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The discovery, synthesis, and role of pyridoxal phosphate: phase I of many phases in the Gunsalus odyssey

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In the period 1943–1945, the discovery of pyridoxal-5-phosphate (PLP) as the vitamin B_6 coenzyme of tyrosine decarboxylase, as well as its synthesis from pyridoxal by Gunny[1] and his associates, Dexter Bellamy [1] and Wayne Umbreit, were seminal events that led to rapid progress in understanding amino acid metabolism and enzymology. It also heralded the entry of Gunny onto the world stage of biochemistry. During that period, Ernest Gale was investigating a preparation from yeast, named codecarboxylase,[2] that activated tyrosine decarboxylation in bacteria [2–4]. Esmond Snell was studying the requirement of lactic acid bacteria for members of the vitamin B_6 group, pyridoxine, pyridoxal ("pseudopyripseudopyridoxine"), and pyridoxamine [5,6]. Karl Folkers [7] was making available to investigators new vitamin B_6 compounds produced by Merck Co. At the same time, Gunny and Bellamy had developed dried cell preparations of *Streptococcus faecalis*, which were devoid of vitamin B_6, but contained an active apotyrosine decarboxylase (and arginine decarboxylase) [8–14]. In addition, they with Wayne Umbreit (a new faculty member) discovered an ATP-dependent pyridoxal phosphorylating system [15,16]. The ability to produce cells devoid of vitamin B_6 was a key step in the discovery of the active form of vitamin B_6. Such cells were used for (a) studying pyridoxal phosphorylation by ATP [15], (b) following the chemical phosphorylation of pyridoxal[3] [15,16], and (c) purifying, recovering, and characterizing PLP [16,17]. The cells also were used to compare synthesized PLP with Gale's codecarboxylase [13,17]. The world's supply of the new coenzyme as the barium salt was lyophilized in 200-μg amounts in small tubes and made available to all. Following that milestone, Gunny and Wayne went on to investigate the location of the phosphate group (either the 3-(phenolic), or 5′-(primary alcoholic) hydroxyl group of pyridoxal [18,19]) (Fig. 1). In addition, a new post doc, Herman Lichtstein, and graduate students Doreen Jeffs and Dan O'Kane joined Wayne and Gunny in demonstrating the role of PLP in glutamic/aspartic transaminases in *S. faecalis* and mammalian tissue [20–22].

Tryptophan synthetase

I joined the lab group in April 1946 as an undergraduate student beginning research (see last section below). The first project with Wayne was to look for a role of PLP in the conversion of indole plus serine to tryptophan in *Neurospora sitophila.*. There was no difficulty in obtaining homogenates that carried out this reaction, as reported by Tatum and Bonner [23]. However, when harvested mycelial mats were stored in the freezer, the tryptophan-forming activity decreased to zero. At Gunny's and Wayne's suggestion, an array of cofactors, extracts, and biological preparations were tested for the ability to rejuvenate the reaction. The result was dramatic. Only PLP restored activity [24].

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[1] Dr. I.C. Gunsalus has always been known by everyone as "Gunny." It is difficult to refer to him otherwise.

[2] The path of identifying codecarboxylase in the Gale laboratory seems to have taken a wrong turn when the elemental analysis failed to detect phosphorus in the preparation.

[3] Gunny tells the story of the fortunate mistake made in the shipping department at Merck Co. when the 25 mg of pyridoxal donated by Karl Folkers arrived as 25 g of pyridoxal, a huge amount in those days. This greatly accelerated the chemical phosphorylation work.

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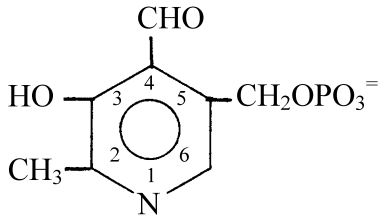

Fig. 1. Structure of pyridoxal phosphate.

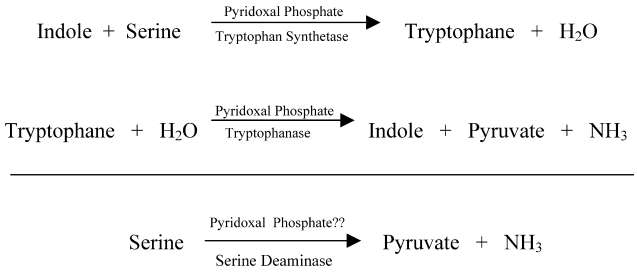

Fig. 2. Serine deamination as the sum of tryptophan synthetase and tryptophanase reactions.

This finding added the tryptophan-forming system, later named tryptophan synthetase, to the list of PLP enzymes and broadened the concepts of PLP function.

Tryptophanase

Based on the requirement for PLP in tryptophane formation, Gunny and Wayne thought that PLP could be involved the degradation of tryptophan by tryptophanase to yield indole, a catabolic reaction characteristic of *Escherichia coli.* Earlier, A.J. Wood and Gunny [25] had developed cultural conditions for growing streptococci with high biochemical activities. In addition, methods were developed in the laboratory for obtaining active cell free extracts by grinding vacuum-dried cells in a ball mill [12,20] or by extracting acetone-dried cells with buffer. Applying these methods to *E. coli* yielded cell-free extracts highly active for tryptophanase. A partially purified enzyme was easily obtained, but the enzyme was inactivated in the final step of the purification by to the accidental addition of sodium cyanide instead of dilute NH_4OH. However, the activity was completely restored by adding PLP [26], thereby converting a mistake into a useful strategy for resolving a PLP-containing enzyme.

There had been considerable speculation and work in other laboratories on the fate of the alanine side chain of tryptophan in the tryptophanase reaction. Several weeks were spent failing to identify the alanine side-chain product formed concomitant with indole. At this point Gunny thought that enough time had been wasted. So, with a little edge in his voice, he asked why pyruvate formation had not been considered? Very shortly thereafter, pyruvate was found in amounts equimolar to the indole produced [26]. Needless to say, Gunny's ability to solve the problem that had eluded so many investigators [23,27,28] impressed this young investigator.

Threonine dehydratase

As a follow-up to this work, Gunny deduced that PLP could be the coenzyme in serine deamination in *E. coli*, a reaction studied by Chargaff and Sprinson [29]. The reason for this can be seen in the sum of the tryptophan synthetase and tryptophanase reactions (Fig. 2). Shortly thereafter, an active serine deaminase was found in a cell-free extract from *E. coli* and purified. In spite of many attempts using the then-known tricks for producing apoenzymes, no evidence was found for a dependence of the reaction on PLP. Instead, adenosine-5′-monophosphate and a thiol-reducing agent were required for activity. At that point the investigation was put on hold while I graduated from Cornell and got married, and all of us cleaned up the mess after a fire had gutted Gunny's and Wayne's research laboratory.

Indiana University

In September of 1947 Gunny became Professor of microbiology at Indiana University. I entered the graduate program in microbiology with Gunny as my advisor. Once the laboratory was established, research was resumed on serine deaminase. It was found that L-threonine was a better substrate than serine. Further, serine irreversibly inactivated the enzyme during catalysis [30]. During that time the deaminase became known, first as threonine dehydrase, then as threonine dehydratase. To Gunny's dismay, relatively large quantities of the then-expensive AMP was being consumed in an attempt to understand how AMP could serve as a coenzyme for serine deamination, but without much success. It was years later when the role of PLP, predicted by Gunny, was demonstrated for both the "biosynthetic" and "biodegradative" forms of the dehydrase by Umbarger and Brown [31] and by Allen Phillips [32], a graduate student in my lab, respectively. Similarly, it was years later when Allen Phillips found that AMP was not a coenzyme in the generally accepted sense, but an allosteric effector which caused oligomerization of the dehydrase, accompanied by a major decrease in K_m for threonine [33,34]. It was also found that the inactivation by serine established in Gunny's lab was due to covalent binding to the enzyme of an active intermediate in serine deamination, presumably aminoacrylate. Later this phenomenon became known as mechanism-based inactivation.

Alanine racemase

In 1949 Gunny pointed out that the data of Snell and Gairard [35] showed that vitamin B_6 is not required for growth of *S. faecalis* in a complex medium provided that DL-alanine is added. Since L-alanine is already present in the complex medium, this suggested that vitamin B_6 is needed to produce D-alanine, which is required for some unknown reason. To investigate this possibility, a manometric method for D-alanine was developed using D-amino acid oxidase purified from pig kidney to follow D-alanine formation. Using that assay it was quickly found that L-alanine was converted into D-alanine in *S. faecalis*. In the absence of D-amino acid oxidase, the purified system formed a racemic mixture from either D- or L-alanine. Pyruvate was neither formed nor utilized, and several α-keto acids plus other amino acids did not yield either D- or L-alanine. PLP was required for activity and pyridoxamine phosphate was inactive. Therefore, transamination to form D-alanine was largely eliminated. Thus, a new type of enzyme, an alanine racemase, was catalyzing the interconversion of alanine isomers. The racemase is specific for alanine, is widely distributed among bacteria, but is not found in other life forms [36]. The role of alanine racemase was later explained when D-alanine was found in the bacterial cell wall peptidoglycan [37]. Later, a glutamate racemase stimulated by PLP was found in *Lactobacillus arabinosus* [38].

Other transaminases

Gunny and and his graduate student Lou Feldman showed that there were many other transaminase reactions in several bacteria which produced glutamate from α-ketoglutarate and a series of amino acids acting as amino group donors. PLP was required when tyrosine or phenylalanine were amino group donors [39].

Reaction mechanism of PLP

Other investigators subsequently reported the function of PLP in a variety of amino acid reactions. These contributions together with those from Gunny's lab led to a unified theory of PLP function based on Schiff's base catalysis in which the amino acid substrate, upon binding to the enzyme, formed an azomethine with enzyme-bound PLP by transaldimination. Subsequent ketamine/aldamine tautomerizations of the azomethine then made possible the wide variety of reactions [40,41]. Thus, PLP reactions provided the basis for, arguably, the first well-established example of how enzymes carry out catalysis.

Transition to other research phases

As research on PLP function matured, Gunny's attention returned to work started at Cornell on "pyruvate oxidation factor" and the nature and mechanism of the pyruvate dehydrogenase complex.

Gunny as my mentor and friend

My eventual participation in the PLP era started when I entered Cornell in 1940 to study bacteriology. I had the good fortune to be assigned Gunny as my faculty advisor. There he was that first day behind the registration table in a white shirt with no tie and sleeves rolled up. He was a 28-year-old new assistant professor of bacteriology, recently made faculty advisor to the bacteriology majors, and I was an 18-year-old incoming freshman. He was a demanding advisor. To my dismay he insisted that I put off taking bacteriology courses the first year in favor of botany, zoology, chemistry, physics, and English. When I protested that I came to Cornell to study bacteriology, he stated that if I did well in the foundation courses, he would let me take bacteriology as a sophomore. That was a fortunate, if not an immediately appreciated, piece of advice on my education! We had a friendly relationship, but I heard the message early that only an excellent performance in top-level courses in physical and biological sciences offered in Colleges of Arts and Science and in Agriculture was going to satisfy him. Later, when I was in the Army, he sent letters describing the research going on in the laboratory. When three bacteriology majors, Paul Vandemark, Tony Castellani, and I, now Army privates, were ordered back to Cornell for an additional semester under the Army Specialized Training Program, we did fermentation balance research projects in Gunny's lab. We were in a military unit and were expected to be present for retreat, which because of our lab work, we regularly missed. In those instances Gunny signed notes to get us squared with the first sergeant and into the mess hall (Williard Straight Hall) for dinner. We were confined to quarters in the evening. However, there was a clandestine weekly evening journal club (with refreshments) at Gunnys' house which increased our ability to understand and use the scientific literature. Based on Gunny's suggestions, we became skillful in finding a means to get out of the barracks and to make our way undetected to his house. Thus, upon returning from Army service in April 1946, I naturally went to Gunny seeking some kind of laboratory activity until the start of the fall semester. Fortunately, he offered me a temporary lab technician position paid for by the National Youth Administration (NYA); I was to work with Wayne Umbreit. Being thrust into the informal but exciting research environment made up of Gunny, Wayne, grad students, and post docs, where new

discoveries were rapidly being made, was a major influence in my aspirations for the future and it propelled me into a career in research. It was in that environment that Gunny said that I should read the philosopher John Dewey and become informed of the intellectual process to be followed when in the course of investigation one encounters an indeterminant situation. Initially, Dewey's writings seemed like lofty prose, but over time I have come to appreciate the philosophical basis for the investigation process as described by Dewey. After Indiana University, both Gunny and I became faculty members in different departments at the University of Illinois, he as professor in microbiology, and I a new assistant professor in dairy science. There he provided invaluable support for my beginning independent research program by referring students and postdoctoral scholars to work in my laboratory. We both used *Pseudomonas putida* in our research, Gunny in the metabolism of camphor and other terpenes, and I in the pathways and enzymes of carbohydrate metabolism. Unquestionably, Gunny had a determining influence on my career in science in the standards he imparted for conduct of research and in what constitutes a good experiment. He was also a friendly helper at the personal level. Because of him I learned to appreciate fine wine and sherry. On Cayuga Lake he also introduced me to sailing, which later became my adventure on the Great Lakes. So I congratulate Gunny on not only his longevity but also on his contributions to science and to the role he played in developing young scientists. The list of those who have benefited from entering his sphere of influence is impressive.

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