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Helmut Sies and the compartmentation of hydroperoxide metabolism

Leopold Flohé [a, b]

[a] Universidad de la República, Facultad de Medicina, Departamento de Bioquímica, Avda. General Flores 2125, 11800, Montevideo, Uruguay
[b] University of Padova, Faculty of Medicine, Department of Molecular Medicine, Viale G. Colombo 3, I-35121, Padova, Italy

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ABSTRACT

The early work of Helmut Sies on mammalian hydroperoxide metabolism is reviewed with particular emphasis on the *in situ* function of catalase and glutathione peroxidase1. Starting out from a catalase-dominated thinking in the middle of the last century, Sies first demonstrated, by whole organ spectroscopy, that H_2O_2 is generated in rat liver and metabolized by catalase. In a joined effort with the author's group, he then worked out that glutathione peroxidase can kinetically compete with catalase in hydroperoxide metabolism *in situ*. In compartmentalized cells, however, the "competition" of the two enzymes turned out to be a mutual complementation because of their different subcellular location. The studies for the first time documented that the metabolism of freely diffusible hydroperoxides is compartmentalized and, thus, paved the way to a better understanding of oxidant challenges and redox regulation. The article, garnished with personal memories, is meant as a nostalgic journey though ancient times of biochemistry with their changing fashions and paradigms, revealing the roots of topical perspectives and controversies in redox biology.

1. The start with an unfriendly letter

It was in the early 1970s when I received a letter from a colleague from Munich named Helmut Sies asking if I had not read his recent publication on the generation H_2O_2 and its metabolism [1]. Being a lonesome post doc left behind by my mentor Friedhelm Schneider at the Tübingen University, I did not know Helmut, yet I recognized the institute where he was working, the prestigious *Institut für Physiologische Chemie und Physikalische Biochemie der Universität München*, headed by Theo Bücher. I could also find out that Helmut had worked with Britton Chance at the Johnson Foundation in Philadelphia, at that time the leading center of research on catalase and mitochondria, as well as of analytical developments. Combining the expertise and skills of these groups, Helmut had managed to monitor the oxidation state of catalase in a rat liver lobe by whole organ spectroscopy. Thereby he had not only for the first time demonstrated that H_2O_2 is generated in an intact organ, but also that it is metabolized by catalase, exclusively, as he believed, since it was kind of a dogma at the Johnson Foundation that no other enzyme can beat catalase in metabolizing H_2O_2. It was this latter assumption that I had challenged in some of my early papers.

At that time, I was working on an enzyme that had been discovered by Gordon C. Mills in 1957 already [2] but practically ignored throughout the 1960s: glutathione peroxide (GPx; now GPx1). As I later learned from Gerald Cohen from the Mount Sinai Hospital in New York, the king of peroxidase research, Britton Chance, had declared this enzyme to be irrelevant or not to exist at all at a Federation meeting [3]. In consequence, almost everybody had dropped the issue like a hot potato, except for few ignorant Europeans. As one of those, I had isolated and characterized the non-existing enzyme as part of my master thesis [4]. Making use of the de Duve technology [5], we also had studied the subcellular distribution of GPx in rat liver and had found it located complementary to catalase, in the cytosol and in the matrix of mitochondria [6]. Our next paper [7], the one that had evidently irritated Helmut, is peculiar in several respects: It owes its existence to a strike at the JFK Airport in New York, it demonstrates for the first time that mammalian mitochondria can generate H_2O_2, and it is the first written publication authorized by Britton Chance that mentions GPx. I had sent a student of mine, Gerriet Loschen, to the Johnson Foundation to work on the kinetics of GPx taking advantage of the fancy stopped-flow equipment developed by Britton Chance. The GPx sample, however, had not survived the strike at JFK in a hot summer. Gerriet, sitting in Philadelphia in midst of brilliant equipment and with all kind of analytical tools to measure minute concentrations of H_2O_2, but without any active GPx, did not

E-mail address: l.flohe@t-online.de.

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waste his time. Stimulated by Nozomu Oshino, he looked for H_2O_2 generation in pigeon heart mitochondria and verified its existence. Under strange conditions at least, when poisoned with antimycin A or when fed with succinate only, mitochondria produced H_2O_2. This finding was essentially enabled by the highly sensitive scopoletin/horse radish peroxidase test I had found in an early Nature article [8] and adapted for elucidating the GPx kinetics [9]. It later became known as the Britton Chance test. When Gerriet came back to Tübingen, he asked me to write up his results, which I did. However, I could not withstand the temptation to smuggle a risky sentence into the discussion: "There are two enzymes which destroy H_2O_2 at a sufficient rate: (1) GSH-peroxidase in the matrix space of mitochondria and in the soluble fraction and (2) catalase in the peroxisomes" [7]. I do not recall how the statement survived the proof reading by my co-authors; after publication, it turned out to be the provocation that called the catalase warriors back to stage.

2. Wine-catalyzed trouble shooting

Considering the reputation of Hemut's academic environment, I could not ignore his letter. However, I was neither prepared to abandon my conviction, nor to ignore the publications [2,10–13] that, although small in number, had convinced me to work on GPx. I answered Helmut's letter with an invitation to defend his view on H_2O_2 metabolism at one of our weekly seminars in Tübingen, yet did not get any answer.

At the next Annual Meeting of the German Biochemical Society, the *Mosbacher Kolloquium* in spring 1972, I met Helmut for the first time in person. I could persuade him to join me to a *Weinstube* instead of listening to the lectures in order to discuss if there were not an experimental way to solve our dispute on the roles of catalase and GPx. My idea was to use Helmut's rat liver system to detect responses indicating an *in situ* GPx reaction, when the organ was perfused with an organic hydroperoxide that cannot be reduced by catalase. By means of parameters thus characterized, it should be possible to decide if also H_2O_2 is metabolized by GPx, when it reaches the liver cell from outside or, more generally spoken, if it is not produced in peroxisomes. One of the possible parameters I had in mind was the export of oxidized glutathione (GSSG), which Srivastava and Beutler had observed in red blood cells [14] and eye lenses [15] and I hoped to also see in the liver. Helmut's key idea was to monitor the fluorescence at the liver surface continuously to detect changes in NADPH levels due to GSSG reduction by glutathione reductase. He was sure to be able to discriminate between NADPH and NADH by measuring the lactate/pyruvate ratio in the perfusate. By the end of the afternoon, each of us had drunken several *Viertele* (= 0.25 L of wine) and happily took along a pile of coasters with notes on reaction schemes, experimental details and homework to be done.

3. The decisive experiment

A few days later only, I drove to Munich together with my PhD student Helmut Menzel, who was familiar with the analysis of GSH, GSSG and hydroperoxides. When we entered the lab next morning, Helmut (Sies) had already installed the experimental set-up along the lines discussed in Mosbach and amended with electrodes to measure pH, potassium concentration and oxygen pressure. Christine Gerstenecker was calibrating the photometers for metabolite analyses. Just the fresh rat liver had to be mounted by 'the master'. Asking what would be my role in the endeavor, Helmut gave me a stop watch and informed me that I would likely have to be a fraction collector for the rest of the week.

In the evening of this first day, we had already the pivotal results. The rat liver survived our drastic treatment (perfusion with a solution being $\geq$0.6 mM in t-butyl hydroperoxide!) for at least 40 min without any sign of damage, as measured by LDH release. Upon infusion of t-butyl hydroperoxide, the liver indeed exported the product of the GPx reaction, GSSG, and the surface fluorescence sharply declined, the latter effect being proportional to the hydroperoxide load. The lactate/pyruvate ratio in the perfusate leaving the liver slightly increased, indicating that the NADH/NAD ratio was not negatively affected. The dramatic decrease in surface fluorescence could, therefore, with confidence be attributed to NADPH consumption due to GSSG reduction (Fg.1). Thus, the experimental system worked as expected and, for the first time, showed that GPx, despite the presence of catalase, works *in situ* and how the GPx action can be monitored [16] (Fig. 1).

To repeat this experiment with H_2O_2 proved to be less trivial than anticipated. The perfused liver turned pale; it seemed to suffer from air embolism. Having a closer look at the entire equipment, we could detect air bubbles already in the infusion syringe. The culprit turned out to be a black rubber of the plunger, which destroyed the H_2O_2 by kind of a catalase activity due to its heavy metal content. A colorless plunger seal solved the problem how to get the H_2O_2 solution undestroyed into the liver. Yet still the oxygen pressure in the effluent rose proportional to the H_2O_2 concentration applied, indicating a catalatic process going on also inside the liver. At the same time, however, GSSG release and the NADPH-dependent decrease of surface fluorescence was observed, which proved the H_2O_2 reduction via GPx. Evidently, the H_2O_2, infused as 0.5–1.0 mM solution, had partially passed through the cytosol, which is full of GPx, and reached the peroxisomes to be destroyed there by catalase [16]. This cannot be a surprise, since the influx of H_2O_2 or t-butyl hydroperoxide, respectively, exceeded the rate of GSSG reduction at least in parts of the liver lobe, as is evident from the massive release of accumulated GSSG and unprocessed hydroperoxide. In short, therefore, also H_2O_2, when entering the cell from outside, was shown to be partially reduced by the glutathione system [16].

4. Follow-up studies

Helmut presented a more detailed version of our short communication [16] at the *Symposium on Glutathione*, held at Tübingen on March 23–25, 1973 [17]. It was the symposium, where *inter alia* the first unequivocal proof of the seleno-protein nature of GPx was reported [18] and where the idea was born that the respiratory chain-linked H_2O_2 formation [19] might result from superoxide dismutation [20], which was soon confirmed [21,22]. Helmut had already complemented our first perfusion studies with analyses of metabolites of the freeze-clamped liver. These early 'metabolomics' proved the correctness of our interpretation of the fluorescence decline upon hydroperoxide challenge as indicating GSSG reduction. Moreover, they demonstrated an activation of the glucose-6-phosphate shunt and gluconeogenesis under the oxidant conditions. He also reported that compounds such as urate or glycolate known to generate H_2O_2 within the peroxisomes, as revealed by catalase compound I formation [23], did not lead to any significant GSSG release [17]. What puzzled us at that time was the observation that also octanoate infusion yielded the same results as typical substrates of peroxisomal oxidases, since it was believed to be oxidized in mitochondria. However, it still appears unclear to what extent the H_2O_2 generated by short chain fatty metabolism is of mitochondrial or peroxisomal origin [24,25]. An unequivocal conclusion that mitochondrial H_2O_2 is primarily reduced by GPx was later provided by a systematic reinvestigation of the roles of catalase and GPx by Oshino and Chance [26]. When perfusing the liver with benzylamine, a substrate of monoamine oxidase, which is located at the outer mitochondrial membrane, they saw a significant GSSG release that was not affected at all by inhibition of