Bioenergetics

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ACADEMIC PRESS INC • NEW YORK • 1957

Library of Congress Catalog Card Number:
57-8382

printed in the united states of america

Foreword

There is but one safe way to avoid mistakes: to do nothing or, at least, to avoid doing something new. This, however, in itself, may be the greatest mistake of all. The selected, who are able to open new roads to science without erring, are very few and the author, certainly, does not belong to them. The unknown lends an insecure foothold and venturing out into it, one can hope for no more than that the possible failure will be a honorable one.

One of the most characteristic features of present-day biochemistry is the coexistence of highlights with darkness, knowledge with ignorance. While we can perform reactions that amount to a "miracle" and, here and there, even improve on nature, we cannot answer many of the simplest and most fundamental questions. We have, for instance, detailed information about the structure of the protein molecules but cannot tell why nature has put those atoms together in that highly specific way, what was the quality she wanted to achieve by doing so. The same holds true for nucleic acids and nucleoproteins. We know most hormones, and many of them we can build ourselves outside of the living body. In a few cases we can even produce more active agents than nature did. But how hormones act, what they do on the molecular level, we do not know; we have not gone beyond symptomatology in the analysis of their action. The same holds true for most of our drugs.

The same duality exists also in our knowledge relating to the high-energy bonds, the main representative of which is the high-energy phosphate bond P—O—P, "~P." Their discovery belongs, undoubtedly, to the most brilliant achievements of modern biochemistry. We know how, at the expense of one ~P, another

endergonic bond is established. We know how, in fermentation, the bonds in hexose or triosephosphate are shifted around till the P's become ~P's which, transferred on to ADP, can support endergonic syntheses. We have an astounding knowledge about the processes in which our foodstuffs are used to build our body, erect the edifice of life, construct its machinery; but how energy is moving this machine, how work, *w*, is done, be it motion, mechanic, osmotic, or electric work, in a word, how energy is *driving* life, we do not know. Dazzled by our successes we even forget to ask.

This *"chiaroscuro,"*[1] "clear-obscure," is one of the most characteristic traits of current biochemistry. Such a schism between the known and unknown suggests that some basic information is missing. This book represents a guess about its nature.

There is one reason why the inquiry into this duality is urgent and imperative. Corresponding to the big lacunas in our understanding there are equally big lacunas in medical science. Most human suffering, at present, is caused by the so-called "degenerative diseases"—the name standing for "diseases we don't understand and, consequently, can do nothing about." The existence of such a closed group of diseases also points towards some major gap in our basic knowledge. Possibly, all these gaps, may they relate to normal function or to disease, have one common denominator, some process which, hitherto, eluded detection. Some fundamental fact, if not a whole dimension, is missing from our biological thinking.

Shortcuts, in science, mostly turn out to be blind alleys and the only safe approach to fundamental questions is that on the basic level. Cures for disease flow out of progress in understanding as

[1] "Chiaroscuro," in painting and the graphic arts, denotes the mixture of highlights and darkness, as often found, for instance, in Rembrandt's etchings.

the natural fruits of knowledge. This will be the leitmotif of this book which contains an attempt to identify the missing link in our knowledge and open alleys to its approach.

Woods Hole, Massachusetts
July 1956

ALBERT SZENT-GYÖRGYI

ACKNOWLEDGMENTS

When letting this book go my thoughts wander gratefully to those who supported my research and shared its risks, in the first place to my associates. The Commonwealth Fund gave its generous help explicitly for the study of energy transmission in spite of my warnings that they might be supporting a gamble. Armour and Co. were my most generous supporters for many years and so were the Heart Association, the Muscular Dystrophy Associations, the Association for the Aid of Crippled Children, who all greatly helped the development of the views presented here by not tying down the author's mind by strings attached to their grants. Much encouragement has been given lately by the National Heart Institute through its Grant H 2042, and by the National Science Foundation as well as the United Cerebral Palsy Fund.

My thanks are due to Dr. William Arnold for his invaluable help in shaping theory and experiment.

My thanks are due to Dr. Oscar Hechter for the patience with which he listened to my gradually developing ideas and criticized this book.

Contents

Abbreviations

A = Ångström = 10^{-8} cm
ADP = Adenosine diphosphate
ATP = Adenosine triphosphate
2,4 D = 2,4-Dichlorophenoxyacetic acid
E^* = Excitation energy
(E) = Bond energy
IDP = Inosine diphosphate
MW = Molecular Weight
~P = High-energy phosphate bond
UV = Ultraviolet
λ = Wavelength
μ = 0.001 millimeter
mμ = Millimicron = 10A = 10^{-6} mm

PART I

General Considerations

"There are more things in heaven and earth,
Horatio, than are dreamt of in your philosophy."
(Hamlet)

1. The Problem Is Stated

The problem is: how does energy drive life? How does it move the living machine? This is one of the most basic problems of biology and, at present, there is no answer to it. So it is possible that the "oscuro," alluded to in the Introduction, is due to our inability to answer this question.

In order to avoid losing ourselves in generalities, we have to take a specific example. I will take a little experiment I made a few years ago. In this experiment I took a strip of muscle (I chose the *musculus psoas* of the rabbit), put it into diluted glycerol, and kept it in the glycerol for a few days in the refrigerator and for a few weeks in the deep freeze. Then I suspended it in 0.1 *M* KCl at room temperature, added a little Mg, and added ATP in the same concentration as the muscle contained it *in vivo*. The muscle contracted and developed the same tension as it developed maximally in the living animal. If we identify life with motion we could say: the muscle came to life again. In this process the ATP was split, losing its terminal phosphate which was linked to it by a P—O—P. Since we know that this link is a so-called high-energy phosphate bond, ~P, and no other energy donor was present, it is evident that the energy which moved the muscle was the energy of this ~P, and so we can narrow our problem down and ask how did the energy of the ~P move the muscle?

Progress in the chemistry of muscle made it possible to simplify the problem even further. I showed almost two decades ago that contraction in muscle is, essentially, the interaction of actomyosin (a complex formed of two proteins, actin and myosin) with ATP and ions. Of the two proteins, myosin is responsible for the elementary act of contraction and so we can simplify our proposition

by considering myosin instead of muscle, and ask how the energy of ~P moves myosin?

We know from the studies of Edsall and Weber that the myosin molecule is a thin filament. So without knowing any more details about it, we can form two different pictures of the process in which the energy of the ~P is transferred to this filament and produces contraction. The one would be to suppose that the molecule carrying this ~P, in our case ATP, enters into some chemical reaction with the myosin, as the result of which a local change is produced in the protein which leads to its folding. An ATP-myosin complex would have to be formed which then splits up, leaving behind phosphate, ADP, and the altered myosin. Such a reaction finds many analogies in the "group transfer reactions" of the intermediary metabolism and, in principle, could be described with symbols of classical chemistry.

The alternative picture is based on the supposition that the ATP molecule does not enter into any such local reaction, but the bond energy of its ~P's becomes released in a more active and mobile form which then is transferred to the myosin molecule, moves through it, and produces in its wake changes which, somehow, lead to contraction and could adequately be described only in terms of quantum mechanics. Compared to the first, this picture is vague, has no analogies in intermediary metabolism, and one may ask why make such hazy pictures if we can make clear ones with deep roots in existing knowledge?

The inadequacy of the earlier classical pictures was brought out by the advances made in the chemistry of myosin. The more we learn about myosin the less we understand it, which suggests that we are looking at it in the wrong way. Continuing some studies made by Gergely, Perry, and Mihalyi, Andrew Szent-Györgyi showed the myosin molecule to be built of two kinds of subunits, "meromyosins" which, within the molecule, stand in a row in series (Lauffer and Andrew Szent-Györgyi). If Laki and Caroll's value of the molecular weight of myosin is correct, one molecule of

myosin contains three meromyosins, while if Weber's value is correct, it contains six. Assuming the smaller value to be correct, the myosin molecule would look something like Fig. 1. Of the two kinds of meromyosins one is somewhat plumper and sediments faster and has been called H-meromyosin, the H standing for "heavy." In Fig. 1, arbitrarily, it is placed into the middle. The

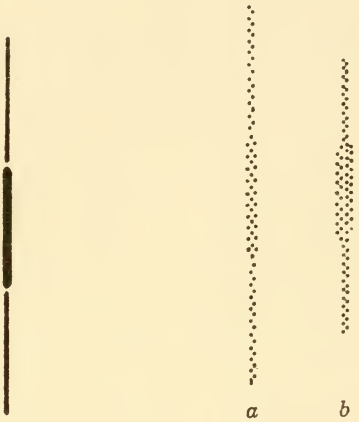


Fig 1 Fig 2

FIG. 1. Schematic representation of a myosin molecule, consisting of one H and two L meromyosins. Sequence of meromyosins arbitrary.

FIG. 2. a: Schematic representation of the myosin molecule of Fig. 1, as consisting of protomyosins. b: Possible rearrangement of protomyosins in contraction.

other two are more slender and have been called L-meromyosins, L standing for "light," these having a lower sedimentation constant. There are two L's for every H.

What makes it difficult to bring this discovery into agreement with earlier concepts is that only the H interacts with ATP, releasing the energy of its ~P's, while there is every reason to believe that the L's are involved in contraction, do the work, and use the energy. The energy would have to get, somehow, from the H's to the L's and it is difficult to see how a bond energy could

do this. There are still possibilities for bringing this structure into line with our earlier concepts. One could suppose, e.g., the ATP to produce some local change on the H which would make the L's fold back on it, producing thus a shortening, or contraction. So there are ways out, though not nice or good ones.

If a theory is good, then any newer knowledge should support it and contribute to clarification, as was the case with intermediary metabolism. With myosin things are going the other way. Andrew Szent-Györgyi and Borbiro showed that the meromyosins also are built of subunits, protomyosins. The protomyosins are of equal size and rather small. Their MW is about 4500 g, which means that one meromyosin is built of a greater number of them, the L of about 20, the H of about 50. These protomyosins are held together by secondary forces only, such as H-bonds, and van der Waal's and electrostatic attractions. If we call a molecule a structure of atoms held together by covalent bonds, then the myosin particle is no molecule at all, only an aggregate. The structure is symbolized in a very crude way in Fig. 2a. It is difficult to see how such a structure could fold; it seems more likely that contraction is not a folding at all, but a rearrangement of protomyosins within the particle, which rearrangement leads to a more rounded, shortened form, as symbolized in Fig. 2b. In order to produce such a rearrangement, many weak forces must be disturbed which keep the protomyosins together. It is impossible to see how a bond energy, enclosed in a ~P, could cause such a disturbance, especially if that ~P is far away, on the H-meromyosin.

We can thus sum up the situation by saying that we do not know how muscle contracts, how it uses bond energy to produce work, and the more we know about its structure the less we understand its function. We might have arrived here at the edge of the chasm which seems to extend through medicine and biology and may be responsible for its "chiaroscuro."

2. A Theory of Energy Transmission

It often happens that, unconsciously, our thinking becomes dominated by certain pictures which we have met too often to question their correctness. In my opinion, our difficulty in approaching the problem of energy transformation in muscle is due to our having been misled by the formalism of our thermodynamic bookkeeping. When making up the energy balance sheets of reactions we usually express both the "potential energy" of a bond and the kinetic forms of energy in calories and so, unconsciously, accept their identity. But there is a very great difference between the two, at least in their biological activity, which we can illustrate by comparing it with the difference between sitting on top of an atomic bomb while its potential is a potential, its bonds are bonds, and its energy is locked up inside its atoms, and then trying to remain sitting on it when these bonds are exchanging their potential for more active, kinetic forms of energy. Though mechanics may find both forms of energy essentially identical we will sense a very considerable difference in their biological activity. The situation with the "energy" of the ~P is analogous to that of the A-bomb. While its energy is enclosed in the bonds of the molecule as a potential, it can be expected to have no outward action (except showing a little extra weight which we could find if our balances were more sensitive). This bond energy may be transferred, as such, from molecule to molecule and from bond to bond in the group transfer reactions of our intermediary metabolism. But if this potential has to go into biological action, produce work or motion, an analogy to the A-bomb, it might be exchanged for more active and mobile forms of energy. Such active and mobile forms of energy, on the molecular level, could hardly be anything

else than some form of molecular excitation, be it electronic, vibrational, or rotational. So what we biologists can safely do without getting into an argument with statistical mechanics is to use different symbols for bond energies which are linked to molecules and have no outward action, and excitation energies which are mobile and may interact with their surroundings. The former I will denote by (E), meaning by E energy and symbolizing by the parentheses that this energy is enclosed within a molecule. Excitational energy I will denote E^*. So I can formulate our problem by asking whether, in muscle the (E) of the $\sim$P in ATP is not exchanged for E^* when it has to go into biological action and produce contraction? Group transfer reactions of intermediary metabolism could be symbolized by writing:

$(E_n) \rightleftharpoons (E_3) \rightleftharpoons (E_2) \rightleftharpoons (E_1)$ where (E_n) stands for the energy of reserve food as fat and carbohydrate while (E_1) stands for the energy of the substance which is directly fed into the muscle machine, in our case ATP. In this row of reactions the potential energy is transferred from bond to bond, from substance to substance. Bond creating bond, these reactions can be expressed by symbols of classical chemistry. The question is whether our inability to understand muscle is not due to the fact that what happens further belongs to a different group of reactions which can no more be described by these symbols, in which (E) is turned into E^*? This duality may hold for all reactions in which work, w is produced, be it mechanical, osmotic, or electric work, etc. While (E) may be the core of reactions in which substances are synthetized and the living machinery is *built,* E^* may be the core of reaction in which this machinery is *driven* and work is produced. This could explain why our notions, derived from intermediary metabolism, did not lead us to a better understanding of muscular contraction.

When supposing a transformation of (E) into E^* we are not lost in the marshes of speculation, for the reaction on which all life is built *is* essentially such a transformation. This reaction is

photosynthesis, in which the solar energy enters into the living world to drive it. In this reaction the radiation is captured by dyes, mostly chlorophyll, in which it produces an electronic excitation. This E^* is then stabilized in the form of (E). Subsequently (E) is shifted from one bond or molecule to another until, eventually, it is stored away in the form of the (E) of carbohydrates or fats. The process of photosynthesis could thus be symbolized by:

$$h\nu \rightarrow E^* \rightarrow (E_1) \rightarrow (E_2) \rightarrow (E_3) \rightarrow (E_n)$$

The reverse process occurs in photoluminescence when, for instance, the firefly emits light:

$$(E_n) \rightarrow (E_3) \rightarrow (E_2) \rightarrow (E_1) \rightarrow E^* \rightarrow h\nu$$

Looking at this row of reactions one cannot fail to notice its identity with that of photosynthesis. Only the order is reversed. If we look upon the production of light by the firefly only as upon an example of production of work, w, then we arrive at the conclusion that the energetics of the living world consist of only two processes: photosynthesis and its reversal.

In muscle E_1, which is directly fed into the contractile mechanism, is the (E) of ATP, and the recent work of Arnon and his associates indicates that ATP plays a very intimate role in the first steps of photosynthesis, while Strehler and Arnold, and Arnold and Davidson have shown photosynthesis to be reversible.

In the above reactions $(E_n) \rightarrow (E_1)$ is what is called "intermediary metabolism." The problem to be dealt with in this book is whether $(E_1) \rightarrow E^* \rightarrow w$ does not represent the reaction which *drives* the living machine and, belonging in the realm of quantum mechanics, can be expressed only in terms of the latter.

Such a question cannot be answered by any single experiment. Only the accumulation of data on various lines can make such a theory acceptable. If correct, this theory should lead us to a better understanding of various biological structures and phenomena, should open new views and suggest new experiments.

3. The Mobility of E^* and Organization

No form of energy can be mobile if there is nothing to conduct it. So if we are looking for mobile forms of energy which could take part in biological energy transmissions we have to consider not only the energy itself, but also the mechanisms which have to conduct it. In this chapter I will review instances of mobility of energy and discuss the qualities demanded of the medium, leaving open the question of which of these mechanisms play a role in living systems. That such transmissions do occur was shown by photosynthesis in which many chlorophyll molecules collaborate in the reduction of one CO_2 molecule (Arnold and Meek).

CONJUGATED SYSTEMS, π ELECTRONS, AND n,π TRANSITIONS

If a molecule contains a system of conjugated double bonds, then it also has π electrons—which are no longer bound to any single atom but belong to the conjugated system as a whole, within which they have a more or less free mobility. If such a π electron accepts energy and is excited to a higher π^* energy level, then its E^* belongs to the whole conjugated system and may produce changes at any of its points. The purine in ATP has such an extensive conjugated system, and so have pyrimidins, isocyclic aromatic compounds, or carotenes with their long chain, built of isoprene units.

Biological catalysts and cofactors often contain N, O, or S atoms in their conjugated system or linked to it. These atoms have their "nonbonded" "lone pair" of electrons which can be excited to the π^* levels and thus contribute to the pool of π^* electrons. Those so-called n,π excitations discovered by McMurry and Mulliken, have specific qualities: their lifetime is considerably longer than

that of π-π^* excitations and their absorption is shifted towards the shorter wavelength with increasing electropolarity of the solvent, while their fluorescence is quenched by strong acids. The heteropolar solvent, with its H-bonding, increases the dissociation energy of these electrons which are immobilized by the protons of the strong acid altogether (Kasha, 1950).

COUPLED OSCILLATORS

If two systems, capable of similar oscillation, are coupled, then they make "coupled oscillators" and the oscillations will tend to pass back and forth between the two. If, for instance, two penduli of the same periodicity are suspended on the same wire and one of them is put into action, it will soon stop while the other takes over the motion. After a while the second pendulum will pass back the motion with its energy, and so it will go on.

If A and B are the two penduli and motion is *, then

$$A^* + B \rightarrow A + B^* \rightarrow A^* + B \rightarrow A + B^* \text{ etc.}$$

The point about this simple experiment is that the energy communicated to the one of the two oscillators does not become divided between the two and thus degraded to lower values, but is periodically transmitted in its entirety. If there were no friction and this transmission of energy would occur without loss, motion could go back and forth forever.

The various atomic groups of the protein are capable of oscillations, vibrations, or rotations comparable to the motion of the penduli. If two such groups with identical frequency are coupled by, say, the polypeptide chain, then they might act as coupled oscillators. It is not impossible that all such groups within the same protein molecule do act as coupled oscillators and so the vibrational energy communicated to one might be taken over by any of them, the whole protein molecule forming one unique system in which the energy communicated to it belongs to the whole, and may appear at any point.

ELECTROMAGNETIC COUPLING

An electronic excitation can also be looked upon as an oscillation. Two molecules, the electrons of which are capable of a similar excitation, can thus act as coupled oscillators. In this case it is not necessary to have a material connection between the two, for the electromagnetic field couples them, provided their mutual distance is not too great (small compared to the corresponding wavelength).

The transmission of excitation energies between molecules through electromagnetic coupling is not a mere matter of speculation. It is one of the basic theories by which classic phenomena, such as the "concentration depolarization" of fluorescences are explained. It has been found, as a general rule, that if a dilute (e.g., 10^{-4} M) solution of a fluorescent substance is cooled to low temperatures to form a solid glass in which the single molecules have a fixed orientation, then the fluorescent light given off by this system will be polarized if polarized light was used for its excitation. The molecules being fixed in space, they will not change the plane of polarization in the interval between the absorption and the emission of the photon. If, however, this experiment is repeated with a high concentration of the fluorescent substance (e.g., 10^{-1} M), then the fluorescent light emitted will not be polarized. The generally accepted explanation is this: owing to the high concentration, the molecules are now close to one another; being coupled by the electromagnetic field, the E^* is passed from one to the other before it is shot out again as a photon. The different molecules visited by E^* have a random orientation, thus the fixed relation of the plane of the primary exciting light to that of the fluorescent light will no longer be maintained and the polarization will be lost. It will be lost if the statistical distance between molecules is smaller than the distance at which such resonance transfer of excitation is possible. Figure 3 shows the relation of concentration to the statistical distance between molecules in any

solution. By determining the concentration at which polarization is lost, the distance can be found at which electromagnetic interaction is possible. This critical distance may even exceed 100 A.

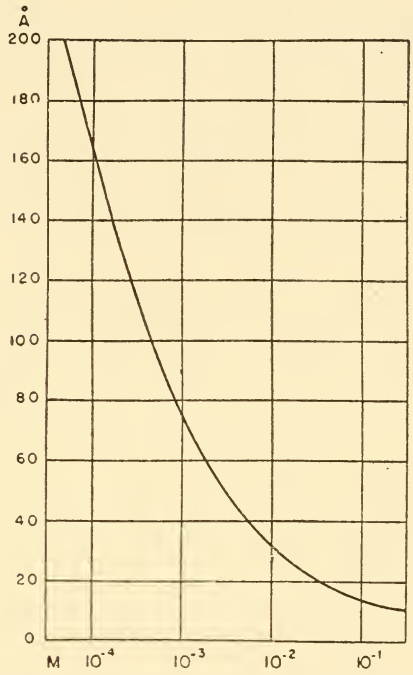


FIG. 3. Relation of statistical distance between molecules and molar concentration. If the solution contains two different substances, then the statistical distance between their molecules is about 70% of the values given by the curve.

which corresponds to a concentration of the order of 10^{-3} *M*. The diameter of many macromolecules is of this magnitude; so E^* can be transmitted between atomic groups of the same macromolecule or of neighboring molecules also by their electrodynamic coupling. One point we must mark well about this transmission: no photons are involved. It is not that the first molecule, absorbing the light, emits a photon again which then is absorbed by the second mole-

cule. The process is photon free, due to the direct electrodynamic coupling of the two molecules.

Transfer of E^* through electrodynamic coupling can occur not only between identical molecules, but also between molecules of different substances. The situation for such a transfer will be the most favorable if there is a wide overlap between the fluorescent spectrum of molecule A with the absorption spectrum of molecule B, which has to take E^* over. Apart from the "overlap integral," the lifetime of A^* and the wavelength influence the result. The distance through which such energy transmission can take place depends on these factors.

Such an energy transfer in biological systems has actually been shown to exist. Arnold and Oppenheimer found that the energy of the light absorbed in green algae by phycocyanin is transmitted through such electrodynamic coupling to the chlorophyll with a high efficiency. The chlorophyll, in its turn, transmits it to the rest of the photosynthetic apparatus which builds carbohydrate with it, transforming E^* into (E). A similar energy transfer has also been found by Duysens from carotenoïds to chlorophyll b in blue and red alga and from chlorophyll b to a in the green alga *Chlorella.*

ENERGY BANDS. THE "CONTINUUM THEORY"

If a physical system has a great regularity in the arrangement of its atoms (as is the case in crystals, or might be the case in proteins), then the atoms disturb one another's orbitals, the energy levels of which may fuse into a common, practically continuous energy band. If such a band is filled, that is, contains the maximal number of electrons allowed ($2n$ if n is the number of atoms), then no energy can be transported by its electrons. If, however, the band is not filled, then its electrons can be accelerated in a definite direction and can transport energy. This is how metals conduct electricity.

If a substance has completely filled bands and above them

empty ones, then it will be an insulator, a dielectric. However, if the distance between the highest filled band and lowest unfilled band is not too great, then electrons may be raised from the former into the latter and make the substance conductant. If the distance between the two levels is small, even thermal agitation may suffice to produce this change, in which case, the substance is a "semiconductor." Absorbed photons may do likewise leading to "photoconductivity." I have pointed out earlier (1946, 1947) that it is possible that proteins may have an electronic structure analogous to that of semiconductors. Evans and Gergely found that the hydrogen bonds, within the protein molecule, may create conditions which could lead to the development of such continuous bands, though their results were not quite conclusive. The distance between the highest filled and lowest unfilled band was found to be rather great, energies of the order of 100,000 calories being needed to raise an electron from the first to the second.

Experimental evidence for the existence of a semiconductor nature of biological material is not missing. Eley, Parfitt, Perry, and Taysum found semiconductivity in plasma albumin, fibrinogen, and edestine. The gap between the highest filled and lowest unfilled band was somewhat smaller than calculated by Evans and Gergely and water was found to decrease the energies needed to lift electrons over it.

W. Arnold (personal communication) showed recently that chloroplasts can "store light," that is, conserve the energy of absorbed photons, which energy they could again shoot out later in the form of light, if heated. This could hardly be explained any other way than by assuming the existence of energy bands. The electrons raised to the higher band seem to have been trapped there in "wells," from which they can be chased out by heat agitation. The differences in the energy of the bands were found to be still lower than found by Eley *et al.*, bringing the energies needed for raising electrons to the higher empty level into the realm of biological possibilities.

Conduction bands need not end at the confines of a protein molecule. If the contact between two molecules is intimate enough, the bands may fuse, thus connecting single protein molecules into a common system.

That energy can move through protein molecules is an experimental fact. The first example was given by the observations of Bucher and Kaspers, who found that the light absorbed by the protein in myoglobin caused the dissociation of the CO bound by the heme. The energy of the photon absorbed by the protein had to travel first through the protein molecule and then through the heme to reach the CO. If there is anything to limit the value of this observation, this is the possibility that the energy might have traveled through the protein in the form of an acoustic wave and not as electronic excitation. This possibility can be excluded by Bannister's discovery that the light absorbed by the protein of phycocyanin is emitted as fluorescent light by the chromophore of this chromoprotein. Equally conclusive are the experiments of Shore and Pardee, who coupled fluorescent dyes to proteins and found photons absorbed by the protein emitted by these dyes as fluorescent light, in certain cases with an almost 100% efficiency. These authors leave open the question of the mechanism of energy transfer but tend to ascribe it to a resonance coupling between the absorbing aromatic amino acids of the protein and the fluorescent dye. In this case it would not be the protein but the electromagnetic field which is responsible for the energy transmission.

As stated at the beginning of this chapter, I will leave the question open, which (if any) of these conductions plays a role in biology. Possibilities are manifold. So, for instance, the columns of heterocyclic purine and pyrimidine bases which, according to the Watson and Crick model, forms the core of DNA (and possibly RNA), may conduct energy by electrostatic coupling in analogy to Sheibe's dyes, the molecules of which form columns and conduct E^*. I have found earlier (1955) that certain proteins have a stronger fluorescence than corresponds to the additive

fluorescence of their constituents, showing that the molecule, as a whole, does something with its E^*. Such systems of energy conduction may, perhaps also explain the disproportionality between the quantity of certain drugs or hormones and the mass of the system they act upon. As a system of conducting electric wires can be rendered inoperative by breaking their isolation or conduction at one point, so biological systems of energy conduction could be rendered inoperative if disturbed at one point, similarly to Sheibe's dye columns, the conduction of which could be broken by single hydroquinone molecules.

4. Absorption, Fluorescence, and Phosphorescence[1]

Molecules send us messages through photons, be it through photons they absorb, be it through photons they emit. This chapter will be devoted to the evaluation and decoding of some of these messages in relation to the problem of biological energy transmission.

If a photon hits a molecule and finds no electron which can take up its energy, then it passes this molecule without leaving a disturbance behind. If it does find such an electron, then this electron absorbs the photon and becomes excited to a higher energy level. In order to transmit E^* a substance must be able to accept it. The light absorption tells us what energies a molecule is capable of accepting.

The fate that the E^* thus communicated to a molecule *in vitro* may meet varies according to the qualities of the excited electron and the molecule which contains it. Electrons of the outer shells of the molecules belong mostly to an individual atom and are bonded to its nucleus. If such a bonded electron is excited, it is likely to cause the nucleus to vibrate around its equilibrium position and spend its E^* which, eventually, becomes dissipated as heat. Evidently, such an electron would be unfit to act as an energy transmitter.

Molecules containing conjugated double bonds have nonbonded electrons which do not belong to single atoms but to the whole conjugated system. These π electrons, as a rule, are more easily

[1] The student interested in fluorescence and related phenomena will find two excellent monographs written on this line by Förster and Pringsheim. The present knowledge about the triplet state has been reviewed lately by Kasha and McGlynn.

excitable than the bonded ones. Not being strongly bonded to any nucleus in particular, they are also less likely to cause nuclear vibrations. However, if the molecule contains any weak bonds which can be broken or distorted by E^* (if the dissociation energy is lower than the excitation energy), then the E^* communicated to the molecule will be used up in breaking or distorting that bond. Evidently, such molecules would be equally unfit to function as transmitters of E^*. If the molecule does not contain such weak spots and is unable to dissipate its E^* (and if there was no collisional deactivation), then it has to shoot out its E^* as a photon, for molecules can hold E^* only for a very short period of the order of 10^{-8} to 10^{-9} seconds. This means that the molecule will be fluorescent. Fluorescence thus tells us that the molecule is capable of accepting energy and does not dissipate it. These are two qualities any molecule must have to be able to act as an energy transmitter. Thus, fluorescence becomes a most important indicator for our studies, though in itself it may have no direct biological meaning, fluorescent light not being different from any other light. Naturally, the biological role of these molecules will not be to *emit* but to *transmit* energy and so in their natural setting they should not be fluorescent at all or should be so only to a small extent (some of the energy being spilled). For example, chlorophyll, the most important energy transmitter, shows a very poor fluorescence in the plant while it displays a most wonderful one in extracted condition.

Naturally, a fluorescence in a tissue extract does not necessarily mean the presence of an energy transmitter, nor does the absence of fluorescence necessarily exclude one. The fluorescence may be covered up by other substances which absorb the light or interfere with excitation. Fluorescence may also be absent because a molecule acquires the structure necessary for fluorescence only in conjunction with other cell constituents such as metals or proteins.

Organic molecules, fluorescent in solution, may belong to different groups of substances but they all can be expected to have a

system of conjugated double bonds. One of the major groups is made up of aromatic hydrocarbons, which have no charges and are unable to accept or give off protons. Accordingly, their fluorescence is independent of pH and redox potential. Their absorption lies mostly in the UV.

Another important group of fluorescent substances is formed by molecules which have charges, the oscillations of which partake in excitation. The fluorescence of these molecules is sensitive to pH

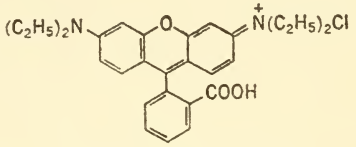


FIG. 4. Rhodamin *B*.

and redox potential; their absorption is mostly at a longer wavelength, in the visible part of the spectrum. Dyestuffs belong to this group. One of the classic members of this group is the dye rhodamin B (Fig. 4), the charge of which oscillates between its two nitrogens. Rhodamin has an absorption band in the UV and one in the visible with a maximum at 554 mμ. So if irradiated by UV it will absorb this light, but according to laws of spectroscopy, it will shoot out its fluorescent light from the lower absorption band. This is symbolized in Fig. 5, in which the line G stands for the normal or ground state of the molecule. The arrow $G \rightarrow S_2$ stands for the excitation of the molecule by the UV light. The arrow $S_2 \rightarrow S_1$ stands for the "internal conversion" in which the electron gives off in a photon-free process its excess energy and drops to the energy level of its lower absorption band, the energy difference being dissipated as heat. The arrow $S_1 \rightarrow G$ symbolizes the shooting out of the energy in the form of the orange fluorescent light, whereby the electron drops back to the ground level, joining its pair. As is generally known, electrons, as a rule, occupy orbitals in pairs, the two electrons spinning in opposite directions.

The quantum mechanical selection rules allow no more than two electrons to occupy one energy level and this only if the two electrons have an opposite spin, in which case the two "coupled" spins neutralize one another's outward electromagnetic actions, making the molecule diamagnetic.

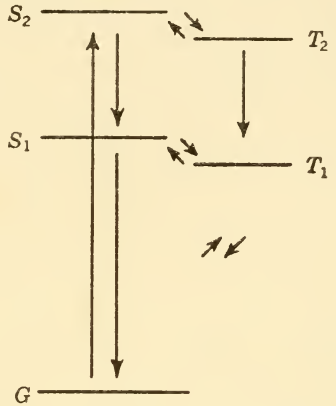


FIG. 5. Schematic representation of an electronic excitation. G: ground level; S_1: lowest singlet level, S_2: next higher singlet level; T_1: lowest triplet level; T_2: next higher triplet level. Vibrational levels are omitted. Short arrows mark forbidden transitions.

Such fluorescence as that of rhodamin can most conveniently be observed in the dark, the solution being illuminated by a high pressure mercury lamp armed with a light filter which allows only near UV light to pass. The UV excites the dye but does not interfere with our observation, being invisible to the human eye. My observations described in this book have been made with such an illumination. The fluorescent light has a somewhat longer wavelength than corresponds to the absorption spectrum, some of the energy being lost on the way. So while the absorption maximum of rhodamin is at 554 mμ, the maximum of its fluorescent band is at 578 mμ.

Such an excitation, in which the electron is simply raised to a higher energy level and then drops back again, is called a "sing-

let excitation" in reference to the single line it generates in the spectrum. However, there is always a small but definite chance that the excited electron may reverse its spin, which then becomes parallel to that of its earlier partner. This state is called the "triplet state" with regard to the three spectral lines into which the singlet line splits up. There is little probability that this will occur, for quantum mechanical selection rules forbid electronic transitions of different multiplicity, that is transition from singlet to triplet and *vice versa*. "Forbidden" just means a low probability.

But once this transition into the triplet has happened, and the excited electron has reversed its spin, it cannot drop back to its original level to join its earlier partner which is spinning in the same direction. The excited electron is thus trapped in the excited triplet level which, as a rule, has somewhat less energy than the corresponding singlet level, some energy being lost in the process. If the energy difference between the two levels is small, heat agitation may raise the electron back to the singlet level ($T_1 \rightarrow S_1$, in Fig. 5). If the electron hereby reverses its spin it may drop back to the ground level emitting its excess energy as *delayed fluorescence*. What mostly happens, eventually, to the electron in the triplet state is that a deactivating heat collision dissipates its excess energy and so the electron disappears from the scene without emitting light. The chances of the electron's falling back from the triplet level directly to the singlet level, emitting a photon, are very small and so a light emission, which corresponds to the arrow $T_1 \rightarrow G$ in Fig. 5 is a rare occurrence. Such a light emission which corresponds to this direct triplet-singlet transition, dropping from the excited triplet to the ground state is termed now, after the pioneering studies of G. N. Lewis and his associates, *phosphorescence*, to distinguish it from *fluorescence* which corresponds to a singlet-singlet transition. Equally rare will be the opposite, a direct transition from the ground state to the excited triplet ($G \rightarrow T_1$ in Fig. 5) under absorption of a photon. This absorption is termed *triplet absorption*.

The electrons going into the triplet will have important consequences. Firstly: the lifetime of the excitation will be greatly lengthened, as a rule about a millionfold (Becker and Kasha). The molecule, which contains the "uncoupled" electrons, will be paramagnetic and will be in an unbalanced, more reactive state. In many ways it will be akin to a free radical.

If we want to study electrons in the triplet state we have to stabilize them, protecting them as far as possible against deactivating heat collision. This can be done by enclosing them in a rigid glass, such as Borax. Cooling will help to some extent too, decreasing heat agitation. We can combine the two and dissolve the substance in question in a solvent such as glycerol and then freeze this solvent by strong cooling in dry ice or liquid N_2 to a rigid "glass." By doing so we can increase the number of electrons present in the triplet state. The more electrons in this state the greater the chances that some will fall back directly to the ground state emitting "phosphorescence." To show that the light actually is phosphorescence and not fluorescence, that it comes from a triplet, we can observe the system in a phosphoroscope. This is an instrument which cuts off alternately the exciting light going from the light source to the phosphorescent material and the light going from the phosphorescent material to the observer. If the time interval between these two is 10^{-3} seconds, then light which the observer sees must have been generated by an excitation which lasted 10^{-3} seconds or more, was thus phosphorescence. Fluorescence only lasts 10^{-8} seconds or so. The author has used two phosphoroscopes in his studies, a fast one with a time constant of 10^{-3} seconds and a slow one which allowed light to pass only if coming from a "long-life-excitation" lasting 0.1 second or more.

There is one more point about the triplet state which might be of prime import to biology, and this is that the probabilities of the singlet-triplet transitions can be modified, are thus accessible to regulatory influences. The probabilities of a singlet turning into a triplet can, for instance, be greatly increased by the presence of

paramagnetic molecules, as O_2, or of heavy atoms with a high atomic number, as iodide, the nucleus of which can create an electromagnetic disturbance in its vicinity. If an excited electron enters it, it may reverse its spin. A beautiful example of this has been given by Kasha (1952), who showed that the almost pure singlet spectrum of dichloronaphthalene went over into an almost pure triplet spectrum if he replaced the Cl by the heavier iodine. But what is even more important—it was not necessary to introduce the iodine *into* the molecule in order to produce this change. It was sufficient to add it *to* the solution in the form of ethyliodide. These effects seem to me especially fascinating because iodine is contained in one of the main regulators of cellular energetics, thyroxine (and related compounds), while O_2, which is one of the very few molecules paramagnetic in its normal state, is most intimately involved in the energetics of life.

The biological energy unit, the energy of the ~P is of the order of 10 Calories, which corresponds to a wavelength of 2–3μ, which is to say that a photon of this wavelength has the same energy as a ~P. This wavelength corresponds to the near infrared. It is thus this spectral region which will have the greatest direct interest for the biologists. It also has an interest of its own. It is here that pure electronic and vibrational excitations meet and transition between the two is the easiest (which might be one of the reasons why the biological quant is located here). Similarly to other borderlines this spectral region, too, is a no man's land, lying beyond the domain of current spectroscopy and on this side of the professional infrared. This region may hold surprises even for the physicists. Its secrets are also guarded by technical difficulties, as the lack of good detectors, and water which has several absorption bands in this region.

5. Triplets and Water

Electronic excitations give us valuable information about properties and reactions of molecules, but whether these excitations are involved in biological reactions and take any part in energy transmissions is a different question. There are reasons to doubt it. Singlet excitations are much too short-lived to allow their utilization or regulation. Moreover, pure electronic excitation, which corresponds approximately to the visible part of the spectrum, demands energy quanta which, as a rule, are too high to be available in biological systems. The longest visible wavelength corresponds to 40 Calories while the biological energy unit, the energy of the ~P, is about 10 Calories.

These difficulties are not present in the case of triplets, of which the lifetime is longer and the energies lower; their energy levels may lie even in the near infrared. However, the forbidden nature, which makes the singlet-triplet transition attractive, seems also to make it useless for the biologist. An excitation which has a small probability of occurring can be of no use to the cell. So we are left empty-handed.

There is one possible hitch in this. The biological solvent is water, not borax or glycerol, and water has many queer and unique properties, such as its strong dipole character. The reasons why the physicist has shunned water as a solvent are evident. On freezing, water cracks, becomes inhomogeneous and unfit for optical measurements. It becomes inhomogeneous both optically and chemically, having a tendency to crystallize out and leave dissolved molecules behind. So the physicist has had good reason not to touch water. The biologist, however, is inseparably linked to it.

Life originated in water, is thriving in water, water being its solvent and medium. It is the matrix of life.

So before discarding electronic excitation as the means of biological energy transmissions, we have to have a look at water and repeat some of the classical experiments in this medium, however unfit it may be for physical measurement. If we cannot eliminate its shortcomings we can diminish them. We can, for instance, try to produce possibly eutectic ice which includes dissolved substances, by cooling our solutions rapidly, using thin-walled test tubes of a small diameter dipping them suddenly into a cooling mixture, such as an ethylene glycol monomethyl ether with dry ice suspended in it.

We can start our experimentation by following the trail of the physicist dissolving various fluorescent dyes and other fluorescent substances first in glycerol. Viewing these solutions under the UV lamp, armed with a filter which allows only UV to pass, we can observe their fluorescence. Cooling these test tubes in our dry ice freezing mixture will not make much difference. The intensity of the fluorescence may somewhat increase, some "delayed fluorescence" may appear but the color, that is the wavelength of the emitted light, as a rule, remains unchanged. The results will be the same if, instead of the glycerol, we use a 10% watery solution of glycerol or 2% methanol.

This situation will change dramatically if we shift to pure water as solvent. At room temperature we will see the usual fluorescence, but on freezing we will find the light emission profoundly altered: it will have disappeared or changed in color. This is illustrated by Fig. 6, which is a colored photograph of test tubes, containing fluorescent dyestuffs, illuminated by the UV lamp. All the tubes contain in their lower half the dye solution in frozen state (cooled in dry ice) and in their upper half the solution in unfrozen, liquid condition.

The first tube on the left contained a 10^{-4} *M* watery rhodamin *B* solution. The upper half of the test tube shows the well known

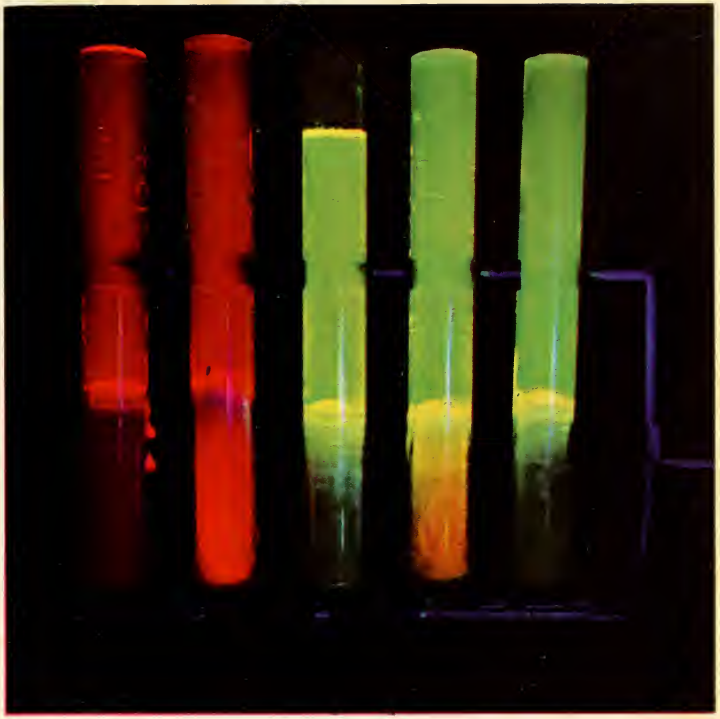


FIG. 6. All tubes contain watery solutions frozen up to the middle. From left to right, first: 0.0001 *M* rhodamin *B*. Second: rhodamin *B* and 0.001 *M* thiamin HCl. Third: 0.001 *M* riboflavine phosphate sodium, no O_2. Fourth: same and atmospheric oxygen. Fifth: same plus 0.001 *M* KI.

brilliant orange fluorescence of this dye, while the lower frozen half shows practically no light emission at all. Ample evidence will be supplied later to show that this change is not due to the dye molecules having become inexcitable nor to their dissipating the energy of their singlet excitation. They are just as excitable as before (as shown by the almost unchanged color) and do not dissipate their energy (the low temperature and solid state also disfavors such dissipation). The change is due to the excited electrons going into the "forbidden" triplet state. The peculiar conditions prevailing in our frozen water make this transition not only an allowed one but the most probable one, and so the electrons go into it wholesale.

The second tube from the left in Fig. 6 contained the same watery rhodamin solution as the first one, with the difference that at the side of the dye also 10^{-3} *M* thiamine hydrochloride was dissolved in it. The unfrozen solution in the upper half shows the same orange fluorescence as before. The difference is in the frozen part. This shows an intense red light emission, comparable in its intensity to the fluorescence of the unfrozen solution. The light emission is red, of longer wavelength (and lower energy) than the orange fluorescence. In the fast phosphoroscope this frozen part of the tube shows luminescence which indicates that the emitted red light comes from a triplet state and is due to the return of the excited electrons from their excited triplet into the ground state. So water, in presence of thiamine, makes this transition possible too, promoting the transition both ways, from singlet to triplet and from the triplet to the ground state.

There are two facts emerging from this experience: that water can promote the singlet $\rightleftharpoons$ triplet transitions and that the probabilities of these transitions can further be modified by added substances. Thus the transitions are accessible to regulatory influences and if these excitations would play a major role in biology they could be controlled by hormones, modified by drugs, or might be disturbed under pathological circumstances, giving rise to disease.

The red phosphorescence of rhodamin has a wavelength which is 35 mμ longer than that of its fluorescence, triplet levels being usually somewhat lower than singlet levels. As has been symbolized in Fig. 5, in the unfrozen state the rhodamin gets excited by our UV to its higher S_2 level, drops in its "internal conversion" to S_1, and from here to G emitting its excess energy as fluorescent light. What happens in the frozen part of the second tube in Fig. 5 is that the electron goes from S_2 to T_1 and then returns from here to its ground state G, emitting energy in the form of phosphorescent light. Whether it gets from S_2 to T_1 *via* S_1 or T_2 can be left an open question.

On closer observation this second tube in Fig. 6 with the rhodamin-thiamine mixture is found to have three zones, a fluorescent liquid one on top, a phosphorescent one on the bottom, and a zone which separates the two and emits no light. This intermediary zone has an intermediate temperature. We can observe this transitory nonluminous phase also by gradually cooling our solution. On cooling, the fluorescence disappears before the phosphorescent light emission appears. Only when the tube reaches a low temperature does the phosphorescent light emission set in. It becomes stronger when we go from dry ice to liquid N_2 which is about 100°C colder. One would have expected radiation to become weaker on cooling and stronger on heating. What happens is the opposite, showing that the light emission was not dependent on activation energies.

The middle tube in Fig. 6 contained a 10^{-3} *M* solution of riboflavine-5'-phosphate.[1] The situation is analogous to that found in the first tube with rhodamin: the upper part shows the well known brilliant yellowish-green fluorescence, while the lower frozen part shows no light emission at all. In this tube the O_2 was expelled

[1] It is necessary to use fresh solutions. The riboflavine is light sensitive but even stored in the dark refrigerator it loses its phosphorescence without diminishing its fluorescence. The former seems to be linked to subtle qualities of the molecule.

prior to cooling by bubbling through N_2. If the cooling is effected in the presence of the absorbed atmospheric oxygen, the result is the one shown in the next tube to the right. The O_2 present made no difference to the fluorescence of the unfrozen part, but made the frozen part emit an orange phosphorescence. The O_2 rendered the triplet state unstable, making it emit light, "allowing" the excited electrons to return from their lower triplet state to the ground state, giving off their excess energy in the form of photons.

The last tube on the right contained besides riboflavine the atmospheric O_2 and 10^{-3} *M* potassium iodide. As the figure shows, the phosphorescence was completely "quenched." Transitions are accessible to regulatory influences both ways, as shown by the opposite action of O_2 and I^-. If the one labilized the triplet state and promoted the $T \rightarrow G$ transition, the other abolished it. Such antagonistic effects can be produced by various substances. Adrenaline, for instance, wipes out the weak phosphorescence of frozen rhodamin in 10^{-5} *M* concentration while acetylcholine promotes it and so the enhancing effect of .0025 *M* acetylcholine can be compensated by 10^{-4} *M* adrenaline. Adrenaline quenches the phosphorescence of riboflavine in 10^{-5} *M* concentration.

The light emission of a watery riboflavine solution, frozen without the previous elimination of oxygen, has been observed previously by Dhéré and Castelli, and interpreted correctly as a phosphorescence.

We can thus sum up by saying that water has brought about a profound change in the excitational states of the two fluorescent substances studied, making forbidden transitions into probable ones, and the question arises whether the behavior of rhodamin and riboflavine represent some general rule or whether we have stumbled here on two substances that behave peculiarly. As far as my experience goes we are faced here with a general rule. All fluorescent substances tested showed such an extraordinary behavior at low temperatures in water. The following examples may be quoted: on freezing of its watery solution in dry ice, the green-

yellow fluorescence of acridine orange gives place to an orange phosphorescence. The same happens with acridine yellow. As we go below 0°C the yellow fluorescence disappears and all light emission vanishes. On further cooling an orange light emission appears and becomes intense around —50°C. In acridine yellow this light emission comes from a long-lived excitation since it is very intense even in the slow moving phosphoroscope. If the test tube is held in the light of the UV lamp and then the illumination is suddenly disconnected, the frozen solution shows a strong afterglow lasting for a second or more.[2] Pyronine B, a dye related to rhodamin B, which shows in liquid solution an orange-yellow fluorescence, loses (like rhodamin) all light emission as soon as the water freezes. No light emission appears even at the temperature of dry ice. However, if 1% glucose is present, below —40°C a strong red light emission appears which comes from a long-lasting excitation as shown by the strong afterglow. Acridine red, eosin Y, tetrabromo fluoresceine, rose bengal, titan yellow, all show similar sudden changes in their fluorescent light emission on freezing. These changes are not limited to dyes. A saturated watery solution of acridine, which has a blue fluorescence at room temperature, emits a strong orange phosphorescence if cooled in dry ice. The blue fluorescence of folic acid disappears on freezing. Atebrin (0.0005 *M*), which has an intense yellowish-green fluorescence in water shows, in the frozen state, an orange-green light emission which can be shown to be composed of a yellow fluorescence and a red phosphorescence by adding 1% glucose to the solution prior to freezing. On direct observation the light emission of the frozen system is found to be green-yellow, while in the phosphoroscope the system shows an intense red phosphorescence, coming from a long-lived excitation. In quinine and quinidine the intense blue fluorescence fades out on freezing to give room, at lower temperatures, to a whitish-blue phosphorescence. "Whitish"

[2] If afterglow had to be observed, or if the light emission was studied in the phosphoroscope, no light filters were shunted between the lamp and the material.

means a mixed color, the yellow component of which can be intensified by freezing the solution of the alkaloid in the presence of 10^{-3} *M* potassium iodide. In lysergic acid (10^{-4} *M*) the blue fluorescent light emission of the watery solution assumes a purplish color on freezing and can be shown to contain, at the side of fluorescence, a strong long-lived phosphorescence in the presence of 1% glucose.

Such changes are not limited to fluorescent substances. Isopentaquine, for instance, emits under the UV lamp a strong orange light if frozen in dry ice. This light emission is composed of a yellow fluorescence (which can be greatly increased by 10% glucose) and a red phosphorescence which can be seen in the fast phosphoroscope and can be somewhat increased by 10^{-3} *M* glutathione. A frozen blue tetrazolium sends out orange-green light, composed of a green fluorescence and a red phosphorescence visible in the fast phosphoroscope. Chlorophyll behaved like other fluorescent dyes: on freezing its watery solution completely lost its brilliant red fluorescence.[3]

In all instances the light emission, observed on cooling, had a longer wavelength than the fluorescence of the unfrozen solutions. Phosphorescence, as a rule, has a longer wavelength than the corresponding fluorescence. Spectra were known to have a tendency to shift towards a longer wavelength on intense cooling, but the fact that water so completely and dramatically changes the situation was, to my knowledge, not known or appreciated.

These experiments are so simple that they ought to be repeated by everybody interested in this field. They should be repeated even in the classroom. They demand no paraphernalia, except an UV lamp and some dry ice. Doing an experiment is different from reading about it. These experiments make the impression of a new, colorful world opening up unexpectedly and promising a deeper insight into the mechanisms of life and their interaction with their matrix, water.

[3] "Chlorophyll, water soluble" of Fisher Scientific Co., New York was used.

6. Excitations and the Biological Matrix

The first question that needs to be answered in connection with the experiments described in the previous chapter is that about the role of water which, together with the low temperatures employed, so completely changed the situation. The results make it clear that the effect of water and cooling was not additive but the two did something specific together. Water, in itself, does nothing, and cooling, in itself, does nothing, for at room temperature the situation is not changed by using water as a solvent instead of glycerol, while cooling does not change the situation if glycerol or a 10% watery glycerol is used as a solvent. That temperature, as such, is ineffective is also borne out by the fact that the behavior of rhodamin or riboflavine is essentially identical whether we use dry ice or liquid N_2 for cooling, though there is more than 100°C difference between the temperature of the two. So evidently, something new and specific had to be produced by the introduction of water *and* cooling. This could hardly be anything else than the formation of ice. That this is actually so is borne out by the temperature dependence of the observed phenomena. We must distinguish here between two reactions: the excited molecule's going into the triplet state and its emitting phosphorescent light. The two depend on temperature in a different way. A rhodamin solution, for instance, cooled to —78°C shows a weak red light emission. If allowed to warm up gradually, the light emission fades out around —40°C. From —40° upwards there is no phosphorescence and until the ice does not melt there is no fluorescence either. There is no light emission at all. Fluorescence sets in only when the ice starts to melt. Thus the triplet is stable until the ice does not melt. We do not need the strong and rapid cooling in our ex-

periment to produce triplets. Freezing at —1°C would be sufficient, only such slow freezing would favor the formation of pure ice which leaves the dye behind in a small volume of mother liquor. The deep freezing is needed to have the dye enclosed in the rapidly forming crystals, but once this has happened we can maintain the triplet state even at the melting point. The same is true for the other substances studied, such as acridine orange, acridine, quinidine, and riboflavine. The light emission fades out in these substances at different temperatures, in acridine orange around —20°C, in riboflavine around —3°C; the blue emission of pure quinidine and the yellow emission in the presence of 0.001 *M* KI fade out around —20°C. So the real color of the triplet can be seen best closer to the melting point where the ice is optically less inhomogeneous.

This shows clearly what is important for the triplet state is not the low temperature but the physical state of water, the formation of ice. Ice is not just solidified water. As is generally known, modern physics puts less emphasis on the idea of "solid" and "liquid" than on "regular" and "random," and the "solid state" owes its specific qualities less to the fact that its particles cannot move relative to one another than to the fact that they can form regular structures, crystal lattices, which is possible only if the relative position of particles is fixed and regularities are not destroyed by heat agitation. Glass, which is rigid and does not flow at room temperature, has no regularity in its structure and so is looked upon by the physicist more as a fluid of high viscosity than a solid. The same is true for glycerol frozen to a rigid "glass" at low temperature, while ice is a real solid with a regular hexagonal crystal structure though it readily changes its shape and "flows," as evidenced by the motion of glaciers and as Helmholz showed. The strong dipole character of the water molecules may endow their crystals with specific qualities and so the most reasonable assumption is that the transition of our excited molecules into the "forbidden" triplet state comes about by an interaction of these mole-

cules and their excited electrons with the crystal lattices of this queer substance, water.

This explains why there was no striking change in the behavior of our fluorescent substances in glycerol on cooling; glycerol may form a rigid mass at low temperatures but does not form crystals. This explains also why an admixture of 10% glycerol or 2% methanol to the water deprived it of its specific action on excitation: because these admixtures destroyed the regularity of the water crystals.

When considering water structures we enter a fantastic and fascinating world. Bridgman, in his studies on high pressure, could distinguish between half a score of different ices. But we need not go to ice to find structures in water. Bernal and Fowler, in their classic paper showed water to have a quartz-like "crystalline" structure which is different from that of common ice which is tridymit-like. Though Bernal's and Fowler's theory has somewhat been modified by later researchers, its essential correctness has never been questioned. In the H_2O molecule the central O holds the two protons on one side, which lends a strong dipole moment to the molecule. The orbitals of the lone pair of electrons are directed towards the other side, contributing to the dipole character (Pople). The two protons can be shared, through H-bonds, with two other water molecules, while the lone pair of electrons may attract two protons from two other H_2O's, so that each water molecule can link up with four others. Since the four orbitals responsible for these links point in nearly tetrahedral direction, a tetrahedral lattice will thus be formed. If water, in this state, is still liquid this is due to the fact that the links can be broken, and, as Pople emphasized, can also be bent easily. Above 0°C, heat agitation does not allow the molecules to settle down to permanent rigid lattice but all the same the tendency to form such a lattice is there, keeping water in a "quasi crystalline" condition. Water seems to have two melting points, one at 0°C, the point at which the water goes from a rigid crystal, so to say, into a liquid one.

The other melting point is between 30° and 40°C, at which temperature, under influence of the intense heat agitation, the crystal structure fades out altogether (Feates and Ives). It is possible that nature stabilized temperature in higher isothermal organisms around 37°C to allow their cells to build crystalline water structures of their own choosing.

The situation becomes more complex still if we consider water structures built around solid surfaces. The tendency of building structure-ordered layers around surfaces reaching deep into the fluid phase seems to be a general tendency of liquids. This problem is also of first-rate industrial importance with regard to lubrication. Henniker and McBain collected, as early as 1947, 175 references to such layers and have come to the conclusion that "deep-surface orientation is the normal behavior of many liquids. The surface zones of liquids are tens and hundreds of molecules deep, rather than monomolecular, as commonly assumed." A clear demonstration of such surface layers of water was given more recently by Palmer, Cunliffe, and Hugh who measured the dielectric properties of water between mica plates and found that water behaved around the mica not as water but as "liquid ice" showing the frequency dependence of the dielectric constant of ice. These layers of ice were found to be several microns deep.

The formation of such water structures should not be confused with the old idea of "bound water." "Binding" involves rather the idea of energy than that of structure. "Binding" means a certain force, energy being needed to remove a molecule from its site. Such "bound" molecules, having their dipole forces engaged, are also unfit to serve as solvents for other molecules. Such a binding is especially strong around free charges, as those of ions. The order thus produced is a "short range order" the number of more firmly held layers of molecules being very small, 1–2. Contrary to this the building of lattices means "long range order" in which the single molecules collaborate collectively.

The building of such water structures is not necessarily linked

to such sharp phase-interfaces as those of water and mica. Such structures can, and probably are formed around any dissolved molecule. Frank and Evans introduced the picturesque expression of "iceberg," trying to express that molecules, dissolved in water are surrounded by such structures. The measurements of Frank and Evans show that not only electropolar molecules, but also apolar molecules surround themselves with such icebergs. As described lately in an attractive article by Buswell and Rhodenbush, the ice formed around homoiopolar molecules is different from common hexagonal ice, having a very loosely packed cubic lattice structure, their formation causing thus a strong expansion. "This behaviour (of water) was called to the attention of chemists in a dramatic fashion by certain surprising natural phenomena. One was the fact that corn sometimes showed frost effects when the temperature was 40°F, well above freezing. Another was the discovery that pipelines carrying natural gas often became clogged with a slushy 'snow,' containing water, at temperatures as high as 68 degrees F." The formation of ice above the freezing point is thus not a theory. It is crude fact which causes trouble in industry.

We can thus suppose water structures to be built around dissolved molecules, structures which may have a different crystalline structure according to the polar or nonpolar nature of the atomic groups on that molecule and the mutual distance of these groups in relation to the lattice constants of the different possible water crystals. It is believable that different spacings promote different crystal forms or, if unfavorable, inhibit order and lattice formation. Possibilities are rich, relations complex.

If structures or molecules are surrounded by "icebergs" then we can expect that the dimensions and the nature of the icebergs will decide how far these molecules can approach one another and also whether a dissolved molecule can pass a pore in an "ice-covered" membrane. Two "icebergs" having an identical ice structure might fuse, allowing a close approach, while the opposite may hold for ices of different nature.

B. Jacobson has studied highly polymer deoxyribose nucleic acid and found that its behavior could be explained better by the assumption of "lattice ordered hydration shells" than by the old idea of rotational ellipsoids. The great number of electropolar groups and free charges on this molecule may strongly favor the formation of water structures. Also the fibrous nature favors ice-formation. A globular shape disfavors it, so that it seems likely that mobile globular protein molecules will be found to be built so as to avoid formations of icebergs which would interfere with mobility. Since structural proteins are fibrous and closely packed, it seems likely that water, between them, is in a highly ordered state. Bernall and Fowler introduced the idea of "structural temperature" which they defined as the temperature at which free water would have the same degree of order as the water enclosed in the specific system under discussion. We could expect this "structural temperature" to be rather low in protoplasmic formations. The building of such water structures, to use Jacobson's words (1955), "involves a change in physical properties of the whole system qualitatively similar to the changes obtained in the properties of pure water when the temperature is decreased." All this put together means that water, within the cell, may not be random water but "liquid ice" which makes it possible that the triplet states, observed on frozen water, also represent the most probable form of excitation in tissues.

No attempts have been made yet to apply our knowledge of water structures to living systems or to demonstrate triplet excitations in biological processes. There are sporadic observations which are suggestive. Gergely (see the author, 1947, p. 101) observed that acridine orange, dissolved in water, showed phosphorescence if actomyosin was added to the solution. The less fibrous myosin or actin were less active. Gergely applied actomyosin in relatively low concentration and so we can expect that at the high protein concentration of muscle triplet excitation would be favored still more. I found that a fresh frog or squid muscle, if suspended in

0.1 *M* KCl and stained with acridine orange (10^{-3} *M*), showed on illumination (at room temperature) the orange phosphorescence of the dye, while the dye seeping out of the muscle into the underlying filter paper showed the usual yellowish green fluorescence. If the muscle was brought into contracture by freezing and subsequent thawing, phosphorescence disappeared indicating a collapse of the water structures. The great quantity of liquid pressed out by such a muscle also indicated that the physical state of water had undergone a profound change. Using 0.1 *M* NaCl, as suspension fluid instead of KCl disfavored phosphorescence. As will be shown later Na disturbs water structure.

The study of water, in its relation to biological structures and electronic excitation processes, opens a fascinating and promising field of inquiry which may lead us far towards the understanding of normal and pathological processes. In fact, these water structures suggest a new outlook on life itself. Hitherto, water was looked upon, more or less, as a neutral medium, filling the space between the structural elements within the cell. In the outlook developed in this chapter, water forms one single unique system with structural elements in which electronic excitations become possible which are highly improbable outside it. The protoplasmic systems which generate these excitations also generate the water structures which are necessary for the production, and possibly also for the propagation of these excitations. Biological functions may actually consist of the building and destruction of water structures, water being part and parcel of the living machinery and not merely its medium, the water structures and their interactions with electronic excitations being intimately connected with the very essence of the "living state."

One of the most basic principles of biology is organization, which means that two things put together in a specific way form a new unit, a system, the properties of which are not additive and cannot be described in terms of the properties of the constituents. As points may be connected to letters, letters to words, words to

sentences, etc., so atoms can join to molecules, molecules to organelles, organelles to cells, etc., every level of organization having a new meaning of its own and offering exciting vistas and possibilities. Living matter seems to be a system of water and organic matter, which forms one single inseparable unit, a system, as the cogwheels do in a watch.

Water is not only the *mater,* mother, it is also the matrix of life, and biology may have been unsuccessful in understanding the most basic functions because it focused its attention only on the particulate matter, separating it from its two matrices, water and the electromagnetic field.

One critical remark may be added to the contents of the last two chapters: there is no conclusive evidence yet available to prove that the long-lived excitations, observed in ice, were actually triplets and not some other unexpected forms of excitation. Triplets are not the only known long-lived excitations, as witnessed by the luminous ciphers on the dial of a wrist watch. The delayed light emissions, in this case, belong to the so-called "crystal phosphors." In crystals, the orbitals of single atoms and molecules may fuse to continuous bands and if electrons are excited to a higher energy band they may be trapped there and drop back to the ground level with a delay, emitting their excess energy in the form of protons. Long-lived excitations are also given by the so-called "gelatin phosphors" which can be prepared by drying a fluorescent dye in the presence of a protein, like gelatin. Should the long-lived excitations, described in the last two chapters, turn out not to be a triplet excitation, but some other exceptional form of E^* this would alter little the validity of the essential conclusion, that within water structures electronic excitation may assume an unusual long-lived form which might be of prime import for biological energy transmissions.

The evidence available at present strongly pleads for the assumption that the observed excitations were triplets which also strengthens the conclusion, arrived at in the second chapter of this

book, that the blueprint of the energetics of the living world consists essentially of two parts only: photosynthesis and its reversal. Increasing evidence is available to show that chlorophyll, in photosynthesis, is excited by light to a triplet state. In our *in vitro* experiments chlorophyll behaved in ice in the same way as other fluorescent dyes, going thus, in all probability, into the triplet state on excitation. So triplets seem to be the main instruments of energy transmission in both, photosynthesis and the biological actions which consume energy, which brings the analogy between the two processes still closer. It seems likely that also within the chloroplasts the triplet excitation of chlorophyll is made possible and stable by the surrounding water structures. This interaction of triplets and water may also open the way to the understanding of the mechanism by which the E^* of chlorophyll is used to decompose water into its elements, which is at the core of photosynthesis. As will be shown later, triplet excitations in ice are stabilized by SH, which may help to fit Calvin's thioctic acid into the picture.

I have considered here only changes which may be induced in the solvent, water, by its freezing, but changes may be induced also in the solute, the fluorescent matter. On freezing, for instance, the water crystallizes out, which may lead to increased local concentrations of the solute with consequent self-quenching or polymerization which may profoundly alter excitational states and light emissions. These possibilities will be studied later.

7. Quenching and Quenchers

When studying a biological process, we mostly try to preserve our material as well as possible. We can also do the opposite and induce damage by poisons, drawing conclusions from the consecutive changes in function. In an analogous fashion, we could also try to find poisons for E^*.

Light emission, be it fluorescence or phosphorescence, indicates electronic excitation and if a substance quenches it, it has to interfere with the underlying E^*. If this E^* is involved in a biological process then the quencher should poison it. So highly active and specific quenchers could help us in our study and the question is how to find them?

It is known that certain atoms or atomic combinations have a quenching action. Such an atom is the iodine and to a lesser degree the bromine atom. SCN and NO_3 are also known as quenchers, suggesting that the combination of atoms with nonbonded "lone" pairs of electrons make quenchers; O, N, and S are such atoms. The activity of these atoms and atom combinations declares itself also by a strong absorption in the UV.

These simple quenchers will not help us much, partly because their activity is not high enough and partly because they have no specific affinities to tissue elements which could support a specific action. Such a specificity demands a more complex chemical structure. In our search for quenchers with such a structure we can make use of the experience that the mentioned simple atoms or atomic groups usually abolish the fluorescence if introduced into a more complex fluorescent molecule. They make the molecule dissipate its energy. Figuratively speaking, they act as a sink for the E^*. The molecule may be able to dissipate not only its own

E^*, but may also be able to take over that of other molecules and do with it likewise. In this case, this molecule will be a quencher. In this way we can hope to arrive at molecules which may have not only the desired quenching activity but may have also selective affinities.

To quote an example: benzene is known to be weakly fluorescent but if NO_2 is introduced into its molecule the resulting nitrobenzene will be fluorescent no more, though it is even more easily excitable than the benzene itself, as witnessed by its strong absorption in the UV and the blue. The NO_2, as a "chromophore" group, enables the molecule to pick up energy still easier and to act with its conjugated double bond system, so to speak, as an antenna, which picks up energies from its surroundings by electrodynamic coupling, taking E^* over from other molecules, acting thus as their quencher, while the NO_2 dissipates the E^* thus acquired.

By introducing additional NO_2 groups at the points most fit for resonance, or by introducing electron donor groups, as OH, or by extending the resonating system by adding one or more benzene rings to it, we can further improve the quenching abilities as well as the specificity of our molecule. Such ideas lead us into fascinating fields of biology. When building such quenchers, we might be following the same route which nature followed when she introduced iodine into extensive resonating aromatic structures creating thyroxine and related substances.

These considerations also lead us to one of the most fascinating puzzles of contemporary biochemistry: 2,4-dinitrophenol. This substance (Fig. 7a) uncouples oxidative phosphorylation, the transmission of the energy from foodstuffs to ATP. Mitochondria, in its presence, go on oxidizing foodstuffs, may do so even at an increased rate, but waste the energy thus liberated as heat. This caused dinitrophenol, a few decades ago, to become a most fashionable drug for reducing body weight. Unfortunately, its use in medicine had to be discontinued because in some cases, mysteri-

ously, it produced cataract and most people prefer being fat or hungry to being blind.

The mechanism of the action of dinitrophenol is the more puzzling because it acts not only on oxidative phosphorylation, but seems to act wherever energy has to be transmitted and put to action. It inhibits various processes involved in photosynthesis and inhibits the "pumping activity" of cell membranes, in which osmotic or electric work is done. These reactions are so different that it was impossible to bring them to a common denominator

(*a*) (*b*)

FIG. 7. a: 2,4-Dinitrophenol. b: 2,4-Dinitro-1-naphthol.

in terms of chemistry. The only point at which all these reactions agree is that transmission of energy is involved, in one way or another. This makes it seem likely that the dinitrophenol is not involved in a definite chemical reaction, but acts rather through some physical principle, as is the quenching of E^*. It may be interesting to note in this connection that both 2,4-dinitrophenol and 2,4-dinitro-1-naphthol (Fig. 7b) have in the near infrared a broad region of absorption. Dinitronaphthol is, essentially, dinitrophenol with an extra antenna added to it.

McLaughlin and the author measured the quenching of the fluorescence of the aromatic hydrocarbon, chrysene, by a number of aromatic nitro derivatives and found dinitrophenol active and dinitronaphthol even more so. Unfortunately, their methods did not allow them to distinguish between true quenching and the simple competition for the light between quenching and quencher. So Karreman and Steele repeated these measurements with more adequate methods. Their results showed that both dinitrophenol and dinitronaphthol are true quenchers. The quenching observed

could not be accounted for by deactivating collisions only and had to be, partly, a resonance quenching which takes place without a collision, through the resonance transfer of E^*. Fluorescence, on the whole, is rather insensitive to quenchers, which can easily be explained by the brevity of the lifetime of the singlet excitation. But fluorescence, as such, is of little direct interest for our problem. If E^* plays a role in biology it has to be in the form of triplets, as shown in the previous chapters. I found triplets most sensitive to quenchers, some of which suppress phosphorescence in

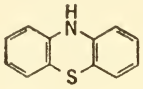


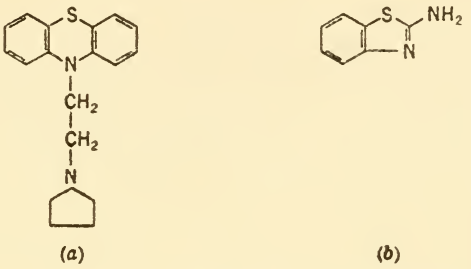
FIG. 8. Phenothiazine.

high dilution. The insensitivity of fluorescence can be used with advantage to show that the quenching of phosphorescence observed was a "true quenching" and the light emission did not simply disappear because the quencher absorbed the exciting light. If this were the case, then fluorescence would have to be quenched as well. The quenchers to be described, in the concentrations applied, did not diminish fluorescence appreciably and so their quenching had to be a "true" one. A quenching activity can have different mechanisms and so for the time being we'd better refrain from an interpretation of the mechanisms involved and content ourselves with the fact that a quenching, that is a suppression of light emission, must involve an interference with E^* and an interference with E^* must mean interference with biological function if E^* has an important role to play in it.

One of the strongest inorganic quenchers is SCN^- which suppresses the phosphorescence of riboflavine in 10^{-4} M concentration. The atom combination SCN plays no role in organic chemistry. However, the closely related SC=CN is the core of phenothiazine (Fig. 8), in which it is a center of an extensive system of conjugated double bonds. Phenothiazine itself is unsuitable for ex-

perimentation, owing to its insolubility in water. Pyrrolazote (Fig. 9a) is more soluble. The phosphorescence of rhodamin, acridine, and riboflavine phosphate was found completely quenched by this substance in 3×10^{-4} *M* concentration, while the fluorescence of the unfrozen solutions remained unchanged.

That this activity of Pyrrolazote is actually due to the combination of the S, C, and N present can be shown by testing 2-aminobenzothiazole, which contains the same atoms but in a different



(*a*) (*b*)

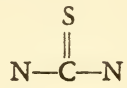
FIG. 9. a: Pyrrolazote. b: 2-Aminobenzothiazole.

cycle and separated only by one C (Fig. 9b). This substance quenches the phosphorescence of acridine in 0.005 *M* and that of riboflavine in 10^{-5} *M* concentration.

Pyrrolazote is not known to have any specific pharmacological activity, which indicates that it lacks a specific affinity for tissues or cell constituents. Naturally, if it interferes with E^* and E^* is important for life, then it should kill the animal if it reaches the concentrations in which it interferes with E^*, and *vice versa,* if it kills the animal by interfering with E^*, then it should quench also *in vitro* in the concentrations which are established in the animal by a lethal dose. The experiment showed that 100 mg injected in mice per kilogram of body weight produced no symptoms, while 200 mg killed the animals; 200 mg per kilogram corresponds to a random concentration of 6.10^{-4} *M*. The phosphorescence of rhodamin and acridine were quenched by 3.10^{-4} *M*.

It would be interesting to know how far the pharmacological

activity of other substances containing S, C, and N atoms in proximity is actually due to the action of this atom combination on E^*. Thiourea and thiouracil contain



6-Mercaptopurine, known for its anticarcinogenic activity, has no action on excitation of rhodamin or fluorescein, but completely quenches the phosphorescence of riboflavine in a 6.10^{-5} M concentration, and has a moderate quenching action on the phosphorescence of acridine. Its action shows thus a certain measure of specificity. It is believable that a mercaptopurine molecule built into the nucleic acid cuts the energy transmission along the column of purine and pyrimidin bases which forms the core of DNA in the Watson-Crick model. Such an action would be analogous to the action of hydroquinone which cuts the energy transmission through Scheibe's columns of isocyanine dyes (see pp. 16, 17).

Another atomic group known to abolish fluorescence, if introduced into the molecule of fluorescent substances, is the N=N. Accordingly, we can expect to find strong quenchers among azo compounds. McLaughlin and the author, while finding diazobenzene a poor quencher, found aminoazotoluene (toluazotoluidine) highly active. The latter substance, as well as butter yellow are difficult to evaluate, owing to their insolubility in water. Both seem to quench the phosphorescence of riboflavine but were inactive with rhodamin.

8. Miscellaneous Observations

In this chapter I will briefly describe a few observations which seemed not to be without interest though their connection with the main theme of this book was not evident in all.

NARCOSIS

Since Overton proposed his theory, narcosis has occupied many research workers. Most of them came up with a new theory of their own. This recurrent production of theories shows that none of them has found general acceptance and we still do not know what narcosis is. If our theory of E^* is correct then it can be expected to make diverse old unsolved problems appear in a new light and so we may ask whether it has anything to suggest about narcosis?

A watery acridine orange solution (10^{-4} M) shows under the UV lamp a greenish-yellow fluorescence. If frozen, the place of fluorescence is taken by a very weak brownish phosphorescence. Similarly to the case of rhodamin, this phosphorescence can be increased by added substances to a vivid red light emission. No long-lived phosphorescence can be observed in this system in the slow moving phosphoroscope.

If instead of pure water we dissolve the dye in a saturated watery solution of cortisone we not only find the weak phosphorescence moderately increased but find its lifetime greatly lengthened. The test tube appears strongly luminescent in the slow moving phosphoroscope or shows a strong afterglow if its illumination is suddenly disconnected. The interaction of water and dye has thus been modified by the sterin which was present in a very low concentration, cortisone being but slightly soluble in water (6.5×10^{-5} M). There could hardly have been a direct interaction between dye and

sterin, the number of the sterin molecules being too small and no specific affinity being known to exist between sterin and the dye. We can expect the sterin molecules to build water structures with a cubic lattice around themselves and it is reasonable to assume that the long life of the excitation is due to the interaction of the excited molecules and their electrons with this cortisone-water system.

Chloroform is very sparingly soluble in water (0.014 *M*). All the same if $\frac{1}{50}$ part of a saturated watery solution is added to the cortisone-dye solution, no long-life phosphorescence will be observed on freezing, although the light emission remained increased, in spite of the presence of the narcotic. It is reasonable to assume that the hydrophobic steroid attracted the chloroform molecules which modified its relation to water and modified with it the water structures responsible for the lengthening of the lifetime of the excited dye molecules. Since the concentration of the chloroform in our system was very low, 3.10^{-4} *M* or less, it is conceivable that this narcotic inhibits the functions of the central nervous system *in vivo* by interfering with the interrelation of lipids and water, disturbing herewith E^* and, possibly, shortening the lifetime of excitations. The total abolition of the long-life phosphorescence is the extreme of an action in this direction. Less drastic actions may lead to subtler changes and may be responsible for the great variation in symptoms produced in the central nervous system by different drugs. In the earlier chapters of this book we considered only direct interactions between two substances A^* and *B*. It seems likely that for an understanding of drug actions an indirect interaction will also have to be considered in analogy with the above experiment with acridine orange, cortisone, and chloroform, in which the drug did not seem to act directly on the dye, but seemed to act by modifying the relations of a third substance to water. Naturally, this does not preclude that the excited molecules themselves should be also directly influenced by a narcotic, if they have an affinity for it. Rhodamin B has such an affinity, as shown by the fact that it can be shaken out from water with chloro-

form as a colorless anhydride. If one-tenth part of a saturated watery solution is added to a watery rhodamin solution then, on subsequent freezing and illumination the rhodamin will not go into the triplet and show its usual brilliant orange fluorescence. That this change is not due to a simple direct disturbance of the water by the chloroform is shown by the fact that a saturation with ether has no such effect although ether is considerably more soluble in water than chloroform. Ether has no affinity for rhodamin but will readily abolish its long-life phosphorescence if $\frac{1}{50}$ part of a saturated watery solution is added to the acridine orange—cortisone system.

ASCORBIC ACID

We know that ascorbic acid is indispensable for life but do not know why, what is the role it fulfills. If allowed to autooxidize it turns into a yellow dye, probably dehydrogulonic acid (see Schiffman, McLaughlin, and the author). Bivalent metals form deeply colored complexes with this oxide.

As has been shown before, as far as experience goes, all fluorescent substances in frozen water go into the triplet state. This seems to hold also for this oxidation product of ascorbic acid. If a methanol solution of ascorbic acid is neutralized with BaOH and stored, then in the presence of air a yellow precipitate is formed which is the Ba salt of the oxide. If this is dissolved in water it shows no fluorescence in the visible. Frozen, it shows a strong blue phosphorescence with a long lifetime. The oxide of ascorbic acid is able to attach itself firmly to proteins without losing its optical reactivity. It seems not impossible that it forms such complexes with protoplasmic proteins also *in vivo,* serving as prosthetic group for the proteins. This assumption finds support in the fact that the two organs which are the richest in ascorbic acid, the adrenal medulla and lymph glands, both contain yellow pigments. When the author worked two decades ago along this line, he had the impression that these pigments could not be separated from

the structural proteins and were not all carotenes. Possibly, the disappearance of ascorbic acid from the adrenal cortex in increased functional activity is due to the formation of such protein complexes. In any case a closer study of these relations seems to be indicated.

LONG-LIFE EXCITATION IN PROTEINS

Frozen animal tissues, in the absence of oxygen, show after illumination with the UV lamp a strong and long-lasting afterglow. Pigments interfere with the observation of this light emission which is thus observable only in organs which are poor in colored matter. If, for instance, the brain of a guinea pig is placed in a test tube, the air is replaced by N_2 and the tube is immersed in the dry ice freezing mixture, if held (after temperature equilibrium is attained) before a high pressure Hg lamp for a second it is found to emit an intense bluish-white light which lasts for half a minute or so, and is followed by a weak one lasting for some time more. In the presence of air, that is O_2, no such phosphorescence is obtained, nor does the tissue show any such emission at room temperature in N_2. Other organs poor in pigment, such as muscle, uterus, intestine, lung, skin, and mammary cancer (mice) show the same phenomenon. The emission is not visible in organs which are rich in pigment, as are kidney, liver, or dark muscle (heart). This long-lived phosphorescence is not a specific property of tissues. A 1% serum albumin solution (serum albumin cryst., Armour), as well as an 1% ovalbumin or gelatin show it too, though in the latter the emission is of shorter duration.

This long-lived phosphorescence was, to my knowledge, not described before, having been overlooked, probably, owing to the quenching action of the atmospheric oxygen. Debye and Edwards described a long-lived phosphorescence in protein solutions, but note that this phosphorescence, which they observed at the temperature of liquid N_2, is very sensitive to the elevation of tempera-

ture and does not occur at the temperature of dry ice (which is about 100°C higher than that of N_2). Debye and Edwards ascribe the phosphorescence to the ejection of an electron which is temporarily caught in a "hole," the light emission being due to the electron's return to its original ground state.

PERTURBATIONS

A watery rhodamin solution has a reddish color as compared with the orange color of a methanol solution. The spectroscope shows that in water both the absorption and emission spectrum are shifted by 50 mμ towards the longer wavelength. Evidently, this shift is connected with the higher dielectric constant of water and the difference between the two solvents would be still bigger if, instead of methanol more nonpolar solvents could be used. This, unfortunately, cannot be done because in such solvents rhodamin forms a colorless internal lactone and the lactone formation makes resonance impossible and so eliminates the color (Lundgren and Ninkley).

What lends interest to this observation is that a similar shift towards the longer wavelength could be observed if to the methanolic solution of the dye were added small amounts of various substances known to have a strong biological activity. One can expect energy levels in biological systems to be tuned rather carefully and so a similar shift in energy levels *in vivo* may disturb the good working order.

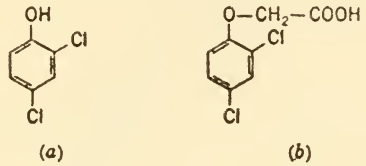
A number of various substances have been tested by McLaughlin (unpublished) for their ability to shift the spectrum of a methanolic rhodamin solution towards the longer wavelength. The substances have been applied in three different concentrations, 0.01, 0.001, and 0.0001 *M*. The results are summed up in Table I where the numbers mark the shift of the absorption spectrum towards the longer wavelength in A. Since acids (1–4) cause a strong shift, all acid substances have been used as neutral sodium salts.

TABLE I

SHIFT OF THE SPECTRUM OF RHODAMIN B (DISSOLVED IN METHANOL) IN A UNITS TOWARDS THE LONGER WAVELENGTH ON ADDITION OF VARIOUS SUBSTANCES AT 0.01, 0.001, AND 0.0001 *M* CONCENTRATION

	Substance	Molar concentration 0.01	0.001	0.0001
1	Acetic acid	72	68	52
2	Sodium acetate	0	0	0
3	Salicylic acid	72	64	4
4	Sodium salicylate	16	0	0
5	Sodium picrate	56	56	8
6	Phenol	4	0	0
7	1 Naphthol	8	0	0
8	*p*-Chlorophenol	0	0	0
9	*o*-Iodophenol	0	0	0
10	*o*-,*m*-, and *p*-Nitrophenol	0	0	0
11	*p*-Nitroaniline	0	0	0
12	*m*- and *p*-Dinitrobenzene	0	0	0
13	*p*-Diiodobenzene	0	0	0
14	2-Amino-4-nitrophenol	0	0	0
15	2,4-Dinitroaniline	0	0	0
16	2,4-Dinitroanisole	0	0	0
17	2,4-Dinitrophenetole	0	0	0
18	2,4-Dinitrophenol	80	80	32
19	4,6-Dinitro-*o*-cresol	68	60	12
20	3,5-Dinitrosalicylic acid sodium salt	4	0	0
21	2,4-Dinitro-1-naphthol	80	72	40
22	2,4-Dinitro-1-naphthol-7-sulfonic acid sodium salt	76	65	15
23	2,4-Dichlorophenol	0	0	0
24	2,4-Dichlorophenoxyacetic acid Na	48	36	8
25	2,4,5-Trichlorophenol	0	0	0
26	2,4,6-Trichlorophenol	20	0	0
27	Pentachlorophenol	60	28	4
28	2,4-Dichloro-1-naphthol	0	0	0
29	DDT	4	0	0
30	3,5-Diiodo-2-hydroxybenzoic acid Na	68	64	8
31	3,5-Diiodo-4-hydroxybenzoic acid Na	48	32	8
32	2,4,6-Triiodophenol	28	8	0
33	Dicumarol	—	—	26
34	Potassium Iodide	0	0	0

The table shows most substances to be inactive, but also shows some of them to be highly active and to cause a maximal shift in 0.001 *M* concentration and still be active in 0.0001 *M*. Among the most active ones we find dinitrophenol and dinitronaphthol, both of which uncouple oxidative phosphorylation. That the two actions might actually have some relation to one another is suggested by the activity of dicumarol (33) and pentachlorophenol (27), both of which are also known to uncouple. Owing to its



(*a*) (*b*)

FIG. 10. a: 2,4-Dichlorophenol. b: 2,4-Dichlorophenoxyacetic acid.

insolubility, dicumarol could not be tested in a higher concentration than 0.0001 *M*. The abolition or substitution of the phenolic hydroxy group, which is essential for the uncoupling activity, also deprived the molecule of its action on rhodamin.

A point worth mentioning is the great difference in the activity of two chemically closely related substances, 2,4-dichlorophenol and 2,4-dichlorophenoxyacetic acid (Fig. 10). Both substances are highly active biologically, but their activity is very different, so it is intriguing to find them different also in this simple *in vitro* experiment (see Chapters 12 and 16). In the present experiment with rhodamin, a tentative explanation for the differences in the activity of the dichlorophenol and 2-4 dichlorophenoxyacetic acid (2,4D) can be found in steric relations. If the rhodamin molecule, as well as the two chlorophenols and the nitrophenols are built of an atomic model, it is found that the active nitrophenol and nitronaphthol can easily be brought into a position in which the phenolic hydroxide and one NO_2 group touch the two resonating N's of the dye. The same holds for the carboxylic O^- and one Cl (4)

of 2,4D, while the 2,4-dichlorophenol cannot be made to touch the two N's of rhodamin with its OH and one of its Cl's.

The activity of the iodo-substituted phenols (30, 31) should also be noted. Here, evidently, the activity is connected with the high atomic number of the iodine and not the dipole moment of the molecule, since the corresponding chloro compounds, though having a stronger dipole moment, are less active. A heavier chloro substitution, as in pentachlorophenol (27), also renders the molecule active, which deserves mention with regard to the insecticide action of substances of this group.

RHODAMIN COMPLEXES

During these experiments, a fascinating reaction of rhodamin with nitro- and chlorophenols was observed. If a chloro- or nitrophenol is added to an acid watery solution of rhodamin, a striking change takes place; the fluorescence which, according to its maximum at 578 $m\mu$ was orange, disappears and the solution assumes the appearance of strong turbidity, without changing color. Looking, however, at the light source through the solution, the latter appears to be limpid and deep blue. The change is due to the formation of an insoluble complex in colloidal dispersion which settles slowly to the bottom of the test tube in a day or so. R. Steele (unpublished) studied this compound—evidently a "molecular complex." He found that an undissociated phenolic group and the presence of water are necessary for its formation. On rendering the solution alkaline or dissolving the complex in anhydrous solvents, such as methanol or acetone, he found that it readily dissociated. There is one quality which may lend biological interest to this reaction—its sluggishness. The formation of the complex, being accompanied by a decrease and a shift of the absorption maximum, can be followed in the spectroscope. Figure 11 (borrowed from Steele) illustrates this point. The first eight curves went through an isobestic point, showing that it was actually the rate of the formation of the complex and not its sedimentation which caused the gradual change, the reaction having taken hours

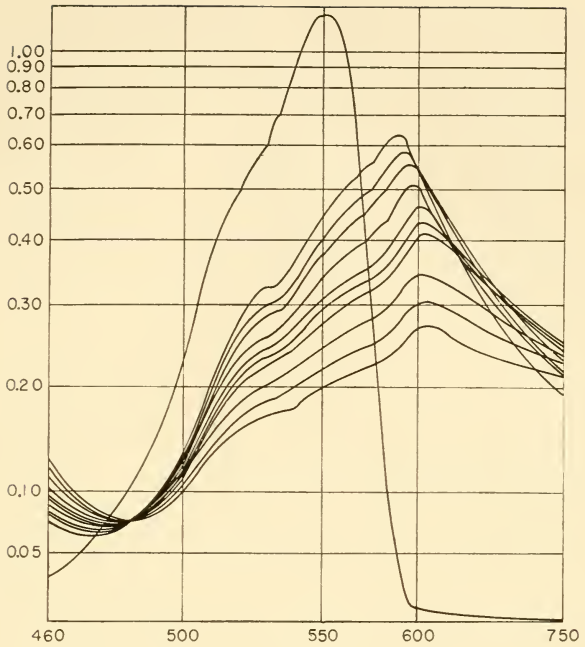


FIG. 11. Reaction between rhodamin *B* (1.8×10^{-5} *M*) and 2,4-dichlorophenol (0.011 *M*) at pH 5.9 as followed in the spectroscope. Abscissa: wavelength in mμ. Ordinate: extinction. The single high curve in the middle is rhodamin alone. (Dichlorophenol alone has no absorbency at these wavelengths.) The family of curves has been obtained by measuring the absorption at a different time after mixing the two reagents. The top curve on the right was taken 7 minutes after mixing. The following lower ones 5, 10, 29, 59, 89, 120, 252, and 312 minutes later. The first eight went through the isobestic point on the left. Only the two last curves were slightly deviating from this point (not visible in the curve), indicating that at this point sedimentation began to influence results (but not before).

for its completion. What may make this sluggishness interesting for the biologist is the fact that thyroxine or triiodothyronine, which forms a similar complex with rhodamin, showed a similar sluggishness. The biological action of thyroxine is known to need 24–48 hours for its full development, and may reach its maximum on the tenth day after ingestion.

PART II

Biological Structures and Functions

"Research is to see what everybody has seen and think what nobody has thought."

9. A Theory of Muscular Contraction

In the present part of this book I propose to review a selected number of biological structures and functions in order to see whether we could understand them better or, at least, see them in a new light by looking at them through the glasses of E^*. To remain faithful to my traditions I will start with muscle.

Theories of muscular contraction are available on the market by the dozen. The author himself is responsible for a few of them. Most of these theories were fatally hurt in the impact between physics, chemistry, physiology, and electron miscroscopy, fitting only the requirements of the science of their author but being incompatible with that of others. The situation with muscle is at present, similar to that of the holy elephant which had ninety-nine names, the real one being the hundredth, known only to the elephant himself. If we fail to understand muscle because its function involves E^* then our experience with E^* should fuse spontaneously with older knowledge into a new theory of contraction. An important place has to be given in this theory to the fact that the formation of the water structures, which the nonpolar groups of a protein can be supposed to build around themselves, goes with a strong expansion, the formed ice having a cubic lattice and a very low density. Also the electropolar groups of myosin and the substances, like ATP, bound to myosin, may take their share in building up water structures.

As far as we know, the elementary act of contraction is performed by the myosin "molecule" which is built of a greater number of small units, "protomyosins" which are held together not by single strong covalent bonds but by a greater number of weaker ones such as H-bonds, or van der Waals or electric attractions.

Unlike covalent bonds, such links have no fixed valency angles. This makes the whole structure comparable to a string of beads, or a bundle of such strings, the most characteristic feature of which is pliability, the lack of resistance to deformation. The forces between protomyosins can be expected not only to hold these particles together, but also to tend to pull them together from the fibrous form into a more compact, rounded, and shortened shape (Fig. 2). If, all the same, we find the myosin particles in solution or in resting muscle stretched out to a straight filament there must be forces which counteract the mutual attractions which tend to shorten the particle. The problem of contraction is thus not what makes the particles shorten but what keeps them extended when at rest, or stretches them out again after contraction?

It is suggested that what keeps the myosin particle extended, counteracting the forces of contraction, are the expanding water structures which the particles build around themselves, while contraction is due to the collapse of these water structures with the consecutive rearrangement of protomyosins into a shortened aggregate. The latter changes would have to be brought about by the energy of ATP. In order to effect such a change the (E) of ATP would have to be transformed into the more mobile E^* which can interact with the water structures.

The theory proposed is, in its simplest form, the following: the myosin particle is kept stretched out by its water structures. Contraction is induced by the collapse of these structures, the reestablishment of which is relaxation.[1] The question is how far is this theory in agreement with known facts about muscle or to what extent can it explain them?

Myosin, in a watery solution, has a high viscosity. This indicates

[1] The fibrous molecules of deoxyribose nucleic acid, dissolved in water, are known to shorten by 30% or so on addition of salts. Jacobson found under these conditions (using NaCl) that the water structure collapsed. Possibly, the collapse of these structures is responsible for the shortening, making the process analogous to muscle contraction, as supposed by the theory presented here.

that its particles are in the extended fibrous form. No ATP is thus needed to keep them in this form. The intimate relations of actomyosin to water are shown by the strong hydration of this complex. In fact, it is difficult to prepare solutions of actomyosin which contain less than 97% water. The high viscosity shows the particles to be in the extended fibrous form. What ATP does to this colloid at a physiological salt concentration is to deprive its particles of their hydration and make them contract. The contracted gel consists of "dry" actomyosin with some water trapped between its particles.

Heat can be expected to destroy the water structures and heat not only denatures actomyosin but makes it contract and develop not inconsiderable tension, as shown by Varga. Possibly, alcohol denatures also by destroying the water structure but produces no contraction because, eliminating the water it makes the system too rigid. The denaturing action of freezing and subsequent thawing on myosin might similarly be due to the loss of hydrate water, the latter being transformed into the hexagonal ice crystals.

Ernst has discovered that muscle decreases its volume in excitation. This "volume contraction" follows on the heels of excitation and precedes contraction. Contraction is thus secondary to a change which entails volume contraction. This volume contraction has never found a satisfactory explanation which could enjoy general acceptance. Our theory demands that there should be such a volume contraction since the collapse of the expanded water structures around the myosin particle should induce such a volume change. Both the cubic and hexagonal water structures have a lower density than random water.

If it is the expanded water structures which keep the myosin particle in its extended state then it follows that an increase of pressure should promote contraction, and contraction should go hand in hand with a decrease of the molar volume of myosin. That this is actually the case has been shown by D. S. Brown who also calculated the decrease of the molar volume of myosin and found

it considerable. The contraction of muscle exposed to high pressures has been known for a long time as "Ebbecke's phenomenon."

Bulky ions, such as I^-, SCN^-, and NO_3^-, which do not fit into the water lattices, should impede the formation of these lattices and thus counteract relaxation, lengthening the "active state" of muscle, and thus increase twitch tension. That this is the case has been shown by Chao and the later analyses of his observations by Kahn and Sandow, Hill and Macpherson, and Ritchie. The action of these ions was found to depend on their position in the "Hofmeister series," that is on their atomic radius. Since these ions are "quenchers," they may have acted also by interfering with E^*.

Needless to say that the function of muscle is not merely to contract, but to contract when contraction is needed, and do so at milliseconds notice, and relax immediately afterward when the job is done. Accordingly, we find in muscle at the side of myosin a triggering mechanism in which actin plays a prominent role. According to the experience of my laboratory, resting muscle contains no actomyosin, but contains actin and myosin side by side, kept apart by the subtle balance of attractive and repulsive forces with a slight predominance of repulsion. These repulsive forces are electric and the ATP (linked to myosin), plays, with its four negative charges, a leading role. This balance of forces is destroyed, for an instant by "excitation," whereupon actin and myosin form actomyosin. In the actomyosin thus formed the terminal $\sim$P of ATP becomes split and its energy put to action. Relaxation involves the rephosphorylation of the ADP into ATP which, with its four charges restored, pushes actin and myosin apart whereupon the free myosin particles rebuild their water structures and stretch out into filaments again, thus becoming ready for a new contraction.

The theory outlined also finds support in the fact that no folding was hitherto revealed in contracting actomyosin filaments by the electron microscope. All one can see (as shown beautifully in

the pictures taken by Spiro) is a thickening. At higher degrees of contraction the X-ray periodicities disappear while regular folding should give rise to new lines.

The theory proposed may be right or wrong. In any case it shows that new pictures can be constructed with the experience presented in this book.

10. The ATP Molecule[1]

Looking at the conventional structure formula of ATP (Fig. 12), one's first impression is that of great complexity. Nature does not indulge in luxuries, so one may wonder why the cell uses such a complex molecule if a P—O—P link is all that is needed. A much simpler inorganic polyphosphate should do just as well.

The molecule has two ends: a phosphate-end and a purine-end. The phosphate-end represents the energy store, (E); one may ask whether the purine-end may not represent E^*, thus providing the molecule with the essential parts needed for the $(E) \rightarrow E^*$ transformation. The purine contains an extensive system of conjugated double bonds with its nonlocalized π electrons and five N-atoms, each with its lone pair of electrons. As will be discussed later, under conditions, this end of the molecule may also become strongly fluorescent and thus conform to our demands of an E^* transmitter. The purine may thus be instrumental in transforming the (E) of $\sim$P into E^*, when this (E) has to go into biological action and drive the living machine. The whole ATP molecule could thus be not only a storage battery but also a transformer.

What is difficult to see in Fig. 12 is how the energy accepted by the purine-end could be transmitted to the phosphate-end, and *vice versa,* since the two are separated by the pentose, which has no conjugated double bonds and no π electrons.

But does the ATP molecule really have this structure? Are we not misled by the visual impression made by a structural formula,

[1] The contents of this chapter were presented at the International Enzyme Symposium, Ford Foundation, Detroit, November 1–3, 1955, and published in "Enzymes: Units of Biological Structure and Function," Oliver H. Gaebler, Ed., Academic Press, New York, N.Y.

the correctness of which we fail to question only because we have seen it too often?[2] The ATP molecule has one C—N, one C—C, and one C—O link (marked in Fig. 12 with arrows), which allow

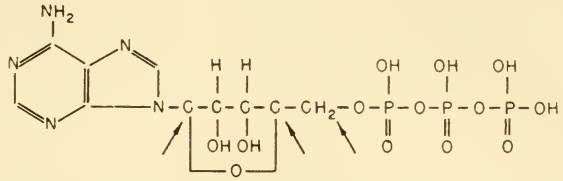
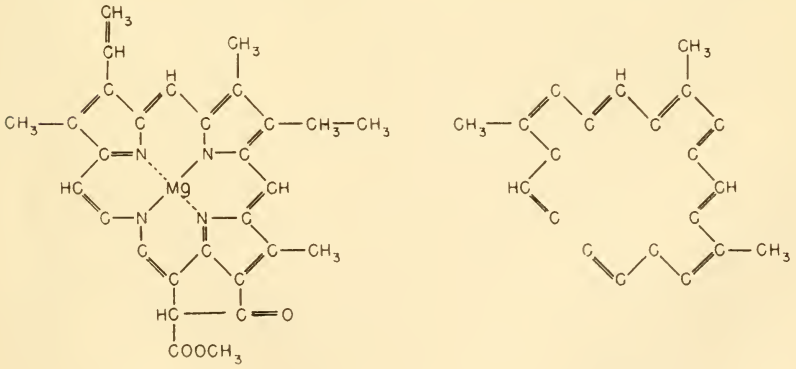


FIG. 12. ATP.

a free rotation so that the molecule has a limited freedom to change its shape and curl up. In fact, the outstretched linear form of the molecule in Fig. 12 is an improbable one and the possibility

[2] Possibly, chlorophyll also offers an example for such an influence of visual pictures which inhibit our thinking in certain directions. Relations between chlorophyll and carotenes have been sought for a long time without definite results.

The Mg-porphyrin part of chlorophyll is reproduced in Fig. 13a, while the chain of beta carotene is shown in Fig. 14a. The two structures seem to



a b

FIG. 13. a: Mg-porphyrin part of chlorophyll without the phytol side chain. b: Same as "a" with Mg, N's, and longer side chains eliminated.

is open that by rotation and the consecutive folding the phosphate- and the purine-end of the molecule may approach one another. Naturally, for an energy transfer to take place between the two ends, an "approach" is not enough, since the E of the $\sim$P which has to be transferred is an (E), that is, a bond energy which has no outward action. For the transference of (E) there must be a close fit, point counter point. The atoms which have to touch one

show no relation, the first consisting of pyrrols, coordinated by a metal, while the latter is built of isoprenes. However, if we eliminate the Mg with the four N's, as well as the longer side chains from the porphyrin, we are left with a chain (Fig. 13b) which is almost identical with the chain of carotene, curled up (Fig. 14b).

If these relations were hitherto not considered, this is probably due to the fact that we have seen the structure formula of pyrrol too often.

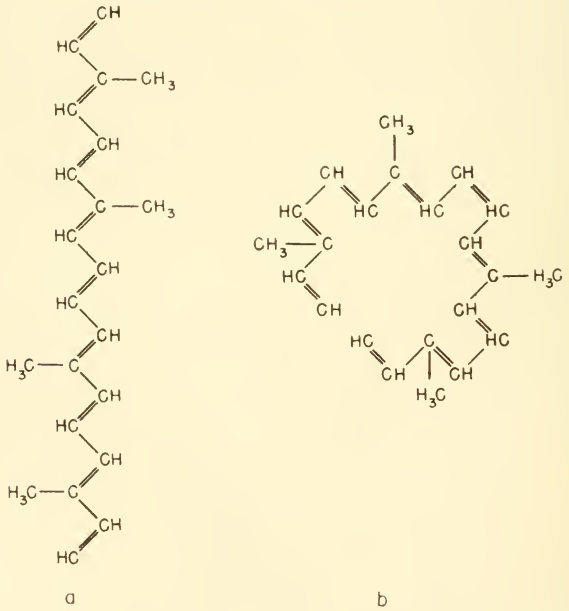


FIG. 14. a: Central chain of carotene without the ionone rings, b: Same curled up.

another are two O^-'s of the dissociated OH groups of the phosphate chain, and two N's of the purine. The two O^-'s are those lying on either side of the terminal $\sim$P, which has to be split and give up its (E), while the two N's of the purine would be, in all probability, the N of the NH_2 group at position 6 (since this N seems to be most involved in the reaction occurring in muscle contraction), and its second neighbor at position 7.

However, free rotation does not mean free motion. Such a C—C bond does not permit the molecule to bend in any way it pleases, for the valency angles have to be kept constant. The motion is not freer than that of the relative motion of two wheels mounted on the same axis. So even with three rotating links the freedom of the ATP molecule is a very limited one and, statistically, the chances that the NH_2 and N_7 would be able to meet the two O^-'s and make a close fit, are very remote. So if the four could meet and make a close fit, it would be probable that this is not mere chance, but has a functional meaning and that the ATP molecule is *made that way and does not happen to be that way.* Whether such a meeting is possible can be decided by building up the molecule of an atomic model which keeps account of atomic radii and limitations of freedom, the rigidity and small flexibility of bond angles. Such a model is the Courtauld atomic model.[3] Figure 15 shows an ATP molecule built up of this model, in its conventional linear form corresponding to Fig. 12. (The molecule is here in its dissociated form, with O^-'s instead of OH's on the phosphates.)

If the molecule is now rotated around the C—C, C—N, and C—O bonds, then the phosphate-end can be folded back so that the O's of the terminal and middle phosphate just touch the N's mentioned. This situation is shown in Fig. 16. If some sort of a link is formed now between the O^-'s and N's, then the P—O—P link, which has to be broken in contraction and supply the energy

[3] Produced by Griffin and Tatlock, London, available in U.S. through the Anglo-American Scientific Import-Export Co., 185 Devonshire Street, Boston 10, Mass.

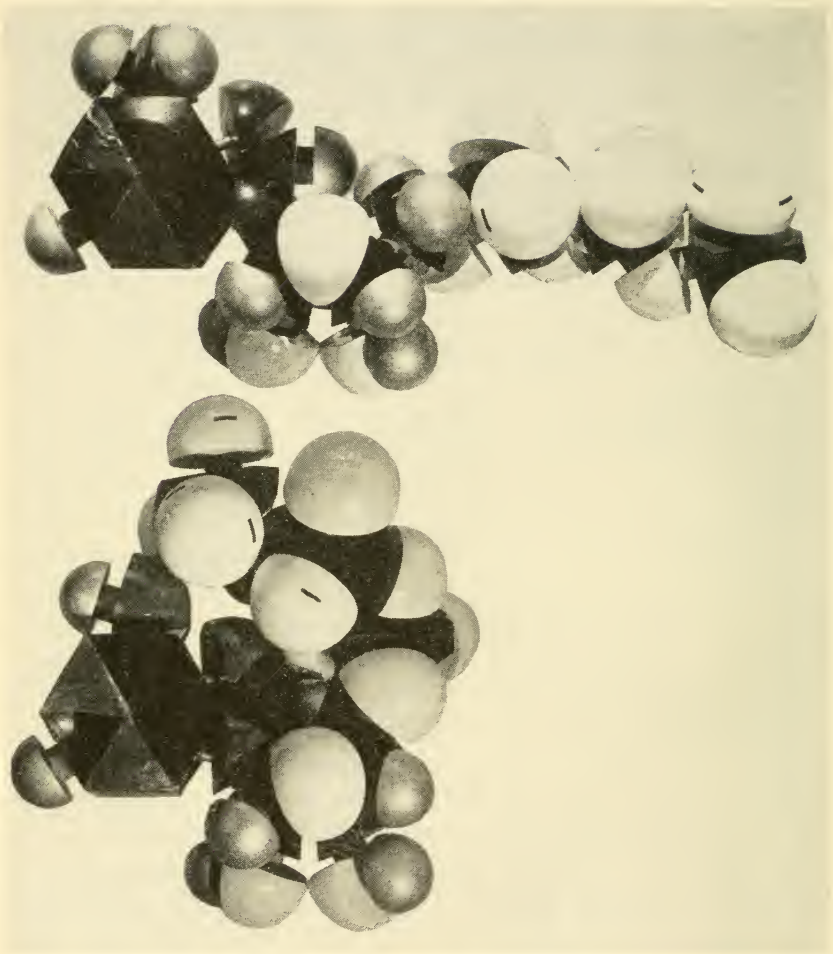


FIG. 15 (*above*). ATP of Fig. 12 built of the Courtauld atomic model.
FIG. 16 (*below*). Same as Fig. 13, folded.

for it, has formed a ring with the purine. Naturally, for energy to pass from the phosphate to the purine, the contact would have to be an intimate one. H-bridges, possibly, formed between the O's and N's might do, having been shown by Gergely and Evans that H-bonding can establish relations in which π orbitals overlap. It is thus possible that the ATP molecule, activated by myosin, connects its two ends, thus opening the way for an energy transmission from one to the other.

This, however, does not explain the role of the bivalent ions, Ca and Mg, both of which can accelerate the ATP-ase activity of myosin. The possible answer to this problem was given by a chance observation. As discussed in Chapter 4, we may expect energy transmitters to be fluorescent. Since there are violent shifts in energy during contraction, the author expected to find in muscle a fluorescent energy transmitter in high concentration, and prepared alcoholic extracts of muscle, expecting them to show strong fluorescence under the UV lamp. They showed none. However, if a bivalent metal, as Mg, Ca, or Zn, was added (as chloride), an intense blue fluorescence appeared. The fluorescent substance was isolated, identified by McLaughlin, Schiffman, and the author and found to be the metal complex of inosinediphosphate, IDP, the substance produced from ATP by the loss of its terminal phosphate and its hydrolytic deamination.

The probable structure of the metal complex is elucidated by the close analogy of inosine and oxyquinoline (Fig. 17a and b). The latter is known to form with Mg in alcoholic solution a very stable, strongly fluorescent chelate (Fig. 17b). Evidently, an analogous chelate was formed by the inosine (Fig. 17a).

Mg and Ca are known to form very stable, coordinative complexes with polyphosphates, so the possibility is given that the metals form with their four coordinative valencies a quadridentate chelate, connecting the two ends of the ATP molecule. This structure is illustrated by Fig. 18. The model in Fig. 16, shows that there is just enough room left for an Mg between the two N's and

two O's. The arrow in Fig. 17b indicates that the metal attracts an electron from the quinolinol (Leverenz). The same can be expected to happen also in the complex of inosine, and if the metal

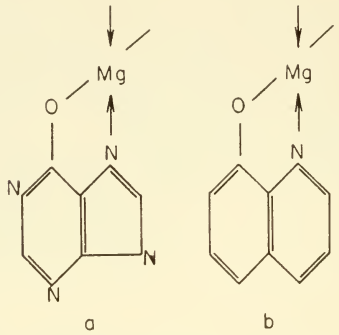


Fig. 17. a: Mg complex of oxypurine. b: Mg complex of oxyquinolinol.

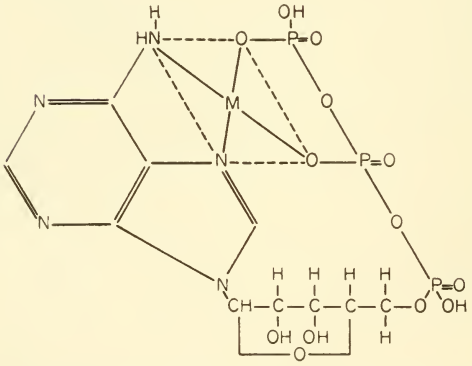


Fig. 18. Possible structure of Mg complex of ATP (interatomic distances are arbitrary).

forms a quadridentate chelate linking up also with the phosphates, it may attract electrons also from the latter. The metal may thus serve as a bridge, across which electrons can pass from phosphate to purine. So the Mg actually not only may connect the two ends of a molecule; it may make one single, unique electronic system of the phosphate chain and the purine with common nonlocalized

electrons which could transport energy, the purine having its system of conjugated double bonds and the phosphate the O's with their nonbonded lone pairs of electrons. The P—O—P, which represents the (E), could merge thus with the adenine in one extensive system of mobile electrons.

This opens the possibility that when such a double chelate is formed and the metal attracts electrons from the phosphates, it

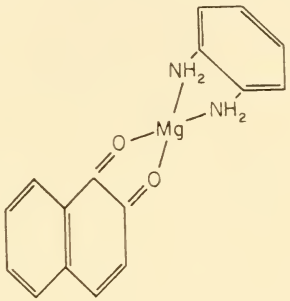


FIG. 19. Mg chelate of *o*-phenylenediamine and naphthoquinone.

decreases the energy and strength of the P—O—P bond, which then falls prey to hydrolytic splitting while its energy appears in the purine ring as E^*, completing the $(E) \rightarrow E^*$ transformation.

That Mg can actually facilitate the passage of electrons from one substance to another with which this metal forms complexes can be demonstrated by mixing an alcoholic solution of 1,2-naphthoquinone and *o*-phenylenediamine (Fig. 19). In this system the quinone oxidizes the diamine very slowly, electrons passing from the latter to the former. This reaction is greatly speeded up by Mg, in analogy to the ATP-ase activity of myosin, which also can occur without Mg, but is greatly accelerated by the metal. The reaction between quinone and diamine is indicated by the darkening of the solution (which can readily be reverted by reducing agents such as ascorbic acid).

One attractive feature of this theory of ATP-ase activity is that it is analogous to E. L. Smith's theory of peptidase activity.

A serious objection may be raised against this theory: the stability constant of the metal chelate of Mg and the adenine or isonine, in water, is very low; the energy of this binding is not great enough to hold the whole quadridentate chelate together against the forces of heat agitation. The Mg-phosphate complex is very stable so that we can expect the ATP to be present in muscle as a Mg complex, there being twice as much Mg in muscle as there is ATP, but links formed by Mg with the adenine or purine can be expected to break up in water. Our model in Fig. 16 only says that such a complex *can* be formed, not that it *is* formed. However, we have to remember that the ATP, when undergoing splitting in muscle, is not free but is linked to the protein, myosin, and is "activated" by it. We do not know what "activation" means. Probably it means a binding of the substrate with its consecutive deformation. So it may be that the myosin holds the ATP molecule in the position required for the formation of this bridge between phosphate and purine and this is actually what we mean by activation. One could go even one step further with the argument and say that if the stability constant of the Mg-purine complex were high enough to bring the two ends of the molecule together and make the energy transmission and the splitting of the ~P occur, then this reaction would be of no use to the muscle, because then the ~P would be split and its energy dissipated senselessly. It is one of the basic principles of nature not to use spontaneous reactions which occur by themselves and cannot be kept in hand. If the energetics of the cell consisted of spontaneous reactions, the whole mechanism would have to run down senselessly as a watch does if released from its regulators. We have to demand from any theory of the ATP-ase activity that it should make the splitting of ~P possible only in ATP molecules, bound and activated by the myosin, when the energy of the ~P can be transmitted to the protein and applied usefully. It is thus just the low stability constant of the hypothetic Mg-purine complex which makes the presented theory acceptable.

A word may be said about the third member of the ATP mole-

cule, the pentose which has to connect the adenine and the phosphates in such a way that they come together exactly in the right position. A hexose would not do. But this may not be all there is to it. Nature often kills more than one bird with the same stone. It may be worth noting in this connection that the United States Patent Office has granted two patents for the hardening of gelatine by pentose, a reaction not shared by hexose.[4] This suggests that the ribose can enter into an intimate reaction with the protein which may very well play an important role in the "activation" of ATP.

One last remark may be made about Ca. It greatly promotes the "ATP-ase activity" without promoting contraction or analogous reactions, such as "superprecipitation." It even inhibits them. One tentative explanation which may be given for this behavior could be that Ca, similarly to Mg, forms chelates and weakens the ~P but is unable to transmit its energy to the purine, owing to the great differences in the energy terms of Ca and N. Accordingly, Ca is also unable to promote the oxidoreduction between 1,2-naphthoquinone and *o*-phenylenediamine.

In Chapter 12 we will return once more to a possible additional role of pentose.

While reading the proof sheets of this book my attention was kindly called to a paper of B. H. Levendahl and T. W. James (*Biochim. et Biophys. Acta* **21**, 298, 1956). Its summary may be quoted without comment: "The rotatory dispersion of adenine, adenosine, AMP, ADP, and ATP have been determined. The data have been interpreted to show that the ATP molecule is folded back on itself in such a manner that bonding is permitted between the last two phosphate groups and the amino group of adenine. This stabilized structure is then proposed as necessary for the action of ATP. Parallel 'ATP-like' action of CTP and UTP and other triphosphonucleotides could be explained by possession of a similar configuration."

[4] USA Patent No. 2,059,817 (Nov. 3, 1956) and Patent No. 2,180,335 (Nov. 21, 1939), the first granted to the Eastman Kodak Co., Jersey City. N.J., the second to the Agfa Ansco Corporation, Binghamton, N.Y.

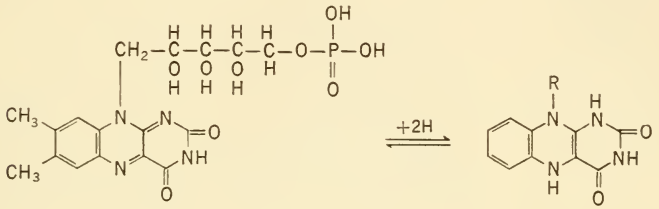
11. Riboflavine

As is generally known, the function of riboflavine-5′-phosphate (which I will briefly call "riboflavine") is to act as intermediary H-acceptor in the oxidative system, accepting H's coming from the foodstuffs, and passing on electrons to the cytochrome system. This function is in accord with its chemistry. Accepting H's in pairs and giving off electrons one by one, it must be able to form a free radical. The ability to do so can readily be demonstrated by reducing it in a strongly acid medium, whereby the greenish-yellow color of the oxidized form turns into the red-brown color of the free radical.

Naturally, the real function of the substance is not merely to serve as a bridge for the H's or electrons to pass over, but to convert the energy, released in its oxido-reduction into ~P's, and our problem here is to find out whether E^* is involved in this process. As has been stated earlier, fluorescence tells us that the molecule does not readily dissipate its E^*, may thus act as an energy transmitter. All fluorescent molecules were also found to be able to go into the triplet state in ice. The well known brilliant greenish-yellow fluorescence of riboflavine may thus be taken as an encouragement.

The structure of riboflavine (Fig. 20) has close analogies with that of ATP. We found the structure of ATP more complex than was needed for its alleged function, that of a phosphate carrier. Similarly, the structure of riboflavine is more complex than would be needed for an intermediary H-acceptor. The molecule of ATP is built of three parts: it has phosphate on one end, a heteroxyclic compound with an extensive system of conjugated double bonds on the other, with a pentose connecting the two. Riboflavine has

an analogous structure with phosphate at one end, an isoalloxazine instead of the purine at the other, and a ribose instead of a ribofuranose in the middle. Only the function of riboflavine is, in a way, the opposite of that of ATP. While the function of ATP is to spend the E of $\sim$P's wisely, the function of riboflavine is to invest E in P's, taking part in oxidative phosphorylation and stabilizing the energy released in this form. ATP accepts phosphates from other phosphate acceptors in the form of a triphosphate



(a) (b)

FIG. 20. a: Riboflavine-5,-phosphate, oxidized. b: Same reduced.

chain, then breaking down this chain, converting (as we supposed) its (E) into an E^* in the purine. The tentative hypothesis thus suggests itself that riboflavine might do the same in the opposite direction and fulfill its role by converting the E^* generated by its oxido-reduction in its alloxazine into $\sim$P's by building up a triphosphate chain on its other end, the phosphates of which it then passes on to other phosphate acceptors, keeping but one for itself.

As to the mechanism of these reactions we supposed that ATP connected its two ends (on the enzyme) by forming a coordinative metal complex between the active groups of its purine and the triphosphate end. So, when studying ATP, our first question was whether the formation of such a complex is sterically possible. If there is the supposed analogy between ATP and riboflavine, then we might ask likewise whether the opposite process is sterically possible, whether the alloxazine could bind two phosphates as metal complexes, then hitch them together and bind them to its

own phosphate by means of the E^* generated by the oxidoreduction in its alloxazine. To do this the alloxazine would have to have three active groups in a vicinal position. It has three such groups, a C=O at position 2, an NH at 3, and another, C=O at 4 (Fig. 20) and so the question is whether it is sterically possible to bring the hypothetic triphosphate formed on the furanose into a position in which one O^- of each of the three phosphates touches upon one of the three active groups of the alloxazine.

The answer is given by Fig. 21, an alloxazine-triphosphate built up of the Courtauld atomic model. The three atoms pointed at by the single-headed arrows are O^-'s of the three phosphates while the three double-headed arrows indicate the =O, NH, and =O on the alloxazine. These are in close touch. If, in ATP, the statistical chances for the possibility of the formation of a complex were remote, they are still more distant here and so we again can say that the structure pleads for the assumption that the riboflavine does not *happen* to be able to form such a complex but is *made* that way. We can also add that any change on the riboflavine, as the replacement of ribose with ribofuranose or a different location of the O's and NH on the isoalloxazine, would make the formation of such a complex impossible. So the structure of the riboflavine molecule becomes accessible to a functional interpretation. The CH_3 groups at 6 and 7 may act as electron donors, resonating with the CO's and NH. For this function we could expect them to be located on the opposite end of the molecule, where they actually are.

Once the formation of a triphosphate is structurally possible, the question arises whether this is possible energetically. The spectral properties of riboflavine give us a lead. The riboflavine has three absorption bands, one at 260, one at 375, and one at 445 $m\mu$. So if the molecule is excited by UV its electrons are raised to one of the UV absorption bands and then drop in an "internal conversion" to the lowest singlet level and emit from here the well known greenish-yellow fluorescence with a maximum of its emission band at 540 $m\mu$ ($S_1 \rightarrow G$ in Fig. 5). To excite electrons to

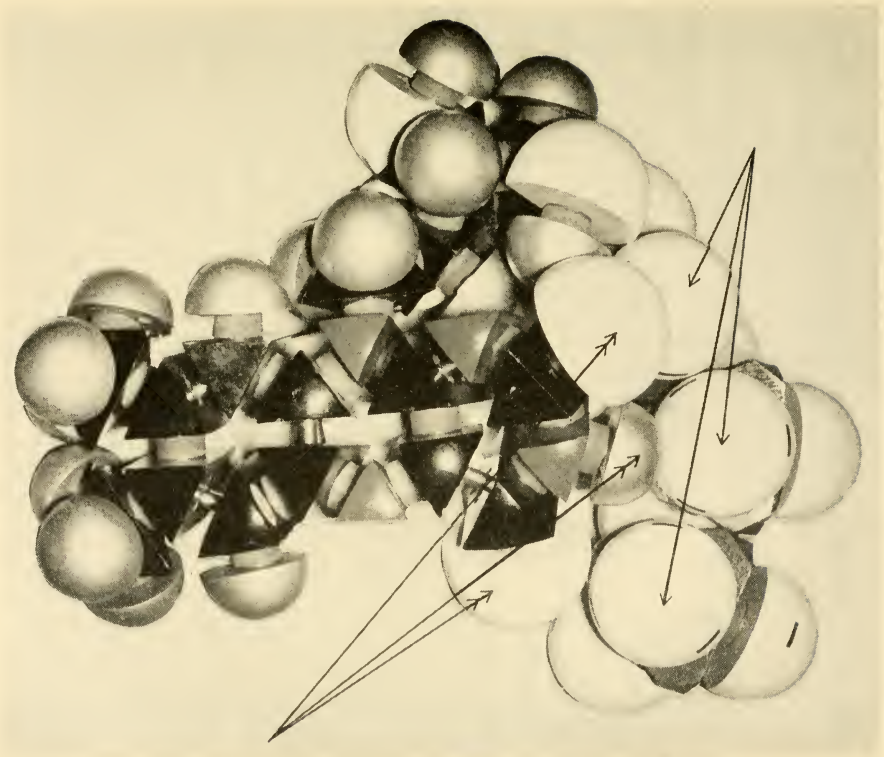


FIG. 21. Hypothetic triphosphate of riboflavine, built of the Courtauld atomic model, folded. For explanation see text.

this 540 mμ level 64 calories would be needed, which are not likely to be available. As has been shown (Fig. 6), in water the excited riboflavine goes into the triplet, the emission of which has its maximum at 605 mμ which corresponds to an energy of 47 calories which is somewhat closer to biological values but seems still to be too high. However, we must not forget that riboflavine fulfills its role in the cell "activated" by the protein and we do not know how far its energy relations are altered by its being linked to the protein. We know that they are altered because the riboflavine bound to its protein loses its fluorescence (which may mean that it goes into the triplet) and that the wavelength of its absorption becomes lengthened by 20 mμ (which means that less energy is needed for its excitation). How far the energies needed for the triplet excitation are altered we do not know. It is possible that they are lowered more considerably.

To return once more to the experiment in Fig. 6, the riboflavine in th frozen tube, on excitation, went quantitatively into the triplet state. Even so this tube did not change color, as observed in daylight. This indicates that it absorbed light by its singlet excitation and if a triplet was formed it was formed through a transition from the singlet into the triplet, as symbolized by the arrow $S_1 \rightarrow T_1$ in Fig. 5. The tube containing KI showed a strong brownish tint which was observable even better if the tube was removed from the freezing mixture and was allowed to warm up to some extent.[1] This suggests that the iodide made a direct transition into the triplet possible, an observation which is interesting for the biologist because iodine, in the form of thyroid hormones, is one of the main regulators of metabolism and its action on E^* is independent of its charge or binding. Iodine is not the only substance which is capable of this action. Serotonin shows this action considerably stronger. Its effect is noticeable even in a 5.10^{-5} M concentration. There are two circumstances which make this effect

[1] On freezing, first the peripheral sheets freeze and crack up making it more difficult to see what is inside the tube. On warming up the opposite happens.

remarkable. The one is that serotonin is a constituent of normal tissues, has a strong biological activity, and various pathological conditions such as hypertension or schizophrenia have been connected with serotonin (Woolley and Shaw). The other circumstance is that serotonin is an indole derivative and is closely related to plant hormones, the ones affecting growth. The derivatives of indole seem to be among the most powerful instruments of living nature and so their action mechanism is one of the most important problems of biochemistry. The discoloration of riboflavine suggests that they can promote the direct transition from the ground state into the triplet. Serotonin seems to be able to do this to some extent even in an unfrozen watery solution, in the absence of structures; 10^{-3} M serotonin added to a solution of riboflavine induces a brown tint in its color. The spectroscope indicates that this change is due to the appearance of a shoulder in the absorption spectrum of riboflavine which according to its position corresponds to the triplet.

Serotonin is not the only substance which can produce such changes. Lysergic acid acts similarly, though more weakly; 1-benzyl-2,5-dimethylbufotenine and 1-benzyl-2,5-dimethylreotonine act likewise.[2] For kindly supplying the last two I am deeply obliged to Dr. D. W. Wooley. These substances seem to increase the probability of a direct transition from the ground state into the triplet and also to increase the stability of this triplet, as shown by the quenching of the phosphorescent light emission induced by O_2 in frozen riboflavine. This emission, in a 10^{-4} M riboflavine solution is completely quenched by 2.10^{-5} M serotonin or lysergic acid. How far the actions on E^* are limited to riboflavine or how far they represent a general ability to alter the probabilities of singlet triplet transitions in favor of the triplet, remains to be shown by more detailed studies which these substances certainly deserve.

[2] The similarity of action was surprising since Woolley and Shaw found these substances to be antagonists of serotonine. Lately (1955), however, Shaw and Woolley found also similarities in biological activity.

12. Ions, Glutathione, Sugars, and Alcohols

While a single experiment might suffice to disprove the theory of E^* only the accumulating mass of data can make it acceptable. It could gain weight by making old problems appear in a new light or by giving tentative answers to questions which have yet found no satisfactory answer or have found no answer at all. Such questions can be asked about ions and glutathione.

IONS

The main intracellular monovalent cation is K^+ while Na^+ is kept out as far as possible by the cell and one of the simple and basic questions of general biology is: why is this so? Buswell and Rodenbush state that the K^+ ions fit well into the holes of the cubic water lattice, formed around nonpolar groups, but ions bigger than K^+ do not do so. The K^+ ions fit also into the lacunas of a defective hexagonal lattice. Their dimensions are very similar to those of the water molecules. So we can expect K^+ to cause no disturbance in the crystalline regularity while other ions, bigger than K^+, can be expected to do so. According to the contents of this book this crystalline regularity is of prime import for cell life because triplet excitations can take their normal course only in an undisturbed orderly system.

In a way, Na^+ is bigger than K^+ because its nucleus is less screened by an electron shell, leaving outward forces unbalanced, which leads to the formation of a bigger hydrate envelope. So we can expect Na^+ to cause trouble, and if this is so then excitation processes could not run undisturbed in its presence and we can understand why the cell keeps the Na^+ out as far as possible, making K^+ its main intracellular cation.

In phosphorescent dyes we have an indicator for the disturbance of water structures and our problem here is to demonstrate with their help that Na actually causes a disturbance which would be incompatible with life if E^* played a major role in it and were dependent on order. Different dyes have a different sensitivity to disorder which declares itself in the length of the lifetime of their excitation. A longer lifetime indicates a greater stability and thus a smaller sensitivity. Riboflavine has a short lifetime, of the order of 10^{-3} seconds, and is thus most sensitive. The lifetime is somewhat longer in rhodamin B, and the longest in acridine orange.

The excitations, on the whole, are less sensitive to the qualities of the anions than to the qualities of the cations present. So the behavior of rhodamin, on excitation, depends only to a small extent on the nature of the anion and is dominated by that of the cation present.[1]

The behavior of excited rhodamin in the presence of varied concentrations of NaCl, KCl, and Cs and RbCl is shown in Fig. 22. The ordinate shows the intensity of phosphorescent light emission in an arbitrary scale, as observed visually. A frozen watery rhodamin solution (10^{-4} M) shows a weak light emission and a disturbance may declare itself in a quenching of this phosphorescence or in its increase.

As the middle curve shows, KCl, up to the limits of its physiological concentrations (0.16 M), causes no disturbance and quenches only above the limits of physiological concentrations. At a very low concentration, below 10^{-2} M, a slight increase in light emission is seen which (as judged by results on riboflavine) might be due to the Cl anion. In contrast to this Na^+ causes a strong disturbance. The difference between Na^+ and K^+ can most convincingly be demonstrated by holding, side by side under the

[1] The difference in the action of the various halogens can be demonstrated in more sensitive dyes such as riboflavine, which behaves differently in the presence of KF, KCl, KBr, and KI.

UV lamp, three test tubes with a frozen rhodamin solution, one containing at the side of the dye 0.1 *M* NaCl, another 0.1 *M* KCl, and the third no salt at all. The latter two will show hardly any phosphorescence, while the tube with NaCl shows an intense red

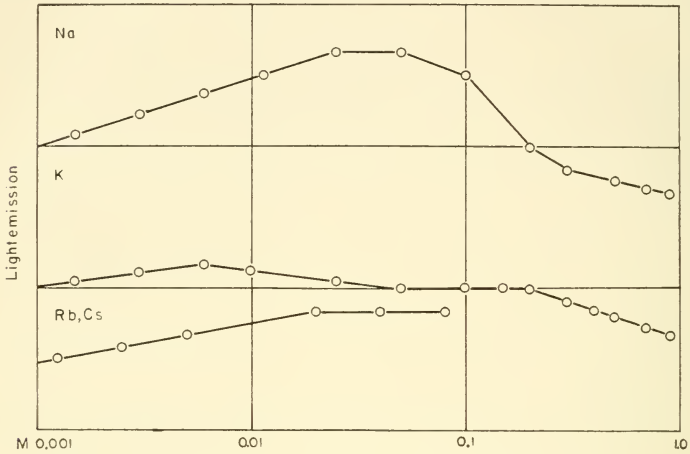


FIG. 22. Light emission of a frozen aqueous rhodamin *B* solution (10^{-4} *M*) in the presence of various concentrations of NaCl, KCl, Rb, and CsCl.

The abscissa corresponds to the weak light emission of the control tube with no salt, so the curve's going below it means quenching.

glow at the temperature of our freezing mixture. It is very impressive, indeed, to see the difference of the two so closely related ions demonstrated in such a striking fashion.

The hydrate shell of Li^+ is still bigger than that of Na^+ and so the disorder and harm caused by this ion have to be still graver. For the biologist the action of Li^+ is not less interesting than that of Na^+, having been discovered by Herbst that it induces monstrosities in developing embryos exposed at an early stage for a short while to relatively low concentrations of Li^+. In rhodamin the graver disorder declares itself in a quenching of light emission. Since the light emission of (salt-free) rhodamin is rather weak this action is not very impressive. For the demonstration of

the difference between Li^+ and Na^+ we better use a less sensitive dye, such as acridine orange. If LiCl, NaCl, and KCl are added in 0.1 *M* concentration to a (0.10^{-4} *M*) watery solution of acridine orange and the solutions are frozen in test tubes and held under the UV lamp, the first, containing LiCl, will show an intense red glow while the others will show practically no light emission. The disturbance caused in the developing embryo could thus have its explanation in the disturbance of the water structures which may be instrumental in the decoding and transmission of the information contained in genes.

The dimensions of the ammonium ion are similar to those of K^+. It was found, accordingly, that NH_4Cl, similarly to KCl, caused no disturbance in the phosphorescent behavior of rhodamin.

GLUTATHIONE

One of the most puzzling cell components is glutathione which is present according to F. G. Hopkins in normal tissues in as high concentrations as one mM. Though various interesting reactions of glutathione have been described its real biological meaning is still unknown. It is assumed that it acts as redox buffer keeping with its own SH groups those of the protein in reduced condition. This, however, only shifts the real problem instead of solving it because then we may ask: what has the SH to do in proteins? It certainly plays a most important role in its function. Many enzymes are "SH-enzymes," the intactness of the SH group of which is indispensable for activity. There are even S-containing hormones (oxytocin, vasopressin, insulin) which may exert their action in a reduced form. So we are faced here with a wider problem, that of the biological meaning of sulfur in general.

Glutathione, when tested for its influence on the excitation of rhodamin in the ways described in the previous pages, yielded no clue. Added to the solutions of this dye it produced the same action as thiamine: increased phosphorescence, an action which is not specific and is mainly a function of molar concentration.

One of the parameters of excitation which may have a major biological importance is lifetime. The lifetime of rhodamin is short, of the order of 10^{-2}–10^{-3} seconds. A greater number of substances have been tested for their ability to prolong this lifetime, with negative results. Only two substances were found to be capable of extending it at a low concentration and extending it strongly into the dimension of seconds: these are glutathione and pyridoxine. Solutions of rhodamin containing 0.01 *M* glutathione showed a strong red phosphorescence in the slow moving phosphoroscope and showed an afterglow if illumination was suddenly disconnected. In 10^{-3} *M* concentration the effect was but somewhat weaker. O_2 had only a moderate quenching effect so that it can be expected not to interfere with this long life excitation at the low O_2 concentrations of tissues.

That the lengthening of lifetime was actually due to the SH group could be shown by the fact that glutathione lost activity on oxidation of this group and that the same effect was produced by cysteine but not by cystine. There was only one difference between the action of cysteine and glutathione: while the former acted in acid solution only (dissolved as cystine HCl) and was inactive at neutral reaction, glutathione showed the opposite pH dependence, being active at neutral but not at acid reaction. Cysteine ethyl ester was found to be inactive indicating that the action of the SH group, somehow, depends on the structure of the whole molecule involving the COOH group and its dissociation. Possibly nature has built the cysteine into a tripeptide and produced glutathione to make the substance more soluble and to enable the SH to do its job at the physiological neutral reaction.

The lifetime of triplet excitations varies within wide limits. Rhodamin has a short one, quinidine and acridine orange a long one. The longer the lifetime originally, the easier it is prolonged into the 0.1 or 1 second dimension. Accordingly it was found that while this effect could be achieved in rhodamin with three substances only, a greater number of known or unknown cell con-

stituents had the same effect with the other two substances. Tissue extracts were found to produce a long-lived excitation with in quinidine at a very high dilution.

There is one conclusion we can draw from this for our later experiments. When studying the question how far E^* is involved in biological action and how far the modifications of this E^* can explain drug or hormonal action, we will have to consider also the lifetime. A drug action may very well consist of modifying the lifetime or modifying the influence of another agent, such as glutathione, on the lifetime of an excitation.

SUGAR AND ALCOHOLS

In the experiment described in Chapter 5 the phosphorescent light emission of rhodamin was increased by thiamine. In the present chapter a similar effect was obtained with sodium. We may ask whether such an increased light emission is due to an increase or a decrease in the stability of the triplet state? These substances may have caused increased light emission by making the triplet less stable and increasing herewith the probability of a return of the excited electron from the triplet into the ground state ($T_1 \rightarrow G$ in Fig. 5). It is equally possible that these substances might have increased phosphorescence by rendering the triplet more stable allowing a saturation of the triplet level. The greater the number of the electrons in the triplet state, the greater the probability that some of them will drop back under light emission into the ground state. In this case we could expect that the electrons emit their photon with a delay and give signs of a long-lived excitation. A long-lived excitation may declare itself in an afterglow, or in a phosphorescence observable in a slowly moving phosphoroscope. The rhodamin, in presence of thiamine or sodium showed no such afterglow so that it seems likely that the increased light emission was due in this case to a reduced stability, or at least to an increased probability of the $T_1 \rightarrow G$ transition.

Several instances were quoted on the previous pages in which

an afterglow was produced by the addition of 1–2% glucose to the watery dye solutions previous to their freezing. Other mono- and disaccharides, such as ribose, galactose, mannose, fructose, saccharose, or maltose acted similarly, while high polymers such as dextrin, starch (soluble or insoluble), or agar had no such action. A tentative explanation is suggested by a simple experiment: if a dye solution is placed in a test tube and is frozen slowly (e.g., by placing it in the deep freeze at —20°C), a sheet of colorless ice is formed in the periphery while the dye is concentrated in the middle. Evidently, the water crystallized out leaving the dye behind. If, prior to freezing, 1–2% sugar is added to the solution, the dye is found more or less homogeneously distributed in the ice. The sugar, so to say, mediates between ice and dye, making the contact between the two more intimate. If it is the water structure which enables the dye to form triplets, then the probability and stability of the triplets must be the greater the more intimate the contact between dye and ice.

The sugar molecules can be expected to build their own "icebergs." In the above experiment these icebergs must have fitted into the water lattices formed on freezing, or else the sugar would have also been eliminated by the freezing water. This system of ice and sugar-icebergs accommodated the dye molecules better than pure ice. However, the glucose added favored not only phosphorescence but caused also a strong fluorescence to appear. While a pure rhodamin solution shows no fluorescence at all, in presence of 1–2% glucose the frozen dye shows on illumination, at the side of phosphorescence, also a strong orange fluorescence, which blends with the red phosphorescence to a red-orange glow. The higher the sugar concentration the stronger the fluorescence. While in the presence of 1% glucose the emission is red-orange, in the presence of 10% glucose it appears purely orange and the strong phosphorescent component can be seen only in phosphoroscope which does not allow the fluorescent light to pass. So glucose seems not only to stabilize the triplet. In a way it seems to render

it also more unstable, preventing the transition of singlets into triplets and making the triplets drop back more easily into the ground state.

Such action of sugars may have its biological bearing. Deoxyribose nucleic acid consists according to the Watson Crick model of a column of heterocyclic bases surrounded by a sheet of pentose and phosphoric acid. If the bases are instrumental in conducting energy then the water structures built by the sugars and phosphates may be instrumental in transmitting this energy to the outside and transmitting with it also the genetic information contained in DNA. Solutions of DNA (as well as those of RNA) show a fluorescence in the visible, which turns into a long-lived phosphorescence on freezing. None of the components of nucleic acid shows such phosphorescence in itself, which indicates some collective activity in relation to the energy communicated to the system. A long-lived phosphorescence in dissolved substances is, without the addition of some stabilizer, such as glucose, a very rare occurrence. Evidently, the nucleic acid contains both components: the phosphorescent heterocyclic bases, and a pentose (and possibly also phosphate) as stabilizer.

The weak, short-lived phosphorescence of a frozen acridine orange solution is turned into a strong and long-lived one also by low concentrations (10^{-3} M) of ATP. This reaction is due to two factors, firstly to the formation of a stable complex between ATP and the dye which readily precipitates if the reactants are present in higher concentration. Adenosine monophosphate behaves similarly though the phosphorescence of its complex was weaker than that of ATP. Phosphorescence was found to be absent with adenine, though it also complexed with the dye. Evidently, icebergs of the pentose and the phosphate were responsible for the stabilization of E^* in the ATP-dye complex, which icebergs may play a major role also in muscular contraction.

It is not enough for a molecule to be hydrophylous and build icebergs in order to produce long-lived excitations in dyes.

Whether such an effect will be produced or not depends evidently on the inner structure of these icebergs, their relation to the bulk of the ice and their relation to the excited molecules. If the icebergs formed around a substance do not fit into the structures of water then the final result will be a disorder which will interfere with triplet excitations. Such disorder seems to be created by mono- and polyvalent alcohols. As mentioned before, no triplets are formed in ice containing 5–10% glycerol. Similar is the effect of monovalent alcohols, such as methanol or ethanol which in 2% concentration completely prevent the formation of triplets. There is a remarkable parallelism between the action of these alcohols on triplet excitations and their biological activity, suggesting that this latter may be due to their interfering with E^*. Two per cent ethanol temporarily stops most of the manifestations of life (Gaddum); it completely abolishes triplet excitations *in vitro*. Rhodamin, for instance, frozen in 2% alcohol, shows only an intense orange fluorescence, and no red phosphorescence whatsoever. A 0.2% alcohol concentration in our body juices make us heavily drunk. *In vitro* it cuts down the long life of the excitations in acridine yellow, while the long-life induced in acridine orange by cortisone is eliminated even by 0.06% ethanol. In rhodamin 0.2% ethanol turns the phosphorescence partly into fluorescence. Lower concentrations of alcohol which only cheer us up and relieve us of our inhibitions increase phosphorescence, making the triplet state less stable. Also the poisonous action of methanol, which cannot be due solely to our inability to oxidize it, is reflected in these experiments. The long-lived excitation of acridine orange in presence of glutathione, which is fairly insensitive to 0.6% ethanol, is cut down by mere traces of methanol; the triplet state of chlorophyll is disturbed by ten times smaller concentration of methanol than ethanol. Equally sensitive is the long-lived excitation of riboflavine, stabilized by glucose.

Results of the attemps to lengthen the lifetime of excitation are summed up in Table II which shows two points: extension of

lifetime may have a certain specificity, as shown by the example of ATP. Secondly: while sugars produce long life only in relatively high concentration, glutathione and ATP produced it in a hundred times lower concentrations; this indicates that glucose

TABLE II
LONG-LIFE PHOSPHORESCENCE IN THE PRESENCE OF GLUCOSE, GLUTATHIONE, AND ATP IN FROZEN SOLUTIONS OF ACRIDINE ORANGE, RHODAMIN B, RIBOFLAVINE AND QUINIDINE

	Glucose (2%)	Glutathione (0.002 *M*)	ATP (0.002 *M*)
Acridine orange	+	+	+
Rhodamin B	+	+	0
Riboflavine	+	+	0
Quinidine SO_4	+	+	+

acts by some general physical influence, like the alteration of solubility, while the other two substances act by means of some more specific mechanism.

The example of glucose and alcohol suggests that substances can, roughly, be divided into two groups: those which fit well into the water structure and, in a way, increase order, and, those which do the opposite. It seems likely that it will be found that the substances which play a major role in cell life and are found in cells in an appreciable concentration, belong to the first group. It seems also likely that substances which create disorder are not admitted into the cell by the membrane or may be even pushed out from the ordered intracellular water, suggesting a new theory of permeability. Possibly, substances which prolong the lifetime of the excitation of another substance, mediating between this substance and water, also will be found to render these substances more permeable.

13. On Oxidative Phosphorylation and Its Uncoupling

One of the sites of most intense energy transmission in the cell is the mitochondrion which oxidizes foodstuffs, transferring their oxidative energy to ATP, stabilizing it in the form of ~P, and so if E^* is involved in biological energy transmissions then we can expect it to have a hand in oxidative phosphorylation. We could approach this problem from either of two directions. We could ask whether substances known to uncouple oxidative phosphorylation have a special action on E^* in concentrations in which they uncouple, and we could ask whether substances known to act on E^* do uncouple oxidative phosphorylation?

When attacking this problem we will have to go back for an instant to the experiment with riboflavine, reproduced in Fig. 6. In this experiment the vitamin was found to go on excitation quantitatively into the triplet state and emit no light, as shown by the middle tube in the figure. This absence of luminosity is in agreement with rules of quantum mechanics which impose a strict prohibition on the radiative transition from the triplet state into the ground state. All the same, as witnessed by the next tube in the same figure, in the presence of atmospheric oxygen, such a transition does take place and the tube emits an orange phosphorescence with a wavelength of 570 mμ. This means that the O_2 present altered transition probabilities, making transitions possible which had practically no probability in its absence. If there is oxidative phosphorylation there is also O_2 and so we can expect the vitamin to take part in this process in a perturbed state with an altered reactivity. The O_2 thus serves not only as a final elec-

tron acceptor but at the same time also tunes the reactivity of this central member of this chain enabling it to perform its function.

This action of oxygen has rather remarkable features. The concentration of O_2 in the atmosphere is low, 0.01 *M*, and its concentration in the water must be considerably lower, owing to the poor solubility of O_2. The experiment shows that while bringing the riboflavine solution prior to freezing into equilibrium with pure O_2 does not increase the light emission, bringing the solution into equilibrium with air, diluted with 10 volumes of N_2, does not decrease it. The action is maximal throughout this wide range of concentrations and so the O_2 can be expected to exert a maximal influence even at the low tension present in the animal tissues.

Most chemical reactions depend on the concentration of the reaction partners. It is thus unusual to find the action of O_2 independent within such a wide range of its molarity. Since the concentration of the O_2 present in the solution, in balance with the diluted air, had to be considerably lower than 10^{-3} *M*, the concentration of the riboflavine present, we can exclude a direct interaction between riboflavine and O_2; we must look out for an unusual mechanism of action, based on some exceptional quality of O_2 which can be brought into a direct relation with the triplet state. O_2 has such a quality, being one of the very rare substances which are paramagnetic in their ground state, and paramagnetic molecules produce a magnetic perturbation of the electromagnetic field which is known to be able to alter transition probabilities between singlet and triplet excitations of electrons.

This explains also the queer dependence on (or rather independence of) concentrations. Until the concentration of the O_2 is high enough to ensure that statistically every riboflavine molecule comes to lie within the action radius of an O_2 molecule, we can expect the influence to be maximal. The action radius of the O_2 could be calculated from the concentration at which it ceases to have a maximal effect. My experiments only allow me to state that this radius must be a rather big one, of the order of 100 A.

Summing up, we can state that aerobic life occurs in a perturbed electromagnetic field and that this perturbation may have a profound influence on the course of reactions taking place within this field. If so, then there is a new possible mechanism for drug action which we have to consider: drugs may act also by eliminating, in one way or another, the influence of O_2. If this influence of O_2 on the riboflavine is an important one, then we can expect that substances which eliminate this influence will interfere with oxidative phosphorylation, since riboflavine plays an important role in this process. So when studying uncoupling agents, we will have to look out for changes in the orange phosphorescence which riboflavine owes to the presence of O_2. We can also turn the argument around and say that if we find that drugs which uncouple oxidative phosphorylation affect triplet excitations specifically, then this pleads for the assumption that E^*, in its triplet form, plays an important role in energy transmissions. If the uncoupler specifically acts on riboflavine, then this will support the hypothesis that riboflavine is involved in oxidative phosphorylation by its triplet excitation.

IODIDE

One of the substances which strongly quenches the phosphorescence of riboflavine is iodide. It quenches completely in 10^{-3}, incompletely in 10^{-4} M concentration. Its action fades out on further dilution. This suggests an action radius of the order of 50 A. Accordingly, iodide should inhibit oxidative phosphorylation in small concentrations. This point was tested by M. Middlebrook and the author, who isolated mitochondria from rat liver in the usual way, and measured their oxygen uptake and phosphorylation in the presence of beta hydroxybutyrate. The chloride in the suspension fluid was replaced to varying degrees by iodide. The P/O ratio varied in the various experiments between 2 and 2.6, the average being 2.2. The results of the experiments are summed up in Fig. 23. As this figure shows, oxidative phosphorylation was

uncoupled completely by iodide in a 0.03 *M* concentration, while O_2 uptake remained uninhibited. Bromide had a similar action, but corresponding to its weaker quenching abilities had to be applied in higher concentration to produce the same effect.

These results are at variance with those obtained by Klemperer, who found iodide without effect on oxidative phosphorylation.

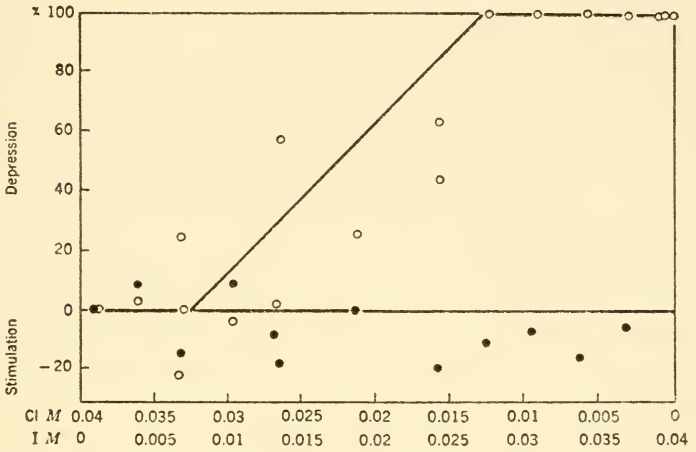


FIG. 23. Effect of replacing the chloride by iodide in the saline on oxidative phosphorylation of rat liver mitochondria, expressed in % of control in 0.04 *M* chloride. Open circles: phosphorylation. Full circles: oxygen uptake.

His technique of isolation of mitochondria was somewhat different and probably superior to ours. Possibly, his negative results were due to the mitochondrial membrane, which might have been preserved better in his experiments and might have kept the iodide out. The seats of oxidations are, apparently, not readily accessible to iodide. This may explain why somewhat higher concentrations of iodide had to be used to arrest phosphorylation than was expected.

As mentioned earlier, riboflavine frozen in the presence of 10^{-3} *M* iodide has a brownish color which suggests a direct transi-

tion from the ground state into the triplet. While O_2 seems to make the triplet unstable and promotes the transition of electrons from the triplet into the ground state, iodide seems to have the opposite effect, making the triplet stable, abolishing light emissions and increasing the probability of the transition $G \rightarrow T_1$ (Fig. 5). Iodide acts the strongest in 10^{-3} *M*. At higher concentrations it begins to acts as a salt, disturbing the water structures, rendering the triplet state unstable.

These effects of iodine are independent of its charge or binding. Bound iodine, as that in 3,5-diiodo-4-hydroxybenzoic acid, had the same effect in isomolar concentrations. It abolished the light emission and changed the color. This indicates that iodine does not produce these changes by a direct chemical interaction but by electromagnetic coupling.

Thyroxine and triiodothyronine were found to act as they were expected to act being compounds of iodine: they suppress the light emission of riboflavine in 10^{-4} *M* concentration to a great extent. Their insolubility renders it impossible to apply them in higher concentration. So the two hormones can also be expected to uncouple oxidative phosphorylation in mitochondria in 10^{-3} to 10^{-4} concentration, which, as is known from the work of Lipmann and Martius and their associates, they actually do.

The quenching or stabilizing action of iodide on riboflavine is not specific. Iodide equally suppresses the phosphorescent light emission of rhodamin B solutions (0.0001 *M*) at dry ice temperature in 10^{-4} *M* concentration. This is in agreement with the assumption that iodide owes its activity to its being a rather big, soft, and fat ion, by which is meant that it has a great number of electrons which are capable of all sorts of transitions between energy levels, enabling the molecule to resonate with other molecules. Excited electrons coming close to its nucleus may also reverse their spin, owing to the high atomic number of this element and the correspondingly high positive nuclear charge.

The assumption that iodide suppressed the phosphorescence of

riboflavine (and, possibly, also suppressed oxidative phosphorylation) by stabilizing the triplet could be supported by the following experiment: a riboflavine solution was frozen in the presence of 0.1 *M* NaCl. Instead of the usual orange phosphorescence a yellowish-white light emission was obtained. The white color indicated the mixed nature of the emission, while its yellow tint showed that fluorescence reappeared. That this actually was the case could be shown by observing the frozen tube in the phosphoroscope which cuts out fluorescence. Here the system showed a strong red phosphorescence, strong as compared to the tube with no added NaCl. What the NaCl did was thus to render the system unstable. The excited electrons of riboflavine could no longer go as readily into the triplet as they did in pure ice, and if they did so the triplet was rather unstable so that more electrons did emit their energy in a radiative process and drop back to their ground level. If KI is added in 10^{-3} *M* concentration in addition to the 0.1 *M* NaCl, the phosphorescent emission becomes weaker and the fluorescence disappears.

2,4-DINITROPHENOL

The classic uncoupler is 2,4 dinitrophenol as shown in the studies of Loomis and Lipmann. It does the uncoupling in low concentration, at 10^{-4} *M*, and may do so without inhibiting oxidation, which may even be increased. It is this action which made nitrophenol into a reducing agent (used for reducing body weight). Its medical dose is 100 mg, which means a random concentration of 10^{-5} *M* in the body (60 kg body weight and 1.2 specific weight). But even smaller single doses, such as 25 mg, can cause a rise of the basal metabolism by 30%, which would mean a concentration in the body of 4×10^{-6} *M*. The molecule of dinitrophenol is simple, has no characteristic solubilities, and so we can suppose it to have no specific affinities to tissues or cell constituents and to exert its pharmacological action at its random concentration. So if we want to support the idea that dinitrophenol

uncouples oxidative phosphorylation by interfering with E^*, then we have to show that it can disturb E^* at these concentrations, at 10^{-3} M, and do something even in a 4×10^{-6} M concentration. The experiment showed that the phosphorescence of a 10^{-4} M riboflavine solution is strongly quenched by 10^{-5} M dinitrophenol and 4.10^{-6} still had a marked effect. So our experiment *in vitro* agrees with the assumption that the oxidative phosphorylation, *in vivo,* is uncoupled by interfering with E^*, and more specifically, interfering with the triplet E^* of the riboflavine, tuned to its function by the O_2 present.

In a way, the quenching action of dinitrophenol on the phosphorescence of riboflavine can be called specific. In 10^{-4} M concentration the nitrophenol did not appreciably diminish the phosphorescence of other substances, such as acridine or atebrin, nor that of rhodamin, even if it was increased by thiamine. In higher concentrations the dinitrophenol absorbs light appreciably and so the decrease in phosphorescence of other substances in its presence could be ascribed to a direct absorption of the exciting light by the nitrophenol. The quenching observed in this case is not a "true quenching."

2,4-DINITRONAPHTHOL

This substance is but a dinitrophenol with an extra antenna, another benzene ring added to it. Accordingly, we can expect it to interfere with E^* somewhat more strongly than did 2,4-dinitrophenol.

The experiment *in vitro* shows that 2,4-dinitro-1-naphthol suppresses the phosphorescence of riboflavine *in vitro* about four times more strongly than does the corresponding dinitrophenol, which is to say that the naphthol can be applied in one-fourth the concentration and produce the same effect. M. Middlebrook compared the action of the two drugs on the oxidative phosphorylation of rat mitochondria. Her results are summed up in Fig. 24. As can be seen, the dinitronaphthol inhibits oxidative phosphorylation

more strongly than the phenol, arresting it at a lower concentration. But the curves also show that while the nitrophenol, at a low concentration, increases the oxygen uptake, the reverse effect

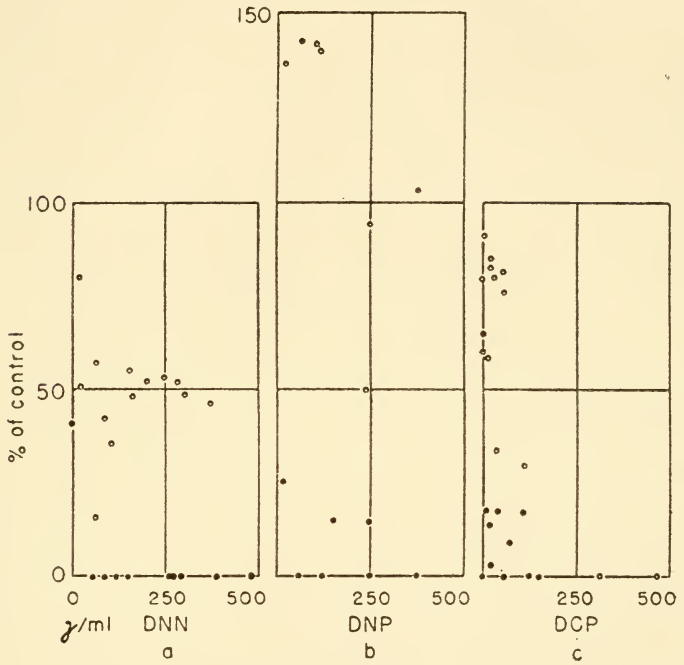


FIG. 24. The effect of dinitronaphthol (a), dinitrophenol (b), and dichlorophenol (c) on O_2 uptake (open circles) and oxidative phosphorylation (full circle) in rat liver mitochondria.

is observed with the naphthol, which moderately decreases oxidation. All the same, the inhibition of phosphorylation could not be ascribed to the lack of oxidation, for the oxygen uptake still present would have sufficed to maintain phosphorylation at its original level. Dinitronaphthol has more specific solubilities than dinitrophenol, having more affinity to lipides and less to water. So it may be accumulated by certain cell constituents and have biologi-

cal effects different from those of the dinitrophenol which is more water soluble.

2,4-DICHLOROPHENOL

Clowes and Krahl found that 2,4-dichlorophenol produces effects in *Arbacia* eggs similar to those induced by 2,4-dinitrophenol, which is to say that both produced an increased oxygen uptake in a low concentration, and decreased O_2 uptake at a higher one with a complete inhibition of cell division. This suggests an action mechanism similar to that of the nitrophenols. However, the experiment *in vitro* shows that while the dichlorophenol has a strong quenching action on the phosphorescence of riboflavine even in 10^{-5} *M*, its action is less specific and it affects the excitational states of other substances as well on which the nitrophenol had no appreciable action. So, for instance, it was found to quench the yellow phosphorescence of acridine (saturated water solution) in a 10^{-4} *M* concentration. The dichlorophenol can thus be expected to suppress oxidative phosphorylation but can also be expected, at the same time, to inhibit other processes. The experiments of M. Middlebrook bore out this expectation. Her results (Fig. 24) showed that the dichlorophenol, similarly to the dinitrophenol, completely inhibits oxidative phosphorylation in a low concentration, but at the same time it cuts down oxygen uptake so that in this case the failure of phosphorylation might also have been secondary to the failure of oxidation.

Salicylic acid has also been shown recently to be a powerful metabolic stimulant (Alexander and Johnson) and to uncouple oxidative phosphorylation in a 5×10^{-3} *M* concentration (Smith and Jeffrey). One gram, the medical dose, taken by an adult corresponds to a random concentration of 10^{-4} *M*. Salicylic acid quenches the phosphorescence of riboflavine in a 10^{-5} *M* dilution.

14. Depolarization of the Cell Membrane

The cell membrane divides the world in two: the "inside" and the "outside." It receives all the messages coming from its surroundings; with its specific permeability and pumping action it decides the composition of the intracellular environment on which function depends. It has an electric gradient, being mostly negative inside and positive outside, and is thus the seat of an electric double layer with its potential energy, which it spends in "excitation." To build up this potential it needs energy and so does it need energy to restore this potential after excitation. It needs energy to perform its pumping action, to move ions or molecules against electric or concentration gradients. Last, but not least, it needs energy for maintaining its structure which, like all living structures, is from a physical point of view an improbable one.

For the study of the cell membrane, the muscle fiber offers a propitious material, because the function of muscle is contraction, a function which entails violent changes in energy, chemistry, and shape, and can thus be registered by means of crude instruments and observed even with the naked eye. These macroscopic changes, summed up as "contraction," are changes in the physical state of the actomyosin system. In living muscle these are elicited by the collapse of the normal membrane potential. Whatever the link between membrane potential and actomyosin may be, the fact stands that the contraction of actomyosin indicates in the intact muscle the collapse of the membrane potential, making, so to say, this collapse visible. Ling and Gerard found that the resting potential of the membrane could be decreased to a certain critical value without eliciting contraction. In the muscles studied this potential was around 55 MV. If this critical potential was reached

the potential collapsed altogether and contraction ensued. This offers a good opportunity to compare different drugs for their depolarizing action, for the ensuing contraction indicates the instant at which the potential was decreased to 55 MV under action of the drug in question, the muscle giving a clear "yes" or "no" answer.

If E^* is involved in the energization of the membrane, then drugs which interfere with E^* should cause contracture. Hajdu and the author observed that 2,4-dinitrophenol causes contracture in the isolated rat diaphragm muscle. Simultaneously, Barnes and Duff, in England, made the same observation. If this depolarizing action of the 2,4-dinitrophenol was due to its interference with E^*, then it could be expected that other related substances which likewise interfere with E^* will have the same effect and produce contracture and that their ability to do so will be proportional to their ability to interfere with E^*; the latter action can be measured *in vitro*.

The results obtained are illustrated by Fig. 25 taken from Hajdu and the author's paper. It shows the kymographic record of a rat diaphragm muscle, excited electrically at two different frequencies. At the arrow, 2,4-dinitro-1-naphthol was added to the saline bathing the muscle. This addition had no immediate effect, but, as the lower curve shows, after a while, the base line started to rise and the muscle went into contracture, the force of which, evenutally, reached twitch tension. These curves give a variety of information. The high tension reached in contracture indicates that there was still plenty of ATP available. As the comparison of the upper and lower curves shows, the contracture, setting in at the same time, was independent of the rate of stimulation. The depolarization leading to this contracture was thus independent of the rapid depolarizations induced by the single electric shocks. Relaxation, i.e., repolarization after these shocks, took place with a normal speed even after the contracture began to set in. The energy transmission involved in the rapid repolarization

of the membrane following excitation was thus not affected by the dinitronaphthol. (This rapid repolarization can be slowed down by substances, such as cyanide, which cut out the whole energy supply.) The dinitronaphthol seems to have acted on some

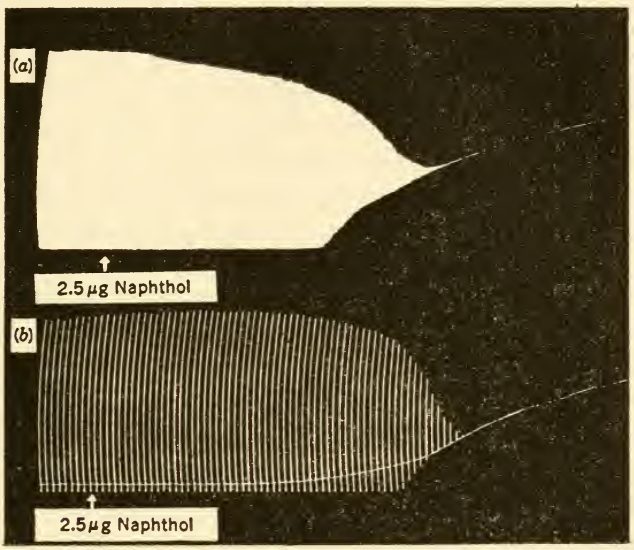


FIG. 25. Dinitrophenol contracture of isolated rat diaphragm muscle. a: Nerve stimulated with a frequency of 12 impulses per minute. b: Same with one stimulus per minute. At arrow 2.5γ dinitronaphthol were added per milliliter.

slower process, interfering, possibly, with the energy supply which is needed for the maintenance of the normal structure of the membrane or its pumping action.

Whatever the nature of the action of the dinitronaphthol may be, we can expect that it acts in an "all-or-none" fashion, that is, produces contracture if it diminishes the potential to its critical value, or produces no action at all. The experiment showed dinitronaphthol to be inactive in a concentration of 1.25 μg. per milliliter, while 2.5 μg. per milliliter produced maximal contracture.

Five μg. of dinitrophenol per milliliter was inactive; maximal action was produced by double this concentration; 2,7-dinitrophenanthrenequinone and 2,6-dinitrothymol gave results between the two, while 2,4,6-triiodophenol was found to be half as active, and 3,5-dinitrosalicylic acid inactive.

According to these data, the dinitronaphthol was four times more active than dinitrophenol. Comparison of their action on the phosphorescent emission of riboflavine showed that the same quenching could be obtained with a concentration of dinitronaphthol one-fourth as great as that of dinitrophenol. 3,5-Dinitrosalicylic acid was found inactive while dinitrothymol was comparable in activity to dinitrophenol. The phenanthrenequinone and triiodophenol could not be evaluated, owing to their extreme insolubility in water. Ten μg. of dinitrophenol per milliliter corresponds to a concentration of 5×10^{-5} *M*. As has been shown in the foregoing chapter, the drug can strongly quench E^* in this concentration, but its action fades out on further dilution.

That the contractures observed were actually due to the depolarization of the membrane was demonstrated by Loewenstein and the author, who measured the resting membrane potential in isolated single muscle fibers and found it to disappear under the action of dinitronaphthol, reaching the critical values at approximately the time when contracture began.

To summarize the results, we can thus say that the experiments supported the conclusion that nitrophenols exert their biological action by interfering with E^*, and that E^* is involved in maintaining the polarized state of the membrane.

The action of dinitronaphthol on the membrane of isolated muscle agreed with the symptoms caused by this drug if ingested parenterally to the animal. If 2 to 3 mg of this substance (in an oily solution) are injected into an adult mouse, the animal shows no symptoms for an hour or so. Then it suddenly drops dead. A few seconds later, all its muscles are found in violent contracture. Death occurs, evidently, when the membrane potentials reach the

critical value of Ling and Gerard. If the dose given was insufficient to decrease the potential to this value the animal survives and shows no symptom whatsoever.

According to our assumptions E^* is involved in various cellular activities; so the symptoms in the whole animal, caused by the ingestion of toxic doses of a substance such as an aromatic nitro compound which interferes with E^*, can be expected to depend on the question to which tissue or cell constitutent the drug has the greatest affinity. According to the experience quoted, dinitronaphthol seems to have a special affinity for the cell membrane. Dinitrophenol seems to have a greater affinity for mitochondria and so its interference with cellular metabolism dominates the picture. Toxic doses may kill the animal in hyperthermia, while smaller and repeated doses produce emaciation.

Substitution of acidic radicals in aromatic compounds is known to interfere with biological activity while leaving optical properties untouched. Possibly, the acidic radical interferes with permeability, slowing down the penetration into the cell. According, it was found that dinitronaphtholsulfonic acid caused no acute symptoms in the animal when ingested in the same doses as the free nitronaphthol, and caused no contracture of isolated muscle though it quenched the phosphorescence *in vitro* with the same intensity as dinitronaphthol. All the same, its biological activity was found to be proportional to its *in vitro* action on E^*, and equal to that of free dinitrophenol: it killed the animal in the same doses but did so with unspecific symptoms in a few days time, following a wasting away of the animal.

PART III

On Drugs, Hormones, and Disease

"God made the little fly,
If you squash it it will die."

15. General Remarks on Drugs and Disease

What characterizes modern medicine as compared to the earlier empiricism is that it is based on our understanding of the processes underlying disease. In order to understand a disturbed structure or function, we have to understand the normal one first. This is why medicine has to lag one step behind the advances in basic knowledge. To compensate for this, any progress in basic knowledge can be expected to bear fruits for medicine as its natural consequence. But conversely, if there is a gap in basic knowledge there must be a bottomless pit in our understanding of disease. There *is* such a pit and the author will dive into it in the hope that having E^* in mind, he will hit bottom, but warns the reader who is adverse to speculation not to follow him. Most of this last part of this book will be pure speculation.

The author's research has always been dominated by the idea that there is but one living matter which has overgrown this globe's surface, taking on different shapes, sizes, colors, and complexities, adapting itself to different conditions. In spite of the great variations in its appearance life is built on the same limited number of basic principles, wherever and in whatever form we meet it; there is no real difference between "cabbages and kings." But if the foundations of normal life are simpler than its appearances, then the same may be true also for disease, and a great variety of symptoms can be caused by disturbance of single basic mechanisms, and the way in which disease declares itself may have no direct relation to the underlying cause. Vitamin B_1, for instance, is a coenzyme, equally important for all cells, animal or vegetable, but all the same, its lack in higher animals causes polyneuritis. If highly unsaturated fatty acids are withheld from the

diet of the rat, then the animal's tail drops off (Burr and Burr). It would be erroneous to conclude that the biological function of these acids is to keep tails in place.

The disturbance of any basic function must lead to disease. The generation and utilization of E^* is such a basic function, the disturbance of which may declare itself in a variety of symptoms or diseases. The last part of this book will be devoted to an attempt to find connections between E^* and "degenerative diseases." Faithful to my traditions I will choose a degenerative disease of muscle for example. The difficulties of this proposition are twofold. *Firstly,* the physical theory of fluorescence and phosphorescence is still in its infancy and so the biologist has no really firm ground to stand on. This applies especially to conditions in water. Since the triplets formed in water have just been found, and the water structures formed around nonpolar substances belong to the latest achievements of science, the interaction of the two necessarily belongs to the blank spaces on our map of knowledge. *Secondly,* even if we could understand these relations we could not state how a certain change should declare itself in biological function, how, for instance, the shortening or lengthening of a lifetime or a change in stability of an E^* should alter cell life. So in our first approach we will have to content ourselves with showing that changes in E^* are related to changes in function, that substances which affect E^* also affect function and that drugs which affect function affect E^*. In order to prove that a drug actually could have exerted its function by affecting E^* we will have to show that the drug is capable of affecting E^* *in vitro* in the same concentration as it exerts its action *in vivo.* Unfortunately, such a narrow parallelism can be expected to exist only with drugs which have no specific affinities, as was the case with 2,4-dinitrophenol. Drugs with more specific structures and affinities can be expected to be accumulated by their target and so exert their biological action at a higher concentration than corresponds to their random distribution. The higher the specificity, the greater the gap will

have to be between the *in vivo* and *in vitro* action, distribution being random *in vitro*. We can circumvent this difficulty in two ways. If a drug exerts its biological action by acting on E^* then it will have to have an active nucleus (conjugated double bonds, NO_2 or SCN groups, iodine, etc.), by which it exerts this action. Our drug in question, if it acts by acting on E^*, will have to produce an effect on E^* *in vitro* at the same concentration as related compounds which have no specific affinities, lacking the specific structural details to which specific affinity is due, but having the same active nucleus. To quote an example: if thyroxine owes its biological action to its iodine, then we can expect it to act on E^* *in vitro* in the same concentrations as does free iodide or other organic compounds of iodine. The other way to circumvent this difficulty would be to look out for the less specific symptoms of the drug in question (for no drug action is entirely specific). If E^* has a general importance for cell life then we can expect any drug acting on E^* to affect all cells to some extent. To produce such unspecific symptoms with a specific drug, we have to administer it in higher doses than are needed to produce its specific action. We can expect that the drug will affect E^* also *in vitro* in the concentration in which it produced its unspecific action *in vivo*. To illustrate this point: chlorpromazine (Chapter 16) has a specific effect on certain nervous centers but in higher doses it affects the whole basal metabolism. If its pharmacological activity is due to its action on E^*, then we can expect it to affect E^* *in vitro* in the same concentration as it affected the basal metabolism *in vivo*.

Unfortunately, when comparing active concentrations *in vitro* and *in vivo*, we will also have difficulty in making final statements about the concentrations in our test tube with its frozen contents. Water has a tendency to crystallize out in pure condition, leaving dissolved substances behind in higher concentration. So, however rapid our cooling may be, there is always a chance that the actual concentrations were higher than corresponds to a random distribution.

Before closing this chapter I would like to return to my point of departure: the apparent independence of symptoms and the underlying disturbance. It seems natural that one should be inclined to connect an increased function with an improved energy supply, an excess of E^*, and a decreased function with the opposite. However, things are more likely to be the other way round. To use a crude comparison: if a car runs too fast downhill, this is more likely to be due to faulty brakes than to an improved motor. Thermodynamically, all biological reactions are "downhill" reactions, that is, go with a decrease of free energy, the driving force of chemical reactions being the trend of any systems to decrease their free energy. It is possible to "enforce" on a substance an increased free energy content, make it go "uphill," but in this case another substance has to go "downhill" even lower to pay the bill. Biological reactions which have to be fast have to be "downhill," that is go with an expenditure of free energy, "downhill" being faster than "uphill." So if the contractile matter or the membranes of the muscle fiber spend free energy in function, they have to be reenergized after and then kept in the high-energy-resting-state to be ready for renewed action. The slower "uphill" work of reenergization is done after completed action, when it can be done at ease, as the recharging of an accumulator. The active state will thus be mostly the low energy state, the energy having been spent in that action. So, for instance, the low energy state in muscle must be the contracted state, energy having been spent to produce work. Similarly, the low energy state of the membrane has to be its discharged state. An impaired energy supply and reenergetization will cause the systems to persist in the active state. So, for instance, if the supply of energy to the muscle membrane is impaired the fiber will have difficulty in raising the membrane potential above the critical value and the muscle will have a strong tendency to drop back into the contracted state. The nervous end plate which would have fired but once in case of a normal resting potential may fire repeatedly, producing a myotonic behavior in

case of a poor energy supply and the ensuing low membrane potential. If the critical potential is 55 MV and the resting potential 100 MV then the end plate has to overcome a barrier of 45 MV. Jerenik and Gerard have shown that the lower this barrier, the poorer the reenergization, the easier the end plate "fires." So we are led to the paradoxical situation that an impaired energy supply causes increased activity and an increased energy supply may cause inactivity making barriers too high to be overcome.

16. On Iodine and Chlorpromazine

The list of drugs, the action of which is not understood, is a long one. So when attempting to correlate pharmacological activity with E^* we can hardly do more than to pick one or two examples and see whether E^* leads us to a better understanding. I will pick "something old and something new": KI and chlorpromazine.

KI

When I was a medical student, iodine in the form of KI was *the* universal medicine. Nobody knew what it did, but it did something and did something good. We students used to sum up the situation in this little rhyme:

> "Wenn Du nicht weisst wo, was, warum,
> Gebe dann Iodkalium."

Freely translated:

> If ye don't know where, what, and why
> Prescribe ye then K and I.

Our medical predecessors, possessing very few and crude instruments only, had to make use of two given by nature (the use of which has since gone out of fashion): eyes and brains. They were keen observers and the universal application of iodide might have been not without foundation.

Since the iodide has no specific solubilities or affinities, we can expect it to act on E^* *in vitro* at the concentration which it reaches in the body when applied in therapeutic doses, provided that it owes its activity to an action on E^*. The single medical dose of KI is 1 gram, which in an adult of 60 kg makes an over-all con-

centration of 2×10^{-4} *M*. So our question is: can I^- affect E^* in concentrations of the order of 10^{-4} *M*?

It has been shown earlier that iodide strongly quenches the phosphorescence of riboflavine in 10^{-4} *M*. It was also indicated as probable that it stabilized the triplet of this vitamin and favored the direct triplet excitation. All the same, its action, *in vivo,* cannot be limited to riboflavine, since KI is not known to affect normal metabolism and the effects of its medication (if any) are more general. The experiment shows that it acts on the E^* of a variety of substances. For instance, KI strongly quenches the yellow phosphorescence of acridine in a frozen (saturated) watery solution at 10^{-4} *M*. Its action on quinidine is more colorful, quinidine itself having a colorful behavior. As mentioned before (Chapter 5), 10^{-3} *M* KI greatly favors the transitory yellow phosphorescence. At the temperature of dry ice 10^{-4} *M* renders the whitish blue phosphorescence deep blue. At this concentration it can also lengthen the lifetime, making the excitation into a long-lived one, especially if the solution of the alkaloid has been "washed out" with pure N_2. In the fast phosphoroscope a lengthening of the lifetime, that is an increased luminosity, is still noticeable in presence of 10^{-5} *M* KI.

KI plays no major role as a therapeutic agent any more. What still lends interest to its action on E^* is the fact that hormones of the thyroid are iodine compounds and they play a major role in the regulation of the normal energy household of our body, as well as in pathological processes. Since the disturbances caused in E^* by iodine are independent of the charge or mode of binding of this atom, we can also expect that the thyroid hormones will be equally capable of disturbing E^* as free iodide is and do this *in vitro* in the same concentration as the equimolar I^-. This point was tested by comparing KI, triiodothyronine, and thyroxine. They were found to have a very similar action. Owing to their insolubility the hormones could not be applied in higher concentrations, but they lengthened lifetime in 5×10^{-5} *M*. Owing to their

specific affinities, in the animal body they are capable of producing effects at a lower concentration, being accumulated by their target.

CHLORPROMAZINE

The drug chlorpromazine has a most colorful, unique action in the animal body. It has come into the limelight lately as one of the main drugs in the treatment of schizophrenia. Needless to say,

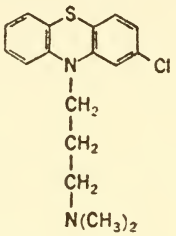


FIG. 26. Chlorpromazine.

if the drug influences schizophrenia, then it must influence some process involved in the pathogenesis of this disease and if it does act by interfering with E^* then, evidently, E^* must be involved in one way or another, in schizophrenia. So our problem here is to find out whether chlorpromazine does act on E^*, whether the concentrations in which it produces changes in E^* *in vitro* are comparable to the concentrations in which it exerts a drug action *in vivo*. Chlorpromazine (Fig. 26) is a phenothiazine and a member of this group has occupied us earlier (pyrrolazote). According to what has been said before, we could expect that in chlorpromazine the phenothiazine ring acts on E^* while the side chain tunes the specific biological affinities.

As is known from the rapidly growing literature on chlorpromazine, this drug produces in the animal symptoms akin to hibernation. In order to be able to compare this action with actions on E^* *in vitro* the basal metabolic rate was measured in mice by the author and S. L. Baird. The results, which have no pretense of

originality, are summed up in Table III.[1] As the table shows, the action has a remarkable feature: while the action of other drugs depends on their concentration, in this case doses varying from 8.5 to 140 mg per kilogram had approximately the same effect, de-

[1] CO_2 production was measured in groups of Swiss albino mice, each group consisting of six animals. Three animals of each group received an identical quantity of "thorazine" injected subcutaneously. The other three served as controls, showing the normal basal metabolic rate. The CO_2 production was measured individually in each animal. The numbers in Table III are the average values of the three identically treated mice.

The apparatus used for the determination of the basal metabolic rate was so simple and worked so well that it deserves a short description. Essentially, the method consisted of sucking the expired air through a known amount of baryta and measuring the time needed for neutralization, thymolphthalein being used as indicator. In order to provide an intimate contact between baryta and air, the latter was passed through a filter disk with fine pores (*F* in Fig. 27). It was found that even this way CO_2 was lost, the air bubbles

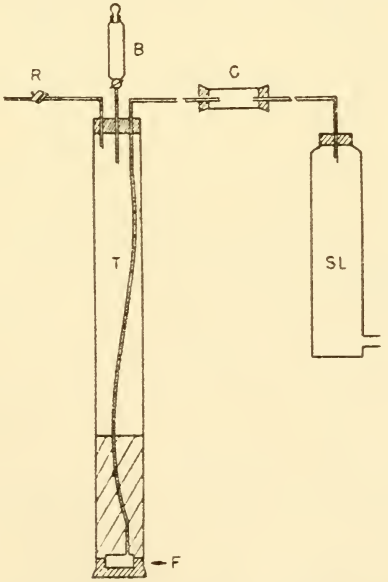


FIG. 27. Apparatus used for the measurement of CO_2 production in mice. For explanation see text.

pressing the basal metabolic rate by about one-half. A dose of 8.5 mg had roughly the same effect as a dose 16 times bigger, suggesting that within this range the drug suppresses a process necessary for the maintenance of the normal metabolic level but not indispensable for life. Increasing the dose further killed the animals, suggesting that at higher concentrations the drug attacked another more vitally important process. Below 8.5 mg per kilogram the action began to fade out altogether. Calculating the molar over-all concentration (taking 1.2 g body weight equal to 1 ml): 0.0013 *M* killed the animal, 0.00073–0.000044 *M* had roughly the same strong effect, while the action began to fade out around 0.00002 *M*. As the table shows, the temperature of the animals dropped during the experiment and so the drop in basal metabolic rate

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being too large and passing too rapidly through the liquid. This difficulty was overcome by adding 5% butyl alcohol to the baryta, which makes the bubbles smaller and makes them rise to the surface much slower. The whole apparatus consisted of six units, making it possible to measure the CO_2 production of six mice simultaneously. Its essentials are shown in Fig. 12, in which only one of the six units is represented.

The experiment was performed as follows: 200 ml of 5% watery *n*-butanol solution were placed into the glass cylinder (*T*), about 60 cm long and 6 cm wide, closed on both ends by rubber stoppers; 10 ml of 0.2 *M* baryta were pipetted into the container *B* with a few drops of a 10% alcoholic solution of the indicator. Then the animal was enclosed in a small cylindrical wire cage, weighed, and placed with its cage into the short glass tube *C*, closed on both ends by rubber stoppers. Air was sucked through this container, liberated of its CO_2 in a soda lime tower (*SL*). The air was led from *C* through a rubber tube and through the stainless steel filter disk *F* (A. H. Thomas, Philadelphia, Catalogue No. 5151 S). This filter disk was mounted into the lower rubber stopper in such a way that air could pass it only through its upper surface. The air was sucked out of the cylinder *T* with a constant negative pressure of 20 cm Hg.

The air, passing through the filter, formed a fine foam which filled tube *T* to about its middle. The rate of airflow was regulated by the clamp-screw *R*. After everything was set, the baryta was allowed to run into *T*, the container *B* rinsed. The measurement began with the running in of the baryta and ended with the complete discoloration of the solution, the interval being measured by means of a stopwatch.

could have been the consequence of the decreased temperature. This does not invalidate our conclusions; in this case we have only to suppose that the drug exerted its action on the regulatory mechanisms responsible for the maintenance of the body temperature.

The chlorpromazine molecule has rare qualities, as far as its own excitational states, its own E^*'s are concerned. If frozen, a

TABLE III
EFFECT OF VARYING AMOUNTS OF THORAZINE (CHLORPROMAZINE) ON THE BASAL METABOLIC RATE AND BODY TEMPERATURE OF SWISS ALBINO MICE

Thorazine injected (mg)	Milligrams per kg	Over-all M concentration in the animal	% Decrease in BMR three hours after injection	Body temperature 4 hours after injection	Time needed for complete recovery
10	251	0.0013	−9%	35°C	+
5	140	0.00073	56%	27°C	48 hours
2.5	80	0.00042	63%	25.5°C	48 hours
1.25	34	0.00018	63%	25.1°C	24 hours
0.63	19	0.00010	71%	24.7°C	24 hours
0.32	8.5	0.000044	51%	26.6°C	12 hours
0.16	3.7	0.000019	16%	34.2°C	6 hours

10^{-3} M solution shows a strong yellow afterglow, a long-life phosphorescence which lasts for about a second. In the phosphoroscope this phosphorescence can be observed even in 0.00003 M, thus in the whole range in which the drug exerts a strong pharmacological action (Column 3 in Table III). The atmospheric O_2 does not abolish this phosphorescence—it only diminishes it. This is evidently the reaction of the phenothiazine ring because pyrrolazote shows it too, though its phosphorescence is more sensitive to O_2 than that of chlorpromazine. Excitations which are very sensitive to O_2, could have no biological function. Such a long life phosphorescence is a rare quality.

Chlorpromazine is capable also of influencing the E^* of other

molecules.[2] In 2×10^{-3} *M* it abolishes the phosphorescent light emission of riboflavine. In 2×10^{-4} *M* it abolishes the phosphorescent light emission of acridine and that of rhodamin B, also if the latter is increased by the addition of substances such as Ritaline or thiamine. So it is capable of suppressing phosphorescence of other substances in the whole range of concentrations in which it exerts pharmacological activity. In a higher concentration, as 0.0014, it is capable of quenching the phosphorescence of a new group of substances, to which quinidine and atebrin belong, and it is possible that it kills the animal in these concentrations because it quenches the excitation of molecules involved in processes indispensable for life. Many biological catalysts are related in their structure to quinidine and atebrin.

Another remarkable property of chlorpromazine is that it is capable of influencing the probabilities of the triplet-singlet transitions of other molecules in presence of low concentrations of alcohol which cause a mixed fluorescence and phosphorescence. In acridine (saturated watery solution plus 0.125% methanol) it favored the blue singlet. Its action was strong at 0.00017 *M* and still noticeable at 5×10^{-5} *M*. In fluoresceine, rhodamin, and riboflavine it favored the triplet.

All this shows the high and colorful reactivity of chlorpromazine in relation to E^* *in vitro* in concentrations in which it exerts its pharmacological action *in vivo*. Taking into account that its molecule is a chemically inert one, this experience strongly supports the assumption that it actually exerts its influence on biological functions by interfering with E^*. If it influences schizophrenic behavior in concentrations smaller than those that affect basic metabolism this may be due either to its accumulation in certain nervous centers, or to the specific sensitivity of those centers to alterations of E^*.

[2] We can expect, accordingly, oxidative phosphorylation to be uncoupled by chlorpromazine. This to be the case has been shown lately by Berger, Strecker, and Waelsh.

The two examples discussed, KI and chlorpromazine, were picked at random. Their strong action on E^* and the similarity of concentrations in which these drugs acted on E^* *in vitro* and exerted their pharmacological activity *in vivo* encourages an extension of the research to other drugs; this may lead to unexpected results, such as actions on E^* where no such were expected. This was the case with morphine, novocaine, and salicylate which were found to quench the phosphorescence of rhodamin strongly in 10^{-4} *M*. Serpasil, the alkaloid of *Rauwolfia* which is also invoked in the treatment of schizophrenia was found to be even more active. It completely quenched the phosphorescence of riboflavine in 10^{-4} *M* and suppressed the yellow phosphorescence of quinidine in 10^{-5} *M* concentration, turning the light emission of quinidine from whitish-blue to deep blue.

17. On Myotonia

What makes myotonia fascinating for the experimental researcher is the fact that this hereditary degenerative disease of man occurs in an experimental animal, the goat. Owing to the generosity of Mr. R. Lombardi, of Philadelphia, Pennsylvania, the author is the happy owner of a small herd of such animals in which myotonia is transmitted from generation to generation. G. L. Brown and Harvey showed that this myotonic behavior is due to the repetitive response of the nervous end plates of muscle. If the nerve is excited by one electric shock, then, instead of producing one wave of depolarization, the end plate fires a whole train of them. Corresponding to this, if a resting myotonic animal wants to make a sudden movement it stiffens up for a while and will then start moving slowly with a spastic gait. In graver cases, the animal may lose its balance and fall and will be unable to get up for a while. As in human myotonia, exercise relieves the symptoms and so once the animal starts moving it quickly shakes off its symptoms and behaves normally. No myotonic behavior can be elicited in animals which find themselves in vigorous motion.

Brown devised a simple method by which the gravity of the disease can be measured and characterized by numbers, without the use of sophisticated equipment or electronic hardware. The method consists of hanging up the animal on belts just off the ground. The animal is kept in this position for 15 to 30 minutes, then is suddenly dropped to the ground. Previously, the animal was trained to run to a certain place, say a corner where it could find food and shelter. In the experiment the time is measured which the animal needs to reach this place. Myotonic animals, under the influence of the shock of the sudden fall, stiffen up and

need more time to reach their corner. My goats needed 7 to 20 seconds instead of 2 or 3. Under standard conditions, the experiment yields for one and the same animal surprisingly reproducible results, the running time differing on various days by no more than 0 to 3 seconds.

Another circumstance which adds to the attractions of myotonia is that the symptoms of this disease can faithfully be reproduced by the administration of a drug, 2,4-dichlorophenoxyacetic acid, commonly called 2,4D. Bucher has discovered that this drug produces myotonic symptoms in laboratory animals such as mice and rats. Eyzaguirre, Folk, Zierler, and Lilienthal showed that the electric behavior of muscle in 2,4D poisoning is similar to that of myotonic muscle, single electric shocks being answered by the firing of a train of waves of excitation. This possibility of reproducing a degenerative disease by means of a drug is most interesting. What lends additional fascination is the fact that 2,4D is also known as a powerful growth hormone for plants and is widely used as a weed killer, showing the basic unity of the principles on which the animal and vegetable world are built.

When trying to connect myotonia with E^* one could start with trying to show that 2,4D does something to E^* and does it in the same concentration as it elicits myotonic symptoms in the animal. Such a demonstration would bring the possibility closer that hereditary myotonia also is, essentially, a disturbance of E^*, related in its nature to the disturbance caused by the drug. According to the earlier workers 300 mg of 2,4D per kilogram ingested parenterally to rats is lethal, while 200 mg causes grave myotonic symptoms. The author repeated these experiments in Swiss albino mice and corroborated them. His results are summed up in Table IV. The table also shows the CO_2 production. The methods used here were the same as the ones used in the study of chlorpromazine. As can be seen, the myotonic symptoms were accompanied by a drop in the basal metabolic rate depressed by 200 mg to about one-third of its normal value; 250 mg per kilogram depressed it to one-

quarter, keeping the animal for days in a comatous condition in which signs of life could hardly be noticed. The drop in basal metabolic rate was accompanied by a steep drop in body temperature to 22°C or so, the whole condition resembling hibernation. The drop in the body temperature made it difficult to say how far the low basal metabolic rate and coma were due directly to the action of the drug. The gravity of the symptoms depended to a

TABLE IV

BASAL METABOLIC RATE AND MYOTONIC SYMPTOMS IN 2,4D POISONING IN MICE

2,4D injected (mg per kg)	CO_2 produced (mg per kg mouse per minute)	Gravity of myotonic symptoms
300	—	Lethal
200	38	+++
100	56	+
50	80	0
25	84	0
0	97	0

great extent on the outside temperature. If the animals were kept warm the symptoms accompanying myotonia were less severe and so the myotonic behavior could more clearly be observed. In any case, the symptoms indicated that the action of the drug was not limited to muscle, and that the drug affected some basic process which is common to all cells, as is energy transmission. The table even shows that the drop in basal metabolic rate (overlooked by earlier workers) is a more sensitive indicator of the drug action than the myotonic syndrome.

Since 200 mg per kg corresponds to a random concentration of 10^{-3} *M* our next question is whether 2,4D can affect *E* in vitro* in 10^{-3} *M* concentration?

It has been shown in Chapter 8 that 10^{-3} *M* concentration of 2,4D shifts the absorption spectrum of rhodamin towards the longer wavelength. It introduced thus some disturbance in the en-

ergy relations of that system which declared itself in the decrease of the excitation energy of the dye. Such a shift in energy values may very well cause trouble in cells where energy levels can be expected to be tuned carefully. Such a lowering of excitation energies for instance, may prevent the excited molecule from going from the singlet into the triplet state, the energy level of which is usually somewhat lower. So if the energy of the singlet excitation is decreased, the energy difference between the two states may become insufficient to allow the transition.

We have found 2,4-dichlorophenol to be a strong quencher of various excitations and so it was surprising to find 2,4D devoid of this action. On the other hand, 2,4D showed other activities not possessed by 2,4-dichlorophenol. One of these consisted of decreasing the lifetime of triplet excitation. As has been shown before, glutathione prolongs the lifetime of rhodamin from 10^{-3} seconds into the dimension of seconds. While a pure rhodamin solution, in the frozen state, showed a very poor phosphorescence in the phosphoroscope, in the presence of 0.01 *M* glutathione it showed a strong phosphorescence even in the slow moving phosphoroscope and showed a strong afterglow. Addition of 10^{-3} *M* 2,4D completely abolished this long life phosphorescence. In its presence the tube showed even in the fast moving phosphoroscope no increased phosphorescence; 2,4D thus completely eliminates the long life excitation caused by 0.01 *M* glutathione or 2% glucose. Since even ten times less 2,4D had a strong effect, the change could not be explained by an interaction of 2,4D with glutathione and the drug must have acted on the excitation, decreasing greatly the stability of the triplet state. If such states are important for the energy supply of the cell because of their long lifetime than it is easy to see that 2,4D has to cause a profound disturbance in energization. The experiments showed that 2,4D could eliminate the long-life phosphorescence even in 10^{-4} *M* concentration; 2×10^{-4} *M* also quenched the yellow phosphorescence of quinidine.

Another additional observation also indicated a labilization of

the triplet state. As has been discussed before, the fluorescent light emission of rhodamin is of an orange color while the phosphorescence is red. It was noticed that the 2,4D changed the light emission from red to orange in all concentrations in which it reduced the lifetime of the excitation. In its presence the rhodamin molecule is thus unable to go into the triplet state, or is able to do so only to a smaller extent than in its absence. The 2,4D made the triplet state an improbable one.

Analogous observations could be made also with riboflavine, in which the fluorescence is greenish-yellow, the phosphorescent emission orange. The experiment showed that 10^{-4} *M* 2,4D turned the color of the light emission of a frozen 0.0001 *M* riboflavine solution from orange into greenish-yellow, which color change indicated that the light emission came to a great extent from a singlet. In the phosphoroscope only a red-brown phosphorescent light emission was observed which indicated that the green fluorescence was not a "delayed" one, did not occur because the excitations were turned back from a triplet into a singlet, but occurred because they could not go into the triplet. In these tubes the phosphorescence was stronger than in the control containing riboflavine alone, which indicated that part of the excitation still went into the triplet but this triplet state was rather unstable.

That hereditary myotonia is, in some way, connected with a defective energy supply was also suggested by the observation of Hajdu and the author that 2,4D-dinitro-1-naphthol, which is a strong "quencher" of E^*, aggravates the symptoms of this disease in goats.

There are observations to indicate that, similarly to the action of 2,4D, the disturbance in hereditary myotonia is not localized to muscle, but is of a more general character. It is known, for instance, that myotonic goats are inclined to stillbirth. I also lost a goat in advanced stages of pregnancy without any apparent reason: the animal simply expired. I also have the impression that my goats become smaller from generation to generation though no

records have been kept on this. There is reason to believe that in man, too, myotonia is but a localized expression of a more generalized disturbance affecting some basic process of cell life. As is generally known, myotonia is often accompanied by cataract, which almost belongs to the classical symptoms of this disease. The disturbance thus strikes even such a relatively inactive organ as the lens. It is worth noting in this connection that 2,4-dinitronaphthol, which does disturb the energy household, also causes cataract;[1] this is the more remarkable since the lens, as I have shown, contains strongly fluorescent proteins. Buchthal and Clemensen, as well as de Jong, found in dystrophic forms of myotonia that the central nervous system was involved, many patients showing a certain dullness which often bordered on idiocy.

Myotonia is a rare disease, is not fatal, and has, consequently, been neglected by medical research, however fascinating it may be. What may lend a major practical importance to it is its close relation to myasthenia gravis. The symptoms of the two diseases are almost identical, only the sign is opposite: what is positive in the one is negative in the other. The two are mirror images. The myotonic muscle does more than it should, the myasthenic less. The myotonic one is worst when starting activity and symptoms fade out with exercise, while the myasthenic muscle does best after rest and tire rapidly. So the two diseases may be expressions of a disturbance of the same basic process, disturbed in the two cases in opposite direction.

The experiments reported in this chapter leave the problem of

[1] This cataract is known for its whimsical character. Sometimes it appeared after a light medication while it was often missing after a prolonged and heavy one. Personal disposition seems to decide. In collecting bullocks' lenses in great numbers from the slaughterhouse, I found most of them colorless. A small number, however, about 2–3%, were yellow, which color seemed to be due to the oxidized state of the flavines present. This color may indicate a sluggishness of the energy household, which might be in connection with the ease by which cataract is contracted under influence of a dinitrophenol medication.

molecular mechanism of myotonia unsolved. It supports the assumption that energy transmissions may be disturbed in this disease, possibly owing to labilization of the triplet state and the consecutive shortened lifetime of triplet excitations. Such an effect may be due to the shortage of stabilizing agents as glutathione, or to the presence of labilizing agents, analogous to 2,4D. In any case, the experiments quoted need to be extended and suggest further experimentation. They suggest, for instance, the search for substances rendering triplets more stable and transition into the triplet state more probable. There are substances which act this way. Some of them have been mentioned before: iodide, or iodine in its organically bound form. There are other such substances, even among normal cell constituents. As has been shown, serotonine is one of them, lysergic acid another. Extensive experimental work is possible on these lines and this work may eventually lead not only to a better understanding of the degenerative diseases of muscle but also may reveal ways of curing them. Thoughts on this line may find encouragement also in the experience that amphetamin and ephedrine were found to produce changes in the excitation of riboflavine similar to those produced by 2,4D. One of these, ephedrine, is known to have a beneficial effect on myasthenia, which may be found to be true even to a higher degree in other drugs, having a similar but stronger action in E^*.

Symptoms of myotonia can be relieved in man by quinidine. It seems worth noting that S. L. Baird (unpublished) found the drug equally active in myotonia, induced in mice by 2,4D. It may be more than a coincidence that while 2,4D shortens the lifetime of excitations, quinidine has a strong tendency to undergo a long-lived excitation in presence of a great variety of substances.

18. On the Thymus

The extirpation of the thymus often benefits myasthenic patients. There has to be thus some relation between this disease and the gland which also shows signs of hyperfunction in successful operations. If myasthenia is connected with the thymus and myotonia is its mirror image, then there might also be some relation between this gland and myotonia, which disease may be connected with a hypofunction of the thymus.

There is a curious disproportionality between the size of the thymus and the "size" of our knowledge about its function. If it is a gland of internal secretion at all, then in early childhood it is the biggest of such glands, while our knowledge about its function is next to zero. Somehow, the thymus failed to excite the curiosity of researchers. Treatises on endrocrinology, such as that of Pincus and Thimann, have not even a chapter on it. This lack of interest is probably due to the fact that the thymus can be extirpated without harmful effects. If it is not taken out, nature herself eliminates it by letting it atrophy in adolescence. If we can live without it, then its function cannot be very important. This logic is impeccable. The question is only whether nature does not follow a logic of her own? Histologically, the thymus is a lymphoid tissue, part of the lymphatic system, together with the spleen, bone marrow, and lymph glands, though it has some peculiarities. So if the thymus is a gland of internal secretion, then it may well be that the whole lymphatic system is one, although it has other functions as well. One could imagine that this gland produces a substance involved in the process of building the body, in synthetizing its protein, which substance is in greater demand when the building is going on most actively, in early childhood. So in this period the lym-

phatic system may become supplemented by the thymus. In adolescence, when the building is completed, the supplementary gland retires. This could also explain why the extirpation of the thymus causes no trouble, even if its function is important—because the rest of the lymphatic system takes over. There are many examples of such compensation. So our logic may have holes and it may just as well be (for all we know) that the whole lymphatic system is one big gland of internal secretion, may be even the biggest and most important of all, a possibility which has hitherto been overlooked only because it is too obvious.

These assumptions might also explain why thymus extirpation benefits only part of the myasthenic patients. If myasthenia is due to the dysfunction of lymphatic tissue, then the success of the thymus extirpation has to depend on the question how far this dysfunction was localized to the thymus.

Myasthenia is characterized by a great fatigability of muscles. I was often impressed by the great fatigability of infants. I would almost say that all infants are myasthenics. An adult dog will outrun almost any other animal, but a three-year-old child (which has nearly the same amount of muscle for body weight) tires in a short walk and is unable to lift a heavy weight. Nature may have seen to it that while the building process is going on, it should proceed undisturbed by exaggerated function. Nature often kills more than one bird with the same stone and it is possible that the same substance which is involved in the building process might also keep the muscles at a low functional level.

The tentative assumption that the thymus (and, possibly, other parts of the lymphatic system) produces some substance involved in protein synthesis finds some support in the observations of Gudernatsch which, a few decades, ago, focused attention for a short while on the thymus. Gudernatsch found that tadpoles, fed on thymus glands, showed a retarded metamorphosis with an increased growth, developing into giant tadpoles. Attempts to isolate the active substance responsible for this effect failed and the in-

terest gradually faded out, leaving the impression that the thymus supplied some "nutritional factor" rather than a growth hormone. The hormonal theory was revived more recently in experiments of Harms, who found general atrophy in salamander larvae after extirpation of the thymus, atrophy which could be prevented by thymus transplants. Gudernatsch also showed that the feeding of thyroid substance had an action opposite to that of the thymus. While the latter retarded metamorphosis and promoted growth, the thyroid promoted metamorphosis and inhibited growth. While giant tadpoles were produced by the former, dwarf frogs were produced by the latter, suggesting some sort of an antagonism between the two.

There is no need for the author to sum up the literature of the subject, this having been ably done by Gudernatsch and Hammar. One could almost say that all possibilities have been expressed about the function of the thymus, the integral being zero.

Techniques of isolation have been perfected so much during the last decade that, as a rule, the isolation of a substance, in itself, does not constitute a major problem. What the isolation depends on is a test for the unknown substance which allows us to estimate its quantity in different fractions in the course of manipulations. The difficulty, in this case, is that we have to find a test for something that has to be found by that test. This is a circle which, somehow, has to be broken.

The author made an abortive attempt in this direction reasoning in the following way: small quantities of biologically active normal constituents of the body usually produce effects in the animal only if there was a deficiency before. Vitamins, for instance, produce striking effects only in an avitaminostic condition. Should myotonia be due to a lack of the hypothetic thymus hormone, then myotonic goats would be the test object for the hormone *par excellence*. So four female myotonic goats were trained according to G. L. Brown for the measurement of their running time. Then they received daily an injection of a thymus extract which con-

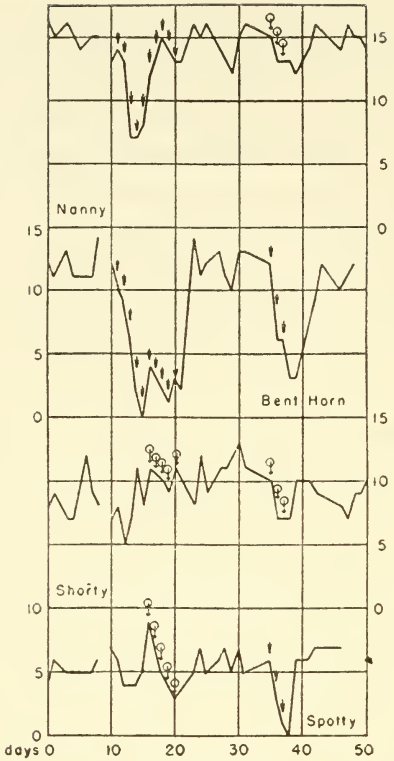

FIG. 28. Running time of myotonic goats. Abscissa: days. Ordinate: running time in seconds. Arrows: injection of thymus extract. Arrows with circle: injection of saline. For details see text.

tained in 5 ml the extractive matter of 150 g frozen calf thymus, which contained the fraction not precipitated isoelectrically, was not thrown down by methanol but was precipitated by an excess of acetone.[1] The results of this experiment are reproduced in Fig. 28. The external conditions were not favorable and so the normal

[1] *Prep. 1.* 16 Kg of frozen calf thymus glands were minced, suspended in 28 liters of water, and sent through a colloid mill. After the addition of 414 ml of glacial acetic acid, the suspension was diluted with 30 liters of water. The undissolved material was separated on a Sharples centrifuge and

running times were not as constant as in previous work with Hajdu.[2]

The results seemed positive in the case of "Bent Horn," "Shorty," and "Spotty," three young goats, about 15 months old. The result was negative with "Nanny," an old goat which belonged to the original flock received in full grown condition four years earlier. Figure 28 suggests the presence of an active substance in our extracts, capable of alleviating or abolishing the symptoms of myotonia. The extracts had no acute effect and developed their action gradually. This action outlasted the injections and disappeared gradually. The active matter present thus did not act as drugs do, which act the strongest when their concentration is the highest.

The myotonic goat is not an ideal test object. The size and smell of the animal are bigger than desired; the expense and labor involved in keeping it are considerable. The measurements are time consuming. So the final isolation of the active agent, if any, will

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the clear liquid concentrated *in vacuo* at 20–23°C in two steps to 1.4 liter. The fluid was stored overnight at —20°C, on thawing the precipitate removed on a small Sharples centrifuge. Four volumes of methanol were added, the precipitate separated, the fluid stored at —20°C, and the newly formed precipitate separated again. The fluid, when liberated from its methanol content, was poisonous to mice, probably owing to its potassium content. Concentrated sulphuric acid was added gradually under strong mixing, whereupon a white crystalline precipitate formed, presumably consisting of K_2SO_4. H_2SO_4 was added until the addition of 10% sulphuric acid caused no appreciable precipitate. The pH at this state was about 5.

The solution was concentrated *in vacuo* to 500 ml. Two liters of acetone were added, the precipitate separated, and shaken out with acetone. The supernates were rejected and the semi-liquid precipitate redissolved in 500 ml water. This fraction was injected after neutralization, a small quantity of penicillin being added to prevent infection.

[2] On the first three days of the treatment, the running time was measured one hour before and one hour after the injection. The two measurements showed no marked difference so it was concluded that the injections had no acute effect and subsequently the running times were measured only before the injections.

have to await the development of a simpler test, or the setting up of the organization needed for a more extensive testing on goats.

The isolation of the active principle of the thymus is the more urgent because it is not impossible that myotonia and muscular dystrophy are related diseases and involve the hormonal activity of the thymo-lymphatic system.

There are also a few observations to suggest that the thymus may be, in some way, involved in the production of nucleotides, and thus also in the production of nucleic acids (which, in their turn, are involved in protein synthesis). The extraordinary wealth of the thymus in DNA may be taken as an indication of this. My thymus extracts gave only one reaction not given by analogous extracts of other organs: they altered the light emission of acridine orange and acridine yellow if these dyes were added in a low concentration (10^{-4} *M*), and gave a precipitate if these dyes were added in a higher concentration. The light emission (as observed under the UV lamp) shifted towards the red in the case of acridine orange and was quenched in the case of acridine yellow. Nucleic acids and adenine nucleotides show analogous reactions and the behavior of my thymus extracts could be explained by their extraordinary wealth in nucleotides. The acridine-dye complexes of nucleic substances are very stable and so it was not surprising to learn that Lewis and Goland found that acridine dyes, like trypaflavine caused an atrophy of the thymus. An attempt seems to be indicated to try acridine derivatives as therapeutic agents in cases of myasthenia in which the thymus is suspected to be the culprit.

The promising yellow dye which I isolated earlier from thymus extracts (1955) turned out to be an oxide of ascorbic acid which is especially abundant in this gland.

19. On Oxidation, Fermentation, and Cancer

Of the two biological energy-producing mechanisms no thought was given in this book to fermentation because there is no reason to suppose that E^* is involved in this process which consists, essentially, in coupling phosphate to carbohydrate and then rearranging the H and O atoms within the molecule in such a way that the energies inside the molecule are shifted and the originally "low-energy" P—O—P becomes a ~P which is then transmitted to other molecules, such as ATP, and aliments biological action. Fermentation, essentially, is a series of group transfer reactions which belong to the realm of classical chemistry, the energies generated being bond energies.

Contrary to this, biological oxidation is the utilization of the thermodynamic potential between H and O and we were led to suppose that in this process E^* plays a role, energy being liberated and transferred at certain points in the form of electronic excitation. We were also led to suppose that this electronic excitation has specific forms which were probable only within the water structures which the protoplasm generates around itself, the electrons forming triplets. We have also seen that the role of O_2, in this process, is not limited to acting as the final electron acceptor but that the oxygen, by means of the perturbations of the electromagnetic field, greatly influences the reactivity, i.e., the transition probabilities of one of the central members of the oxidative chain. Transition probabilities of triplets greatly depend on the perturbations of the electromagnetic field and so have to depend on the perturbations caused by O_2. But the triplets themselves also cause such a perturbation, which can be expected to influence the formation and reactions of other triplets. Aerobic cell life has thus as

its foundation a perturbed electromagnetic field, the perturbation of which might be one of the most important parameters in biology.

This brings us to the intriguing interrelation of oxidation and fermentation, usually summed up as the "Pasteur effect." By this is meant a certain interdependence of the two processes, the classical example of which is found in yeast. Under anaerobic conditions yeast lives on fermentation, but if oxygen is admitted it shunts over to oxidation. The mechanism of this "shunting over" has occupied biochemistry to a great extent and many theories have been proposed for its explanation. Our considerations offer a new, and maybe simpler one. If O_2, by its mere presence alters the reactivity of central catalysts, as that of riboflavine, and makes new electronic transitions possible, then it might seem natural that in its presence chemical events should take a new course, the priming action being done by the magnetic disturbance caused by the paramagnetic O_2 molecule. No substance other than riboflavine has shown a similar behavior towards oxygen. This behavior is thus specific and it is reasonable to think that it has its biological meaning and is not accidental.

Another intriguing interrelation is that of pathological growth with fermentation and oxidation. As is known from the classical studies of O. Warburg, cancer cells live to a greater extent on fermentation than do normal cells, and Warburg ascribes a primary role to this change in the genesis of cancer. If oxidation and fermentation represent merely two alternate pathways of energy production then it is difficult to see why a shift in the balance of the two processes in favor of fermentation should lead to pathological growth.

The considerations presented in this book offer an explanation. If excitation energies play a major role in biological energy transmissions and the various biological processes are actually driven by the energies of triplet excitations then events will greatly depend on the perturbation of the electromagnetic field; this pertur-

bation is started up by oxygen and is contributed to by the triplets formed under its priming action. Aerobic life thus means qualitatively a different type of life, which has the perturbed electromagnetic field as its foundation and triplet excitations as its instrument. Warburg has repeatedly emphasized the importance of structure, oxidation being bound to structure, while fermentation is not. We could also use, instead of "structure," the words "solid state," by which physics means an orderly state with periodicities. In this book we have extended this idea of structure and orderliness beyond protein or protein-lipin complexes into the water and shown that the two structures form one single unique system. It seems likely that only structures can build structures, that is that only the structure to which oxidation is bound can build around itself extensive water structures which complete it. The water structures thus generated play then a basic role in the handling of the excited electrons and can thus be expected to play also a fundamental role in the energy transmissions between oxidation and the systems which are driven by its energy. There are reasons to suppose (or no reasons to exclude) that there are more direct connections between oxidation and biological functions than the connection over ~P's, and it seems rather likely that oxidative energies need not necessarily be invested into ~P's before they can be used by the cell but that biological systems may also be coupled more directly to oxidation and use its excitation energies. The immediate cessation of activity in the central nervous system on removal of oxygen or the poisoning of its activator by cyanide plead for such direct relations. So if the energy of triplet excitation is used also to maintain the system of structures which generates these excitations, the whole system has to collapse, structures must disintegrate in a vicious circle if anything goes wrong.

Fermentation and oxidation thus do not mean merely alternate pathways of energy production. They mean a different way of living. Fermentation, to which the cell is driven under anaerobic conditions, is based on group transfer reactions which demand no

structures, neither structures of water nor of solid matter, nor a perturbed electromagnetic field. The other way of living, the aerobic one, demands order, both order of the solid matter and order in the water, and has a perturbed electromagnetic field for its foundation.[1] It seems reasonable to suppose that triplet excitations, and energy transmissions which presuppose an orderly structure, play a role not only in oxidation but also in the most varied biological functions and their regulations, and thus demand not only structure and order but also a perturbed electromagnetic field, which is established by oxidation and its triplets under the priming action of O_2. Cell division may belong to these functions, its orderly mytotic way being linked to order, structure, and what goes with it.

Looking at cancer and its relation to oxidation and fermentation from this angle, things may seem somewhat clearer. If O_2 is withheld from tissues of a higher organism for a longer period, death follows. If, however, O_2 is withheld but periodically, as in the experiments of Cameron and Goldblatt, and restored before death ensues, then we can expect not only that the oxidative system will become disorganized but also that there will be degeneration of all those higher functions and regulations which demand structure and a perturbed electromagnetic field for their function and ener-

[1] It would be interesting to measure the magnetic susceptibility of aerobic, living, and respiring cells and compare it with the susceptibility of anaerobic, as well as dead, cells. If the suggestions contained in this chapter are correct, the first should be found slightly paramagnetic, beyond the paramagnetism induced by the O_2 present, while the latter should be diamagnetic. A shift in the latter direction should be found in cancer cells.

The dielectric constant of cancer should show less frequency dependence than that of normal tissues, cancer having a lower degree of organization and with it also less water structure.

No light emission was produced in illuminated anaerobic frozen riboflavine solutions by placing them in a magnetic field of about 20,000 oersted. So it is not a simple static disturbance of the field which causes the emission in the presence of oxygen but there are more involved interactions between the O_2 molecule and the excited riboflavine.

gization. The cell will thus revert to a more primitive way of life with its lower level of organization, which is not dependent on ordered structures, which also lacks the subtle regulations and has thus (as in yeast) unlimited proliferation as one of its characteristics, and can depend for its energy supply only on fermentation.

20. Conclusion

After having been submerged in details through nineteen chapters, we may conclude by coming to the surface in order to look around, find our bearings, and ask what all this was about.

Going back to our point of departure, to the "chiaroscuro," we should see its outlines somewhat clearer now. Biochemistry is not an independent principle; it is dependent for its advance on progress made in other fields; in the first place in pure chemistry. There are definitely two periods discernible in the history of this science. The first was heralded by Lucretius and Epicurus, who professed that matter was built of small, indivisible units, atoms. What chemistry has done in the subsequent two milleniums has been, essentially, to find out how many different sorts of atoms there are —about a hundred—to isolate them, name them, and establish the ways and forms in which they are linked together. The different atoms have been symbolized by letters, their links by dashes, so that by means of letters and dashes we can write symbols on paper which give a fairly clear picture of the structure of complex molecules.

Biochemistry, following in the footsteps of chemistry, has been wonderfully successful in analyzing structures and reactions which could be described in these terms and by their symbols, by letter-dash-letter (as P—O—P), following up the various atoms and bonds in their way through the maze of biological reactions.

The second step of the history of chemistry was heralded by Bohr and his orbits in 1913. The theory came of age in 1927 with Schrödinger's wave equation. In this theory, the atom is no more an indivisible unit, but a cloud of electrons, or more exactly, a cloud of the probability-densities of electrons. Molecules are

clusters of such clouds of phantastic and changing shape. A great number of changes can occur within these clouds which may all be covered by the same letter-dash-letter symbol, which thus is inadequate for their description. Biological phenomena, possibly, are to a great extent the expression of such subtler changes which take place in dimensions unknown to classical chemistry. They belong to the realm of quantum mechanics and can be described only in its language.

Biochemistry has taken no cognizance of this progress yet, or has done so only to a small extent, sporadically, and is still a Lucretian or Epicurian letter-dash-letter science. Accordingly, it has made no progress in the analysis of the reactions which cannot be described by these symbols. Hence the "oscuro" of the "chiaroscuro."

This is not meant as a reproach to the biochemist. The author spent half a year at the Institute for Advanced Studies, at Princeton, enjoying this wonderful institution's boundless hospitality. He did so in order to be able to rub elbows with those who know most about electrons, hoping to find help for a better understanding of biological phenomena. He found a profound and sympathetic interest in biology. However, when he revealed that living systems contain more than two electrons, physicists turned their backs on him in terror, mathematical difficulties becoming insurmountable.

Lucretian biochemistry involves the assumption that no interaction can take place between molecules without their touching one another. Support is given in this book to the idea that manifold interactions can take place without such bodily contact, either through energy bands or through the electromagnetic field, which thus appears with water and its structures as the matrix of biological reactions.

In accordance with the basic concepts of Lucretian chemistry, biology dealt with molecules and their aggregates as isolated units, separated by the water which fills the space between them. The

material presented in this book pleads for the assumption that water is more than this: it is part and parcel of the living matter itself. One of the main functions of protoplasmic structures may be to generate in water those specific structures which make forms of electronic excitations and energy transmissions possible which would be improbable outside these structures. The solid matter and the water of the cell form together that unique system which has the queer property of being alive.

I have no doubts that the coming century will witness a profound revolution, extension of biology, the establishment of a quantum mechanical biochemistry, built on top of the Lucretian one. This book may be but one of the early swallows of this spring.

The biologist, venturing into this new field, can do so only at the danger of errors, many of his interpretations being found at fault later. He can only hope that through the door, left ajar, researchers will follow him who are more qualified to deal with quantum mechanical problems and find him at fault at many points. His only reward will be the fleeting glance he could throw at a future biochemistry, from the elevation of which the letter-dash-letter symbols will look like skeletons which can tell us no more about the real nature of life than the fossil bones of a dinosaurus can tell us about that animal's reflexes or sexual relations.

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