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X-ray analysis of haemoglobin

Nobel Lecture, December 11, 1962

Forty years ago William Lawrence Bragg came here 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 unravelling the atomic arrangement in crystals of common salt and other simple compounds.* Today 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 honour.

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 distancesa. Soon afterwards Sir Lawrence Bragg was appointed Cavendish Professor. My X-ray diffraction pictures of haemoglobin at once fired his imagination and enthusiasm. He was fascinated by the idea that the powers of X-ray analysis might be extended to 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 such highly complex structures, and I know that Bragg is overjoyed by the success and the honours that have now rewarded us.

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. I) 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
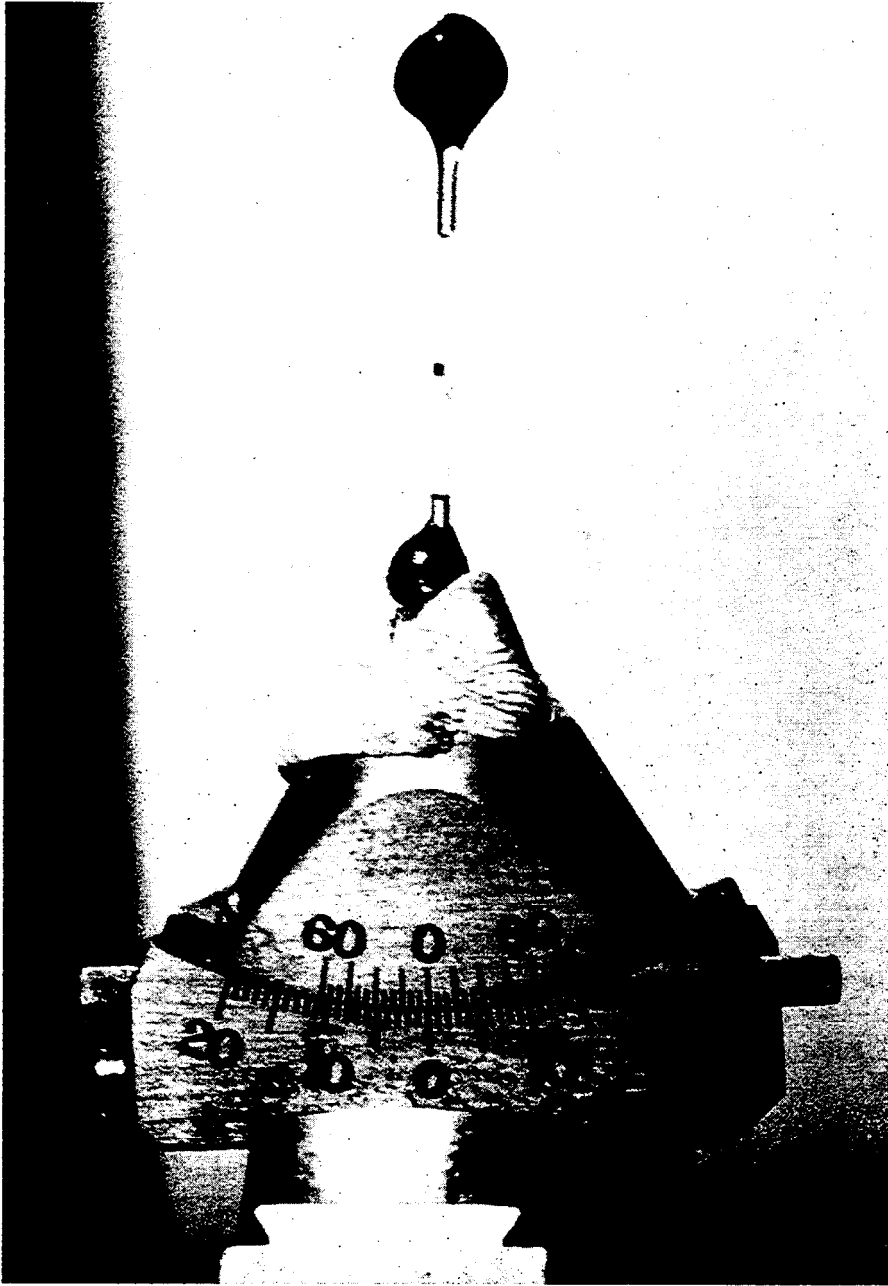
Fig.I. Haemoglobin crystal in equilibrium with its mother liquor, mounted for X-ray
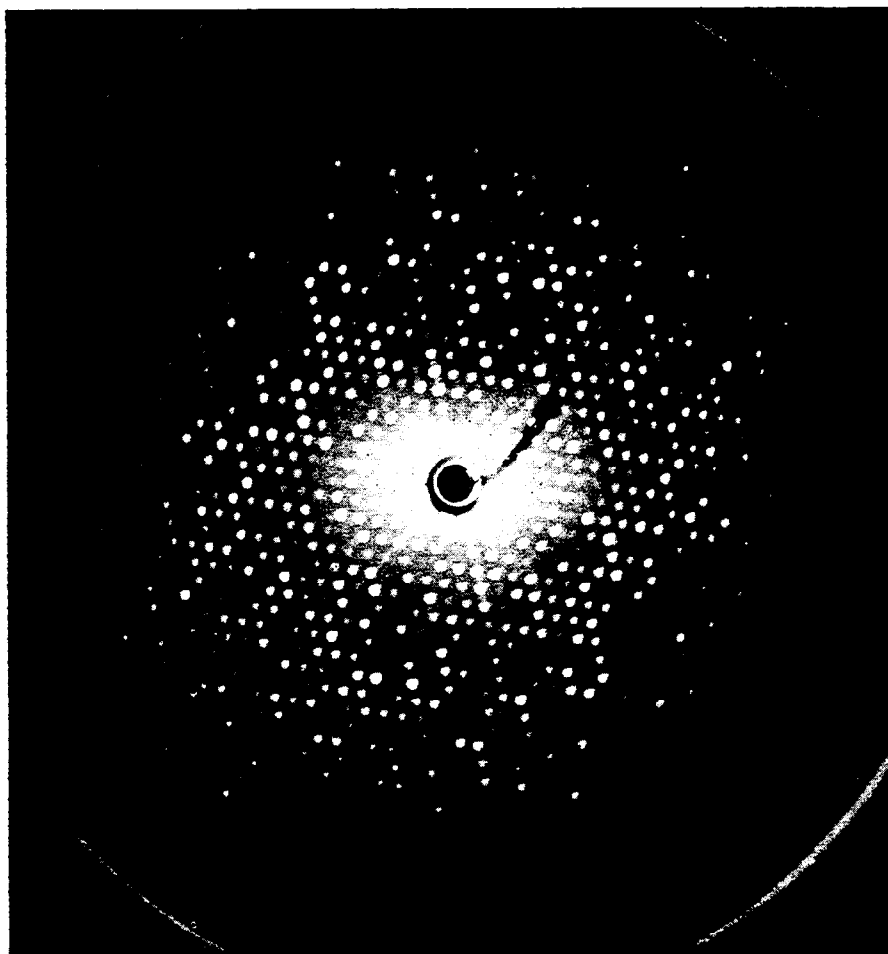analysis. (The glass capillary is about I mm wide.)

Fig. 2. X-ray diffraction pattern from a haemoglobin crystal, taken with a Buerger Precession Camera. The pattern fades out near the rim of the picture, which corresponds to a spacing of 1.8Å.

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. In his Nobel Lecture *(Les Prix Nobel en 1921-1922)* Sir Lawrence Bragg explained this relationship in the following words:

"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
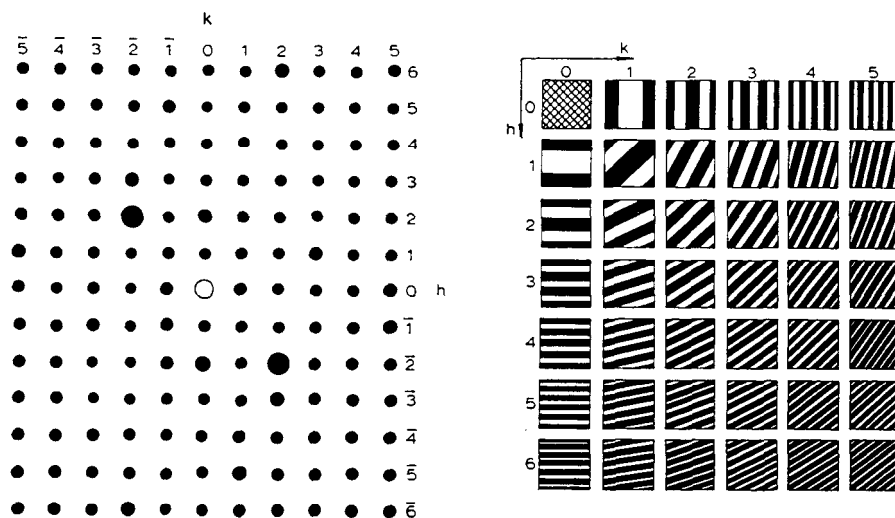
Fig. 3. *(Left)* : Array of spots in an X-ray diffraction pattern. The varying intensities are represented by circles of varying size. The orders are given on the margin. *(Right) :* Sets of fringes produced by pairs of spots in the diffraction pattern which are symmetrically related about the origin at the centre. The order of the fringes *h* and *k* corresponds to the order of the spots used to produce them. *(Reproduced, by permission, from W. de Beauclair, "Verfahren und Geräte zur mehrdimensionalen Fouriersynthese", Akademie-Verlag, Berlin 1949.)*

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 influence 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 arrange-

ment. 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, using either calculation or optical analogues, to give the required image (Fig. 4a).

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 a set of fringes of given
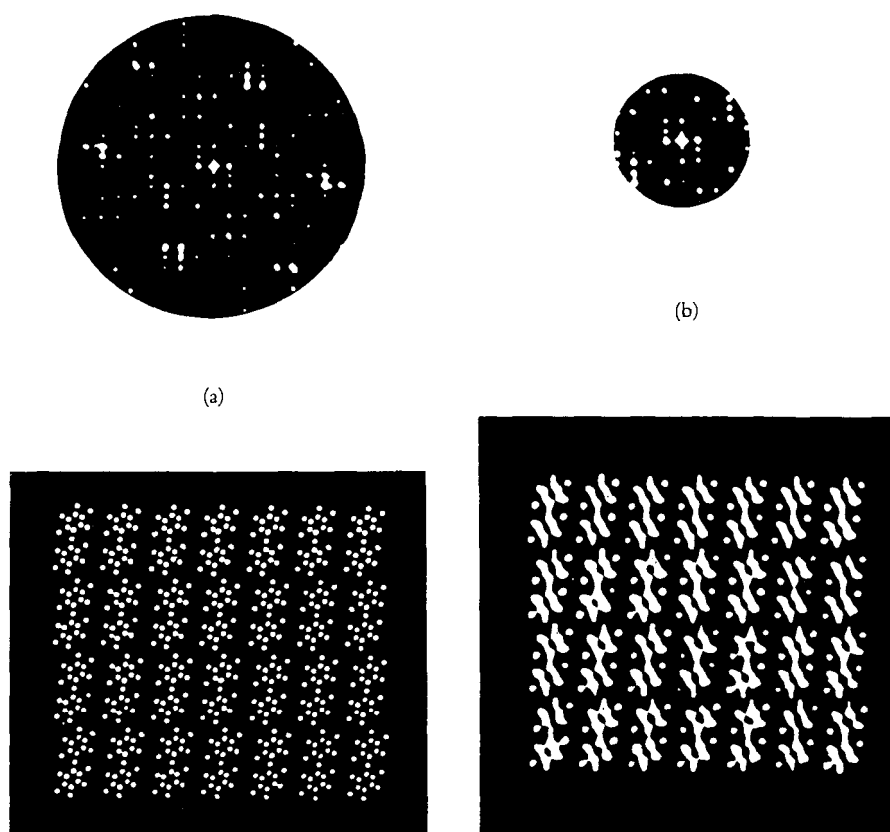

(a)

(b)

Fig. 4. (a) Image of hexamethyl benzene (bottom) reconstituted by recombination of the fringes produced by the X-ray diffraction pattern (top). The recombination is done in an optical diffraction apparatus. (b) Lack of resolution of image (a) caused by covering the outer part of the diffraction pattern. (By courtesy of Dr. C. A. Taylor.)
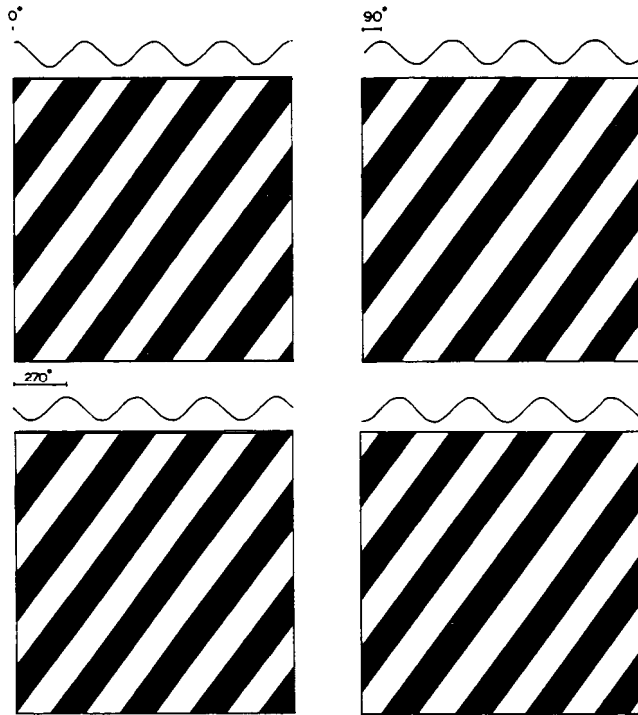
Fig. 5. Set of fringes related by different phase angles to the origin at the top left-hand corner. The phase angle marks the distance of the wave crest from the origin in degrees, one complete wavelength being taken as 360°.

amplitude, as shown in Fig. 5, 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 the amplitude, but not about the phase angles of the fringes to be generated by each pair of spots, so that 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 I, and yet another containing the protein with a heavy atom attached to a different position, say 2 (Fig. 6)[3-6].

The presence of heavy atoms produces measurable changes in the intensities of the diffraction pattern which allow information to be gathered 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, thus giving the magnitude of the phase angle, with the heavy atom serving as a common origin. Unfortunately, this still leaves an ambiguity of sign, since we cannot tell whether the phase angle is to be measured in the forward or backward direction, but by examining the diffraction pattern from the second heavy atom compound, giving the distance of each wave-crest from heavy atom No. 2, the ambiguity can be resolved, provided that 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[7,8]. As long as the number of heavy atoms associated with each protein molecule is not too large, it can be overcome by calculation of the Fourier series[9] indicated in Fig. 6d.

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 perpen-
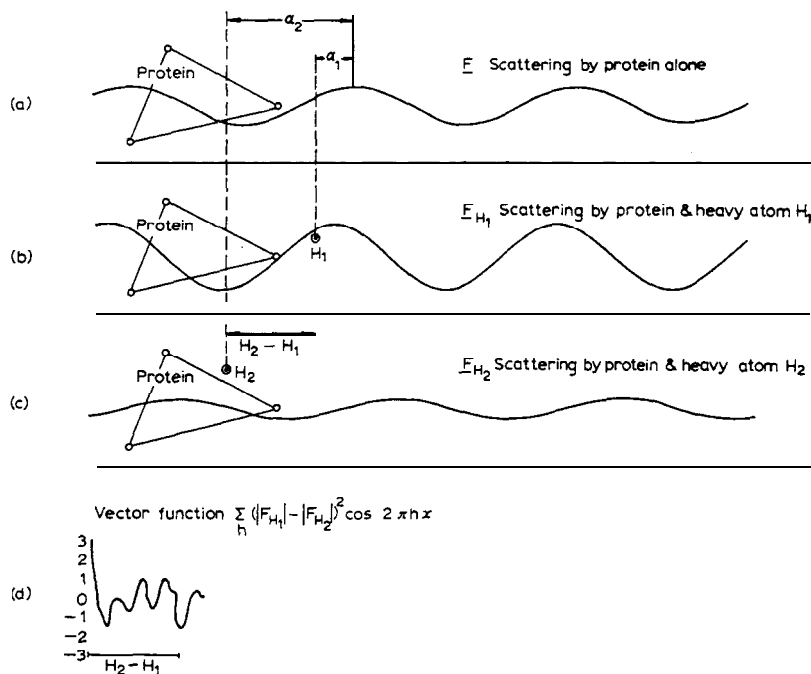
Fig. 6. (a) Diffraction by triangle representing a protein molecule. *(b)* and (c) Change in amplitude and phase of the diffracted wave caused by presence of heavy atoms H_1 and H_2. (d) Vector function devised by M. G. Rossmann. The Fourier sum shows a marked dip at a distance from the origin which corresponds to the vector H_1-H_2. Normally this function would be evaluated in three dimensions.
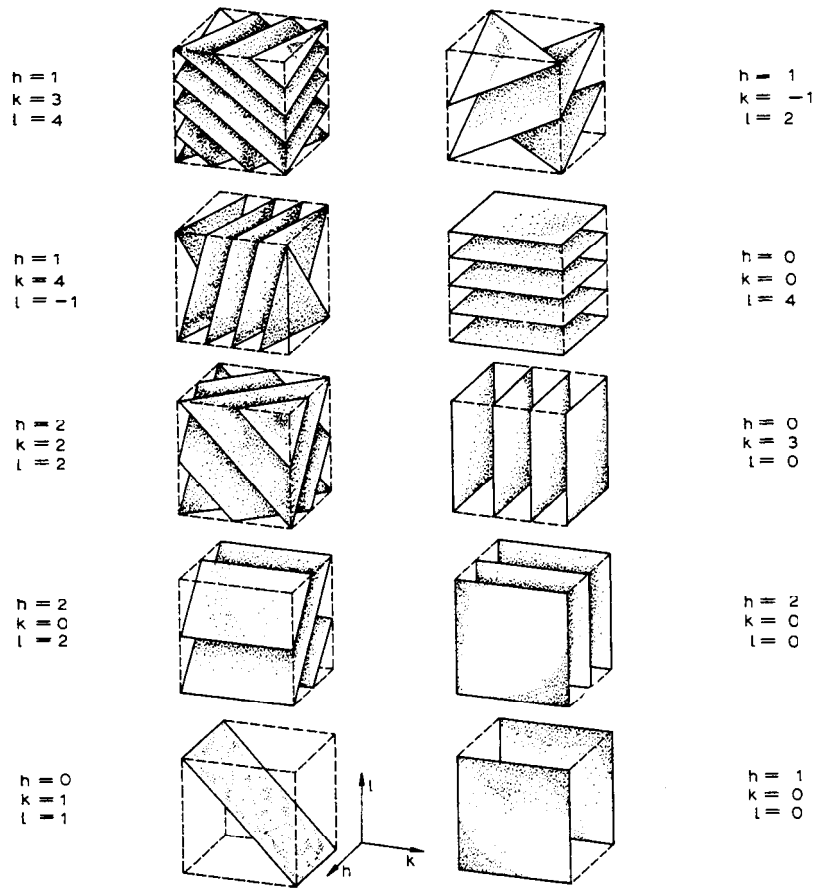
Fig. 7. Set of three-dimensional fringes used to build up image of the electron density. *(Reproduced, by permission, from W. de Beauclair, "Verfahren und Geräte zur mehrdimensio-ualen Fouriersynthese", Akademie-Verlag, Berlin, 1949.)*

dicular 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. Fig. 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 thousand times smaller scale (Fig. 8).

Finally the question of resolution has to be faced. The resolving power of
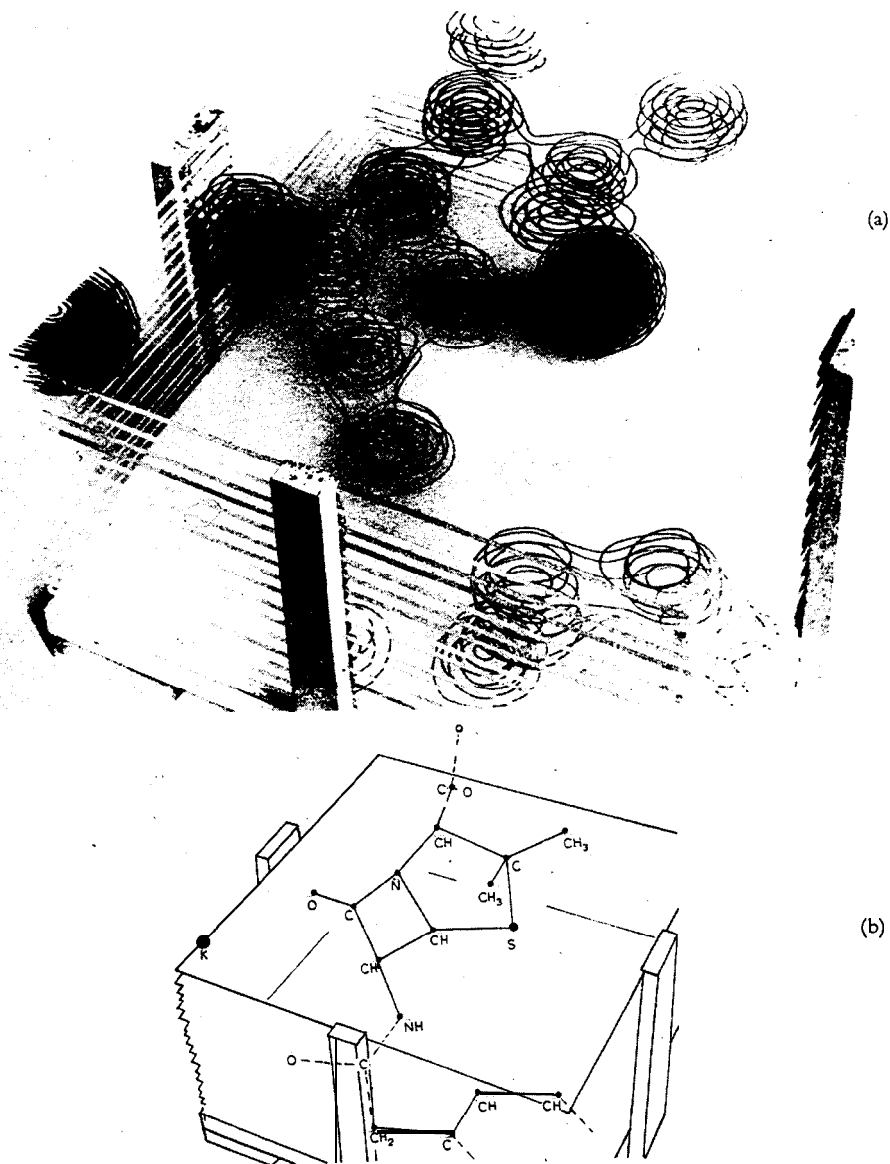
(a)

(b)

Fig. 8. Three-dimensional representation of the electron density distribution in penicillin, with resolution sufficient to separate individual atoms. *(Reproduced, by permission, from D. Crowfoot et al., Princeton University Press).*
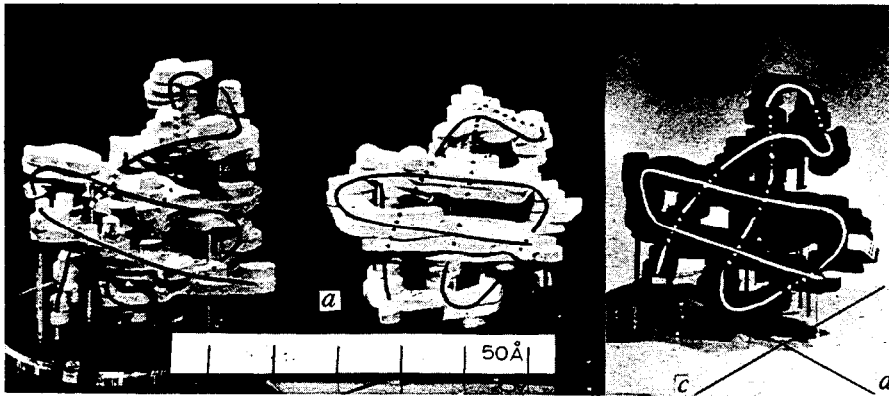
Fig. 9. Comparison of myoglobin (a) with the white and black units of haemoglobin
(b) and (c).

the image is roughly equal to 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. 4b). 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, corrected by various geometric factors and finally used to build
up an image by the superposition of tens of thousands of fringes. For instance,
the calculation of a three-dimensional image of myoglobin at 2 Å 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 sub-
tracted[10]. 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 haemoglobin

Vertebrate haemoglobin is a protein of molecular weight 64,500. Four of its
10,000 atoms are iron atoms which are combined with protoporphyrin to
form 4 haem groups. The remaining atoms are in four separate polypeptide
chains, each containing just over 140 amino acid residues. In human haemo-

globin their sequence is now known[11,12]. In horse haemoglobin it has been partly elucidated and differs from the human form in only a small number of residues[13,14].

Haemoglobin acts as a carrier of oxygen from the lungs to the tissues and of carbon dioxide back to the 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 haemoglobin when it has given up its oxygen. Conversely the presence of acid in the tissues speeds the liberation of oxygen by haemoglobin[15]. The haemoglobin molecule may therefore be regarded as an enzyme with two functions and several active sites,
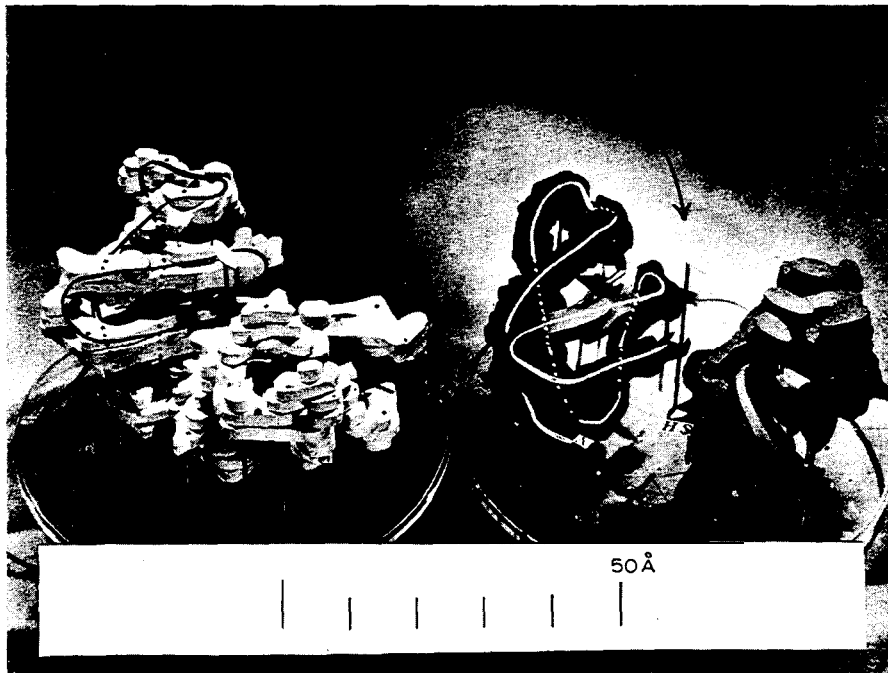

Fig. 10. Two pairs of chains symmetrically related by the dyad axis. The arrow shows how one pair is placed over the other to assemble the complete molecule.
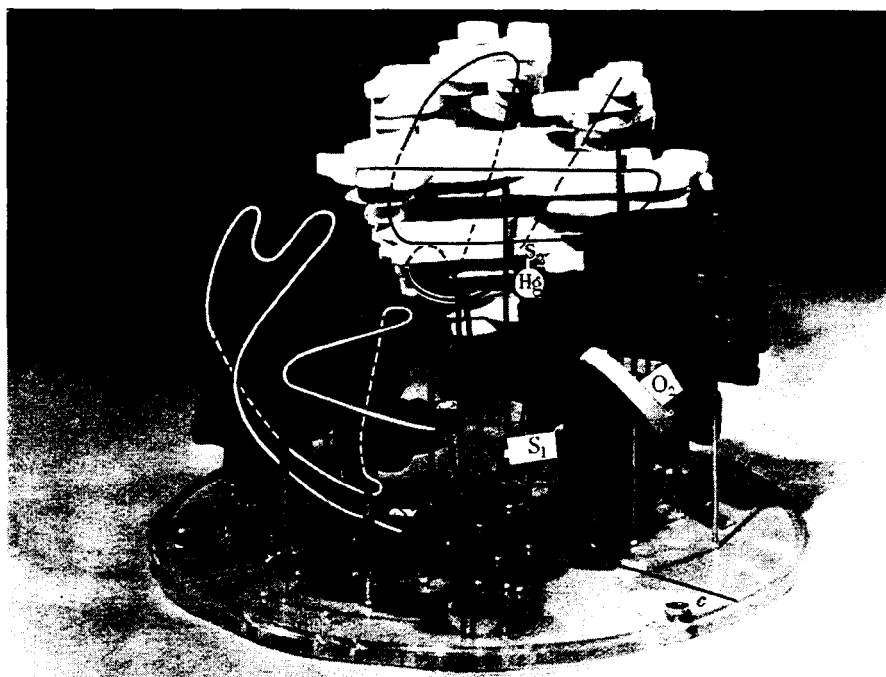
Fig. II. Partly assembled molecule showing two black chains and one white. The *white ball* indicates a mercury atom attached to one of the "hidden" cysteines (S_2). S_1 indicates one of the reactive cysteines.

which interact in a complex and sophisticated manner. The explanation of this behaviour is one of the main objects of our research.

We have carried out three-dimensional X-ray analyses of the oxyhaemoglobin of horse and of the reduced haemoglobin of man. The resolution so far attained is 5.5 Å, which is sufficient to show thecourse of the polypeptide chains and the positions of the haem groups, but does not allow individual amino-acid residues to be seen. Nevertheless the results show that the reduced and the oxygenated forms differ markedly in structure, and they suggest that a molecular rearrangement accompanies the reaction of haemoglobin with oxygen.

The structure of horse oxyhaemoglobin has been published some time ago and I need merely remind you of its main features[16,17,18]. In agreement with the chemical evidence, the electron density maps show four haem 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[10] whose molecular architecture will form the subject of Dr. Kendrew's discourse. He will show you that its single polypeptide chain is made up of eight helical stretches interrupted by comers or non-helical regions, and that the N-terminal end of the chain is at the bottom left (Fig. 9).

The haemoglobin molecule is assembled by first matching each chain with its symmetrically related partner (Fig. 10), then inverting the pair of white chains and placing it 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 haem groups arranged in separate pockets on the surface of the molecule (Figs. 12 and 13). The wide distances separating the haem groups was perhaps the greatest surprise the structure presented to us, for one would have expected the chemical interaction between them to be due to their close proximity. As it stands, the structure of oxyhaemoglobin leaves its physiological properties unexplained.

The oxygen-free form of haemoglobin, called somewhat inappropriately reduced haemoglobin, has long been known to differ from oxyhaemoglobin
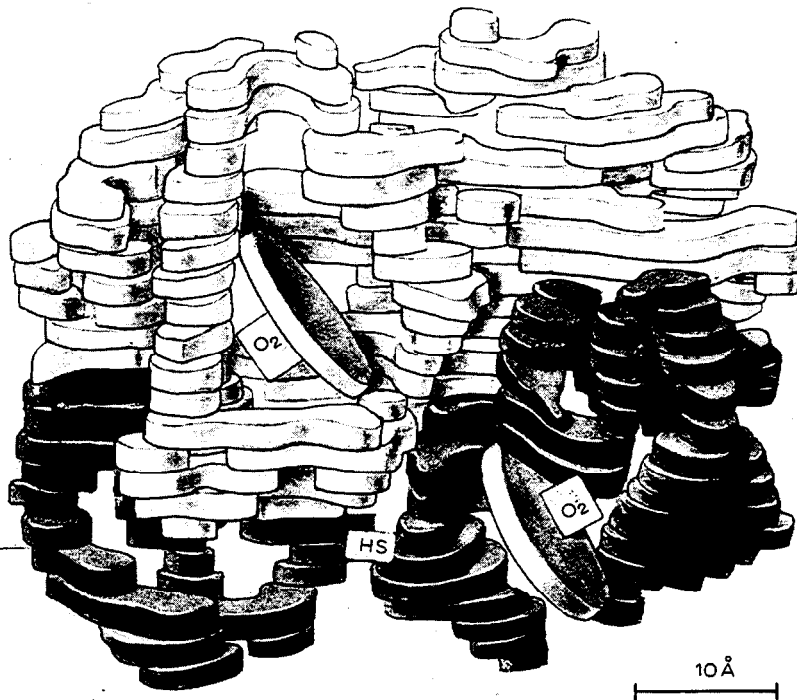

Fig. 12. Complete haemoglobin molecule. The haem groups are indicated by grey disks.
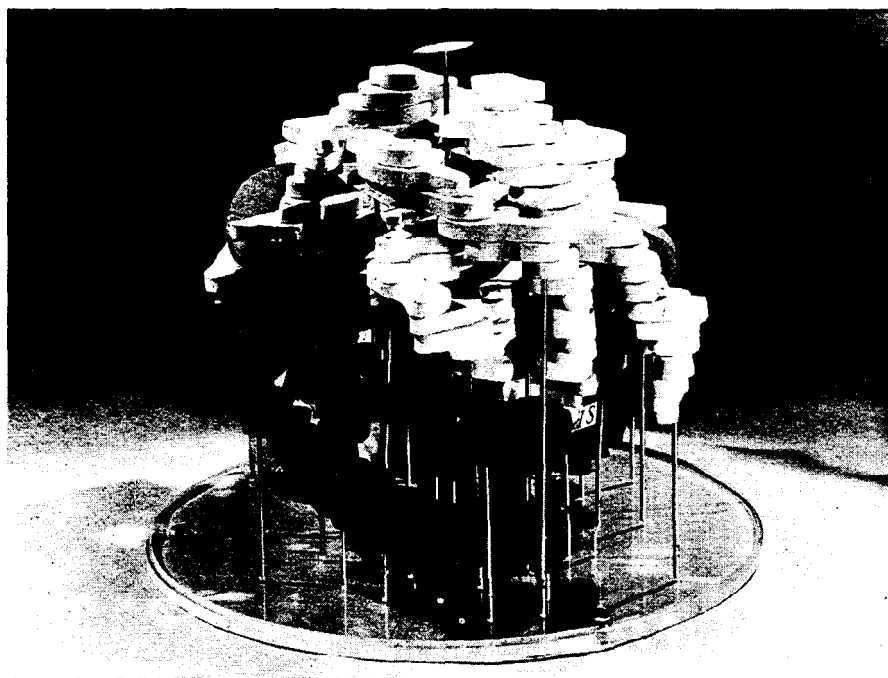
Fig. 13. Haemoglobin molecule viewed in another direction.

in its solubility, crystal structure,[19-21] and other properties, which suggested that the explanation should perhaps be sought in a structural rearrangement between the two forms. Unfortunately, reduced haemoglobin of horse, crystallizes in a form unsuitable for detailed X-ray analysis, so that we turned to human haemoglobin which is more amenable. This choice leaves a gap in the argument, because the structure of human oxyhaemoglobin is still unknown and might conceivably differ from that of horse oxyhaemoglobin in the same manner as the human reduced form. 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. (See note on p. 673.)

So far the structure of human reduced haemoglobin is based on the X-ray analysis of only a small number of isomorphous heavy atom derivatives, as compared to the six used for horse oxyhaemoglobin, and for technical reasons these particular derivatives are insufficient to determine the phase angle accurately[22].

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 oxyhaemoglobin (Fig. 14), but there is a striking rearrangement of the two black subunits, involving an increase in the distance between symmetrically related features by over 7 Å. The relative arrangement of the white subunits is unchanged (Fig. 15).

Chemically, the white units are known as the α- and the black as the β-chains[23]. The rearrangement of the two β-chains in reduced haemoglobin 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 present resolution, 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 error, in human reduced and in horse oxyhaemoglobin, and whatever structural changes take place within the individual chains must be too small to be detected at the present resolution.

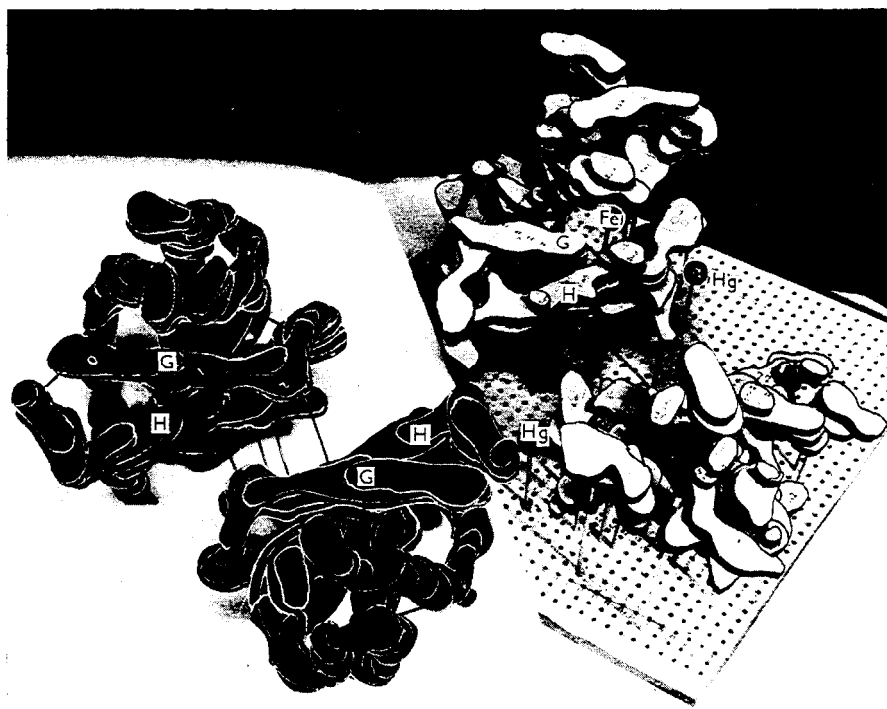We may hope that that interaction, and the acid shift on which the res-

Fig. 14. Comparison of black β-chains of horse oxyhaemoglobin with the corresponding ones of the human reduced form. (Note the helical regions marked G and H.)
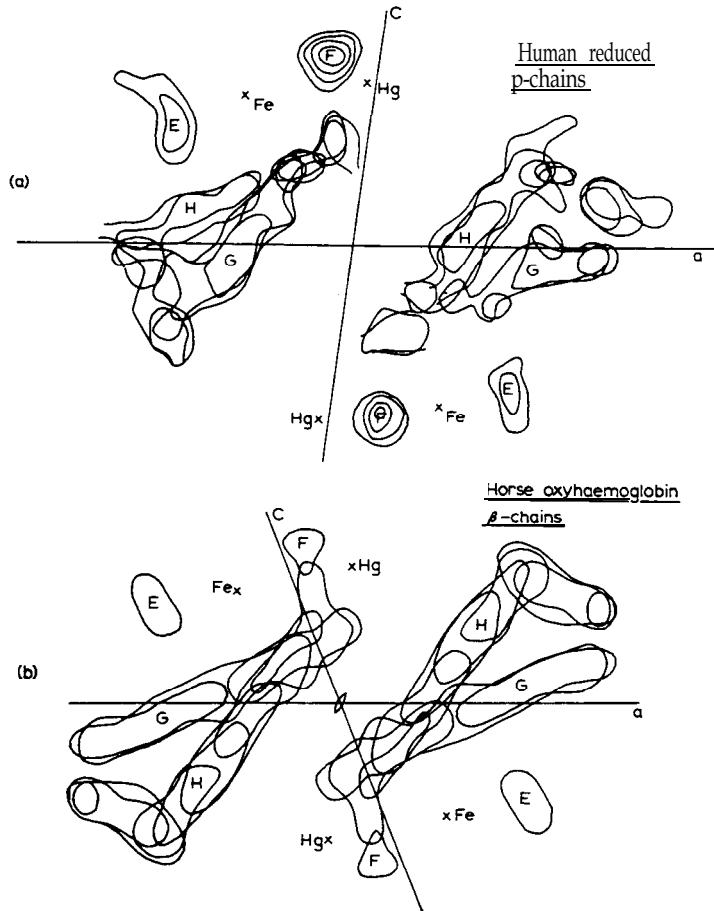
Fig.15. (a) Projection of the helical regions G and H, together with the positions of the iron and mercury atoms (attached to cysteines) and sections through the helices E and F, in human reduced haemoglobin. (b) Same for horse oxyhaemoglobin. (*Fig. 15 continued on pp. 669 and 670.*)

piratory functions of haemoglobin depend, will eventually find their explanation in terms of the structural changes of which these new results have just 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. Due to the enormous amount of labour involved this may take some time, but not much, perhaps, compared to the 22 years needed for the initial analysis.

The discovery of a marked structural change accompanying the reaction of haemoglobin 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.

Please forgive me for presenting, on such a great occasion, results which are still in the making, but the glaring sunlight of certain knowledge is dull and one feels most exhilarated by the twilight and expectancy of the dawn. Thank you again for your magnificent award.

Acknowledgements

I have already alluded to the great part played by Sir Lawrence Bragg in supporting this work, but I also owe 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
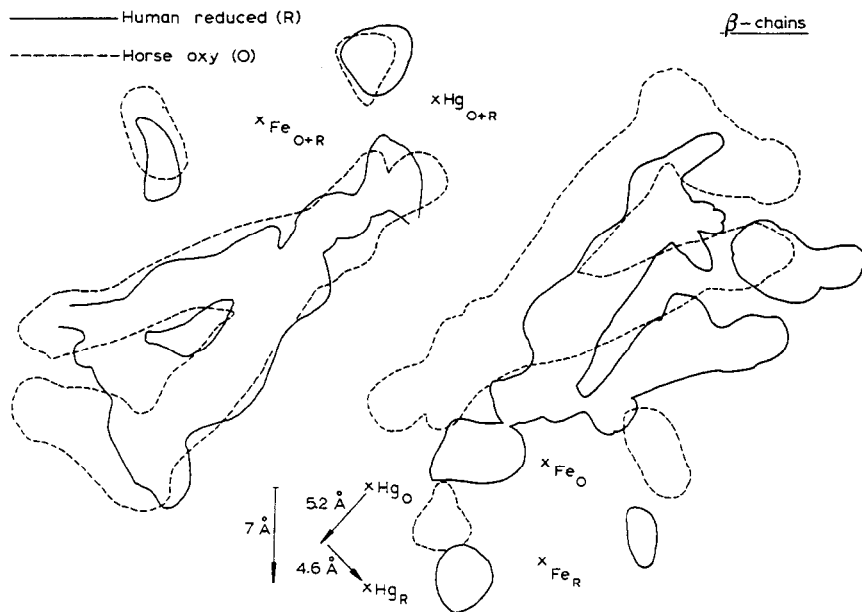

Fig. 15 *(ctd.)* : (c) Superposition of (a) and (b) m outline, showing difference in structure between the two forms. At the *top left,* one member of each pair has been superimposed so as to bring the iron and mercury positions into coincidence. The *bottom right* shows the shift of the two chains relative to each other.
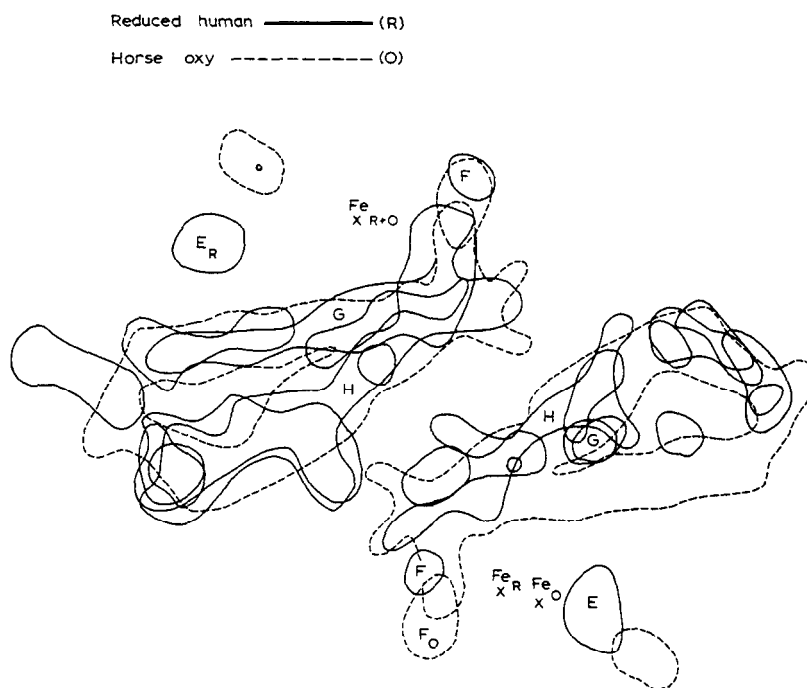
Reduced human ——————— (R)

Horse oxy ———————— (O)

Fig. 15 *(ctd)* : *(d)* Superposition of white chains, showing little change of structure.

Contributors to the Work on the Structure of Haemoglobin

Name	Contribution	Reference
David M. Blow	Development of experimental and theoretical methods for the determination of phase angles by multiple isomorphous replacement.	5
D. A. G. Broad	Design and construction of rotating anode tubes used to produce the high-intensity X-ray beams essential for X-ray diffraction work on proteins. He also designed many other instruments used in this work.	24
Ann F. Cullis	acted as my research assistant during the crucial years when the three-dimensional X-ray analysis was being done.	16, *17,* 18,25
Howard M. Dintzis	introduced new methods for attaching heavy atoms to haemoglobin.	25
John A. L. Fasham	reconstructed and improved the microdensitometer used in measuring the intensities of the X-ray reflexions.	
Michael Fuller	maintained the rotating anode tubes.	
David W. Green	did some of the early work on reduced human haemoglobin and helped me with the first Fourier projections of horse oxyhaemoglobin.	4,21
L. G. Hayward	made the rotating anode tubes and many other instruments used in the work.	
Eric R. Howells	helped me to interpret the X-ray pattern of one of the forms of horse haemoglobin. Hc also did much other useful work on the structure.	27,28
David J. E. Ingram	determined the inclination of the haem groups by electron spin resonance.	26
Vernon M. Ingram	prepared the first crystalline heavy atom derivatives of haemoglobin.	4
Ann Jury	worked as my research assistant in 1959/1960.	
Christian K. Møller	worked on the infrared dichroism of haemoglobin.	
Hilary Muirhead	developed a computer programme for calculating the phase angles in horse oxyhaemo-	16-18,22

Name	*Contribution*	*Reference*
	globin. She also did most of the work on reduced human haemoglobin reported here.	
M. Murayama	made one of the doubly substituted heavy atom derivatives and also improved our chemical methods.	
A. C. T. North (Royal Institution, London)	determined the absolute intensity of the X-ray reflexions, and the relative intensities of all the stronger reflexions by counter spectrometer methods.	16-18
D. C. Phillips (Royal Institution, London)	measured the absolute intensities of reflections from haemoglobin cystals.	
Michael G. Rossmann	developed the mathematical methods used for determination and refinement of the heavy atom parameters and also devised most of the computer programmes used in this work.	9, 16-18
Larry L. Steinrauf	tried to develop new methods of introducing heavy atoms into haemoglobin.	
Ian F. Trotter	did much of the early work on reduced human haemoglobin.	21
Georg Will	took the first set of three-dimensional X-ray photographs of horse haemoglobin and worked out the geometric correction factors.	16

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Note added December 1963. When I received the Nobel Prize a year ago, Miss Muirhead and I had just arrived at the first tentative solution of the structure of human reduced haemoglobin. Since then we have obtained a much clearer set of electron density maps which confirmed the differences between the structures of horse oxy and human reduced haemoglobin just described. However, we were unable to decide whether these were due to the oxygenation reaction, as we believed, or merely to the difference in species[22].

Recently I discovered a new crystalline form of horse reduced haemoglobin and found its structure to be similar to that of human reduced, rather than to that of horse oxyhaemoglobin. Thus the rearrangement of the "black" chains is really due to the oxygenation reaction: a most remarkable effect.