University campus a few miles away, on how to proceed. It’s easy, he advised: mix acetyl chloride and the silver salt of adenylic acid, remove the insoluble silver chloride, and collect the mixed anhydride from the fluid. Within a week or so the first ever batch of pure acetyl adenylate was available and I could verify that the enzyme converted it rapidly and quantitatively to ATP in the presence of only PPi and to acetyl-CoA with added CoA. The overall reaction could then be explained as the result of two successive steps.

\[
\text{ATP} + \text{acetate} \leftrightarrow \text{acetyl adenylate} + \text{PPi},
\]
\[
\text{Acetyl adenylate} + \text{CoA} \leftrightarrow \text{acetyl-CoA} + \text{AMP}
\]

A nagging concern was my inability to detect or accumulate acetyl adenylate with only ATP and acetate as the substrates. Perhaps, I thought, it remained bound to the enzyme and existed only in amounts stoichiometric with the amount of enzyme. To test that surmise, I resorted to the use of hydroxamic acid. Acetyl phosphate was known to react with that reagent to form acetyl hydroxamate, and I confirmed that a similar reaction occurred with synthetic acetyl adenylate. I was elated to detect an accumulation of acetyl hydroxamate and PPi when the enzyme was incubated with ATP, acetate, and hydroxamic acid. Evidently, acetyl adenylate formed in the reaction remains tightly bound to the enzyme when the acetyl acceptor, CoA, is absent.

The novelty of acetyl adenylate as the intermediate in the formation of the acetyl-CoA reaction caused a stir when I reported it at the annual Federation meeting in 1955. Lipmann
and Lynen were considerably embarrassed, as they confessed to me later. More than likely the problem with their experiments was the impure state of the enzyme. Having obtained their relatively crude enzyme from yeast grown with acetate as the carbon source, it’s likely that acetate contaminated their preparations. Kornberg’s reminder of Efraim Racker’s dictum not to waste clean thoughts on dirty enzymes was vindicated.

Soon thereafter, the formation of acyl adenylates accounted for the production of the longer chain fatty acyl-CoAs as well as of other carboxylates, e.g. lipoic acid. However, I was curious about another ATP-PP_i exchange activity encountered in the earlier fractions of the acetyl-CoA synthetase, one stimulated by methionine (7). It appeared not to be related to the enzyme discovered by Giulio Cantoni that forms S-adenosylmethionine because of the relative irreversibility of that reaction. Soon other enzymes catalyzing amino acid-dependent exchanges of ATP with PP_i were detected, and their purification revealed that each was specific for a different amino acid. The ability to trap aminoacyl hydroxamates with hydroxamic acid in the reaction was consistent with the likelihood that these enzymes also catalyzed the formation of enzyme-bound aminoacyl adenylates. Reasoning by analogy with the mechanism of the acetyl-CoA synthetase, it seemed probable that there was a naturally occurring acceptor for the aminoacyl moiety, a counterpart to CoA. I asked Jim Offengand, my first graduate student, to search for such an acceptor.

To our surprise, his search led to the discovery of RNA as the aminoacyl acceptor (8). Purification of the acceptor RNA revealed that it was relatively small and probably identical to the sRNA that Zamecnik and Hoagland had found to stimulate the in vitro incorporation of amino acids into protein (9). Further work by Offengand and Jack Preiss, my first postdoctoral fellow, established that a single and highly specific enzyme converts each amino acid to an enzyme-bound aminoacyl adenylate and then transfers the aminoacyl group to a specific RNA molecule, now referred to as tRNA. At Gobind Khorana’s invitation, I spent the delightful summer of 1956 at his British Columbia Research Council laboratory in Vancouver, Canada learning to synthesize aminoacyl adenylates chemically. With those in hand, each of the purified enzymes was shown to convert the cognate synthetic aminoacyl adenylate to ATP with PP_i and to aminoacyl tRNAs in the presence of tRNA (10). Because the aminoacyl acceptor property of the tRNA preparation could be successively saturated by each amino acid, we inferred that each amino acid is transferred to only a limited set of tRNA molecules. It took other experiments to establish that each tRNA molecule had a single acceptor site, somewhat surprisingly one of the two 3’-terminal hydroxyl groups. This stoichiometry (one amino acid per tRNA) squared with Francis Crick’s surmise that the aminoacyl tRNAs serve as “adaptors” for matching amino acids to their cognate mRNA codons during ribosome-mediated assembly of polypeptide chains (11).

Adenylylation of the acyl groups of fatty acids and amino acids by ATP proved to be the first discovered of a general class of enzymatic reactions. Proteins (for example, DNA ligase and glutamine synthetase) are also adenylylated by ATP. In the case of the ligase, adenylylation of the protein enables it to catalyze the covalent joining of DNA ends, and adenylylation of glutamine synthetase regulates the responsiveness of the enzyme to feedback regulation. In each case, the rapid hydrolysis of the eliminated PP_i to P_i drives the adenylylation reactions to completion.

These discoveries, spaced over a period of about 5–6 years, were most satisfying because they happened at the beginning of my career when I still performed many of the experiments with my own hands. Coincidentally, they also provided the confidence that I could do creditable research independently. Despite my earlier aversion to living in St. Louis, I learned that interacting with remarkable colleagues in a stimulating setting trumped the occasionally unpleasant summer and winter weather, and over the 6 years of my stay there, the city overcame its legacy of racial prejudice and revealed a vibrant social and cultural life.

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