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Cellular and Molecular Life Sciences

Memories of a Senior Scientist

Early days of Structural Biology

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The Royal Institution and Lysozyme

I began my graduate work in 1962 at the Royal Institution, London, having completed a first degree in Physics at University College London. The Royal Institution (RI) is housed in a beautiful building with a neo-classical front of 14 Corinthian pillars and situated off Piccadilly in the centre of London's West End.

Figure 1.

It was founded in 1799 by Lord Rumford (Benjamin Thompson) as "a public institution for promotion of science and diffusion and extension of useful knowledge". Its distinguished directors had included Humphrey Davy and Michael Faraday, who had well fulfilled the ideals of the founders with their fundamental discoveries in electro-chemistry and electromagnetism. In my day the Director was Sir Lawrence Bragg (born 1890), who was then in his 70 s. His early work, which had won him the Nobel Prize in 1915 when he was aged 25, had laid the foundations of X-ray crystallography and thus the determination of structures at the atomic level. He had retired from the Cavendish Laboratory at Cambridge, where he had overseen the initial studies in protein crystallography by John Kendrew on myoglobin and Max Perutz on haemoglobin, and in his retirement was keen to set up protein crystallography at the RI.

When I joined in 1962 the structure of myoglobin, the first protein structure to be solved at the atomic level in 1959, was known and it was time to move to a new problem. Lysozyme had been selected almost accidentally when Roberto Poljak (originally from Argentina) arrived in 1960 from Massachusetts Institute of Technology bringing with him crystals. It was a happy choice. Lysozyme from chicken egg white could be obtained commercially, it crystallised readily in a tetragonal crystal form with high symmetry, which helped the precision of data measurements and the search for heavy atoms, and it was an enzyme with antibacterial activity. In 1964 Pierre Jollès and R. E. Canfield, working independently, determined the amino acid sequence of lysozyme, a significant achievement that was crucial for the interpretation of the

electron density map. The senior members of the lysozyme team when I arrived were David Phillips, Tony North and Colin Blake. They were all expert crystallographers with additional skills in instrumentation (Phillips), computing (North) and chemistry (Blake). None of us had much knowledge of biology. To a large extent we learnt our biology from lysozyme. I was given the task of determining the crystal structure of N-acetylglucosamine, part of the oligosaccharide component of lysozyme's substrate, the bacterial cell wall. This took me 2 years (nowadays it would take 1 day). I used visual estimation to measure the X-ray intensities recorded on photographic film, I used the RI workshop to produce a modification of the Weisenberg camera to record simultaneously intensities on the zero level and higher levels of the reciprocal lattice, and I wrote my own structure factor programme. This was a great training exercise. Later Gareth Mair joined us and he brought with him a crystallographic least squares refinement programme which, when implemented on our Elliott 803B computer in 1963, greatly assisted the structure determination[1]. The crystal structure indicated that there was a mixture of α- and β-configuration sugars in the crystal lattice, a conclusion that I was able to substantiate with optical rotation measurements, and which was later disputed but found to occur with other sugars. All this prepared me for lysozyme, when I began my work on inhibitor binding studies in 1964. The team solved the structure of lysozyme in 1965. David Phillips and Uli Arndt had built a linear diffractometer, which was based directly on an analogue version of the reciprocal lattice. This instrument was capable of measuring intensities automatically, initially one reflection at a time but subsequently three and then five reflections simultaneously. These modifications greatly increased the speed and reliability of the measurements. The prototype diffractometer had been built in the RI workshop and with it the lysozyme team were able to outperform other protein crystallographers with the precision and speed of intensity measurements. A 2 Å data set required 14 crystals and took over 2 weeks to measure. (Nowadays a lysozyme dataset can be collected in less than 5 minutes at a synchrotron source). Lysozyme, the second protein structure to be solved, was remarkable. In addition to α-helices, already recognised in the myoglobin structure, lysozyme showed for the first time β-sheet in a three dimensional structure, connecting loops between secondary structural elements with a variety of conformations including a 3_{10} helix, and right- and left-handed disulphide bridges.

My task was to determine the location and structure of the catalytic site. I began binding studies initially with N-acetylglucosamine, and then longer oligosaccharides such as tri-N-acetylglucosamine (from John Rupley, Arizona) and fragments of the bacterial cell wall oligosaccharide (from Nathan Sharon, Weizmann Institute). These experiments worked like a dream, paving the way for subsequent ligand binding and drug binding studies with other systems that have become so productive and informative in macromolecular crystallography. I once compared notes with Lubert Stryer who had done a ligand binding study on azide bound to myoglobin in 1964. We both agreed that we had been extremely fortunate to be in the right place at the right time. In science, luck is an important component of success.

The structural studies with the tri-saccharide at high resolution (2 Å) allowed deductions to be made on the mechanism of action of lysozyme on its bacterial cell wall substrate. This was a marvellous leap of imagination by David Phillips. The mechanism was based on the structural evidence, on what we had learned from the biochemistry of lysozyme's catalysis, and significant input on organic carbohydrate chemistry from Charles Vernon. It showed for the first time that knowledge of structure could lead to knowledge of biological function. Although there were important papers published on the mechanism and structure, the complete story of how lysozyme was solved was only published in 2001 [2].

The first year of graduate research was hard, especially coming from a physics background where the subject was based on definite knowledge. When experiments did not work, I would envy the girls who worked at the Harrods department store, which I used to pass each day on my bus journey to work. They knew what they had to do and could go home in the evening and forget about work. However once an experiment started to work and I felt that I had discovered something new that no one else knew, the effect of research was addictive. It was the small triumphs and the acquisition of new skills that hooked me to a life in research.

Yale and ribonuclease

In 1966 I moved to Yale University to join Fred Richards and Hal Wyckoff who were working on the structure on ribonuclease, in competition with at least two other teams. Richards had made the observation that the proteolytic enzyme, subtilisin, digested ribonuclease into two fragments with the cleavage of the peptide bond between residues 20 and 21. Neither fragment had enzymic activity but when they were mixed together activity was restored. We now know that this is because the two essential histidines, His12 and His119, are required to form the active site.

Richards' work was the first quantitative example of the specificity in macromolecular assembly indicating that the S-peptide (residues 1–20) had sufficient recognition properties to bind in the correct conformation to the S-protein (residues 21–124). Ribonuclease-S, the subtilisin cleaved protein, crystalised well. Cesium chloride (6 M) was included in the crystallisation media in order to increase the density of the solution so that crystals floated and produced three-dimensional growth. The cesium was later removed by washing the crystals. The Yale team solved the structure in 1967 [3,4]. Ribonuclease was one of three protein structures (ribonuclease, chymotrypsin and carboxypeptidase) to be reported in that year.

Yale was an eye opener for me. In the shielded environment of the RI, I had been fortunate to make a significant contribution. I knew all about lysozyme but very little about other aspects of biochemistry. I had come to Yale to learn. There was a wealth of seminars and opportunities. Many of my colleagues were older than myself but were still working for their PhDs. They knew much about many subjects but had not yet made a major discovery. I have often pondered on the best way to train graduate students. In my 40 years of training graduate students at Oxford I have tried to ensure that both components are included, an aspect of original discovery and independence (even if it is a small step forward) and a broad training in several disciplines.

The Yale lab needed to develop software for protein crystallography. Thus I wrote a programme to compute the phases using the Blow and Crick weighting system to account for errors in observation to produce the best electron density map. Both Richards and Wyckoff encouraged innovation. Together with Richard Perham, who was a Helen HayWitney scholar in Richards' lab at Yale, I used neutron activation to detect platinum binding to ribonuclease. Platinum dichloroethylendiamine had proved a useful heavy atom derivative for ribonuclease-S and appeared to bind very strongly. It could not be removed from the crystals by washing. We wanted to know where it was bound. We performed a tryptic digest of the platinum labeled ribonuclease and separated the peptides by chromatography. We then irradiated the chromatography paper with neutrons at the Brookhaven Graphite Research Reactor. The Pt^{196} isotope could be activated to Pt^{197}, which decays to Au^{197} with a half life of 18.3 h emitting characteristic β and γ radiation. This enabled us to detect the labeled peptide. Later the crystallographic work showed the platinum compound bound to methionine 29. The major problem in the neutron activation experiment was that the sodium in the chromatography paper was also activated to Na^{24} making the sample extremely radioactive, but fortunately the isotope of the activated sodium decayed more quickly than that of the activated platinum. Nowadays there are easier methods to detect platinum, such as proton induced X-ray emission.

Oxford and the Laboratory of Molecular Biophysics

I returned to the UK in 1967 and joined the Laboratory of Molecular Biophysics at Oxford, where the lysozyme team under David Phillips had moved following the retirement of Bragg. The Laboratory was housed in the Zoology Department. This was a somewhat unusual location, which arose because the Biochemistry Department, the more logical home for biophysics, was hostile to the new subject, with the notable exception of Sir Hans Krebs, the Whitley Professor of Biochemistry, who was excited at the new field. Others in Biochemistry could see little relevance of the study of enzymes in the crystalline state, despite the results from Fred Richards' lab, which had shown that enzymes retained their activity in the crystalline state. Professor John Pringle, Professor of Zoology, had a vision of zoology extending from the whole animal to the molecular level. This was exemplified by his work on the *Lethocerus* insect flight muscle, a synchronous muscle. During the early 1970 s Richard Tregear from Zoology and Andrew Miller from Molecular Biophysics collaborated with a marvellous X-ray study in which, exploiting the synchronous nature of the muscle, they could detect the fluctuations in intensity of the 145 Å reflection that was dependent on the myosin cross-bridge movement generating the sliding filament mechanism for muscle contraction. As the years went by, the resistance of the Biochemistry Department towards Molecular Biophysics weakened. We were allowed to teach in biochemistry and were found not to corrupt the young. In fact our teaching became valued so that today the structure and function of macromolecules comprises about a quarter of the course in the degree for Molecular and Cellular Biochemistry. When I was appointed to the David Phillips Chair of Molecular Biophysics in 1990, the laboratory moved from Zoology to Biochemistry. In many ways we were sorry to leave Zoology that had been an interesting home for us with interaction with colleagues in animal behaviour and ecology, but our more fruitful collaborations were now with Biochemistry.

I joined the University of Oxford as a Departmental Demonstrator, the lowest rung on the academic ladder. Under the grant system at that time, the Department received a block grant allocation from

which it was able to fund the research of its staff. Accordingly I did not have to write a grant application until 5 years later. This was a valuable time to explore new projects. With my graduate students I started crystallographic studies with triosephosphate isomerase, aldolase and transition state analogue binding to lysozyme before beginning my work with glycogen phosphorylase. The freedom to explore was most valuable but it meant that the effort on any one project was small. Nowadays, new faculty members have less chance to explore preliminary projects but are required immediately to support their work by their own grant applications. If successful this gives them more substantial funding to build up a team for major projects, which on the whole is advantageous, but the value of a start up grant not tied to a specific project should not be underestimated.

One result of these early explorations was electron microscope experiments with my research student Peter Eagles on protein crystals and with adrenalin storage chromaffin granules. I had been asked to take over the running of the electron microscope unit from John Baker. Baker was a distinguished zoologist and cytologist. The unit had an enormous number of early micrograph plates some of which dated from the days when Baker had pioneered the transition from optical microscopy to electron microscopy, showing how some details could be resolved by EM but not by optical microscopy. When I asked Baker what should be done with these old micrographs, his reply was "throw them all away; it is the scientific method; everything should be reproducible". We threw away most of the plates but I kept a few for the archives.

In February 1969, the UK Science Research Council published a report that recommended enzyme chemistry and technology as worthy of special encouragement. Oxford was well placed to respond because already there was an informal grouping of scientists that became the Oxford Enzyme Group. Under the chairmanship of Professor Rex Richards, Professor of Physical Chemistry, this group of 22 scientists from 8 different Departments met regularly with scientific presentations and discussion, preceded by a dinner at one of the colleges. The Group promoted interdisciplinary collaborations and it led to the strong foundation of biological NMR in Oxford. Later in 1988 when the Conservative Government called for Interdisciplinary Research Centres with the expectation of links with industry, Oxford was again able to respond, building on the success of the Oxford Enzyme Group, to form the Oxford Centre for Molecular Sciences under the chairmanship of Professor Jack Baldwin, Professor of Organic Chemistry. This grant, renewed on several occasions, provided large scale infrastructure funding including X-ray diffraction equipment for Molecular Biophysics. The grant was closely linked to collaborative research projects. This funding mechanism for providing large-scale infrastructure was enormously advantageous. Such schemes have continued in various guises and their importance for the health of University research are enormously important.

Phosphorylase and synchrotron radiation

I began work with glycogen phosphorylase in 1971. Phosphorylase was to occupy most of my research effort for the next twenty years. It was instigated following a visit of David Phillips to Seattle where he brought back a portfolio of papers from Eddie Fischer and Ed Krebs. It sounded a fascinating problem for structural biology because the enzyme exhibited a variety of non-covalent and covalent regulatory mechanisms. Monod, Wyman and Changeux [5] had cited the work of Ernst Heilmeyer and Carl Cori (1964) [6] on phosphorylase in their classic 1965 paper as a key example of allostery. Neil Madsen from Edmonton supplied our first crystals. They were grown from ammonium sulphate and had a unit cell 119 x 190 x 88 Å, a very large unit cell that gave weak intensities our home rotating anode X-ray generator. We started to prepare the enzyme ourselves with the help of the Oxford Enzyme Group's enzyme preparation laboratory. We succeeded in growing a new tetragonal crystal form that diffracted most beautifully. The crystals had appeared overnight in a 100 ml measuring cylinder from a preparation that had included AMP and magnesium chloride but no conventional precipitant. The stock of crystals lasted almost six months but we were unable to repeat the crystallization, until Neil Madsen visited us and found that the solution from which we obtained the original crystals contained IMP and not AMP. Crystals grew readily with IMP. Evidently our more vigorous extraction of phosphorylase from rabbit skeletal muscle had led to inclusion of AMP deaminase in the purified preparation that over the period of crystallization had converted AMP to IMP. This was a fortunate accident. IMP is a weak activator of phosphorylase and does not induce the T to R state allosteric transition. Hence our early work was with the T state enzyme. Although with great patience and hard work we could obtain data to 3-Å resolution with our in-house facilities, we needed high-resolution data to understand the atomic detail.

The breakthrough came in 1978 when I sent my graduate student, Enrico Stura, to visit Roger Fourme at the LURE synchrotron near Paris. The photographs he obtained were heart stopping and I knew then that

synchrotron radiation would be the way forward for challenging problems in structural biology. With our home source the images required 12 hours exposure and the crystals were severely damaged. But at LURE synchrotron the crystals diffracted to 2-Å resolution and a photograph could be recorded in 6 minutes. When the new UK synchrotron, the Synchrotron Radiation Source at Daresbury opened in 1981, the first source to be dedicated to the production of radiation for experiments rather than high energy particle physics, we were among the keen first users. Using the experimental station 7.2 built by John Helliwell and his team, and with the leadership of Keith Wilson from my group, we were able to carry out both structure determination and ligand binding studies. These studies led eventually to the high-resolution structure with Ravi Acharya and David Stuart driving the work forward. Later Janos Hajdu started time resolved studies and Laue diffraction, allowing us to follow catalysis in the crystal [7].

The significance of synchrotron radiation for structural biology has been immense. In 2003 I was privileged to become Director of Life Sciences at the Diamond Light Source project, the UK's new third generation synchrotron source built on the Harwell Science and Innovation Campus near Didcot and to lead the way through encouragement of the talented staff to create outstanding resources for future research.

For many years the active form of glycogen phosphorylase, the R state, escaped us. In 1986 David Barford bravely returned to the ammonium sulphate crystal form and showed that here the sulphate ions had acted to mimic phosphate and promoted the R state, an assumption that he was able to substantiate by going on to crystallise phosphorylase a, the active phosphorylated form of the enzyme, and solving its structure in 1990 [8,9].

By 1991, the structural studies with rabbit muscle phosphorylase, both our work and those of others notably Bob Fletterick, Stephen Sprang and Betsy Goldsmith, had led to a molecular understanding of most of phosphorylase's biological properties. But there was one outstanding feature. How did phosphorylase recognize a polysaccharide bound at the catalytic site and what was the arrangement of the substrate inorganic phosphate molecule with respect to the co-factor pyridoxal phosphate 5'-phosphate group? To answer this question we used biology to help us. The mammalian phosphorylase recognizes glycogen, which is too large to bind in the crystals, but the *E. coli* maltodextrin phosphorylase, which is 46 % identical to the mammalian phosphorylase and exhibits similar catalytic properties, is designed to recognize oligosaccharides. With the help of Dieter Palm from Ernst Helmreich's group at Wurzburg, Kim Watson succeeded in crystallizing the maltodextrin phosphorylase. By using a thio-oligosaccharide that cannot be phosphorylysed, she was able to determine the crystal structure of the ternary complex of enzyme, oligosaccharide and inorganic phosphate. The results demonstrated the arrangement of the inorganic phosphate substrate hydrogen bonded to the 5'-phosphate of the co-factor and in a position to act as a general acid to promote cleavage of the glycosidic bond. As anticipated from model building, the oligosaccharide had an unusual torsion angle between the two terminal sugars that facilitated the attack and allowed the reaction to proceed with retention of configuration [10].

Concluding remarks

In this article I have left for a later date a description of more recent studies with protein kinases and other systems, the history of the Laboratory of Molecular Biophysics and an account of the scientists who have contributed and the young scientists who have trained there. The Laboratory of Molecular Biophysics derives much of its strength by being a close-knit society with the supporting umbrella of the parent Biochemistry Department and an energetic outreach to research collaborators both within and outside Oxford. My role has been as an enabler to keep the Laboratory strong and to promote the research and teaching careers of its members. I should like to pay tribute to all those who have worked with me over the years for their patience and enthusiasm, and whose technical skills far surpassed my own. I have been 40 years in Oxford, which shows either great contentment with the place or lack of initiative. It is the former that has kept me in Oxford. It is reported that Humphrey Davy was once asked to define his most important discovery. He replied that he considered his most important discovery to be Michael Faraday. In many ways I feel that my most important discoveries have been those with whom I have worked and trained.

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