ELECTRONIC ASPECTS OF BIOCHEMISTRY

PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM HELD AT RAVELLO, ITALY, SEPTEMBER 16–18, 1963, SPONSORED BY NATO.

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Introduction

The International Symposium on the "Electronic Aspects of Biochemistry," the Proceedings of which are being published in this Volume, was held in Ravello, Italy, from September 16 to September 18, 1963, at the end of the first NATO Advanced Summer Institute in Molecular Biology which lasted from September 2 to September 15. Like the Summer Institute itself it was sponsored by NATO and it is my duty and pleasure to express our gratitude to the Scientific Bureau of NATO and in particular to its Head, Dr. Helms, and to Dr. Coleby both for their generous contribution and for their efficient help in other aspects of the organization of the Institute and of the Symposium.

The penetration of the electronic concepts of modern chemistry and physics into the biological sciences is becoming more and more pronounced every day and, as was the case with organic chemistry some 40 years ago, this penetration leads to a much deeper understanding of the basic phenomena involving living matter than would have been possible otherwise. This state of affairs is particularly evident in biochemistry where the application of the methods of quantum chemistry not only permits the elucidation, at the fundamental level, of the mechanism of known phenomena but leads to most useful generalizations and predictions.

Without being exhaustive, far from it, of the numerous possibilities for the application of electronic concepts and of quantum procedures to biochemistry, these Proceedings indicate some of the fields in which useful applications have been made. I wish to thank all the contributors for their cordial collaboration.

BERNARD PULLMAN

May, 1964

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Opening Remarks

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The success of the Summer School which just finished bears ample testimony to the interest that biologists now take in the electronic aspects of their science.

These aspects are manifold. The most obvious is the utilization of optical, electrical, and magnetic properties of matter for the analysis of substances present permanently or transiently in the cells, as well as for the study of micro and submicro cellular morphology. These techniques are growing as fast as physicists can conceive of them and engineers can materialize devices permitting more and more precise and automatic measurements.

A second electronic aspect of biochemistry concerns the relation between structure and function. A biological process once described in chemical language remains to be understood in the physical sense. For example, substances participating in metabolic reactions are often sufficiently well known that one might attempt to understand, from their structure and by quantum-mechanical methods, why and in what manner they undergo transformation; in other words, to understand the facility of passage to transition states, the free energy changes corresponding to their degradation or dissociation, the aptitude for the transfer of certain groups, and so forth.

In fact, the introduction of these methods into biochemistry has been rapid. The recent book of B. and A. Pullman shows how extensive are the data already collected, at least for highly conjugated molecules. Charge distribution in phosphorylated compounds and in the coenzymes permits one to explain many aspects of their behavior. Data relative to the bases composing the nucleic acids are no less suggestive, and these, among others, might constitute the basis for interpretations of mutagenesis and carcinogenesis. In the latter case, relations between biological effect and electron density distribution have been observed before the acceptor cellular system has been identified.

In spite of these achievements, the task of the theoretical biochemist remains of considerable magnitude. To speak only of enzyme action, it is evidently not enough to understand the functioning of the coenzyme; one should also get an insight into that of the apoenzyme. Here the problem is not limited to the simple chemistry of active groups. It is

recognized that the specificity depends on apoenzyme configuration. Numerous facts suggest that the catalytic power is also conditioned by tertiary structure. It is even probable that reversible modifications of tertiary structure and the accompanying entropy effect play an important part in substrate activation. Now, tertiary structure is determined by the geometry of amino acid residues and intramolecular van der Waals interactions, as shown by the recent interesting work of Professor Liquori and others.

In the study of the stability of tertiary structure, the electronic background comes up only in the last analysis. On the contrary, it becomes of prime importance in processes involving energy transport through a chemical structure which remains apparently unmodified. This third electronic aspect of biochemistry will have a large place in this symposium and we all hope that it will be the subject of fruitful discussions. In fact, it is not only concerned with phenomena originating from high-energy excitation, such as photosynthesis, vision, and the effects of various radiations. Following the stimulus given by Szent-Györgyi, the question of energy transport in the course of ordinary metabolic reactions has been proposed and has given rise to many interesting theoretical and experimental studies. Protein semiconductivity, particularly, has been the subject of experimental measurements as well as of theoretical calculations. The question, while remaining open, is of great importance, since it concerns essential processes like the passage of electrons from one link to another in the respiratory chain, or from one prosthetic group to another inside a protein molecule.

Modern biology attributes so many functions to protein and nucleic acid molecules that, while trying to understand, one is tempted to think of them as possessing some exceptional properties. Interest aroused during the past few years by the experiments of Blumenfeld and the controversy that followed are eloquent reminders of this.

The papers at the symposium cover almost all electronic aspects of biochemistry. I am sure that they will bring forth many ideas and much new light, especially under the influence of the wonderful atmosphere of the Villa Cimbrone. The beauty and serenity of its gardens, its parlors, its cloisters, all call for fruitful discussions even outside regular sessions.

Triplet States in DNA

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I. Experimental Evidence for Triplet Exciton Migration

Robinson and co-workers have given evidence for the migration of triplet excitations in crystals containing isotopically substituted molecules (Nieman and Robinson, 1962; El-Sayed *et al.*, 1962). For example, in crystals containing 99.2% C_6D_6, 0.4% C_6H_5D and 0.4% C_6H_6, the fluorescence intensities of the C_6H_5D and C_6H_6 were comparable but the C_6H_6 phosphorescence was ten times as strong as that of C_6H_5D. In addition, Nieman and Robinson (1962) and Robinson and Frosch (1962, 1963) have shown that the easy migration of the triplet excitation is really to be expected on theoretical grounds.

Bersohn and Isenberg (1963, 1964) have observed triplet excitation migration in DNA and potassium polyadenylate. Their specific observation was that the phosphorescence but not the fluorescence of DNA was almost totally quenched by the addition of one Mn^{2+} ion to every ten base pairs. The phosphorescence had previously been shown to originate in the purine bases, guanine and adenine. Presumably, the Mn^{2+} ion quenches because its unpaired electrons facilitate radiationless triplet to singlet conversion; Mg^{2+} and Hg^{2+} do not quench appreciably. It is known from electron resonance studies of solid crystals that the spin density associated with a paramagnetic ion does not penetrate beyond the first- and second-nearest neighbors. Inasmuch as the spin density cannot reach the molecule which is excited to a triplet state, the triplet excitation must be able to migrate. The emission from the DNA is composite, resembling the emission of a mixture of guanine and adenine. We can therefore infer that two types of excitons exist in the system, the adenine excitons and the guanine excitons. The shape of the emission intensity vs. wavelength does not change very much as paramagnetic ions are added to the DNA, which suggests that the two types of excitons are quenched at about the same rate.

II. Theory of Triplet Exciton Migration

A very simple theory, which is essentially identical to that of Nieman and Robinson (1962), can be used to explain the diffusion of the triplet excitation. Consider for definiteness an adenine triplet excitation whose wave function is $\phi_A{}^T$. We suppose that this excitation can be transferred with great speed from the given adenine to a nearest-neighbor adenine but an intervening guanine provides an appreciable barrier. Two intervening guanines would impose an even higher barrier, and so on. We are thus led to consider the sequence of structures AGA, AGGA, AGGA, AGGGA, . . . and we may ask how many G's constitute an impossible barrier for the adenine exciton. (Nothing need be said about the pyrimidine bases in this primitive calculation.)

Consider first the states $\psi_{A_1}{}^T\phi_G\psi_{A_2}$ and $\psi_{A_1}\phi_G\psi_{A_2}{}^T$. The jump rate for the adenine exciton over one guanine will be of the order of $(\langle\psi_{A_1}{}^T\phi_G\psi_{A_2}|H|\psi_{A_1}\phi_G\psi_{A_2}{}^T\rangle)/h$. The wave functions of the excited molecules overlap their nearest neighbors to a small extent because the base pairs are only 3.4 Å apart. The wave functions of the triplet states as perturbed by this overlap are

$$\psi_A{}^{T\prime} = \psi_A{}^T + \epsilon\phi_G{}^T$$

where

$$\epsilon = \frac{\langle\psi_A{}^T|H|\phi_G{}^T\rangle}{\Delta E} = \frac{\beta}{\Delta E}$$

and $\Delta E \equiv E_G{}^T - E_A{}^T$ is the difference in triplet state excitation energies of guanine and adenine. A key question is the value of β, and recent calculations (Nieman and Robinson, 1962; Katz *et al.*, 1963) show that these integrals for distances of the order of those found in benzene and naphthalene crystals which are comparable to those found in DNA are of the order of 10^{-3} eV. For ΔE we may take the value 1 eV, which cannot be wrong by an order of magnitude. For ϵ, we then have 10^{-3}. It should be noted that, in DNA, ΔE is not particularly small in contrast to the case of isotopically diluted crystals where it is 100–200 cm^{-1}.

The desired integral is given by

$$\langle\psi_{A_1}{}^T + \epsilon\phi_G{}^T|H|\psi_{A_2}{}^T + \epsilon\phi_G{}^T\rangle \cong 2\epsilon\langle\psi_{A_1}|H|\phi_G{}^T\rangle = 2\epsilon\beta$$

Inasmuch as 1 eV corresponds to a frequency of 2.4×10^{14}/sec, $2\epsilon\beta$ corresponds to a frequency of 5×10^8/sec or a time of 2×10^{-9} sec.

Consider next the states $\psi_{A_1}{}^T\phi_{G_1}\phi_{G_2}\psi_{A_2}$ and $\psi_{A_1}\phi_{G_1}\phi_{G_2}\psi_{A_2}{}^T$. The matrix element between these two states must be evaluated using states whose wave functions are corrected for overlap, i.e.,

$$\psi_{A_1}{}^{T'} = \psi_{A_1}{}^{T} + \epsilon\phi_{G_1}{}^{T}$$
$$\psi'_{A_2} = \psi_{A_2}{}^{T} + \epsilon\phi_{G_2}{}^{T}$$

so it is written

$$\langle\psi_{A_1}{}^{T} + \epsilon\phi_{G_1}{}^{T}|H|\psi_{A_2}{}^{T} + \epsilon\phi_{G_2}{}^{T}\rangle = \epsilon^2\langle\phi_{G_1}{}^{T}|H|\phi_{G_2}{}^{T}\rangle \cong \epsilon^2\beta$$

One can show in general (McConnell, 1961) that if there are n intervening guanines, the jump rate will be given by $\epsilon^n\beta/h$. The correlation times for the various jumps will be given by the reciprocal of the jump rate and—for the above numerical values—will be 2×10^{-9} sec, 2×10^{-6} sec, 2×10^{-3} sec, and 2 sec for jumps over one, two, three, and four guanines, respectively. These values are given for purposes of illustration only; obviously they are very sensitive to the values assumed for β and ΔE. Nevertheless, one may draw the following important conclusions: A singlet adenine or guanine exciton, lasting only about 10^{-8} sec, can by this mechanism jump over at most one neighbor of a different type, although by dipole-dipole interaction much longer jumps are possible. The triplet excitons which have lifetimes of about 1 sec (guanine) and about 2 sec (adenine) can jump over a number, perhaps four or five, of successive unlike neighbors in a time comparable to the decay time.

The actual degree of delocalization of the triplet exciton will depend on the structure of the DNA. If there are many long sequences of identical purine bases, the excitons of the opposite type will be trapped between these reflecting walls. If, on the other hand, such long sequences are rare, the exciton will be able to diffuse over long distances before it decays by radiation or otherwise.

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The Use of Fluorescence in Studies of the Structure and Interactions of Proteins

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I. Introduction

The fluorescence of natural proteins and their conjugates has been the subject of intensive study in recent years. The subject is of interest both for its own sake and for the information which it may yield about the structure and interactions of proteins. In the latter connection, fluorescence techniques are becoming increasingly popular because of their great sensitivity and the ease and convenience of measurements.

Virtually all natural proteins display an ultraviolet fluorescence. This property is conferred by the aromatic residues tyrosine and tryptophan, whose emission bands are at 300 mμ and at 330–350 mμ, respectively. When both residues are present the emission of tyrosine appears in general to be suppressed, by a mechanism as yet imperfectly understood. Emission from a third fluorescent amino acid, phenylalanine, has not been observed in proteins.

The basic experimental parameters which characterize fluorescent systems are the spectral distribution, the polarization, and the quantum yield. The ultimate objective of studies of the ultraviolet fluorescence of proteins is the relation of these quantities to explicit structural

features of the protein, especially those related to the immediate environment of the emitting groups.

While native proteins do not fluoresce at visible wavelengths, they can be made to do so by coupling with 1–2 residues per molecule of a fluorescent dye. While a large number of such conjugates have been prepared, most investigations have utilized proteins labeled with 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS), fluorescein isocyanate, or fluorescein isothiocyanate.

The excited lifetimes of the aromatic residues of proteins are so short that the polarization of their fluorescence is not influenced by the Brownian rotation of the molecule. However, synthetic conjugates can be prepared whose lifetime is sufficiently long to permit the use of fluorescence polarization in studies of the over-all characteristics of the molecular domain (Section II).

II. The Polarization of the Fluorescence of Protein-Dye Conjugates

The basic theory relating the polarization of fluorescence to the size and shape parameters of the emitting molecule was developed by Perrin (1926, 1929, 1934, 1936). The most general equations of Perrin are too complicated to be useful, but assume a simple form, provided that (a) the hydrodynamic properties of the fluorescent molecule are those of a rigid ellipsoid; and (b) the axes of absorption and emission of the fluorescent oscillator are randomly oriented with respect to the coordinates of the molecule.

Condition (a) is an assumption common to all the hydrodynamic methods for studying proteins. Condition (b) is likely to hold for the case of a protein labeled with a small dye molecule, although there can be no guarantee that deviations may not occur in particular instances.

For unpolarized incident light the simplified form of Perrin's equation is as follows:

$$\frac{1}{P} + \frac{1}{3} = \left(\frac{1}{P_0} + \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \tag{1}$$

Equation (1) has been independently derived by Weber (1952a).

Here ρ_h is the harmonic mean of the three characteristic rotational relaxation times of the ellipsoid, about its principal axes, i.e.,

$$\frac{1}{\rho_h} = \frac{1}{3}\left(\frac{1}{\rho_1} + \frac{1}{\rho_2} + \frac{1}{\rho_3}\right)$$

The polarization (P) is equal to $(I_v - I_h)/(I_v + I_h)$, where I_v and I_h are the vertical and horizontal components of the fluorescent radiation observed at 90° to the incident beam. τ is the mean lifetime of the excited state of the fluorescent species. P_0 is a constant (for a single

electronic transition) and depends formally on the angle (λ) between the (linear) absorption and emission oscillators. The equation of Jablonski (1935) relates the two quantities. For unpolarized incident light,

$$P_0 = (3\cos^2\lambda - 1)/(7 - \cos^2\lambda) \tag{2}$$

In the limiting case where the molecule has spherical symmetry ($\rho_1 = \rho_2 = \rho_3 = \rho_0$), the Perrin equation assumes the form:

$$\left(\frac{1}{P} + \frac{1}{3}\right) = \left(\frac{1}{P_0} + \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_0}\right) \tag{3}$$

For a sphere ρ_0 is equal to $3\eta V/RT$ where V is the molar volume; η is the viscosity of the solvent; R, the gas constant; and T, the absolute temperature. Consequently $(1/P + \frac{1}{3})$ should vary linearly with T/η if the form of the molecule is invariant in the range of T/η investigated. In practice P_0 is obtained from the intercept at $T/\eta = 0$ of a linear plot of $(1/P + \frac{1}{3})$ *versus* T/η. The value of ρ_h at 20° may then be computed from Eq. (1).

Any deviation of the form of the molecule from the limiting case of a rigid unhydrated sphere will result in a value of ρ_h/ρ_0 differing from unity. The variation of ρ_h/ρ_0 with axial ratio for oblate and prolate ellipsoids of revolution has been derived by Weber (1952a).

With macromolecules capable of existing in a multiplicity of configurations of varying degrees of internal constraint, it should be possible to define a parameter which can distinguish between these states without specifying them. Most native globular proteins appear to be rigid and relatively symmetric. Flexibility arises by the destruction of portions of the network of internal weak bonds (as hydrogen bonds, hydrophobic bonds, etc.) which endows the native form with its unique structure. The origin of ratios of ρ_h/ρ_0 less than unity may be sought in the altered configurational states of proteins which have been modified by some form of treatment. It seems reasonable to assume that values of ρ_h/ρ_0 can vary within limits corresponding to the completely rigid state of the native molecule and to a completely structureless form in which all weak bonds have been lost. These states will define the maximum and minimum values of ρ_h/ρ_0.

In view of the agreement between the relaxation times of serum albumin and ovalbumin computed from polarization of fluorescence and from dielectric dispersion data (Weber, 1952b), it appears likely that labeling with 1–2 moles of DNS does not influence the hydrodynamic behavior of these proteins. In the case of enzymes, preferential labeling of the active site may result in loss of activity. This appears to be the case for α-chymotrypsin (Massey *et al.*, 1955).

If selectivity of labeling exists it is of course possible for the apparent relaxation time to be unduly influenced by the purely local behavior of the molecule near the site. This may be the origin of the anomalous behavior of aldolase, for which ρ_h/ρ_0 varies with the degree of labeling.[1]

It is an important feature of the polarization-of-fluorescence method that the observed value of the polarization is not influenced by a large number of experimental variables, including charge, ionic strength, and protein concentration. Consequently, the versatility of the method is considerably enhanced in comparison with others which are subject to a host of nonspecific effects. Weber found that the polarization of ovalbumin, at a given temperature, was independent of protein concentration between 0 and 1%, of pH between 1.5 and 13, and of salt concentration between 0 and 0.2 M (Weber, 1952b). He also found, in accordance with Perrin's equation, a linear dependence of $1/P$ on T/η between 3 and 45°C. In a subsequent study also designed to evaluate the validity of the method, Steiner and McAlister labeled three crystalline proteins each with three dyes of different lifetimes (Steiner and McAlister, 1957). Perrin's law was obeyed in all cases. In addition, the computed values of relaxation times for each protein were shown to be independent of the degree of conjugation, the nature of the conjugate, the lifetime of the dye, and the wavelength of the exciting radiation.

In addition to the advantages in experimental methodology discussed above, fluorescence polarization measurements often appear to be able to resolve minor modifications in structure where other methods are insensitive. Thus the polarization of bovine γ-globulin was found to be constant between pH4 and 8.5 (Steiner and Edelhoch, 1962). An approximately linear decrease in polarization occurred between pH8.5 and 12. When the viscosity, sedimentation, optical rotation, and solubility (denaturation) were examined, no change in these properties was found between pH4 and 10.5. At more alkaline pH values, all the properties changed. Johnson and Richards have reported similar behavior with legumin (Johnson and Richards, 1962). The marked sensitivity of the polarization technique may reside in its ability to respond to an increase in torsional oscillations in polypeptide segments which do not result in changes in hydrodynamic volume. The rupture of the weakest class of internal bonds may result in such a situation. Thus incipient changes in structure which precede the breakdown of larger sections of the protein appear to be resolvable by polarization measurements.

Since polarization measurements may be carried out quickly, this type of measurement should provide an excellent method of monitoring protein behavior under a variety of environmental conditions. Weber

[1] Edelhoch, H., and Feigelson, P. Unpublished results.