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Metabolism of vitamins in microbes and mammals

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Contacts and dedication

Before elaborating on some of my research that may relate to the interests of Professor Irwin C. Gunsalus, it seems appropriate to outline how I came to know and appreciate the man. My first awareness of some early work by Gunsalus was in 1958 when I was a newly arrived NIH postdoctoral fellow in the laboratory of Professor Esmond E. Snell in the Biochemistry Department of the University of California at Berkeley. In beginning my research there, I perused the literature on the ATP-dependent phosphorylation of vitamin B_6. I encountered the pioneering work of Gunsalus and his colleagues that indicated a responsible kinase in *Streptococcus faecalis* [1,2]. Other investigators had subsequently found similar enzymatic activities in eucaryotic cells and tissues, e.g., Hurwitz with yeast [3,4], Roberts and Frankel with brain [5], and Trufanov and Kirsanoba with liver [6]; however, the isolation and characterization of these kinases from any source were lacking and became my starting point on a trail initiated by Gunsalus.

After I left Snell's lab and began on the faculty at Cornell University in Ithaca, my choice of a research problem was to determine if the phosphorylation of riboflavin in mammals was perhaps also due to a kinase as indicated by Kearney to be the case in yeast [7,8], rather than transphosphorylation by alkaline phosphatase as proposed by Yagi and others [9]. This led me toward flavins and flavoproteins. With a seminar visit by Gunsalus to Cornell, his early post for graduate training in microbiology and first faculty appointment, I was introduced to "Gunny" by my colleague, Professor Lemuel D. Wright. Gunny convinced me that a visit to the University of Illinois in the summer of 1963 would be of interest. I would teach the general course in biochemistry and work on my newly synthesized FAD analogs.

Gunny made other visits to Cornell at a time (1965–1973) when Lem Wright and I were investigating biotin metabolism. We had a pseudomonad that had been isolated from soil by enrichment-culture technique to grow on biotin as a sole source of C, N, and S. Our initial publication on this bacterial degradation of biotin was in *Biochem. Biophys. Res. Commun.* [10] when Gunny was an editor and referee. This opened the door to studying the catabolism of this vitamin, again an area where little work had been done. Partial destruction of ureido carbonyl-labeled biotin in rats was reported by the Fraenkel-Conrats [11], breakdown of carboxyl-labeled biotin was shown with kidney cortex slices used by Baxter and Quastel [12], and work out of Jud Coon's lab demonstrated that extracts of liver and a soil bacterium could activate biotin to its adenylate and Coenzyme A ester forms [13]. We used our bacterial system to systematically elucidate the pathway for biotin catabolism before documenting the more limited oxidations and degradations that occur in mammals. When Gunny paid us a visit, we used him as a sounding board for some of our ideas and findings. Eventually I sent him a culture of our *Pseudomonas* species to add to the Illinois collection. More than this, I also sent Gunny one of my students, John Tsibris. John worked with Gunny from 1965 to 1971 when, with such colleagues as Beinert, Orme-Johnson, and Munck, the non-heme iron–sulfur complex of putidaredoxin was elucidated. As an aside, I would also mention that I sent Gunny slides of photomicrographed crystals of enzymes that were in the slide collection of Professor James Sumner, a Nobel laureate enzymologist at Cornell during Gunny's earlier days there.

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A furtherance of our research on bacterial metabolism and its extension to mammals was with lipoic acid, where again only a little was known of its catabolism from some Japanese work [14,15]. Jason Shih in my lab at Cornell isolated *Pseudomonas putida* LP, which was used to elucidate the pathway for degradation of this cofactor, which is a vitamin for some microbes. Its essential function in mammals was of interest to Lester Reed, a former associate with Gunny, who did much to unravel the transacylation reactions involving lipoyl residues in α-keto acid dehydrogenase complexes. Our work on lipoate metabolism in bacteria and rats spanned the period of 1974–1980. The redox behavior of lipoate and its side-chain shortened metabolites is currently of interest to pharmacologists and those concerned with antioxidant activity of natural compounds.

Though my trail of cofactor research entails considerably more than alluded to in the above [16], it is in the area of vitamin metabolism, both conversion to functional forms and their turnover, that I found common ground with some of Gunny's interests. This is particularly the case as it involved the use of prokaryotic bacteria to help unravel pathways common to themselves and, at least in part, to the eucaryotic human and other mammals. It is with respect and thanks that I dedicate this paper to Gunny.

Comparative metabolism of microbes and humans

It is a long-held tenet of biochemistry that what is learned of metabolic events occurring in bacteria, often *Escherichia coli*, applies in whole or in part to higher organisms such as the rat or human. Because pseudomonads are chemotrophic and capable of converting numerous organic compounds to life-sustaining metabolites, it is not surprising that these organisms are often isolated from soil by culture enrichments that incorporate less-than-usual, energy-utilizing nutrients in the medium. Gunny, an expert on the genus *Pseudomonas*, used a *putida* species to study the metabolism of camphor and isolate and characterize metabolites and enzymes involved. Some of this is covered in other articles of this dedicatory issue. Snell and his associates also used this technique to isolate catabolites of vitamin B_6 from cultures of two pseudomonads; this subject is covered in detail by Richard Burg [17]. For my own work with cofactors, including vitamins and coenzymes, it was similarly rational to isolate pseudomonads capable of living on certain vitamins as a food and energy source. We could then obtain and chemically identify the metabolites derived from the vitamin as well as characterize those enzymes responsible for the metabolic conversions. Findings from microbial systems, where relatively large quantities of material allowed facile isolations, were for us often a first step toward determining the case for mammals. Findings that relate more closely to the title of this paper are summarized in the following text.

Vitamin B_6

Pyridoxal (pyridoxine, pyridoxamine) kinase

Isolation of and comparative studies on both pro- and eucaryotic forms of pyridoxal kinase allowed us to delineate general properties, including the first substantiated role of Zn^{2+} in preference to Mg^{2+} in the cosubstrate ATP complex for the mammalian phosphokinase and led to circumscription of inhibitory aspects, including the potent action of carbonyl reagents and such drugs as are known to bind to the kinase.

Pyridoxine (pyridoxamine) 5′ phosphate oxidase

Japanese researchers had succeeded in enrichment of the FMN-dependent oxidase responsible for conversion of the kinase-derived phosphovitamin B_6 to coenzymic pyridoxal 5′-phosphate, but we succeeded in the first complete purification of the oxidase from liver. More facile affinity purification and assays were developed and structural requirements for substrate and coenzyme specificities delineated. The oxidase requires the 5′-phosphate for substrate but is fairly tolerant of substitutions on the 4-aminomethyl function. Systematic elucidation of the homodimeric subunit association, active-site amino acid residues, the different kinetics for the two natural substrates, and ultimately mechanistic delineation of stereochemical aspects have provided definitive information on the way this essential flavoprotein operates in the ionic abstraction of a substrate hydrogen. The oxidase depends upon the flavin status of an organism and participates in the regulation of B_6 metabolism. The sequences for this oxidase from several organisms have been determined. An important interface between vitamin B_2 (riboflavin) and B_6 is now clear.

The scheme given in Fig. 1 outlines the sequential roles of kinase and oxidase in the interconversions of B_6 vitamers toward the coenzyme pyridoxal 5′-phosphate.

Riboflavin

Flavocoenzyme biosynthesis

The phosphorylation of riboflavin to form its 5′-phosphate (FMN) in mammalian tissues and elsewhere was shown by us to be catalyzed by flavokinase, which is another Zn^{2+}-preferring enzyme. Its enrichment from

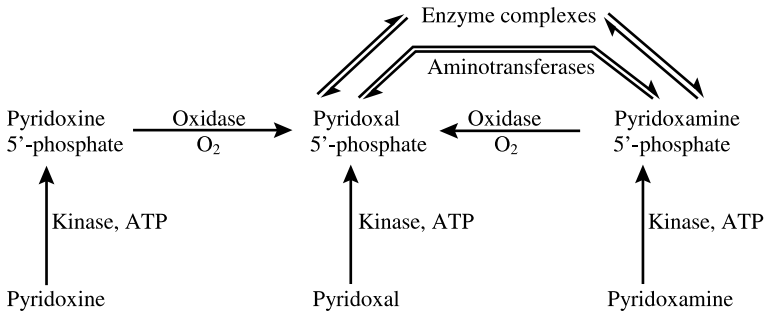

Fig. 1. Vitamin B_6 conversion to its coenzyme. Reactions occur in numerous organisms, both pro- and eucaryotic.

liver was accomplished by classic techniques and then purification done with affinity chromatography. Detailed studies on the specificity of this enzyme helped clarify the biological activities of flavin analogs. Investigations of substrate induction and thyroid hormone stimulation led to recognition of the "active" and "inactive" forms, which are poised at the regulation site for flavocoenzyme biosynthesis.

We elaborated the substrate specificity of mammalian FAD synthetase and accomplished its complete purification using FMN-agarose. Interestingly this synthetase is not a nucleotide pyrophosphorylase as earlier reported by Arthur Kornberg for the yeast enzyme [18]. Further work on the Mg^{2+}-preferring enzyme led to more detailed characterization of the cooperatively interactive kinase-synthetase system from liver and to the kinetic order as regards substrate addition and product removal. In *Brevibacterium ammoniagenes*, there is a single polypeptide with kinase activity stimulated by Zn^{2+} and synthetase by Mg^{2+} [19].

Flavin turnover

Nonspecific acid and alkaline phosphatases in many organisms have been separated and generally characterized as degradative hydrolases responsible for breakdown of flavocoenzymes.

A bacterial ribityl side-chain oxidizing enzyme that had been called a "hydrolase" was found by us to have relative specificity, whereas another enzyme narrowly specific for riboflavin was molecularly cloned and sequenced from *Schizophyllum commune*. We showed this 5′-hydroxymethyl oxidase produces both aldehyde and acid products, which had been named "schizoflavins" [20].

We have helped detail the overall metabolic fate of riboflavin in mammalian tissues, urine, and milk. The finding that some of the flavins in milk are potentially antivitaminic is of interest, as is the occurrence of an 8α-sulfonyl flavin in human urine as a result of the turnover of the thioether-linked FAD in monoamine oxidase. The predominant catabolite of riboflavin to appear in blood plasma following ingestion of the vitamin is 7α-hydroxyriboflavin. The in vivo kinetics of riboflavin absorption and disposition have been quantitated in both rats and humans.

The scheme given in Fig. 2 outlines comparative aspects of riboflavin metabolism.

Biotin

Biosynthesis

Proof that biotin is formed directly from desthiobiotin was accomplished by our use of the radiolabeled precursor of the vitamin presented to *Aspergillus niger*.

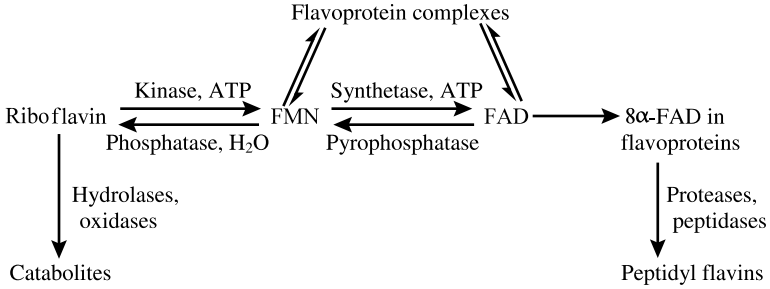

Fig. 2. Riboflavin metabolism. Biosynthesis and turnover of flavocoenzymes occur in most cells of both pro- and eucaryotic organisms.

Catabolism

The fate of biotin and some of its analogs when wholly degraded in a pseudomonad and partly degraded in a fungus and in the rat was elaborated in our laboratory. Also a discriminating colorimetric reaction for biotin and analogs was developed. Present knowledge of the metabolism of biotin is based on these detailed studies. Whereas a soil pseudomonad forced to use biotin as the sole source of C, N, S, and energy can effect extensive degradation of the vitamin, including the bicyclic ring system, mammals, including humans, operate more sparingly, mainly on side-chain β-oxidation and oxidation of the ring sulfur.

Based on the numerous biotin catabolites we have isolated and the known function of ε-lysyl-linked biotin in carboxylases, an overview of events is summarized in Fig. 3.

Lipoate

Similar studies have been conducted on the catabolic fate of lipoate, an essential cofactor that becomes covalently linked to the ε-amino functions of specific lysyl residues of transacylases. Lipoate is a vitamin for some microbes but not the mammal, which can biosynthesize it from the level of octanoate. We first detailed degradation of lipoate in a pseudomonad isolated by enrichment culture from soil and then examined the fate of lipoate in the rat. Syntheses and delineation of the properties of critical side-chain-shortened catabolites, e.g., bisnor- and tetranorlipoates, were also accomplished as were HPLC separations of metabolites.

The routes for function and catabolism of lipoate are shown in Fig. 4

Applications and conclusion

Even before it became commonplace for university administrators to encourage their faculties to consider extensions of their research that might lead to patents (and hence shared monies), it was inherent in the nature of those who funded research to expect that at least some findings would lead eventually to applications that would directly or indirectly improve the lot of humankind. During investigations of cofactors, metabolites, and enzyme systems that have occupied my attention and that of my colleagues [16], several extensions of our basic findings have led to useful applications [21]. Though few such achievements are the result of only one individual or group, we have played at least a significant and literature-documented role in pioneering biochemically specific ("affinity") absorbents, exemplifying transporter-enhanced drug delivery with vitamins, correcting and expanding means for calculating and graphing modifications of biopolymers (e.g., enzymes), photoinactivating pathogens using riboflavin, and improving the bases for dietary recommendations for certain vitamins. Again, at least an intellectual connection to such applications as derive from Gunny and his use of pseudomonads is the fact that some of these bacteria can degrade crude oil, useful as a fuel and starting material for other petroleum products but a real source of annoyance when fouling coastal areas. When Gunny left Illinois to work at the U.S. Environmental Agency in Gulf Breeze, Florida, I felt confident he would agree with my conservationist views.

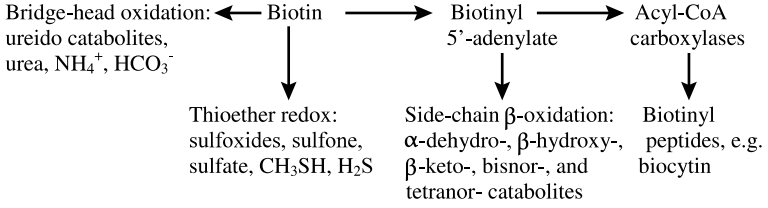

Fig. 3. Biotin metabolism. Side-chain β-oxidation and S oxidation occur in both bacteria and mammals, but extensive degradation of the bicyclic ring system occurs only in certain bacteria.

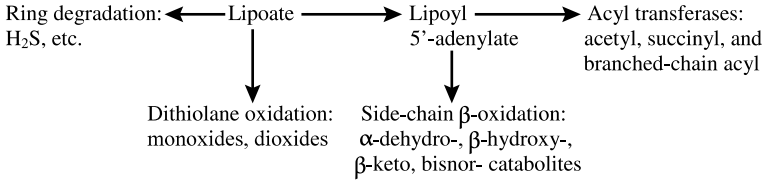

Fig. 4. Lipoate metabolism. Side-chain β-oxidation and S oxidation occur in bacteria and in mammals, but more extensive degradation of the dithiolane ring occurs in bacteria utilizing lipoate as a sole source of S and C than mammals.

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