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X-ray Analysis of Hemoglobin

The results suggest that a marked structural change accompanies the reaction of hemoglobin with oxygen.

M. F. Perutz

Forty years ago William Lawrence Bragg came here to Stockholm to thank you for awarding the Nobel prize to himself and to his father, and to tell you how they had brought into being a new and fruitful branch of physics by unraveling the atomic arrangement in crystals of common salt and other simple compounds (*1*). To-day Sir Lawrence Bragg ranks as one of the fathers of x-ray crystallography, but he has also been something of a scientific father to me personally, and I feel immensely proud that it should now be my own turn, as his former pupil, to thank you for having bestowed on me this supreme honor.

I started my x-ray work on crystalline proteins in the Cavendish Laboratory at Cambridge under J. D. Bernal in 1937, just after he and Dorothy Hodgkin had demonstrated that protein crystals can be made to yield sharp x-ray diffraction patterns which extend to spacings of the order of interatomic distances (*2*). Soon afterward Sir Lawrence Bragg was appointed Cavendish professor. My x-ray diffraction pictures of hemoglobin at once fired his imagination and enthusiasm. He was fascinated by the idea that the powers of x-ray analysis might be extended to study of the giant molecules which form the catalysts of living cells. Bragg's determined support made it possible for me, and later for both Kendrew and myself, to carry on with our studies over the many years that were needed to develop x-ray methods suitable for investigating such highly complex structures, and I know that Bragg is overjoyed by the success and the honors that have now been our reward.

The author is chairman of the British Medical Research Council's Laboratory of Molecular Biology, Cambridge, England. This article is adapted from the lecture which he delivered in Stockholm, Sweden, 11 December 1962, on receiving the Nobel prize in chemistry, a prize which he shared with John C. Kendrew. It is published with the permission of the Nobel Foundation. It will also be included in the complete volume of Nobel lectures in English which is published yearly by the Elsevier Publishing Company, Amsterdam and New York.

Physical Principles

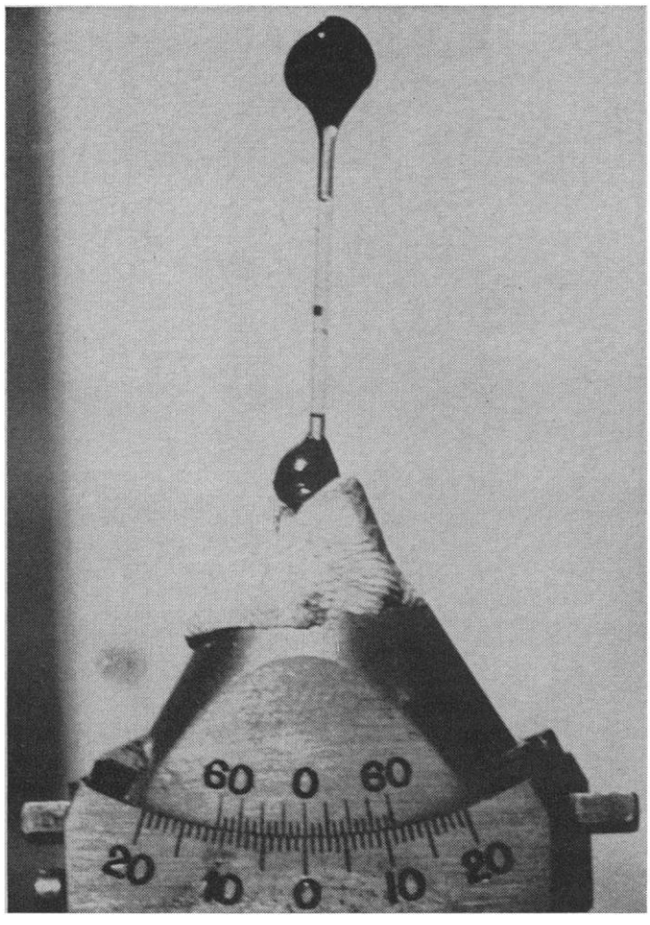
X-ray analysis of proteins is often regarded as a subject comprehensible only to specialists, but the basic ideas underlying our work are simple. A small protein crystal is mounted in a glass capillary to keep it wet (Fig. 1) and is illuminated by a narrow pencil of x-rays. Upon rotating the crystal in certain ways, a regular pattern of diffracted x-rays is produced on a photographic film placed behind the crystal (Fig. 2). The spots are seen to lie at the corners of a regular lattice which bears a reciprocal relationship to the arrangement of molecules in the crystal. Moreover, each spot has a characteristic intensity which is determined in part by the atomic arrangement inside the molecule. Bragg himself explained the reason in his Nobel lecture.

It is well known that the form of the lines ruled on a grating has an influence on the relative intensity of the spectra which it yields. Some spectra may be enhanced, or reduced, in intensity as compared with others. Indeed, gratings are sometimes ruled in such a way that most of the energy is thrown into those spectra which it is most desirable to examine. The form of the line on the grating does not influence the positions of the spectra, which depend on the number of lines to the centimetre, but the individual lines scatter more light in some directions than others, and this enhances the spectra which lie in those directions.

The structure of the group of atoms which composes the unit of the crystal grating influences the strength of the various reflexions in exactly the same way. The rays are diffracted by the electrons grouped around the centre of each atom. In some directions the atoms conspire to give a strong scattered beam, in others their effects almost annul each other by interference. The exact arrangement of the atoms is to be deduced by comparing the strength of the reflexions from different faces and in different orders.

Thus there should be a way of reversing the process of diffraction, and of getting back from the diffraction pattern to an image of the atomic arrangement. In order to produce such an image, each pair of symmetrically related spots in the x-ray pattern can be made to generate a set of fringes, each fringe having an amplitude proportional to the root of the intensity of the spot (Fig. 3). The fringes should now be superimposed, by means either of calculation or of optical analogs, to give the required image (Fig. 4, *a* and *b*).

However, at this stage a complication arises. To obtain the *right* image, each set of fringes must be placed correctly relative to some arbitrarily chosen, common origin (Fig. 5). At this origin the amplitude of the fringe may show a crest or a trough, or some intermediate value which is referred to as the phase angle. It is almost true to say that with sets of fringes of given amplitude, as shown in Fig. 3, an infinite number of different images can be generated, depending on the choice of phase for each of the fringes. By itself, the x-ray pattern tells only about



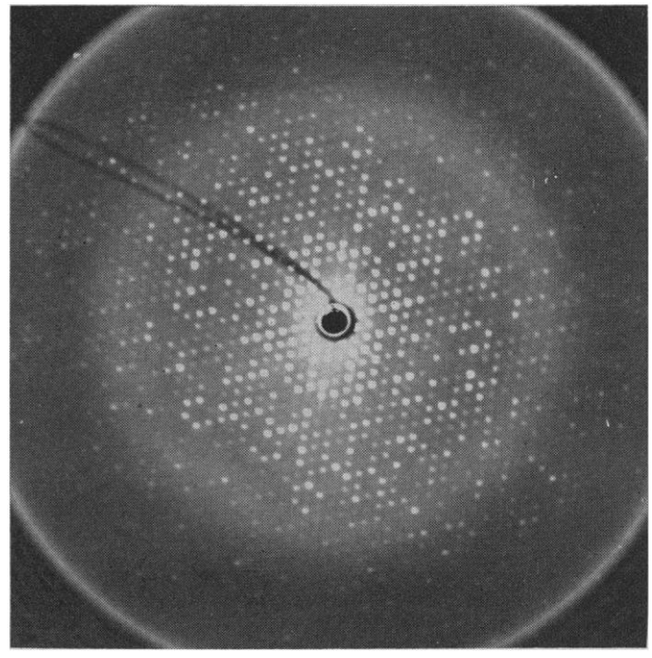


Fig. 1 (left). Hemoglobin crystal in equilibrium with its mother liquor, mounted for x-ray analysis. The glass capillary is about 1 millimeter wide.

Fig. 2 (above). X-ray diffraction pattern from a hemoglobin crystal. The picture was taken with a Buerger precession camera. The pattern fades out near the rim of the picture, which corresponds to a spacing of 1.8 angstrom.

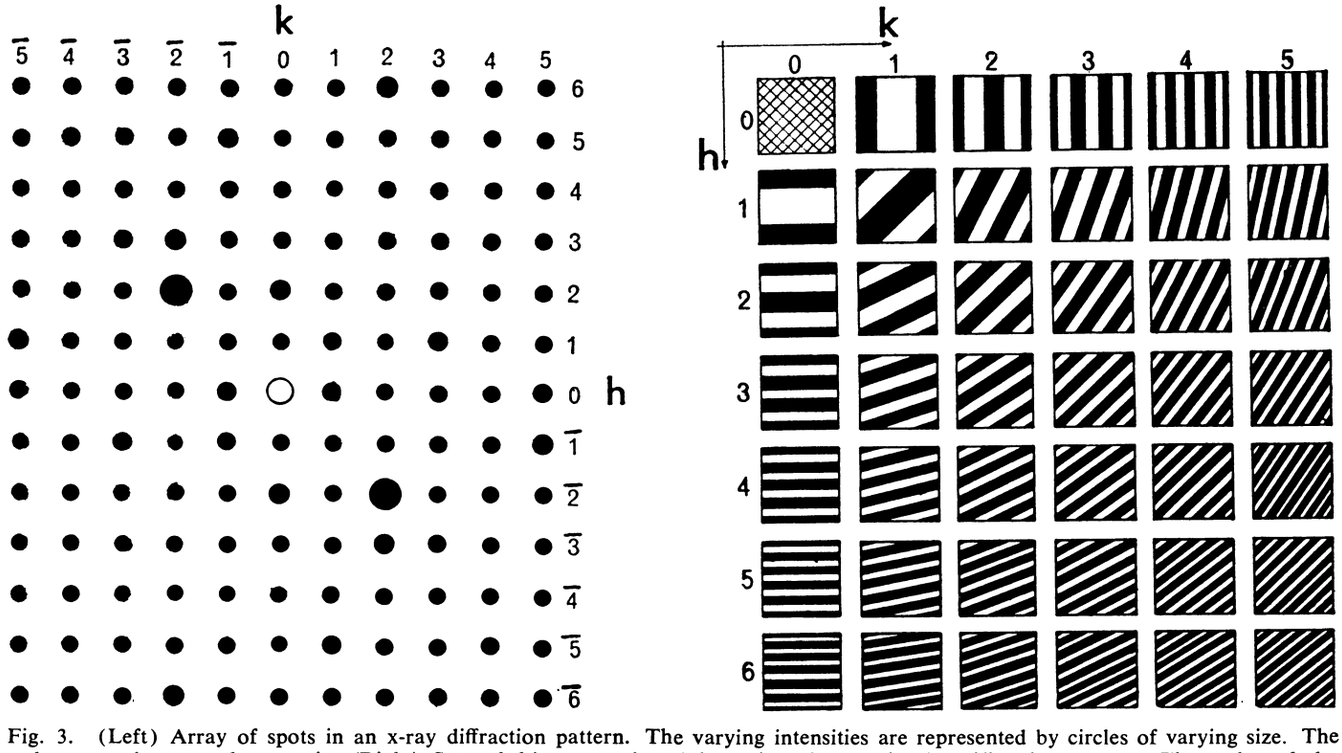


Fig. 3. (Left) Array of spots in an x-ray diffraction pattern. The varying intensities are represented by circles of varying size. The orders are given on the margin. (Right) Sets of fringes produced by pairs of spots in the diffraction pattern. The order of the fringes h and k corresponds to the order of the spots used to produce them, for instance, the fringes $h = 2$ and $k = 5$ being produced by diffraction from the pair of spots $h = 2$, $k = 5$ and $h = -2$, $k = -5$. [From W. de Beauclair, *Verfahren und Geräte zur mehrdimensionalen Fouriersynthese* (Akademie-Verlag, Berlin, 1949), reproduced with permission.]

the amplitude, not about the phase angles of the fringes to be generated by each pair of spots, so half the information needed for production of the image is missing.

The phase problem can be solved by the preparation of isomorphous crystalline compounds, one containing the protein alone; another containing the protein with a heavy atom, such as mercury, attached to the protein in some definite position, say 1; and yet another containing the protein with a heavy atom attached to a different position, say 2 (Fig. 6) (*3*).

The presence of heavy atoms produces measurable changes in the intensities of the diffraction pattern which make it possible to gather information about the values of the phases. This is done in the following way. From the difference in amplitude in the presence or absence of the heavy atom, the distance of the wave crest from the heavy atom can be determined for each set of fringes, and thus, with the heavy atom serving as a common origin, the magnitude of the phase angle can be obtained. Unfortunately, this still leaves an ambiguity of sign, since we cannot tell whether the phase angle is to be measured in the forward or the backward direction, but by examining the diffraction pattern from the second heavy-atom compound, and thus determining the distance of each wave crest from heavy atom No. 2, the ambiguity can be resolved, provided the vector H_1–H_2 is also known. The determination of that vector is one of the vital and often difficult steps in the x-ray analysis (*4*). As long as the number of heavy atoms associated with each protein molecule is not too large, the difficulty can be overcome through calculation of the Fourier series indicated in Fig. 6*d* (*5*).

Having solved the phase problem, we now have to consider the formation of the image. In simpler structures the atomic positions can often be found from two-dimensional representations projected on two mutually perpendicular planes, but in proteins a three-dimensional image is essential. This can be attained by making use of the three-dimensional nature of the diffraction pattern. Figure 2 can be regarded as a section through a sphere which is filled with layer after layer of diffracted spots. When these are recorded, each pair of symmetrically related spots can be made to generate a set of three-dimensional fringes, and these are then superimposed to build up the image of the protein (Fig. 7). The image is represented in the form of a series of sections through the molecule, rather like a set of microtome sections through a tissue, only on a scale a thousand times smaller (Fig. 8).

Finally, the question of resolution has to be faced. The resolving power of the image is roughly equal to the shortest wavelength of the fringes used in building it up, and that wavelength stands in a reciprocal relation to the angle which the corresponding diffracted ray makes with the incident x-ray beam (Fig. 3). If the image is built up from fringes corresponding to only part of the diffraction pattern, the resolution is impaired (Fig. 4, *c* and *d*). In the x-ray diffraction patterns of protein crystals the number of spots runs into tens of thousands. These all have to be measured in several isomorphous compounds, then the results are corrected by various geometric factors and finally used to build up an image through the superposition of tens of thousands of fringes. For instance, the calculation of a three-dimensional image of myoglobin at 2-angstrom resolution involved the recording and measuring of about a quarter of a million spots, and in the final calculation about 5×10^9 figures had to be added or subtracted (*6*). Clearly this would have been impossible before the advent of high-speed computers, and we have in fact been very fortunate, because the development of computers has always just kept in step with the expanding needs of our x-ray analyses.

Function and Structure of Hemoglobin

Vertebrate hemoglobin is a protein of molecular weight 64,500. Four of its 10,000 atoms are iron atoms which are combined with protoporphyrin to form four heme groups. The remaining atoms are in four separate polypeptide chains, each containing just over 140 amino acid residues. In human hemoglobin their sequence is now known (*7*). In horse hemoglobin it has been partly elucidated; horse hemoglobin differs from the human form in only a small number of residues (*8*).

Hemoglobin acts as a carrier of oxygen from the lungs to the tissues and of carbon dioxide back to the

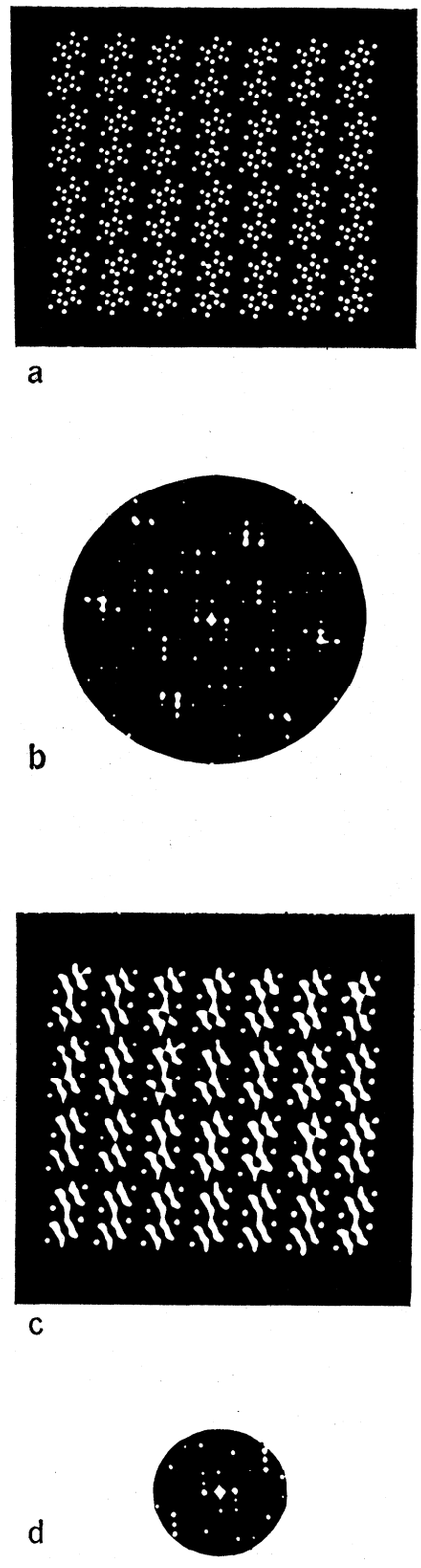


Fig. 4. (*a*) Image of hexamethyl benzene structure reconstituted by recombination of the fringes produced by the x-ray diffraction pattern (*b*). The recombination is done in an optical diffraction apparatus. Lack of resolution of image (*c*) caused by covering the outer part of the diffraction pattern (*b*) and using only the fringes produced by the innermost spots as shown in (*d*).

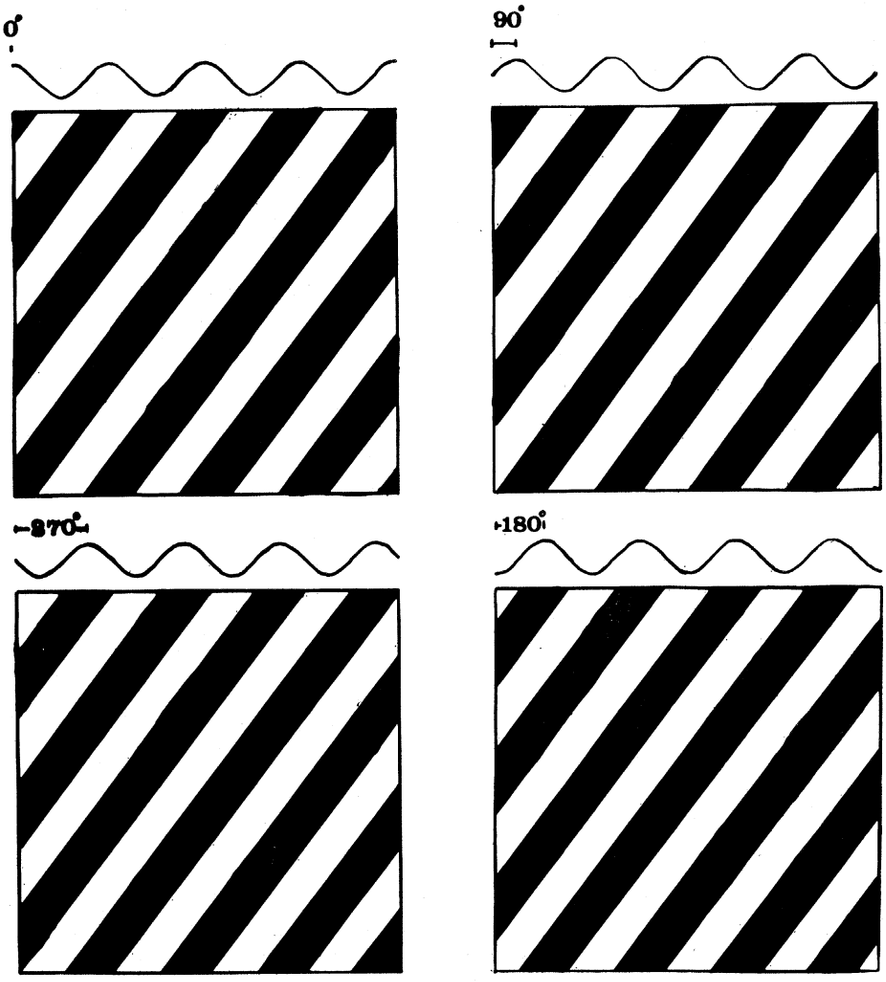


Fig. 5 (top). Four identical sets of fringes related by different phase angles to the origin at top left-hand corner. The phase angle marks the distance of the wave crest from the origin, in degrees, one complete wavelength being taken as 360 degrees.

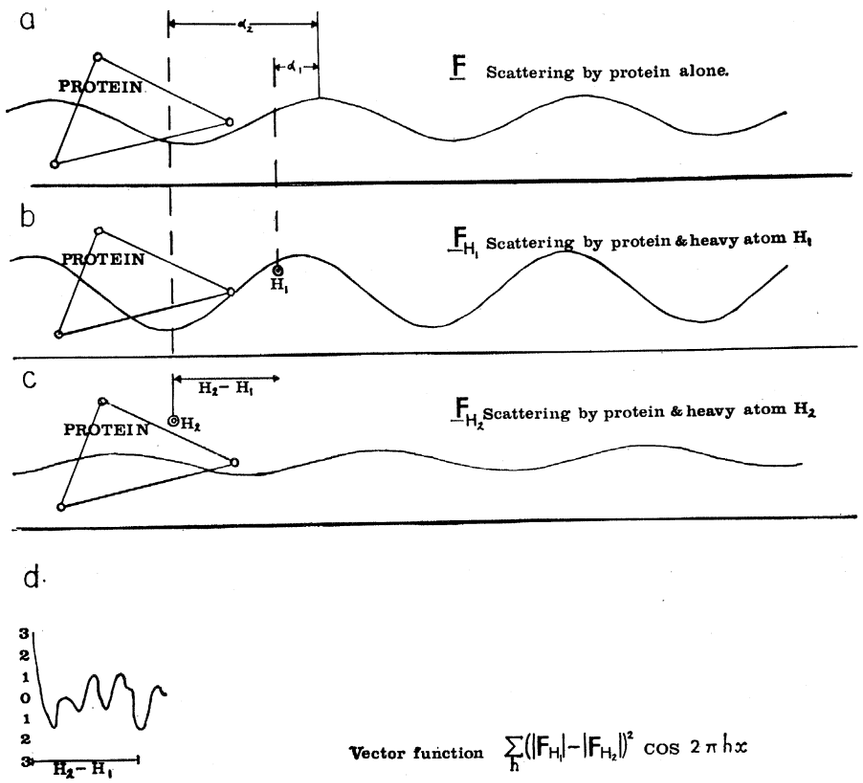


Fig. 6 (bottom). (*a*) Diffraction by a triangle representing a protein molecule. (*b* and *c*) Change in amplitude and phase of the diffracted wave caused by the presence of heavy atoms H_1 and H_2, respectively; α_1 and α_2 are the phase angles of the set of fringes *F* related to H_1 and H_2 as origins. (*d*) Vector function devised by M. G. Rossmann. The Fourier sum shows a marked dip at a distance from the origin which corresponds to the vector H_1–H_2. Normally this function would be evaluated in three dimensions.

lungs. The four iron atoms are in the ferrous state, and each is capable of combining reversibly with one molecule of oxygen without itself becoming oxidized in the process. The four iron atoms interact in a physiologically advantageous way, so that the combination of any one of them with oxygen increases the rate of combination with oxygen of its partners. A similar interaction takes place when the oxygen is given up. Carbon dioxide is not carried by the iron atoms directly, but its uptake by the red cells or the serum is facilitated by the disappearance of an acid group from each quarter-molecule of hemoglobin when it has given up its oxygen. Conversely, the presence of acid in the tissues speeds the liberation of oxygen by hemoglobin (*9*). The hemoglobin molecule may therefore be regarded as an enzyme with two functions and several active sites, which interact in a complex and sophisticated manner. The explanation of this behavior is one of the main objects of our research.

We have carried out three-dimensional x-ray analyses of the oxyhemoglobin of horse and of the reduced hemoglobin of man. The resolution so far attained is 5.5 angstroms; this is sufficient to show the course of the polypeptide chains and the positions of the heme groups but does not allow individual amino acid residues to be seen. Nevertheless, the results suggest that the reduced and the oxygenated forms differ markedly in structure, and that a molecular rearrangement accompanies the reaction of hemoglobin with oxygen.

The structure of horse oxyhemoglobin was published some time ago,

and I need merely remind you of its main features (*10*). In agreement with the chemical evidence, the electron density maps show four heme groups and four separate chains which are identical in pairs. They are very similar in structure, and each chain bears a strong resemblance to sperm whale myoglobin (*6*), whose molecular architecture forms the subject of Kendrew's discourse (*11*). He shows that its single polypeptide chain is made up of eight helical stretches interrupted by corners or nonhelical regions, and that the N-terminal end of the chain is at the bottom left (Fig. 9).

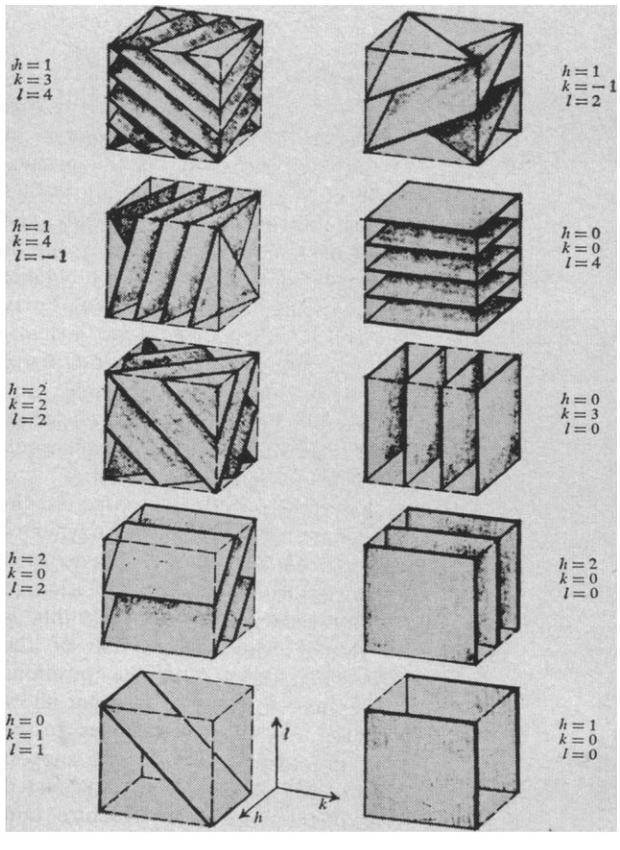
The hemoglobin molecule is assembled by first matching each chain with its symmetrically related partner (Fig. 10), then inverting the pair of white chains and placing them over the top of the pair of black ones (Fig. 11). The resulting arrangement is tetrahedral, the four subunits forming a compact spheroidal molecule, with the heme groups arranged in separate pockets on the surface of the molecule (Figs. 12 and 13). The wide distances separating the heme groups were perhaps the greatest surprise the structure presented to us, for one would have expected the chemical interaction between the groups to be due to close proximity. As it stands, the structure of oxyhemoglobin leaves the physiological properties unexplained.

The oxygen-free form of hemoglobin, somewhat inappropriately called reduced hemoglobin, has long been known to differ from oxyhemoglobin in solubility, crystal structure (*12*), and other properties; this suggested that the explanation should perhaps be sought in a structural rearrangement between the two forms. Unfortunately, reduced hemoglobin of horse crystallizes in a form unsuitable for detailed x-ray analysis, so we turned to human hemoglobin, which is more amenable to x-ray analysis. This choice leaves a gap in the argument, because the structure of human oxyhemoglobin is still unknown and may conceivably differ from that of horse oxyhemoglobin in the same manner as human reduced hemoglobin. In view of the close similarity between the amino acid sequences of the two species this seems unlikely, but obviously the point remains to be proved.

So far the model for the structure of human reduced hemoglobin is based on x-ray analysis of only a small number of isomorphous heavy-atom derivatives, as compared to the six used as a basis in studies of horse oxyhemoglobin, and for technical reasons these derivatives are insufficient for accurate determination of the phase angles (*13*).

Despite these imperfections, several features stand out clearly. The molecule is made up of four subunits which appear to be very similar in structure to those found in horse oxyhemoglobin, but there is a striking rearrangement of the two black subunits, involving an increase of over 7 angstroms in the distance between symmetrically related features. The relative arrangement of the white subunits is unchanged.

Chemically, the white units are



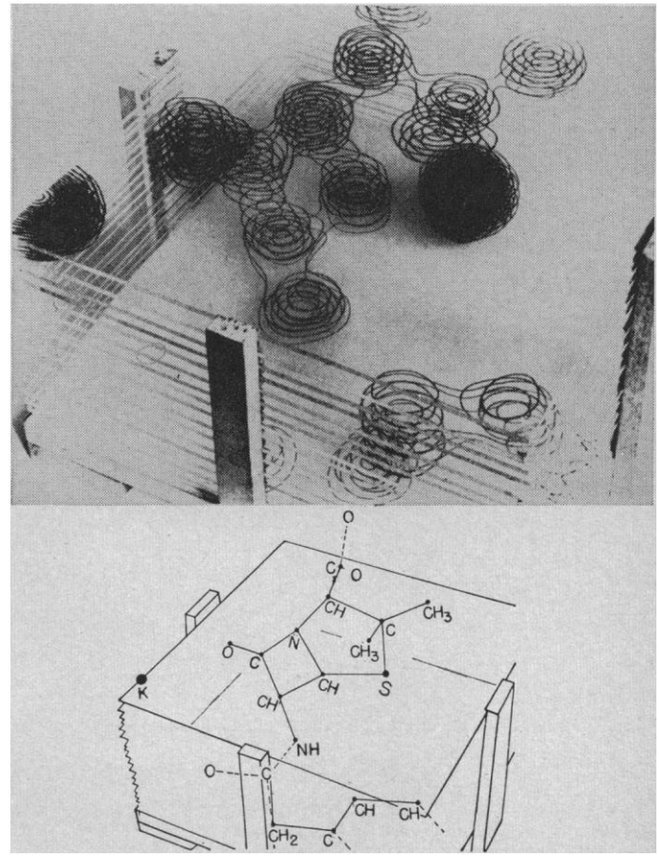


Fig. 7 (left). Set of three-dimensional fringes used to build up the image of the electron density. Each set is generated by a pair of spots with indices h, k, l, and $-h$, $-k$, $-l$ in the x-ray diffraction pattern. [From W. de Beauclair, *Verfahren und Geräte zur mehrdimensionalen Fouriersynthese* (Akademie-Verlag, Berlin, 1949), reproduced with permission]. Fig. 8 (right). Three dimensional representation of the electron density distribution in penicillin, with resolution sufficient to separate individual atoms. Contours represent levels of equal electron density. [From D. Crowfoot *et al.* (Princeton Univ. Press), reproduced with permission].

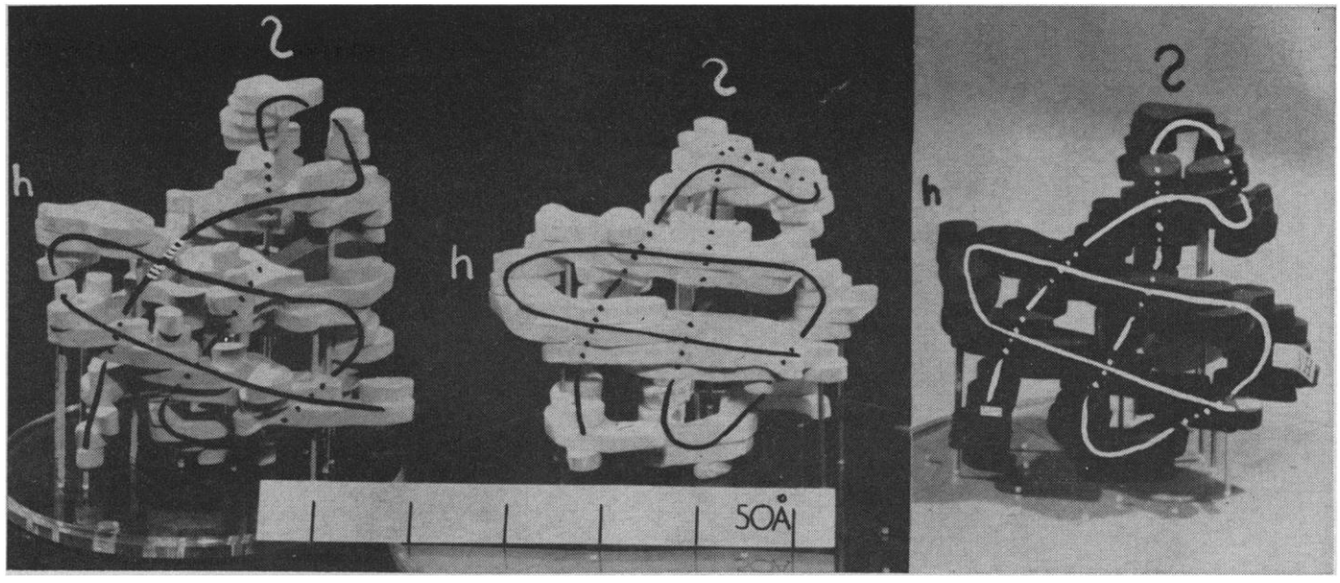


Fig. 9 (top). Comparison of myoglobin (left) with the white and black units of hemoglobin (middle and right).

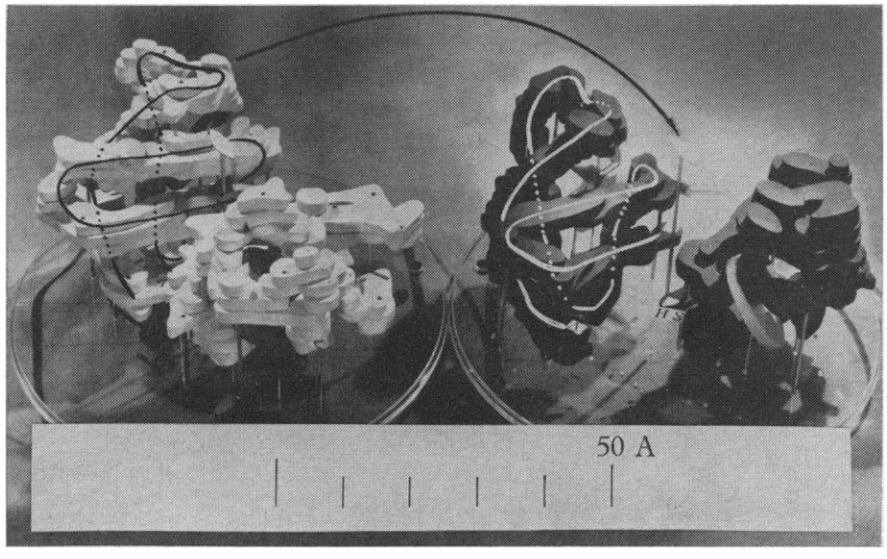


Fig. 10 (middle). Two pairs of chains symmetrically related by the dyad axis. The arrow shows how one pair is placed over the other in assembly of the complete molecule.

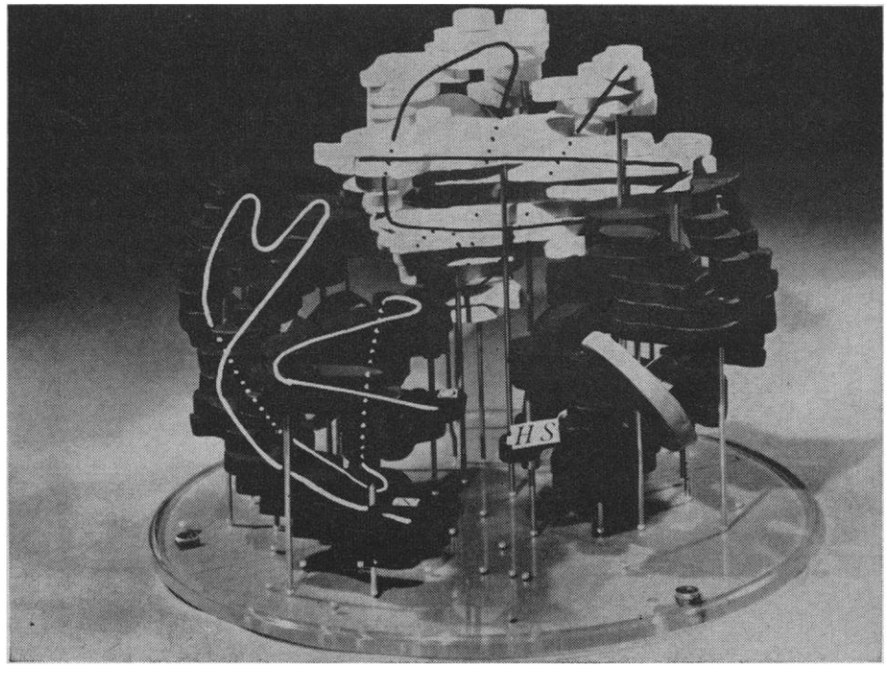
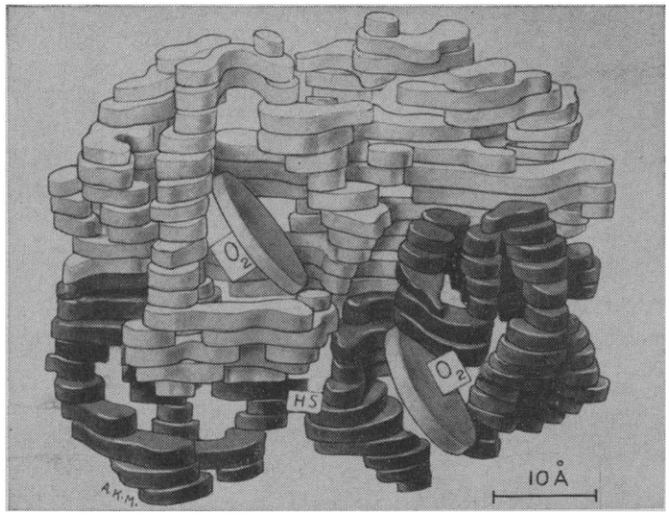


Fig. 11 (bottom). Partly assembled molecule, showing two black chains and one white.

known as the α-chains, and the black, as the β-chains (*14*). The rearrangement of the two β-chains in reduced hemoglobin must of course have come about as the result of some structural rearrangement within them, or possibly at the points of contact between the α- and β-chains. Small structural changes cannot be distinguished at the resolution now attainable but it should be possible to detect any major change —for instance, in the angles between the helical segments. However, these angles appear to be the same, within the limits of experimental error, in human reduced hemoglobin and in horse oxyhemoglobin, and whatever structural change takes place within the individual chains are too small to be detected at the present resolution.

We may hope that heme-heme interaction, and the acid shift on which the respiratory functions of hemoglobin depend, will eventually be explained in terms of the structural changes of which these new results have given us a first glimpse, but it may well be necessary to solve the structure of at least one of the two forms in atomic detail. Because of the enormous amount of labor



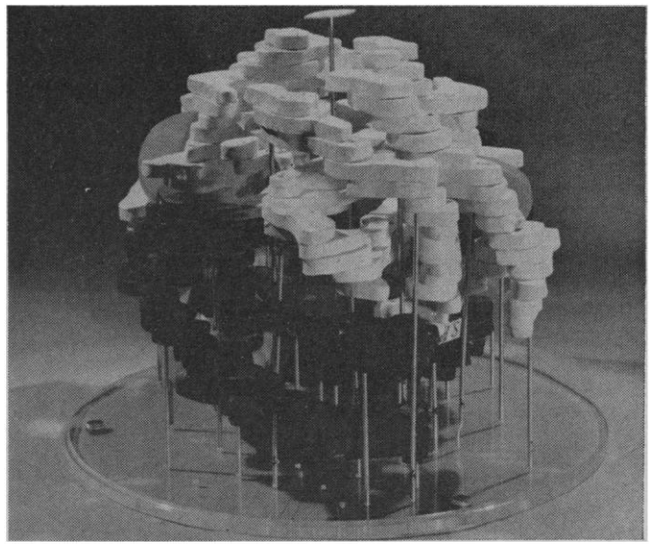


Fig. 12 (left). Complete hemoglobin molecule. The heme groups are indicated by grey disks. Fig. 13 (right). Hemoglobin molecule viewed from another direction.

involved, this may take some time, but not much, perhaps, as compared to the 22 years needed for the initial analysis.

The discovery of a marked structural change accompanying the reaction of hemoglobin with oxygen suggests that there may be other enzymes which alter their structure on combination with their substrate and that this may perhaps be an important factor in certain mechanisms of enzymatic catalysis (*15; 16*).

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16. I have already alluded to the great part played by Sir Lawrence Bragg in supporting this work, but I also owe him an immense debt for the many ideas he contributed and for his astute criticism. Sir Harold Himsworth, as secretary of the Medical Research Council, made sure that we never lacked any of the funds or equipment needed for our work. Without his consistent support, research on the required scale could not have been done. Dr. G. R. Pomerat, as one of the directors of the Rockefeller Foundation, helped us to cover any additional expenditure which the Medical Research Council had difficulty in meeting. Moreover, the Rockefeller Foundation supported me in the early years; without this support the work would probably not have got beyond its initial stages.