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JB Reflections and Perspectives

Esmond E. Snell—the pathfinder of B vitamins and cofactors

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Esmond E. Snell (1914–2003) was a giant of B-vitamin and enzyme research. His early research in bacterial nutrition had lead to the discovery of vitamins such as lipoic acid and folic acid, and an anti-vitamin avidin. He developed microbiological assay methods for riboflavin and other vitamins and amino acids, which are still used today. He also investigated the metabolism of vitamins, discovered pyridoxal and pyridoxamine as the active forms of vitamin B_6 and revealed the mechanism of transamination and other reactions catalysed by vitamin B_6 enzymes. His research in later years on pyruvoyl-dependent histidine decarboxylase unveiled the biogenesis mechanism of this first built-in cofactor. Throughout his career, he was a great mentor of many people, all of whom are inspired by his philosophy of science.

Keywords: pyridoxal/pyruvoyl group/vitamin/bacterial nutrition/bioassay.

Abbreviations: PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PN, pyridoxine.

In 1971, Esmond E. Snell had a sabbatical stay at the Department of Biochemistry, Faculty of Medicine, Osaka University. One day, after his lecture, a student came to him, showed a fancy paperboard and asked him to write a message—or 'motto'—that would encourage and inspire the students. He wrote:

> Hard work on interesting problems is enjoyable and preferable to aimless wasting of leisure time. It may also lead to unexpected findings that give insights into important related problems. Such unexpected findings—sometimes called 'luck'—frequently happen to the active researcher, but only rarely to those who prefer talk to study and work. So one should study and work hard, on interesting problems of any nature, with the purpose of explaining nature and helping others.

This calligraphic message was handed on to Hiroshi Wada (1928–2003), and had been hung on the wall of his office room until his retirement from Osaka University (1992). Many Japanese and American scientists have copies of this statement. The memories of Snell always remind us of this message; we realize how deeply this message was connected to his attitude towards science, as we will see further.

In writing this review, we are indebted to the autobiography by himself (*1*) and the excellent biographical articles by Miles and Metzler (*2*), and by Hackert *et al.* (*3*). This text was written in gratitude of over 100 scientists and students who worked with him, only few of whom could be mentioned here.

Bacterial nutrition and discovery of vitamins

Snell started his carrier in William H. Peterson's laboratory at University of Wisconsin–Madison as a graduate student studying the nutritional factors of lactic acid bacteria. His first publication (1937) described the requirements for growth of *Lactobacillus delbrueckii* (*4*). He recalls in 1993: 'Since almost all of the substances later identified as essential nutrients were either unknown, unavailable, or both, we could either depend on luck to find a crude medium in which a single nutrient was growth limiting, or, alternatively, attempt to remove or destroy one or more of the essential unknown growth factors of an adequate medium without lowering others below the concentrations required for growth' (*1*). He found that potato extract stimulated the growth of *L. delbrueckii* on peptone, and showed that the nutritional factor existed in the acid-ether extract of potato extract (*4*). Although the factor was not characterized further, he found that acetate can replace it. He then added acetate to all media used in subsequent studies. In 1951, his lifelong friend Lester J. Reed of the University of Texas at Austin identified the nutritional factor and named it 'lipoic acid' (*5*). Since lactic acid bacteria do not have the citric acid cycle, they can grow without lipoate if acetate is supplied to make acetyl-CoA necessary for biosynthesis. Unless acetate was added to media at that time, the reaction synthesizing acetyl-CoA limited the bacterial growth, making the response to other nutritional factors worse. This would have resulted in the delay of identification of many bacterial nutritional factors.

Every time the new nutritional factor was discovered, it was added to the basal media in a pure or concentrated form to replace crude ingredients, and through this process another new nutritional factor was discovered. Riboflavin was added to the medium for *L. casei*, in which the peptone was treated with alkali in advance to destroy the inherent riboflavin.

No growth was observed, indicating that some nutritional factors were removed by the alkali treatment. In 1939, he purified the factor ~1000-fold from liver extract and showed its identity with 'pantothenic acid' (*6*), which had been reported in 1933 as a yeast nutrient and named for its ubiquitous occurrence by Roger J. Williams (*7*), then at Oregon State University. As an attempt to make a more purified medium, a basal medium containing acid-hydrolysed, purified casein, glucose, salts, tryptophan, cystine, acetate, riboflavin and pantothenic acid was prepared. *Lactobacillus casei* was expected to grow on this medium, but no growth was observed. Niacin was found to stimulate the growth in the first culture, but the growth failed on subculture, indicating depletion of additional factors necessary for growth (*6*). Pursuit of the still unidentified factors yielded the 'Norit filtrate factor' and 'Norit eluate factor'. He purified the 'Norit eluate factor' 1000-fold before he left Wisconsin.

Based on these works, he developed a microbiological assay of riboflavin (*8*). Riboflavin was completely removed from the alkali-treated peptone–yeast extract medium by precipitation with lead acetate and photolysis. By measuring the stimulating effect on the rate of growth of *L. casei* on this medium, the content of riboflavin in a sample could be determined. The method was sensitive and quantitative, and served as a prototype for the following microbiological assay of B vitamins and amino acids.

In 1939, Snell moved to the University of Texas at Austin to join Williams' group as a postdoctoral fellow. During the study of the identity of biotin and 'biotic acid' found by Williams, he showed that the 'Norit filtrate factor' was the newly discovered vitamins biotin and pyridoxine (PN). He developed a yeast assay system for biotin, and using this isolated the protein in egg white that tightly binds biotin and causes 'egg white injury' (*9*). He named it 'avidin' because of its peculiar biotin-binding capacity.

Now that 'biotic acid' had been shown to be identical to biotin, which was not good news for Williams' group, Snell began investigating the nutrition of less-studied lactic acid bacteria. He soon found a new nutritional factor for *Streptococcus faecalis* R in a rich local source, spinach (*10*). He processed 'four tons' of spinach and concentrated the active principle. He named this compound 'folic acid' following Latin *folium*, leaf. The 'Norit eluate factor' turned out to be folic acid.

Perspectives

When Snell started the study of bacterial nutrition, the only reliable method was the biological assay using animals. It was expensive, time consuming and required significant amount of the sample material. In addition, reproducibility of the results was unsatisfactory. The idea that bacterial growth factors may also be vitamins for animals was 'hardly acknowledged at that time' (*1*), and nobody considered that bacteria can replace animals in the bioassay of vitamins. It became apparent only later that bacteria and animals share many vitamins in common, just like Jacques Monod had said in 1954, 'What is true for *E. coli* is true for an elephant.' Despite the development of physicochemical assay methods of vitamins, the contents of vitamin B_6, pantothenic acid and folic acid in foods are often determined by the microbiological assay originally developed 60 years ago by Snell. When new physicochemical assay methods are developed, they are usually compared with the microbiological assay method to show their validity.

Vitamin B_6: discovery of pyridoxal and pyridoxamine

Snell extended the microbiological assay methods to other B vitamins [summarized in (*2*, *3*)]. He constructed an assay system for PN using *S. faecalis*. However, the PN content in rat tissues determined using this system was several thousand times higher than those obtained by yeast growth, rat growth or colorimetric methods. He postulated that *S. faecalis* was responding to a substance (he called it tentatively 'pseudopyridoxine') that far surpasses PN in physiological activity for *S. faecalis*. He then found that 'pseudopyridoxine' was derived from PN in rat and human bodies. Finally, he found that PN was, quite unexpectedly, not absorbed by *S. faecalis* (*11*). These results indicated that PN was not active by itself in promoting the growth of *S. faecalis* and must be converted to 'pseudopyridoxine' prior to be utilized by the bacteria (*11*). Important findings followed: filter-sterilized PN required large amounts to support growth of *S. faecalis*, and when media supplemented with PN was heat sterilized, progressively smaller amounts of PN were required as the autoclave period was increased. Collectively, it was concluded that a very small fraction of PN is converted to 'pseudopyridoxine' by autoclaving the media, but a large fraction of PN is converted to 'pseudopyridoxine' in animal tissues. This resulted in the 'apparently high values of PN' in tissue samples.

Snell assumed oxidation to an aldehyde and amination to an amine as possible routes of conversion of PN to 'pseudopyridoxine'. In collaboration with Karl Folkers at Merck & Co., he showed that both 4-aldehyde and 4′-amine were highly active for *S. faecalis*, 4-aldehyde was highly active for *L. casei*, whereas PN, 4-aldehyde and 4′-amine are equally active for a yeast *Saccharomyces carlsbergensis* (*12*, *13*). Here, it became clear that the nature of 'pseudopyridoxine' is not a single compound but a mixture of 4-aldehyde and 4′-amine derivatives of PN. He and Folkers named them 'pyridoxal' (PL) and 'pyridoxamine' (PM), respectively.

Perspectives

The discovery of PL and PM opened the way to elucidation of the biological function of PN. After he had noticed the presence of 'pseudopyridoxine', Snell took a strategy not usually employed in identifying nutritional factors. Instead of directly isolating the substance 'pseudopyridoxine' itself, he first confirmed that 'pseudopyridoxine' can be chemically formed

from PN, and then sought for more effective chemical procedures for transforming PN to 'pseudopyridoxine'. We can see here a happy marriage of biology and chemistry. Through logical induction and deduction used in classical biology such as genetics, Snell showed the presence of 'pseudopyridoxine', and through chemical consideration, predicted its structure—actually, it was comprised of two compounds. Thus, Snell was a true 'biochemist', although he was reluctant to define 'biochemistry' in a narrow sense (*1*). Today, biochemistry is becoming more and more biological. For long, chemical structural formulas have been replaced by colourful photographs of cells on the pages of biochemistry journals. When we talked with Snell, we were always impressed by his vast knowledge on every aspect of science—not only science but also literature, music, art and so on. He told us not to restrict our interest to narrow, specific subjects, and simultaneously, not to forget to keep up with the current research of our own field and related fields. One of us (H.H.) once asked him how to satisfy both of the two seemingly incompatible matters. He laughed and replied 'Keep yourself busy', in a gentle voice as usual. But he always looked like in comfort.

Vitamin B_6: mechanism of catalysis

Using the two bacteria, *S. faecalis* and *L. casei*, it became possible to quantitate separately PL and PM. It was a natural course for him to test the effect of heat sterilization on media supplemented with PL or PM. The results were remarkable: they showed conversion of PL to PM in the media. Amino acids were the possible source of the amino group of PM. He confirmed it by heating PL and glutamate and observing the formation of PM and α-ketoglutarate (*14*). This was the first discovery of non-enzymatic transamination. The transamination with PL occurred with other amino acids. Combination of these reactions (Equations 1 and 2) yielded a reaction in which amino group is transferred between different amino acids (Equation 3).

$$\text{PL} + \text{glutamate} \rightleftharpoons \text{PM} + \alpha\text{-ketoglutarate} \quad \mathbf{1}$$

$$\text{PL} + \text{alanine} \rightleftharpoons \text{PM} + \text{pyruvate} \quad \mathbf{2}$$

$$\text{glutamate} + \text{pyruvate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{alanine} \quad \mathbf{3}$$

The transamination reaction in animal tissues was first discovered in 1937 by Braunstein (*15*) of Institute of Molecular Biology in Moscow, Russia (then Soviet Union), as an amino group transfer between glutamate and alanine in the extract of pigeon muscle. Based on Equations (*1–3*), Snell predicted in 1945 that PL acts as a catalyst in the transamination reaction (*14*).

In support of this notion that PL is a biocatalyst, Gunsalus of Cornell University showed that the tyrosine decarboxylase activity of the dried cell enzyme preparation from *S. faecalis* was slightly stimulated by the addition of PL, but was markedly stimulated by the addition of both PL and ATP (*16*). This was the first to suggest that pyridoxal phosphate (PLP) acts as a coenzyme. Rabinowitz and Snell (*17*) discovered another phosphorylated form of vitamin B_6, pyridoxamine phosphate (PMP), in dried yeast, liver powder and dried grass.

After his Coast Guard service, Snell spent the postwar years (1945–1951) at University of Wisconsin–Madison, where he became Professor of Biochemistry in 1947. In 1949, he and his graduate student David E. Metzler started a series of studies on non-enzymatic catalytic reactions of PL, which continued after his laboratory moved to the University of Texas at Austin. Metzler recalls in his memoir (*2*) that, one day, Snell suggested him to investigate the effect of EDTA, then a new compound, on the non-enzymatic reaction. It inhibited strongly, showing that the reactions were accelerated by metal ions. Then, by adding a metal ion such as copper, iron or aluminum to the buffer, they could establish conditions to obtain reproducible results. The first publication was the transamination reaction of Equations (1) and (2) in the presence of Al^{3+} (*18*). When other amino acids were used, various reactions proceeded depending on the substituent group of the amino acids. Those included dehydration of serine and threonine, aldol cleavage of threonine, racemization and decarboxylation. Experiments using various analogues of PL, synthesized by Miyoshi Ikawa, a *Nisei* chemist, determined the minimum structural requirements for the catalytic reaction: the pyridine ring, the 3-hydroxy group and the 4-formyl group. These findings lead to the proposal of a generalized catalytic reaction mechanism of PL (*19*). This article was the fourth of five consecutive papers from Snell's laboratory on non-enzymatic reactions of PL, crowning the beginning of the issue 3, volume 76 of the *Journal of the American Chemical Society*, 1954. The array of these papers fascinated and influenced many researchers in the world. Among them was the young Hiroshi Wada. He met Snell at the International Symposium on Enzymes, held in Tokyo, 1958, and asked if he could join his laboratory, now at University of California, Berkeley. Snell accepted the proposal. This was the beginning of the continued contributions of Japanese biochemists to his research projects (Fig. 1).

Despite the plausible mechanism for transamination proposed from non-enzymatic reactions (*14*), there was still a question whether aminotransferases actually undergo catalysis in a similar way as shown in Equations (*1–3*). Jenkins and Sizer (*20*) of Massachusetts Institute of Technology purified aspartate aminotransferase from pig heart and proved the existence of the PLP and PMP forms of the enzyme, which are converted to each other upon reaction with amino acids and keto acids. Still, however, there was an uncertainty about the role of the 5′-phosphate group of PLP and PMP in catalysis, because non-enzymatic reactions proceeded without the phosphate group and yet enzymes required the phosphorylated form of the coenzyme. Wada and Snell (*21*) showed that the apo form of aspartate aminotransferase catalysed the reaction of Equation (1). Here, PL and PM are loosely bound to the enzyme and act as substrates rather than coenzymes. They further isolated and crystallized PM–pyruvate aminotransferase

Fig. 1 Esmond E. Snell receiving a testimonial from the Japanese Biochemical Society at the celebration of his 70th birth anniversary held in St Louis, June, 1984. To his left is Hiroshi Wada. Photograph taken by S.T.

from *Pseudomonas* MA, which showed activity comparable with that of aminotransferases containing PLP as the coenzyme (*22*). It then became clear that the phosphate group of PLP is not involved in catalysis, although it is important for tight binding to the enzymes as a coenzyme.

Perspectives

The final proof for the Ping Pong mechanism of aminotransferases was provided by obtaining the PLP and PMP forms of the enzyme. In a sense, Jenkins and Sizer had 'isolated the reaction intermediates' and demonstrated the conversion from one intermediate to the other; this is the most rigorous strategy of investigating mechanisms that has been employed up to now. However, this does not negate the importance of kinetics. To know 'how the structure functions' is an important and eternal subject of biochemistry. In order to elucidate the function of the phosphate group of PLP, Wada and Snell 'cleaved' the phosphate group (actually, they used PL and PM), as reported in their first paper. This may be considered to be a methodology leading to today's site-directed mutagenesis. The result was positive. However, Snell was not satisfied with that. They continued studying to see whether Mother Nature has the 'mutation'. The result was again positive, showing that the observation in their first paper was not an artefact.

Enzyme structure and function

Since the discovery of PLP as the coenzyme of tyrosine decarboxylase (*16*), a large number of enzymes catalysing a variety of reactions had been shown to be PLP dependent by the early 1950s (*23*). Snell became interested in enzymatic β-elimination and β-replacement, and studied tryptophanase and tryptophan synthase. Newton and Snell showed that tryptophanase is a multifunctional enzyme catalysing both β-elimination and β-replacement reaction of a variety of β-substituted amino acids (*24*). Morino and Snell (*25*) showed kinetic evidence that all these reactions proceed via a common intermediate, the enzyme-bound α-aminoacrylate. Tryptophan synthase was later crystallized and its structure was solved as the first enzyme among β-lyases/synthases by Snell's former student Edith Wilson Miles and co-workers (*26*).

Another enzyme Snell investigated in detail was bacterial histidine decarboxylase. Unlike other amino acid decarboxylases, histidine decarboxylase from *Lactobacillus* 30a was independent of PLP (*27*). Riley and Snell (*28*, *29*) identified the prosthetic group as an N-terminal pyruvoyl group, which was derived from serine but not from the metabolite pyruvate. Recsei and Snell showed that the pure proenzyme isolated from a mutant of *Lactobacillus* 30a underwent a full conversion to the mature enzyme in first-order kinetics, indicating that the pyruvoyl group is derived from a serine residue in the proenzyme in an autocatalytic manner (*30*). They continued the work after Snell moved to Austin (1976), and showed that the precursor polypeptide is cleaved at Ser81–Ser82, first by formation of an intramolecular ester bond, then by elimination of the ester as carboxylate, leaving the residue 82 as an α-aminoacryloyl group, which is hydrolysed to form the pyruvoyl group (*31*).

The pyruvoyl group as the catalytic centre was the first example of the 'built-in cofactors', which result from chemical conversion of normal amino acid residue(s) in the protein. Snell called it a 'new type of zymogen'. Since then, many examples belonging to this category have been reported. These include 4-methylene-1*H*-imidazol-5(4*H*)-one (formerly believed to be dehydroalanine residue) of phenylalanine and histidine ammonia-lyases (*32*) and quinone cofactors in amine oxidases, which had been believed to be copper-containing PL enzymes (*33*).

Perspectives

The discovery of PL and its coenzyme form PLP had made clear many of the unsolved questions about the action of enzymes involved in amino acid metabolism. However, a new question arose: how does a specific type of reaction proceed in PL enzymes, while a variety of reactions can be catalysed by PL in non-enzymatic model reactions? Dunathan (*34*) of Harvard University proposed that among the three possible scissile bonds around Cα, the bond that is perpendicular to the plane formed by the imine bond and the pyridine ring is preferentially cleaved, because of the maximum σ–π overlap. This proposition, although chemically reasonable, was rather harshly criticized by biochemists. Morino recalls: 'When we had read that article (*34*), we thought it was a kind of ''daydream fantasy'' of an

organic chemist who does not know much about enzymes. But, only—as far as I know—Esmond highly evaluated that proposition.' Later, Ayling *et al*. (*35*) showed a stereospecific proton transfer in PM–pyruvate aminotransferase. Today, crystallographic structures of many PL enzymes have been elucidated; the reaction mechanism revealed by these structures showed, without exception, the labilization of the bond around Cα proceeds in a manner proposed by Dunathan [see a review by Eliot and Kirsch (*36*)].

Snell loved logic. But he was never hindered by any dogma. Although he established the wonderful world of PL catalysis, he later changed his interest to the catalytic mechanism of pyruvoyl-dependent histidine decarboxylase. His passion was even more augmented by the discovery of PLP-dependent decarboxylases for histidine, which was equally active as pyruvoyl-dependent enzymes (*37*, *38*). Why is PL necessary at all, if a pyruvoyl group can substitute for PL? Snell repeatedly talked about this issue when we were in his laboratory in the early 1980s. We think this was essentially solved by the subsequent studies by Snell, Hackert and their colleagues, which are summarized in (*39*). Although the substrate histidine forms a Schiff base with the pyruvoyl group and the substrate carboxylate group seems to be perpendicular to the plane of the pyruvoyl group, a large part of the driving force for decarboxylation comes from the hydrophobicity around the substrate carboxylate group and ionic repulsion of the carboxylate group with a glutamate residue. Thus, the pyruvoyl group does not seem to act as an electron sink. From this point of view, it is conceivable to see the facts that no pyruvoyl enzymes catalyse the same reaction as catalysed by PL enzymes other than decarboxylases, and in proline and glycine reductases the significance of forming a Schiff base with the pyruvoyl group is to convert the amino group to a good leaving group (*39*). Activation of bonds by σ–π overlap is often observed in biochemical reactions. However, too much emphasis on this is sometimes misleading. We can see a typical example in the reaction mechanism of orotidine monophosphate decarboxylase, which is still elusive but clearly does not involve resonance stabilization of the unstable carbanion (*40*).

Another long-standing problem is concerning the α-aminoacrylate and relevant intermediates formed during the catalytic reaction of β-lyases and β-synthases. Snell repeatedly questioned us why these enzymes escape inactivation while α-aminoacrylate was considered to be responsible for suicide inactivation of other PL enzyme, and why β-synthases do not undergo transaldimination to form pyruvate. The first question was solved in part by the finding of a novel inactivation mechanism involving a condensation of α-aminoacrylate and PLP rather than Michael addition by Likos *et al*. (*41*), and a subsequent demonstration of a similar inactivation mechanism for histidine decarboxylase (*42*). The second question is equivalent to why the active site lysine residue cannot attack the α-aminoacrylate Schiff base but can attack the product amino acid Schiff base, which is not answered by simple chemical logic. The enzymes must be able to distinguish the subtle difference of the intermediates, and elucidation of the mechanism will bring about invaluable knowledge in the field of mechanistic enzymology.

Metabolism of vitamins

Snell studied the metabolism of pantothenic acid and vitamin B_6 as his lifework. The outline of degradation pathway of pantothenic acid had been established in 1966 based on the enzymatic reactions involved in the pathway (*43*). Two similar but distinct pathways were identified for the degradation of PN; pathway I for *Pseudomonas* MA (*44*) and II for *Pseudomonas* IA and *Arthrobacter* Cr-7 (*45*). The enzymes involved in these pathways have been purified and characterized. They include several unique enzymes; in particular, PM–pyruvate aminotransferase as described above, and 3-hydroxy-2-methylpyridine-5-carboxylic acid oxygenase, which was the first enzyme catalysing a ring-opening reaction of heterocyclic compounds (*46*). In spite of these excellent achievements, his studies on vitamin metabolism do not seem to be appreciated properly compared with his other works. However, one should note the fact that since his discovery of PM and PL, Snell had been interested in every aspect of vitamin B_6, including its synthesis, transport, interconversion, function and degradation, and that PM–pyruvate aminotransferase, which played a crucial role in elucidating the function of PL and PM and stereochemistry of PL catalysis, is a successful outcome of these integrated studies. This is a reflection of his attitude towards science.

Epilogue

Papers published form Snell's laboratory were concise and to the point. Snell always told us that the value of a paper is not proportional to its length. As reflected in his writing style, Snell preferred simple chemical mechanisms. If we are allowed to make a mechanism as complex as we want, it is relatively easy to explain any experimental results with that mechanism. On the contrary, it is not easy to present a mechanism that is simple but accounts for various experimental results. However, the truth often resides in these simple mechanisms.

Pursuing such simple mechanisms, however, sometimes results in too much contemplation and less studying and working. Snell warned this. Only through compilation of experimental results, we can reach the truth.

Revisiting the works of Snell, we realize that, probably enzymes are not as complex as we think, but are not as simple as we expect, and re-acknowledge the importance of his attitude in doing science. Many students and postdoctoral fellows of Snell were exposed to his philosophy of science. It influenced their following lives.

Conflict of interest
None declared.

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