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METAL IONS IN BIOLOGICAL MOLECULES

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24 November 1967

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METAL IONS IN BIOLOGICAL MOLECULES

I. INTRODUCTION

Biological systems are composed mainly of light elements (e.g., H, C, N, O), but small concentrations of metallic elements -- Mg, Fe, Co, Cu, Zn, as well as others -- are also present and they appear to be quite essential for many processes. As charge carriers, metal ions occur in certain transport processes in membranes, but such phenomena will not be considered here. Of far greater subtlety is their role in enzymes, where typical metallic concentrations are of the order of a few atoms in 10^4 while the importance of these few atoms is entirely out of proportion to their concentrations. Two examples will illustrate the point: The enzyme carboxypeptidase is a protein with a molecular weight of 34,000; it contains one atom of zinc, which, if removed, causes the activity to be completely abolished. Hemoglobin, another protein with a molecular weight of 68,000, contains four atoms of iron; the ability of hemoglobin to bind oxygen reversibly and thereby to transport oxygen from the lungs to the tissues is totally dependent on the presence of the iron atoms, to which the oxygen molecules are attached.

The presence of metallic atoms (most often in ionic form) in a biological molecule confers upon it certain special properties which extend the range of possible experimental and theoretical approaches. Thus, there are often new absorption bands, or the molecule may become paramagnetic, or, in special cases, may exhibit Mössbauer resonance. It is largely because of these properties, as well as others, that the subject of biological metallo-organic systems has attracted and fascinated investigators from a variety of fields, including biochemistry, inorganic chemistry, biophysics, chemical physics and solid state physics. Although a great deal of information regarding such systems has been developed, particularly in the last decade, it would be premature to suppose that we understand what is going on.

The material for this report is based on the following:

1. Visit to Physics Department, Univ. of Birmingham, P.B. Moon, D.A. O'Connor, 3 Aug 1967
2. Visit to Nuclear Physics Division, Atomic Energy Research Establishment, Harwell, Berks., L.G. Lang, C.E. Johnson, 18 Aug 1967

3. Participation in an Oxford Inorganic Discussion on Metal Ions in Biological Systems, Oxford Univ., 28-29 Sep 1967

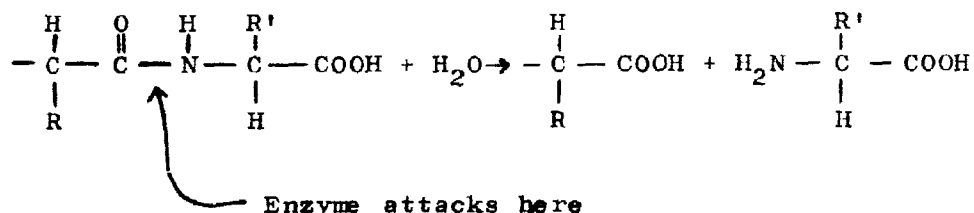
4. Visit to Physics Department, Technische Hochschule, Munich, Germany, R.L. Mössbauer, A. Mayer, A. Trautwein, 2-5 Oct 1967

5. Visit to Laboratory of Molecular Biology, Cambridge Univ., M.F. Perutz, 20 Oct 1967.

II. THE OXFORD INORGANIC DISCUSSIONS

Some 200 people representing varied disciplines gathered for a two-day meeting (28-29 Sep 1967) at Oxford University to discuss "Metal Ions in Biological Systems." The topics and speakers are listed in Appendix I. We give a brief summary of the properties of two molecules which received special attention -- carboxypeptidase (CPD), discussed by B.L. Vallee of Harvard Univ., and ferredoxin (Fd), discussed by F.R. Whatley of King's College.

CPD is a single chain protein of molecular weight 34,300; it consists of 302 amino acids and one atom of zinc. As an enzyme, CPD exhibits peptidase activity, i.e., it will rupture peptide bonds in other proteins by hydrolyzing the CO-NH bond:



CPD has a dual specificity in that it also exhibits esterase activity, a process analogous to the above. The enzymatic activity of CPD follows the zinc atom both in removal and restoration. Also, if the zinc ion is replaced by cadmium or mercuric ions, the esterase activity is enhanced while the peptidase activity disappears. It has been established that the active site consists of a grouping of zinc and the amino acids tyrosine and histidine. The activity is sensitive to minor structural alterations in the molecular architecture.

Ferredoxin (Fd) is a protein with a molecular weight between 6 and 14,000, depending on its source. It is found in photosynthetic green plants and photosynthetic bacteria; it also participates in the conversion of atmospheric nitrogen to NH_3 by nitrogen-fixing bacteria. A molecule of Fd contains

between 2 and 7 iron atoms, depending on the species. The iron can assume either a ferrous or ferric oxidation state, and in plant ferredoxin at least, the iron is not bound in a heme (iron porphyrin).

Other speakers dealt with other systems like xanthine oxidase (R.C. Bray, Chester Beatty), special techniques like spectroscopy (R.J.P. Williams, Oxford), nuclear magnetic resonance (R.E. Richards, Oxford), and Mössbauer absorption (C.E. Johnson, Harwell). Certain aspects of the Mössbauer technique will be discussed later, but first some general remarks regarding the Oxford meeting may be appropriate.

Although the meeting focused attention on the metallic ions present in many enzymes, the basic question being asked was: How does an enzyme work? It was stressed repeatedly that there were many odd features to enzymatic reactions when compared with reactions in other branches of chemistry; that, despite the vast number of enzymes that are known in greater or lesser detail, we are completely mystified by the high rates of reaction and by the high degrees of specificity. Because our knowledge can be said to be almost entirely phenomenological -- a conclusion which carries the implication that there are no concepts to provide useful guidance -- it is necessary to exercise extreme care in the employment of models or model compounds for experimental or theoretical purposes. Conclusions based on a model have frequently turned out to be irrelevant, insofar as the real compound was concerned. Finally, in a metallo-enzyme, it is not fruitful to weigh the relative importance of the metal vs. the organic part; the system acts as a unit and one constituent without the other is worthless.

The metallo-enzymes -- important in their own right in various biological processes -- occupy a unique position in that they can be investigated by a wider arsenal of techniques, which, it is assumed, increases the probability of ultimately arriving at a fundamental understanding of their mechanism. Success in this regard would constitute a major breakthrough in biology, fully comparable with that which followed the discovery of the structure of DNA.

III. MOSSBAUER ABSORPTION IN PROTEINS

Since the discovery in 1958 of recoilless emission and absorption of gamma rays in certain nuclei -- an effect named after its discoverer, R.L. Mössbauer -- there has arisen a vast literature on applications as varied as general relativity and biological molecules.

The central idea behind the various applications of the Mössbauer effect may be described as follows: Under suitable conditions, certain radioactive nuclei emit gamma rays that are extremely monochromatic. For example, the excited nucleus Fe^{57} emits, among other things, gamma rays having an energy of 14,400 eV and a monochromaticity to about one part in 10^{12} , i.e., the line width is of the order of 10^{-8} eV. Moreover, there exist relatively simple means of varying the gamma ray energy over several (or more) line widths. It is therefore quite feasible to do absorption spectroscopy in which the absorbing entities are other nuclei of Fe^{57} (in their ground state) and whose energies may have been altered, through various interactions by amounts of the order of 10^{-8} eV. The Mössbauer effect thus provides a method for measuring extremely small changes in energy, and from such measurements we attempt to draw conclusions regarding the interactions which have brought about these energy changes. In other words, we are using a nucleus as a probe to sample its local environment, which consists of electric and magnetic fields established, generally, by electrons in the vicinity of the nucleus. In the simplest case, a Mössbauer spectrum consists of only one line; more often, in actual cases of interest -- particularly for biological molecules -- the spectrum is quite complex and usually becomes more so as the temperature is reduced.

The Harwell group (L.G. Lang, C.E. Johnson, and W. Marshall) have been concentrating on the low-temperature region where the magnetic hyperfine interaction is revealed. This is an interaction between the magnetic moments of unpaired electrons and the magnetic moment of the nucleus, and is described by a Hamiltonian of the general form

$$\mathcal{H} = \vec{I} \cdot \mathbf{a} \cdot \vec{S} \quad (1)$$

in which \vec{I} and \vec{S} are the nuclear and electronic spin operators; \mathbf{a} is a tensor which contains: (1) the interaction of the nucleus with the electronic orbital angular momentum; (2) the dipole-dipole interaction between nuclear and electronic spins; and (3) the Fermi contact interaction with s-electrons which may also give non-vanishing contributions by indirect effects such as exchange interactions with d-electrons.

A necessary condition for (1) to be valid is that the magnetic moments of the electrons must not change direction too rapidly. Stated more precisely, the average time between flips -- the relaxation time -- of the electronic moments must be long compared with the period of the Larmor precession of the nuclear

moments in the effective field at the nucleus. Otherwise, the field at the nucleus averages to zero and there is no magnetic hyperfine interaction. In ferric compounds (high spin) where the above condition is usually satisfied mainly because of the absence of orbital angular momentum, the effective field at the nucleus lies in the region of several hundred kilogauss. Such fields give rise to a nuclear Zeeman effect which may or may not be fully resolved in the Mössbauer spectrum.

Additional information may be obtained by applying an external magnetic field, as the Harwell group have clearly demonstrated. However, here it is necessary to distinguish between weak and strong fields as well as between short and long relaxation times. In this context, a magnetic field is said to be strong if it is sufficient to decouple the nuclear and electronic spin systems; in the compounds of interest here -- those containing iron -- the coupling is weak, and magnetic fields greater than about 100 gauss are considered to be strong. Thus, the direct contribution of the applied field to the effective field at the nucleus is negligible; the major effect is to hold the electronic spins fixed in magnitude and direction.

In the presence of an applied field, considered to be strong in the above sense, the computation proceeds in one of two possible directions. If the relaxation time is long, the total Mössbauer spectrum is the sum of contributions from each spin state, whereas for short relaxation times it is necessary to obtain a thermal average of the spins and then to compute the Mössbauer spectrum.

The Harwell group have worked with a variety of hemoglobin derivatives, the ferric compounds hemoglobin cyanide (HiCN), azide (HiN_3), hydroxide (HiOH), and methemoglobin (HiH_2O), as well as the ferrous compounds oxyhemoglobin (HbO_2), deoxyhemoglobin (Hb), and carbon monoxide hemoglobin (HbCO). To avoid possible structural alterations associated with the removal of water, they worked with frozen aqueous solutions in the temperature range of 1.2 to 195°K. Also to obtain good intensities in the Mössbauer absorption lines, they enriched their samples with the stable isotope Fe^{57} . More recently, their work has been extended to include such compounds as the cytochromes, ferredoxin, and special varieties of hemoglobin. A few references to the Harwell work are:

- G. Lang and W. Marshall, "Mössbauer Effect in Some Hemoglobin Compounds," *Proc. Phys. Soc.* 87, 3 (1966).
- G. Lang, "Biological Applications of Magnetism," *J. Appl. Phys.* 38, 915 (1967).
- J.E. Johnson, "Mössbauer-Effect Studies of Fe^{57} in Biological Compounds," *Oxford Inorganic Discussions*, Sept. 1967.

The Mössbauer work on biological substances at the Physics Dept. of the Technische Hochschule in Munich represents another aspect of research in this area. The efforts of this group (A. Mayer, A. Trautwein, H. Vogel) have been concentrated on instrumental matters. They have developed a constant velocity drive system which is useful when one wishes to examine with high precision a small portion of a Mössbauer spectrum. By this means they have obtained quite accurate values of the quadrupole splitting and the isomer shift for methemoglobin, oxyhemoglobin and deoxyhemoglobin at several temperatures. A reference on this work is:

- H.M. Kappler, A. Trautwein, A. Mayer and H. Vogel, "Mechanical Constant Velocity Drive for Mössbauer-Spectroscopy of Hemoglobin," *Nuclear Instr. & Methods* 53, 157 (1967).

It is probably premature to attempt a serious assessment of Mössbauer spectroscopy in the biological area. At best, two trends are discernible and they appear to be parallel to those in older and more established forms of spectroscopy. On the one hand, the accumulation of spectra on a wide range of compounds over a wide range of conditions permits the technique to be used as a diagnostic tool. Thus, given a particular compound, we might learn to identify certain properties like the oxidation state or the spin state, purely on the basis of recognizable features of the Mössbauer spectrum. Such tools are of unquestioned utility, and all branches of spectroscopy are frequently used in this way. The other aspect is the attempt to understand the spectra in a fundamental way, i.e., in terms of the contributions from various interactions, and to deduce from them information regarding structure, charge distribution, spin densities, etc. The second aspect is, of course, the more difficult and progress has been slower. Nevertheless, research along these lines is the one more likely to result in improved understanding of the relation between biological processes and purely physical properties.

IV. DETERMINATION OF STRUCTURE ON THE BASIS OF INTERFERENCE BETWEEN RAYLEIGH AND MOSSBAUER SCATTERING

When we are given a particular crystalline structure and we have available the necessary atomic parameters -- positions of atoms, charges, scattering factors, etc. -- it is possible to compute, by a fairly straightforward process, the full details of the diffraction pattern that results from the illumination of the crystal with X-rays. When, however, we are given the full details of a diffraction pattern, complete to within the limits of observation, and we are asked to compute the structure which gave rise to the pattern, there is, in general no solution to this problem. We wish to enlarge upon this notion in order to show how, in principle, the Mössbauer effect may be employed in certain special cases to obtain a solution to the problem of how to determine a structure from its diffraction pattern.

A typical X-ray diffraction pattern produced on photographic film (or recorded by counters) consists of an arrangement of spots (diffraction maxima) of varying intensities. The spatial arrangement of the maxima is governed primarily by the symmetry properties of the space lattice, that is, by the distribution of units (e.g., molecules) in space; the integrated intensities of the diffraction maxima, on the other hand, are mainly determined by the charge distribution in an individual unit, i.e., the molecular structure. Now, for each set of parallel planes in the crystal, labeled by their Miller indices $(h k l)$ there corresponds, generally, a diffraction maximum whose intensity is proportional to the square of a certain function $F(h k l)$ known as the structure factor. It is defined by the expression:

$$F(h k l) = \text{const} \times \int_V \rho(x, y, z) e^{2\pi i \left(\frac{hx}{a} + \frac{ky}{b} + \frac{lz}{c} \right)} dx dy dz \quad (2)$$

in which $\rho(x, y, z)$ is the density of charge at x, y, z referred to an arbitrary coordinate system, and the integration is carried out over a unit cell of dimensions a, b, c and volume V . When the charge density may be approximated by a set of well-separated atoms, the integral in Eq. (2) is replaced by a sum over the atomic positions.

Since, then, the diffraction maxima are proportional to $|F(h k l)|^2$ and $F(h k l)$ is fully given by Eq. (2) (or an equivalent sum), it is possible to compute the diffraction pattern when the structure is known. We note that $F(h k l)$ is

a complex quantity; in a more compact notation it is convenient to write

$$F(h\ k\ l) \equiv F_H = |F_H| e^{i\alpha_H} \quad (3)$$

to emphasize that $F(h\ k\ l)$ is determined only when the absolute value, $|F_H|$, and the phase angle, α_H , are both known.

When we come to the consideration of the inverse problem, namely, the retrieval of the physical structure from its X-ray diffraction pattern, we are confronted with the problem of calculating $\rho(x, y, z)$. This function, as has already been mentioned, describes the distribution of charge in the unit cell and, if we are considering a molecular crystal, $\rho(x, y, z)$ gives the positions of the atoms, i.e., the molecular structure. This is the main objective of most crystallographic work, particularly in biological applications. Since we are dealing with a crystal, $\rho(x, y, z)$ is a regular, periodic function and may therefore be represented by a three-dimensional Fourier series

$$\rho(x, y, z) = \text{const} \times \sum_{h, k, l=-\infty}^{\infty} F_H e^{-2\pi i \left(\frac{hx}{a} + \frac{ky}{b} + \frac{lz}{c} \right)} \quad (4)$$

That the coefficients in the series are indeed the F_H is evident from Eq. (2). But the only things we can measure which relate to the F_H are the intensities of the diffraction maxima, and these provide us with information regarding $|F_H|^2$ or $|F_H|$ but not regarding the phase angle α_H . It is therefore not possible to sum the series in Eq. (4) and $\rho(x, y, z)$ remains unknown.

This is a statement of the "phase problem" in crystallography; the problem remains regardless of the method used for detecting the scattered X-rays.

Before discussing how the Mössbauer effect may contribute to the solution of the phase problem, it is appropriate to comment that the non-existence of a general mathematical solution does not mean that we are completely without resources -- if that were so, there would be no science of crystallography. By various ingenious devices, it is often possible to make an educated guess of the structure; the diffraction pattern is then computed and compared with the observed pattern. By successive approximations one arrives at the real structure. This procedure works for molecules that are not too large -- for biological molecules, e.g., proteins, it's hopeless, and one must resort to other tricks, the most important of which is the heavy atom technique (vide infra).

Let us now suppose that the crystalline material contains a resonant atom, that is, an atom whose nucleus is capable of displaying the Mössbauer effect, for example, Fe^{57} . When such an atom is illuminated with X-rays (issuing from a Mössbauer source, not an X-ray tube), two kinds of scattering processes take place. One has its origin in the interaction of X-ray photons with the electrons (Rayleigh scattering) and the other involves an interaction with the nucleus (Mössbauer scattering). In both cases, the energy of the scattered radiation is the same as that of the incident radiation -- only the direction, or the momentum, has been changed. The essential point is that the two types of scattering are coherent, that is, the radiation scattered by the Rayleigh process can interfere with that scattered by the Mössbauer process.

We must now comment on certain properties of the two types of scattering. Rayleigh scattering, which applies only when the photon energy is well away from an absorption edge is a non-resonant process. This means that, in the very narrow range of energies within which the Mössbauer effect occurs, the cross section for Rayleigh scattering is independent of photon energy and depends only on the angle of scattering. Also the phase of the Rayleigh scattered radiation, relative to the incident beam, remains constant over the small energy interval and may be taken as zero. According to our previous discussion, the amplitude of the scattered radiation from the crystal (by the Rayleigh process) is given by:

$$F(h\ k\ l) = |F_H| e^{i\alpha_H}.$$

We note that the phase angle α_H is governed by purely geometrical considerations associated with the position of the atom with respect to the coordinate system.

By contrast, Mössbauer scattering is a resonant process; that is, the amplitude of the scattered wave A_M is described by an expression of the form

$$A_M = \frac{K}{E(v) - E_0 + \frac{1}{2}i\Gamma} \quad (5)$$

K is a constant; E_0 is the resonance energy of the Mössbauer line whose line width is Γ ; $E(v)$ is the energy of the incident (and scattered) photons. $E(v)$ may be varied over a number of line widths by imparting to the source a velocity v ,

thereby causing a Doppler shift in the photon energy. In this case, it is important to note that, in the vicinity of resonance, the phase of the scattered radiation relative to the incident radiation will depend strongly on v . This quantity is, of course, under the control of the experimenter, who may vary v as he chooses.

In a more compact notation we write

$$A_M = \frac{P}{\mathcal{E}(v) + 1} \quad (6)$$

Combining the two types of scattering amplitudes, Rayleigh and Mössbauer, we obtain the total amplitude of scattering from the crystalline planes ($h k l$)

$$A_H(v) = |F_H| e^{i\alpha_H} + \frac{P_H}{\mathcal{E}(v) + 1} \quad (7)$$

In the general case there would also be a phase factor associated with the second term arising from the positions of the atoms. However, assuming there is no more than one resonant atom in each unit cell, it may be put at the origin of the coordinate system. This is tantamount to choosing a reference phase as that of the Rayleigh scattering from the resonant atoms. With this choice, P_H is real and positive.

The intensity of the scattered radiation is then given by

$$\begin{aligned} I_H(v) &= |A_H(v)|^2 \\ &= |F_H|^2 + \frac{2|F_H|P_H[\mathcal{E}(v) \cos\alpha_H + \sin\alpha_H] + P_H^2}{\mathcal{E}^2(v) + 1} \end{aligned} \quad (8)$$

An equivalent expression is derived by P.J. Black (Nature 206, 1223 (1965)).

We shall now outline a procedure for obtaining the necessary quantities.

1. Choose a velocity v far from resonance -- a few line widths away is sufficient. In this case, we expect no contribution from Mössbauer scattering, and as seen from Eq. (8),

$$I_H (v \rightarrow \infty) \equiv I_H(\infty) = |F_H|^2 \quad (9)$$

2. Write Eq. (8) in the form

$$\begin{aligned} Y_H &\equiv [I_H(v) - |F_H|^2] [\mathcal{E}^2(v) + 1] \\ &= 2 |F_H| P_H \mathcal{E}(v) \cos \alpha_H + [P_H^2 + 2 |F_H| P_H \sin \alpha_H] \quad (10) \end{aligned}$$

We see that Y_H is a linear function of $\mathcal{E}(v)$; therefore a minimum of two velocities in the vicinity of resonance (preferably on either side) will determine this function, giving

$$2 |F_H| P_H \cos \alpha_H = a \quad (i)$$

$$P_H^2 + 2 |F_H| P_H \sin \alpha_H = b \quad (ii)$$

in which a and b are the slope and intercept, respectively, of the linear function Eq. (10).

3. For an inverse reflection ($\bar{h} \bar{k} \bar{l}$), that is, for a diffraction maximum associated with a set of planes $(-h, -k, -l)$, we have

$$\alpha_{\bar{H}} = -\alpha_H, \quad F_{\bar{H}} = F_H, \quad P_{\bar{H}} = P_H$$

Eq. (10) then becomes

$$\begin{aligned} Y_{\bar{H}} &\equiv [I_{\bar{H}}(v) - |F_H|^2] [\mathcal{E}^2(v) + 1] \\ &= 2 |F_H| P_H \cos \alpha_H + [P_H^2 - 2 |F_H| P_H \sin \alpha_H] \quad (11) \end{aligned}$$

Repeating the procedure in item 2 above, for the same two velocities, we obtain a third relationship,

$$P_H^2 - 2 |F_H| P_H \sin \alpha_H = c \quad (iii)$$

Equations (i), (ii), and (iii) are sufficient to determine all the unknown quantities:

$$|F_H| = \sqrt{I_H(\infty)} \quad (12a)$$

$$P_H = \sqrt{(b + c)/2} \quad (12b)$$

$$\cos \kappa_H = \frac{a}{I_H(\infty) \sqrt{2(b + c)}} \quad (12c)$$

The literature on this subject is not very extensive; in Appendix II we have listed a number of references.

Two groups are known to be active in this area. One is in the Physics Dept. of the Univ. of Birmingham (P.B. Moon, D.A. O'Connor, P.J. Black), and another is at the Technische Hochschule in Munich (R.L. Mössbauer, W. Hoppe). It is also likely, based on one known publication, that work of this sort is going on at the Physical Faculty of the Moscow State University (R.N. Kuz'min, A.V. Kolpakov, G.S. Zhdanov).

Finally, it is necessary to comment on certain practical aspects. The most formidable technical difficulties that must be overcome before the theoretical possibilities can be realized are associated with intensity. Since it is necessary to use Mössbauer-type sources which, typically, consist of radioactive materials diffused into a metallic foil, the brightness of such sources is several orders of magnitude below that of X-ray tubes. This means that the time required to obtain a diffraction pattern increases in the same ratio, and for most applications, the necessary durations of exposure are prohibitive.

It is partly for this reason that the work of the Birmingham group is largely concerned with certain experimental and theoretical aspects and only secondarily directed toward crystallographic applications. Indeed, it should be noted that the first experiments to demonstrate the existence of interference between Rayleigh and Mössbauer scattering was accomplished by this group, and they have continued this activity along both experimental and theoretical lines.

The Munich group is making a concerted effort to overcome, or at least improve, the problem of intensity in order to attempt crystallographic work, particularly for biological molecules. The attack on the problem is along two lines -- to improve the sources and to concentrate the scattered radiation. They hope to

produce a source of 100 mCi of Co^{57} in a spot size of about 1 mm in diameter. To concentrate the scattered radiation is not an easy matter, since the index of refraction of X-rays, for almost any material, differs from unity by 10^{-6} to 10^{-5} ; ordinary optical systems are therefore useless. However, because the scattered radiation has a very narrow line width there exists the possibility of designing what may be called "resonant" optical devices in which the index of refraction for X-rays is significantly different from unity.

V. STRUCTURE OF HEMOGLOBIN

Much has been written about the Laboratory of Molecular Biology at Cambridge University. With this Laboratory are associated the names of M.F. Perutz, J.C. Kendrew, F.H.C. Crick, J.D. Watson, M.H.F. Wilkins, F. Sanger -- all Nobel Laureates. It is perhaps no exaggeration to say that the spectacular advances in biology during the last decade were pioneered by these men, and, in a certain sense, Perutz led the way. For it was in 1937, as a graduate student in Cambridge, that Perutz undertook the ambitious project of determining the structure of hemoglobin by X-ray diffraction methods. As Perutz tells it (*Scientific American* 211, 64 (1964)), "Fortunately the examiners of my doctoral thesis did not insist on a determination of the structure, otherwise I should have had to remain a graduate student for 23 years."

To appreciate the magnitude of the problem, it is necessary to recognize that hemoglobin consists of 574 amino acids, or some 10,000 atoms, organized into four intricately wound chains. A heme group, containing one iron atom, is tucked into each chain, and it is at the iron that a molecule of oxygen attaches itself and is transported to the tissues. Clearly the phase problem, in a system as complicated as this one, could not be attacked by trial and error; another approach had to be found and the one that ultimately succeeded was the heavy atom method. In this method, one attempts to incorporate heavy atoms into the molecule without at the same time altering its structure or the arrangement of molecules in the crystal. The heavy atoms, by virtue of their much higher scattering power, affect the intensities of the diffraction maxima in a way which enables the phase constants to be deduced.

In hemoglobin, this was finally accomplished by attaching mercury atoms to the sulfur atoms in the amino acid cysteine. Even so, the task was formidable -- tens of thousands of spots had to be measured and the data computed. It was indeed fortunate that high-speed computers came into being at about the time when the technical problems with the

heavy atom technique were solved. The first structure appeared in 1959; it had a resolution of 5.5 Å. To get better resolution more spots must be read, but the gain is slow; thus to double the resolution, the number of spots to be read increases by a factor of 2³. It is therefore not surprising that it has been only in the last few months that the structure of hemoglobin to a resolution of 2.8 Å has emerged.

It is generally assumed that structure and function are intimately related; it is therefore appropriate to inquire whether the knowledge of the structure of hemoglobin leads to an understanding of its function. As has already been stated, hemoglobin binds oxygen; the quantitative aspects of this process are contained in a binding curve in which the percent of bound oxygen is plotted against the partial pressure of oxygen. This curve has a sigmoid shape, i.e., at low oxygen pressures the curve is non-linear -- the percent of bound oxygen increases faster than the first power of the oxygen pressure. There follows an approximately linear portion and finally, at high oxygen pressures (> 60 mm Hg) the curve saturates. The interesting region is the low pressure region because the non-linearity is a manifestation of cooperative effects among the four heme groups. It seems that when oxygen is attached to one heme, the affinity of the other hemes to oxygen is increased.

The cooperative phenomenon in hemoglobin has long been a puzzle, for the hemes are separated by distances of 25 to 40 Å, a distance far too large for known interactions to be effective. The puzzle in hemoglobin became even more interesting when it was recognized that it was not an isolated phenomenon. In a large number of enzymes there are several active sites -- locations in the molecule which interact with the substrate. It was discovered that when one site is occupied by a substrate, the catalytic activity of the other sites is influenced. Such enzymes are known as allosteric enzymes (J. Monod, J.P. Changeux, F. Jacob, J. Mol. Biol. 6, 306 (1963)); the general notion which is invoked is that when one site is occupied, the molecular structure is altered in such a way as to alter the affinity of the substrate to other active sites. Since the structure of enzymes is generally not known (altogether the structure of only a handful (< 10) of biological molecules is known to various degrees of detail), the hypothesis remains in an uncertain state. Could we then look to hemoglobin, where the structure is known, as a possible model for an entire class of molecules that exhibit cooperative phenomena?

Perutz and his co-workers made an accurate comparison of the structure of hemoglobin containing oxygen (oxyhemoglobin) with hemoglobin not containing oxygen (deoxyhemoglobin). They indeed found structural differences which can be described by small

rotations of each of the four sub-units about separate axes. Unfortunately, these structural differences, which leave the region around each heme unaltered, do not seem to lead to an understanding of the cooperative effects. In fact, with the advent of the more refined structure, at the 2.8 Å resolution, it became more of a mystery how an oxygen molecule manages to move between the heme and the surface of the molecule, since the packing is so close that there is no obvious passageway.

Paradoxically, it appears that as our knowledge of the structure of hemoglobin has improved, the mysteries of its function have deepened. Perhaps, as in other contexts, we may expect it to be darkest just before the dawn.

APPENDIX I - Program of Oxford Inorganic Discussions, 1967,
Metal Ions in Biological Systems, Oxford Univ., Oxford,
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2. B.L. Vallee, Harvard Univ., The Active Center of Metallo-Enzymes
3. R.E. Richards, Oxford Univ., The Application of Nuclear Relaxation Time Measurements to Biological Systems
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APPENDIX III - Some Recent References on the Structure of Hemoglobin

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The main topics discussed in this report are:			
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2. The Mössbauer effect as a tool in investigating biological molecules containing iron,			
3. The combination of Rayleigh scattering with Mössbauer scattering as a possible method for molecular struc- ture analysis,			
4. Recent improvements in the resolution of the hemo- globin structure and its implications for allostericity.			

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