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Hydrogen peroxide and central redox theory for aerobic life
A tribute to Helmut Sies: Scout, trailblazer, and redox pioneer

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ABSTRACT

When Rafael Radi and I wrote about Helmut Sies for the Redox Pioneer series, I was disappointed that the Editor restricted us to the use of "Pioneer" in the title. My view is that Helmut was always ahead of the pioneers: He was a scout discovering paths for exploration and a trailblazer developing strategies and methods for discovery. I have known him for nearly 40 years and greatly enjoyed his collegiality as well as brilliance in scientific scholarship. He made monumental contributions to 20th century physiological chemistry beginning with his first measurement of H_2O_2 in rat liver. While continuous H_2O_2 production is dogma today, the concept of H_2O_2 production in mammalian tissues was largely buried for half a century. He continued this leadership in research on oxidative stress, GSH, selenium, and singlet oxygen, during the timeframe when physiological chemistry and biochemistry transitioned to contemporary 21st century systems biology. His impact has been extensive in medical and health sciences, especially in nutrition, aging, toxicology and cancer. I briefly summarize my interactions with Helmut, stressing our work together on the redox code, a set of principles to link mitochondrial respiration, bioenergetics, H_2O_2 metabolism, redox signaling and redox proteomics into central redox theory.

1. Intercellular versus intracellular O_2 gradients

My PhD research in the early 1970's was on the biochemistry of hypoxia with Howard S. Mason in Portland, Oregon; by the time I graduated I was introduced to the leading redox biochemistry research of the day, including recent research on superoxide dismutase by McCord and Fridovich, chemiosmotic coupling by Mitchell, and catalase function in liver by Sies and Chance. Arguments abounded at that time about whether cytochrome P-450 and cytochrome P448 were really two enzymes or only one. This was before molecular biology; enzymology was king. Methods were relatively primitive for protein purification, computers were very limited (1K of memory cost $1000), and advanced spectroscopic methods were rapidly being development. The major discovery of my dissertation research was that under hypoxic conditions, the mitochondrial O_2 consumption in hepatocytes was sufficient to establish intracellular concentration gradients of O_2 to mitochondria while O_2 dependence of cytochrome P450 in the endoplasmic reticulum experienced only a minimal O_2 gradient [1]. This research directly linked me to Helmut Sies, who was also studying tissue O_2 gradients at the time.

And this connection continues to current times. At the recent Oxygen Club of California Meeting in Valencia, Spain, Salvador Moncada delivered a splendid keynote address in which he described his work with a newly developed device which he described as an "oxystat". As I walked by after his presentation, I overheard Helmut cordially telling Salvador that his group had developed an "oxystat" a few decades earlier [2]. I could not resist teasing Helmut that he was late with his development, that I had described an oxystat earlier as part of my PhD research [3]. Helmut seized the moment and commented that we should certainly obtain a photo of this historic oxystat trio (Fig. 1).

Upon receipt of my PhD, I went to Cornell University in Ithaca to study enzymology of stearoyl-CoA desaturase with Jim Gaylor. The nature of this enzyme was only poorly characterized at this point in time, and Jim was interested in gaining an understanding of a protein fraction that stimulated desaturase activity. Generation of hydrogen peroxide upon addition of NADPH to microsomes was known from Lars Ernster's research, and Jim was interested in whether the auxiliary protein was functioning to change the stoichiometry of the desaturase from an H_2O_2-producing to a non-H_2O_2-producing activity. My first stoichiometry studies on the

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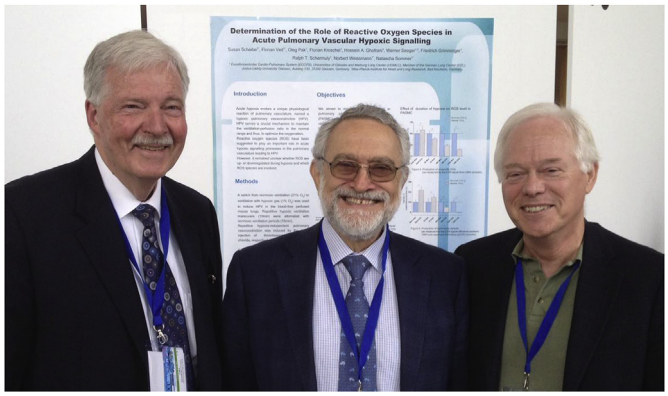

Fig. 1. Legendary Oxystat Trio. Author with Professor Helmut Sies (Left) and Professor Salvador Moncada (Center) following Professor Moncada's keynote presentation at the 2015 Oxygen Club of California meeting in Valencia, Spain.

desaturase activity showed that the enzyme produced no H_2O_2. But because I had no positive control, i.e., an enzyme which definitely produced H_2O_2, I did not have a satisfactory answer. So I proceeded to develop a different H_2O_2 production assay, and finally a third H_2O_2 production assay, before I finally concluded that H_2O_2 was not produced by stearoyl desaturase [4]. In the process of these "failed" experiments, I became an authority on measurement of H_2O_2 production. When I moved to Stockholm the following year to continue my studies with Sten Orrenius, I was already an expert in measurement of H_2O_2 production.

The time at Cornell was doubly serendipitous. During this period of time, Britton Chance invited me to the University of Pennsylvania to work with him on respiration-dependent O_2 gradients in hepatocytes (which I published later independently from my own laboratory; [5]). This gave me hands on experience with the methods that Helmut Sies had used in his pioneering studies on H_2O_2 production in perfused rat liver [6]. In the process of my studies with Britt, I obtained independent confirmation of O_2 gradients in hepatocytes. More importantly, Efraim Racker, a Cornell biochemist noted for his pioneering work on the mitochondrial ATPase (Complex V, ATP synthase), was hosting Helmut Sies as a visitor to the Department. Efraim knew that I had worked with Britt and felt that this would be useful for me to meet Helmut.

As I recall, Helmut and I had a good discussion. After I explained my PhD research and conclusions, however, he told me that he had already shown this and he would send a copy of his paper. As imaginable, I was totally deflated. But then when I received his paper [7], I found that even though he referred to intracellular indicators in his title, he had only concluded that there were "intercellular" O_2 gradients in liver. Thus, my detection of "intracellular" O_2 gradients, was the first publication showing heterogeneity of O_2 concentration within cells [1]. I followed this with studies showing O_2 gradients in cardiac myocytes [8] and proximal kidney cells [9], ATP concentration gradients in hepatocytes [10], pH gradients in proximal tubule cells [9], and H_2O_2 gradients in hepatocytes [11]. These provided the foundation for the concept that kinetic limitations are central to the redox organizational structure as we recently included in the redox code (described below; [12]).

Years later, when I recalled this first meeting to Helmut, he had no recollection. As I guess this meeting should have been: he was much more impressive to me than I was to him. At least he remembered me from the Glutathione meeting in Schloss Reisensburg the following year (see: [13]).

2. H_2O_2 production in hepatocytes

I had the remarkable good fortune to join Sten Orrenius in 1977, just after he had purchased an Aminco-Chance dual beam spectrophotometer. I arrived with experience using Britt's homemade prototype instrument and also had my experience measuring H_2O_2 during studies of the stoichiometry of the desaturase. Don Reed was visiting on sabbatical at the time and had recently developed a sensitive HPLC method for GSH and GSSG [14]. Within a very short period of time, we were able to show that certain cytochrome P450 substrates stimulated H_2O_2 production in hepatocytes [15] as measured by both the spectrophotometric method that Helmut had pioneered [6] and by stimulation of GSSG efflux, a process also described by Helmut [16].

I pursued research on H_2O_2 metabolism when I set up my own laboratory at Emory. These studies included collaboration with Sten showing that H_2O_2 metabolism is spatially resolved, with low rates of H_2O_2 production in peroxisomes being metabolized in peroxisomes and low rates of H_2O_2 production in the endoplasmic reticulum being metabolized by GSH peroxidase rather than peroxisomal catalase [11]. Years later, Michel Toledano concluded that specificity in H_2O_2 signaling is obtained by localized H_2O_2 generation and metabolism [17], apparently without realizing that local generation and metabolism of H_2O_2 was established decades earlier [11]. From our series of studies on intracellular gradients, we also derived an equation to predict when intracellular gradients will occur [18]. Application of this to redox signaling shows conditions under which substantial local H_2O_2 gradients exist for redox signaling (Fig 2).

My controlled titration studies [19] provided an independent confirmation of Helmut's finding that intracellular H_2O_2 concentration is in the low nanomolar range. More recent modeling studies of H_2O_2 metabolism also support this interpretation [20]. Thus, the modern view of H_2O_2 generation and metabolism is largely developed from Helmut's pioneering research published in 1970. Available evidence indicates that H_2O_2 is a ubiquitous metabolite under aerobic conditions. As we developed in the redox code, this steady-state concentration of H_2O_2 provides a context to maintain redox organization and function.

3. Functions of GSH

Helmut had already been studying GSH for a few years [21] before I began to appreciate its importance [15]. I was well aware of Helmut's research on subcellular compartmentation of NADH/NAD and NADPH/NADP systems in liver [22]. He established the steady-state values for these couples in mitochondria, cytoplasm and nuclei, and these data provided a foundation for research in my laboratory to subsequently determine steady-state redox potentials for GSH/GSSG and thioredoxin couples within the different compartments [23–26].

My introduction to GSH research came from the nutritional story of the selenium requirement for GSH peroxidases [27]; my introduction to Helmut's research came from his studies on GSH and GSSG release from liver [28]. Helmut had made important contributions on Se deficiency effects on GSH peroxidase and cellular GSSG release from liver in studies with Ray Burk [29]. For my research, his series of prescient experiments showing the disequilibrium between the NADPH/NADP couple and the GSH/GSSG couple in liver [21] were probably most important. In a conversation that I had with Sir Hans Krebs in the late 1970's, it was apparent that Krebs understood the fundamental importance of Helmut's research. Krebs described regulation of the pentose phosphate pathway by GSSG as a mechanism to stimulate NADPH supply to maintain GSH [30].