

The story of insulin crystallography

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Early efforts

The discovery of insulin by Banting and Best¹ in the early twenties of this century was a major event in the history of therapeutics. Insulin has since been involved in many landmarks in the development of biology. For instance, insulin was the first protein to be sequenced² and also chemically synthesized³⁻⁷. Insulin has also been involved, long before these developments, in the initiation of macromolecular crystallographic studies. In fact, it was the second protein to be subjected to preliminary X-ray crystallographic studies. Pepsin was the first. The history of biological macromolecular crystallography began in 1934 at Cambridge when J. D. Bernal and his young Ph D student Dorothy Crowfoot (subsequently Hodgkin) studied the diffraction pattern from the crystals of the digestive enzyme, pepsin⁸. Bernal observed that the crystals lost birefringence when removed from the solution of crystallization and dry crystals did not give a diffraction pattern. Therefore, he mounted crystals, along with the mother liquor, in Lindemann capillaries, a practice followed since then, except when the recently introduced technique of flash freezing is used.

On her return to Oxford, twenty-five-year-old Dorothy took up the X-ray analysis of insulin as the first problem in her independent research career. Bovine insulin had earlier been crystallized by Abel in 1926 (ref. 9). The method of crystallization in the presence of salts of zinc and other metals, was standardized by Scott in 1934 (ref. 10). It is this method that Dorothy used for growing crystals. She could then record a series of X-ray diffraction photographs from these crystals¹¹. The excitement of seeing the first diffraction photograph is best captured in her own words¹²: 'The moment late that evening about 10 pm – when I developed the photograph and saw the central pattern of minute reflections was probably the most exciting in my life. I waited while the film was washed, hung it up to dry, and wandered out, too excited to return to college immediately. I wandered South to the Broad and there

was stopped by a policeman. I said I was just going back to college and slowly turned west. Next morning I woke up with a horrid fear – perhaps the objects I photographed were not insulin? I hurried straight to the laboratory and tested my crystals for protein in the xantho protein reaction – yellow with nitric acid, brown with ammonia – it worked. I went gladly back to college and breakfast' (Figure 1).

During the next couple of years, Dorothy carried out detailed investigations, as detailed as could be done in those very early days of protein crystallography, on the crystals of insulin and the diffraction patterns from them¹³⁻¹⁵. She painstakingly measured the density of the crystals. They belonged to the rhombohedral system, a crystallographer's nightmare in the pre-computer days. Yet, she successfully indexed all the diffraction spots recorded using a primitive oscillation camera and measured their intensities using eye estimation. By then Patterson had discovered the synthesis named after him^{16,17}. He showed that a Fourier synthesis computed with the intensities as coefficients would give a representation of the interatomic vectors in the structure. A Patterson function would obviously correspond to objective reality. The problem is the deconvolution of the

vector density (vector set) into electron density (the structure). If there are n atoms in the structure, the vector set would contain n^2-n non-origin peaks. In the absence of non-random features like heavy atoms, deciphering the positions of the n atoms from the n^2-n peaks is an impossible task, even for the structure of a moderately-sized organic molecule. The task is utterly hopeless when the structure contains hundreds or thousands of atoms, as in a protein. Yet Dorothy undertook to calculate a Patterson map manually using Beavers-Lipson strips^{18,19}, the magic weapon in the armoury of crystallographers for computing Fourier series in the pre-computer days. At the end of this stupendous effort, the Patterson function did not reveal any meaningful pattern that Dorothy might have hoped to see. However, the preliminary X-ray work, as a whole, showed the molecular weight of the protein in the unit cell to be 37,600 Da, a value remarkably close to the weight of an insulin hexamer as we know it today. The work also demonstrated that the unit cell with this weight had trigonal symmetry¹⁴.

Pepsin and insulin were not the only proteins to be examined using X-ray crystallography in the thirties. Dorothy took up the study of lactoglobulin along with Riley²⁰. Max Perutz had also started his monumental work on the X-ray analysis of haemoglobin²¹. These were heroic efforts of the pioneers, with no reasonable prospects of success. That was a time when one did not even know what proteins were. Furthermore, it was a time when the solution of the crystal structure of even a small organic molecule involved formidable intellectual challenge. Yet, it was these heroic efforts that laid the foundations of macromolecular crystallography, which now forms an indispensable component of modern biology.

Gathering momentum

Dorothy herself drifted away from insulin in the late thirties to other problems. She had already been involved in the



Figure 1. Dorothy Crowfoot in the late thirties.

structure analysis of cholesterol and other sterols. During the next couple of decades, she solved the structures of many important molecules, including penicillin and vitamin B₁₂. The structure solution of vitamin B₁₂, which even today shines through as a great intellectual achievement and exhibition of sheer crystallographic prowess, fetched a Nobel Prize. Yet insulin remained the problem closest to her heart. Come back she did to insulin in the fifties, while Perutz and Kendrew had been demonstrating through their X-ray analysis of haemoglobin and myoglobin, that protein structures could be solved using the isomorphous replacement method.

The last primary publication during the early phase of insulin crystallography appeared in 1939. The next crystallographic paper on insulin co-authored by Dorothy Hodgkin was published in 1966 (ref. 22), a remarkable instance of Dorothy's ability to pick up the threads of a problem after long years of comparative neglect. The primary structure of insulin had been determined by then². Also, Schlichtkrull had systematically grown four different crystal forms of pig insulin; one cubic, another monoclinic and two rhombohedral²³. One of the rhombohedral forms corresponded to the crystals originally photographed by Dorothy (Figure 2). The rhombohedral forms were called 2Zn insulin and 4Zn insulin as they contained two and four zinc ions respectively, per insulin hexamer. It is the preliminary X-ray studies on these crystal forms, spanning several years^{24,25}, that were reported in the 1966 paper marking the resumption of serious crys-

tallographic work on insulin. The rhombohedral forms, particularly 2Zn insulin, received special attention.

In the meantime, Rossmann and Blow^{26,27} had developed their rotation and translation functions, which formed the basis of the molecular replacement method; currently perhaps the most widely used method of structure solution of proteins. Rotation and translation functions seek to establish the geometric relation between chemically identical or similar, but crystallographically non-equivalent protein molecules in the same crystal or different crystals. This essentially involves rotation and translation of vector sets as visualized by Patterson maps. Application of the two functions to 2Zn insulin and 4Zn insulin was described in a second paper published in 1966 (ref. 28). The unit cell – the basic unit, the repetition of which in three-dimensional space produces the crystal – of 2Zn insulin has an internal threefold symmetry and contains an insulin hexamer and two zinc ions in addition to the solvent. The two ions are located on the threefold axis with a distance of about 17 Å between them. The three dimers that constitute the hexamer are related to one another by the crystallographic threefold axis. The rotation and translation function studies showed that the two monomers in the dimer are related to each other by a non-crystallographic twofold axis perpendicular to and passing through the crystallographic threefold axis. The insulin hexamer thus has 32 (D₃) symmetry (Figure 3). The results of the calculations on 4Zn insulin were similar, but the two-

fold axis appeared to be about 1 Å from the threefold axis.

The rotation and translation functions enable the delineation of the basic framework of the quaternary structure. But heavy atom derivatives were necessary, the situation still substantially continues to be so, to determine the atomic positions in a protein of totally unknown structure. Perutz was the first to demonstrate that the presence of one or a few heavy atoms such as mercury, uranium or lead per protein molecule in a crystal, would lead to measurable changes in the intensities of X-ray diffraction spots²⁹. These differences can then be used to calculate the phase angles of the amplitudes which can be readily obtained from the intensities. Once the amplitudes and the phase angles are known, the electron density can be calculated, and hence the structure obtained, by computing a Fourier synthesis. In principle, two independent heavy-atom derivatives are necessary to solve a protein structure³⁰. In practice, several are desirable. The preparation of heavy-atom derivatives involves the attachment of heavy atoms or groups containing them to the protein molecules in a coherent manner, without altering the structure of the molecules and their arrangement in the crystal. The derivatives so produced are called isomorphous heavy-atom derivatives and the method of solving structures using them is called the multiple isomorphous replacement method. Heavy-atom derivatives are prepared by soaking or co-crystallization. The preparation of heavy-atom derivatives is facilitated by the presence of large regions containing water between protein molecules in the

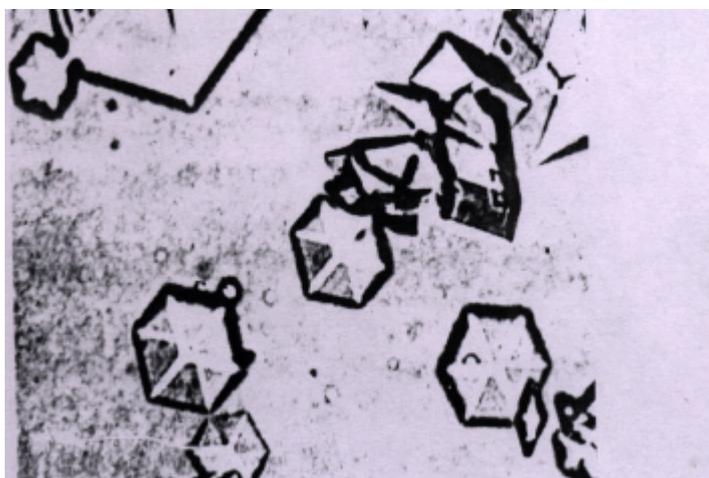


Figure 2. Rhombohedral 2Zn insulin crystals.

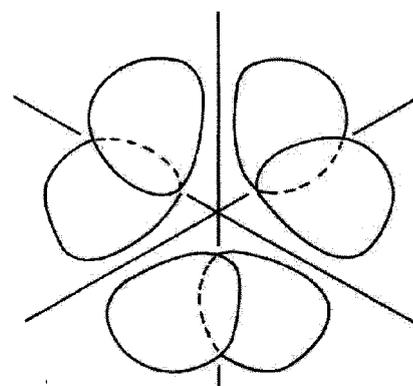


Figure 3. Schematic representation of zinc insulin hexamer.

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crystal. Typically, about 50% of the volume of a protein crystal is made up of water. These watery regions permit the heavy atoms or groups containing them to diffuse into the crystal and lodge themselves at appropriate locations.

Preparation of heavy-atom derivatives turned out to be the Achilles heel of insulin crystallography. The insulin molecules in the hexamer are tightly packed. The hexamers themselves are tightly packed in the crystal with a solvent content as low as about 35%. This meant that it was difficult to diffuse heavy atoms into the crystal without disrupting it. Furthermore, insulin does not have free sulphhydryl groups which are popularly used to bind mercury atoms. Thus all the early attempts to produce heavy-atom derivatives resulted in no heavy-atom substitution or disruption of the crystals. The first breakthrough came with the preparation of a zinc-free lead derivative. This derivative was prepared by removing zinc from 2Zn insulin crystals in 0.1% EDTA, and then soaking the zinc-free crystals so obtained in a buffer containing lead acetate³¹. The lead ions presumably occupied positions originally occupied by the zinc ions and possibly other sites as well. It was clear that the preparation of the derivative involved some changes in the arrangement of molecules in the crystal. However, the diffraction pattern exhibited more changes than could be accounted for by non-isomorphism. Furthermore, anomalous differences (more about it later) could be observed in the pattern, clearly indicating the presence of lead in the crystals. Attempts to solve the structure using this single derivative failed, though at times they looked hopeful.

The breakthrough

That was where things stood in 1968 when the most exciting phase of insulin crystallography began to unfold. In the meantime, Dorothy's insulin group had already moved to the Laboratory of Molecular Biophysics headed by D. C. Phillips and located in the Zoology building, while her small-molecule crystallography group remained in the Chemical Crystallography Laboratory. That brought all the protein crystallography efforts at Oxford under one roof (Figure 4). Much of the work involving

Zn-free lead insulin formed part of the D Phil thesis of Margaret Adams³² who obtained her degree and left for Purdue in early 1968. Guy and Eleanor Dodson, who were in the Oxford laboratory from the early sixties constituted the backbone of Dorothy's insulin team. Tom Blundell and Ted Baker joined Dorothy in the second half of 1967. Ted had a special responsibility of setting up and running the newly acquired Hilger-Watts 4-circle diffractometer. I joined the insulin group in January 1968. To start with I was entrusted with the responsibility of re-collecting accurate data from the native 2Zn insulin and Zn-free lead insulin crystals to get the maximum out of the only available derivative. That involved collecting anomalous data as well using special strategies. Use of the 4-circle diffractometer constituted the utilization of the then best available technology for data collection, and that made a difference. Guy and Tom, among other things, busied themselves with preparation of additional heavy atom derivatives. Eleanor had the overall responsibility for computational operations. Although each person had special responsibilities, essentially all of us worked together and got involved in almost all aspects of the work, an experience that stood us in good stead in our future career.

By 1968–69 winter, everything appeared to fall in place. The new data allowed a fuller elucidation of the heavy-atom

parameters in Zn-free lead insulin. Also, four more derivatives became available. One involved the soaking of 2Zn insulin crystals themselves in a solution containing lead acetate. Then there were uranyl acetate and uranyl fluoride derivatives. The fifth derivative involved mercury benzaldehyde. The derivatives were not independent among themselves. There were common heavy-atom sites. Some of them could be considered as variations of the same theme. In such a situation, the conventional strategy of cyclical calculations involving phase evaluation using all but one derivative and the use of these phases in the refinement of the heavy-atom parameters in the derivative left out, does not work satisfactorily. This problem was solved through the extensive use of anomalous scattering.

In X-ray crystallographic analysis, atoms are treated as spheres made up of free electrons. The scattering amplitude, or the form factor, of an atom then is a real positive number which decreases as the scattering angle increases on account of the finite size of the atom. In such a situation, the intensity of a diffraction spot specified by h, k, l is the same as that of a spot specified by $-h, -k, -l$. This is referred to as the Friedel's law and the spots specified by h, k, l and $-h, -k, -l$ are referred to as a Friedel pair. Thus the diffraction pattern is centric, irrespective of whether the crystal itself is centrosymmetric or not. However, when X-rays used in the study have a wavelength



Figure 4. Photograph taken in the late sixties showing (from left) David Phillips, Dorothy Hodgkin, Tony North, Thomas Hodgkin, the author, Tom Blundell, Ted Baker, and (partially visible) Eleanor Dodson.

close to that of an absorption edge of one or more atoms in the crystal, the assumption of atoms being spheres of free electrons becomes substantially invalid. The form factors of such atoms, referred to as anomalous scatterers, become complex. Consequently, the intensities of diffraction spots h, k, l and $-h, -k, -l$ become unequal or, in other words, Friedel's law breaks down. Anomalous scattering has other effects as well, but violation of Friedel's law was the effect that was first used widely for phase determination³³. Heavy atoms have several absorption edges and therefore, are always anomalous scatterers. The difference between the intensities of a given diffraction spot from a derivative crystal and the native crystal is referred to as the isomorphous difference. The difference in the intensities of the two members of a Friedel pair is called the anomalous difference. An isomorphous difference gives a partial measure of scattering by the heavy atoms (anomalous scatterers). So does an anomalous difference. An estimate of the heavy atom scattering can be obtained by combining the two³⁴⁻³⁶. Such estimates provide a means for determining and refining the heavy-atom parameters in each derivative independently. The pitfalls in determining and refining heavy-atom parameters in derivatives with common features, as in the case of insulin, could then be avoided. Anomalous differences strengthen phase-angle calculations as well. In the early days of protein crystallography when computational approaches were much less sophisticated than they are today, data corresponding to centrosymmetric projections were heavily relied upon, as the phase angles in such data can be only 0 or 180°. Rhombohedral 2Zn insulin, with only a threefold axis as a symmetry element, has no centrosymmetric projections. This disadvantage was effectively compensated by the use of anomalous scattering. The relative weights to be given to isomorphous and anomalous differences, while combining them, was a major concern. New approaches developed by us and those developed by others were carefully used for this purpose. Overall, the effective and extensive use of anomalous scattering was the most important feature of 2Zn insulin crystallography^{37,38}.

By the middle of 1969, the analysis of heavy-atom derivatives was complete, and a 6 Å map appeared promising (Figure 5). Soon afterwards, a 2.8 Å electron-

density map based on isomorphous replacement and anomalous dispersion was ready (Figure 6). Electron-density maps were dealt with in a much cruder fashion in those days than we do today. Interactive computer graphics was not even in the horizon. Even devices and

software for preparing contour maps were not available. One usually printed out the number field in each section through the density in the form of letters and numbers, each character representing a level of electron-density. The grids in the vertical and horizontal directions in

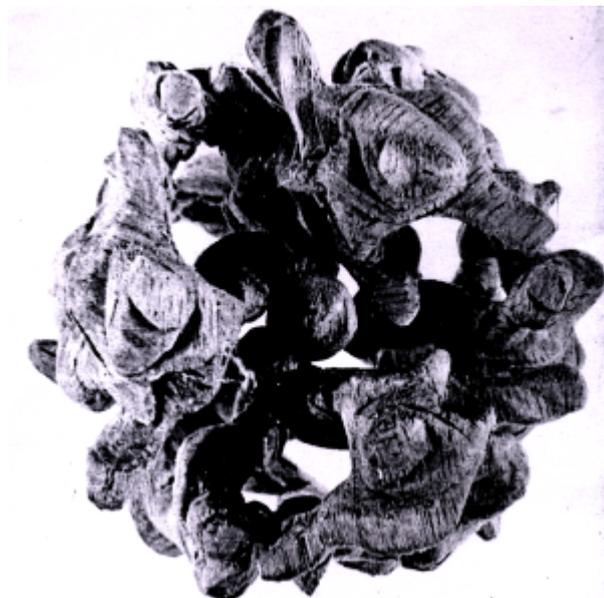


Figure 5. Balsa wood representation of the electron density corresponding to zinc insulin hexamer at 6 Å resolution.

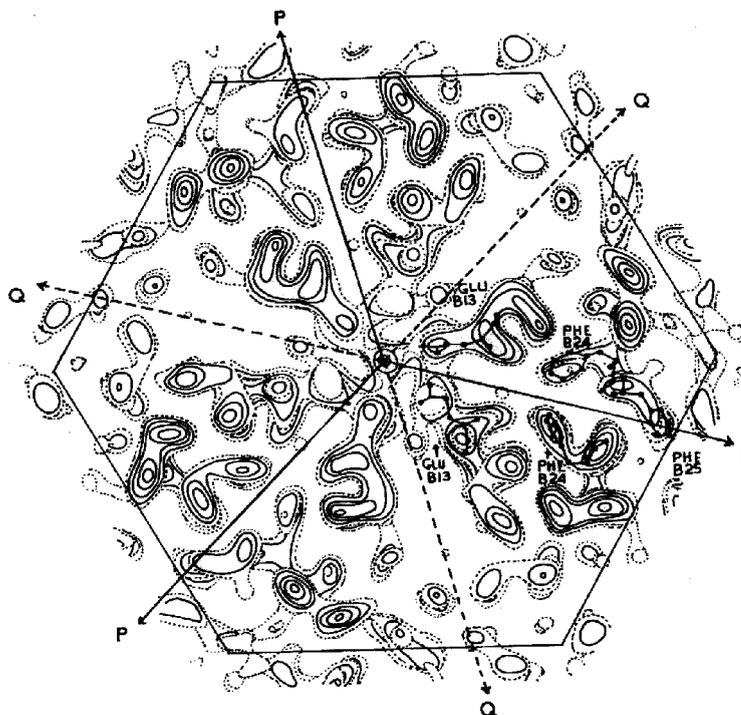


Figure 6. Superposition of a couple of sections through the 2.8 Å electron-density map.

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each section and the spacings between characters were adjusted such that the physical dimensions of the map section corresponded to the appropriate unit-cell dimensions. The number field was then contoured by hand. The contours were then traced on a mylar sheet which was then stuck onto a perspex sheet of appropriate thickness. The perspex sheets were stacked over a light box with appropriate spacers between them, to give a three-dimensional image of the electron-density. This method of producing usable electron-density maps obviously involved a great deal of tedious semi-skilled operations. Dorothy often commandeered the help of the wives of research students and postdocs for this purpose. Dorothy's laboratory, therefore, often gave the impression of families working together. For a period of time, an impressive item in the work place was a beautiful playpen for toddlers to play in while their young mothers worked!

In 1969, the number of proteins with known three-dimensional structures could be counted on one's fingers. Therefore, the solution of a new protein structure was of considerable historical importance. Furthermore, no structure of a protein or peptide hormone was then known. In addition, the importance of the protein and Dorothy's long and deep involvement with the work, led peers to eagerly look forward to the structure solution of insulin. Therefore, it was with great excitement and anticipation that we went through the final stages of the preparation of the map. The day on which we finally stacked the map sections, Dorothy happened to be away from Oxford. Guy and I were the first to examine the map. Neither of us had had any experience in interpreting protein electron-density maps. However, we had no difficulty in recognizing a helical stretch in the map. That turned out to be the B₁₀-B₁₉ α -helix. Starting with the helix, we could build a substantial part of the B-chain, although we made one major mistake. We mistook a tyrosine side chain for a stretch of peptide. The model building was done in an approximate manner. There were no means for 'fitting' the model into the electron density. Essentially, one examined the electron density in bunches of stacked perspex sheets and built the model using Kendrew model bits in a frame placed next to the map.

Dorothy returned to the lab next morning, examined the map, confirmed the

correctness of much of the polypeptide chain that Guy and I had constructed, and corrected the mistake we had made. For the next few days, our almost exclusive occupation was the building of the model. The logistics was such that we had to be on our feet to do so. Along with us, Dorothy stood hours on end, often with swollen feet. In parallel, we prepared several structural diagrams, by hand of course, using different kinds of templates. The first round of model building was completed by early August (Figure 7). Max Perutz came to Oxford from Cambridge along with a few colleagues on 5 August to congratulate Dorothy and the rest of us. Dorothy and Guy left for the US on the 6th to participate in the Crystallography Congress at Stonybrook. Tom was already in the US. Guy stayed on for the Biophysics Congress at Boston which started in late August. I joined him there.

When we all assembled again at Oxford in September, the task was to improve the model which was built in a hurry to beat the deadline of the Stonybrook Crystallography Congress. However, there was pressure on Dorothy from the editor of *Nature* to publish the insulin structure in the centenary issue of the

journal, scheduled to appear in November. Thus preparing the manuscript was the immediate task. With typical Hodgkin generosity, Dorothy included earlier workers, who had worked for long years on insulin without results, also as authors³⁹. Then there were seminars to give at Oxford, Cambridge and other places. The laboratory also had a stream of visitors. In the meantime, Dorothy's group had to move out of the old Zoology building. The final destination was the new Zoology/Psychology building. The space allotted to Dorothy in the new building was not ready for occupation. As a temporary measure, we moved to a cold, yet cosy, dilapidated building in South Parks Road, which earlier used to be the Commonwealth Services Club, and which was later demolished to construct a car park. That again was a great distraction. It was only after settling down in the new abode that we could start serious re-building of the model. In the meantime, another postdoctoral fellow, Dan Mercola had also joined us.

F. M. ('Fred') Richards spent a sabbatical in 1967-68 at the Laboratory of Molecular Biophysics in Oxford. During this period, he designed and constructed a device for fitting the molecular model

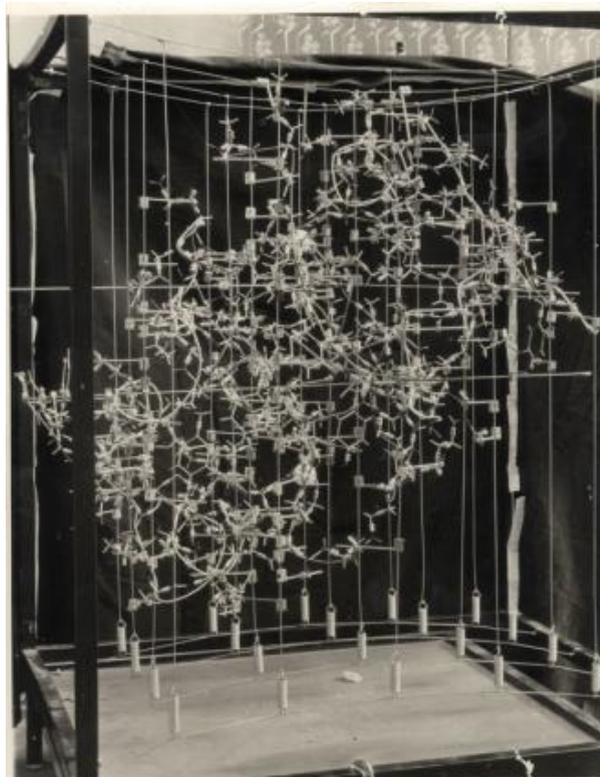


Figure 7. Model of an insulin dimer built using Kendrew model bits.

of a protein into the corresponding electron-density map⁴⁰. In a typical set-up, it essentially involved a set of transparent map sections stacked horizontally and a frame for constructing the model vertically upwards, with a half-silvered mirror inclined at 45° in between. A line passing through the centres of the map sections horizontally and that passing through the centre of the model vertically, should ideally intersect at the centre of the half-inclined mirror. Then, a viewer looking directly into the map sections through the half-silvered mirror would see the model fitted into the map. Of course, several details have to be attended to when constructing the device, variously described as Richards' box, Richards' comparator or Fred's folly (with its double meaning!). Though simple in concept, the device revolutionized map interpretation and remained an indispensable component of any protein crystallography laboratory, until the use of computer graphics for map interpretation became popular.

The first thing we did after moving to the building was to construct a Richards' box and begin accurate model building. The fitted coordinates were subjected to real space and energy refinement, and the

refined coordinates were again fitted into the electron density, operations in which John and Sue Cutfield, who joined the group late in 1970, also took part. The results were published in *Nature* in 1971 (ref. 41), a publication which could be considered as a sequel to the hurriedly prepared paper in the centenary issue of the journal. That marked the end of a phase, undoubtedly the most important phase, of insulin crystallography. By then, I had returned to India.

The structure of insulin is now textbook material. Also, a detailed discussion of the structure and its biological implications⁴²⁻⁴⁵ is perhaps not appropriate in a historical account like the present one. Briefly, the hexamer in 2Zn insulin crystals is organized around a crystallographic threefold axis (Figure 8). Incidentally, electron microscopic studies indicate that the arrangement of hexamers in the crystal is similar to that in the *b*-islet cells of the pancreas, where they are stored as zinc-containing hexamers⁴⁶. The hexamer is made up of three dimers, the monomers in each of which are related by a non-crystallographic twofold axis perpendicular to and passing through the threefold axis (Figure 9). One monomer is coordinated to one of

the two zinc ions situated on the threefold axis, while the other is coordinated to the second zinc ion on the threefold axis. In each monomer, the longer B-chain has a V-shaped structure with an extended tail at the amino terminus (Figure 10). Much of the amino-terminal half of the 'V' is made of the B₁₀-B₁₉ helix, while the carboxy-terminal half is an extended chain involving B₂₂ to B₃₀. The A-chain by itself has a compact structure with helices at the two ends. In the molecule, it is the B-chain that is involved in quaternary interactions. The carboxy-terminal strands of the two monomers associate through hydrogen bonds in an antiparallel manner in the dimer. In fact, the dimer is the primary structural unit with a common hydrophobic core made up of residues from both the monomers. The amino-terminal strand of the B-chain, on the other hand, is involved in dimer-dimer interactions in the hexamer. These interactions, in addition to zinc coordination, stabilize the hexamer.

The aftermath

Liang Dong Cai from China came to Dorothy Hodgkin's laboratory in 1966 to

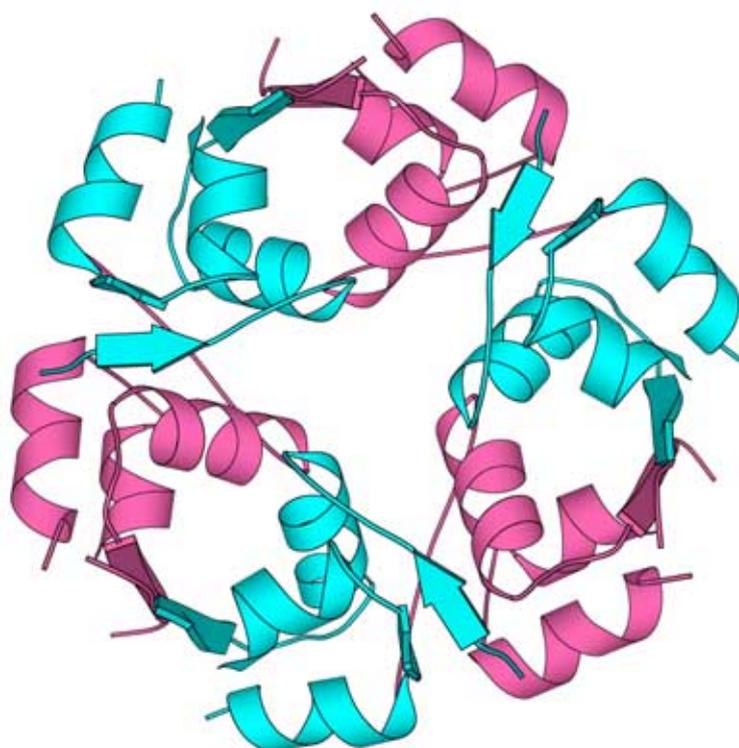


Figure 8. Ribbon diagram of the 2Zn insulin hexamer. The ribbon diagrams have been prepared using MOLSCRIPT⁵⁶.

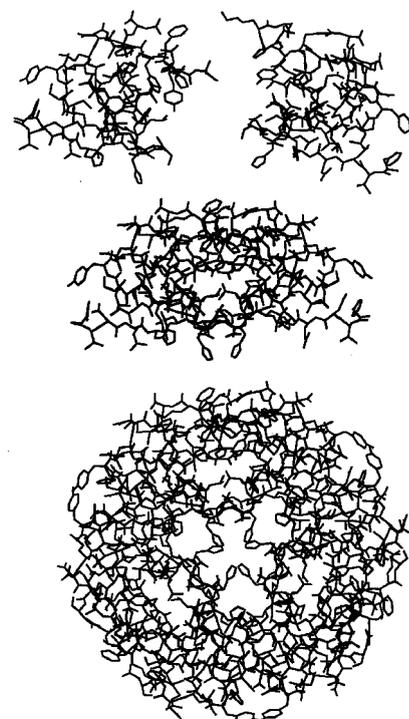


Figure 9. Association of insulin monomers into a dimer and dimers into a hexamer.

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gain experience in protein crystallography, with a view to initiating work in the area in China. The cultural revolution started soon afterwards and Liang was called back to Beijing. For a few years, nothing much was known about him and his activities. However, even during the cultural revolution, work was in progress on the structure of insulin in Liang's Institute of Biophysics at Beijing, independent of the Oxford investigations. Shanghai was another centre of insulin work. In fact, work on insulin was protected at the highest level to a substantial

degree even during the height of the cultural revolution. The Beijing group independently determined the structure of insulin in the early seventies and subsequently refined it^{47,48}. Dorothy encouraged them and was generous in her praise for their work⁴⁹ (Figure 11).

In the meanwhile, independent work on the structure of insulin had started at Japan as well under the leadership of Noriyoshi Sakabe at Nagoya. The Nagoya group was eventually in touch with the Oxford group. In fact, Sakabe and his wife Kiwako worked in Doro-

thy's laboratory for a couple of years in the first half of the seventies. Subsequently, the Japanese group worked on insulin, partly in collaboration with the Oxford group and partly independently⁵⁰.

At Oxford itself, work on insulin was pursued on several fronts. A major result that emanated from Oxford in the first half of the seventies was the structure of rhombohedral 4Zn insulin⁵¹. Rather unexpectedly, it turned out that the two monomers in the dimer had somewhat different conformations, especially in the amino-terminal stretch of the B-chain, in 4Zn insulin (Figure 12). This eventually led to interesting investigations on the 'tense' and the 'relaxed' states of insulin and transformations between them.

While working on several other forms of insulin, the main emphasis at Oxford in the seventies was on the refinement of the structure of 2Zn insulin. By then the refinement of the structure using the least squares minimization of the differences between observed and calculated intensities (or structure factors) of diffraction spots, had become a routine technique in the crystallography of small molecules. The least squares technique is effective only when, among other things, the number of observations is several times the number of parameters being refined. This condition is easily met in the crystallography of small molecules. Protein crystals, however, diffract only to comparatively low resolutions and consequently, would have a low observation-to-parameter ratio. Also, unlike in the case of most crystals of small molecules, the order in or the definition of the structure in different regions in a protein molecule differs markedly. Furthermore, the refinement of protein structures, with thousands of parameters, posed, especially in those days, a formidable computational problem. On account of these problems, refinement of protein structures was a relatively unexplored terrain in the early seventies. In fact, 2Zn insulin was among the first protein structures to be subjected to rigorous refinement.

After solving the structure at 2.8 Å resolution, X-ray data from 2Zn insulin crystals were extended to 1.5 Å resolution through data collection at different stages. The structure was refined using two approaches. The first approach based on difference Fourier syntheses was pursued by Guy Dodson, Eleanor Dodson and others at Oxford. In the meantime, Agarwal had adapted the Fast Fourier

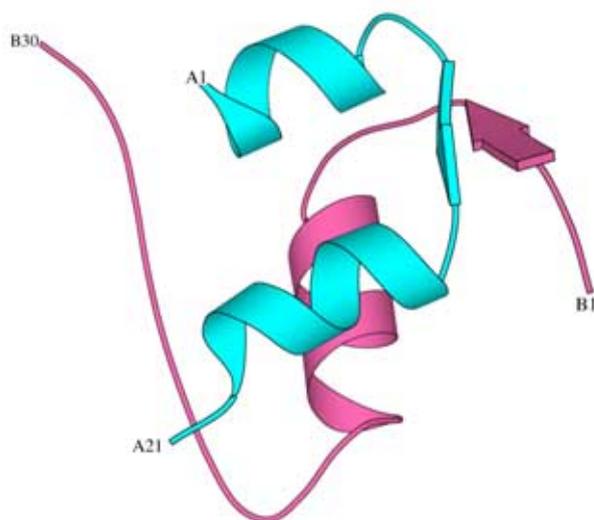


Figure 10. Ribbon diagram of an insulin monomer viewed approximately perpendicular to the threefold axis.

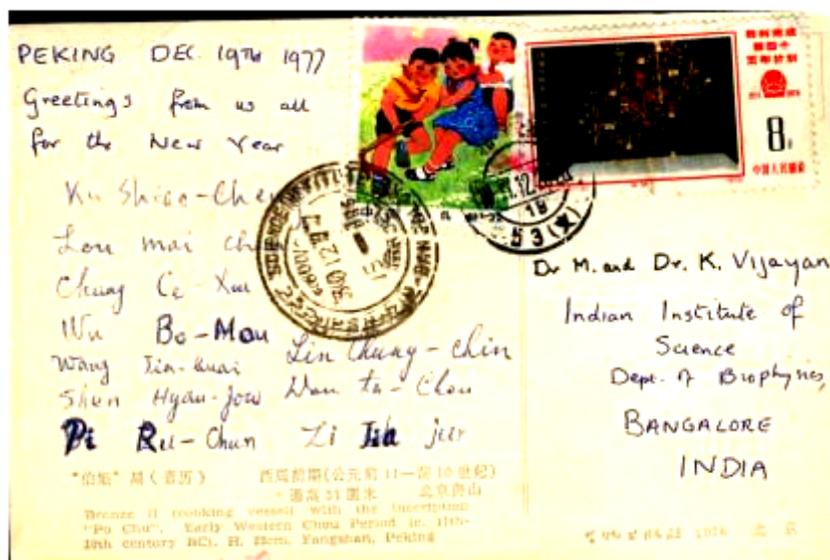


Figure 11. Post card sent from China in December 1977 by Dorothy Hodgkin and several members of the Beijing insulin group. A postal stamp depicting an insulin dimer is clearly seen.

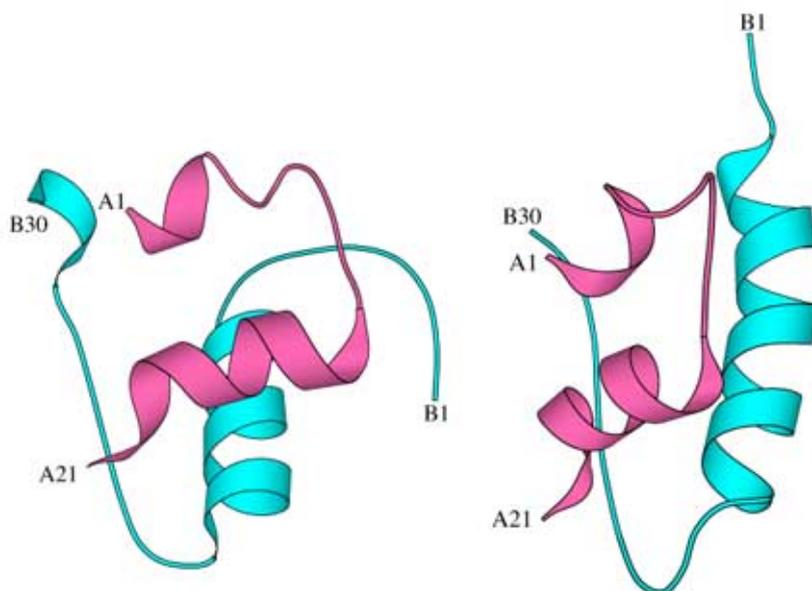


Figure 12. Ribbon diagram of the two monomers in a dimer in 4Zn insulin.

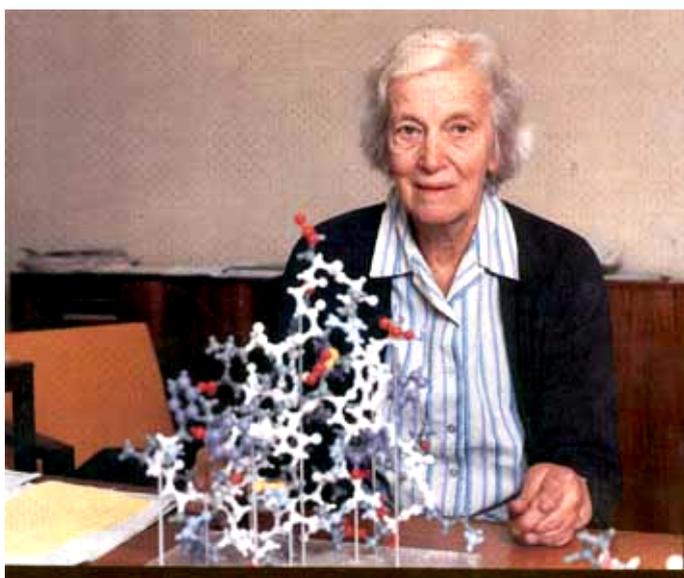


Figure 13. One of the last photographs of Dorothy Hodgkin with an insulin model.

Transform (FFT) algorithm for calculating structure factors, making the calculations very fast⁵². Neil Isaacs and Agarwal at the IBM Thomas J. Watson Research Center, New York, used this development to refine the structure employing least squares⁵³. The two refinements were carried out in parallel.

When I again went to work with Dorothy during 1976–77, just prior to her formal retirement at the age of 67, my mandate was to combine the results of

the two independent sets of refinements and prepare the paper. However, to our surprise and dismay, we found significant differences between the two sets of coordinates. Both the refinement calculations were individually internally consistent, but the results were not consistent with each other in details. That clearly showed that automatic refinement calculations could be unreliable when dealing with proteins, for reasons mentioned earlier. It was also noticed that the

results of each cycle of refinement depended upon the past history of calculations. It turned out that systematic errors in coordinates, once introduced, are difficult to detect and remove by automatic computational procedures⁵⁴. In subsequent years a great deal of attention has been paid by the macromolecular crystallography community to methods for elimination of systematic errors, and validation procedures. The more immediate concern at that time was to reconcile the two sets of coordinates. This we sought to do by careful examination of different types of maps preceded or followed by trial calculations of various kinds. (By this time there were computer programs for preparing contour maps.) Here again, Dorothy's extraordinary facility with electron-density maps was much in evidence. On holidays, she often took bundles of maps to her Ilmington home, spread them over beds and carefully examined them. She always had new ideas when she returned to the lab. We discussed them, compared notes and planned further calculations.

By the time Dorothy formally retired in 1977, much work was completed, but there were still things to be done. Also, the methods for refining protein structures had started improving dramatically. She did much of the subsequent work in association with Guy and Eleanor Dodson who had moved to York in 1975, and who indeed worked earlier for the longest period of time with her at Oxford. She often carried the insulin maps with her during her travels abroad. I vividly remember poring over maps along with Dorothy during her visits to Bangalore. The final paper on 2Zn insulin was published in 1988, and it covered an entire issue of the *Philosophical Transactions of the Royal Society*⁵⁵. The paper contains an extraordinarily detailed account of the structure of insulin. Much work has been done since then and even earlier, without the involvement of Dorothy, particularly by Guy and Eleanor at York. However, the *Philosophical Transactions of the Royal Society* paper carried an air of finality. Dorothy published her first paper on insulin in 1935 and her last in 1988, an eventful period of 53 years (Figure 13).

Many have cut their protein crystallographic teeth on insulin under Dorothy's motherly guidance. Most of them are thriving now in different parts of the world in their chosen areas of work, car-

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rying forward the Dorothy Hodgkin tradition, a tradition that flowered with the insulin structure.

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