Review

Spectroscopy of succinate dehydrogenases, a historical perspective

Helmut Beinert *

Institute for Enzyme Research and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, 1710 University Avenue, Madison, WI 53705-4098, USA

Received 10 May 2001; received in revised form 27 August 2001; accepted 12 October 2001

Abstract

An attempt is made to retrace, from personal experience, the discovery of redox-reactive non-heme iron in living matter, which turned out to occur in the form of iron–sulfur (Fe–S) clusters, and then to recount the immediate application of this knowledge in exploring the composition of the mitochondrial respiratory chain, and in the rather detailed description of the workings of its components and, for the purposes of the present volume, of succinate dehydrogenase. The relationship of these events to the general status of technology and the available methodology and instrumentation is considered in some detail, with the conclusion that there scarcely was a way that these discoveries could have been made earlier. It is then shown how methods, techniques and interpretations of results were developed and evolved during the applications that were made to a complex problem such as that of the composition, structure and functioning of succinate dehydrogenase. A tabulation of the most significant events – concerning specifically spectroscopy and its interpretations – in this development is given up to the year 2000. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Iron–sulfur cluster; Electron paramagnetic resonance spectroscopy; Magnetic circular dichroism spectroscopy; Resonance Raman spectroscopy; Mössbauer spectroscopy; EXAFS spectroscopy

1. Introduction

If we consider, as I plan to do, the development of a branch of science over a period of several decades, close to 100 years, we must also look at the simultaneous developments, trends or fashions in life in general, in attitudes, and more specifically in progress of the supporting infrastructure and technology. I will follow trends in science and in related circumstances that eventually funneled into what we now call Bioenergetics, which also encompasses the subfield to which this issue of the Journal is devoted. I want to go back to the origins of this subfield, however limiting the extent to the period to which my own experience reaches back in time. I must say though that prior to this period metal proteins were by no means an unknown subject; aside from heme proteins, laccase was recognized as a metal protein – although initially the wrong metal (Mn) was assumed – and other polyphenol oxidases became known, also proteases that required heavy metals for activity; and the difference between proteins with tightly bound metals and metal-activated proteins was appreciated.

The first decades of the century then witnessed the
feud shaping up between those emphasizing the activation of oxygen in biological oxidations and those seeing the specificity of hydrogen activation of substrates as the primary condition. I have still heard the two foremost proponents of the latter concept, Thorsten Thunberg and Heinrich Wieland, lecture on this topic. Thunberg’s pioneering studies [1] were the starting point for the subsequent work that resulted in the purification and eventually isolation and characterization of various dehydrogenases in the 1930s and on. These studies had clearly shown that, according to their different sensitivity to heat and freezing, there was a variety of specific dehydrogenases with distinct properties.

2. Comments on infrastructure and status of techniques through the century

Before we continue along these lines we must consider the state of the infrastructure and supporting technology. Seen from today it was dismal. I am afraid, spoiled as we are now, we would simply refuse to work under such conditions. Work we did, nevertheless, partly because we did not know any better; we only knew we were better off than our predecessors! To mention a few examples: protein extraction was sometimes aided by proteolytic enzymes, and purification was mainly dependent on precipitation with lead or mercury salts, which were then eliminated with H₂S; later, ammonium sulfate or organic solvents, such as acetone, ethanol or ether were used as precipitants. Separation was largely by filtration or decantation. Slowly, custom-made, non-commercial low-speed centrifuges in the liter range became available in the 1920s and on. Column separations were only developed in the 1950s, mainly with various inorganic gels as supporting material. Butanol extraction came on the scene and preparative electrophoresis or electro-decantation also became an option. The use of polyacrylamide as a medium was developed slowly from the 1950s on. On the bright side, however, the pioneering war-time effort of Edwin J. Cohn and his group on the separation of blood proteins had generated much useful knowledge of protein separation and physical chemistry, which found its way into everyday laboratory life. The analytical tools were also very limited. Colorimetry, when suitable color reactions were available, was one of the main tools. Thanks to Arnold Beckman, a useful pH meter and later a manual, battery-operated spectrophotometer, ‘the Beckman’, became available in the 1940s, similarly did high-speed, but low-volume centrifuge attachments. Analytical procedures were usually carried out on a 5–20 ml scale, with a common step too often being: ‘fill up to the mark in a 100 ml volumetric flask and use an aliquot;’ micropipettes were uncommon, except in circles that had had contact with the Carlsberg Laboratory of Linderstroem-Lang. During my work on CoA and derivatives [2], which we had to produce from scratch in our own lab, the necessity for scaling down volumes became apparent, so as not to use up much of our precious products in analyzing them.

I, therefore, introduced microliter pipettes and microcuvettes in our section of the Enzyme Institute; and, when our interest in metal proteins arose, I then undertook to work out semimicro-analytical methods for the determination of Fe and Cu [3] and later of sulfide [4] on the scale of nanomoles, which have found much use in the course of the years. It should also be mentioned that, as was the case with CoA, biochemicals, and actually much more common ones than CoA, were not commercially available; they had to be made in the laboratory or in the best case borrowed from someone, who had once made the effort to prepare them. I remember that, at either the 1950 or the 1951 Federation Meeting at Atlantic City, a New York firm, for the first time, offered AMP; ATP was what we needed! Sigma then soon followed suit, Boehringer in Germany started offering some useful biochemicals later in the 1950s and other companies arose in quick succession. There still was a lack of appreciation of the purity of reagents, which is, of course, an important aspect for work on metals as, at least Fe and Cu, are universal contaminants, as we all experienced later. Ultrapure buffers and reagents for biochemists are only a more recent development.

It is against this background that we have to evaluate the work of the years that led to the definition and description of an enzyme, such as succinate dehydrogenase (SDH), and we will recognize that, what may appear from today’s viewpoint as modest advances, were in fact major accomplishments, such as the preparation of a soluble enzyme [5,6], the find-
ing of covalently bound flavin [7] and tightly bound iron, the separation and definition of the electron transport complexes of mitochondria [8], the discovery of ubiquinone [9] and its identification as a quinone and a membrane-bound cofactor in electron transport [10], which all was accomplished in the 1950s.

3. A surge of interest in metal flavoproteins

This work and progress in related areas of biochemistry led to a surge of interest in metal proteins in general, but particularly in metal flavoproteins. There was actually one of the then famous McCol- lum–Pratt meetings at Baltimore in 1955 devoted to ‘Nitrogen fixation; function of metallo-flavoproteins’ [11] in which topics such as Mo and Fe in xanthine oxidase and nitrogenase, Cu in butyryl-CoA dehydrogenase (which turned out to be a contaminant), and iron in SDH and NADHDH were discussed. It happened to be a coincidence, and maybe an unfortunate one, that at the same time there was great interest in metal chelation [12]; the ‘versenes’ (EDTA derivatives) made headlines and were used in almost every laboratory. It is, therefore, not surprising that the function of the metals in enzymes was brought in connection with chelation and some claims were made that flavin was a good metal chelator [13,14] which was soon refuted [15]; thus, it was thought that a metal–flavin chelate was the actual electron transfer agent.

Concomitant with this development was the finding at the Enzyme Institute that there was a large amount of iron in all preparations of mitochondria and submitochondrial particulate fractions that could not be accounted for as heme iron [16]. It was toward finding an answer to this problem that my methods for metal analysis were aimed. Ernest Page and I made an effort to exclude the possibility that this iron could merely be a contaminant from reagents and tools used. We found by the use of radioactive 59Fe that there was indeed contamination, but far from the extent expected, if it were to account for the non-heme iron in mitochondria [17]. We also found that a reagent like EDTA would remove contaminating Fe, while the bulk of the non-heme Fe remained, i.e., it was tightly bound in the particles. The work on metal flavoproteins such as xanthine and aldehyde oxidases gained renewed significance in what is to follow, because it potentially could furnish clues to the iron and sulfide stoichiometry in metal flavoproteins, with the easily determinable flavin concentration as point of reference. Optical absorption or oxidation–reduction difference spectra of metal flavoproteins were not very useful [18], as long as the contribution of the metal component was uncertain. However, it was possible to determine an extinction coefficient for the putative iron chromophore, because in some cases the flavin could be removed by mild methods [19], so that the Fe–S absorption remained; and it turned out that this absorption was almost identical to that of ferredoxins ([17], Fig. 1). Particularly dihydroorotate dehydrogenase of Zymobacterium oroticum, which does not contain Mo, as do xanthine and aldehyde oxidases, became something like the smallest common denominator [20], because it had a single flavin and only two irons and two sulfides; it thus furnished another hint (see below) that the smallest unit that could...
have the features of the unknown iron chromophore had to have two irons.

After the first enthusiasm for metal or iron flavoproteins had worn off, there was actually some backlash to these concepts. Experiments using chelators specific for \( \text{Fe}^{3+} \) or \( \text{Fe}^{2+} \) to determine valency changes of the iron, and with chelators to remove the non-heme iron, had yielded ambiguous results [21–23] as one might have expected: this led to the conclusion that non-heme iron in enzymes is not involved in the catalytic steps of the respective enzymatic reactions. There were also attempts to show with bacteria and yeasts that they could live without non-heme iron and that this iron was not involved in energy producing metabolism [24]. Some more detail and references can be found in [25] (p. 23). This may seem particularly ironic at the present time, when we learned [26] that \textit{Saccharomyces} can live quite well without making ATP by oxidative phosphorylation, but they do need the proteins required for Fe-S protein synthesis to survive! To conclude this discussion on a positive note: as early as at the 1965 meeting on ‘Non-heme iron proteins: role in energy conversion’ at Yellow Springs, the editor of the volume emerging from the meeting says in the preface, without any hesitation: “The non-heme iron proteins ... function as electron carriers in a number of biological systems, including mitochondrial electron transport, photosynthetic electron transport, and nitrogen fixation” [27].

4. Circumstances, opportunities, and luck

Prior to my interest in this non-heme Fe I had been involved in work on the flavoproteins of fatty acid \( \beta \)-oxidation [28] and had been fascinated by the colored compounds that they formed on addition of substrate [29]. There was some background to this in my past, as I had been a student of Richard Kühn at Heidelberg, who was a pioneer in the early flavin work [30]. Free radicals in chemistry and then, of course, also of flavins, had always fascinated him. Were the colored compounds that I found with the acyl-CoA dehydrogenases by any chance free radicals? Flavin free radicals have similar but not identical absorption spectra [31]. I decided to settle this problem with a specific method that is independent of optical phenomena, and such a method had just come to the scene, namely electron paramagnetic resonance (EPR). In 1957 I applied to Varian Associates, who offered some customer service, flew out to Palo Alto and was met by a young physicist, who had just made his thesis in physics on EPR with George Pake [32] at St. Louis and had moved to Stanford with him. We found no significant radical formation with the acyl-CoA dehydrogenases, when the colored compounds appeared [33]. At least an answer, but much more important was my contact with Dick Sands, the young physicist, who actually had made his thesis on EPR of Fe in glasses [34]. I told him about our non-heme iron problem and about Cu in cytochrome oxidase – another problem I had been interested in – and asked him whether we could not try to look for these metals by EPR and, maybe, detect valency changes. So we made plans. However, before we could pursue them, there was another development: Dick accepted the position of Assistant Professor of Physics at Ann Arbor, Michigan, and built his own EPR spectrometer there. This was a lot closer to home and would save a lot of time and money. In 1959 we were able to run the first...
samples and luck happened to be with us ([35–37], Figs. 2 and 3).

In retrospect I recognize now how many favorable circumstances had to come together to make the early work, on what turned out to be Fe–S proteins, possible: my background, however modest, with EPR of free radicals; my interest in non-heme iron and experience with iron determination, and for that matter also with iron contamination; meeting a young, eager physicist, namely Dick Sands, who had been brought up with EPR and even with EPR of iron, and who was able to build his own EPR spectrometer, and also had EPR facilities at Varian Associates at his disposal, while I had the Enzyme Institute as a home base, with a whole group of clever young colleagues such as Joe Hateé, Dan Ziegler, Fred Crane, Tom Singer and Edna Kearney and their collaborators in David Green’s division, producing mitochondria, submitochondrial fractions and purified components of the respiratory chain, which were waiting to be investigated. I am sure, with the best of intent, a single investigator with the usual size group could not have moved nearly as fast as we did. As soon as commercial instruments became available, I acquired my own instrument in 1960 with some logistic support from David Green, which simplified matters considerably. I also had the luck that I was able to enlist the help of a clever instrumentation specialist, Raymond E. Hansen, with whom we developed our EPR setup further and also a number of accessory techniques.

In the first few years we could only work close to liquid nitrogen temperature, as neither liquid nitrogen nor helium was available in Madison; we were lucky to get some liquid air off and on, and even that not without some hassles. Thus, we could not detect with EPR spectroscopy Fe–S clusters with a spin relaxation much more rapid than that of most 2Fe clusters; but at least we knew we were on the track and had found what we set out to look for. Unfortunately, I did not pay sufficient attention to the presentation of spectra, i.e., I did not know any better and, apparently, neither did the referees of our publications. Thus the spectra in our early papers, up to 1964, do not conform to the presently used convention, namely that the absorption is presented as positive, as in optical absorption spectroscopy. If the EPR spectra are shown as the first derivative of the absorption spectrum – representing the slope of the absorption spectrum – as is usually the case, the derivative spectrum must initially rise above the baseline for the absorption spectrum to be positive. Also, the magnetic field direction is mostly reversed, i.e., right to left, in most of the early spectra. So those comparing our early spectra to more recent ones will have to consider these shortcomings.

All evidence pointed to the interpretation that the material we were looking at by EPR was a reduced transition metal ion. Chemical and spectrographic analyses left little choice other than iron. Proof that the observed signals really were due to iron
was provided with a ferredoxin from *Azotobacter*\(^1\) that had been grown in a medium containing \(^{57}\)Fe, which has a nucleus of spin 1/2 and should lead to line broadening, as we indeed observed ([38], Fig. 4). However, no EPR signals of ferrous iron were known and they should, according to theory, not be detectable under the conditions at which we were working [39]. When we published our conclusion that we must have a ferrous species in our samples, I received a handwritten, very brief letter from John S. Griffiths, the brilliant theoretician of ‘The Theory of Transition Metal Ions’ [40], which said that it was absolutely impossible that we were looking at Fe\(^{2+}\). Dick was aware that it could not be simply Fe\(^{2+}\), and he proposed that the signal could also come from two interacting iron ions, when we discussed with him our contribution to the 1962 Brookhaven symposium [41]. He did not elaborate on the kind of interaction required to produce EPR spectra as we found them, and it remained for John Gibson and his collaborators to put such an interaction, namely between a ferric and a ferrous iron, into solid numbers and equations in 1966 [42]. This was one of the milestones in the development of the Fe–S field. In four substantial papers Dick Sands and his collaborators confirmed the Gibson–Thornley model for the 2Fe cluster by Mössbauer (MB), electron-nuclear double resonance (ENDOR) and magnetic circular dichroism (MCD) spectroscopies and measurements of magnetic susceptibility [43–46] and most of this on home-built instruments; this was the next milestone [47]. Similar MB data were reported by Debrunner et al. [48] and Rao et al. [49]. At this time there also appeared the first report of a well characterized synthetic analog of the (4Fe–4S) cluster by Herskowitz et al. [50].

### 5. Ferredoxins in plants and bacteria

Before we continue along this line, we must go somewhat back in time and consider developments in areas of research which turned out to be related to the problems discussed above. Work aimed at obtaining soluble protein fractions, which were able to carry out nitrogen fixation, led to the discovery of small, brownish colored proteins that were required as electron transfer agents in that process. They were found to contain non-heme iron and were named ferredoxins [51]. Proteins of similar properties were encountered in research on photosynthesis and the name ferredoxin was then also applied to these [52]. By that time it had been found that SDH contained what was called ‘labile sulfide’ [53], defined as inorganic sulfide that could be liberated as \(\text{H}_2\text{S}\) on acidification of these proteins. Labile sulfide was then also found in other metal flavoproteins such as xanthine and aldehyde oxidases and dihydroorotate dehydrogenase [19,20] and also in the ferredoxins [54,55]. There had been strong evidence that cysteines were also part of the underlying structure(s). It was, therefore, significant that it was ascertained that on acidification \(\text{H}_2\text{S}\) was not liberated from cysteines in proteins as had been claimed [56]. Thus it became clear that the non-heme iron

---

\(^1\) The designation ‘ferredoxin’ is now generally used for small soluble Fe–S proteins with a molecular mass in the range of 6–14 kDa, which contain 2-, 3-, or 4-Fe clusters and are involved in single-electron transfers in the low-potential range. The specificities for their electron donors or acceptors are often not as clearly defined as they are for the chloroplast ferredoxin in photosynthesis or for ferredoxin I of *A. vinelandii* in nitrogen fixation. High-potential Fe–S proteins, HiPiPs, are in essence 4Fe ferredoxins that operate in a higher potential range at about 100–450 mV.

---

**Fig. 5.** Temperature dependence of EPR signals of spinach ferredoxin, and *Azotobacter* ferredoxins II and III. The spectra were recorded at a microwave power of 27 mW. The high- and low-field peaks are located at \(g=1.89\) and 2.05 for spinach ferredoxin; at \(g=1.90\) and 2.04 for Fe protein II and at \(g=1.92\) and 2.01 for Fe protein I of *A. vinelandii*. It can be seen that the three proteins exhibit a very different behavior over a relatively narrow temperature range. Reproduced from [57], with permission from Academic Press, ©1968.
that was present in the brownish ferredoxins, in iron flavoproteins and in those showing the EPR signal at $g = 1.94$ was associated with the presence of inorganic sulfide. It was therefore fairly obvious to conclude from these findings that there was a whole new family of iron proteins in living matter containing an iron–sulfide complex of unknown structure. However, there was one obstacle to the general acceptance of this conclusion: why did the mitochondrial and other iron flavoproteins show the distinctive EPR signal that led to their discovery and the ferredoxins did not? It had been noticed by those recording the EPR spectra of Fe–S proteins, as we will call them now, that the intensities of these spectra showed a range of different temperature dependences, which is due to differences in electron spin relaxation ([57], Fig. 5); and this in turn depends on the immediate environment of the unpaired spins. Thus it seemed possible that the spectra of the compounds with the most relaxed spin systems had not even been detected yet. This was indeed the case, and after the introduction of helium technology (see below) it could be readily shown that temperatures below $\sim 30$ K were needed to record spectra from most ferredoxins [58,59], unless they were present in concentrations not accessible with large proteins [60]. It will be of importance for later developments that for the detection of strongly interacting Fe–S clusters even lower temperatures and high microwave powers are required. The described development then completed the unification of the field, in that it established that all the phenomena observed with the proteins under consideration here were due to the same basic iron sulfur structure.

6. Is the Fe–S cluster of SDH catalytically competent?

On first principles, and particularly as there were attempts to show that in yeast the signals interpreted as originating from respiratory chain components were in fact not related to energy producing metabolism ([24,25], see above), it was necessary to show that the phenomena observed by EPR occurred on a time scale that was compatible with the turnover of the enzymes. We prepared for providing an answer to this by building, with the help of Bob Bray of then London now Sussex [61], a rapid freeze–quench set-up, which was not commercially available at that time. We had already shown by combined optical reflectance [62] and EPR spectroscopy on frozen samples of submitochondrial particles from beef heart that on addition of substrate, the signals of the reduced Fe–S clusters appeared concomitant with the bands of the reduced cytochromes ([41], Fig. 6). With the rapid freeze–quench setup we were then successful in showing that the Fe–S clusters of NADH dehydrogenase were reduced within the turnover time of NADH dehydrogenase when we used a substrate analog that did not react as rapidly as NADH [63]; NADH itself reacts faster than in the $\sim 10$ ms dead time of the freeze–quench apparatus.
However, with SDH the situation was clouded with some sticky problems, which had to be solved first. Preparations of soluble SDH available at that time were only partially in the active state and had to be ‘activated’ before they could be used for the planned experiments [64]. Usually they reverted slowly to the inactive state. It was eventually found that the culprit is tightly bound oxaloacetate, which arose from turnover of malate, a slow substrate of SDH and a spurious contaminant arising from the tissue used in the preparations. Removal of inhibitory oxaloacetate could be achieved by incubation with substrate or inhibitors that compete for the substrate binding site of SDH. However, such an approach would be self-defeating, if one wanted to test for enzyme turnover by rapid freezing. Eventually some cumbersome ways were found to achieve activation with harmless reagents [64]; however, complete activation was never achieved and this has remained a persistent complication until today. There had also been a second problem that was a source of serious objections to the work with the then available SDH preparations, namely: were they reconstitutively active? This meant: would they have the complement of protein and/or prosthetic groups necessary to reconstitute succinate oxidase activity in an alkali-extracted Keilin–Hartree preparation [65]? Preparation of a soluble enzyme had to be carried out under anaerobic conditions and in the presence of substrate. In practice either condition, namely complete removal of oxaloacetate or complete protection from aerobic degradation, was rarely or never achieved. The discovery, in 1957, of ubiquinone (UQ) as a component of the respiratory chain [9] and the advent, in 1959, of succinate-UQ reductases [66] as the naturally occurring forms of the enzyme then at least gave the reconstitution problem a new twist and much interest was now centered on this enzyme. First it was found that on addition of dithionite to samples reduced by succinate, there appeared additional signal intensity around $g \approx 2$ and $g \approx 1.94$ [35–37,68]. It was not spectacular and depended somewhat on the preparations used, but was too consistent and intense to be easily explained away ([69], p. 256). It was attributed to an Fe$^-S$ center 2, which was thought to be a second [2Fe–2S] center. In

7. Advent of the helium age

In the meantime, thanks to the space program which required lots of liquid oxygen, liquid nitrogen had become available at affordable prices, and even liquid He was now produced on many university campuses for work in atomic physics. Thus, the next major step forward became possible, which was to show that at temperatures in the 10–30 K range all ferredoxins, with which signals had previously not been detected at liquid nitrogen temperature, now gave EPR signals at $g \approx 2.0$ and $g \approx 1.94$ [58–60], thus making it clear that these signals were a common property of all compounds that contained iron and sulfide in about equal amounts. Helium technology now also made it possible to see additional Fe–S clusters in the enzymes of the respiratory chain. First it was found that on addition of dithionite to samples reduced by succinate, there appeared additional signal intensity around $g \approx 2$ and $g \approx 1.94$ [35–37,68]. It was not spectacular and depended somewhat on the preparations used, but was too consistent and intense to be easily explained away ([69], p. 256). It was attributed to an Fe–S center 2, which was thought to be a second [2Fe–2S] center. In

![EPR signal of center 3 as observed with a reconstitutively active, soluble preparation of SDH, recorded at 13 K and 2.7 mW. Reproduced from [72] with permission from Academic Press, ©1977.](image-url)
1974 a third feature was seen at low temperature (< 30 K) in the oxidized form of complex II, the succinate-UQ reductase, with an intense, rather isotropic signal at $g = 2.01$ ([70,71], Fig. 7, [72]), which was thought to originate from a high-potential Fe–S center, akin to those seen in bacterial high-potential Fe–S centers (HiPIPs). As far as shape and $g$ values are concerned, this signal was not very similar to the signals given by HiPIPs, but it certainly stemmed from a compound with a relatively high potential, compared to those of other Fe–S proteins; its redox potential was measured to be +60 mV in complex II [71]. Thus, the designation HiPIP was generally and readily accepted, unfortunately one of the assumptions that turned out to be wrong. HiPIPs all have $[4\text{Fe}–4\text{S}]^{3+–2+}$ clusters. As analyses of relatively intact preparations of SDH indicated the presence of close to eight irons and eight sulfides per bound flavin, it was then thought that two 2Fe and one 4Fe, namely the ‘HiPIP’ cluster, would account for the metal and sulfide present. There was still uneasiness in some quarters about cluster 2, because it had never been seen in the expected intensity and its presence had in some instances been mainly postulated from its effect on the spin relaxation of center 1, which was indeed very dramatic ([68,72], Fig. 8). However, it was not clear how a 2Fe cluster could have the required high spin relaxation rate to produce such an effect or require helium temperatures for its detection. The suggestion that it could be a 4Fe cluster was fairly obvious, but as it did not agree with the analytical data, most did not dare to seriously speak out on this, because, at that time, the analytical data were taken more seriously than the EPR arguments; after all, according to the then available information, the analyses made in several laboratories would have had to be off by some 20%.

Before we move on to progress on these matters we must consider another windfall of the helium age, namely the discovery of relatively broad signals flanking the center region ($g = 2$) of the spectra that arose during reduction of complex II ([73], Fig. 9). As they again disappeared on further reduction, they were obviously arising from a two-electron acceptor. The only known components of complex II that fulfill this condition were flavin and UQ, which is always bound in the complex. Extraction of the preparations with hexane, which removes UQ, eliminated these signals and addition of UQ restored them [73]. As such broad satellite signals are typical of spin-
spin interactions, we concluded that UQ in the preparation interacted with another paramagnet. Flavin radicals, the ‘HiPIP’ component or a second UQ molecule would qualify for such a role. Simulations of the spectra were most compatible with there being a UQ pair [73]. Ingledew and Ohnishi made similar observations and ruled out flavin and the HiPIP on account of potentiometric titrations [74]. These observations point out a possible direction in which progress with respect to function might be made by ENDOR spectroscopy.

A more unusual, but very interesting application of EPR spectroscopy was reported by Salerno et al. [75], namely the use of oriented membranes as samples on a rotating stage. It is surprising that even at a time when the identity of cluster 2 was not certain, the conclusions as to the position with respect to the membrane and the sequence of the Fe$^S$ clusters were in essence not very different from present ideas gleaned on the basis of the X-ray structures. More detail about that work with oriented membranes is found in [76].

8. Cluster identification by the extrusion method and its fallacies

In the late 1970s there seemed to be some light at the end of the tunnel: the cluster extrusion (or exchange) procedure [77] had been refined to a state that, technically, it could qualify as a reliable analytical method, and several papers appeared that seemed to fulfill expectations, namely that clarity on the number and kinds of clusters present in Fe–S proteins would result. For instance, the single Fe–S cluster in trimethylamine dehydrogenase which, on account of analyses and its EPR spectrum and relaxation behavior, had been assumed to be a 4Fe cluster, was shown to be extruded as a 4Fe cluster [78]. Then, by application of the extrusion method to SDH, there were found two 2Fe clusters and one 4Fe cluster [79], which was as expected at the time. However, then came the surprise: the cluster of aconitase ‘as isolated’ was extruded as a 2Fe cluster [80], which would have meant that a [2Fe–2S]$^{3+}$ cluster must exist; as there are already two ferric irons at the 2$^+$ state, where would the lost electron come from? Thus it became obvious that, while the extru-

dition method seemed to be working, at least one of the assumptions on which it was based did not stand up, namely: there should not be interconversions between cluster types during the process.

When we read two review articles from the early 1980s, namely one by myself and Albracht [69] and one by Singer [81], we realize that the ratio of words used to firm conclusions arrived at in these articles is typical for the lack of a major piece of information and of utter confusion on the most critical issues that were to be discussed. If these articles had been written only one year later, they might have been much more decisive and useful rather than mainly illustrating our ignorance. Actually in the article that I and Albracht wrote, we were able to inject, in proof, some of the missing information, but, of course, could not rewrite the whole piece, as I would have preferred.

9. The three-iron cluster

The answer came in the early 1980s: the missing information was the existence of yet another kind of cluster, namely the 3Fe cluster. In a spell of ingenious insight, Münck and his colleagues [82] concluded from the MB and EPR spectra of several proteins, such as FdII of Desulfovibrio gigas, FdI of A. vinelandii and aconitase, that the spectra are only compatible with each other if the proteins have clusters with 3Fe (or a multiple thereof). MB is insensitive toward sulfur and up to that point all Fe–S clusters had shown an equal number of irons and sulfides. Therefore, and also because there was a relatively low resolution structure of Azotobacter FdI [83], which suggested that the 3Fe cluster could be a benzene-like ring structure, [3Fe–3S], with alternating sulfides and irons and relatively long Fe–Fe distances, this interpretation was provisionally accepted. However, shortly thereafter extensive iron and sulfide analyses and EXAFS on aconitase [84] showed that the 3Fe cluster was in fact a [3Fe–4S] cluster, i.e., a 4Fe cluster with one corner Fe missing and with the usual short Fe–Fe distances. Resonance Raman spectroscopy led to the same proposal [85]. It became obvious that, under reducing conditions, the 3Fe cluster could be readily converted to a 4Fe cluster, even without added Fe or sulfide, with the needed Fe
arising from decaying 3Fe clusters [86]. In addition, it was shown by means of the linear electric field effect in EPR applied to complex II ([87], Fig. 10) that the \( g = 2.01 \) signal of this complex has the unmistakable properties of typical [3Fe-4S] clusters [88]. An important contribution to this development was the determination of the spin state of the reduced 3Fe cluster by variable temperature (VT) MCD through evaluation of magnetization data [89], which yielded the value \( S = 2 \). Suddenly, the confusion cleared up and it became fairly obvious that SDH in fact contained one 3Fe and one 2Fe cluster, accounting for five Fe, and then presumably a 4Fe cluster, with the strongly relaxing properties; this would make 9 Fe, which is a much more acceptable deviation from the analytical values of 8 Fe than 10 Fe. Now, with EPR having failed to be conclusive in this instance, it was only necessary to confirm the presence of a 4Fe cluster by an independent method. MB might have been ideal, but the necessity of incorporating \( ^{57}\)Fe into the protein and the presence of three different clusters made this option unattractive. It remained then for the MCD spectroscopist to provide the answer.

Before we pursue this topic further, we must discuss one aspect, which had clearly come out in the preceding section on cluster extrusion, namely that there can be ready interconversions between cluster types. It had been pointed out in the first ever review article on 3Fe clusters [90] that, at the time of writing in 1982, there was no clear-cut information on whether 3Fe clusters could exist as such in living cells or were all artifacts of purification efforts. It seemed though that their presence in particulate preparations, separated under relatively mild conditions [87], would speak against the latter alternative. This uncertainty was removed in 1984–85, again by spectroscopy, when it was shown by EPR and low-temperature MCD [89] that reconstitutively active SDH preparations contained the three Fe-S clusters, previously recognized in SDH, namely clusters 1, 2 and 3, whereas in preparations that could not be reconstituted, much less cluster 3 could be detected. In preparations reduced by succinate or dithionite, cluster 3 was identified in the reduced state by its characteristic MCD spectrum and magnetization behavior. MCD also indicated that cluster 2 was a 4Fe cluster with MCD features akin to classical 4Fe ferredoxins [91]. Analogous results concerning cluster 3 were obtained with the fumarate reductase complex and with soluble enzyme preparations. There was no evidence that cluster 3 was involved in any cluster interconversions [92]. Finally, \( E. \ coli \) cells were grown anaerobically on fumarate and glycerol after plasmid-amplified expression of fumarate reductase.

**Fig. 10. Effect of a linear electric field on the EPR spectra of Fe-S proteins.** The LEFE method is based on electron spin echo measurements. On the abscissa the applied magnetic field is given in Gauss (= 0.1 mT) and the ordinate shows the ‘shift parameter’ \( \sigma \) with the electric field \( E \) either perpendicular (\( E \perp H \)) or parallel (\( E \parallel H \)) to the applied magnetic field. \( \sigma \) was calculated from the electric field required to reduce the spin echo amplitude to one half of its value in the absence of a perturbation [88]. (A) Aconitase from beef heart; (B) \( A. \ vinelandii \) ferredoxin I; (C) \( T. \ thermophilus \) ferredoxin; (D) \( E. \ coli \) glutamate synthetase; (E) spinach ferredoxin; (F) \( B. \ polymyxa \) polypeptide; (G) \( G. \ gelatinosa \) HiPIP; (H) succinate-UQ reductase. Reproduced and combined from [87] and [88], with permission from Am. Soc. Biol. Chem. Mol. Biol., ©1983 and 1984, respectively.
When excess fumarate was added anaerobically to these cells, the EPR signal of the \([3\text{Fe}^4\text{S}]^+\) cluster \((g = 2.016)\) with its typical temperature and micro-wave power characteristics was shown to be present \cite{93}. All this work, by the same research group, established beyond reasonable doubt that 3Fe clusters \((3\text{Fe}^4\text{S})^1\); \(0\) can be genuine constituents of enzymes and are not necessarily products of experimental manipulations, which they certainly may be in many instances.

10. The final word on cluster 2

Even with the help of yet another highly discriminating technique, such as VTMCD, it was not an easy victory and required an extensive study on different types of preparations, the use of MCD difference spectroscopy, determination of the temperature dependence of magnetization and all the strategic, technical and interpretative skills of a spectroscopist familiar with the Fe-S field to come to a firm conclusion \cite{91}; and this conclusion was that there must be a 4Fe cluster with strong spin relaxation properties. If this were true, there had to be an EPR signature of this cluster. A determined search was indeed successful with the finding of two pairs of faint satellite lines flanking the center of the \(g = 2.026, 1.935, 1.912\) principal signal, which could be sufficiently enhanced at low temperature and high power to make them measurable \cite{94}, Fig. 11); these lines were at the \(g\) values of 2.064, 1.847 for the soluble enzyme at 13 K and 100 mW and at \(g = 2.27, 2.06, 1.84, 1.63\) for complex II at 5 mW and 9.5 K. Particularly convincing was the demonstration by EPR that the extra lines increased dramatically in intensity when, in potentiometric titrations of the samples, the midpoint potential attributed to cluster 2 was reached. Fig. 12 shows a spectrum that we had obtained with complex II 10 years earlier \cite{67} at high power and 13 K, which shows the \(g = 2.07\) and 1.85 lines clearly. However, at that time there was no encouragement to pursue this, because under the conditions of recording, when the main signal is orders of magnitude stronger than the satellite signal, the danger of observing impurities is real. We note that, in the MCD work reported in 1985 \cite{91}, when preparations presumably were of higher purity, there was still a cytochrome impurity in the sample, interfering with MCD (Fig. 13 in \cite{91}). We were worried, at the time, about a slight contamination with complex I, which has several 4Fe clusters. It would have been hard to prove the point and we wanted to be cautious.

It is clear from all the studies on various interactions between the components of SDH or complex II that, in the reduced state, there is an interaction between clusters 1 and 2, but, because the signal of
cluster 1 is only little perturbed, it is now thought that clusters 2 and 3 also interact [94]; and reduced cluster 3 with a spin $S = 2$ is a stronger magnet than cluster 2. This picture is in excellent agreement with the available data on X-ray diffraction, namely that cluster 2 is in the middle between clusters 1 and 3 with about 10 Å between each pair. It is then also likely that cluster 3 interacts with the neighboring UQ and this in turn interacts with the distant UQ (see above). All this experience may be encouraging to those in the search for the yet missing clusters in complex I [95].

11. Afterthoughts

Spectroscopy certainly has contributed significantly to almost all phases of research on SDH, and knowledge about many details concerning the properties of this protein has uniquely come from spectroscopy of its metal clusters. In turn, many applications of the various spectroscopies have been refined and extended in the course of these efforts. The story of SDH is, like probably all research, one of hard work, luck and disappointments, agreements and controversies, perseverance along promising or blind alleys, ingenious thought and mastering of elaborate techniques and, above all, it is also a fascinating story of molecular evolution.

As with all accomplishments, there were many building blocks and builders needed to achieve the level from which we can now look down on the path along which we moved up. Many of the steps that it took were mentioned or discussed in preceding sections of this chapter on spectroscopy; however, it seems appropriate at a period like ours, when time is becoming one of our most precious commodities, to recall those events that moved our knowledge, or our understanding what this knowledge meant, forward in leaps rather than steps. We may subdivide this brief overview into two parts: one that is concerned with Fe–S clusters in general and which, of course, is applicable to the Fe–S components of SDH also, and a second part that is concerned specifically with spectroscopy of SDH. The understanding of 4Fe clusters developed more slowly and it is not as easy to point out specific leaps as for the initial discoveries.

Fe–S clusters in general:

- **1956–58**: Tightly bound non-heme iron in tissues and particularly in mitochondria and their subfractions [16].
- **1960**: EPR signals attributable to this non-heme iron [35,36].
- **1966**: Theoretical explanation of these signals as due to antiferromagnetic coupling of one Fe$^{3+}$ and one Fe$^{2+}$ and derivation of the observed $g$ values [42].
- **1971**: Experimental verification by various spectroscopies, particularly MB, of the Gibson–Thornley model [43–49].
- **1972**: Structure of synthetic model for [4Fe–4S] cluster [50].
- **1974**: The crystal structure of Chromatium HiPIP [96].
- **1980**: Model for the 4Fe cluster with extensive delocalization of electrons and substructure of two inequivalent pairs of Fe atoms from MB on a 4Fe ferredoxin [97].
- **1981**: Use of VTMCD magnetization curves to determine spin state [89].
- **1983**: Demonstration by analyses and EXAFS that the 3Fe cluster is a [3Fe–4S] not a [3Fe–3S] cluster [84].
- **1987**: Recognition that for the reduced 3Fe cluster ‘double exchange’ (or ‘spin-dependent delocalization’) must be considered, a concept which also applies to 4Fe clusters having a mixed-valence pair of Fe atoms, i.e., for the 1+ and 3+ states [98].
- **2000**: Explanation of the often unusual signal shapes of oxidized 3Fe clusters in terms of antisymmetric exchange [99].
- **2001**: Quantitative determination of the degree of covalency in iron–sulfur bonds by sulfur K-edge X-ray spectroscopy [100].

SDH specifically:

- **1955**: Covalently bound flavin and bound iron [5–7].
- **1957**: Labile sulfide [53].
- **1957**: Ubiquinone in mitochondria [9].
- **1959**: Succinate ubiquinone reductase [66].
1960: Center 1 of SDH by EPR [35].  
1973: Indications of a center 2 by EPR [68].  
1974: Pronounced change in saturation of the signal of center 1 on reduction by dithionite [72,101]; indications for spin–spin interaction between centers.  
1974: Center 3 of SDH [70,71].  
1975: UQ pair by EPR [73].  
1984: Center 3 of SDH is a 3Fe–4S cluster by LEFE EPR [88].  
1985: EPR satellite lines due to center 2 found at high power and low temperature [94].

Acknowledgements

I am particularly indebted to my friend Richard Sands for joining me, over 40 years ago, in a time-consuming and costly venture of uncertain outcome. I acknowledge the generous support of my colleagues and collaborators at the Enzyme Institute by supplying proteins and information and also the active participation of the late T.P. Singer and his research group in later phases of our work. I thank Dr. B.A.C. Ackrell and B.B. Buchanan for their helpful comments on the manuscript as well as the reviewers. The author’s own work on the subject was supported for many years by the Institute for General Medical Sciences of the National Institutes of Health through research grants and a Research Career Award. Thanks are due to Janet Dewane and R.H. Beinert for their indispensable assistance in the production of the manuscript, and to Laura Vanderploeg for the illustrations.

References