On the Electronic State of Iron in Hemoglobins, With a Short Introduction to Problems of Quantum Biophysics.*

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PART I

MOLECULAR biophysics is a rapidly growing field of science—a field in which what has already been understood constitutes a very minute portion compared to what still remains to be studied. Up to the present, molecular biology is mainly concerned with problems in which molecules can be regarded as compounds of atoms as units. Quite recently, however, electrons are playing more and more important roles in basic problems in this field. The importance of electronic aspects has been emphasized particularly by Szent-Györgyi, who introduced the term "submolecular biology."[1] Quantum mechanics is the essential tool in studies in this challenging field of science, so that it may well be called "quantum biophysics."

The list of problems of quantum biophysics is fairly long; it includes, among others, the studies of elementary electronic processes underlying various functions of biological macromolecules.

1. Migration of Electrons and Holes

Semiconduction of dried proteins was measured extensively by Eley and his collaborators,[2] but normally the conduction is observed at too high a temperature to be biologically significant. If, however, a strongly electrophylic reagent is complexed to proteins or nucleic acids, charge transfer may create holes which can travel along the chain of the macromolecule. We have a few phenomena which seem to suggest the possibility of charge migration within a protein molecule. Firstly, EPR experiments showed that in irradiated proteins the positive holes seem to settle down to some specific positions in the protein, namely, in the neighborhood of cysteine.[3] This suggests the presence of mobility of holes in a single molecule. Secondly, in the oxidation–reduction process carried out by cytochromes, one electron has to be carried away from, or carried to, the Fe ion of the heme. Since the heme in cytochrome is regarded as coordinated by imidazoles from both sides, this phenomenon suggests the possibility of electron migration in a protein molecule.

In studying electron migration, we have to distinguish between intermolecular and intramolecular migration. In dc conduction measurements the intermolecular carrier mobility is the deciding factor, but the intramolecular movement of carriers could be studied through ac experiments with sufficiently high frequencies. In this connection, recent studies on ac conductivity of inorganic impurity semiconductors[4] and of DNA seem to be worthy of careful attention.

It is natural to discuss carrier migration on the band theory of the linear quasicrystalline structure of biological macromolecules. Calculations of energy bands on Hückel molecular scheme or Pariser–Parr–Pople method have been carried out by several authors.[5] The band widths, thus calculated are usually of the order of $0.1 \sim 0.5$ eV, so that the effective mass of the carrier is several times the mass of an electron. Under these circumstances, the vibronic interaction and hopping mechanism of charge migration may play important roles.

2. Excited States of Biological Macromolecules

Understanding of the excited states of peptides, amino acids, and nucleotides (or bases) is a prerequisite to the interpretation of excitons in proteins

* The title of the lecture at the Hylleraas Conference, as originally assigned to me, was "Some Problems in Biophysics," but, with Professor Löwdin's permission, I touched very briefly on various electronic problems in biophysics, and I spent most of the time in discussing a specific problem, i.e., the problem of electronic structure of hemes in hemoproteins, particularly in hemoglobins. In the present paper, I put even more emphasis on the hemoglobin problem.

[1] A. Szent-Gyorgyi, *Introduction to a Submolecular Biology* (Academic Press Inc., New York, 1960).

[2] D. D. Eley, *Horizons in Biochemistry*, edited by M. Kasha and B. Pullman (Academic Press Inc., New York, 1962), p. 341.

[3] W. Gordy, *Symposium on Information in Biology*, edited by M. Yockey, R. Platzman, and H. Quastler (Pergamon Press, Inc., London, 1957), p. 241.

[4] M. Pollak and T. H. Gaballe, Phys. Rev. **122**, 1742 (1961); S. Tanaka and H. Y. Fan (to be published).

[5] M. G. Evans and J. Gergely, Biochim. Biophys. Acta **3**, 188 (1949); J. Ladik and K. Appel, Quantum Theory Project, University of Florida, Gainesville, Florida (unpublished).

and nucleic acids. When the macromolecules take ordered structure such as α helix or double-stand helix, the excited states form bands, and we have selection rules characteristic of helical structures. Furthermore, transition probability in ordered structure is much influenced by the interaction between transition dipoles,[6] and the resulting change of absorption intensities can be utilized as a detector of ordered structure in macromolecules (hyper- and hypochromism in DNA). Exciton bands in polypeptides are also responsible for optical rotatory dispersion, the measurement of which is a powerful means of detecting α-helix structure.[7]

Excitation transfer between different chlorophyll molecules in grana is an important process in the initial stage of photosynthesis. Suggestions have been made that the quasicrystalline ordered arrangement of chlorophylls is essential and methods of solid state physics may be applicable.

3. Studies of Biological Molecules by Magnetic Methods

Paramagnetic molecules can be studied by electron paramagnetic resonance, susceptibility measurement, etc. Since magnetic properties are intimately related to electronic structures, the magnetic methods are powerful means of studying biological molecules which have unpaired spins. Semiquinones and other free radicals formed during the course of biochemical reactions belong to this category. Another group of paramagnetic molecules are those containing transition metal ions, among which hemoproteins have been most extensively investigated. Detailed studies of electronic structure of hemes provide us with information about numerical values of a number of parameters, and, through theoretical interpretation of these parameters, we may be able to understand the electronic processes basic to the biological functions (oxygenation, enzyme activities) of these molecules.

A specific problem in this field is discussed in Part 2.

In conclusion, I must admit that this short list of problems in quantum biophysics is incomplete and many interesting subjects, e.g., problems on correlations between charge distribution in molecules and their biological activities, have not been mentioned at all. Quantum processes related to protons, such as postulated in Löwdin's theory of spontaneous mutation, should also be included in quantum biophysics.

PART II

In this section, I discuss a specific problem concerning the electronic structure of hemoproteins, in which I happen to be interested, i.e., the electronic structure of iron in hemoglobin derivatives. The electronic state of iron in hemoglobin and related molecules is a rather unique one, because the iron can take different spin values according to the kind of molecule or radical coordinated at the sixth position; in some cases the spin value changes even with the temperature or the pH value. In the case of ferrohemoglobin, the iron is in the ferrous state with six d electrons; it has $S = 2$ when the coordinated molecule is water, but the resultant spin vanishes when the ligand is the oxygen molecule. Here already, we have an interesting problem: how to account for the fact that two systems with $S = 2$ and $S = 1$ (oxygen molecule) are combined into a system with $S = 0$.

In the oxidized state, in which the iron is in the ferric form and has the configuration $(3d)^5$, we have $S = \frac{5}{2}$ (high spin) in some cases and $S = \frac{1}{2}$ (low spin) in some other cases. For instance, ferrihemoglobin fluoride has "high" spin, while ferrihemoglobin azide has "low" spin. In certain cases, particularly in ferrihemoglobin hydroxide, magnetic susceptibility measurement gives the magnetic moment apparently corresponding to the intermediate spin $S = \frac{3}{2}$, but George, Beetlestone, and Griffith carefully examined the temperature dependence of magnetic susceptibility and optical-absorption spectrum of this substance, and showed that this may be regarded as a thermal mixture of $S = \frac{5}{2}$ molecules and $S = \frac{1}{2}$ molecules.[8] In the cases of a few other ferrihemoglobins having apparently intermediate values of the magnetic moment, similar examination still remains to be done, so that we cannot definitely eliminate the possibility of the intermediate spin. In this paper, however, we pay attention to high-spin and low-spin cases.

The possibility of low-spin normal states for ferrous and ferric complexes was first explained by Pauling using the valence-bond theory, and later by Van Vleck using the crystalline-field theory. Furthermore, Van Vleck showed that the molecular-orbital theory can account for this situation, and is more general than valence-bond theory and crystalline-field

[6] I. Tinoco, J. Chem. Phys. **34**, 1067 (1961); W. Rhode, J. Am. Chem. Soc. **83**, 3609 (1961).

[7] W. Moffitt, J. Chem. Phys. **25**, 467 (1956); W. Moffitt, D. D. Fitts, and J. K. Kirkwood, Proc. Natl. Acad. Sci. **43**, 723 (1957); P. Urnes and P. Doty, Advan. Protein Chem. **16**, 401 (1961).

[8] P. George, J. Beetlestone, and J. S. Griffith, *Haematin Enzymes*, edited by J. E. Falk, R. Lemberg, and R. K. Morton (Pergamon Press, Inc., London, 1961), p. 105.

theory.[9] There are a few MO studies of the heme molecule as a whole, but the incorporation of metallic orbitals into MO presents some difficulty in the case of ferric states. Hence, I prefer to discuss the problem on the semiempirical-ligand-field theory, which is the modern version of the crystalline-field theory.

In the ligand field theory we have several parameters to express the energy levels. They are: Slater's F_2 and F_4, or Racah's B and C; separations between orbital energies of $3d$ electrons; and the spin-orbit coupling constant a.

B and C and a can be taken over from their values determined for the case of free ions, if we disregard the modification of orbital functions due to partial covalency. Orbital-energy separations can be expressed in terms of 4 parameters in the most general case, but if we may assume the cubic symmetry $3d$ is split into two levels $d\epsilon$ and $d\gamma$, so that a single parameter $E_\gamma - E_\epsilon \equiv \Delta(=10Dq)$ suffices. Although the symmetry of the heme is far from cubic, still the cubic part of the ligand field may be regarded as most important. In the ligand-field theory at the present stage these orbital energy differences are determined emprically from a reasonable set of experimental data.

The diagram showing the dependence of the low-lying excited states of $(3d)^5$ configuration on the strength of the cubic ligand field was given by Tanabe and Sugano.[10] In the limit of vanishing Δ, the lowest state is ${}^6S$, so that for smaller values of Δ the normal state must be a sextet, ${}^6A_1$. On the other hand, when Δ is very large the lowest state comes from $(d\epsilon)^5$ and must necessarily be a doublet. This is ${}^2T_2$. Therefore,

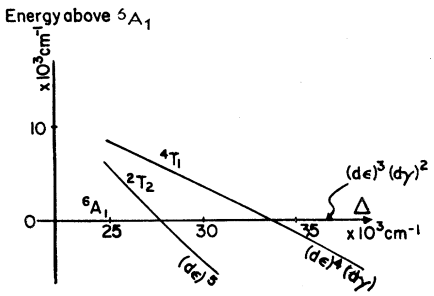

FIG. 1. Crossing of high-spin and low-spin states in Fe^{3+} in cubic liquid field.

the crossing of ${}^6A_1$ and ${}^2T_2$ must occur at an intermediate value of Δ. From the consideration given above, the appropriate value of Δ for hemoglobins must be near to its value Δ_c corresponding to this crossing point. In order to obtain a quantitative picture of lower energy levels in this neighborhood, I have calculated ${}^6A_1$, ${}^2T_2$, and ${}^4T_1$, taking $B = 1133$, $C = 3883$ (free ion values). It is important to note that in this region of Δ, $\sim\Delta_c$, the ${}^4T_1$ level comes fairly low, the excitation energy being only $\sim$6000 cm^{-1}.

So far I have assumed the ligand field to be cubic. The symmetry felt at the position of Fe^{3+} in the free heme will be approximately tetragonal, but when the imidazole ring of histidine is coordinated at the fifth position, the symmetry is reduced to rhombic, since the azimuth of the imidazole plane, with its π-electron system, characterizes a distinguished direction within the heme plane. Hence, in hemoglobins 3 orbitals

$$\xi \propto R(r)Yz\,, \quad \eta \propto R(r)zx\,, \quad \zeta \propto R(r)xy\,,$$
$$(z \perp \text{to the heme plane})$$

have different orbital energies ϵ_ξ, ϵ_η, and ϵ_ζ. Knowledge of the separations between these levels is very valuable, since we can derive from these values some information concerning the interaction between the heme and the imidazole. Such knowledge can be obtained by analyzing results of electron paramagnetic resonance (EPR) experiments.

First, consider the low-spin case, in which ${}^2T_2$ is the lowest in cubic approximation. EPR experiments carried out by Gibson and Ingram on single crystals of ferrihemoglobin azide yielded the following principal values for the g tensor[11]:

$$g_{xx} = 1.72\,, \qquad g_{yy} = 2.22\,, \qquad g_{zz} = 2.80\,.$$

If we knew $\epsilon_\xi - \epsilon_\eta$, $\epsilon_\eta - \epsilon_\zeta$, and a, we could calculate, by solving a secular equation, energies of 3 split levels arizing from ${}^2T_2$, the corresponding wave functions, and their principal g values. [We assume that ${}^2T_2$ belongs to the $(d\epsilon)^5$ configuration, and neglect configuration interaction.] Actually, we have the reverse situation: we know the principal g values for the lowest Kramers' doublet. From this set of data, we can calculate back and determine $\epsilon_\xi - \epsilon_\eta$, $\epsilon_\eta - \epsilon_\zeta$ in terms of a.[12,13] The result is shown in Fig. 2. Thus, the lowest excitation energy is $\sim 2.6a$, which is about 1000 cm^{-1} if a is assumed to be $\sim$400 cm^{-1}. This is much higher compared with the thermal energy, so that the observed magnetic susceptibility comes from the normal doublet only. Nevertheless, the effective magnetic moment μ_{eff} in-

[9] J. H. Van Vleck, J. Chem. Phys. **3**, 807 (1935).
[10] Y. Tanabe and S. Sugano, J. Phys. Soc. Japan **9**, 766 (1954).
[11] J. F. Gibson and D. J. E. Ingram, Nature **180**, 29 (1957).
[12] J. S. Griffith, Nature **180**, 30 (1957).
[13] M. Kotani, Progr. Theoret. Phys. (Kyoto) Suppl. No. **17**, 4 (1961).

creases as the temperature increases, on account of the presence of the so-called temperature independent paramagnetism. Actually, we get for the mean value of μ_{eff}^2:

$$(3.87 + 1.29\, kT/a)\beta^2 \qquad (\beta \text{ is the Bohr magneton}) .$$

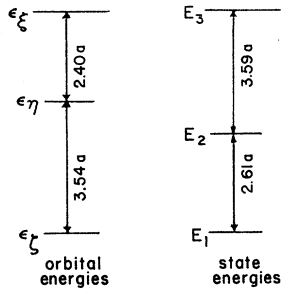

FIG. 2. Orbital energies and state energies of low-spin ferric ion in hemoglobin azide.

According to this formula μ_{eff} increases from 1.97β to 2.13β as T increases from 0°K to room temperature. This is somewhat smaller than the experimental value 2.3β, but is evidently larger than the spin-only value 1.73β.

We proceed to discuss the high-spin case of ferrihemoglobins, with $S = \frac{5}{2}$. In this case the normal state ${}^6A_1$ is not split in the first order even in the rhombic ligand field. EPR experiments,[14] however, showed very clearly that g is highly anisotropic: $g_{xx} = g_{yy} = 6$, $g_{zz} = 2$. These values can be explained if we assume that the sextet level is split into 3 doublets, that the lowest level corresponds to $S_z = \pm\frac{1}{2}$, and that the higher doublets are separated from the lowest one by more than 10 cm^{-1}. Thus, if we represent the energy levels by a spin Hamiltonian DS_z^2, D must be positive and $\gtrsim 5$ cm^{-1}.

It is remarkable that D takes such a large value in high-spin ferrihemoglobin. In almost all compounds containing Fe^{3+} or Mn^{++} the lowest ${}^6S$ state is very insensitive to low-symmetry field, and D is $\sim$0.01 cm^{-1} or $\sim$0.1 cm^{-1}.

This anomaly is related to the presence of the very low excited ${}^4T_1$ state mentioned above. If we assume that this ${}^4T_1$ is one of genuine $(d\epsilon)^4(d\gamma)$, its 3 spatial functions are

$$\xi^2\eta\zeta u' , \quad \xi\eta^2\zeta u'' , \quad \xi\eta\zeta^2 u''' , \quad (u',u'',u''' \subset d\gamma) .$$

[14] J. F. Gibson, D. J. E. Ingram, and D. Schonland, Disc. Faraday Soc. 26, 72 (1958).

Accordingly ${}^4T_1$ is split into 3 quartets when ϵ_ξ, ϵ_η, and ϵ_ζ become different. Now, these sublevels of ${}^4T_1$ interact with the normal state ${}^6A_1$ through spin–orbit interaction, and remove the sixfold spin degeneracy of ${}^6A_1$, in the second order of a. The result can be written as follows:

$$DS_z^2 + E(S_x^2 - S_y^2) ,$$

where

$$D = \frac{fa^2}{4}\left(\frac{2}{\Delta + E_z} - \frac{1}{\Delta + E_x} - \frac{1}{\Delta + E_y}\right) ,$$

$$E = \frac{fa^2}{4}\left(\frac{1}{\Delta + E_x} - \frac{1}{\Delta + E_y}\right) .$$

In these formulas E_x, E_y and E_z are energies of three sublevels of ${}^4T_1$ mentioned above, chosen so as to satisfy $E_x + E_y + E_z = 0$ and f is the relative amount of ${}^4P$ contained in ${}^4T_1$. If we assume ${}^4T_1$ to belong to $(d\epsilon)^4(d\gamma)$, f is equal to $\frac{2}{5}$. If we may further assume that the separations $E_x - E_z$, $E_y - E_z$ are of the same order of magnitude as $\epsilon_\xi - \epsilon_\zeta$, $\epsilon_\eta - \epsilon_\zeta$ for the low-spin azide case, we find

$$D \sim 2.9 \text{ cm}^{-1} .$$

Thus, the sign and the anomalous magnitude of D can be explained semiqualitatively in a rather straightforward way, although quantitatively our estimate cannot be accurate on account of neglect of differences in energies of $d\gamma$ orbitals u', u'', u''' and other approximations.

If we can find the value of E, say by microwave or far-infrared spectroscopy, we may expect to obtain the values of ϵ_ξ and ϵ_η. Dependence of these orbital energy values on physico-chemical conditions of the environment of the iron would provide us with a means to study the nature of heme–imidazole interaction, which will be important for the understanding of the role of the globin part on the biological activities of the heme. For the study of biological activities, however, the electronic structure of ferrous ion has to be investigated; unfortunately, the low-spin species is diamagnetic and EPR experiments have not yet succeeded for the high-spin species. The possibility of EPR experiments for the latter case was discussed.[13] It can be expected, however, that the results obtained for the ferric case will contribute to the understanding of the situation in the ferrous case in various ways.