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My association with Dr. Gunsalus in the EPA lab and beyond

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On the way to work with Dr. Gunsalus

When I finished my M.Sc. final exam in chemistry at Guwahati University, Assam, India, it was time to decide exactly in which direction I should build my career. Most commonly, the M.Sc. is the gateway to a research, teaching, federal, or state administrative service position or a job in a private company. Most of my friends joined the teaching profession, a secure job which was quite easy to get at that time. However, I was less interested in teaching but rather deep in my mind, I had high admiration for research, one of the most challenging professions. But the difficulty was to obtain entry into a quality research program, which was not easy in India at that time. The best bet was to qualify in the National Entrance Test (NET) conducted jointly by the Council for Scientific & Industrial Research and University Grant Commission, Government of India, for the award of a Junior Research Fellowship. In that case, the fellowship amount was quite good by Indian standards, and, once selected, there was every chance to pursue research for a Ph.D. at any research institute/university in greater India. However, with little confidence, I took the NET (1988) during my university final year. Although I performed well within my limitation, I had very little hope because only a couple of hundred would be selected throughout the nation covering all the disciplines. Fortunately, luck was with me to qualify, which relieved me from all sorts of thoughts of compromising with various professions in career building.

Subsequently, I shifted to Calcutta and joined the Bose institute, one of the most prestigious research institutes in India, to enroll in the Ph.D. program under Dr. Timir B. Samanta in the Department of Microbiology. There, I was supposed to work on the P450-mediated hydroxylation of steroids by filamentous fungi. Since P450 always goes with Dr. I.C. Gunsalus (popularly known as Gunny in the scientific world), I heard a lot about him from Dr. Samanta, who had worked with him in the early 1980s at the Urbana campus. Slowly, I became interested in learning about his work [1] and was excited by his huge number of publications and also the citations of his work by others. After I was awarded the Ph.D., I wrote to Gunny about the possibility of a postdoctoral position. At that time, he was at the U.S. Environment Protection Agency Lab at Gulf Breeze, Florida. After a couple of months of complete silence, I received a letter from Gunny suggesting that I write to the National Research Council (NRC) with a proposal for a research associate position. I became excited and immediately sent my application to the NRC to work with Gunny as my adviser in Gulf Breeze. Almost 6 months later, I got a congratulatory message from Gunny, and felt like a dream had come true, but things turned in the wrong direction when I was refused a visa for no apparent reason. I became tense because of the number of U.S. visa applications of my friends in Calcutta turned down during those days. I wrote to Gunny for help. He was visiting New Delhi, to attend the 16th International Congress of Biochemistry and Molecular Biology, in September of 1994, and I asked to see him there. I was thrilled to meet the great man in person for the first time, but I was concerned about the visa matter and the future ahead. During our short discussion in New Delhi, he indicated that he would try to sort out the matter when returning to the States. However, on my second attempt I got the visa, and I celebrated with my friends.

My association with Dr. Gunsalus: professional and personal

I made my trip to the U.S. on November 2, 1994, and was received at the Pensacola airport by Dr. Peter

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Chapman, who worked at the EPA Lab and was one of Gunny's students in the 1960s. I stayed for a month in Gunny's apartment at Pensacola Beach, and then moved to a nearby apartment. I felt lonely, but got inspiration looking at Gunny in his eighties, living alone and doing great! When I used to live at his place, on a couple of occasions, I would find him working with his computer even late at night. However, to keep himself physically fit, every morning, he used to go for a swim at the nearby hotel, finish his breakfast and newspaper reading, and come back to work early.

The EPA Laboratory at Sabine Island was 1.5 miles away from my apartment. The lab is located on a small island in the Gulf of Mexico very close to Pensacola Beach, one of the tourist spots in Southern Florida, famous for its white sand. A seasonal hurricane is the only disadvantage of this place. I can remember that during 1995–1996, the beach residents including Gunny and I had to leave twice because of the danger and the water was so deep that the entire beach area, including the EPA Lab remained closed for more than 10 days.

I rode a bicycle until I managed to get a driving license (on my second attempt) and buy a car. During my bicycling period, Gunny used to help me with shopping and also gave me a lift to the lab, particularly at night for my experiments. I started to look at the possible involvement of a P450 monooxygenase in the catabolism of *p*-cymene. Gunny was interested in P450cym since the problem was initiated in the late phase of his long career at the University of Illinois, though a detail characterization of the enzyme system involved remained unresolved [2]. I found a couple of strains from the collections of Gunny and Peter which were found to degrade *p*-cymene as sole carbon source. Gunny is well known for his extensive investigations on P450cam, the camphor monooxygenase from *Pseudomonas putida*, the first well-studied bacterial P450, which was later established as a model system [3–8]. Soon after exploring the details of oxidation of the bicyclic terpene, camphor, he managed to find another bacterial P450 for linalool, an acyclic terpene [2,9,10]. So I had the feeling that he had a great interest in finding one for a monocyclic terpene, such as *p*-cymene, which might help clarify the structure–function relationships of bacterial P450s in the oxidative metabolism of terpenes in general. A couple of months of effort to find a carbon monoxide difference spectrum characteristic of P450 monooxygenases for *p*-cymene did not succeed. As a time-bound project, ultimately, we had to leave the idea of identifying a P450 but rather became focused on resolving the catalytic model of the methyl hydroxylase in *p*-cymene metabolism.

Among the strains explored, a *Pseudomonas aureofaciens* strain was the most potent and was chosen to study the metabolism of *p*-cymene. The strain was among the most active, carried a broad-host-range conjugal fertility, and expressed the oxidation of methyl substituents of other aromatics as well. The oxidative pathway beginning with the methyl group, followed by ring dioxygenation and fission, was ascertained from the characterization of metabolites formed both in spent culture and in resting cells. To understand the structure–function relationships of methyl monooxygenases, enzyme separation and purification were assumed to be most important at that time. While I was preparing for that, Gunny advised me to visit Prof. Sligar's lab at the Beckman Institute in Urbana to work on protein purification. I took the opportunity, and Prof. Sligar was kind enough to allow me to use his resources and also provided one of his postdoctoral students to help me. Within a couple of weeks, I was able to partially purify the reductase associated with the *p*-cymene methyl hydroxylation enzyme system. Finally, back at the EPA lab, the reductase was purified to homogeneity by a combination of conventional column chromatography and FPLC and characterized as having two irons, two sulfurs, and one FAD per molecule.

The N-terminal sequence as well as the intermediate sequences from the tryptic/CNBr digest of the purified protein allowed me to construct degenerate oligo(s) for cloning and characterization of the genes responsible for the upper metabolic pathway [11]. Here, I must mention Stephen Francesconi (Steve), the other NRC research associate and a good friend of mine, Richard Eaton, and Richard Devereux of the EPA Lab, who helped me as needed. Subsequently, PCR analysis followed by cloning and sequencing of a 1-kb fragment characterized as the partial reductase gene was used as the probe for characterization of the complete upper pathway by molecular hybridization techniques. Functional analysis indicated that the *p*-cymene methyl hydroxylase system has three components: a reductase, a terminal oxygenase with a catalytic iron center, and a small intermediate protein of 164 amino acids. The small component was found to enhance hydroxylation (unpublished data).

During that time, most attention was focused on hydrocarbon-associated, iron-containing mono- and dioxygenases and on a smaller number of flavin-dioxygen complexes, including those leading to luminescence. Among the prominent iron prosthetic groups documented were the P450 heme-thiolate center, the di-iron oxy-bridged center found in methane and ring monooxygenases, the dissociable ferrous, non-heme oxygenases, and the ferric and ferrous aromatic ring fission centers. Electron transfer proteins were believed to serve in the pathways of respiration, photosynthesis, and a variety of oxygenations. Although the enzymes function similarly, they were found to have diverse molecular makeups, dependent on the terminal electron acceptor and catalysis toward various substrates. Increasing numbers of gene–enzyme superfamilies were evident from sequence data. With a view to understanding the

effect of the electron transport protein superfamily on biosphere chemistry, Gunny, Stephen Francesconi, and I had several discussions to explore the possibility. In this effort, molecular analysis and sequence alignment of the best documented 54 amino acid sequences of the reductase revealed that two broad groups exist in nature and retain characteristic binding motifs and conserved domains for flavin and pyridine nucleotide. Some of the reductases were found covalently linked with electron transfer enzymes, indicating several forms of gene organization. The two-iron, two-sulfur forms include chloroplast/plant and Rieske types with four ligands, either Cys_4 or Cys_2His_2. Crystal structure data of a number of reductases have revealed folding patterns characteristic of the two groups of reductases. Phylogenic analyses revealed the evolutionary relationship of the reductases originating from two basic lineages, and gene origins appear to be common to both pro- and eukaryotes, with frequent horizontal gene exchanges (unpublished data).

Mostly, I discussed research problems with Gunny but I was also lucky to get the advice of Peter, who always gave me his valuable time. I also had a good time with Sergey Selifonov, a big guy of Russian origin, who used to work with Peter. We discussed science a lot, relaxed on the weekends fishing on Sabine Island, and played chess. We thought about the possibility of looking at the biodegradative potential of methyl-substituted naphthalenes. Eventually, we found that 2,6-dimethylnaphthalene (2,6–DMN) and a number of its isomers served as growth substrates for *Sphingomonas paucimobilis* (strain 2322), a strain from Peter's collection, isolated from a creosote-contaminated site by enrichment on phenanthrene as sole carbon source. The methylsalicylate pathway of 2,6-DMN metabolism observed in this study had been demonstrated clearly. Interestingly, the metabolic intermediate 1,2-dihydro-6-methyl-1,2-diol-2-naphthoate detected in the culture broth indicates the dioxygenation of 6-methyl-2-naphthoate and the probable involvement of a benzoate/toluate-type dioxygenase. The complete pathway for 2,6-DMN assimilation was ascertained from various metabolites obtained from spent culture medium and/or resting cell transformations. Different dimethylnaphthalene isomers metabolized by 2,6-DMN-grown cells indicated the presence of benzoate/toluate-type and naphthalene/phenanthrene-type reductive dioxygenases specific for the metabolic pathway of DMNs with a methyl group on each ring or two methyl groups in a single ring furnishing methyl- and dimethylsalicylates respectively. Further, the degradation of various monomethylnaphthalenes by 2-6-DMN-grown cells furnishing (methyl)salicylate and phthalate indicated the involvement of multiple metabolic pathways [12].

I had a very good time with Gunny. Very often, we went out to dine, and he also used to come to my place for dinner. Gunny was very fond of having a variety of dishes and liked spicy Indian food. Surprisingly, he had no problem with hot preparations. The photographs in Fig. 1 capture one of those moments. The other faces in the photographs are members of Gunny's clan visiting us, who used to work with Gunny years back, and Stephen Francesconi of the EPA lab. At the dinner table, apart from discussion about research, I had the opportunity to learn a lot about science in the early days, Gunny's association with different scientists during his research career of more than 60 years, their contribution to science under difficult situations etc., and also about countries and cultures around the world, including the United States.

Fig. 1. Dr. I.C. Gunsalus with others at dinner parties. Clockwise, beginning with Gunny (upper right): Dr. S. Francesconi, the author, Dr. A. J. Ullah, and Dr. D. Ghosh.

After a stay of 2 years in the EPA Lab, I visited India for a month and during that period was married. Gunny was very happy about this, and immediately after our return, he arranged a welcome party for my wife, Moutusi, at the Dune Hotel. All of our colleagues in the EPA lab were invited. Gunny has a great interest in art and classical music, and so has my wife, and she was highly impressed by Gunny's art collection. A party was arranged for Gunny at my place on his 87th birthday at which my wife presented him with a watercolor painting of the crystal structure of P450cam, to his delight.

Beyond the EPA Lab

After the completion of my 3 years as NRC research associate, I left the country as part of the condition of the assignment. Later, I contacted Dr. Shigeaki Harayama in Japan, whom Gunny knew very well, and joined his laboratory at the Marine Biotechnology Institute at Kamaishi, in the northern part of Honshu. I worked there on the fate of crude oil in the environment to learn more about natural processes such as evaporation,

dispersion, photooxidation, and microbial degradation. I developed a couple of methods there for the analysis of certain classes of petroleum products [13,14] and studied the role of photooxidation, and microbial degradation of crude oil [15,16]. Later, I decided to settle permanently in my home country and accepted an offer of a faculty position back at the Bose Institute in Calcutta. These days, I am trying my best to work in the areas of environmental microbiology and microbial biotechnology within our limited resources [17].

Finally, it is an esteemed privilege to participate in this issue honoring Dr. Irwin C. Gunsalus to remember with gratefulness the amicable hospitality with which he treated me during my postdoctoral years (1994–1997) in the US EPA lab. My career and, indeed, my life were profoundly influenced by Gunny and I hope I can work with him again some day. I am still in regular contact with Gunny. Our communications beyond are about science as well as other subjects. He always encourages me in good science and better living. I wish him fine health to be active as ever.

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