labour for decades on the structure determination of natural products. Instead, a single crystallographer could solve such a structure in a period of months.

Improvements in data-collection devices have also had a major impact. Until the mid-1950s, the most common method of measuring intensities was by visual comparison of reflection 'spots' on films with a standard scale. However, the use of counters (used, for instance, by Bragg in 1912) was gradually automated and became the preferred technique in the 1960s. In addition, semi-automatic methods of measuring the optical densities along reciprocal lines on precession photographs were used extensively for early protein-structure determinations in the 1950s and 1960s.

1.2.3. The first investigations of biological macromolecules

Leeds, in the county of Yorkshire, was one of the centres of England's textile industry and home to a small research institute established to investigate the properties of natural fibres. W. T. Astbury became a member of this institute after learning about X-ray diffraction from single crystals in Bragg's laboratory. He investigated the diffraction of X-rays by wool, silk, keratin and other natural fibrous proteins. He showed that the resultant patterns could be roughly classified into two classes, α and β, and that on stretching some, for example, wool, the pattern is converted from α to β (Astbury, 1933).

Purification techniques for globular proteins were also being developed in the 1920s and 1930s, permitting J. B. Sumner at Cornell University to crystallize the first enzyme, namely urease, in 1926. Not much later, in Cambridge, J. D. Bernal and his student, Dorothy Crowfoot (Hodgkin), investigated crystals of pepsin. The resultant 1934 paper in *Nature* (Bernal & Crowfoot, 1934) is quite remarkable because of its speed of publication and because of the authors' extraordinary insight. The crystals of pepsin were found to deteriorate quickly in air when taken out of their crystallization solution and, therefore, had to be contained in a sealed capillary tube for all X-ray experiments. This form of protein-crystal mounting remained in vogue until the 1990s when crystal-freezing techniques were introduced. But, most importantly, it was recognized that the pepsin diffraction pattern implied that the protein molecules have a unique structure and that these crystals would be a vehicle for the determination of that structure to atomic resolution. This understanding of protein structure occurred at a time when proteins were widely thought to form heterogeneous micelles, a concept which persisted another 20 years until Sanger was able to determine the unique amino-acid sequences of the two chains in an insulin molecule (Sanger & Tuppy, 1951; Sanger & Thompson, 1953*a*,*b*).

Soon after Bernal and Hodgkin photographed an X-ray diffraction pattern of pepsin, Max Perutz started his historic investigation of haemoglobin.* Such investigations were, however, thought to be without hope of any success by most of the contemporary crystallographers, who avoided crystals that did not have a short (less than 4.5 Å) axis for projecting resolved atoms. Nevertheless, Perutz computed Patterson functions that suggested haemoglobin contained parallel α-keratin-like bundles of rods (Boyes-Watson *et al.*, 1947; Perutz, 1949). Perutz was correct about the α-keratin-like rods, but not about these being parallel.

In Pasadena, Pauling (Pauling & Corey, 1951; Pauling *et al.*, 1951) was building helical polypeptide models to explain Astbury's α patterns and perhaps to understand the helical structures in globular proteins, such as haemoglobin. Pauling, using his knowledge of the structure of amino acids and peptide bonds, was forced to the conclusion that there need not be an integral number of amino-acid residues per helical turn. He therefore suggested that the 'α-helix', with 3.6 residues per turn, would roughly explain Astbury's α pattern and that his proposed 'β-sheet' structure should be related to Astbury's β pattern. Perutz saw that an α-helical structure should give rise to a strong 1.5 Å-spacing reflection as a consequence of the rise per residue in an α-helix (Perutz, 1951*a*,*b*). Demonstration of this reflection in horse hair, then in fibres of polybenzyl-L-glutamate, in muscle (with Hugh Huxley) and finally in haemoglobin crystals showed that Pauling's proposed α-helix really existed in haemoglobin and presumably also in other globular proteins. Confirmation of helix-like structures came with the observation of cylindrical rods in the 6 Å-resolution structure of myoglobin in 1957 (Kendrew *et al.*, 1958) and eventually at atomic resolution with the 2 Å myoglobin structure in 1959 (Kendrew *et al.*, 1960). The first atomic resolution confirmation of Pauling's β structure did not come until 1966 with the structure determination of hen egg-white lysozyme (Blake, Mair *et al.*, 1967).

Although the stimulus for the Cochran *et al.* (1952) analysis of diffraction from helical structures came from Perutz's studies of helices in polybenzyl-L-glutamate and their presence in haemoglobin, the impact on the structure determination of nucleic acids was even more significant. The events leading to the discovery of the double-helical structure of DNA have been well chronicled (Watson, 1968; Olby, 1974; Judson, 1979). The resultant science, often known exclusively as molecular biology, has created a whole new industry. Furthermore, the molecular-modelling techniques used by Pauling in predicting the structure of α-helices and β-sheets and by Crick and Watson in determining the structure of DNA had a major effect on more traditional crystallography and the structure determinations of fibrous proteins, nucleic acids and polysaccharides.

Another major early result of profound biological significance was the demonstration by Bernal and Fankuchen in the 1930s (Bernal & Fankuchen, 1941) that tobacco mosaic virus (TMV) had a rod-like structure. This was the first occasion where it was possible to obtain a definite idea of the architecture of a virus. Many of the biological properties of TMV had been explored by Wendell Stanley working at the Rockefeller Institute in New York. He had also been able to obtain a large amount of purified virus. Although it was not possible to crystallize this virus, it was possible to obtain a diffraction pattern of the virus in a viscous solution which had been agitated to cause alignment of the virus particles. This led Jim Watson (Watson, 1954) to a simple helical structure of protein subunits. Eventually, after continuing studies by Aaron Klug, Rosalind Franklin, Ken Holmes and others, the structure was determined at atomic resolution (Holmes *et al.*, 1975), in which the helical strand of RNA was protected by the helical array of protein subunits.

1.2.4. Globular proteins in the 1950s

In 1936, Max Perutz had joined Sir Lawrence Bragg in Cambridge. Inspired in part by Keilin (Perutz, 1997), Perutz started to study crystalline haemoglobin. This work was interrupted by World War II, but once the war was over Perutz tenaciously developed a series of highly ingenious techniques. All of these procedures have their

* Perutz writes, 'I started X-ray work on haemoglobin in October 1937 and Bragg became Cavendish Professor in October 1938. Bernal was my PhD supervisor in 1937, but he had nothing to do with my choice of haemoglobin. I began this work at the suggestion of Haurowitz, the husband of my cousin Gina Perutz, who was then in Prague. The first paper on X-ray diffraction from haemoglobin (and chymotrypsin) was Bernal, Fankuchen & Perutz (Bernal *et al.*, 1938). I did the experimental work, (and) Bernal showed me how to interpret the X-ray pictures.

counterparts in modern 'protein crystallography', although few today recognize their real origin.

The first of these methods was the use of 'shrinkage' stages (Perutz, 1946; Bragg & Perutz, 1952). It had been noted by Bernal and Crowfoot (Hodgkin) in their study of pepsin that crystals of proteins deteriorate on exposure to air. Perutz examined crystals of horse haemoglobin after they were air-dried for short periods of time and then sealed in capillaries. He found that there were at least seven consecutive discrete shrinkage stages of the unit cell. He realized that each shrinkage stage permitted the sampling of the molecular transform at successive positions, thus permitting him to map the variation of the continuous transform. As he examined only the centric (*h*0*l*) reflections of the monoclinic crystals, he could observe when the sign changed from 0 to π in the centric projection (Fig. 1.2.4.1). Thus, he was able to determine the phases (signs) of the central part of the (*h*0*l*) reciprocal lattice. This technique is essentially identical to the use of diffraction data from different unit cells for averaging electron density in the 'modern' molecular replacement method. In the haemoglobin case, Patterson projections had shown that the molecules maintained their orientation relative to the *a* axis as the crystals shrank, but in the more general molecular replacement case, it is necessary to determine the relative orientations of the molecules in each cell.

The second of Perutz's techniques depended on observing changes in the intensities of low-order reflections when the concentration of the dissolved salts (*e.g.* Cs_2SO_4) in the solution between the crystallized molecules was altered (Boyes-Watson *et al.*, 1947; Perutz, 1954). The differences in structure amplitude, taken together with the previously determined signs, could then map out the parts of the crystal unit cell occupied by the haemoglobin molecule. In many respects, this procedure has its equivalent in 'solvent flattening' used extensively in 'modern' protein crystallography.

The third of Perutz's innovations was the isomorphous replacement method (Green *et al.*, 1954). The origin of the isomorphous replacement method goes back to the beginnings of X-ray crystallography when Bragg compared the diffracted intensities from crystals of NaCl and KCl. J. Monteath Robertson explored the procedure a little further in his studies of phthalocyanines. Perutz used a well known fact that dyes could be diffused into protein crystals, and, hence, heavy-atom compounds might also diffuse into and bind to specific residues in the protein. Nevertheless, the sceptics questioned whether even the heaviest atoms could make a measurable difference to the X-ray diffraction pattern of a protein.* Perutz therefore developed an instrument which quantitatively recorded the blackening caused by the reflected X-ray beam on a film. He also showed that the effect of specifically bound atoms could be observed visually on a film record of a diffraction pattern. In 1953, this resulted in a complete sign determination of the (*h*0*l*) horse haemoglobin structure amplitudes (Green *et al.*, 1954). However, not surprisingly, the projection of the molecule was not very interesting, making it necessary to extend the procedure to noncentric, three-dimensional data. It took another five years to determine the first globular protein structure to near atomic resolution.

In 1950, David Harker was awarded one million US dollars to study the structure of proteins. He worked first at the Brooklyn Polytechnic Institute in New York and later at the Roswell Park Cancer Institute in Buffalo, New York. He proposed to solve the structure of proteins on the assumption that they consisted of 'globs' which he could treat as single atoms; therefore, he could solve the structure by using his inequalities (Harker & Kasper, 1947), *i.e.*, by direct methods. He was aware of the need to use three-dimensional data, which meant a full phase determination, rather than the sign determination of two-dimensional projection data on which Perutz had concentrated. Harker therefore decided to develop automatic diffractometers, as opposed to the film methods being used at Cambridge. In 1956, he published a procedure for plotting the isomorphous data of each reflection in a simple graphical manner that allowed an easy determination of its phase (Harker, 1956). Unfortunately, the error associated with the data tended to create a lot of uncertainty.

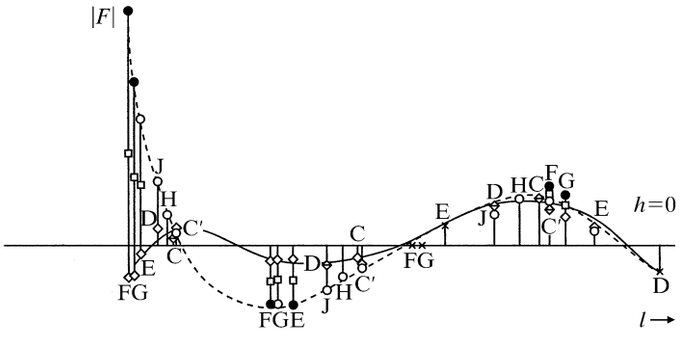

Fig. 1.2.4.1. Change of structure amplitude for horse haemoglobin as a function of salt concentration in the suspension medium of the low-order *h*0*l* reflections at various lattice shrinkage stages (C, C′, D, E, F, G, H, J). Reprinted with permission from Perutz (1954). Copyright (1954) Royal Society of London.

In the first systematic phase determination of a protein, namely that of myoglobin, phase estimates were made for about 400 reflections. In order to remove subjectivity, independent estimates were made by Kendrew and Bragg by visual inspection of the Harker diagram for each reflection. These were later compared before computing an electron-density map. This process was put onto a more objective basis by calculating phase probabilities, as described by Blow & Crick (1959) and Dickerson *et al.* (1961).

One problem with the isomorphous replacement method was the determination of accurate parameters that described the heavy-atom replacements. Centric projections were a means of directly determining the coordinates, but no satisfactory method was available to determine the relative positions of atoms in different derivatives when there were no centric projections. In particular, it was necessary to establish the relative *y* coordinates for the heavy-atom sites in the various derivatives of monoclinic myoglobin and in monoclinic horse haemoglobin. Perutz (1956) and Bragg (1958) had each proposed solutions to this problem, but these were not entirely satisfactory. Consequently, it was necessary to average the results of different methods to determine the 6 Å phases for myoglobin. However, this problem was solved satisfactorily in the structure determination of haemoglobin by using an $(F_{H1} - F_{H2})^2$ Patterson-like synthesis in which the vectors between atoms in the two heavy-atom compounds, H1 and H2, produce negative peaks (Rossmann, 1960). This technique also gave rise to the first least-squares refinement procedure to determine the parameters that define each heavy atom.

Perutz used punched cards to compute the first three-dimensional Patterson map of haemoglobin. This was a tremendous computational undertaking. However, the first digital electronic computers started to appear in the early to mid-1950s. The EDSAC1 and EDSAC2 machines were built in the Mathematical Laboratory of Cambridge University. EDSAC1 was used by John Kendrew for the

* Perutz writes, 'I measured the absolute intensity of reflexions from haemoglobin which turned out to be weaker than predicted by Wilson's statistics. This made me realise that about 99% of the scattering contributions of the light atoms are extinguished by interference and that, by contrast, the electrons of a heavy atom, being concentrated at a point, would scatter in phase and therefore make a measurable difference to the structure amplitudes.'

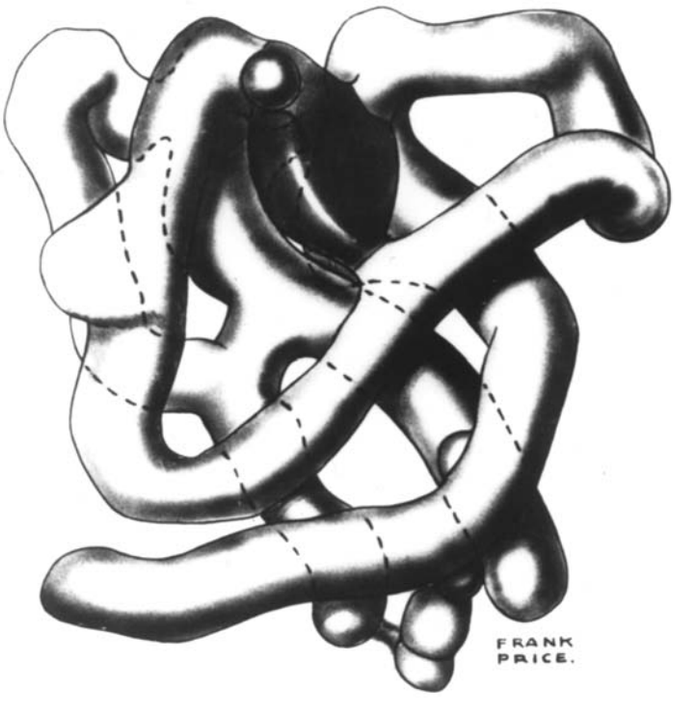

Fig. 1.2.5.1. A model of the myoglobin molecule at 6 Å resolution. Reprinted with permission from Bodo *et al.* (1959). Copyright (1959) Royal Society of London.

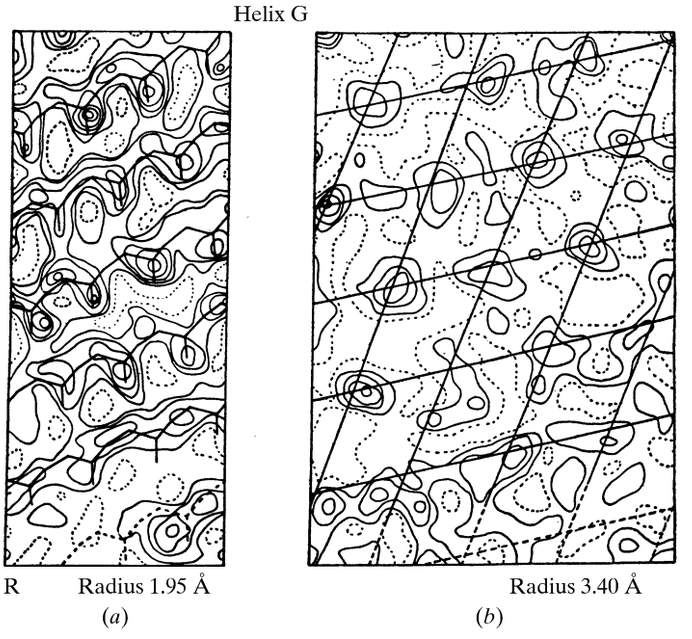

Fig. 1.2.5.2. Cylindrical sections through a helical segment of a myoglobin polypeptide chain. (*a*) The density in a cylindrical mantle of 1.95 Å radius, corresponding to the mean radius of the main-chain atoms in an α-helix. The calculated atomic positions of the α-helix are superimposed and roughly correspond to the density peaks. (*b*) The density at the radius of the β-carbon atoms; the positions of the β-carbon atoms calculated for a right-handed α-helix are marked by the superimposed grid (Kendrew & Watson, unpublished). Reprinted with permission from Perutz (1962). Copyright (1962) Elsevier Publishing Co.

6 Å-resolution map of myoglobin (Bluhm *et al.*, 1958). EDSAC2 came on-line in 1958 and was the computer on which all the calculations were made for the 5.5 Å map of haemoglobin (Cullis *et al.*, 1962) and the 2.0 Å map of myoglobin. It was the tool on which many of the now well established crystallographic techniques were initially developed. By about 1960, the home-built, one-of-a-kind machines were starting to be replaced by commercial machines. Large mainframe IBM computers (704, 709 *etc.*), together with FORTRAN as a symbolic language, became available.

1.2.5. The first protein structures (1957 to the 1970s)

By the time three-dimensional structures of proteins were being solved, Linderström-Lang (Linderström-Lang & Schellman, 1959) had introduced the concepts of 'primary', 'secondary' and 'tertiary' structures, providing a basis for the interpretation of electron-density maps. The first three-dimensional protein structure to be solved was that of myoglobin at 6 Å resolution (Fig. 1.2.5.1) in 1957 (Kendrew *et al.*, 1958). It clearly showed sausage-like features which were assumed to be α-helices. The iron-containing haem group was identified as a somewhat larger electron-density feature. The structure determination of haemoglobin at 5.5 Å resolution in 1959 (Cullis *et al.*, 1962) showed that each of its two independent chains, α and β, had a fold similar to that of myoglobin and, thus, suggested a divergent evolutionary process for oxygen transport molecules. These first protein structures were mostly helical, features that could be recognized readily at low resolution. Had the first structures been of mostly β structure, as is the case for pepsin or chymotrypsin, history might have been different.

The absolute hand of the haemoglobin structure was determined using anomalous dispersion (Cullis *et al.*, 1962) in a manner similar to that used by Bijvoet. This was confirmed almost immediately when a 2 Å-resolution map of myoglobin was calculated in 1959 (Kendrew *et al.*, 1960). By plotting the electron density of the α-helices on cylindrical sections (Fig. 1.2.5.2), it was possible to see not only that the Pauling prediction of the α-helix structure was accurately obeyed, but also that the C_β atoms were consistent with *laevo* amino acids and that all eight helices were right-handed on account of the steric hindrance that would occur between the C_β atom and carbonyl oxygen in left-handed helices.

The first enzyme structure to be solved was that of lysozyme in 1965 (Blake *et al.*, 1965), following a gap of six years after the excitement caused by the discovery of the globin structures. Diffusion of substrates into crystals of lysozyme showed how substrates bound to the enzyme (Blake, Johnson *et al.*, 1967), which in turn suggested a catalytic mechanism and identified the essential catalytic residues.

From 1965 onwards, the rate of protein-structure determinations gradually increased to about one a year: carboxypeptidase (Reeke *et al.*, 1967), chymotrypsin (Matthews *et al.*, 1967), ribonuclease (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967), papain (Drenth *et al.*, 1968), insulin (Adams *et al.*, 1969), lactate dehydrogenase (Adams *et al.*, 1970) and cytochrome *c* (Dickerson *et al.*, 1971) were early examples. Every new structure was a major event. These structures laid the foundation for structural biology. From a crystallographic point of view, Drenth's structure determination of papain was particularly significant in that he was able to show an amino-acid sequencing error where 13 residues had to be inserted between Phe28 and Arg31, and he showed that a 38-residue peptide that had been assigned to position 138 to 176 needed to be transposed to a position between the extra 13 residues and Arg31.

The structures of the globins had suggested that proteins with similar functions were likely to have evolved from a common precursor and, hence, that there might be a limited number of protein folding motifs. Comparison of the active centres of chymotrypsin and subtilisin showed that convergent evolutionary pathways could exist (Drenth *et al.*, 1972; Kraut *et al.*, 1972).

references