PHYSICAL TECHNIQUES IN BIOLOGICAL RESEARCH

edited by

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PREFACE

These volumes have resulted from collaborative effort, in which a large number of physical techniques that have been found useful in biology are discussed briefly from both the theoretical and the practical viewpoint. No claim to encyclopedic range is made, but it is believed that from these volumes the reader can get a fairly comprehensive idea of the present place of the techniques of physics and of physical chemistry in biological research.

During the last two decades there has been unprecedented broadening of the scope of attack on fundamental problems of physiology. This is largely a consequence of the increasing use by biologists of modern physical techniques, some of which are of the most advanced types and characterized by precision and delicacy rarely employed in their application in physics. These refinements have not always resulted from the collaboration of a biologist and a physicist, as one might suppose. Fully as often the biologist has turned physicist and has himself adapted, or indeed sometimes developed, physical techniques suited to his needs. Thus, Martin and Synge developed adsorption experimental procedures into the enormously useful paper chromatography; Holter and Linderstrøm-Lang made from the diver of Descartes an apparatus delicate enough to obtain analytical data from single cells; while in the microtome electron microscopists have designed an engineering marvel that cuts slices about one order of magnitude above the range of monomolecular films.

Workers with any of the powerful new aids to biology from physics cannot avoid feeling strongly encouraged to continue to elaborate new methods and to broaden the range of biological problems to which the techniques are applicable. The present work has been conceived in the hope of accelerating such development and wider use. Every specialist in one of these techniques is constantly called upon to help his fellow biologist to decide whether, or how, a particular physical technique can serve a biological use more or less unlike that for which it was originally designed. It is also not an infrequent experience of a physicist or a physical chemist to have a biologist come to him with a difficulty which he hopes can be overcome if he can find the right physical approach. The success of such conferences depends to a large extent upon both participants having a fair knowledge of the details of the techniques and the biological uses to which they have already been put; and one of the aims of the present work is to serve such a need. For every such biologist who makes a vigorous effort to use new physical methods,

there must be many who have a vague feeling that their researches might proceed better if reoriented in a physical direction, but who hesitate to attempt this because of timidity. For such workers it is felt that the many examples of simple methods will be helpful. Finally, in a broad sense, it is hoped that this treatise will serve as a real orientation for biologists and for chemists and physicists who may be potential biologists. In these volumes each author, an expert in his field, has written in such a way that a biologist can see whether he may start to employ the technique, or whether the application to his particular biological problem demands collaboration with a physicist or a physical chemist. The latter, on the other hand, should be able to assess in realistic terms the possibility of fruitful and exciting application of his special training to the baffling problems of biology.

The arrangement of material has been determined primarily by biological considerations. Volumes I and II deal with theory and methods applied to relatively pure preparations of biological substances that are obtained from cells or other tissue elements. The optical approaches, so favored in biology, are in Volume I, while in Volume II are a wide variety of nonoptical techniques (the only exception is the chapter on X-ray diffraction which is in Volume II). Volume III deals with the application of physical techniques to cells and tissues.

The editors wish to express their appreciation to the authors for their contributions.

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I. Principles of Photochemistry

1. The Light Absorption Act

Photochemistry is the study of chemical reactions initiated by radiant energy. The statement that the energy must be absorbed in order to induce chemical events is often called the first law, or Grotthus' law, of photochemistry. Radiant energy is absorbed by matter in discrete amounts called quanta. The energy of a single quantum, ϵ, is proportional to the frequency of the radiation, ν.

$$\epsilon = h\nu \tag{1}$$

Planck's constant, h, has the numerical value of 6.62×10^{-27} erg seconds. A more practical unit for the biologist is the energy of a mole of quanta, or an einstein, $N\epsilon$, where N is Avogadro's number, 6.02×10^{23}. In more familiar terms, the energy of an einstein in kilocalories per mole, E, is related to the wave length in Angstrom units, λ, by equation (2).

$$E = \frac{2.859 \times 10^5}{\lambda} \tag{2}$$

a. Molecular Energy Levels

Absorption of light by a molecule results in a conversion of radiant energy into the energy of internal motions within the molecule. These motions can be classified as rotations of the molecule as a whole, vibrations of the atomic nuclei with respect to each other (bond stretching or bending), and motions of the electrons. The ground state of a molecule is that state in which the energies of all these motions have their minimum values. Other states of the molecule are called excited states, and the transition of a ground state molecule to an excited state with the absorption of energy is called excitation. The reason that only certain wave lengths are absorbed by a given type of molecule is that the energies of these internal motions can have only certain discrete values, and the energy of an absorbed quantum must exactly equal the excitation energy for some allowed excited state. The excitation energy is equal to the difference in the total internal energy of the molecule between the excited and ground states.

Ordinary chemical reactions take place in the dark when a molecule absorbs a critical amount of vibrational energy as the result of collisions with other molecules. If the energy must be absorbed in the form of light, as is the case in photochemical reactions, it is necessary that excitation occur in the electronic motions. The spectral regions in which molecular electronic absorption occurs are the ultraviolet, the visible, and the very near infrared. For polyatomic molecules the transition to an excited elec-

tronic configuration is characterized by a fairly broad absorption band, in which varying degrees of vibrational and rotational excitation are superimposed upon a fundamental electronic excitation.

b. Action Spectra

If we are confined to the easily accessible near ultraviolet, above 2400 A, then the molecular groupings giving rise to the important absorptions are the familiar chromophores: unsaturated linkages, aromatic compounds, and other resonating structures. Thus, proteins are most likely to be photochemically active at the tyrosine, tryptophane, and phenylalanine residues; nucleic acids are universally ultraviolet sensitive because of the purine and pyrimidine moieties; and so on. The absorption spectrum thus provides a suggestive pattern in choosing the wave length for a photochemical reaction. In many biological processes, however, the situation is reversed. That is, a photochemical phenomenon is observed before the definite identity of the active absorbing molecule is known. In such a case it is very useful to measure the wave length dependence of the phenomenon. The plot of the quantitative biological or chemical response as a function of wave length is called the action spectrum. In order to make measurements for such a plot a dilute sample should be chosen which absorbs only a small fraction of the light incident upon it. The experiment must be performed at various more or less narrow wave length regions. The two common conventions for recording such data are either (1) the amount of response per unit amount of incident energy, or (2) the amount of response per unit number of incident light quanta. The energy multiplied by the average wave length in the selected light region gives a product which is proportional to the number of quanta. If the total wave length span of the action spectrum is not large, the two curves will have almost the same shape. The wave length correction inherent in the type (2) curve makes this a convenient form for comparison with the absorption spectrum, plotted as the extinction coefficient against wave length. For purposes of comparison the values of the ordinates on the action spectrum should all be multiplied by a constant normalizing factor chosen so that the action spectrum will have the same numerical value as the absorption spectrum at some arbitrarily chosen wave length. Then if the photochemical response, or action, is proportional to the number of quanta absorbed, irrespective of wave length, the normalized type (2) action spectrum determined at less than 10% absorption will coincide with the absorption spectrum at all wave lengths. This can be shown in Eqs. (3) and (4), where D is the optical density, k is a constant, I_0 is the incident intensity, I is the transmitted intensity, and I_{abs} is the absorbed intensity.

$$\text{action per unit of incident radiation} = \frac{kI_{abs}}{I_0} = k\left(1 - \frac{I}{I_0}\right) = k(1 - 10^{-D}) = k(1 - e^{-2.3D}) \quad (3)$$

When D is very small this expression can be simplified by MacLaurin's theorem.

$$\text{action per unit of incident radiation} = 2.3kD \quad (4)$$

Since D is proportional to the extinction coefficient, the action per unit of incident radiation is also proportional to the extinction coefficient.

The useful feature of the action spectrum is that it has the shape not of the absorption spectrum of an overall mixture but of the absorption spectrum of only those components that absorb energy used in the photochemical reaction. If there is only one active component it can often be identified directly in this manner. Thus the action spectrum for human scotopic vision can be fitted to the absorption spectrum of rhodopsin (Hecht, 1944); the action spectrum for phototaxis in *Rhodospirillum rubrum* can be fitted to the absorption spectrum of bacteriochlorophyll (Milatz and Manten, 1953); and the action spectrum for the photoreactivation of *Streptomyces griseus* previously exposed to ultraviolet light can be fitted to the absorption spectrum of a porphyrin (Kelner, 1951). There are often more complicated cases where more than one component may contribute absorbed energy for the photochemical purpose or where an active and an inactive component may both absorb at the same wave length. An example is an analysis of the relative roles of various plastid pigments in photosynthesis (Emerson and Lewis, 1943).

2. Utilization of Absorbed Light Energy

There are several different courses that the energy of absorption may take after it has appeared as internal energy of molecular excitation. That the energy does not remain long in the absorbing molecule is proved by the simple experimental fact that the color of most substances does not change during a period of illumination. This must mean that the excited molecules are quickly returned to the ground state where they are prepared to absorb the same wave lengths as at the first instant of irradiation.

a. Fluorescence

The simplest type of dissipation of excitation energy is the emission of light. Such an emission, if it occurs very soon after the absorption, less than about 10^{-6} seconds, is called fluorescence. The ratio of the number of quanta emitted as fluorescence to the number of quanta absorbed is known as the fluorescence yield. Even if the fluorescence yield is one, there are probably no cases of organic molecules in solution or in tissues which can

convert 100 % of the absorbed energy into fluorescent light. This is because the average fluorescent quantum is at a longer wave length and thus has less energy than the absorbed quantum. The peak of the emission spectrum for fluorescence usually occurs at longer wave lengths than the peak of the absorption spectrum, and the tail of the fluorescence curve spreads out even further toward the red. This red shift, and hence energy deficiency, of the fluorescent light is explained in terms of the Franck-Condon principle. According to this principle the most probable electronic transitions are those for which the average interatomic distances are the same in the initial and final states. The absorption of light does not necessarily bring a molecule to the lowest vibrational state in the new electronic configuration because the interatomic distances in that state are not necessarily the same as in the ground state. Instead, the primary excitation will leave the molecule in a vibrating state for which the average interatomic distances correspond most closely to the distances in the ground state of the molecule. The excess vibrational energy in the higher electronic state will be dissipated as heat by rapid impacts with the solvent or other surrounding molecules. Fluorescence will then occur mainly from the lowest vibrational level of the excited state and the fluorescence transition will occur preferentially to a vibrating state having the proper match of interatomic distances. The excess vibrational energy of the molecule will again be dissipated to the milieu as heat. Another important aspect of the fluorescent spectral band is that it is independent of the absorption frequency selected for excitation. If a molecule has an absorption band in the near ultraviolet and another in the green, the fluorescence will be only in the green, slightly displaced by the usual shift. The radiationless transition from the second to the first electronic excited state occurs by internal conversion, discussed in *c*, below.

b. Phosphorescence

Many organic molecules, particularly those with highly conjugated structures, have in addition the ability to radiate after passing through an electronic state intermediate in energy between the normal ground state and the fluorescent state. This type of luminescence, called phosphorescence, occurs with a time delay of as much as several seconds after the absorption act. The phosphorescent light at normal temperatures has the same spectrum as fluorescence, but at low temperatures a new luminescence spectrum may appear at a considerably longer wave length. The first type of phosphorescence results from the non-radiative transition from the phosphorescent state to the fluorescent state. For several cases the phosphorescent state has been shown to be a triplet state, that is, a biradical in which two electrons have unpaired spins. The importance of the phosphorescent state in biology is that it is much more reactive chemically than the fluorescent state, both on account of its free radical nature and

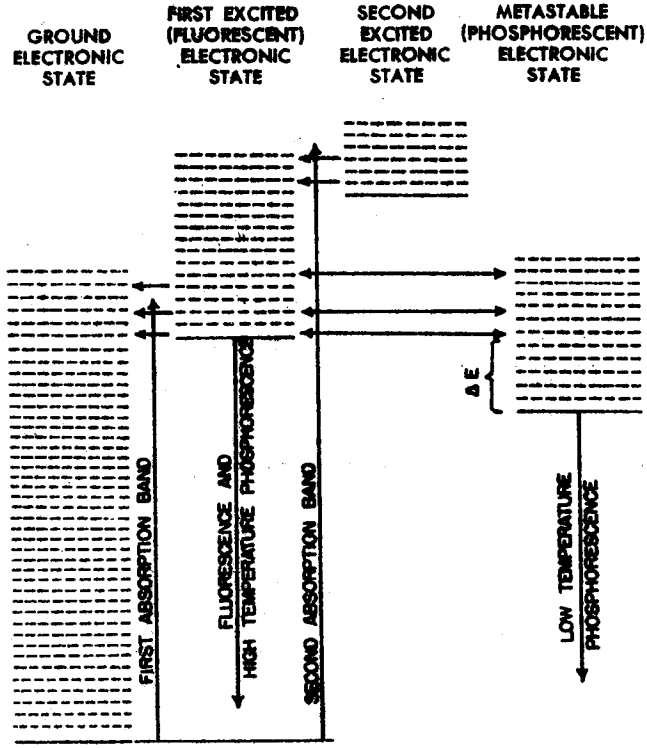

FIG. 1. Schematic diagram of molecular energy levels. This is an energy level diagram in which vertical distances are proportional to the differences in internal energy between various states. Solid horizontal lines represent the electronic states without excess vibrational excitation. The broken lines represent varying degrees of vibrational excitation superimposed on the electronic energies. Each upward arrow represents the strongest transition within an absorption band. Each downward arrow represents the strongest transition within an emission band. The length of each vertical arrow on the energy scale is related by Eq. (2) to the wave length at the peak of the absorption or emission band. The horizontal arrows represent internal conversion transitions. ΔE represents the minimum energy dissipation in the passage from the fluorescent to the phosphorescent state.

also because of its relatively long life. For example, most of the dyes that sensitize the photochemical oxidations constituting photodynamic action have well-known phosphorescent properties (Blum, 1941). The relationship between absorption, fluorescence, and phosphorescence is illustrated graphically in Fig. 1.

c. Internal Conversion

Vibrational dissipation of absorbed energy is observed not only as part of the emission process but often in exclusion to any luminescence. Most

organic molecules in solution have a fluorescence yield considerably less than one. This means in most cases that much of the energy of electronic excitation is dissipated as heat. This occurs because of the internal conversion from a higher electronic state to a lower electronic state (Franck and Livingston, 1941). This conversion occurs between combined electronic-vibrational states of equal energy and is followed by stepwise dissipative losses of the excess vibrational energy to the medium. The chemical consequences of internal conversion are very important because the high content of vibrational energy following this process produces a "hot molecule," the type produced by high temperature collisional activations. Thus a photochemical reaction could result in products similar to ordinary reactions, but now the light replaces thermal agitation as the source of the energy of activation.

It might appear at first glance that most molecules should decompose if a quantum of absorbed visible or ultraviolet light should be completely converted internally, since such quanta have energies, calculated by Eq. (2), in the range of the typical chemical bond, 50 to 150 kcal. Actually, simple bond rupture is a rare consequence of internal conversion because rupture requires the localization of the requisite energy in a particular bond. The vibrational energy of a molecule after internal conversion is distributed over many possible modes of vibration of a variety of atoms in the molecule. Especially for complicated molecules the amount of vibrational energy in any one pair of adjacent atoms is a small fraction of the total energy. Rather than simple bond rupture, another type of chemical result of internal conversion requiring less energy is an intramolecular rearrangement in which bonds are both broken and formed or in which stereo-isomerization occurs by rotation about a rigid bond (Franck and Sponer, 1948). The overall energy requirement for such a process is not so large as for a simple dissociation.

It has been known for a long time that *cis-trans* isomerization often occurs in ultraviolet light. At a given temperature and wave length a photostationary state can be established in which there is a constant ratio of *cis* to *trans*. In general this stationary distribution will not be the same as the equilibrium distribution achieved in the dark with a catalyst. Some important biological reactions involve such a photo-isomerization. One of these, the isomerization of vitamin A aldehyde, retinene, will be discussed in Section 3*b*. (Hubbard and Wald, 1952). Kuhn and Winterstein (1933) reported the complete conversion in the light of the carotenoid, *cis*-crocetin dimethyl ester to the *trans*-isomer. This reaction is reported to be one of the principal mechanisms by which light exercises an influence on the mating characteristics of the green alga, *Chlamydomonas* (Kuhn *et al.*, 1938; Moewus, 1950).

Another chemical consequence of photochemically produced hot mole-

cules has been proposed by Franck and Platzman (1954). They believe that the heating in the immediate neighborhood of a hot molecule, resulting from the absorption of radiation, might cause local disturbances of the intermolecular structure in biological tissues. According to their picture, the hydrogen bonds which are responsible for the shape of protein molecules might easily be vulnerable to such heating effects. It is important to point out here that the energy of excitation, initially located in the electronic system of a particular absorbing chromophoric group, may spread quickly after internal conversion, by the strong coupling of molecular vibrations, to the extremities of the molecule and even to the first sphere of the solvated molecules of the environment.

d. Chemical Reaction

Photochemists usually use a separate term for those reactions in which the electronic energy of absorption is transferred, without general dissipation, into the vibrational energy of a particular bond. A resulting bond rupture is called dissociation or pre-dissociation. If the molecule were a normal saturated structure, the primary products would be free radicals.

Methods exist for detecting the intermediacy of free radicals in photochemical reactions. Free radicals are known to initiate polymerization processes in solution. If a reaction mixture containing a monomeric substance is resistant to polymerization in the dark but undergoes polymerization in the light, the photoproduction of free radicals is strongly suggested. Uri (1952) has used this technique to demonstrate radicals in some photochemical reactions of chlorophyll. In applying this method it is necessary to choose a wave length that is not absorbed by the test monomer. Sometimes, chemical reactions of free radicals can be used for identification. Bawn and Mellish (1951) have used the stable radical, diphenylpicrylhydrazyl, as a trapping reagent for certain other radicals. The reagent is highly colored and is bleached upon reaction with another radical. The presence of benzoyl peroxyl radicals can be shown in this manner. Calvin and Barltrop (1952) have used the same reagent to test for a biradical formed by the rupture of a disulfide bond when zinc tetraphenylporphin was illuminated in the presence of *n*-propyldisulfide. Calvin (1953) has used the dehydrogenation of tetralin as another test for the photosensitized formation of the disulfide biradical. In any of these tests for photochemically produced radicals that require the addition of a test reagent to the reacting system, controls must always be made to be sure that the reagent does not interfere with the primary reaction being investigated. Sometimes, bond rupture does not lead to free radicals, but to stable molecules. An example is the photochemical liberation of carbon monoxide from carbon monoxide-iron porphyrin complexes (Warburg and Negelein, 1928). In all

cases of bond rupture, it must be emphasized that the particular bond that is ruptured is not necessarily the closest bond to the absorbing chromophoric group. Because of the coupling of the molecular vibrations and the complexity of the excited electronic state of polyatomic molecules, it is difficult to predict in advance which, if any, bond will break. It should be no surprise, therefore, that absorption by a phenylalanine moiety in a polypeptide results in the rupture of a peptide bond several atoms removed from the phenyl ring (McLaren, 1949). It is quite unnecessary to invent an explanation of this reaction in terms of the very unlikely migration of an electron through the molecule.

The first law of thermodynamics provides a necessary, but of course not sufficient, condition for bond rupture. In other words the wave length of the light must be low enough to satisfy the requirement of the bond dissociation energy according to Eq. (2). Table I lists some bond dissociation energies, based on measurements with simple molecules. The values for this table are based on a review by Szwarc (1950) and on measurements of Franklin and Lumpkin (1952). Such a table should be used only for approximation because the energy for a given type bond is not constant from molecule to molecule.

TABLE I

Bond Dissociation Energies (in kcal. per mole)

O—H	118	C—N (amide)	98
S—H	87	C—N (amine)	77
N—H	104	S—S	64
C—H (aliphatic, primary)	100	C—S	72
C—C	82	C—O	90
		C=O	145

Bimolecular reactions in which the molecule after absorbing radiation transfers its excitation energy to a second molecule are termed collisions of the second kind. It is sometimes difficult to make a sharp distinction between uni- and bimolecular reactions when any molecule, as in a condensed system, is in a permanent state of impact with its surroundings. If the overall reaction involves permanent changes in both the absorber and the second molecule, the two possibilities are (1) unimolecular bond rupture followed by a reaction in the dark between one of the fragments and the second molecule; and (2) chemical rearrangement within a bimolecular complex formed by collision of the excited molecule with the second molecule. Hydrogen transfer is a particularly common type of photochemical reaction involving two molecules. Chlorophyll is known to be formed in higher plants by the photochemical dehydrogenation of protochlorophyll (Smith, 1948). Also synthetic chlorins analogous to chlorophyll in their