Photobiology

BIOLOGY
COLLOQUIUM
1958

OREGON STATE CHAPTER OF PHI KAPPA PHI
OREGON STATE COLLEGE • CORVALLIS • 1958

Nineteenth Annual Biology Colloquium
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Photobiology

EDITED BY
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Foreword

The Biology Colloquium is conducted in a spirit of informal discussion and provides opportunity for participation from the floor. The colloquium is sponsored by the Oregon State Chapter of Phi Kappa Phi with the collaboration of Sigma Xi, Phi Sigma, Omicron Nu, and Phi Lambda Upsilon. Sigma Xi assumes special responsibility for the colloquium luncheon. The other three societies provide afternoon teas. The college Library arranges special displays of the writings of colloquium leaders and notable works on the colloquium theme.

In 1956 the National Science Foundation provided a grant of money to help finance the colloquiums for a three-year period. Theme selected for next year is "Marine Biology."

Grateful acknowledgment is made of the cooperation and interest of the several faculties of Oregon State College concerned with biology, of those biologists contributing to the program, of Chancellor John B. Richards, President A. L. Strand, and other executives of Oregon State College.

The first Biology Colloquium was held March 4, 1939, with Dr. Charles Atwood Kofoid of the University of California as leader, on the theme "Recent Advances in Biological Science." Leaders and themes of succeeding colloquiums have been:

1940, Dr. Homer LeRoy Shantz, chief of the Division of Wildlife Management of the United States Forest Service, theme "Ecology."

1941, Dr. Cornelis Bernardus van Niel, professor of microbiology, Hopkins Marine Station, Stanford University, in collaboration with Dr. Henrik Dam, Biochemical Institute, University of Copenhagen, theme "Growth and Metabolism."

1942, Dr. William Brodbeck Herms, professor of parasitology and head of the Division of Entomology and Parasitology, University of California, theme "The Biologist in a World at War."

1943, Dr. August Leroy Strand, biologist and president of Oregon State College, theme "Contributions of Biological Sciences to Victory."

1944, Dr. George Wells Beadle, geneticist and professor of biology, Stanford University, theme "Genetics and the Integration of Biological Sciences."

1945 colloquium omitted because of wartime travel restrictions.

1946, Dr. Robert C. Miller, director of the California Academy of Sciences, theme "Aquatic Biology."

1947, Dr. Ernest Antevs, research associate, Carnegie Institution of Washington, theme "Biogeography."

1948, Dr. Robert R. Williams, Williams-Waterman Foundation, theme "Nutrition."

1949, Dr. Eugene M. K. Geiling, head of the Department of Pharmacology, University of Chicago, theme "Radioisotopes in Biology."

1950, Dr. Wendell M. Stanley, in charge of Virus Laboratory, University of California, theme "Viruses."

1951, Dr. Curt Stern, professor of zoology, University of California, theme "Effects of Atomic Radiations on Living Organisms."

1952, Dr. Stanley A. Cain, conservationist, University of Michigan, theme "Conservation."

1953, Dr. Wayne W. Umbreit, head of the Department of Enzyme Chemistry, Merck Institute for Therapeutic Research, theme "Antibiotics."

1954, Dr. Daniel Mazia, professor of zoology, University of California, theme "Cellular Biology."

1955, Dr. Ernst Mayr, curator, Museum of Comparative Zoology, Harvard University, theme "Biological Systematics."

1956, Dr. Henry Borsook, professor of biochemistry, California Institute of Technology, Pasadena, theme "Proteins."

1957, Dr. Ira L. Wiggings, director, Museum of Natural History, Stanford University, theme "Arctic Biology."

Colloquium Participants

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Colloquium Leader

F. W. WENT, Ph.D., Director, Earhart Plant Laboratory and Professor of Biology, California Institute of Technology, Pasadena.

Colloquium Speakers

JAMES A. BASSHAM, Ph.D., Radiation Laboratory, University of California, Berkeley.

FREDERICK CRESCITELLI, Ph.D., Professor of Zoology, University of California, Berkeley.

DONALD S. FARNER, Ph.D., Professor of Zoophysiology, State College of Washington, Pullman.

C. S. FRENCH, Ph.D., Director, Department of Plant Biology, Carnegie Institution of Washington, Stanford.

F. A. GILFILLAN, Ph.D., Dean, School of Science, Oregon State College, Corvallis.

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LEO VERNON, Ph.D., Associate Professor of Chemistry, Brigham Young University, Provo.

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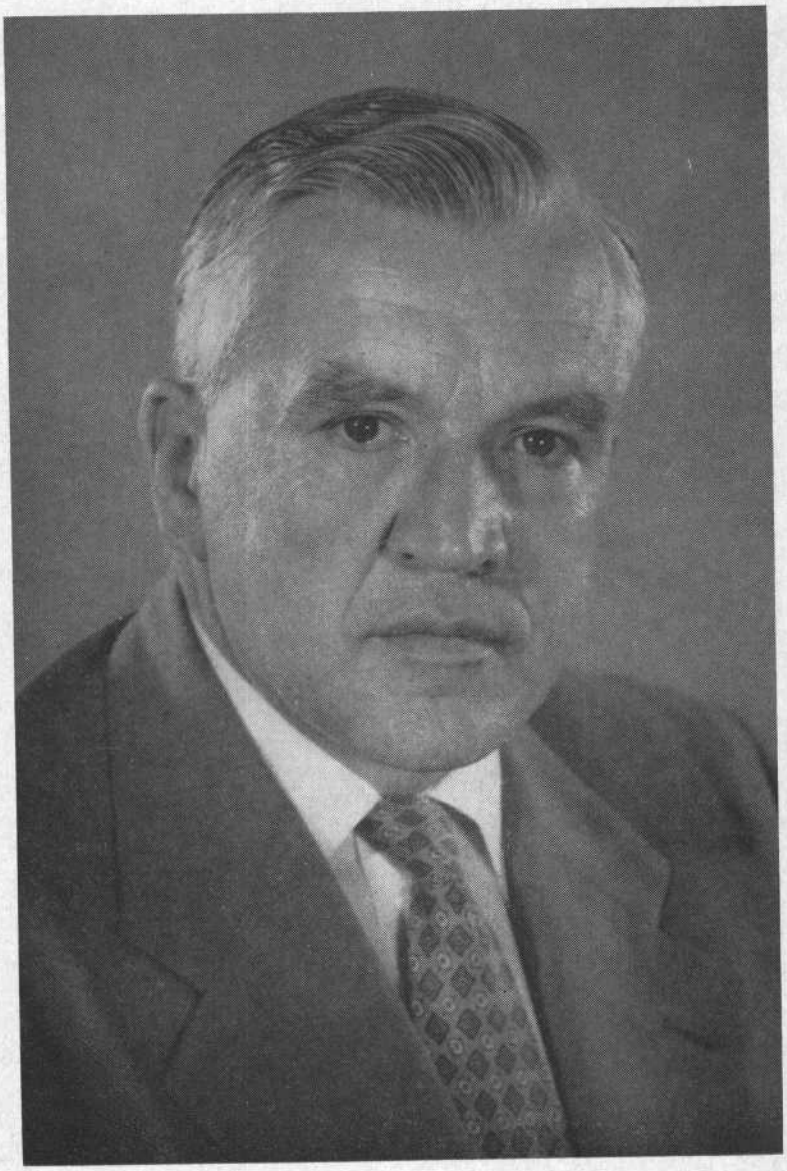

F. W. WENT, Ph.D.

Leader of Nineteenth Annual Biology Colloquium

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Nineteenth Annual Biology Colloquium

Theme: PHOTOBIOLOGY

Opening of the Colloquium

President Strand: We welcome you to the 19th Biology Colloquium of Oregon State College. We have a good subject, a distinguished leader, a very good supporting cast, and no doubt we will have a high standard of particpation from the audience. Even the weather is propitious.

A colloquium, or a *conversation*, about biology may sound as if it had been invented, or revived, by Dr. Hutchins, but I assure you he had nothing to do with it. However, it's consistent with "the Great Conversation," part of which certainly should include biology.

The idea was generated on this campus in the late 1930's and there must have been some survival genes involved in the conception, for the Colloquium has been going, and growing, since that time. It was a good stroke of scholarship promotion by Phi Kappa Phi and ably supported by Sigma Xi, but the most distinctive feature of this meeting is usually found in the audience. A good proportion of those who attend are not professional biologists; some are not even scientists. They come from many fields. They are people who are interested in biology, as everyone should be who is alive and thinks.

Through the years some great scientists have come to lead the Colloquium or to join in the program, and the discussions. Lately, some very distorted ideas are being voiced by persons who evidently know little about science and have had little or no personal acquaintance with scientists. A good example is to be found in last week's *Time*. The dean of the Pacific School of Religion—located so close to the California Radiation Laboratory that it probably gets hit by maverick nuclear particles now and then—thinks that scientists in our civilization compose a new sort of religious order, and set themselves apart from the world—in discipline, language, and attitude. Their attitude, he says, is that of *contemptus mundi*, a scorn for the ordinary pleasures and privileges of life. He charges that scientists, like priests, are disposed to claim infallibility, "but the most virtuous priest may have virtue corrupted by stupidity and ignorance, and the most intelligent scientist may have his wisdom distorted by pride and arrogance, and the lust for personal prestige."

I haven't found the scientists I have known to be that way at all. There have been some cases where a scientist made a mistake, or made a mistaken claim, early in life and spent the rest of his years defending a lost cause. But none of these was great; they were just great lessons to younger men. The scientists I have known, and others I have read about, have been humble men and more to be criticized for their extreme tentativeness rather than for any wisp of infallibility about them.

Scientists are subject to variation as everything else. Some are more worldly than others. Most have the capacity to lose themselves in their work and accordingly place a much lower value on the "ordinary pleasures and privileges of life." All the great ones have a devotion to truth and a regard for excellence that, from the time of Copernicus and Vesalius, have given meaning to Western culture.

I think these are the very values the degree of regard for which in America, during the years which lie immediately ahead, will determine the kind of education we have and the kind of a country we will be living in. If we continue to honor the pleasures and privileges of life more than we honor merit and excellence, our schools will deteriorate at the very time they should be improving rapidly.

Rather than blasting the scientists, or falsely categorizing them, we should emulate some of their characteristics in our society and heed the advice that some of them are giving us.

I trust this will be a stimulating and profitable meeting for all of us. I thank Dr. Went for serving as leader and all the others who have come to joint in the program. We're glad you're here.

Plant Growth in Response to Light

F. W. WENT
Director, Earhart Plant Laboratory, California Institute of Technology

When we look over the impressive list of important Biology Colloquia organized over the last 20 years here in Corvallis, we see that many of them were concerned with *particular biological processes,* such as nutrition, or growth; or *areas of investigation,* such as Arctic or aquatic biology, or conservation, or special *biological components,* such as proteins or viruses. This time the unifying principle of the Colloquium is, we might say, a technique, like a previous Colloquium on Radioisotopes. For we are talking about Photobiology, and we are this time not concerned with light production by organisms, but with effects of light.

Biological reactions are practically always coupled reactions in which, through the intermediary of an enzyme, molecular transformations are performed. The amount of energy required to remove a hydrogen atom attached to one oxygen atom is enormous, but when it can be coupled with the transfer of another radical to the oxygen, the amount of required energy is much less since all energy comes in discrete packages or quanta, and only quanta of the proper energy level can be used to cause these coupled reactions.

Planck has shown that light also is quantised, and that each photon carries a specific amount of energy ($h\nu$), the quantity depending on the wave length. It now turns out that the energy required for coupled biological reactions is usually that of a visible light quantum. Lower energy quanta, in the infrared e.g., are insufficient, whereas higher energy quanta, such as in the far ultraviolet or of X-rays, are too high in energy, and can break chemical bonds without coupled reactions. They can therefore produce free ions, and those short wave radiations are called ionizing radiations.

Usually the energy necessary for chemical reactions in the cell is provided by molecular resonance, that is to say that one molecule with the right amount of energy transmits this energy to an adjacent molecule or enzyme. But provided light quanta can be absorbed by a pigment, this light, by way of the activation of the pigment, can be transferred to other molecules and thus drive reactions. This means that only pigments which are closely associated with other cell constituents, the absorbed pigments such as chlorophyll, carotenoids, cytochromes, can transfer energy, whereas the pigments dissolved in water, such as the anthocyanins and flavones, cannot.

All this explains why visible light is so effective in biological reactions, and why it can produce such specific effects: (1) because of its specific energy level and (2) because it is absorbed only in specific parts of the cell where the absorptive pigments are located.

Among all experimental tools which the biologist has at his disposal, and with which he can probe into the secrets of the cell and of the organism as a whole, light is certainly the most remarkable. It not only allows him to observe his objects, but he can manipulate processes and reactions in a completely specific way. This is due to specific absorption of certain wavelengths by specific pigments, which may be located in certain parts of cells or organs, and much of our discussion in the next two days will center around such absorption phenomena.

Before starting the discussion of my specific subject, the effects of light on growth, it might be interesting to answer the question why we should be concerned specifically with light within the *visible* range of wavelength. After all, the visible range is only a very narrow band in the whole spectrum of electromagnetic radiations. Actually two reasons can be given. The first one is that the energy of light quanta in the visible range is such that it can cause the shifting of electrons within a molecule but it usually is insufficient to cause their liberation, such as is possible with the far ultraviolet and shorter wavelengths. For this reason energy quanta in the visible light fall within the range of biological reactions such as hydrations, reductions, and oxidations, but they are not destructive. Longer wavelengths, such as in the infrared, carry quanta of insufficient energy to affect biological reactions.

There is another reason why the visible light coincides so closely with the wavelength range which stimulates biological reactions. Since the light absorbed by our visual pigments can be perceived only through the chemical transformations which are induced in the retina, only wavelengths which can cause reversible reactions inside the re-

tina will be effective and thus the spectral ranges for influencing chemical reactions and for inducing vision must necessarily be the same.

Light is effective in a large number of totally different reactions and processes in plants. Actually the number of light-sensitized reactions in plants seems to be far greater than in animals, where especially in the higher ones most light responses are perceived by the eye. In the plant we have, in addition to photosynthesis, also photoperiodism, phototropism, photomorphosis, etc. The two former ones will be discussed in detail by the next speakers and, therefore, I will restrict myself to the problems of photomorphosis and phototropism.

Let us start with a consideration of the effects of light on growth in length. We have here a multitude of responses which belong to very different types. In the first place the phenomenon of etiolation exists. This means that a plant which is growing in complete darkness, provided it has a sufficient amount of storage food, will grow excessively in length. Already very small amounts of light suffice to reduce this growth and make the plants develop normally, that is to say with relatively short internodes and large leaf surfaces. As we increase the amount of light, growth may be stimulated again apart from its effect on photosynthesis, while finally at light intensities in the range of full sunlight stem elongation is severely reduced.

Let us first consider the phenomenon of etiolation. This has been studied in a number of different objects and on a number of different processes. The most generally known effect of light on seedlings grown in darkness is that of reduction of stem elongation. When, for instance, peas are germinated in darkness, they will elongate into ivory white, spindly, sprawling stems. Amounts of light which are hardly in the visible range of intensities can reduce the stem elongation from 10 to 50%. This effect is in the form of an induction, that is to say, the reduced growth can occur hours after the light exposure. This means that not just the growth process as such is affected by the light but that factors leading to the growth reaction are influenced. Since we already know a few of the chemical steps involved in this growth reaction, it becomes interesting to ask in which of these steps light takes a part.

The first problem is how the light is absorbed. This has been investigated by the study of the action spectrum of the light which can prevent etiolation. For peas and for grass coleoptiles it was found that red light was particularly effective, whereas green and blue were practically ineffective. This means that the light must be absorbed by a pigment absorbing in the red region of the spectrum with perhaps a slight secondary absorption in the blue.

Another expression of etiolation is found in leaf growth. Bean and pea seedlings when grown in complete darkness develop only very small leaves. Light exposures which will reduce stem growth also increase leaf development. The action spectrum for this phenomenon has been determined as well and was found to be practically the same as that for the prevention of stem etiolation, that is to say the red wavelengths are also far more effective than the other spectral regions.

Another phenomenon which is connected with etiolation is that of the straightening of the plumular hook of so many seedlings. As long as they grow in the soil the growing point and the enclosing cotyledons are bent backward 180° and thus the upper part of the hypocotyl leads in the breaking of the seedling through the soil. As soon as the plumule emerges from the soil it straightens up. This phenomenon requires only very small amounts of light as investigated especially by Withrow. As for the effect of light on leaf growth, the Product law holds, that is to say, the same quantitative response is obtained when the product of light intensity and exposure time is the same, or in other words, when the same amount of light energy has been applied.

In the case of the prevention of stem etiolation, one single exposure to a small amount of light is not very effective but a series of repeated light exposures at 24-hour intervals are most effective. The reason for this ineffectiveness of a single exposure is that while the internode which is exposed to the light has reduced growth the next internode shows compensatory growth and thus the overall reduction in length is very slight. However, daily repeated light exposures prevent compensatory growth and thus strong growth retardations are found.

When tomatoes or other plants are grown in monochromatic light or in light of a restricted wavelength range a completely different set of growth reactions occur. Whereas etiolation is prevented by small amounts of red light, continued exposure of tomato and other plants to red light results in excessive elongation, poor leaf development, and in general a weak condition. This occurs despite the fact that the light intensity may

have been sufficient for normal photosynthesis. When similar plants are grown in blue light of high intensity their internode length remains small which is exactly opposite to the effects of low intensities of blue light which hardly affect stem elongation. To get normal development of such plants it is necessary to grow them in light which is high in both red and blue parts of the spectrum. The conclusion to be drawn from this is that whereas all etiolation phenomena can be explained on the basis of the effects of light which is absorbed by a single pigment, normal development of plants grown at high intensities is possible only when both red and blue light are available. Either one is not enough. This means that a pigment absorbing both in red and blue, such as chlorophyll, is not sufficient and cannot explain this phenomenon.

Ever since Sachs for the first time recorded growth in length of different plants over a 24-hour period, it is known that usually stem growth occurs predominantly during night and that during day, plants like tomato, corn, etc. have a strongly reduced growth rate. Recordings of their growth rate in air-conditioned greenhouses have shown that these fluctuations are not due to temperature differences between day and night but are the result of inhibition of stem elongation by very high light intensities. This inhibition occurs only in full sunlight. Whereas on cloudy days growth occurs as well during day as during night, yet the total growth per 24 hours tends to be the same for sunny and cloudy days.

Let us now inquire as to the physico-chemical basis for these light effects. Ever since the first plant growth hormone, auxin, was known, light effects on stem elongation have been attributed to effects of light on auxin. Although I had found that light did not destroy auxin and also Kögl and Haagen-Smit had shown the same thing with their crystalline auxins, there was a strong tendency to attribute inhibitions of stem growth to auxin destruction. However, in spite of much experimental work, there is no evidence today that auxin inside the plant is inactivated or destroyed at either high or low light intensities. We, therefore, will have to look in another direction for an explanation for growth retardations by light. More recently it was found that gibberellin can cause pea plants to elongate in light as much as dark-growing seedlings do. Therefore, it seems most interesting to analyze the effect of light on the gibberellins which are normally present inside the plant. There is a chance that light would affect them and thus cause a decrease in growth. Yet *in vitro* gibberellin certainly is stable towards light. There exists also the possibility that the effect of light can be explained not by the different chemical components which together induce growth but that light influences their reaction rates inside the cell. This is a good possibility which has not been investigated as yet.

Whereas all the previously discussed phenomena could be put under the heading of photomorphosis and could all be considered as an adjustment of the plant to its environment, another group of light-induced reactions enable the plant to react more specifically to the light condition of its surroundings, the same way as the higher animals respond to the external stimuli of light, gravity, sound, etc. These are the phototropic reactions which we find in the majority of plants. Different organs respond in a different way and thus the majority of stems bend toward the light, many roots turn away from light, and leaves place themselves perpendicular to the incident light. Most of these responses are due to differential growth of the different sides of the organ and therefore are relatively slow, but in a few cases phototropic movements are caused by turgor changes in cells and these may be relatively fast, such as the leaf movements of legumes.

Once it was recognized that plants responded to the direction of the light, and it also was recognized that their phototropic movements were caused by differential growth, a parallelism was sought between etiolation and phototropism. Already 125 years ago, De Candolle suggested that the phototropic movements of the sunflower were due to the faster growth of the flower stalk at the side away from the sun, very much as if this side were etiolated. This theory was revived by Blaauw and he made this into an all-embracing theory of phototropism. The phototropic curvature of an organ was considered to be entirely due to differential growth of the two sides of the organ, because of different growth responses of the side toward, and the side away from the light as a result of a light gradient between those sides. Blaauw went so far as to say that the problem of phototropism had become empty and that the only real problem was the effects of light on stem elongation. In spite of the fact that Blaauw brought a large body of experimental facts to support his contention the so-called Blaauw theory was never fully accepted by plant physiologists and it became at least partly replaced by the Cholodny-Went theory.

Let us first consider what is known about the short-time light effects on stem elongation, the so-called light-growth response. This was studied by Blaauw, Koningsberger, Van Dillewijn and many others, especially in my father's laboratory in Utrecht. As a typical example the effect of short illumination on the growth of the oat seedling will be discussed. An exposure of only 10 to 100 foot-candle seconds suffices to cause a growth inhibition within ½ hour, followed by an acceleration of approximately equal magnitude. Therefore, the overall growth remains practically unchanged by such a small light exposure. With larger amounts of light the growth reduction becomes more pronounced but even with the largest amounts of light the growth rate of the seedling remains fairly even over a period of several hours. This growth reduction followed by an acceleration resembles a phenomenon described for the pea in which compensatory growth occurs after a very small light exposure. It also resembles the pea in that red light can induce this response. These two properties of the light-growth response make it impossible to consider them the basis of a phototropic curvature. It is known that phototropism in the oat seedling can be induced only by blue light, whereas red is completely ineffective in inducing phototropic curvature. Besides, we find that a phototropic curvature not counteracted by gravity will continue to increase for a period of several hours, whereas the total amount of growth over a period of one or a few hours is hardly affected by light even though a transient growth retardation occurs. Therefore, we see that we have to look for an explanation of phototropic curvature in an entirely different direction and we cannot use the effects of light on straight growth as a basis of an explanation of phototropism such as explicitly stated in the theory of Blaauw.

Perhaps the most compelling reason to reject the theory of Blaauw as a general principle according to which phototropic curvatures occur is already indicated by the early experiments and observations of Darwin. He had shown that it was not necessary to illuminate that portion of the seedling which responds to unilateral light with a curvature but that it was predominantly the tip which perceived the light stimulus. When a seedling of Panicum, for instance, was buried up to its tip in sand and then the tip illuminated from one side, the buried portion of the plant would curve toward the light. This means that the portions of the plant which show differential growth responses are not a direct result of differential growth of cells exposed to different light intensities.

A great advance in our knowledge about phototropism was made by Boysen Jensen who showed just 50 years ago that a phototropic stimulus can be transmitted from an illuminated tip to the regions which respond, even after the tip has been cut off and has been glued onto the stump again. This showed, like Darwin's experiments, that there is a transmission of the phototropic stimulus from the light-perceiving tip to the responding cells lower down. It was then Paal who in 1918 suggested that this transmission of the stimulus was caused by the downward movement of a growth-promoting substance which is normally produced in the tip of grass seedlings.

The just cited experiments tell us much about the mechanism of phototropic curvature. They suggest that there is produced a differential in the amount of auxin moving from the tip to the base. There are several possibilities in this respect. It might be suggested, for instance, that the auxin is destroyed predominantly on the light side of the tip but continues to move downward along the unilluminated side. That this view is incorrect is shown in the first place by the light stability of auxin mentioned earlier, and it also is negated by the occurrence of the negative phototropic curvatures which occur when plants have been exposed to unilateral illumination of approximately 1000 foot-candle seconds. In this case the illuminated side actually received more auxin than the side away from the light.

Since the role of auxin in the growth of the Avena coleoptile is well known and has been studied in a quantitative manner by many investigators it is obvious that we should look into the question as to whether an auxin is involved in a phototropic curvature. It was found that in the coleoptile there is no growth without auxin and also that growth is strictly proportional to the amount of auxin supplied. In line with what was said about the effects of light on stem elongation there was probably no change in the amount of auxin produced by the coleoptile tip after light exposure, yet when the auxin was diffused from the 2 sides of the coleoptile tip after unilateral illumination an enormous difference in the diffused auxin was noticed. In the course of one to two hours after illumination the majority of auxin produced in the coleoptile tip moves downwards along the side away from the light, whereas practically nothing moves along the light side. This redistribution of auxin is quantitatively sufficient

to explain the phototropic curvatures which are produced.

Not only have we seen that there are quantitative differences in the effects of light on growth in length on the one hand and the phototropic curvature on the other hand, but it turns out that the action spectra for these two phenomena are radically different. For growth in length we have seen that the red part of the spectrum is most effective, whereas the blue shows only slight activity. In phototropism it was already known since Blaauw that it is particularly the blue part of the spectrum which is effective and this has been investigated in a number of other laboratories, always with the same result. Therefore, we can say that the absorption spectrum of the pigment involved in the phototropic curvature very closely resembles that of carotene with two absorption peaks in the blue, and a very sudden drop off towards the yellow part of the spectrum with no effectiveness of the red rays whatsoever.

The amounts of light necessary to produce phototropic curvature are very small. If we assume that every light quantum moves one auxin molecule from the light to the dark side, then there is serious discrepancy; that is to say, about a hundred times more auxin molecules move under the influence of light than there are light quanta available. However, it is quite certain that there is no direct connection between the light quanta and the movement of the auxin molecules, for it has been found that the lateral movement of auxin continues for several hours after completion of the light exposure. Therefore, the light induces a polarity in the coleoptile tip which persists for a long time and it is this polarity which causes the lateral displacement of auxin. This means that light is only the first step in a chain of reactions which ultimately leads to the auxin displacement, and we do not need to look for any stoichiometrical relationships.

The Mechanism of Photoperiodism in Plants

KARL C. HAMNER
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Photoperiodism is defined as the response of plants to variations in day length or to the relative lengths of day and night. There have been many thousands of publications on this subject and numerous recent reviews, (2-20) so no attempt will be made here to review the literature or to cite individual publications, except as such citations may seem pertinent to the discussion. While there are many types of photoperiodic response, the discussion here will be confined to the effect of photoperiod on the initiation of flowers in plants. This seems justified, not only because of the fact that many plants growing on an unfavorable photoperiod tend to remain vegetative indefinitely, whereas transfer to a favorable photoperiod causes prompt initiation of flowers, but also because of the general interest in the whole problem of physiology of flowering.

Garner and Allard reported on their discovery of photoperiodism in 1920. On the basis of their early work, they classified all plants into three general groups: the short-day plants, the long-day plants, and the day-neutral plants, a classification which still holds. On a natural "day," (i.e. a twenty-four hour cycle) a short-day plant is one which flowers at day lengths[1] shorter than a certain critical day length; a long-day plant is one which flowers at day lengths longer than a certain critical; and the day-neutral plants are those which flower over a wide range of day lengths, showing no particular critical. In classifying plants into one of these three groups, one must be careful to adhere to Garner and Allard's original definition. Failure to do so has led to some confusion in the literature. For example, the critical day length of most short-day plants lies between 14 and 16 hours, whereas the critical day length of most long-day plants lies between 9 and 11 hours. Thus, in general, the long-day plants have a shorter critical day length than do the short-day plants. The critical day length of some plants is rather precise and can be measured within fifteen minutes or less; it may be only slightly affected by temperature variations. A given plant species may contain strains which vary widely in the critical day length. In general, however, a species will tend to contain strains of the same photoperiodic classification, differing from one another only in the critical day lengths. For example, a species which contains strains that are definitely short-day plants will, in general, not contain strains which are long-day plants and vice versa. If there are any exceptions to this rule, they are extremely rare. However, species which contain short-day strains may also contain day-neutral strains and, similarly, species which contain long-day strains may also contain day-neutral strains. In some species the differences between a short-day or a long-day strain and a day-neutral strain involve a single gene.

There are some exceptions to the above classification. Some plants seem to have two clear-cut critical day lengths above which or below which they will not flower, flowering only over a narrow range of day lengths. There are other plants which may be considered as long-day short-day plants or short-day long-day plants. Such plants will flower only when grown under a given day length and then transferred to another day length. For example, some plants must be grown under short-day conditions for a period of time and then transferred to long-day in order for flowers to be induced. They will not flower if grown continuously under long-day. Similarly, other plants must be grown under long-day for a period of time and then transferred to short-day in order for flowers to be induced. They will not flower if grown continuously under short-day. These exceptions will not be considered further in the present discussion.

It has long been known that the leaves are the organs which perceive the variations in day length. This is true both for long- and short-day plants. The responses of the cocklebur, *Xanthium pennsylvanicum*, may be used to illustrate this point. *Xanthium* is a short-day plant with a critical day length of 16 hours. If grown continuously at day lengths longer than this it will remain vegetative indefinitely, growing into a small tree. Transfer of the plant from long-day conditions to short-day results in prompt initiation of flower buds; the influorescences assume macroscopic size within two weeks. In fact, transferring the plants from

[1] Day length as used here refers to the length of the light period in a twenty-four hour day.

long-day conditions to only a single short-day results in prompt floral initiation, even though the plants are subsequently grown continuously on long-day. This brief treatment with a favorable day length, causing floral initiation, is called photoperiodic induction. Removal of the leaves of a vegetative plant of *Xanthium* does not result in floral initiation, even though the defoliated plant is placed under short-day conditions continuously. However, if as little as one square centimeter of leaf tissue is allowed to remain on the defoliated plant, prompt floral initiation occurs when the plant is placed under short-day. In fact, initiation is approximately as rapid as if all of the leaves were present. An undefoliated vegetative plant of *Xanthium* growing on long-day may be induced to flower simply by placing one leaf under short-day conditions. It appears, therefore, that the leaf tissue perceives the photoperiodic change and sends the message to the meristems which respond as a result.

The fact that exposure to the appropriate day length results in the generation in the leaf of the stimulus, which is transmitted through the stem to various actively growing buds, may be indicated in a variety of ways. One may grow a vegetative plant of *Xanthium* with two branches of approximately equal size by removing the terminal bud and permitting the buds in the axils of the cotyledons to grow out. If we place one branch of such a plant on short day, that branch will promptly flower. The second branch, which is maintained continuously on long day, will also flower, indicating the transmission of this stimulus for flowering from one branch to the other. In fact, exposure of a single leaf of *Xanthium* to short day, may cause several branches of a single plant to initiate flower buds. In this regard, *Xanthium* is more sensitive than most other plants, since in most photoperiodically sensitive plants the initiation of flowers tends to be limited to the branch or portion of the plant which is exposed to the appropriate day length. However, with the proper techniques, it has been possible to demonstrate that the photoperiodic response of all plants involves the generation of a stimulus in the leaves, which is transmitted to the buds.

It is also possible to obtain transmission of the stimulus from one plant to another across a graft union. One may take two vegetative plants of *Xanthium* and remove a longitudinal slice from the stem of each plant. If the injured surfaces of the two plants are brought into contact by bending the plants together and bound tightly, a graft union is promptly formed. If one of the plants is then exposed to short day, that plant flowers, and the graft partner subsequently flowers, indicating the transmission of a stimulus across the graft union. The question arises as to whether or not tissue contact between the two graft partners is necessary for successful transmission of the stimulus. To study this question, numerous barriers have been placed between the two injured surfaces of the "graft partners." Interposing such things as cellophane, agar, and gelatin have caused failure in the transmission of the stimulus. We have had transmission of the stimulus across a lens paper barrier, but lens paper is very coarse, and we are not sure that the tissues of the two plants have not grown through the lens paper, providing direct contact between the living cells of one plant and the other. It may be, therefore, that the stimulus moves only from one living cell to another living cell in direct contact with it.

Utilizing the grafting technique, certain information concerning the behavior of the stimulus has been obtained. If a vegetative *Xanthium* plant is exposed to seven short days, it will promptly initiate flower buds. If it is then returned to long day, it may be grafted to a vegetative *Xanthium* plant, and the latter will flower, indicating that the plant which has been exposed to short day continues to supply the stimulus long after the original photoperiodic stimulation has ceased. Furthermore, the stimulus may be effective a long distance from the original point of perception. For example, one may graft several two-branch plants of *Xanthium* in series, grafting one branch of the first plant to one branch of the second plant, and the second branch of the second plant to the first branch of the third plant, and so on, resulting in a chain of plants grafted together. If, then, the branch of the plant at the end of the chain is exposed to the short day, the flowering response will travel slowly down the chain of plants, across the several graft unions. It has been possible to follow this stimulation from one plant to another through five plants. We do not know just how far this stimulus will travel from the original point of perception, since we have always run into difficulty with continued successive grafts, for technical reasons of one sort or another.

The above gives some of the specific responses of *Xanthium*. In general, in both long- and short-day plants, the leaves are the organs which perceive the photoperiodic stimulation, and the stimulus moves from the leaves through the stem to the buds, and will move across a graft

union from one plant to another. The stimulus will not only move across a graft union from one plant to another plant of the same species, but it will move across a graft union from one species of plant to another species. It has been possible to demonstrate transmission of this stimulus when two species of short-day plants are grafted one to another, or two species of long-day plants are grafted together. It will also move from day-neutral species to either short- or long-day species. Moreover, it has been possible in a few cases, to demonstrate the transmission of the stimulus from a long-day plant to a short-day plant, and vice versa. For example, a long-day plant may be used as a scion and a short-day plant as a stock. When this is done, and the graft pair is placed on short day, the short-day plant may produce a branch which flowers, and the long-day scion will also flower. On the other hand, if we put the graft partners on long day, both plants will also flower. Transmission of the stimulus takes place, therefore, from the short-day stock to the long-day scion and vice versa. It is, of course, possible to use the long-day plant as the stock and the short-day plant as the scion, and get similar results.

The above results, which were obtained a number of years ago, were very exciting since they indicated the existence of a flower-inducing hormone which was common to many plants if not all plants in the plant kingdom, a hormone which we should be able to extract, crystallize, identify, and apply to plants, causing them to flower at any time we desired. In fact, this hypothetical hormone was named "florigen" by a Russian investigator. Many thousands of attempts have been made since 1938-39 to extract and identify this hormone, completely without success. There have been some reports in the literature that active extracts have been obtained, but it has been impossible even for the investigators doing the original work to repeat these results. If there is a hormone that is responsible for the transmission of the stimulus from the leaves to the buds and from one plant to another, it is difficult to extract. For this reason many of the investigators who have been interested in these problems have decided to study the changes which take place during photoperiodic induction, in the hope that more information on the nature of the stimulus might be obtained. As a result there are many reports in the literature dealing with the changes which take place in the leaves as they perceive the photoperiodic stimulation, and the factors which affect the movement of the stimulus from the leaves to the buds.

Because of its extreme sensitivity, most of the work on the mechanism of photoperiodic induction has involved the cocklebur or *Xanthium*. The real advantage of the use of *Xanthium* in these investigations is that it may be induced to flower as a result of exposure to only one short-day or one photoinductive cycle. This provides an opportunity to dissect the cycle to see which part is most effective. Is it the light period, or is it the dark period, or are both involved?

It was soon found that very low intensity illumination given as an extension of the short light period of a naturally occurring short day produced a long-day response. However, a striking result was the fact that a small amount of light (as little as one second) given during the middle of what would otherwise have been a long dark period would also produce the long-day response. For example, while *Xanthium* would be induced to flower with only one short day (a short light period and a long dark period) it would not flower when repeatedly exposed to treatments comparable to the short day except for the fact that the long dark period was interrupted with a very brief exposure to light. When these results were first obtained it seemed to indicate that the dark period was the important part of the photoinductive cycle. In other words, it was the critical length of the dark period that determined whether or not a plant would respond photoperiodically.

However, the following series of experiments showed clearly that the main light period was also participating in the photoperiodic reaction. *Xanthium* plants which had received a main light period were placed in a dark room and given a small amount of illumination every three hours. The plants were thus exposed to a main light period followed by short cycles consisting of three hours of dark and a brief exposure (three minutes) to light. If such plants were returned to long day, regardless of the number of such short cycles of treatment they had received, they remained strictly vegetative. If, however, plants were removed from the short-cycle treatment and given a long dark period before returning to long day, they might or might not flower depending upon the number of short cycles given before exposure to the long dark period. The plants would usually flower when returned to long day if, prior to the exposure to the long dark period, they were treated with fewer than five to seven short cycles (i.e., from 15 to 21 hours of short-cycle treatment. However, if the plants were given more than 21 hours of the short-cycle treatment

and then exposed to long day they did not flower when returned to long day. Thus, a long dark period is not effective in photoperiodic induction if it is preceded by short cycles consisting of short light periods and short dark periods. However, if we took such plants which had been exposed to 24 hours of these short cycles and before giving them the long dark period we exposed them to a brief period of bright light (30 minutes of sunlight), then the plants would flower when returned to long day. Thus, a long dark period is effective in photoperiodic induction of *Xanthium* only when preceded by a period of high intensity illumination. In *Xanthium*, therefore, an effective short day consists of an exposure to high intensity illumination followed reasonably closely by a continuous dark period longer than a certain critical duration. All other short-day plants that have been critically examined have indicated a similar response. We can say, therefore, that in the photoperiodic reaction of short-day plants there are high intensity light reactions, dark reactions, and an interaction among them.

Photoperiodic induction of long-day plants also involves high intensity light reactions and dark reactions as is the case in short-day plants. There have been no reports of long-day plants which may be induced to flower by exposure to a single long day and therefore it is not possible to study the mechanism of the reaction as critically as is possible in *Xanthium*. However, long-day plants which are induced to flower by exposure to a series of long days must receive high intensity light during the photoperiod of each long day if the treatment is to be effective. In some cases, intensities of over 700 foot candles are required. The response of long-day plants to darkness is diametrically opposite to that of the short-day plants. A dark period longer than a certain critical duration is inhibitory to flowering rather than stimulatory, as is the case in the short-day plants. A brief period of low intensity illumination given during the middle of the dark period may destroy its inhibitory effect. Utilizing cycles other than 24 hours it has been possible to show that long-day plants will flower if the light periods are longer than a certain critical duration even though accompanied by dark periods of long duration. In both long- and short-day plants, therefore, the photoperiodic induction involves high-intensity light reactions followed by dark reactions and presumably an interaction among them. Let us now consider the information available concerning these partial reactions in the induction process.

It appears that the high intensity light reactions in both short- and long-day plants involve photosynthesis, since if we withhold carbon dioxide during the light period no flowering results. However, it appears that there may be additional high intensity light reactions involved since as little as one second of high intensity light during each cycle of a photoinductive treatment may be effective in certain short-day plants. In *Xanthium* the requirement for high-intensity light may be eliminated if the plants are provided with an external supply of sugar or of organic acids of the Krebs' cycle. It appears, also, that the high intensity light reaction may be nullified by the application of a suitable concentration of auxin. Information is not available concerning the action spectrum of the high intensity light reaction.

There are at least two dark reactions involved in photoperiodic induction. The first of these consists of those reactions which determine the length of the critical dark period. The length of the critical dark period in *Xanthium* is 9 hours. If, immediately following a main light period, *Xanthium* plants are exposed to 9 hours of continuous darkness they fail to flower. However, if the plants are exposed to 9 hours and 30 minutes of continuous darkness before returning to long day there will be 100% flowering. A dark period of 9 hours and 15 minutes is usually sufficient to cause nearly 100% flowering. There is, therefore, within the plant some mechanism whereby it can tell time rather precisely. This particular portion of the photoperiodic reaction may be termed the "timing mechanism." Whatever this timing mechanism is, it seems to have a low temperature coefficient since changes in temperature affect the length of the critical dark period only slightly. Of particular interest is the effect of irradiation upon the timing mechanism. A brief exposure to low intensity red light seems to upset the timing mechanism completely. For example, if *Xanthium* plants are exposed to a dark period shorter than the critical and then exposed briefly to red light, they must be exposed to another dark period longer than the critical if they are to flower subsequently. The action spectrum for this light interruption has been studied in detail. If, immediately after exposing the plant to red light, the plants are exposed to far red illumination (wave lengths just at the end of the visible spectrum) the effects of this red light may be completely nullified. If plants, during the middle of an otherwise effective dark period, are exposed to red light followed by far red light they will respond as if they had been

maintained in a continuous dark period. On the other hand, if, after exposure to red light followed by far red, they are again exposed to red light, the dark period is not effective. In other words, it is the treatment which is given last which determines the response. This red-far red interaction is a phenomenon exhibited in many other responses of plants. It was discovered in studies of the germination of lettuce seeds which is stimulated by red light and inhibited by far red. It also affects the etiolation response of plants. These latter responses are not photoperiodic phenomena. Intensive research is going on at present to determine the nature of the pigments involved in this red-far red interaction.

A second dark reaction apparently takes place when the plants are continued in darkness beyond the critical period, i.e., subsequent to the satisfaction of the "timing mechanism." As has been mentioned, a dark period 30 minutes longer than the critical may result in 100% flowering response. However, if rate of inflorescence or flower development is used as a measure of the response, it is found in *Xanthium* that the magnitude of the response increases with increasing length of dark period from 9 up to 15 hours where the response reaches a maximum. Thus, the second dark reaction results in a quantitative expression of the response rather than a qualitative one.

The discussion of the mechanism of photoperiodic induction so far has centered around the reactions which take place while the plant is actually exposed to a photoinductive cycle. Such a treatment is always terminated by returning the plants to the light period of a nonphotoinductive cycle. In *Xanthium,* for example, the effects of a photoinductive cycle are measured by returning the plants to long-day and observing the floral development approximately two weeks after the treatment. Since the long dark period of an effective photoinductive cycle is always terminated by exposure to light of the first cycle of long day, the question arises as to whether or not this light period participates in the photoperiodic reaction. The answer to this question seems to be in the affirmative. For one thing, movement of the stimulus out of the leaves takes place over a period from 8 to 16 hours following a photoinductive treatment. This may be demonstrated by proper defoliation experiments in *Xanthium.* Furthermore, during the first five hours of exposure to high intensity light following a photoinductive cycle, there appears to occur a stabilization of the stimulus, which may be demonstrated in the following way in *Xanthium.* If at the end of what would otherwise be an effective photoinductive cycle, the plants are exposed briefly to illumination and then given a second dark period shorter than the critical, i.e., from 5 to 8 hours, the effectiveness of the photoinductive cycle may be completely nullified and the plants fail to flower. On the other hand, if the plants are exposed to 5 hours of high-intensity light following a photoinductive cycle, then a second short dark period does not produce an inhibitory effect. This stabilization reaction has a low temperature coefficient since changes in temperature affect the length of time necessary for stabilization only slightly. Proper defoliation experiments indicate that this stabilization is not simply the movement of the stimulus out of the leaves.

In the discussion so far, reliance has been placed primarily upon the responses of the cocklebur, *Xanthium.* Recent work with Biloxi soy beans, initiated by Blaney (1), has indicated that the responses may be far more complicated than indicated above. His work indicated that there is in Biloxi soy beans an endogenous rhythm operating on approximately a 24-hour cycle which determines to a large extent the photoperiodic response. Such a phenomenon had been indicated in prior research and has been substantiated in subsequent work. In fact, additional work has indicated the possibility that endogenous rhythms are participating in the photoperiodic responses of many, if not all, photoperiodically sensitive plants.

The following responses of Biloxi soy bean have led to the conclusion that endogenous rhythms are involved in its response. Biloxi soy bean is a short-day plant, but it does not flower as a result of exposure to a single short day. It is not as sensitive as *Xanthium.* It will respond if given 3 short days, but the 3 short days must occur in direct succession. If we give the plant one short day followed by a long day and then another short day and a long day, and so on, we may continue this treatment for weeks without any flowering response. Furthermore, if we give Biloxi soy beans one, two, three, four, five, six, or seven short days, we get a quantitative response between two and seven short days, using the number of nodes producing flowers as our measure. The response curve is as near a straight line as may be expected of any biological response. We can use the number of nodes bearing flowers as a quantitative measure of the effectiveness of a particular experimental treatment.

All of the subsequent discussion of responses

of Biloxi soy bean will involve experiments where plants were given seven cycles of treatment. In comparing the responses of plants to different treatments it is necessary to describe the conditions prevailing in only one cycle of a given treatment, since all seven cycles are exactly the same. It should be realized that cycles other than of 24-hour duration were used in most of these experiments. Experiments were conducted in which the dark period of the various treatments was constant and the length of the light period varied. Conversely, other experiments were conducted in which the light period of the various treatments was constant and the length of the dark periods varied. Of particular interest to this discussion are those experiments in which the light period of the various treatments was of 8 hours duration and the length of the dark period varied between treatments.

With the light period of 8 hours duration in each treatment, and with variations in length of the dark period between treatments, there are of course concomitant variations in the length of the cycles of the various treatments. Cycle lengths of from 10 to 72 hours have been used (involving dark periods varying from 2 to 64 hours in length). When the flowering response is plotted against the cycle length, a curve is obtained with oscillations of 24 hours duration. There is of course no flowering with cycle lengths shorter than 18 hours, since such cycles contain dark periods shorter than 10 hours, which is the critical length of the dark period for Biloxi soy bean. As the cycle length is increased from 18 hours to 24 hours the response curve increases to a maximum. On a 24-hour cycle the plants are exposed to an 8-hour light period and a 16-hour dark period, a typical short day. As the cycle length is increased beyond 24 hours, the flowering response decreases until it reaches zero at 32 hours, i.e., when the plants are receiving 8 hours of light and 24 hours of darkness. As the cycle length is increased beyond 32 hours, the flowering response again increases to a maximum at a 48-hour cycle. Still further, increasing the length of the cycle results in a minimum of flowering at a cycle length of approximately 58 hours, and additional increase in length of this cycle results in a further increase to a maximum at 72 hours. Cycle lengths longer than 72 hours produce indefinite results since many plants die presumably because of lack of photosynthesis and food reserves.

There seems but little doubt, therefore, that the effectiveness of a photoperiodic treatment in Biloxi soy bean depends upon an endogenous rhythm that has a 24-hour oscillation. The maximum response is obtained with cycles of 24, 48, and 72 hours duration. It should be noted that the three maxima are of approximately the same height; in other words the amount of flowering obtained at all three of these cycle lengths is about the same. On the other hand, the two minima occur at about 32- and 58-hour cycles. The first minimum results in zero flowering, whereas at the second minimum flowering may be 40 to 50% of the maximum. Since the maxima of the response curve seems to remain at the same level, whereas the dips seem to be fading away, it appears that there is an endogenous rhythm of *inhibition* which fades away quickly with increasing length of cycle. Data have been obtained on the effects of temperature on the oscillations. Variations in temperature do have an effect upon the height of the maxima, but do not have any appreciable effect upon the time of oscillation or the distance between the maxima. The endogenous rhythm therefore has a low temperature coefficient. Studies have been made of the effects of applications of chemicals on the rhythmic response of Biloxi soy beans, but these results do not seem pertinent to the discussion at the moment.

Many years ago, Bünning presented an hypothesis, that photoperiodism involved an endogenous rhythm. His information or data came primarily from studies of leaf movements. Some plants, for example, will lower and extend their leaf blades during the light period and fold them during the dark period. Bünning found that such plants continued these leaf movements for some period of time after being placed in complete darkness. He postulated that the photoperiodic responses of plants were also based upon endogenous rhythms comparable to those which caused the continuation of the leaf movement. In both long-day plants and short-day plants, flowering response presumably depended upon the oscillations of these rhythms in relation to the time at which the plants were exposed to light. For example, long-day plants flowered if they were exposed to light each time the rhythm reached a phase in which the plants were responsive to light. On the other hand, the short-day plants failed to flower under identical conditions. He called one phase the photophile phase, and the other phase, the scotophile phase. This hypothesis was not widely accepted during the years subsequent to its first presentation, because of the lack of positive evidence, and the apparent abundance of negative

evidence. Additional evidence, rapidly increasing at the present time, seems to indicate that something remarkably close to his original hypothesis may be the true situation.

The results with Biloxi soy bean, discussed previously, indicate that this short-day plant exhibits a photoperiodic response very similar to that postulated by Bünning. Very recent work by Finn (9) indicates that at least one long-day plant may respond in a manner which would seem to support the hypothesis. Working with the long-day plant, *Hyoscyamus niger,* Finn used treatments very similar to those which Blaney used with Biloxi soy bean. Comparisons were made between the responses of plants exposed to photoperiodic cycles of various durations. In any given comparison, the length of the light period of each cycle in all of the treatments was the same. Variations in the length of the cycle were associated with variations in the length of the dark period in each cycle. The length of the dark period varied from 0 to 60 hours, resulting in cycle durations varying from 9 to 72 hours. In different experiments, the length of a light period used varied from 9 to 12 hours. Most rapid flowering occurred, of course, in those plants exposed to continuous light. This treatment may be considered that with the shortest cycle of treatment, namely, 0 dark period. With increasing length of dark period in each treatment, the amount of flowering decreased until no flowering or a minimum response was obtained on a 24-hour cycle. Increasing the duration of the cycle, simply by increasing the length of the dark period in each cycle, resulted in a subsequent increase in the rate of response until a maximum was reached and further increasing the length of the cycle resulted in another decrease in the rate. The flowering response of *Hyoscyamus,* therefore, exhibits a rhythmic pattern associated with length of cycle. The minimum of flowering was exhibited on a 24-hour cycle, a cycle length at which the short-day plant, Biloxi soy bean, exhibited a maximum. Long-cycle durations resulted in flowering in spite of the fact that during each cycle the plants were exposed only to a shortlight period. Thus the response of this long-day plant seems to be associated primarily with cycle-duration, rather than with the length of the light period. The results with one short-day plant, Biloxi soy bean, and one long-day plant, *Hyoscyamus niger,* seem, therefore, to support the general hypothesis of Bünning.

It is unfortunate that critical experiments similar to those carried out above have not been carried out with a large number of photoperiodically sensitive plants. It seems possible that many of them would respond in a manner similar to the above two. A superficial examination of the many results with *Xanthium* might seem to indicate that this plant does not respond in a manner corresponding to the Bünning hypothesis. In fact, the results with this plant have been used as a primary argument against the hypothesis. On the other hand, a supporter of the Bünning hypothesis could argue that *Xanthium* does exhibit an endogenous rhythm and that this rhythm determines the length of the critical dark period. In other words, as the plant is placed in darkness, it enters the first phase or the inhibitory part of the rhythm, and that the length of this inhibitory phase determines the critical length of the dark period. It could be argued further that the endogenous rhythm fades away very quickly in this plant, and that, in fact, there is only one oscillation associated with the photoperiodic response. Recent unpublished work in our laboratory has indicated that indeed this may be the case. When *Xanthium* plants are given a 9-hour photoperiod and then given a single dark period of varying length, there is no flowering until a dark period of 9½ hours is used. As the length of the dark period is increased above this critical, there is an increase in the rate of flowering until a dark period of 15 to 16 hours is reached. With dark periods longer than this, periods of from 20 to 30 hours, there is a slight decrease in the rate of flowering. A second maximum in the rate of flowering is reached with dark periods of 39-40 hours in length. This rhythmic response is especially apparent when the dark periods are given at low temperatures. It seems possible, therefore, that the "timing mechanism" of the photoperiodic reaction (the mechanism which determines the length of the critical dark period) is simply an expression of an endogenous rhythm.

Many organisms in both the plant kingdom and the animal kingdom exhibit endogenous rhythms, whereby they show rhythmic responses which may be timed very precisely. Bünning has done a great deal of work with plants. One particular response of one plant may be used to illustrate, in general, the responses that have been obtained in many other plants. Bean plants, when growing under a normal day, exhibit leaf movements which are on a diurnal cycle. The leaves assume one position during the light period, and another position during the dark period. If the plants are placed in

continuous darkness, these leaf movements continue for several days, and then gradually cease. If bean seeds are planted in complete darkness, the seedlings, which may be photographed with infra-red light, exhibit no typical diurnal leaf movements. However, if the young seedlings are exposed briefly to bright light (1 hour is entirely sufficient) then the seedlings exhibit leaf movements and these leaf movements are on a 24-hour rhythm. It is emphasized that these plants were never exposed to a 24-hour cycle. These movements will continue for several days and then disappear to be reinduced by another brief exposure to light. Many other plant responses, such as root pressure, and stomatal movements, exhibit similar rhythms.

I was fortunate enough to attend a conference at Gatlinburg, Tennessee, at which the endogenous rhythms of many organisms were discussed in considerable detail (21). Relying upon my memory, I wish to cite one or two examples which illustrate how precisely the endogenous rhythms may enable an organism to tell time. I was fascinated by the story of one particular mouse, which exhibited a rhythm of, as I recall, 23 hours and 52 minutes. This mouse was placed in complete darkness in a cage with an activity wheel. The normal cycle of events was for the mouse to get up in the "morning" and immediately go to the exercise wheel and exercise for some period of time. It would then leave the wheel and eat and come back to the wheel and exercise some more intermittently during the "day." Finally, its activity would die down and it would go to sleep until the next "morning," when it would again be active. Its activity on the wheel was plotted on a kymograph. The length of time from the beginning of activity in one cycle to the beginning of activity in the next cycle, was so precise that it could be anticipated to the minute, and perhaps, even to the second. At Gatlinburg, in casual discussions, I also gained the understanding that the navigation of birds involved an endogenous rhythm—that the birds could tell time instinctively, and so precisely that they could navigate by the stars, not only by latitude, but by longitude.

It seems possible to me that there is some basic timing mechanism or endogenous rhythm in all living organisms, and that this basic mechanism is participating in the photoperiodic reaction.

The above more or less completes what I have to say on the mechanism of the photoperiodic reaction. However, I am sure there are a few aspects of the problem which you have been expecting to be discussed. First, there is the problem of the participation of the gibberellins. Gibberellic acid will, insofar as I know, induce flowering in all but one of the long-day plants that have been studied, even though the long-day plants are grown continuously on short days. One might be inclined to speculate therefore that gibberellic acid is the flower hormone for long-day plants. There has been only one report that gibberellic acid stimulates flowering in short-day plants, and this will need confirmation. All other short-day plants that have been tested do not respond to gibberellic acid. This means that we cannot say that gibberellic acid is the flower hormone for short-day plants. As has been discussed previously, it has been shown that, by grafting long-day plants and short-day plants together, the flower stimulus will travel from one to the other in either direction. If we assume one flower hormone for the entire plant kingdom, we cannot say that gibberellic acid (or the gibberellins) is the flower hormone.

Other chemicals which have been found to induce flowering in some plants are indoleacetic acid and related auxins. These chemicals induce flowering in the pineapple plant and in several other plants. However, they will not induce flowering in most photoperiodically sensitive plants that have been tested. The auxins, including indoleacetic acid, inhibit flowering in *Xanthium*. At one time it was thought that indoleacetic acid concentration affected the dark reaction of the response. However, it now appears that application of indoleacetic acid affects the high-intensity light reaction, or light product. Since this exhausts my time allotment I will not go into details on any of these latter points.

DISCUSSION

Question: What happens in these endogenous rhythms or with the responses when you vary the starting time of each cycle?

Answer: It is very difficult to obtain an answer to this question with Biloxi soy beans since the treatments are repeated for seven cycles. With *Xanthium*, however, it is possible to vary the starting time of the treatment in relation to the naturally occurring day. We have done this and have also varied the termination of the treatment with respect to lighting conditions in the greenhouse. With *Xanthium* there is no indication that the time of starting or terminating an experiment with relation to the natural day has any effect on the ultimate results or upon the rhythm itself.

Question: Is this a clock mechanism within the

organism that goes on more or less indefinitely, or is it an hour-glass timing sort of thing?

Answer: All of our evidence indicates that the clock continues for more than one cycle and it would not be an endogenous rhythm, to my mind, if it did not so continue. In that regard the situation with respect to some organisms that have been studied in much more detail with respect to the endogenous rhythms shows many complications. I have indicated that the rhythm may be initiated by a perturbation of light. This "tickle" starts the rhythmic response and it may continue for as much as a week and then fade away. It may be started over again with another perturbation of light. Now let us say that the rhythm is under way and we give a light perturbation at some point differing by some fraction other than a multiple of 24 hours from the previous light perturbation. In some organisms this will start the rhythm anew and the new rhythm is out of phase with the previous one by some fraction of 12 hours. In other organisms this second perturbation will produce what are called transients, or rhythms other than 24 hours until finally the organism is back on the old phase. These perturbations may be induced not only by light but by a change in temperature. If an organism is shifted from one temperature to another, this may cause transients until the organism is adjusted to the new temperature and then the distance between the peaks of the rhythm remains 24 hours or whatever is characteristic of that organism. It should be emphasized that one of the characteristics of the endogenous rhythms in all of the organisms studied is the low temperature coefficient. Changes in temperature have little effect on the distance between the oscillations except for the appearance of these transients as mentioned.

Question: What evidence in addition to the grafting experiments of long- and short-day plants do we have of the identity of "florigen" for the long-day plants and the short-day plants?

Answer: I would say that we have no evidence one way or the other that would hold up.

Question: Is there any evidence for a common origin of long- and short-day plants?

Answer: Of course there is a common origin for plants, but as I understand your question, the answer is negative. In the course of evolution the sensitivity to day length seems to have recurred many, many times. In fact, we have many cases where a given species may contain photoperiodically sensitive strains differing from the day-neutral strain by a single gene, or mutation. Orange Flare Cosmos was a mutation from Klondike Cosmos. Orange Flare is a day-neutral plant while Klondike is a very sensitive short-day plant. When I first started photoperiodic work, I used Klondike but soon had to give it up because of the impossibility of getting a good seed source. When I would send away for a Klondike seed I would get Orange Flare. We have many examples in given species where photoperiodically sensitive strains differ from insensitive strains by single genes. In general, however, we do not find long-day plants and short-day plants in the same species. If we have a species containing a long-day strain we may have a number of long-day strains with different critical day lengths in that same species, and day-neutral strains in the species, but we are unlikely to have a short-day strain in that species.

Question: Is there any difference in geographical distribution between long- and short-day species, or strains?

Answer: The geographical distribution follows a pattern related to the critical day length. At latitudes near the equator you will find plants with shorter critical day lengths than those growing at higher latitudes. It is easy to understand why this is so. For example, if the day length at which you plant your seeds never exceeds the critical in a short-day plant, the seeds will simply come up and flower while the plants are very small. With commercial crops under such conditions you wouldn't get a harvest, since the plants would not be above the critical day length long enough to produce the vegetative growth necessary for good production. On the other hand, I know of no differences in distribution of long-day plants or short-day plants with respect to geography or latitude, with the exception that at the equator there tend to be more day-neutral plants.

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Photoperiodism in Animals With Special Reference to Avian Testicular Cycles[1]

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In the realms of zoophysiology and animal ecology, photoperiodism has acquired the somewhat restricted meaning of the control of annual physiologic cycles by mechanisms in some way driven, or at least maintained in phase, by the changing duration of the natural daily photoperiod. Even with this restricted meaning, photoperiodism encompasses the control of a variety of cyclic functions in many kinds of animals. The number of known examples is increasing steadily.

It is now possible to make useful generalizations concerning the survival value of photoperiodic mechanisms and also, to some extent, concerning the properties of a photoperiodic response system. On the other hand, the variety of cycles photoperiodically controlled, the variety of animals involved, and the generally primitive state of our knowledge do not allow synthesis of useful generalizations concerning basic mechanisms. Consequently, this discussion will be divided into two parts. Attention will be directed initially to some generalizations concerning the selective value of photoperiodic mechanisms and the known extent of their occurrence among animals. The latter will be illustrated briefly by means of selected examples. Subsequently attention will be turned to a single example of a photoperiodic mechanism, that involved in the control of the avian testicular cycle. This example has been selected because it is better understood than other photoperiodic mechanisms in animals; it is also one with which I have some experimental experience.

For species subject to marked annual fluctuations in important environmental factors, survival requires a scheme of controls causing critical functions to occur during appropriate seasons. Thus in most temperate-zone birds, it appears that the young must be hatched during spring and summer when food is abundant and days are of sufficient length to allow procurement of adequate food for the young. Although a rapidly growing body of evidence indicates that biological clocks are widespread among animals (1,2), it appears that most annual physiologic cycles are either controlled directly through mechanisms activated by periodic environmental functions or are at least kept in phase by such (3). A periodic environmental factor which functions in this sense has been designated as a Zeitgeber by Aschoff (3). Obviously many environmental functions may have such a role as an external timer or Zeitgeber. We shall consider here only the cyclic changes of the daily photoperiod.

As an external timer or Zeitgeber, the change in daily photoperiod has an obvious advantage in its fixed annual periodicity. Furthermore, as a uniform function of time, it can operate through a sustained effect on physiologic process or development, rather than by means of a trigger effect. Although trigger types of photoperiodic mechanisms may exist, it appears that the sustained-effect type of mechanism is certainly more prevalent.

Photoperiodic mechanisms must exist primarily because of their survival value to the species or populations involved. These "causes" of the improved probability of survival are the ultimate "causes" of Baker, or the ultimate "factors" of Thomson (4). It is probable that the following are among the ultimate factors in the evolution of photoperiodic mechanisms.

1. *Seasonal synchronization of certain events.* Obviously a mechanism causing the production

[1] Material included herein on photoperiodism in *Zonotrichia leucophrys gambelii* is from investigations supported by funds provided for medical and biological research by the State of Washington Initiative Measure No. 171, by contract Nonr-1520(00) with the Office of Naval Research, and by a research grant (G3416) from the National Science Foundation. Some reference is made to unpublished investigations conducted by or with James R. King, A. C. Wilson, Fred I. Kamemoto, Andreas Oksche, Donald F. Laws, Harold E. Cheyney, and Conrad A. Donovan. I wish to express my sincerest appreciation to them, not only for the performance of the experiments, but also for their many stimulating suggestions.

of young during the most favorable season increases the survival probability of a species. Similarly, a mechanism which causes the initiation of migration, diapause, or hibernation before the advent of the unfavorable season increases the survival ability of the species.

2. *Seasonal synchronization of events within a population.* A mechanism assuring the simultaneous development of reproductive activity in both sexes is obviously essential to the survival of a species. Apparently many species of birds must migrate in flocks; in such cases, there is value in a mechanism that brings all individuals into a migratory state simultaneously. Obviously, the same mechanism may serve in both the first and second categories.

3. *Mutual exclusion of physiologically incompatible events.* It is advantageous in birds, for example, to have a timing mechanism which places the annual molt at a time of the year when no energy is being spent on reproductive activity, when expenditure for thermoregulation is minimal, and when food is easily procurable. This thesis has been developed convincingly by Kendeigh (5), Seibert (6), and Wallgren (7).

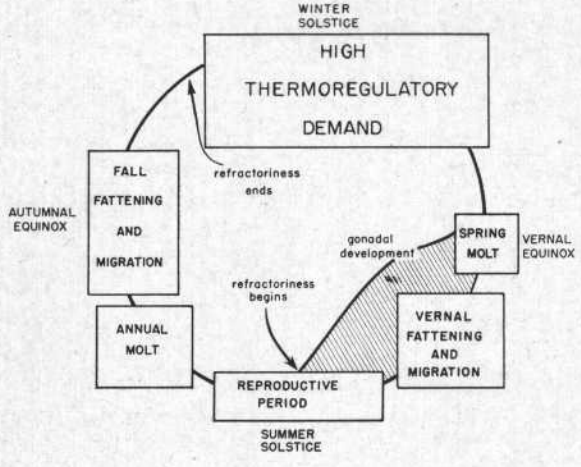

Figure 1. A schematic diagram of the annual cycle of the white-crowned sparrow *(Zonotrichia leucophrys gambelii)* illustrating the essential seasonal synchronization of reproduction and migration and the spacing of physiologically incompatible events.

Figure 1 provides a schematic illustration of the first and third categories of ultimate factors as indicated by the annual cycle of the white-crowned sparrow (*Zonotrichia leucophrys gambelii*).

In considering the evolution of photoperiodic mechanisms, we must also always bear in mind two more general, basic principles in organic evolution:

1. *A principle of phylogenetic restriction.* The evolution of structures and functions occurs by alteration of previously existing structures and processes. Thus, photoperiodic mechanisms probably have developed primarily by alteration of functional relationships among previously existing structures and processes.

2. *A principle of multiple solutions.* Similar structures or functions may evolve from different structures and functions. This implies that superficially similar functions, as we see them now, may involve quite different mechanisms. It is therefore very possible that photoperiodic mechanisms, even within a relatively lower taxon, may represent a battery of cases of convergent evolution. Thus generalizations concerning mechanisms must be made very cautiously.

Photoperiodism Among Animals in General

Although photoperiodic control of annual cycles is probably widespread among animals of mid- and high latitudes, our knowledge is confined largely to arthropods and vertebrates. However, it is apparent that in at least one species of snail (*Lymnaea palustris*) oviposition is photoperiodically controlled (8).

Among the crustaceans it appears that photoperiodic mechanisms are involved in control of the ovarian cycle, development of secondary sex characteristics, and molting cycle of the crayfish (*Cambarus*) (9-11), and in the ovarian cycle of the fresh-water prawn (*Palaemonetes paludosus*) (12).

The insects provide numerous examples of photoperiodic control of seasonal dimorphism (13) and of induction of diapause at all stages of the life cycle (13-15). Indeed, the first experimentally established example of photoperiodism in animals was the demonstration by Marcovitch (16) in 1924 that short days stimulate the development of sexual forms in the strawberry root aphid (*Aphis forbesi*). Photoperiodic mechanisms are involved in the induction of diapause in red-spider mites (17). The photoperiodic mechanism is often a part of a more complex scheme involving changes in temperature and/or other environmental factors. Some examples of photoperiodically controlled functions in arthropods are presented in table I.

Although photoperiodism may be a very common phenomenon among temperate zone vertebrates of all classes (18), it has been investigated most extensively among fish, birds, and mammals.

TABLE I
PHOTOPERIODISM AMONG THE ARTHROPODS
Selected Experimentally Substantiated Examples

Function	Stage stimulated	Species	Effective experimental photoperiod	Reference
Production of sexual forms	parthenogenic females	Strawberry root aphid *Aphis forbesi*	short days	Marcovitch (16)
Production of sexual forms	parthenogenic females	Black bean aphid *Aphis fabae*	short days 8 hrs., <19°	de Fluiter (84)
Production of sexual forms	parthenogenic females	*Brevicoryne brassicae*	short days 8-10 hrs., <22°	Bonnemaison (85)
Pupal diapause	larvae	Noctuid moth *Acronycta rumicis*	short days <15 hrs.	Danilevskii (86)
Larval diapause	larvae	Oriental fruit moth *Grapholitha molesta*	9-13 hrs. 24°	Dickson (87)
Development of females which lay diapause eggs	egg, larva	Silk worm *Bombyx mori* (bivoltine or quadrivoltine)	long days >15 hrs.	Kogure (88)
Development of females which lay diapause eggs	deutonymph, adult female	Fruit-tree red-spider mite *Metatetrarhynchus ulmi*	short days <13 hrs., 15°	Lees (17)
Diapause in adult female	adult female	Common greenhouse red-spider mite *Metatetrarhynchus telarius*	short days <12 hrs., 15°	Lees (17)

Several species of fish apparently have photoperiodic mechanisms involved in control of the annual reproductive cycle (19-20). In some, such as one of the minnows (*Phoxinus phoxinus*), the photoperiodic mechanism functions only when the temperature is within relatively restricted limits (21), whereas in other species, such as the three-spined stickleback (*Gasterosteus aculeatus*), the photoperiodic mechanism is operative over a much wider range of temperature (20). Baggerman's (20) interesting investigations of the three-spined stickleback also suggest that its annual vernal migration from salt water to fresh water is photoperiodically controlled by a stimulation of increased thyrotropic hormone secretion. This causes increased secretion of the thyroid hormone which, in turn, causes development of a preference for fresh water. Selected examples of photoperiodically controlled functions in fish are presented in table II.

TABLE II
PHOTOPERIODISM IN FISH
Selected Experimentally Substantiated Examples

Function	Species	Effective experimental photoperiod	Reference
Completion of gametogenesis, nest-building, oviposition	Three-spined Stickleback, *Gasterosteus aculeatus*	16 hrs., augmented by high temperature 20°	van den Eeckhoudt (89), Baggerman (20)
Vernal migration to fresh water	ditto	16 hrs., augmented by high temperature	Baggerman (20)
Completion of gametogenesis, nuptial color, reproductive behavior, spawning	Banded Sunfish *Enneacanthus obesus*	15 hrs., 22°	Harrington (19)
Maturation of ovary and testes	Minnow, *Phoxinus phoxinus*	long days, temp. >10°	Bullough (21)
Completion of gametogenesis, nuptial color, sexual behavior, spawning	Bridled shiner, *Notropis bifrenatus*	17 hrs., 19°	Harrington (90)

Photoperiodism among mammals is involved primarily in the molting and reproductive cycles. Photoperiodic regulation of molt and growth of hair appears to occur in several mustellid species (table III) and probably also in domestic sheep (22), domestic horses (23), and cattle (24). Although reproductive functions are probably photoperiodically controlled in both sexes, our knowledge largely concerns the female cycle. This is, in part, a consequence of the direction of experimental investigations. However, it should be noted that usually it is the timing of estrus that is critical in fixing the breeding season of the species.

Photoperiodic mechanisms involving long days appear to be implicated in the timing of estrus in the raccoon (*Procyon lotor*) and ferret (table III), as well as the domestic horse (23). In the marten (*Martes americana*), long daily photoperiods control the season of parturition by controlling the duration of the period of delayed implantation (25). Development of estrus seems to be caused by short daily photoperiods in domestic goats (26), several breeds of sheep (27), and possibly also, by a delayed response, in mink (*Mustela vison*) (28).

TABLE III
PHOTOPERIODISM AMONG THE MAMMALS
Selected Experimentally Substantiated Examples

Function	Species	Effective experimental photoperiod	Reference
Breeding	Raccoon, *Procyon lotor*	gradual increase *ca.* 12 to 17½ hrs.	Bissonnette and Csech (91)
Shortening of period of delayed implantation	Marten, *Martes americana*	gradually increased to 16 hrs.	Pearson and Enders (25)
Estrus	Ferret	*ca.* 14 hrs., 16 hrs.	Bissonnette (92), Hart (93), Hammond (94), Thomson and Zuckerman (37)
Molt of winter pellage	ditto	ditto	Bissonnette (95)
Molt of winter pellage	Long-tailed weasel *Mustela frenata* Ermine *Mustela erminea*	increasing day length (6 to 20 hrs.)	Bissonnette and Bailey (96)
Molt of summer pellage	ditto	decreasing day length (15 to 6 hrs.)	
Development of winter pellage	Mink, *Mustela vison*	Short days 6 hrs.	Bissonnette and Wilson (97), Hammond (28, 98)
Estrus ("delayed response")	ditto	6 hrs.	Hammond (28, 98)
Estrus	Domestic sheep (several breeds)	short days, 8 hrs.	Hart (99), Hafez (27)

The mechanism of photoperiodic control of the development of estrus in ferrets has received considerable experimental attention (table III). It is a common experience among the investigators that long photoperiods imposed in midwinter will result in a premature development of estrus. That this regulation is superimposed on another regulatory scheme is evident since estrus will occur eventually, although with protracted delay, even in continuous darkness. The role of a photoperiodic mechanism in providing a more precise timing for an event also subject to other less precise control mechanisms may be more widespread than is currently suspected.

Occurrence of irregular testicular cycles in male mallards in continuous light or continuous dark (29, 30), even though normal control of the testicular cycle is obviously primarily photoperiodic (31-33), is another example of this relationship. Indeed, Thomson (34) has questioned the necessity of the photoperiodic mechanism in estrus timing in ferrets; however, his data do not include information for more than a single season after sectioning the optic nerves. The general elements of the photoperiodic mechanism of the female ferret may be represented schematically as in figure 2. This is primarily on the basis of interpretations by Donovan and Harris (35, 36).

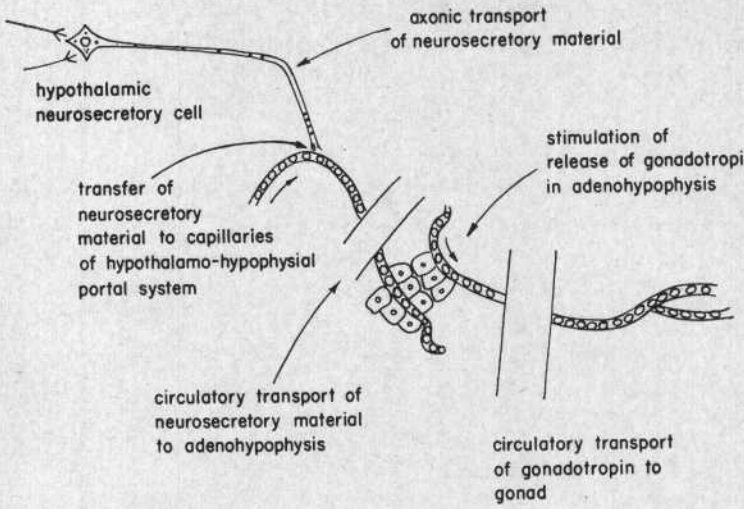

Figure 2. A schematic representation of the photoperiodic mechanism in the female ferret, largely according to Donovan and Harris (35, 36). Photoreception is ocular.

A critical series of events in this scheme is the production of a stimulatory substance in the hypothalamus, the transfer of this substance from the axons of the neurosecretory cells to the blood of the capillaries of the hypothalamo-hypophysial portal system, and its subsequent effect on the adenohypophysis. This requires an intact hypothalamo-hypophysial portal system. The scheme as outlined in figure 2 has been challenged by Thomson and Zuckerman (37) on the basis of photoperiodically induced estrus in ferrets in which they report complete disruption of the hypothalamo-hypophysial portal system. Indeed the disagreement between these two teams of investigators appears to hinge largely on the relatively difficult task of ascertaining when, experimentally, there has been a complete disruption of the portal system. Although the problem of adenohypophysial innervation is perhaps not completely settled, there seems very little reason to expect that it could be involved in a photoperiodic mechanism. The scheme outlined in figure 2 is doubtless oversimplified in light of more recent investigations by Donovan and van der Werff ten Bosch (38). These suggest that long daily photoperiods suppress a hypothalamic center, which during short days inhibits the hypothalamic cells producing the stimulating substance subsequently transported to the adenohypophysis.

Investigations of photoperiodism in birds now outnumber those on all other groups of animals. Although this is partly a consequence of the relative ease with which photoperiodic responses can be studied in birds, it probably also reflects the importance of photoperiodic controls in temperate zone birds.

Functions known to be photoperiodically controlled can be considered conveniently in three groups: reproduction and associated functions; vernal migration and related metabolic adjustments; and molt (table IV). The relationships of these groups of functions in an annual cycle are shown in figure 3. Among the three groups of functions, photoperiodic control of reproductive cycles has been studied most. There is evidence of photoperiodic control of reproduction in at least 35 species. In general, caged males can be caused to produce spermatozoa and to develop reproductive behavior solely with artificially increased day length. However, females usually require that the increased day length be followed by other stimuli if development is to proceed to egg-laying (41, 42). The photoperiodic mechanism may vary from being absolutely essential, as it appears in *Zonotrichia leucophrys gambelii* (43), to serving only as a timer for an event which would occur eventually anyway as in testicular development of domestic ducks (29).

It should be emphasized that control of the reproductive cycle involves an element in addition to the photoperiodic mechanism which operates in response to long days. This is development of refractoriness of the response mechanism as a consequence of the previous photoperiodically induced gonadal development. This period of refractoriness assures termination of reproductive activity, thus providing a period in late summer and early autumn for molt and migration. Natural termination of refractoriness appears to be caused in some way by the short daily photoperiods of winter (39, 44-46). Refractoriness apparently continues indefinitely in some species with con-

TABLE IV
PHOTOPERIODISM AMONG BIRDS
Selected Experimentally Substantiated Examples[1]

Function	Species	Effective experimental photoperiod	Reference
Testicular development, spermatogensis Initial phase of ovarian development Vernal premigratory fat deposition Vernal migration	Slate-colored Junco, *Junco hyemalis*	>10 hrs.	Rowan (100), Wolfson (44), Jenner and Engels (101)
Testicular development, spermatogensis Initial phase of ovarian development	English Sparrow *Passer domesticus*	>9 hrs.	Kirschbaum (102), Polikarpova (41) Vaugien (45, 83), Bartholomew (103)
Testicular development, Migratory behavior	European Robin *Turdus erithacus*	>15 hrs.	Schildmacher (104), Putzig (105)
Spermatogenesis Oogenesis and egg-laying	Bobwhite Quail *Colinus virginianus*	>10 hrs.	Clark *et al.* (106), Bissonnette and Csech (107), Kirkpatrick (108)
Protracted egg-laying period; more eggs	Domestic fowl	long days	
Egg production	Guinea fowl	16 hrs.	Karapetian (109)
Testicular development	Domestic duck	(13 hrs.)	Benoit (31, 32), Benoit and Assenmacher (69, 70)
Testicular development, annual molt	Domestic duck	17.5 hrs.	Svetarov and Straich (63)
(?) Incubation behavior (but not egg-laying)	Domestic pigeon	>8 hrs.	Anarova (110), Larionov and Anarova (11)

[1] For a more extensive resume, see Farner (40, 59).

tinued long daily photoperiods (44, 47-50), although, as noted above, this is not the case in ducks (30). Nevertheless, it does appear that the natural scheme of timing in many temperate zone species involves two photoperiodic mechanisms: a *long-day mechanism* causing vernal development of the gonads, and the *short-day mechanism* which causes elimination of refractoriness in fall or winter. Obviously, however, the situation is more complex in transequatorial migrants (51-53).

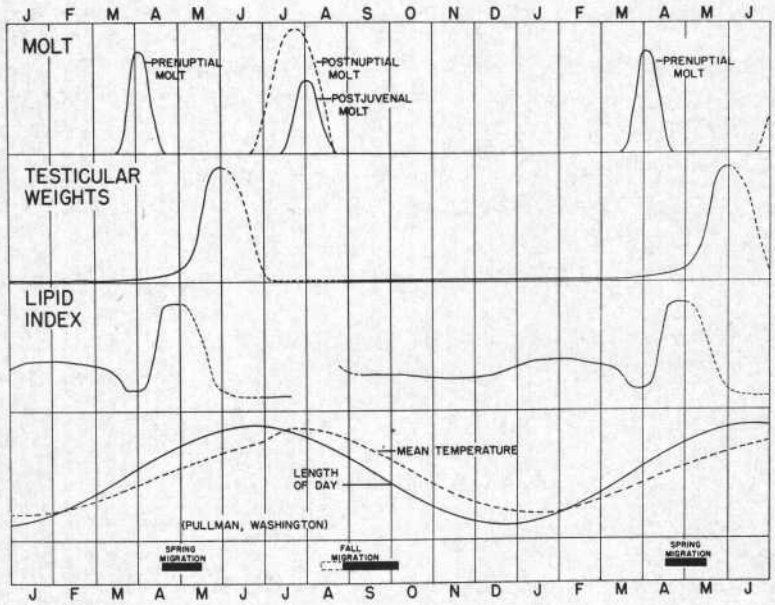

Figure 3. The annual cycle of the male white-crowned sparrow *(Zonotrichia leucophrys gambelii)* indicating the temporal relationships of testicular development, molt, and migration. (Prepared by L. R. Mewaldt)

Vernal migration, in many species at least, is preceded and accompanied by an altered metabolism manifested in marked adiposity. In the white-crowned sparrow (*Zonotrichia leucophrys gambelii*) this involves a photoperiodically stimulated hyperphagia (54, 55) and an altered intermediary metabolism that shifts the storage of energy from glycogen to fat (56). Although interrelationships among these phenomena associated with vernal migration are poorly understood, there can be little doubt that their development is photoperiodically controlled in several species (44, 45, 54, 55, 57-59), although this view is by no means universally held (60). Also, the relation of the vernal migration complex to gonadal development is uncertain. However, it does appear (59) that the relationship is not direct, at least beyond the hypothalamic level, although a direct role of gonadal hormones in the stimulation of migration cannot be completely precluded at this time (61).

There can be little doubt that there is some sort of photoperiodic control of molt and development of plumage in a variety of species. Evidence of this has been recorded for the mallard (*Anas platyrhynchos*) (62, 63), bob white (*Colinus virginianus*) (64), willow ptarmigan (*Lagopus lagopus*) (65), starling (*Sturnus vulgaris*) (66, 67), slate-colored junco (*Junco hyemalis hyemalis*) (45), white-crowned sparrow (*Zonotrichia leucophrys leucophrys*) (45), white-throated sparrow (*Zonotrichia albicollis*) (64), white-eye (*Zosterops japonica*) (68), and others. It can only be noted here that molt, including those cases in which it is obviously photoperiodically controlled, is a process involving functional interrelationships at least among the gonads, the thyroid gland, and the feather follicles. It is beyond the scope of this discussion to formulate a working hypothesis of the over-all mechanism, even if such were possible.

Avian Photoperiodic Testicular Response

The best known photoperiodic mechanism in animals is that involved in the vernal development of the avian testes. It must be emphasized that the testicular photoperiodic response is only one of a rather complex series of photoperiodic responses. This series, as it occurs in the white-crowned sparrow (*Zonotrichia leucophrys gambelii*) is summarized briefly in table V. The photo-

TABLE V
HYPOTHETICAL HIERARCHY OF EXTERNAL CONTROLS IN THE ANNUAL CYCLE OF *Zonotrichia leucophrys gambelii*

Category	Factor	Function	
		Male	Female
Primary timer (primary proximate factor)	Increasing daily photoperiod	*Essential for most events of annual cycle.* Causes spermatogenesis, development of Leydig cells, premigratory fattening (hyperphagia and shift in intermediary metabolism), migratory disposition, sexual behavior, prenuptial molt; also causes development of refractoriness.	*Essential for most events of annual cycle.* Initiates development of ovary; causes prenuptial molt, premigratory fattening (hyperphagia and shift in intermediary metabolism), migratory disposition; also causes development of refractoriness.
Secondary timer	Short daily photoperiod	Ends refractoriness	Ends refractoriness
Essential supplementary factors	(?) migration releaser migration-guiding stimuli migration terminator	presumably the same in both sexes	
	"psychic" factors	territorial behavior, care of young	final development of gonads, sexual behavior, nesting, incubating, care of young
Modifying factors	temperature, etc. food supply	modification of *time* schedule within relatively restricted limits.	

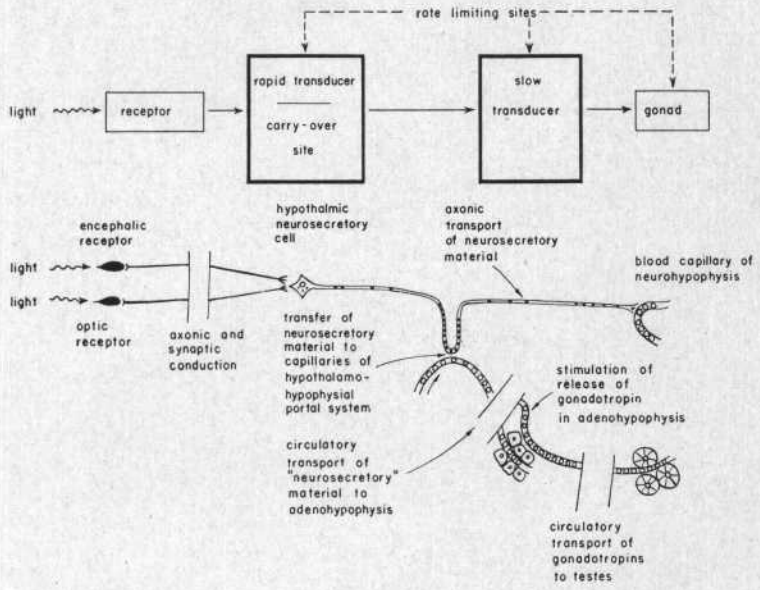

Figure 4. A schematic representation of the photoperiodic mechanism which controls avian testicular development. The lower half of the figure is a functional anatomical scheme based primarily on the investigations of Benoit and Assenmacher (33, 69, 70). The upper half of the figure illustrates the minimum number of hypothetical rate-limiting functions required to rationalize the quantitative relationships between photoperiod and testicular growth in the white-crowned sparrow *(Zonotrichia leucophrys gambelii)*.

periodic control of the testicular cycle will now be described as a composite picture drawn from investigations of several species, but principally from those on ducks by Benoit and his colleagues (32, 33, 69, 70) and from those on white-crowned sparrows (*Zonotrichia leucophrys gambelii*) in our laboratory.

Let us examine first the elements of the mechanism as we presently know them (lower part of figure 4). From the very ingenious studies of Benoit and Assenmacher (69, 71, 72) it appears certain that two sets of receptors are involved; one set is obviously ocular whereas the other is encephalic (rhinencephalon and hypothalamus).

The action spectrum (red and orange are effective; green and blue are ineffective) (33, 39, 40, 73) and the nature of the response of the mechanism to interrupted light (40, 74-77) indicate that ocular receptors may be neither rods nor cones. However, caution must be applied to reasoning based on the action spectrum since the effectiveness of a given wave band is a function of its tissue penetrability (78). Information on the rate of response as a function of intensity is confined almost exclusively to white light; data from several investigations and several species indicate that the mechanism is sensitive at orders of intensities somewhat below one lux with maximal responses in all cases at intensities of the order to 30 - 50 lux or less (40). It appears probable that galliform birds may have lower intensity requirements than passerine species. It should be noted that this sort of intensity function precludes excessive variation in response with variations in atmospheric conditions.

The pathways between the receptors and the hypothalamus are not known; nor is it known definitely which hypothalamic nuclei are eventually involved. In our laboratory we (79) have observed changes in the Gomori-positive material of the cells of the supraoptic nucleus which seem correlated with photoperiod duration. The brilliant experiments of Benoit and Assenmacher (69) on ducks have demonstrated that there must be axonic transport of a stimulatory substance (identical to, or associated with, the Gomori-positive material) to the median eminence with transfer there to the capillaries of the hypothalamo-hypophysial portal sytem and subsequent transport to the adenohypophysis. The anatomical arrangement of the portal system and the hypophysial tracts in birds makes them particularly useful subjects for this type of experiment. This relationship has been confirmed in the domestic fowl (80). In our laboratory (81) we have found that there is a depletion of neurosecretory material in the axonic loops of the median eminence in birds subjected to long daily photoperiods, suggesting the possibility of a more rapid movement out of the loops. The essential role of the adenohypophysis in the production of the necessary gonadotropins has been amply demonstrated (40).

We have given considerable attention in our laboratory to the quantitative aspects of the photo-

periodic responses of the male white-crowned sparrow. Of particular usefulness, in this respect, is the testicular response (75-77, 82). When males are subjected to a constant daily photoperiodic treatment, the growth of the testes, from resting state to a combined weight of about 200 mg. (figure 5), resembles closely the logarithmic growth curve,

$$\log W_t = log\ W_o + kt \qquad (1)$$

where t is the duration of the treatment with constant daily photoperiods, W_o is the initial testicular weight, and W_t is the testicular weight at time t. The slope constant k is then a measurement of the rate of response and is useful in quantitative comparisons of the responses to different experimental treatments. This, for example, has allowed an empirical evaluation of the role of the duration of the daily photoperiod. Thus k, as a function of the duration of the daily photoperiod, may be expressed approximately,

$$k = 0.009\ (p - 9.1) \qquad (2)$$

where p is the duration of the daily photoperiod (supramaximal intensity) in hours and k is in days^{-1}. This empirical relationship holds approximately within the limits, p=9.1 hours to p=17 hours.

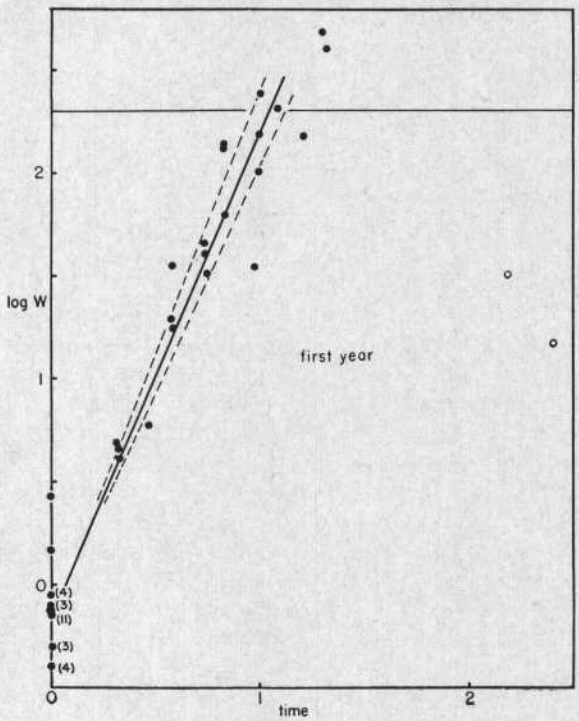

Figure 5. Testicular growth as a function of time with constant daily photoperiodic treatment in the white-crowned sparrow *(Zonotrichia leucophrys gambelii)*. Abscissa is the logarithm of the weight of both testes in milligrams. Ordinate is time in arbitrary units. Closed circles represent developing testes; open circles represent regressing testes. Broken lines represent upper and lower 95%-fiducial limits of the slope. (Courtesy, *Biological Bulletin*)

This relationship has been invaluable in our studies on the effect of dividing the daily light ration into several or many periods by which a much greater value of k may be obtained with a given quantity of light (40, 75-77). For example, when the daily photoperiod is divided in equally spaced 50-minute photoperiods, the effect becomes much greater and may be represented approximately as

$$k = 0.009\ (p_d - 1) \qquad (3)$$

where p_d is the total duration of light (supramaximal intensity) divided in equally spaced 50-minute photoperiods; (3) holds between the limits of p_d = 4 hrs. and p_d = 10 hrs. (77).

A series of experiments with interrupted light (40, 73, 75-77) leads us to the hypothesis that the mechanism has at least three rate-limiting elements (upper half of figure 4).

(1) The first and most apparent of these is designated as a slow transducer. Its existence is indicated by the fact that a photoperiodic treatment, regardless of its nature, must be applied for a minimum continuous period during a 24-hour cycle to be effective. For white-crowned sparrows this minimum period is about nine hours, possibly less since k may not be a linear function of p at lower values of p. At present, the site of this slow transducer can be only conjectural. It could be in the adenohypophysis; if so, the minimum treatment time could be the time required to elevate gonadotropin concentration in the blood to an effective level.

(2) The second rate-limiting element, designated as a rapid transducer, is apparent only when the birds are subjected to patterns of interrupted light. It is apparent also, only when the period of such treatment exceeds the minimum time of about nine hours.

Among our investigations with interrupted light is a series of experiments with two-second flashes of supramaximal intensity. When these two-second flashes were used in 10-second cycles, that is, two seconds of light followed by eight seconds of dark in continuous cycles, the rate of response (k) was near maximum; similar results were obtained with two-second flashes in one-minute cycles. However, there was no response with two-second flashes in 15-minute cycles, although seven-second flashes in 15-minute cycles were effective. These results argue that the effec-

tiveness of very short photoperiods is, in part, a function of the intervening dark period. They have led us to the hypothesis that somewhere (rapid transducer, figure 4) in the response mechanism there is a rate-limiting reaction. During the light period this reaction very rapidly produces a substance essential for the photoperiodic response and during the dark period, this substance decays at a rate much slower than its rate of formation. Thus, stimulation of testicular development persists temporarily after the end of the photoperiod. We (74) have referred to this period of continued effect during the dark as the "carry-over period."

Our data require the assumption that k is effectively a positive function of the concentration of the hypothetical substance only up to some concentration lower than equilibrium concentration during the photoperiod. The data suggest that several seconds are required for the process to go from dark concentration to the equilibrium concentration of the photoperiod, and that the time required for the substance to decay back to minimum dark concentration is probably of the order of a few hours (75, 77). The hypothesis, which has value in considering the nature of the response mechanism and in the design of experiments, is consistent with all the data from a wide variety of photoperiodic treatments of *Zonotrichia leucophrys gambelii*. It appears to be consistent with the data of other investigators using interrupted light (40). The actual site of the rapid transducer is conjectural, although the experiments with flashing lights suggest that it could be at the level of the primary photoreceptors.

(3) Finally it is to be noted that there is a maximum rate of testicular development not to be exceeded regardless of the kind of light treatment. Nor can it be exceeded with supplemental hormone treatment. This, and the fact that k, as a function of p, increases at a markedly decreasing rate as p becomes greater than 20 hours, suggest that the third rate-limiting element may be in the testis itself.

In addition to the relationships and rate-limiting functions discussed above, there are at least two additional properties of the system bearing on its quantitative performance. Both are poorly characterized at present. First, there is an increase in sensitivity of the system as a function of time after the end of the refractory period (75, 83). We had overlooked this because with white-crowned sparrows it is difficult to demonstrate using single long daily photoperiods. However, it is very evident with flash-lighting treatments. Second, the minimal effective value of p is a function of the length of light-dark cycle, or possibly more properly, some function of the duration of the intervening dark period. For example, 13-hour photoperiods in a 24-hour cycle are effective in stimulating the development of the testis whereas 13-hour photoperiods in a 39-hour cycle are ineffective. Whether this is to be explained only in terms of the slow transducer, in the relationship between the slow transducer and the testis, or as some property of the testis is a matter for further investigation.

In summary the photoperiodic control of the annual testicular cycle in birds may be conceived in terms of three mechanisms.

1. The long-day photoperiodic mechanism, normally operating in the spring, involves activation of as yet unspecified photoreceptors, hypothalamic secretion of a stimulatory substance, axonic transport of this material to the hypothalamo-hypophysial portal system, and transport of the material therein to the adenohypophysis which is then stimulated to release additional gonadotropins. Testicular response can be studied quantitatively as a function of the various parameters of photoperiodic regimen as a useful basis for characterization of the over-all mechanism. Such studies suggest at least three rate-limiting functions and a seasonal change in sensitivity of the mechanism.

2. Each period of testicular development is followed by a period of refractoriness in which the long-day photoperiodic mechanism fails to respond to photoperiodic treatments which are ordinarily effective. The nature of this internal refractogenic mechanism is unknown.

3. A short-day photoperiodic mechanism, which normally operates in late autumn and in winter, in some manner eliminates the refractoriness of the long-day photoperiodic mechanism thus placing it in a functional state for stimulation by the vernal increase in day length.

This hypothetical scheme represents a rationalization of our knowledge concerning a few temperate zone species. It is not anticipated that it will have general application even among temperate zone species and certainly not for transequatorial migrants.

DISCUSSION

Question: Have you attempted to make electrical models of this last block diagram?

Dr. Farner: Yes, as a matter of fact, I had a slide to bring, but I wasn't too satisfied with it, partly because there is still an additional thing in here that I didn't talk about. That is, there is also a quantitative function that we don't understand yet which depends on the ratio of the photoperiodic period—whether it is interrupted or continuous—to the intervening dark period, and we got hung up on that. For example, I mean that 13 hours of treatment in 24 cycles is stimulatory but 13 hours of treatment in a 39-hour cycle doesn't affect the thing. This ran into an additional order of complication and I don't know how we stand with electrical bottles.

Question: As the retinal pigments are related to caratonoid pigments in plants, could the two have a common photo receptor?

Dr. Farner: We have explored this some; the possibility that cephalic receptors involve caratonoid pigments exists, but the action spectrum isn't right. In the second place, although this is not strong evidence, we fail to show that we could get any shift of a spectrum in a soup made out of hypothalis when we expose it to light. This point has been raised before, and I think that as matters stand now there is just no reason to presume that the primary photochemical reaction involves caratonoids; I wouldn't want to rule it out.

Question: On what basis did you arrive at the selection of this peculiar avian hybrid for your experiments?

Dr. Farner: The level at which the hybridization was effected, I think, has been misunderstood. When I say about 1/2 duck, 1/6 white ground sparrow, 1/6 sparrow, etc., this approximately indicates the relative contributions of Benoit's laboratory, our laboratory, Bissonnette's laboratory, etc. to this systematic diagram which I have on the board.

Question: Fish "prefer" fresh water under influence of photoperiodic thyroid gonadal system. Is this preference due to an alteration in the ability to regulate salt as by a salt ion?

Dr. Farner: That's really a loaded question and I just don't know the answer to it. I think that I can refer the person who asked this question to Dr. Mans' excellent dissertation which was published last year. This matter is discussed but I don't think if my memory serves me correctly that she has reached any firm conclusion on this.

Question: Would you care to elaborate on the interrelationship between the hypothalamus and the pituitary?

Dr. Farner: With respect to the hypothalamic-pituitary relationship there are a couple of additional points that could be added to strengthen this concept of humoral control of the adenohypophysis by the hypothalamus. An excellent example comes from the tissue culture studies of Heremont who has shown that cultures of adenohypophysis after an interval, I believe a week, no longer release adrenocorticotrophic hormone (ACTH). But if he adds to these cultures some juice from hypothalamus, particularly in the region of the paraventricular and supraoptic nuclei, the culture then begins to produce ACTH which, I think, rather firmly ties down the point that this humoral effect does occur. Now, I should add also that Bald or Hilt who was formerly of Bergman's laboratory has studied supraoptic nuclei cells in tissue culture and he has recorded at least once the movement of one of these granules along in a cone; a granule of the right size. Of course, it couldn't be stained to be sure that it is what we call neurosecretion, but it was the right size and he could see the thing moving along the axions of the neurosecretory cells. He was able to increase the amount of movement of such materials along neurosecretory cells simply by increasing the osmotic pressure of the tissue culture. Now, this makes good sense because as you know, the antidiuretic hormone is produced in these nuclei as figments in response to increasing osmotic pressure. So, I think that we can best size up the relationship between the hypothalamus and anterior pituitary in these terms; that is, anterior pituitary is definitely controlled humoral, not by nerve impulses, humorally by nuclei in the hypothalamus. But the nature of the humoral control, I think, still remains to be worked out. In other words, if we are still going to speak of the pituitary as a master gland we now have to speak of the hypothalamus as a super master or a second order master or something like that.

Question: Is there a difference between the effects of gradually changing photoperiod and those of repeated cycles of the same photoperiod?

Dr. Farner: In white ground sparrows absolutely not. We have worked this out on a good quantitative basis and we have determined testicular growth rates for various photoperiods when the birds were subjected to constant daily photoperiods. That is, each bird got ten hours of light per day, or twelve hours of light per day, etc.

Then from these rates we predicted how the testes would grow in nature where it is subjected to gradualy increasing photoperiods and we missed by something less than ten days in our calculations and we overlooked one variable (well, we didn't overlook it, we didn't know about it at that time) so that if we recalculated, we would probably come even closer. So, as far as we know, in birds the mere fact that light is changing in its duration each day is not what counts. It is the total duration of the light that counts. Now, I wouldn't want to say this with respect to mammals; there is a large argument as to exactly what the situation is in sheep, as to whether it is the changing daylight or the absolute daylight that actually counts.

Question: Does a blood transfusion from a treated bird to an untreated bird have an effect on testicular developments?

Dr. Farner: So far as I know, this has not been tried. At least it has not been published so I claim ignorance on this question.

Question: Will it be possible to measure the amounts of photoactive light reflected in order to determine the amount of light absorbed?

Dr. Farner: Mr. Kaiser is getting at a question that has disturbed us a great deal because we would like to know exactly the quantity of light that is involved. There are many difficulties concerning this. First of all, we can't really be sure that all of the effective light is going through the eye. Presumably that would stimulate the brain, go through the eye, and right through the skull into the surface of the hypothalamus. But, we are not absolutely certain about this and I haven't thought about the possibility of measuring reflective light and subtracting, which I think is what he has in mind. I don't know whether it is possible or not.

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The Natural History of Visual Pigments*

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Many of the visual pigments located in the outer segments of the visual cells of the vertebrate retina can be brought into solution and analyzed spectrophotometrically. Such studies have revealed that these chromoproteins have the same characteristic broad-banded spectra (1), and that the photosensitivity, insofar as it has been determined, is not grossly different for pigments from different animals (2). One important point pertains to the spectral location of these substances. Are there only a limited number of spectral locations for these compounds, such as the region of 500 mμ for the rhodopsins, 522 mμ for the porphyropsins and 562 mμ for the iodopsins, or are these substances distributed over broader regions of the spectrum? The answer to this question has biological significance, not only with respect to theories of color vision, but also to the wider problem of the adaptation of organisms to their environment.

Recent work from a number of laboratories has, in fact, revealed the existence in animals of a multiplicity of visual pigments (3,4,5). We have been especially interested in the types of visual pigments found in nature, and our results, summarized in this report, have suggested that within the setting of a system founded on the two retinenes (retinene$_1$ and retinene$_2$) nature has devised a variety of compounds located over rather broad regions of the spectral scale. It is the purpose of this report to illustrate the nature of this diversity for several vertebrate classes and wherever possible to suggest the biological implications. Some of this work has already been published (6,7,8,9,10,11). Other work is being presented for the first time.

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Analytical Methods

The results of an analysis with an extract of the retina of the three-toed sloth (figure 1) will serve to illustrate the general procedures employed in locating the spectral position of a visual pigment within 2 to 3 mμ. The absorption spectrum obtained with an alkaline (pH 8.2 to 8.4) digitonin extract of the retinae, outer segments, or whole eyes (when the eyes were very small) was first plotted (curve 1, full line).

In the case of the sloth extract the solution was relatively pure, the ratio of the density at minimum to density at maximum being 0.24. Assuming that only one photosensitive component was present (which, in fact, was the case for this extract) the wave length for maximal absorption (λ_{max}) of the visual pigment from the three-toed sloth is at about 493 mμ. This direct method was employed only when a pure extract was available and when the extract contained only one photolabile component. The homogeneity of the extracts was verified by differential bleaching with light at different wave lengths, employing for the initial light exposure a light at the red end of the spectrum. In the absence of a pure solution, difference spectra were employed. The thermally stable, alkaline extract was exposed to a light of selected wave length. Changes in density at different wave lengths throughout the visible spectrum were plotted as a function of wave length. Curve 3 (figure 1) represents such an alkaline difference spectrum obtained with the sloth extract. This difference spectrum is characterized by a spectral maximum for loss of density at about 497 mμ, a value slightly greater than the λ_{max} of the spectrum (curve 1) of the unbleached extract. The maximum for the negative portion (increase in density) is at about 377 mμ, in the region characteristic of the retinene$_1$ pigments.

The addition of NH_2OH to the extract was helpful in several ways. The difference spectrum obtained after bleaching in the presence of this aldehyde-trapping reagent fits the absorption spectrum of the pure extract over a greater portion of the wave length range than is the case for the alkaline difference spectrum. In the case of the ex-

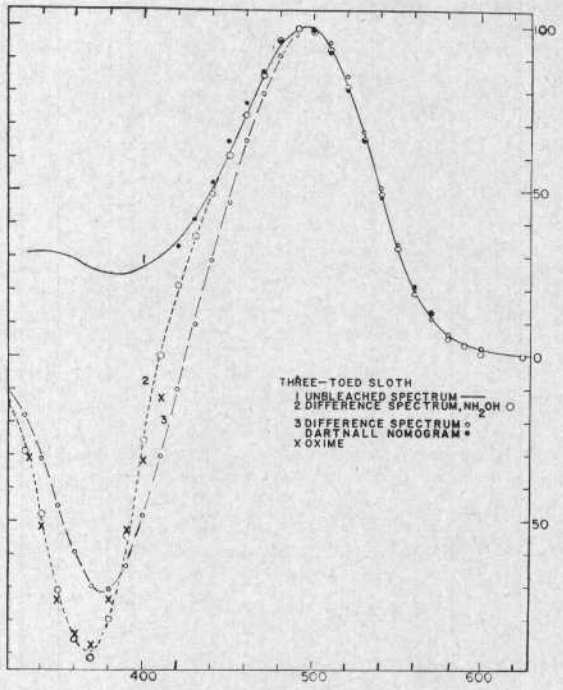

Figure 1. Visual pigment of three-toed sloth. Curve 1 (full line): absorption spectrum of unbleached extract. Curve 2 (larger open circles): NH_2OH difference spectrum after an exposure to light at 430 mμ. Curve 3 (smaller open circles): alkaline difference spectrum after an exposure to light at 606 mμ. The points given by the symbol X give the absorption spectrum which resulted when crystalline, all-trans retinene$_1$ (kindly donated by Dr. Grove Baxter of Distillation Products Industries) was allowed to react with NH_2OH in 2% alkaline digitonin solution. The filled-in points give the curve constructed from the nomogram on the basis of a λ_{max} at 493 mμ. All curves have been scaled so that their maxima coincide.

tract of the three-toed sloth retina, the NH_2OH difference spectrum (curve 2) agrees with the absorption spectrum (curve 1) down to about 440 mμ. The λ_{max} is indicated as well by this NH_2OH difference spectrum as by the absorption spectrum. In addition, the maximum of the negative portion, since it is due to a definite substance (the oxime), is rather precisely located and is useful in differentiating between retinene$_1$ and retinene$_2$ pigments. In the case of the sloth pigment the λ_{max} for the product (curve 2) was at 366 mμ. This indicates a retinene$_1$ pigment since crystalline, all-trans retinene$_1$, when allowed to react with NH_2OH in alkaline digitonin solution, gave a curve (noted as X) which agreed with the negative NH_2OH difference spectrum.

One additional criterion was employed in analysis of these extracts. This was a comparison of the analytical data with the curve constructed by use of the Dartnall nomogram (1). Dartnall pointed out that the alkaline difference spectra of several visual pigments, when plotted with frequency rather than wave length as the independent variable, were all similar in shape. On the basis of this fact he devised a nomogram with which the absorption spectrum of any visual pigment can be deduced once the λ_{max} is known. This last value may be obtained with accuracy, as has been shown, from the NH_2OH difference spectrum. In the case of the sloth extract (figure 1) the filled points represent the values obtained from the Dartnall nomogram on the assumption of a pigment λ_{max} at 493 mμ. These points agree well with both the absorption spectrum (curve 1) and with the NH_2OH difference spectrum (curve 2). Occasionally it was found that the reconstructed curve and the absorption curve of a pure extract did not agree exactly. This is explainable on the basis of the presence of more than one photosensitive component in the extract. In several such cases the method of differential bleaching did in fact reveal the occurrence of two such pigments.

Another experiment, this time utilizing retinal extracts of the Australian gecko, *Oedura monilis*, will illustrate the application of the method of differential bleaching in discovering the presence of more than one photosensitive component. Two analyses, one with and one without NH_2OH, were carried out, with results leading to the same conclusion. The alkaline extract without NH_2OH (figure 2) yielded an absorption spectrum (curve 1, upper section) characterized by a maximum at about 512 mμ, a minimum at about 432 mμ, and a ratio of minimum to maximum densities of 0.57.

The extract was first exposed to light at 660 mμ for 129 minutes. The absorption spectrum changed as shown by curve 2 (upper section). The difference spectrum (curve 2, lower section) revealed a selective loss in density maximal at about 520 mμ and a selective gain in density maximal at about 382 mμ. The loss at 520 mμ was 0.035. The solution was next illuminated for 205 minutes with light at 640 mμ. This involved a relatively large change to curve 3 (upper section). The difference spectrum obtained as a result of this second bleaching (curve 1, lower section) was similar to that resulting from the initial exposure although the density loss at 520 mμ was 0.187. The extract was next bleached for 15 minutes with light at 606 mμ. This caused the spectrum to alter to curve 4 (upper section). The difference spectrum of this bleaching (shown by symbol X, lower section) was identical with the initial change.

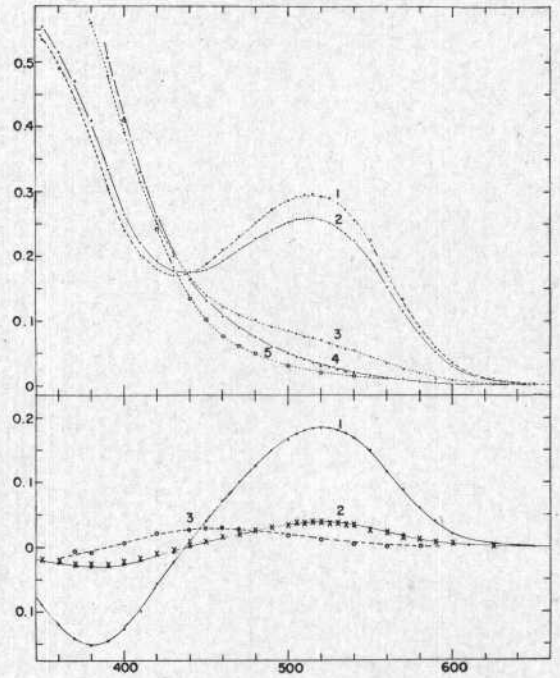

Figure 2. *Upper section.* Results of an analysis of an extract from *Oedura monilis*. Curve 1: absorption curve of unbleached extract. Curve 2: result of exposure for 129 minutes to light at 660 mμ. Curve 3: result of exposure for 205 minutes to light at 640 mμ. Curve 4: result of exposure for 15 minutes to light at 606 mμ. Curve 5: result of exposure to tungsten light (40 watts) for 10 minutes. Plot of optical density versus wave length.

Lower section. Corresponding difference spectra resulting from above-indicated succession of bleaches. Curve 1 is the 2-3 difference spectrum. Curve 2 is the 1-2 difference spectrum (full line) and the 3-4 difference spectrum (shown as X). Curve 3 is the 4-5 difference spectrum. Positive figures indicate loss; negative figures indicate gain, in density as a function of wave length.

In fact the two effects were superimposable. This equality of results obtained before and after the greater portion of the pigment had been bleached is good evidence that, within this range of bleachings, only one photosensitive component was involved.

Indications of the existence of an additional photosensitive pigment in the *Oedura* extract were obtained from the results of a final exposure of the solution to white light. This caused a spectral change to curve 5 (upper section) which involved a selective loss of density at about 457 mμ and a gain at about 358 mμ (curve 3, lower section). This action of the white light could have been the result of the presence in the extract of a blue-sensitive component or, alternatively, of the action of this light on the products of bleaching of the main pigment. The second experiment in which NH_2OH was added to the extract was performed in order to further elucidate this effect. This experiment is summarized by figure 3.

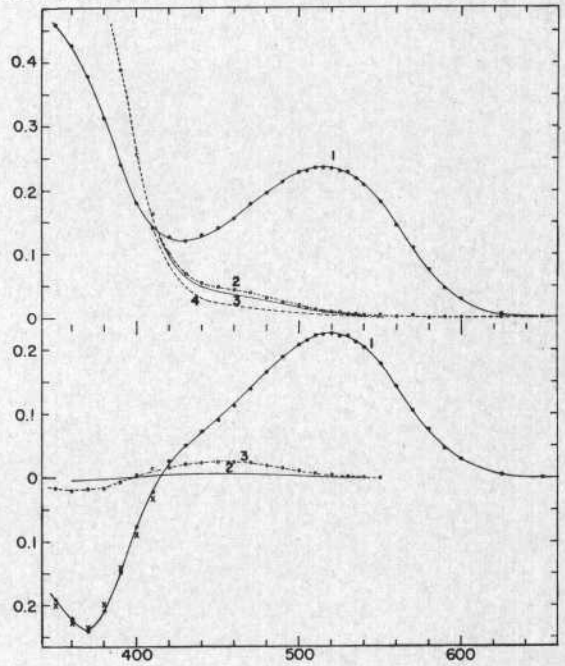

Figure 3. *Upper section.* Results of an analysis of an extract from *Oedura monilis* in the presence of NH_2OH. Curve 1: absorption curve of unbleached extract in the presence of NH_2OH. Curve 2: result of exposure for 124 minutes to light at 606 mμ. Curve 3: result of exposure for 125 minutes to light at 560 mμ. Curve 4: result of exposure to tungsten light (40 watts) for 10 minutes.

Lower section. Corresponding difference spectra. Curve 1 is the 1-2 difference spectrum. The points indicated as X show the data (adjusted to match at maximum) obtained with a retinene$_1$ oxime in 2% digitonin. Curve 2 is the 2-3 difference spectrum. Curve 3 is the 2-4 difference spectrum.

The spectrum of the unbleached solution in the presence of NH_2OH (curve 1, upper section) is not significantly different in form from the corresponding spectrum of the first *Oedura* experiment. In other words, there is no evidence of a destruction or change of the pigment by the NH_2OH. This is generally true of most of the visual pigments with which we have worked, although we have encountered a few such pigments which were slowly bleached in the presence of NH_2OH. After obtaining the spectrum (curve 1, upper section) the solution was exposed for 124 minutes to light at 606 mμ. This resulted in complete bleaching of the main pigment. The difference spectrum (curve 1, lower section), unlike

the alkaline difference spectrum, has a distinct break between the positive and negative portions of the curve. This is characteristic of other visual pigments bleached in the presence of NH_2OH, and is the result of the fact that the product (retinene oxime) has an absorption spectrum located sufficiently away from the spectrum of the visual pigment that the two substances are clearly differentiated by the density changes resulting from exposure to light. In this instance the presence of the break offers the important information that the NH_2OH had in fact reacted with the product of bleaching.

The results of this first bleaching lead to two conclusions regarding the main pigment in this extract. The first is that the absorption maximum of this substance is close to 518 mμ, the wave length of maximum density loss, and the second is that the chromophore is retinene$_1$. This second result follows from the fact that the oxime of retinene$_1$ formed by adding hydroxylamine to a solution of all-trans, crystalline retinene$_1$ gave a spectrum (indicated by the symbol X, lower section) which agrees well with the product portion of the difference spectrum.

The spectrum (curve 2, upper section) obtained after the initial bleaching with light at 606 mμ is especially informative because it possesses a distinct upward inflection in the region of 460 mμ. This suggests the presence, in the solution from which the main pigment had been removed, of a component absorbing in the blue region. The next two exposures will point out that this inflection was abolished by light at wave lengths shorter than 606 mμ. The first of these exposures was for 125 minutes to light at 560 mμ. This caused a very small change (figure 3) but the important information conveyed by this bleaching was that the main pigment at 518 mμ had in fact been completely removed by the previous bleaching with light at 606 mμ. The final exposure was for 10 minutes to a 40 watt tungsten light. This led to a disappearance of the inflection (curve 4, upper section) and to a difference spectrum (curve 3, lower section) with a maximal loss of density at 457 mμ.

The results of both experiments with the *Oedura* extract point to the fact that following removal of the main photosensitive component there occurred in response to appropriate illumination a selective change as described. The important feature of this finding is that it was obtainable in an extract in the presence of NH_2OH, a substance which has been found to be useful in preventing isomerizing or other side reactions of the products of bleaching. In a previous study of another gecko (7) it was found, for example, following removal of the main pigment, that there resulted in response to white light a spectral change in the violet region. This effect was different in form from that reported here and in addition was abolished by NH_2OH. It was very likely an action of the white light on the products of bleaching. In contrast, the *Oedura* effect was significantly different in form and persisted in the presence of NH_2OH. Moreover, the presence of a clear inflection in the spectrum following the removal of the main pigment is evidence not easily ignored. The only point in question is whether the blue-absorbing pigment was a component of the original unbleached extract or a product formed as a result of bleaching the 518 pigment. The pigment at 457 mμ could, for example, have been a result of regeneration. The fact that the NH_2OH had combined with the retinene opposes this view. In any case no evidence of regeneration was obtained during the 45-minute period required to determine the absorption curve.

The evidence suggests the presence of two separate photosensitive substances in the retina of this gecko. The main pigment at 518 mμ is undoubtedly a visual pigment. Its high concentration in a retinal extract, its photosensitivity, and the presence of retinene$_1$ are all features supporting this conclusion. Moreover, the experimental data fit a curve (figure 4A) constructed from the nom-

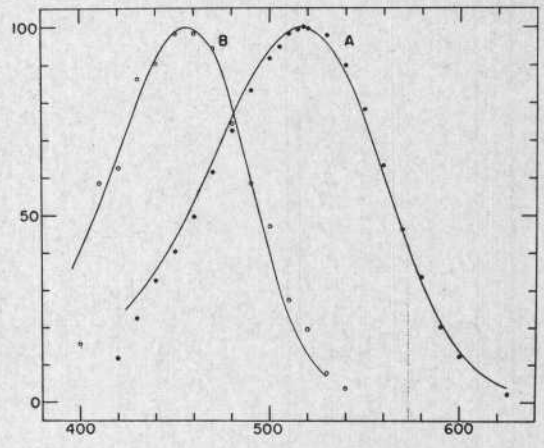

Figure 4. Curves constructed from the Dartnall nomogram for visual pigments. Curve A for a pigment with λ_{max} at 518 mμ; curve B for a pigment with λ_{max} at 457 mμ. Points are difference spectra data from the hydroxylamine experiment. The filled points are the 1-2 difference; the open points are the 2-4 difference—all adjusted so that the maximum is set at 100%.

ogram assuming a λ_{max} at 518 mμ. The conclusion with regard to the 457 mμ pigment is not as certain. It is photosensitive, but riboflavin, a photolabile compound, has been detected in the vertebrate retina (12). Pigment 457 is probably not riboflavin. This follows from the results of an experiment with a solution of riboflavin in 2% digitonin. After illumination of this solution a difference spectrum was obtained with two peaks, one at 373 mμ and the second at about 453 mμ. The 453 mμ peak was significantly narrower than the 457 difference spectrum of *Oedura*. A comparison of the difference spectrum of the 457 mμ pigment with a curve constructed from the nomogram assuming a λ_{max} at 457 mμ is made in figure 4B. The data are in reasonable agreement so we are forced to the conclusion that pigment 457 of *Oedura monilis* may in fact be a visual pigment. This experiment with extracts of *Oedura monilis* illustrates not only the usefulness of the method of differential bleaching, but also some of the problems one encounters in analyzing retinal extracts.

To summarize, the procedure involved the following steps.

(A) In cases where a pure extract was available, the λ_{max} was determined directly, differential bleaching being employed to test the homogeneity of the preparation.

(B) In both pure and impure extracts, alkaline difference spectra obtained after selected monochromatic bleachings were useful in describing the long wave-length portion of the absorption spectrum and in locating approximately the wave length of maximum absorption. Providing the extract was stable the λ_{max} obtained in this way was the same for solutions of varying purity. This fact was pointed out by Crescitelli and Dartnall (8) and we have had many occasions to discover the usefulness of this approach in the case of extracts which for various reasons were impure.

(C) Difference spectra obtained in NH_2OH experiments were employed in locating the λ_{max} precisely and in describing the absorption spectrum down to about 430-450 mμ. In addition, the position of the product peak permitted a statement as to the nature of the retinene. Retinene$_1$ pigments yielded product maxima in the region of 366-368 mμ, while retinene$_2$ chromoproteins resulted in comparable maxima at 385-387 mμ.

(D) The absorption spectrum of the unbleached pure extract and the positive portions of the difference spectra, when in agreement with the curve deduced from Dartnall's nomogram, provided reassurance of both the homogeneity of the extract and of the absorption spectrum of the visual pigment.

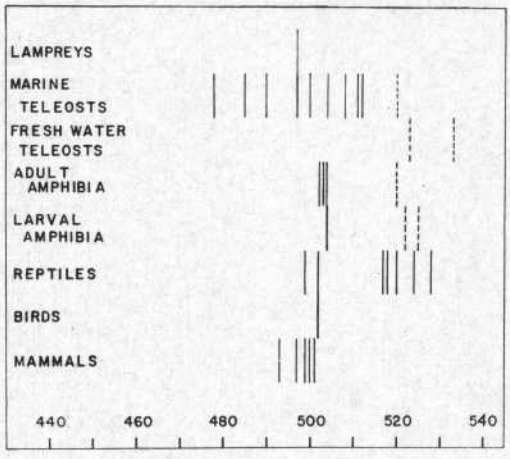

Figure 5. The λ_{max} values of visual pigments from various vertebrates. Most of these results are based on NH_2OH difference spectra. The full line indicates retinene$_1$ pigments; the dotted line indicates retinene$_2$ pigments. This is an incomplete summary of the work since for many visual pigments the NH_2OH technique has not yet been applied. It is likely that the diversity is even greater than shown by these data.

Evidence of Diversity

The general picture which emerged as a result of the application of the above methods is summarized in figure 5 which pictures the wave length positions for maximum absorption for some of the pigments examined in our laboratory. Most of these results are based on analyses by the NH_2OH difference spectrum technique. The results were quite unexpected. Instead of a simple rhodopsin-porphyropsin display, these visual chromoproteins from only this very limited sampling of the animal kingdom range themselves over a considerable portion of the spectrum. The diversity is greater for certain groups of vertebrates than for others. It is especially striking for the marine teleosts in which group the work of Munz (13) revealed a spread from 478 mμ to 520 mμ. It is also striking in the reptiles in which a retinene$_1$ system of pigments was noted in the range from 499 mμ to 524 mμ. Diversity has developed in both aquatic and terrestrial vertebrates. Even the mammals revealed some variation from about 493 mμ to 502 mμ.

In spite of this, the basic idea of Wald (14) that the vertebrate visual pigments are associated

with two retinenes is supported by the evidence. This is indicated by the data of figure 6 in which the spectral locations of some of the pigments studied by the NH_2OH difference spectrum procedure are indicated, along with the positions of the oxime formed after bleaching. It is clear that the pigments, though diverse spectroscopically, yielded but two products: retinene$_1$ (oxime at about 368 mμ) and retinene$_2$ (oxime at about 387 mμ). Nature has devised a mechanism for obtaining considerable spectral adjustment of the visual protein while utilizing only two prosthetic groups. The nature of this mechanism is not known but the protein portion of the molecule is probably implicated.

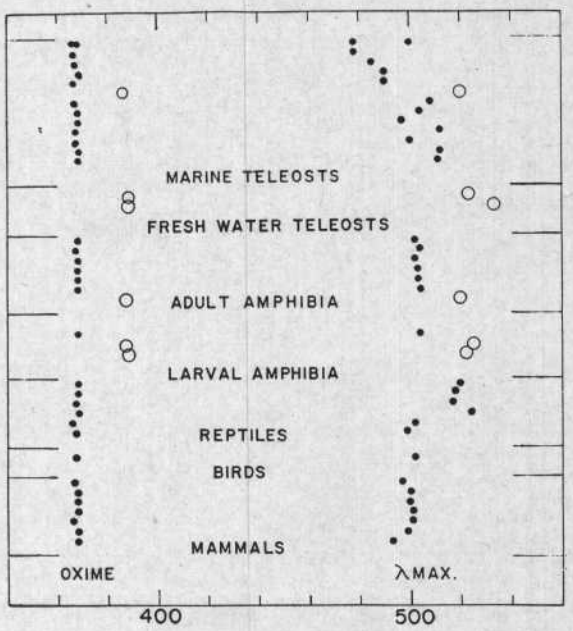

Figure 6. Summary chart indicating absorption peaks of visual pigments deduced from the NH_2OH difference spectra and the nature of the retinene. Open circles indicate the retinene$_2$ system and filled-in points indicate the retinene$_1$ system. A dual system based on the nature of the chromophore is suggested.

Visual Pigments of Specific Vertebrate Groups

1. Lampreys

Cyclostomes are of interest (a) because of their phylogenetic antiquity and (b) because their life cycle involves profound ecological, morphological, physiological, and biochemical changes. No direct objective analysis of the visual pigment system of these animals was made until recently when Crescitelli (6) reported the occurrence of classical rhodopsin in the land-locked Great Lakes sea lamprey. (*Petromyzon marinus*) and in the Pacific Coast lamprey, *Entosphenus tridentatus.*

This finding was surprising since Wald (15) had previously concluded, on the basis of vitamin A analyses, that the sea lamprey (*P. marinus,* sea-run population) had a preponderance of the porphyropsin system. For his analyses Crescitelli employed recently transformed downstream migrants of the land-locked sea lamprey and upstream migrants of the Pacific lamprey on their way to the spawning grounds. Wald had used upstream migrants of the sea lamprey. Recently, Wald (16) reexamined this problem with due regard to the stage of metamorphosis of the animals under examination. He first confirmed Crescitelli's finding of rhodopsin in the downstream migrants of the land-locked *P. marinus.* In the upstream migrants of the sea-run form of *P. marinus* Wald found only porphyropsin which in this animal possessed a peak at 518 mμ. These results were interpreted by Wald as indicating the presence of a dual system of pigments in the sea lamprey with rhodopsin as the characteristic system of the downstream migrant and of the parasitic phase, and with a change to porphyropsin occurring as the animals approach sexual maturity. Wald's case could have been stronger if he had compared the same ecological population as well as the same species. Assuming, however, that the land-locked and sea-run populations behave in the same manner, there is still the larger question of whether all lampreys possess this dual system of pigments and show this metamorphic shift. Since my original publication on this subject I have examined downstream migrants and upstream migrants of *E. tridentatus* and I have always found the classical rhodopsin system (figure 7). I have not yet had the opportunity to test lampreys gathered from their nests so as to assay the pigment of sexually mature adults. It may be that *E. tridentatus* will turn out not to have the metamorphic change suggested by Wald for the sea lamprey. A more thorough study of lampreys in general needs to be made before definitive conclusions about this group of vertebrates are set down.

2. Marine Teleosts

Analyses of extracts of the retinae of marine teleosts have demonstrated the presence of a pigment system predominantly of the retinene$_1$ type. This is in accord with the concept proposed by Wald (14). Spectroscopically, however, these pig-

ments are located, not merely in the region of 500 mμ, but all the way from about 478-525 mμ. Is there any biological significance to the array of pigments in these fish? Munz (13), who recently completed a study of 35 species of marine or euryhaline teleosts, has made the following observations.

(a) There is no overall obvious phylogenetic significance to this array. Taxonomic position is not correlated with the spectroscopic type of visual pigment possessed by a fish. There appears to be some consistent relationship within the family grouping, at least for some teleost families. Thus the species, *Oncorhyncus kisutch* and *Oncorhyncus tshawytscha,* in the family Salmonidae, showed the presence of a retinene$_1$ pigment at 507 mμ. In the family Gobiidae, three species, *Clevelandia ios, Eucyclogobius newberryi,* and *Gillichthys mirabilis,* revealed another retinene$_1$ system at 512 mμ. One species in this family, *Coryphopterus nicholsii,* was found to be unusual in the possession of a 500$_1$ pigment, using the subscript to indicate the type of chromophore. Two members of the family Embiotocidae, *E. jacksoni* and *Hyperprosopon argenteum,* showed a 506$_1$ pigment; and the cottids, *Cottus sp.* and *Leptocottus armatus,* were found to have a 511$_1$ compound. Genetic factors, probably involving the nature of the protein, may be important in these relationships.

(b) Perhaps the most intriguing suggestion which emerged from the recent work on marine

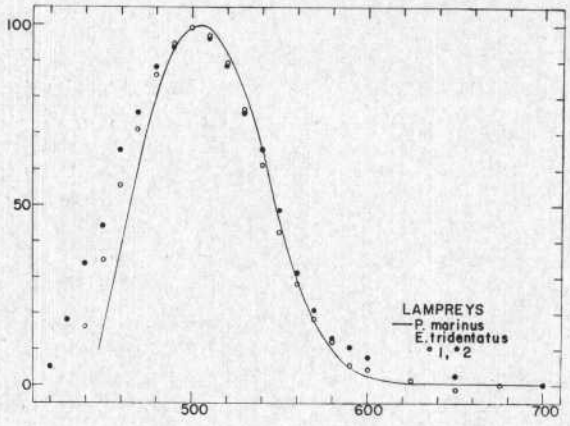

Figure 7. Positive portions of alkaline difference spectra of lampreys. Full line: *P. marinus* (land-locked Great Lakes population); downstream migrants; line is mean of two bleachings each at 630 mμ. Open circles: *E. tridentatus;* upstream migrants; mean of two bleachings, one at 630, the other at 600 mμ. Filled-in points: *E. tridentatus;* upstream migrants; mean of two bleachings, one at 631 mμ, the other at 606 mμ. Downstream forms of *E. tridentatus* gave similar results but these are not shown.

teleosts is the idea that these pigments, in some species at least, may be adapted to the quality of the light in the environment. This idea arose directly out of the studies of the pigments in certain deep-sea fishes. Even before these pigments were known, however, it had been predicted from studies of the spectral composition of the light transmitted by sea water that deep-sea fish might have spectral sensitivity curves with maxima in the blue-green region (17,18). Denton and Warren (19) were the first to report the presence in deep-sea fish of retinal photosensitive pigments with absorption maxima in the region of 480 mμ. These substances, called chrysopsins by Denton and Warren, were not studied by the method of extraction, but by means of densitometry applied to the excised retina. At the time that information about Denton and Warren's work reached us, Munz was at sea collecting deep-sea fish for study. He prepared extracts of the retina, and his results confirmed the findings of Denton and Warren and reported the additional datum that these compounds belong to the retinene$_1$ system (10, 11). Wald, Brown, and Brown (20) have also extracted such pigments from the retinae of certain deep-sea fish. They demonstrated that the original photosensitive pigment is regenerated in the dark upon addition to the protein of the neo-b retinene$_1$.

The spectral location of these chromoproteins well below 500 mμ is best suited to the quality of the light transmitted to these oceanic depths. Jerlov (21) reported that light penetrating to 200 meters has a maximum at 475 mμ and that at this depth only a narrow spectral band remains. It could be, however, that the retinal pigment is also adapted to light of biological origin, which at sufficient depths may, in fact, be the only light. The treatises of both Harvey (22) and Johnson (23) suggest that the bioluminescence of deep-sea fish, though variable from species to species, tends to be predominantly bluish or bluish-green. Recently Kampa and Boden (24) studied the spectral quality of light at various depths of the San Diego trough. They found the light flashes at 25 and 60 meters to be bluish-green in quality, the mean spectral emission curve having a peak at 478 mμ. Kampa and Boden made the point that the intensity of the flashes was often as much as 1000 times the level of the background illumination and the overall level of flash illumination actually increases with increasing depths. These investigators also measured the spectral distribution of the light emitted by two crustaceans, *Euphausia pacifica*

and *Pyrosoma atlantica.* For *Euphausia* the spectrum was bimodal with a peak at 476 mμ and a lower peak at 515-530 mμ. For *Pyrosoma* a similar curve was recorded with peaks at 482 mμ and 525 mμ. Such a "match" between the color of the emitted light and the absorption spectrum of the photon-capturing substance of the retina could be important in such processes as food capture, mating, and schooling.

These pigments of deep-sea fishes are present in high concentrations within the visual cells. Denton and Warren (25) reported densities between 0.19 and 1.21 in various fishes. This is obviously an adaptation for vision in an environment with low light levels. Such high densities, however, lead to greater thermal breakdown and, it has been assumed, to higher noise levels. Such an effect is partly compensated for because of the low temperatures of the deep-sea fish environment. It has also been assumed that a spectral shift to lower wave lengths will reduce the rate of thermal breakdown, leading to a lower noise and thus improving the ability of the eye to detect signals at low light levels (26). According to this concept the spectral positions of the visual pigments of deep-sea fishes, relative to other fishes, has resulted in a visual system with greater inherent stability. Recent work, however, has questioned the view that light and heat bleach rhodopsin by the same mechanism and has suggested the idea that only the light effect is specific for the excitatory process (27). The importance of experiments to determine the *in vivo* noise level and the visual threshold in fishes with a high concentration of a pigment absorbing in these lower spectral regions is suggested by the recent work.

Although the visual pigments of marine teleosts are predominantly of the retinene$_1$ system, a strictly marine fish is occasionally found with a retinene$_2$ type of chromoprotein. Munz has noted this phenomenon but the experiments are still incomplete. Wald (28) himself admitted an exception to the general rule when he found in the retina of two species of labrids, *Tautoga onitis* and *Tautogolabrus adspersus,* a great preponderance of vitamin A_2 over vitamin A_1. In our laboratory, however, Munz (29) examined the visual system of the sheep-head, *Pimelometopon pulchrum.* In this labrid Munz found only a retinene$_1$ system. The labrids are obviously worthy of further examination.

In the retinal extracts of several marine teleosts, not one, but two photosensitive pigments with the characteristics of visual substances have been noted. The scup (*Stenotomus chrysops*) has been found to contain rhodopsin and another retinene$_1$ photosensitive component with a λ_{max} in the region 525-530 mμ (30). This second component was found to be present in much smaller quantities. From the tautog the same authors (30) reported the presence of a dual retinene$_2$ system with porphyropsin (522 mμ) and a smaller quantity of a pigment at about 550 mμ. Munz (13) has also noted the occurrence of such dual systems in a number of marine teleosts.

Perhaps the most interesting of these double systems was the one found in the deep-sea fish, *Bathylagus wesethi* (11). This was revealed by the method of differential bleaching and illustrates clearly the ability of this method to detect heterogeneity. From the hatchet fish (*Argyropelecus affinis*) a relatively pure extract was prepared. The absorption spectrum of this extract and a curve constructed from Dartnall's nomogram, assuming a pigment with λ_{max} at 478 mμ, agreed perfectly down to about 450 mμ. Selective bleaching in the presence of NH_2OH yielded successive difference spectra which were the same following successive exposures to light of 606 mμ, 580 mμ and 560 mμ. The λ_{max} values of these difference spectra were constant at 478 mμ and the product peak (at 365-367 mμ) was characteristic of a retinene$_1$ system. No evidence of any other component in the extract was obtained.

In contrast were the results obtained with a retinal extract of *Bathylagus.* The extract of the same degree of purity as that from *Argyropelecus* yielded an absorption spectrum which could not be fitted by a curve deduced from the nomogram. The two curves when matched at the peaks showed serious departure at the yellow-orange portion of the spectrum, the absorption spectrum being broader. Selective bleaching in the presence of NH_2OH revealed inhomogeneity. An initial exposure to red light (631 mμ) produced a typical difference spectrum with a λ_{max} at 500 mμ and a product peak indicative of retinene$_1$. The positive portion of this difference spectrum was adequately fitted by a curve read off the nomogram assuming a λ_{max} at 500 mμ. Five successive bleachings with light at 606 mμ, 606 mμ, 580 mμ, 580 mμ, and 560 mμ resulted in a λ_{max} change to 497 mμ, 485 mμ, 480 mμ, 478 mμ, and 478 mμ, and all the while the product peaks remained constant at the retinene$_1$ position. The final difference spectrum was adequately fitted by a nomogram-derived curve with maximum at 478 mμ. These data were interpreted to mean that there are two

retinene$_1$ visual pigments in the retina of *Bathylagus,* one at about 500 mμ, the other at about 478 mμ. Munz calculated that the 500$_1$ pigment constituted about 20-25% of the total photosensitive system. The idea that such a dual system may have arisen in association with the habit of vertical migration, which some of these fish are known to possess, is merely an intriguing thought. No other deep-sea fish has yet been shown to yield, by these methods, two visual pigments. A similar experiment with the sheep-head (a labrid) has disclosed another retinene$_1$ system of two pigments, one at about 520 mμ, the other at about 497 mμ (29).

Duality of another type also occurs in fish and this involves the possession of both a retinene$_1$ and a retinene$_2$ system. Wald (28) is the originator of the idea that euryhaline fishes are characterized by such duality. Bridges (31) put this idea to direct test when he examined extracts of the rainbow trout by means of selective bleaching. In this euryhaline fish Bridges noted the presence of two photosensitive components, one at 533 mμ with retinene$_2$; the other at 507 mμ with retinene$_1$. In our laboratory we also have obtained clear evidence of such duality in salmon. The interesting query as to whether a change in proportion of the retinene$_1$-retinene$_2$ system accompanies the migrations of fishes such as the salmons and the eel has not yet been put to test.† The same sort of lacunae in knowledge exists here as for the lampreys. It should be pointed out, however, that euryhalinity *per se* is not necessarily associated with such a duality. The mudsucker, *Gillichthys mirabilis,* is able to tolerate great changes in salinity (32). Extracts of the retinae of this fish have revealed only one photosensitive component, a retinene$_1$ pigment at 512 mμ (9). This conclusion was reached from an analysis of the absorption spectra of rather pure extracts and from the results of differential bleaching.

3. Fresh Water Teleosts

The theme of this paper—multiplicity and diversity of visual pigments—arose originally in connection with the visual pigments of fresh water teleosts. Willy Kühne, who in fact made the first study of the natural history of visual pigments, saw a difference in color between the retinae of certain fish and the retinae of other animals. He, together with Sewall, noted that the retina of the bream (*Abramis brama*) tends to be more violet than purple (33). They observed that the bream retina is distinctly different in color from the eel retina which in turn is similar to the frog retina except that it is more saturated. From the difference in appearance in different spectral lights, they contrived a crude sort of absorption spectrum for the *Abramis* pigment and compared it with a similarly contrived spectrum for the frog pigment. The spectrum of the bream pigment was shown shifted bodily toward longer wave lengths relative to the position of the frog visual purple. Utiliizng various portions of the sun's spectrum, these workers also noted the difference in bleaching effectiveness of light of different wave lengths and the fact that light of different wave lengths acted differently on the frog and bream pigments. In these brilliant studies Kühne anticipated and stimulated much of the modern work on absorption spectra and on selective bleaching of visual pigments.

The difference in the visual pigments of fish and other vertebrates was confirmed spectrophotometrically by Köttgen and Abelsdorff (34) who employed solutions of these compounds in bile. Employing difference spectra these workers made the important observation that whereas mammals, an owl, and amphibia have visual pigments at about 500 mμ, the corresponding pigments for the fish were at about 540 mμ. The importance of Köttgen and Alebsdorff's contribution lies, not in precisely locating the positions of the visual systems with which they worked (for in certain aspects recent work has brought out errors), but in the fact that it pointed the way to a discovery of a fundamental natural difference between visual pigments.

This discovery was made by Wald. After noting that certain marine fish (35) have visual purple located at 500 mμ and not at 540 mμ, Wald turned to fresh water fish (36,37). These were found to be different in that a new visual protein —named porphyropsin because of its color—was found in place of the rhodopsin of marine fishes. Wald observed that a new retinene (retinene$_2$) was extracted from bleached retinae and that on standing, these retinae yielded a new substance which turned out to be similar to a new compound extracted from the livers of fresh water fish by Lederer and Rosanova (38). This was vitamin A$_2$. Thus, a new visual system based on retinene$_2$-vitamin A$_2$ was discovered to be present in the fresh water fishes. It was soon realized that the fishes examined by Köttgen and Abelsdorff (34)

† Carlisle and Denton have noted that the retina of the eel (*Anguilla anguilla*) is golden-colored in the mature stage whereas it is purple-colored in the immature fish. This information is given in a recent paper by Denton and Walker (73).

were fresh water teleosts and so their discovery of a pigment in fishes distinct from that in mammals, birds, and amphibia was explained. Wald (37) questioned the significance of the 540 mμ position of the pigments studied by Köttgen and Abelsdorff. He considered their use of difference spectra to be meaningless. To Wald porphyropsin meant not only the visual pigment of fresh water fishes, but also the retinene$_2$ pigment with a maximum at 522± 2 mμ. It is clear that originally Wald held rigidly to this spectroscopic definition. Recently he has broadened the scope of the word porphyropsin to include the visual pigment of the upstream stage of the sea lamprey which was found to have a λ_{max} at 518 mμ. In any case it is doubtful if the pigments extracted by Wald (37) all had absorption peaks at 522 mμ. For his conclusion Wald utilized only the absorption spectra of the unbleached extracts. These were of varying degrees of purity. As Crescitelli and Dartnall (8) pointed out, the absorption peaks of such extracts are not truly indicative of the pigment peaks. Dartnall (3) noted, for example, that in the case of Wald's pickerel extract, which was the most impure, correction gave a figure of 532 mμ for the pigment maximum. In addition, Wald did not test his extracts for homogeneity, assuming that only one photosensitive component was present. Recent work has demonstrated that such an assumption cannot be made; the homogeneity of an extract must be proved experimentally.

The most recent studies, especially by Dartnall (3), have revealed the occurrence of considerable diversity in the visual pigments of fresh water fishes. In some cases a pigment is found located in the classical position defined by Wald. Such a case is that of the carp, *Cyprinus carpio* (8). In other fishes, pigments have been detected at spectral positions significantly different from the 522 mμ location. Such a case is that of the tench (*Tinca tinca*). In this fish Dartnall obtained a component with a peak at about 533 mμ (39). To satisfy myself that a real difference exists in the position of the carp and tench pigments, I recently analyzed, by means of NH_2OH difference spectra, an extract of carp retinae and an extract of tench retinae kindly sent to me by Dr. Dartnall. These two extracts were of about the same degree of purity. Bleaching each of these extracts with red light uncovered the following information (figure 8). The carp pigment difference spectrum had a maximum for density loss at about 520 mμ and a maximum for density gain at about 387 mμ. The corresponding figures for the tench extract were 533 mμ and 387 mμ. The long wave portions of the tench and carp difference spectra are seen to be systematically displaced from each other with the tench segment located closer to the red end of the spectrum. The results clearly indicate that while both of these pigments belong to the retinene$_2$ system, they are separated spectrally by more than 10 mμ.

Dartnall has also reported the occurrence of 533_2 pigments in the pike (39), (*Esox lucius*), and in the bleak (40) (*Alburnus lucidus*), but these results have not been confirmed by means of NH_2OH difference spectra. There is also some evidence of two (39) and even of three (40) photosensitive components in retinal extracts from certain fresh water fish, but these results have not been accepted by all investigators in the field. These claims are important enough to merit further investigation.

In general, fresh water fish have been insufficiently examined. Some attention should be devoted to a study of fish from many more different families and from diverse habitats. It would be of interest, for example, to know whether in fresh water fish there is a situation with respect to deep-lake dwellers which is analogous to the deep-sea fish story. One of the most intriguing of recent publications in the visual pigment field is the

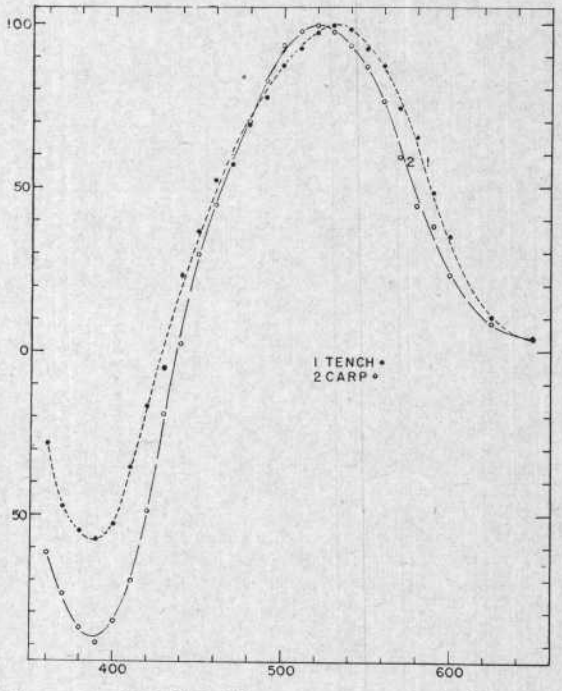

Figure 8. NH_2OH difference spectra obtained from retinal extracts of carp and tench. Explanation in text.

report of the Japanese investigators (41) who measured the spectra of individual cones of the carp retina by means of microspectrophotometry. After showing that individual rods of the frog and of the carp yielded absorption spectra roughly similar in spectral location to rhodopsin and to porphyropsin, they examined 73 cones, 44 of which bleached to give difference spectra located at 420-430 mμ, 490-500 mμ, 520-540 mμ, 560-580 mμ, 620-640 mμ, and 670-680 mμ. In several cases they were able to record two different difference spectra from the same cone. This work is bound to arouse much discussion.

Table 1. VISUAL PIGMENTS OF ADULT AMPHIBIA

Column 1 is the λ_{max} of the alkaline difference spectrum. Column 2 is the λ_{max} of the NH_2OH difference spectrum. Column 3 is the product maximum of the alkaline difference spectrum. Column 4 is the product maximum of the NH_2OH difference spectrum.

	1	2	3	4
Rana muscosa	503		378	
Rana pipiens		502		368
Rana aurora	503		382	
Microhyla olivacea	507		378	
Microhyla olivacea		504		368
Bufo boreas	502		375	
Bufo boreas		502		368
Bufo marinus	502		375	
Bufo marinus		503		368
Bufo terrestris	505		375	
Bufo terrestris		503		368
Hyla regilla	503		377	
Hyla cinerea	504		381	
Xenopus laevis	521		398	
Xenopus laevis		520		387
Xenopus laevis	522		398	
Necturus maculosus	526		406	
Necturus maculosus	526		404	
Necturus maculosus		522		388
Taricha torosa	505		384	
Taricha torosa		502		367

4. AMPHIBIA

The amphibian visual pigments which have been extracted by digitonin solution are of the two general types according to whether they are associated with retinene$_1$ or retinene$_2$. In some amphibian species only one of these types was found throughout all developmental stages. In other species, pigments of both retinene systems were noted in the extracts. In some of the latter amphibia a systematic developmental change from the retinene$_2$ to the retinene$_1$ system was observed to accompany the metamorphic transformation from larva to adult.

Adult amphibia. The adults of the bullfrog, frog, toad, and tree frog all have in their retinae the classical rhodopsin. This statement is based on the absorption spectra of relatively pure extracts, on alkaline difference spectra and on NH_2OH difference spectra. The NH_2OH difference spectra were all characterized by a density loss maximal at 502-504 mμ and a density gain maximal at about 368 mμ (table I). Selective bleaching indicated that rhodopsin was the only photosensitive component present in the extracts. A summary of the amphibian data is given in table I while figure 9 shows some selected data for an adult frog (*Rana muscosa*), a toad (*Bufo canorus,* and a tree frog (*Hyla cinerea*). These graphical data illustrate the positive portions of the alkaline difference spectra obtained as a result of exposure to light of longer wave lengths.

The interesting feature of the amphibian rhodopsin system is its location within a very restricted region of the spectrum. This is in contrast to the retinene$_1$ pigments of fish, reptiles, and even of mammals, all of which have an extremely broad representation over the spectral scale. This spectral constancy of amphibian rhodopsin holds true in spite of wide variations in habitat. The same spectroscopic pigment has been noted, for example, in essentially aquatic species, such as *Rana catesbeiana* and *Rana aurora draytonii,* and in species such as *Scaphiopus hammondi, Bufo boreas halophilus, Bufo canorus,* and others in which the adults are terrestrial in habit. Again the rhodopsins from the Yosemite toad (*B. canorus*) and the western toad (*B. boreas halophilus*) are spectroscopically indistinguishable. Yet the Yosemite toad inhabits the higher altitudes of the Sierra Nevada of California (above 6500 feet) where low night and early morning temperatures enforce a degree of diurnal activity not common to other toads. In contrast, the western toad is found at lower altitudes (down to sea level) and is essentially a nocturnal species. This spectroscopic fixity of amphibian rhodopsins suggests a genetic basis for the determination of the spectral properties of these visual pigments.

It is recognized, however, that some amphibia may also have additional photolabile substances within the rod outer segments. By means of photomicrographic densitometry applied to the isolated fresh retinae of adult frogs (*Rana temporaria*), Denton and Wyllie (42) were able to detect within the so-called green rods a pigment absorb-

ing strongly in the blue end of the spectrum, bleaching readily in blue light, and after bleaching fading in the blue region. Green rods apparently have, in addition to the photolabile, blue-absorbing substance, a stable material. This was detected in bleached green rods by Hanaoka and Fujimoto (41). They reported in such rods a spectrum with minimal absorption in the green and with increasing absorption on either side of the green. These investigators considered that this effect might have been due to an interference phenomenon rather than to the presence of a pigment. In relation to the photolabile pigment of the green rods, Dartnall (3) presented the results of preliminary analysis of a retinal extract from *Rana esculenta.* After complete bleaching of the visual purple, a further spectral change was found, following exposure to white light, in which density loss was maximal at 430-440 mμ. It was suggested that this might have been due to bleaching of the pigment of the green rods. No NH_2OH experiments were reported, and the possibility of an isomerizing effect of white light was not ruled out.

Not all adult amphibia were found to have the rhodopsin system. Certain of the more aquatic forms, such as *Xenopus laevis* and *Necturus maculosus,* were discovered to have visual pigments whose absorption spectra, compared to the spectrum of classical rhodopsin, are located nearer to the red end of the spectrum. This is indicated by the graphical summary of figure 9 which includes, in addition to the results already mentioned, the positive difference spectra found in connection with retinal extracts from *Necturus, Xenopus,* and *Diemyctylus viridescens.* It is clear that, compared with the results given by extracts of frogs, toads, and tree frogs, the visual pigments of the more aquatic adult amphibians are located some 20 mμ further toward the red.

The pigment of the mud puppy will be described first since this does not appear to have received any attention, although Wald (43) reported finding only vitamin A_2 in the retinae of these animals. After several unsuccessful attempts I finally prepared a stable solution of the visual pigment of *Necturus.* Three successive bleachings of the extract with light at 700 mμ, 630 mμ, and 500 mμ resulted in typical alkaline difference spectra which were the same for all three exposures. The maximum density loss in each case was at about 526 mμ, while maximum increase in density occurred at about 405 mμ. A bleaching experiment in the presence of NH_2OH yielded a difference spectrum with density loss maximal at 522 mμ and density gain maximal at 388 mμ. These facts all point to the presence of a porphyropsin in the retinae of this amphibian. No evidence was found for the presence of any other photosensitive pigment in the extracts. The experiments with *Xenopus* furnished similar results. Alkaline difference spectra obtained after bleaching with light at 630 mμ and 420 mμ were characterized by maximum density losses at 522-523 mμ and maximum density gains at 396-398 mμ. The NH_2OH difference spectrum had comparable figures of 519-521 mμ and 387 mμ. In addition, it was possible to extract from bleached extracts of *Xenopus* retinae a pigment giving an absorption band at 410 mμ in chloroform, the position of retinene$_2$ in chloroform. All these data point to the occurrence of a retinene$_2$ system in *Xenopus.* The visual pigment system of *Xenopus* was also examined by Dartnall (44, 45) who first reported the presence of a photosensitive pigment for which the λ_{max} of the alkaline difference spectra was at 520 mμ. Later he amended the account by asserting that *Xenopus* extracts have a mixture of a pigment located at 523 mμ and another at 502 mμ. These were present to the extent of 92% and 8%, respectively. I have not observed this in *Xenopus* extracts but no special efforts were made to look for this small amount of a second pigment. Wald (46) has reported the occurrence of both vitamin A_2 and vitamin A_1 (95/5) in *Xenopus* retinae.

Newts and salamanders. Studies of the visual pigments of newts and salamanders are still in progress, but some preliminary statements are worthy of inclusion in this summary. *Taricha torosa,* the Pacific Coast newt, is a large newt which after metamorphosis becomes terrestrial. Adults of this species were collected and extracts were prepared. The alkaline difference spectra were in all respects typical of classical rhodopsin. One NH_2OH difference spectrum showed a loss of pigment maximal at 502 mμ and a product peak at 367 mμ. No special search was made to discover whether any other pigment was present in much smaller quantities but studies of this newt are continuing.

In contrast to *Taricha,* adults of the spotted newt, *Diemyctylus viridescens,* are aquatic. Wald (43) pointed out that this aquatic adult contains in its retina only vitamin A_2. I have repeatedly tried to obtain from the retinae of these animals solutions which would permit satisfactory analysis. The results so far have not been good. There are indications, however, that a major photosensitive component in the general region of 520 mμ was

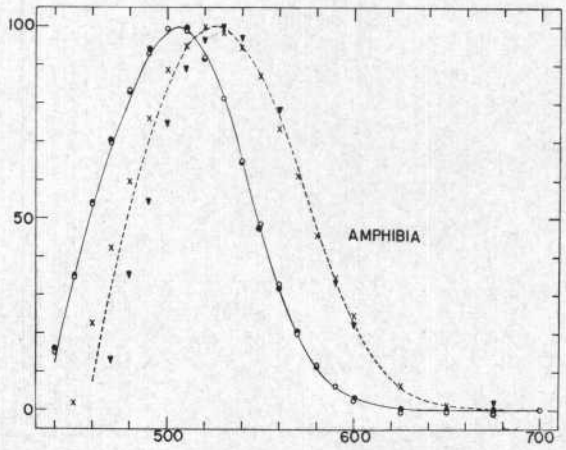

Figure 9. Positive portions of alkaline difference spectra of adult Amphibia. Full line: *R. muscosa,* bleaching with 606 mμ. Open circles: *B. canorus,* bleaching with 600 mμ. Filled-in points: *H. cinerea,* bleaching with 631 mμ. Interrupted line: *N. maculosus,* bleaching with 700 and 630 mμ. Triangles: *D. viridescens,* bleaching with 640 and 606 mμ. Symbols X:*X. laevis,* bleaching with 660 and 640 mμ.

present in the extracts (figure 9). It was not possible to identify the product peak. The indications are that in these two newts, one terrestrial, the other aquatic, there occur the two visual systems of retinene$_1$ and retinene$_2$, respectively. I have also examined the red eft stage of *D. viridescens.* This is often considered to be a terrestrial subadult stage between the aquatic larva and the aquatic adult (47). The results were not conclusive but there was some evidence of the occurrence in the extract of both retinene$_1$ and retinene$_2$ systems. It will be recalled that Wald (43) obtained evidence for the existence of both vitamin A_1 and vitamin A_2 in the retina of the red eft. It is also important to remember that not all populations of this newt pass through the terrestrial, red eft stage. Noble (48), for example, discovered that *Diemyctylus* from Long Island does not have an intermediate terrestrial stage but passes from the larva to the adult through an aquatic stage. Whether this is environmental or genetic is not known, but the study of the visual pigments in these different races or populations might offer a neat quantitative measure of relationships.

Visual Pigments During Metamorphosis

1. *Bufo boreas halophilus.* This is an example of an amphibian in which the retinene$_1$ system was found for both the aquatic larval stage and the terrestrial adult. Tadpoles of this toad were collected from a local pond and segregated into two groups: (a) animals with tails and no limbs and (b) animals with tails and with hind limbs in various stages of development. As it was not possible to remove the retinae from the eyes of these small animals, extracts of the whole eyes were prepared. These extracts were found to have a photosensitive pigment which, on exposure to light, bleached in a typical manner. The difference spectra suggested the presence of a typical rhodopsin and selective bleaching produced no evidence for the presence of a retinene$_2$ component. No spectroscopic differences were found in the pigments from the two larval groups or between the larval and adult chromoproteins. In fact, the difference spectra obtained in the experiments with tadpoles and adult toads were superimposable (figure 10). It is possible, of course, that still earlier larvae than were employed might have revealed some porphyropsin but the technical difficulties of such an experiment discouraged attempts in this direction. In any case, it is certain that during the developmental stages when certain other amphibia were found to possess a porphyropsin to rhodopsin change, *Bufo boreas halophilus* showed only a single system. Such a transformation is obviously not essential to the process of emergence from water to land. These results offer confirmation in a toad for the idea of Collins, Love and Morton (49) who obtained visual pigment solutions from tadpoles of *Rana temporaria* and *Rana esculenta.* The solutions were impure and no selective bleachings were carried out but they concluded that rhodopsin was the only pigment in the extracts.

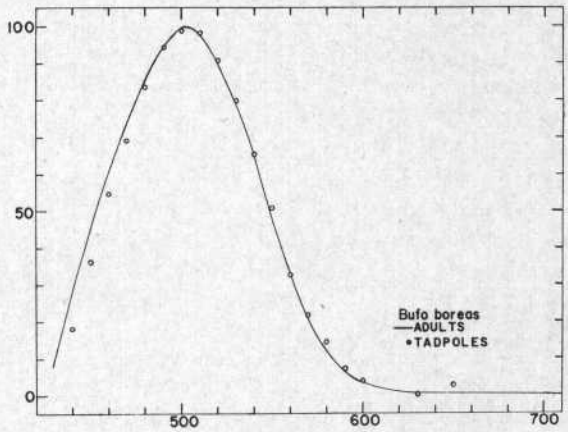

Figure 10. Adult and larval stages of *B. boreas.* Positive portions of alkaline difference spectra. Full line: adult, bleaching with 631 mμ. Open circles: tadpoles, mean of three bleachings with white light, 560 mμ and 580 mμ.

2. *Hyla regilla.* In contrast with the above results, the Pacific tree frog provided definite evidence of a metamorphic shift from a larval retinene$_2$ to an adult retinene$_1$ system. Tadpoles of this species were obtained from the same pond and at the same time as were the toad tadpoles. These larvae were segregated into four groups: (a) larvae with tails but no limbs, (b) animals with tails and hind limbs, (c) tadpoles with tails and four limbs, and (d) emerging animals with four limbs and with tails either gone or reduced to a stub. The animals of this fourth group were in fact beginning their terrestrial phase of life. Extracts were made of the whole eyes and the method of selective bleaching revealed clear evidence of more than one photosensitive component. This fact can be illustrated by citing the results obtained with the fourth group of animals.

Following an initial bleaching with light at 640 mμ, the alkaline difference spectrum which was plotted showed a maximum loss of density at about 521 mμ and a maximum gain in density at about 389 mμ (figure 11A). A second bleaching with light at 606 mμ led to a difference spectrum with corresponding maxima at 506 mμ and 368 mμ (figure 11B). The density loss resulting from the first bleaching was 0.071; that resulting from the second was 0.076. A final exposure to white light resulted in a further loss of 0.039 with maximum at about 505 mμ. This analysis, though not precise in spotting the spectral positions of these two pigments is clear enough in demonstrating the presence in the retinal extract of both retinene$_1$ and retinene$_2$ systems. In contrast, the extract prepared from the retinae of adult tree frogs behaved, in response to selective bleaching, as expected for a homogeneous solution (figure 11). Only a single photosensitive component with a maximum at about 503 mμ was detected. The retinene$_2$ system is lost from the retina, not before emergence of the transformed tadpole, but some time after this event.

Analysis of the other extracts suggests that in the very young tadpole only a retinene$_2$ system was present and that as development occurred, rhodopsin appeared and increased in quantity while the original pigment gradually decreased. Bleaching the extract from group (a) larvae with light at 660 mμ resulted in a selective density loss maximal at 524 mμ. Further exposure to light, even to white light, revealed no further change. The animals of the second group responded to selective bleaching by a change of which about 83% was due to a retinene$_2$ system and 17% to

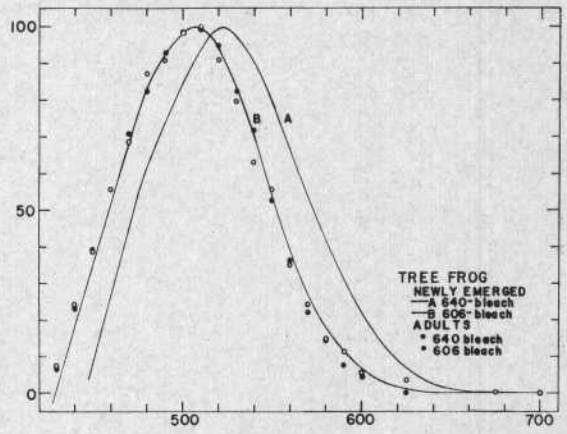

Figure 11. Adult and larval stages of *H. regilla.* Positive portions of alkaline difference spectra. Curve A: newly emerged tree frogs, extract bleached with 640 mμ. Curve B: same extract bleached with 606 mμ following the 640 mμ exposure. Open circles: extract of adult bleached with 640 mμ. Filled-in points: extract of adult bleached with 606 mμ following the 640 mμ exposure.

rhodopsin. The animals of the third group yielded three good extracts for study. An initial exposure to light at 640 mμ led to an alkaline difference spectrum with maxima for density loss and density gain at 523 mμ and 396 mμ. Further bleaching at 640 mμ yielded a very small change. A final bleach at 606 mμ produced a considerable loss in density with maxima at 506 mμ and 380 mμ. In these extracts the rhodopsin system constituted approximately 25 to 40% of the total photolabile component. The data are clear in pointing to a progressive change during metamorphosis from a retinene$_2$ chromoprotein in the early larva to a retinene$_1$ system in the adult.

This positive finding of a dual system in *Hyla regilla* in contrast to the negative findings obtained with *Bufo boreas halophilus* is interesting from several points of view. Both groups of animals were secured from the same pond at the same time. From the time of hatching they had been reared in the same environment. Presumably their food was identical. The aquatic period of life (egg and larval stages) is of about the same duration in both these species. Storer (50) gives a figure of 4-6 weeks for *Bufo boreas halophilus* and 6-8 weeks for *Hyla regilla.* In addition, both these species emerge from water at the termination of metamorphosis and become, as adults, essentially terrestrial and nocturnal animals. All of these facts suggest that the possession of a dual system of this kind is an innate characteristic. An investigation of this dual visual pigment system of am-

phibia in terms of hormonal effects would appear to be a subject worthy of attention.

3. *The bullfrog.* It was the study of this amphibian which originally led Wald (51) to the thesis of a metamorphic shift of visual pigments. Analysis of the A vitamins produced evidence that both vitamin A_1 and vitamin A_2 were present in the bullfrog tadpole in different proportions according to the stage of metamorphosis. An extract of the retinae of tadpoles was prepared. The pigment was purple in color and the absorption spectrum had a maximum at 516 mμ, "as though porphyropsin mixed with a little rhodopsin were present." I was not satisfied with this untested statement of the pigment situation in the bullfrog and so about two years ago I prepared solutions from the retinae of bullfrog tadpoles. I found that Wald was correct in all respects in his interpretation of the data (figure 12). In one experiment, for example, I removed the eyes from 60 tadpoles in various stages of development. The retinae were then obtained, the outer segments were shaken off and an extract was made of these. The absorption spectrum of this solution was characterized by a density at minimum to density at maximum of 0.69 and by a maximum absorption at 516 mμ. Bleaching this solution with a light at 670 mμ produced a typical change with maximal density loss at 525 mμ and maximal density gain at 395 mμ. A second bleaching, this time with light at 430 mμ, produced a much smaller change with comparable maxima at 506 mμ and 370 mμ. Experiments like those with the tree frog were also performed in sufficient number to demonstrate that a change from the retinene$_2$ to the retinene$_1$ system occurs in the bullfrog as in *Hyla regilla.* It is perhaps worth mentioning that the length of time spent in metamorphosis is not a factor as to whether the dual pigment system is present or not. The bullfrog requires at least two years to complete larval development whereas in *Hyla regilla* the comparable period is 6-8 weeks. It is not likely that the absence of this dual system in *B. boreas halophilus* and in the frogs used by Collins, Love and Morton (49) is correlated with the short duration of larval development in these amphibians.

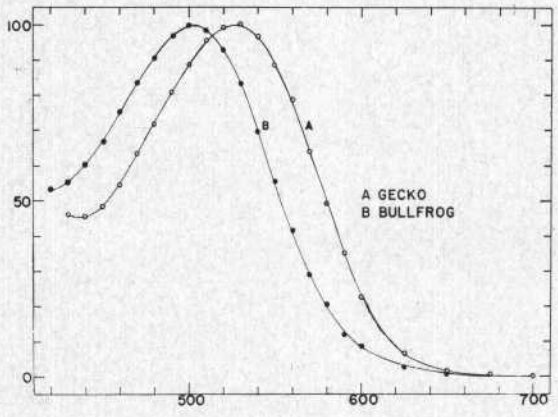

Figure 12. Absorption spectra of unbleached extracts of bullfrog tadpoles (A) and of bullfrog adults (B).

It is clear that amphibians as a class have developed a dual system of scotopic visual pigments. Within this general class plan there are variations among different amphibians. Some, like *B. boreas halophilus,* have a retinene$_1$ system throughout larval and adult life. Others, like *H. regilla* and *R. catesbeiana,* possess a retinene$_2$ chromoprotein as larvae and this is replaced, as development occurs, by rhodopsin. In others, such as *Xenopus laevis* and *N. maculosus,* the retinene$_2$ system persists to the adult stage and, apparently, though this has not yet been thoroughly investigated, the larval type of pigment persists. This persistence occurs both in *Necturus,* which retains many larval features and in *Xenopus,* which undergoes considerable metamorphic change. The distribution of these visual proteins in amphibia is not obviously correlated with taxonomy or with environment. In terms of the ecology of amphibia, it is difficult to understand what advantage there is in the possession of two visual pigments separated by only 20 mμ. One can speculate that the possession of a retinene$_2$ system by the retina during the critical larval period of cellular reorganization is significant in terms of the metabolic competition for vitamin A between the visual cells and other cells. By utilizing a vitamin A_2 while other cells are employing vitamin A_1, the all-important visual cells are effectively removed from the competition for the general pool of vitamin A and are thus placed in a favorable position to complete their functional development. This is sheer speculation, of course, for we do not know what the metabolic requirements of cells for vitamin A are and whether or not cells can utilize vitamin A_2 equally as well as vitamin A_1. In addition, there is the fact that some amphibia do not have a retinene$_2$ pigment. The only reasonable explanation possible in the present state of our knowledge is that this dual system is a phylogenetic accident in terms of the piscine origin of the amphibia.

Are there any correlations of this metamorphic shift with visual processes? Considerable morphological changes have been reported to occur in

the visual cells of the frog (*R. temporaria*) during metamorphosis (52). Cones were seen to differentiate first, followed by the appearance and development of the rods. Correlated with these morphological changes was a shift in spectral sensitivity, as the tadpoles developed, from a maximum sensitivity in the yellow to a peak in the blue-green. This frog was found to have only a retinene$_1$ protein (49) and so the above spectral shift was probably a change from a photopic to a scotopic system. Evidence of a metamorphic shift in spectral sensitivity from a retinene$_2$ to a retinene$_1$ system was recently obtained in *R. pipiens* (53).

5. Reptiles

Until recently, attempts to study the visual pigments of reptiles have yielded poor returns. Kühne, obviously a careful observer, was unable to discover any evidence of a bleachable pigment in the retinae of certain snakes and lizards. He concluded that visual purple was absent from the visual cells of these animals (54). Köttgen and Abelsdorff (34) were similarly unsuccessful in detecting visual purple in the European tortoise. Similar failures have been recorded in more recent times (55,56). On the other hand, Walls (57) reported that he saw a pink, red, or lavender color in the retinae of certain snakes and that this color was bleached on exposure to light.

Successful extracts of visual pigment from reptiles were made by Crescitelli (6). From the rattlesnake (*Crotalus viridis helleri*), rhodopsin was obtained with a spectral maximum at 500 mμ (figure 13). The NH_2OH difference spectrum also showed an increase in density maximal at about 366 mμ (figure 13). From the retinae of an alligator *(Alligator mississippiensis)* an extract was prepared whose absorption spectrum had a ratio of density at minimum to density at maximum of 0.28. The absorption maximum of this relatively pure solution was at 500 mμ. Bleaching this solution in the presence of NH_2OH resulted in a loss of density maximal at 499 mμ and a gain in density maximal at 367 mμ (figure 13). These results were in harmony with the classical idea that the scotopic pigment of terrestrial animals is a retinene$_1$ protein with an absorption peak in the neighborhood of 500 mμ. The nature of the visual pigment in the alligator was confirmed by Wald, Brown, and Kennedy (58) who made the additional interesting observation that whereas the alligator rhodopsin was synthesized following the addition of neo-b retinene to the bleached product, the rate of regeneration, unlike that of frog, cattle, and chicken rhodopsin, was rapid. The rate of dark adaptation of the alligator eye was similarly rapid. Both processes were more similar in kinetics to the corresponding events of cone pigments and of cones (frogs, mammals, and birds) than to rod pigments and to rods.

An entirely new view of reptilian visual pigments was opened up when Crescitelli (6) began to study geckos. Stimulated by Denton's finding (59) that the spectral sensitivity curve of *Gekko gekko*, instead of being at 500 mμ, was shifted significantly toward longer wave lengths, Crescitelli made retinal extracts of several geckos. In these extracts retinene$_1$ photolabile pigments were discovered. The spectral location of these gecko chromoproteins was found to be, not in the region of 500 mμ, but significantly displaced away from 500 mμ toward the red end of the spectrum. For the pigment from the Australian species, *Phyllurus milii*, the location was 524 mμ (figure 13).

Since the original work on geckos was published, several other species from various families of the *Gekkonoidea* have been obtained and their visual pigments studied. With the exception of a few species, which will be considered later in this discussion, all of them were observed to have, not classical rhodopsin, but retinene$_1$ compounds with absorption maxima located in the broad region 518 mμ to 530 mμ (table 2). A comparison of the absorption spectra of the retinene$_1$ pigment from the gecko, *Tarentola mauritanica* (A) and the retinene$_1$ pigment of the bullfrog (B) is given in figure 14. Differential bleaching indicated that only one photosensitive component was present in

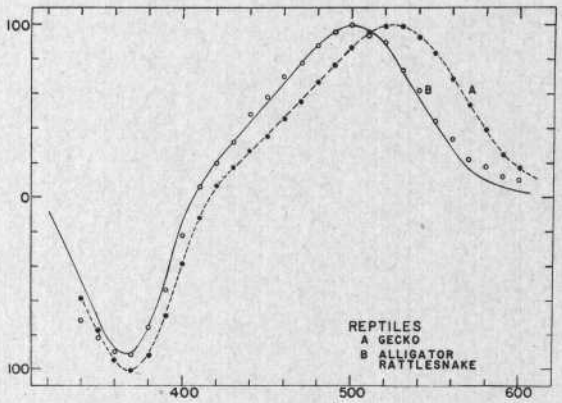

Figure 13. NH_2OH difference spectra (curve A) for a gecko (*Phyllurus milii*), for the alligator (B, full line) and for the rattlesnake (B, open circles).

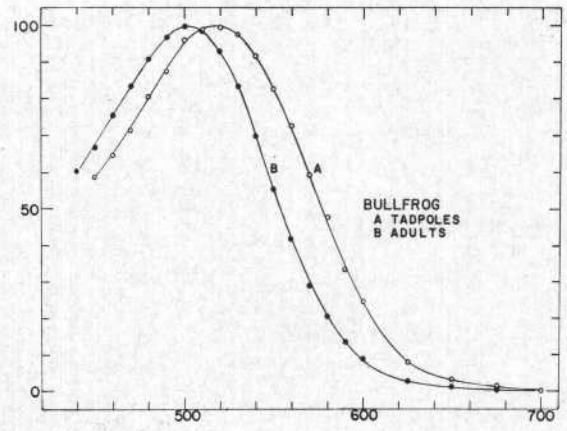

Figure 14. Absorption spectra of unbleached extracts of *Tarentola mauritanica* (A) and of bullfrog (B).

this gecko retinal extract and in the bullfrog extract. In the light of this fact it is clear that in *Tarentola* a pigment spectroscopically distinct from classical rhodopsin was found. Another gecko pigment of the same general type was described in connection with the analysis of the extract of *Oedura monilis* (figures 2 and 3). The *Phyllurus* pigment is therefore not an isolated quirk of nature but is rather an example of a consistent, lawful phenomenon.

What is the possible biological significance of this unusual visual system of geckos? On this point one can only speculate, but as a basis for such speculation we have the phylogenetic history of the gecko visual cells as expressed in the transmutation theory of Walls (60, 61). This theory assumes as one of its postulates (the one which is relevant to this discussion) that cones are the ancestral type of visual cell, and that throughout evolution, transmutation of cones to rods has taken place in association with the change from a diurnal to a nocturnal habit. The evidence for this theory stems from the finding of morphologically intermediate cell types logically dispersed between typical cones and typical rods. Applied to geckos, which are considered to demonstrate this intermediacy to a varied and dynamic degree, the facts are as follows:

a. Double visual cells are commonly found in the retinae of these lizards (62).

b. Though species of geckos have been found without oil droplets (60) in the visual cells, the oil droplet is often a characteristic feature of both the single and the double visual cells (62). This is considered a diagnostic sign of cone origins.

c. The occurrence of a fovea has been noted in some species of geckos (62).

d. The work of Walls (60) suggests that even in geckos in which transmutation to rods has progressed to a marked degree, there is little summation of rods to bipolar cells. I have a private communication from Dr. Katharine Tansley mentioning this small neural summation of the gecko retina.

e. In contrast to these cone-like characteristics the outer segments of many geckos are enlarged to varying degrees and contain visual pigment in sufficient concentration to be seen or to be analyzed. The gecko retina appears to have gained sensitivity by enlarging the outer segment and by forming a large concentration of visual pigment. By refraining from using the trick of neural summation, geckos have retained a respectable degree of visual acuity. It is of some interest to compare some of the morphological observations with the biochemical results which I have obtained. *Gonatodes fuscus* and *Sphaerodactylus argus* have outer segments which are small and cone-like. I have not yet extracted the retinae of *Gonatodes* but in retinal extracts of *S. argus* no evidence of photosensitive pigment has been secured. *S. parkeri,* though showing a fovea (62), has droplet-free visual cells with stout rod-like outer segments. In *S. parkeri* I was able to get evidence for a retinene$_1$ visual pigment with maximum at 528 mμ (alkaline difference spectrum). *Aristelliger praesignis* has a fovea-free retina whose visual cells have colorless oil droplets and whose outer segments are significantly enlarged. In one specimen of *Aristelliger,* kindly sent me by Mr. Underwood, I was able to detect a photosensitive retinene$_1$ pigment with maximum at about 530 mμ (alkaline difference spectrum). In *Coleonyx variegatus,* which is a nonspectacled, nocturnal gecko with greatly elongated outer segments and with visual cells without oil droplets, a visual pigment has been noted with absorption maximum at about 516 mμ.

Considered in its entirety the evidence is good that the gecko retina is in a state of intermediacy. Is there any physiological evidence of cone-like characteristics of this retina? Little work has been carried out in this area but one investigation is worthy of note. This is the study of Crozier and Wolf (63) in which the critical fusion frequency contour of *S. inaquae* was found to resemble that of a cone response. We can now add the biochemical observation that the visual pigment of geckos, though spectroscopically diverse among different geckos, is intermediate in position between iodopsin and classical rhodopsin, both retinene$_1$ pigments. This intermediacy may be simply a biochemical consequence of an evolutionary change from a cone-type visual pigment to a rod-type visual pigment. Diversity among different species of geckos may be just the result of the occurrence of independent evolution in different gecko stocks.

In terms of the ideas developed above it would not be unexpected to find, in some geckos, visual pigments located at or about the classical 500 mμ

TABLE 2. VISUAL PIGMENTS OF GECKOS

	1	2	3	4
1. Eublepharidae				
Coleonyx variegatus	.....	516	.....	367
Coleonyx variegatus	518	.....	382	.....
Coleonyx variegatus	518	.....	386	.....
2. Sphaerodactylidae				
Sphaerodactylus parkeri	528	.....	378	.....
3. Gekkonidae				
a. Diplodactylinae				
Aristelliger praesignis	530	.....	.....	.....
Phyllurus milii	525	.....	378	.....
Phyllurus milii	525	.....	380	.....
Phyllurus milii	.....	524	.....	367
Oedura lesueuri	518	.....	382	.....
Oedura monilis	520	.....	382	.....
Oedura monilis	.....	518	.....	368
b. Gekkoninae				
Gehyra mutilata	518	.....	373	.....
Gehyra variegatus	528	.....	376	.....
Hemidactylus frenatus	.....	520	368	.....
Hemidactylus turcicus	523	.....	384	.....
Tarentola mauritanica	528	.....	382	.....
Tarentola mauritanica	528	.....	376	.....
Tarentola mauritanica	528	.....	384	.....

Columns 1, 2, 3, and 4 represent, respectively, the λ_{max} of the alkaline difference spectrum, the λ_{max} of the NH_2OH difference spectrum, the product maximum of the alkaline difference spectrum and the product maximum of the NH_2OH difference spectrum. The classification followed in this table is from Underwood (72).

position. As a matter of fact some evidence has already been collected which supports this idea. In one retinal extract of *S. parkeri,* bleaching with light at 631 mμ yielded an alkaline difference spectrum with maximum at about 528 mμ. Density loss at maximum was 0.071. The extract was next exposed to light at 560 mμ. This caused a selective change with a maximum loss of 0.015 at about 500 mμ. The negative portions of the alkaline difference spectra of both these pigments were typical of retinene$_1$ chromoproteins. From *Hemidactylus frenatus* an extract was prepared which in the presence of NH_2OH was initially exposed to light at 640 mμ. The density loss at maximum (520 mμ) was 0.039 and the product formed was characteristic of a retinene$_1$ system. An exposure to white light was then employed and this resulted in a density loss of 0.022 with maximum at about 490 mμ. The product was again indicative of a retinene$_1$ compound.

An unusual finding was encountered in an extract from the retinae of two specimens of *Oedura robusta.* No evidence was found for the presence of a typical gecko pigment. Instead, selective bleaching with light at 631 mμ and 606 mμ showed the presence of a component with maximum density loss at about 490 mμ. Perhaps the most interesting of these additional gecko pigments is the one located at approximately 457 mμ which was found in extracts of *Oedura monilis* (64) and evidence for which was presented earlier in this report (figures 2, 3, 4). This pigment is of special interest because of its location in the short wavelength region of the spectrum in which region there is evidence for the human retina of the existence of specific blue or violet receptors (65, 66). The literature on visual pigments includes a number of references (39, 42, 67, 68) which suggest the occurrence of blue-sensitive pigments in the retinae of various vertebrates. Some of these claims are based on questionable experimental procedures and it is not surprising that they have

not received general acceptance. The report of the *Oedura* pigment, which is the first account of a blue-sensitive component in the retinae of lizards, is unique for two reasons: (a) isomerizing actions, which could confuse the interpretation, were reduced to a minimum and (b) the pigment in question was demonstrated to be present in the extract before the bleaching exposure which removed it. All of these results on the presence of additional pigments in retinal extracts of geckos are provisional since the lack of animals has prevented completion of these studies. The results are suggestive of a visual pigment system in these lizards more diverse than has been hitherto suspected. It is even possible that geckos have developed color vision mechanisms.

There is no reason to assume, at present, that transmutation of cones to rods occurred only in geckos. In formulating his transmutation theory, Walls (60) conceived this concept as being of general biological significance. Although intermediacy of visual cell types and of visual pigments is perhaps most strikingly evident in geckos, other vertebrates have demonstrated similar signs of evolutionary change. Retinene$_1$ pigments with spectral locations in the region 525 mμ to 530 mμ have been reported for several marine fish (30). From this laboratory Munz (29) has reported the occurrence of a retinene$_1$ pigment at about 520 mμ in the labrid fish, *Pimelometopon pulchrum*. Unlike the case of most geckos so far studied, these fish pigments were found in the extracts as second components in addition to the classical rhodopsin. Munz, for example, found the ratio of the 497$_1$ to the 520$_1$ pigments in *Pimelometopon* to be 85/15. It may be of some relevance to point out that Lyall (69) has interpreted the loss of single cones in the trout retina during growth as being the result of transmutation into rods. He ventured the opinion that a few visual cells which he observed might have been in a stage of transition between cones and rods. We have here the extension of transmutation to ontogeny, a concept which strikes still closer to the prerogatives of the duplicity theory.

The visual pigment of the alligator has special interest in this connection. Though this pigment appears to be a classical rhodopsin by the usual criteria of spectroscopic location and retinene composition (6, 58), it shows an interesting biochemical similarity to iodopsin which on good evidence is considered a cone pigment of several animals (70). Alligator rhodopsin was found to regenerate, not slowly as does frog rhodopsin, but at a rate comparable to that associated with iodopsin (58). This was not an *in vitro* artifact because the rate of dark adaptation in the alligator, as measured by means of the electroretinogram, was similarly rapid. The alligator rhodopsin shows a very subtle molecular relationship to iodopsin which is quite independent of the evidence of spectral location. It is logical to speculate that this molecular similarity has significance in terms of the phylogeny of the alligator rods.

The story of the geckos is just beginning and these animals will be of special interest to visual physiologists. The work of Walls and of Underwood has pointed the way to the unique position of the gecko visual cells. Now that a unique pigment has been found replacing the usual rhodopsin of terrestrial animals, physiologists would do well to reexamine these lizards in the light of the duplicity theory. Crozier and Wolf have pointed the way in this field. Electrophysiological, cytological, biochemical, and behavioral studies could well be carried out, and with profit, on these interesting animals. The possibility that geckos possess true color discriminating mechanisms might well be a subject for experimentation. The geckos are numerous and diverse. The main problem, as this writer has sadly learned, is to get them alive from the places where they occur to the places where investigators are located. Nature has not disposed wisely in this respect.

6. Birds and Mammals

Traditionally, the scotopic pigments of birds and mammals have been considered to belong to

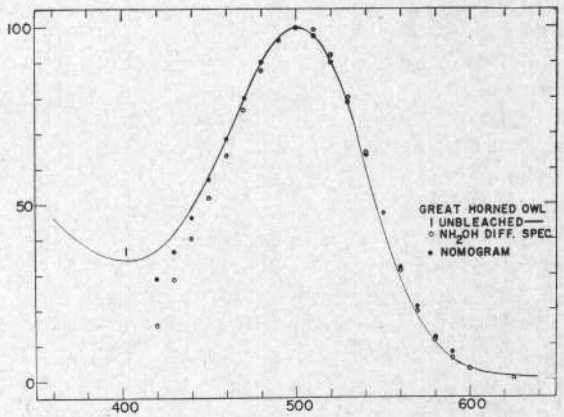

Figure 15. Analysis of an extract from the great-horned owl. Full line: absorption spectrum of unbleached extract. Open circles: NH_2OH difference spectrum after bleaching with 606 mμ. Filled-in points: curve constructed from nomogram assuming a curve with λ_{max} at 502 mμ.

TABLE 3. VISUAL PIGMENTS OF BIRDS AND MAMMALS

	1	2	3	4
Chicken *Gallus gallus*	504	.....	379	.....
Great-horned Owl *Bubo virginianus*	504	.....	378	.....
Great-horned Owl *Bubo virginianus*	506	.....	378	.....
Great-horned Owl *Bubo virginianus*	502	.....	378	.....
Great-horned Owl *Bubo virginianus*	.....	502	.....	367
Screech Owl *Otus asio*	505	.....	380	.....
Gull *Larus occidentalis*	503	.....	376	.....
Pelican *Pelicanus occidentalis*	504	.....	375	.....
Man *Homo sapiens*	497	.....	378	.....
Chimpanzee *Pan sp.*	.....	491	.....	368
Three-toed sloth *Bradypus tridactylus*	497	.....	377	.....
Three-toed sloth *Bradypus tridactylus*	.....	493	.....	366
Sea lion *Zalophus californicus*	497	.....	377	.....
Sea lion *Zalophus californicus*	501	.....	382	.....
Racoon *Procyon lotor psora*	502	.....	377	.....
Opossum *Didelphis virginiana*	.....	493	.....	366
Opossum *Didelphis virginiana*	.....	495	.....	366
Merriam kangaroo rat *Dipodomys merriami*	.....	501	.....	367
Merriam kangaroo rat *Dipodomys merriami*	504	.....	376	.....
Desert kangaroo rat *Dipodomys deserti*	.....	501	.....	366
Mohave kangaroo rat *Dipodmys mohavensis*	.....	501	.....	366
Mohave kangaroo rat *Dipodmys mohavensis*	504	.....	376	.....
Little pocket mouse *Perognathus longimembris*	503	.....	378	.....
Little pocket mouse *Perognathus longimembris*	.....	501	.....	367
Grasshopper mouse *Onychomys torridus*	502	.....	376	.....
Grasshopper mouse *Onychomys torridus*	.....	500	.....	366
Wood rat *Neotoma lepida*	499	.....	378	.....
Wood rat *Neotoma lepida*	.....	498	.....	367

Column 1 is the λ_{max} of the alkaline difference spectrum. Column 2 is the λ_{max} of the NH_2OH difference spectrum. Column 3 is the product maximum of the alkaline difference spectrum. Column 4 is the product maximum of the NH_2OH difference spectrum.

the system of classical rhodopsins. The work in our laboratory, so far, supports this traditional view and nothing significantly new or different has appeared. The data in table 3 summarizes the essential findings while an individual experiment (with the great-horned owl) is pictured in figure 15. A number of specific points are also worthy of brief mention.

(a) Extracts of the retinae of 12-, 16-, 19-, and 20-day-old chick embryos have not yet revealed the presence of rhodopsin. This work is still in progress and more data are required before it can be concluded that visual purple appears in the visual cells some time after hatching.

(b) No photosensitive component has yet been detected in the cone-dominated retinae of ground squirrels. It may be that special methods and approaches will be required to obtain information on photopic pigments.

(c) Mammalian visual pigments display some degree of spectral diversity though they are not as versatile in this respect as are the pigment systems of fish and geckos. Some of the mammalian visual proteins are noteworthy because of the relatively low value of the wave-length for maximum absorption. In the three-toed sloth, the opossum, and the chimpanzee the pigment was located below 495 mμ, at about the same position at which the human visual substance is located (71). This location below 500 mμ of many mammalian visual pigments has been noted by others. In the case of several rodents, however, the visual chromoproteins were found in the region slightly above 500 mμ (table 3).

Summary

The well-known view that vertebrate rods utilize two discrete colored substances, rhodopsin and porphyropsin, is quite obviously not supported by the results presented in this summary. It is impossible to defend such a generalized view of nature in the light of a pigment system which, just for the retinene$_1$ compounds alone, is spread from 478 mμ (in deep sea fish) to 524 mμ (in geckos). This span of 46 mμ is greater than the separation of the classic rhodopsin system from the classic porphyropsin system. In contrast, the generalized concept of duality in terms of the chromophores, retinene$_1$ and retinene$_2$, is thoroughly supported by the evidence. Nature appears to have achieved simplicity and generality in terms of the carotenoid composition, yet versatility and biological usefulness in terms of the protein moieties of the visual pigments. Again the proteins emerge as the purveyors of biological specificity.

Such a diversity of substances only emphasizes the difficulties and limitations of present systems of nomenclature for these compounds. The terms chrysopsin, rhodopsin, porphyropsin, etc. were adequate for a system of pigments located at just a few discrete and narrow spectral regions. These terms are neither accurate nor useful for a system almost continuously variable over a large segment of the visible spectrum. What is the logic in referring to the pigment from a gecko as "gecko rhodopsin," as was done recently (27)? It doesn't appear red; in fact, the *Phyllurus* pigment at 524 mμ looks exactly like carp porphyropsin. Yet the term porphyropsin is not sufficiently descriptive or discriminating either since the *Phyllurus* pigment is associated with retinene$_1$ and the carp pigment with retinene$_2$. The writer is of the opinion that a system giving the spectral location and the nature of the chromophore, along with the name of the animal in parentheses, is simple, accurate, and sufficiently descriptive. Thus the pigment from the gecko *Oedura monilis* would, according to this system, be 518$_1$ (*O. monilis*). This system will work in cases where more than one pigment is found in the same species as in the case of the deep sea fish, *Bathylagus wesethi*. More complicated systems conveying more information could be devised but at this stage of developments a practical system should be simple as well as accurately descriptive.

DISCUSSION

Question: Dr. Farner wishes to know whether there is a visual pigment in cephalopoda which correlates with possible biological environmental changes or differences.

Dr. Crescitelli: The squid visual pigment has been studied and it has been found to be located at about 493 mμ. It would be awfully nice if it were possible to study the visual pigments of deep sea squid because such squid do occur at great depths and I have often wondered what these would show. The visual pigment of the squid, however, is difficult to study in the usual way; first, because it has to be separated from a lot of melanoid pigments; second, because it doesn't bleach at ordinary temperatures.

Question: Dr. French asks whether in the case of intermediate pigments there might not be a mixture of two pigments present together.

Dr. Crescitelli: I am glad you brought up that point; I didn't have time to go into that. In the case of certain euryhaline fish (salmon, eel, trout), as many of you know, two pigments have been found. The best example of this is in a paper by Bridges in which he found a retinene$_2$ pigment at 533 mμ and a retinene$_1$ pigment at 507 mμ. The important question there, I think, is this: (a) do these pigments change in quantity throughout life as for certain amphibia and (b) do these pigments, when they are present, occur in the same visual cell, or do they occur in separate cells? Is there an enzyme here that shifts retinene$_2$ to retinene$_1$, for example? I think there is a whole field here that could be explored both from the enzymatic and hormonal points of view.

Question: Dr. Farner wishes to know whether the action spectrum using the pupilary reflex is a good indicator of the absorption spectrum of the pigment.

Dr. Crescitelli: Here is another point, of course, that I immediately thought of when you mentioned your retinal receptors. In the case of most vertebrates, in addition to rods being concerned with the visual process, some of them, at least, are linked to other reflexes which involve the pupilary reflex. But this is not true for all animals, I understand. In some elasmobranchs the iris has independent receptors which respond to light directly; in fact, I thought of that point in connection with your results and wondered if possibly instead of the visual cells you might not look at the iris and the cells in the iris because there you might get an entirely different action spectrum. But not too much has been done with these. I think it would be worth studying some of these elasmobranchs from that point of view.

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The Variability of Chlorophyll in Plants

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Introduction

For this symposium on photobiology it is unnecessary to spend any of our time in pointing out the importance of photosynthesis to plant and animal life or to elaborate on the fact that photosynthesis depends on chlorophyll for the absorption of light which is transformed into a usable form of energy as food. Much has been written about the properties of chlorophyll, the green substance that could well be called the most obvious organic substance on the face of the earth. Nevertheless, we are really ignorant about its fundamental chemical nature in the form that exists and functions in living plants.

All students in elementary biology and botany courses are shown a vial of green liquid made by extracting chlorophyll from a leaf in alcohol. They are told at the time that extracted chlorophyll is somehow or other a little bit different from chlorophyll in the leaf, and when it is taken out of the leaf it does not do photosynthesis any more, but at least it is green.

We will go into somewhat more detail about the differences between extracted and natural chlorophyll as it exists in living leaves. One of the most easily detected and significant contrasts between extracted and intact chlorophyll is the difference in absorption spectrum. This is simply a corollary of an actual chemical difference between the two substances. Absorption spectra characterize chemical compounds as fingerprints characterize people. The chemistry of extracted chlorophyll is well known; that of chlorophyll in the leaf has been practically impossible to investigate by direct chemical means. We will restrict the discussion of the properties of native chlorophyll to things that can be learned from spectroscopic measurements. Our main topic will be the several different forms of native chlorophyll which are beginning to be recognized as existing together in living plants. Is there really more than one form of native chlorophyll *a*? If so, how many individual forms can be identified and what are their characteristics?

Varieties of Recently Formed Chlorophyll

The last step in chlorophyll formation is the conversion of pale green protochlorophyll into the darker green chlorophyll. This step involves the addition of only two hydrogen atoms to chlorophyll and is caused by light which the protochlorophyll absorbs. Shibata found it possible to follow the transformation of protochlorophyll into chlorophyll by direct measurement of the absorption spectrum of a leaf grown in the dark and then exposed to light.

The dashed line in figure 1 from the work of Dr. James H. C. Smith shows the absorption spectrum of protochlorophyll as it exists in a bean leaf that has been grown in the dark. This curve has overlapping protochlorophyll peaks at 650 and at 635 mμ. Only the 650 mμ form transforms to chlorophyll. The heavy curve, labeled 682, is the absorption spectrum of chlorophyll formed from protochlorophyll by a short exposure to light. This curve was measured immediately after the brief illumination period. Less than one minute of light is adequate to cause this transformation. The dotted line of figure 1 is labeled 670. This spectrum

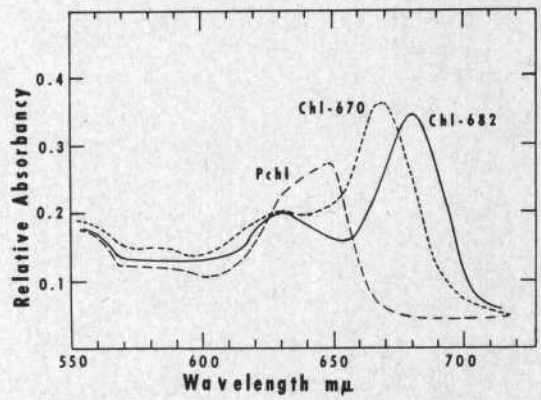

Figure 1. The absorption spectrum of a bean leaf grown in the dark showing the absorption due to protochlorophyll (Pchl). The same leaf immediately after brief exposure to light showing the spectrum of freshly formed *in vivo* chlorophyll (Chl-682) and again after 30 minutes in dark after the chlorophyll has changed to another form (Chl-670). From J. H. C. Smith.

was measured for the same leaf after it had been kept dark for about 30 minutes following the other measurements. During this dark time the freshly formed chlorophyll changed into a form absorbing at shorter wavelength. Before Dr. Shibata did experiments like this it was thought that the chlorophyll formed immediately by illuminating an etiolated leaf had its absorption peak at 670. This erroneous idea was simply based on the fact that the C682 intermediate had always been turned over into the C670 form before it could be detected.

Shibata found that if the leaf is extracted with alcohol immediately after illumination the extract contains ordinary chlorophyll. This is also true for the leaf that has been allowed to stand in the dark until the native chlorophyll with the long wavelength absorption, C682, has shifted to the C670 form. The extracted chlorophyll corresponding to either of these two curves is the same. There must be some difference, therefore, in the state of chemical combination of chlorophyll in the leaf that changes very rapidly with time. Here we have definite proof for existence of two forms of chlorophyll although both are not present together in stable equilibrium. If the leaf is allowed to stand several hours in the dark or given another illumination period the peak shifts toward longer wavelengths and in normal mature leaves usually lies between 675 and 680 mμ.

Evidence For Two Forms of Chlorophyll *a*

Photosynthesis is a delightful field of research because all its literature has been thoroughly reviewed in the three-volume work of Rabinowitch. I will therefore not attempt to give the history of this subject but will mention only a very few of the many articles that bear directly on the problem. The general idea of there being variations in the peak positions and shape of the absorption curves of chlorophyll *a* in living plants goes back to Lubimenko. It was supported by Seybold and Egle. Some of the literature on the subject has been reviewed by French (1959). While everyone agrees that there must be various forms of chlorophylls in plants, the evidence is not very direct, and Rabinowitch summarizes the situation (p. 1848): "No convincing spectroscopic evidence of a doublet structure of the red absorption band in mature plants has been published." This statement taken out of its context sounds a little more discouraging than the situation really is. Another way of putting it is that published data are perhaps best interpreted by assuming several forms of chlorophyll, but other hypotheses might also be used to explain the variability in shape of the spectral curves.

One of the clearest arguments for the real existence of both active and inactive forms of chlorophyll *a* is shown in the upper part of figure 2, redrawn from Haxo and Blinks (1950). This figure gives comparative measurements of the action spectrum and the absorption spectrum for a red photosynthetic alga. The peak at about 675 mμ in the absorption spectrum is due to chlorophyll *a*. Fortunately, this alga does not contain chlorophyll *b*. In spite of the large absorption of chlorophyll in the region around 675 mμ, the activity is lower than in the middle of the spectrum where the light is absorbed by the accessory red pigment phycoerythrin. Unless the unlikely

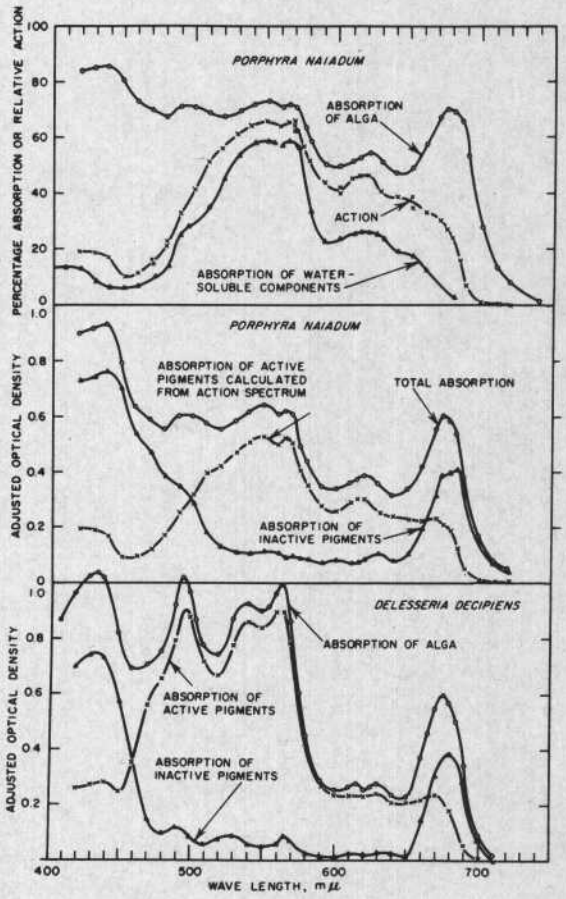

Figure 2. *Top:* The absorption and photosynthesis action spectra of a red alga. *Middle:* The same data replotted to show the spectra of the active and the inactive pigments. The peak position for the inactive chlorophyll is at a longer wavelength than for the active form. *Bottom:* The same for another red alga. Data of Haxo and Blinks, replotted by French and Young.

assumption is made that the active form of chlorophyll is less efficient than phycoerythrin there must be both active and inactive forms of chlorophyll *a* in these plants.

French and Young (1956) calculated an absorbance curve for the active and the inactive pigments from this data of Haxo and Blinks. The center curves of figure 2 show a chlorophyll peak for the inactive pigment that appears to be at a slightly longer wavelength than the absorption band of the total chlorophyll in the alga. The lower part of figure 2 presents a similar calculation from the data of Haxo and Blinks for another red alga.

Great progress in understanding the factors affecting the variation in wavelength position of the red absorption peak of chlorophyll in its natural state has been made by Krasnovsky and his group. (Translations by Rabinowitch and by Milner are available.) The thought is that the longer wavelength forms of chlorophyll absorbing in the neighborhood of 680 mμ may be inactive in photosynthesis while forms absorbing near 670 mμ may be the photosynthetic and also the fluorescent forms of chlorophyll. Krasnovsky has put forward the theory that these forms may differ merely by being monomeric versus polymeric forms of the chlorophyll-*a* complex. This interpretation may well turn out to be an oversimplification, but it does clarify the nature of the problem. Krasnovsky and Kosobutskaya, and Vorobeva and Krasnovsky have done some remarkably interesting experiments on the bleaching of C670 by briefly illuminating crude chloroplast suspensions prepared in the dark. The absorption peak shifts toward longer wavelengths after a short exposure to light. This is taken to mean that C670 bleaches while C682 remains intact.

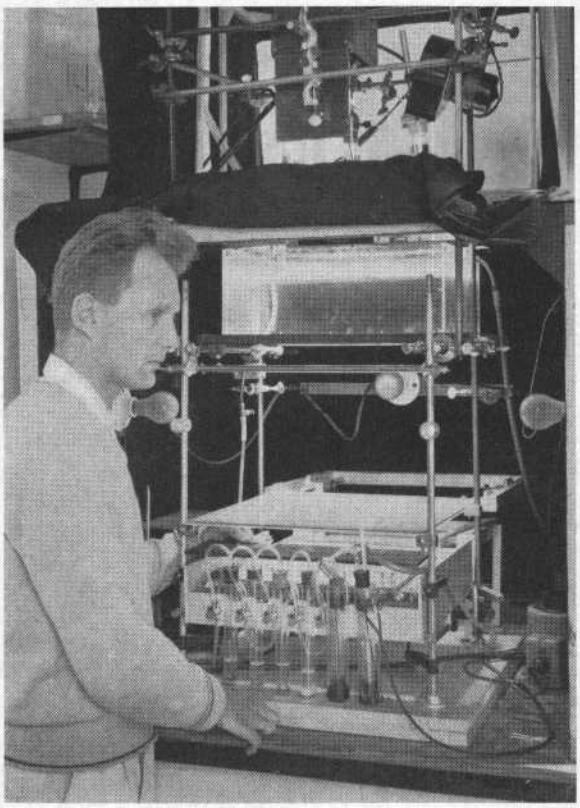

Figure 3. Apparatus for growing algae in a crossed gradient field with temperature on one axis and light intensity on the other being operated by Dr. Per Halldal.

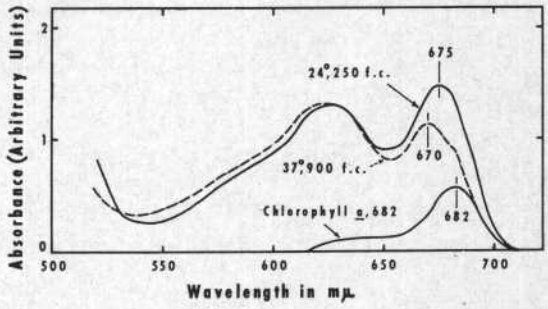

Figure 4. Absorption spectra of the blue-green alga *Anacystis nidulans* grown under two sets of conditions in the apparatus of figure 3. The difference between the two upper curves as given by the bottom curve is taken to approximate the spectrum of one form of *in vivo* chlorophyll *a*. From Halldal.

Some years ago Dr. Young and I measured the fluorescence spectrum of a red alga that had been illuminated with light of several different intensities until the fluorescence had reached a constant intensity. At that time we were not concerned with the differences in kinds of chlorophyll, but only concluded from the data that chlorophyll fluorescence and not fluorescence of the accessory pigments decreased after a bright light exposure (French and Young 1952). Vorobeva and Krasnovsky have pointed out that these curves contain far more interesting information than we had realized at the time. The fluorescence peak of chlorophyll *a* in the low intensity curve comes at about 680 mμ, whereas the difference between the high and the low intensity curves representing the fluorescence that goes away in bright light has a shorter wavelength. This difference of peak position clearly indicates that there must be two forms of *in vivo* chlorophyll differing in their fluorescence spectra.

Dr. Halldal grew a blue-green alga under different conditions of light intensity and temperature to study the formation of accessory pigments. He used a large flat agar surface with a temperature gradient from left to right and a light intensity gradient from front to back, figure 3. (Halldal and French 1958). A blue-green alga, *Anacystis*, grown in this micro-phytotron differs not only in growth rate at various places on the plate but also in color. Dr. Halldal cut out pieces of this agar layer and measured the absorption spectra of the live algae grown under diverse conditions. Figure 4 from Halldal (1958) shows a different shape for the chlorophyll part of the spectrum under two growth conditions, whereas the phycocyanin part of the two spectra are nearly identical. Subtracting one of these curves from the other gives a curve presumed to be the absorption spectrum of the C682 *in vivo* form of chlorophyll. The difference between these two curves is much larger than is usually found between comparable plant spectra. What we really need for investigations of this type is some method of making much better measurements of the shapes of absorption spectra.

Figure 5 shows how the red absorption band of chlorophyll *a* in a red alga might be composed of two overlapping components with their peaks at different wavelengths. The upper curve is the sum of the two lower Gaussian probability curves. (The sum of these curves is a reasonably close approximation to the chlorophyll *a* absorption band of *Porphyra naiadum*.) Now, by varying the relative proportions of these two components, summated curves having very different shapes may

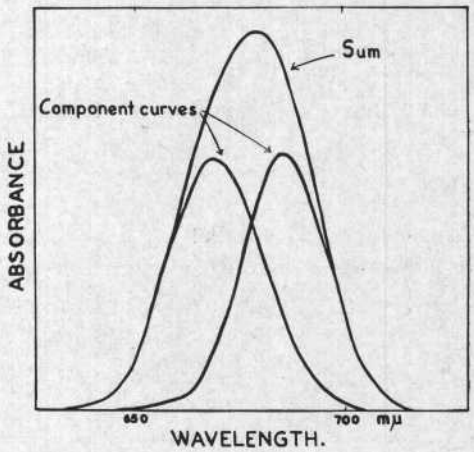

Figure 5. The addition of two curves to illustrate the way chlorophyll *a* absorption consists of two or more overlapping individual components.

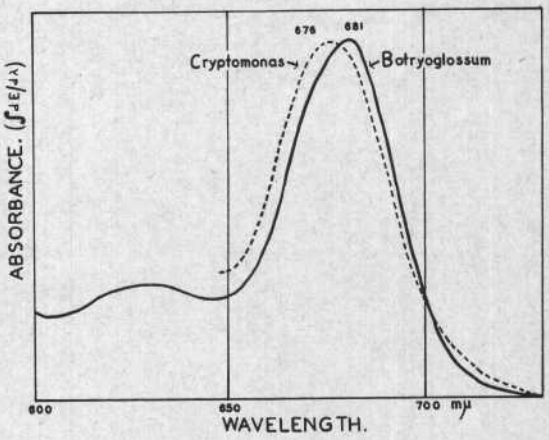

Figure 6. The red absorption band of chlorophyll *a* in two algae. The curves differ in shape as well as in peak wavelength.

be obtained. Curves like those in figure 6 for two algae having only chlorophyll *a* could thus be fitted. It is easy to see that raising the proportion of one component or changing the separation interval would put a bulge on one side of the summated curve. Some of the interesting surprises in store for those who try to match curves this way have been described by Vandenbelt and Henrich. The possibility of thus analyzing complex curves into simpler components leads to the thought that the variations in shape of absorption spectra of the chlorophyll *a* band in live plants may be due to differing relative proportions of two or more components. The presence of a bulge on one side suggests that the component on that side is present in more than the amount required to give a symmetrical summated curve.

Derivative Spectrophotometry

The detection of small bulges on measured absorbance curves is difficult even though the curves are measured with precision. We have therefore built a machine which plots not the height of the absorbance curve but rather its slope. Small differences of slope on the side of steep absorption bands are very hard to see. However, if the slope is recorded directly the detailed structure of an absorption band becomes much more clearly evident (Giese and French). I will first describe the way in which such derivative measurements are made and then present some results obtained with this derivative spectrophotometer which bear on the problem of variation of chlorophyll *a* in living plants. The machine has a vibrating slit that rapidly alternates over a 3 mμ range of the spectrum. The AC

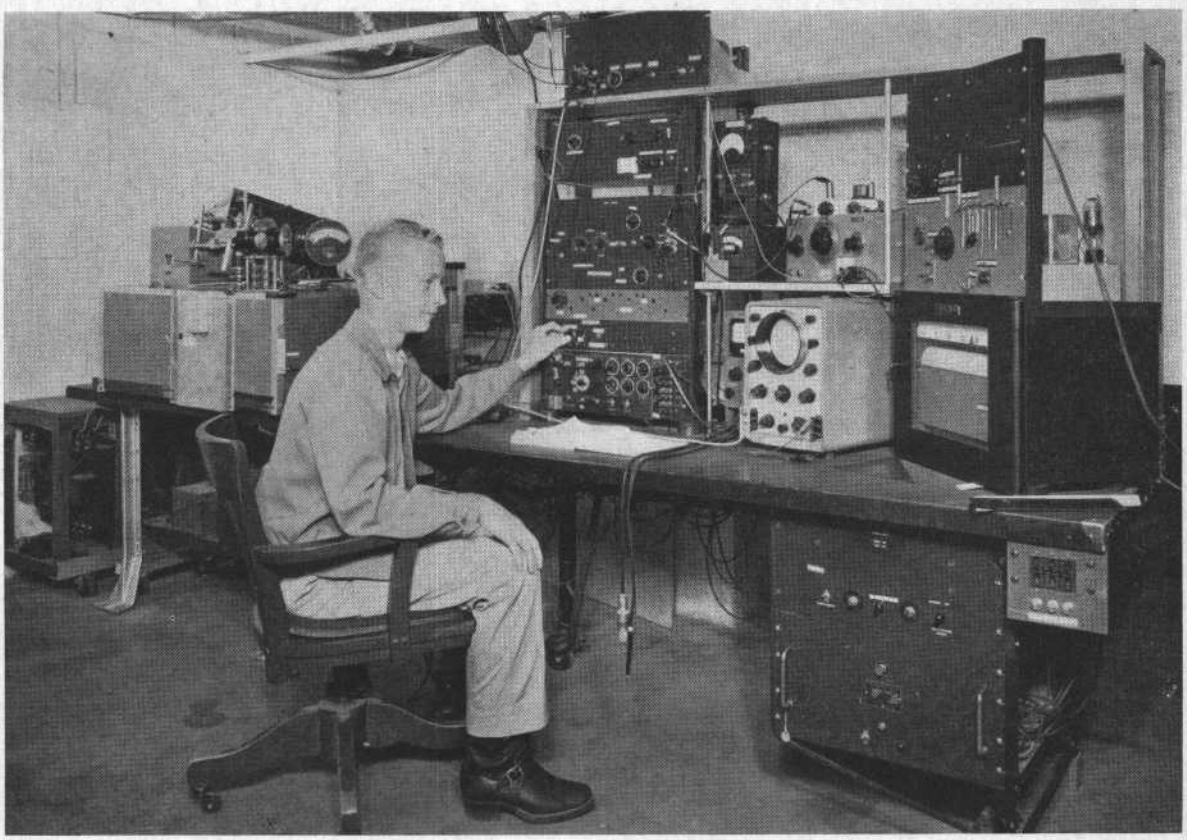

Figure 7. The derivative spectrophotometer operated by Mr. Gordon Harper.

voltage from the photocell thus varies in amplitude in direct proportion to the difference in intensity of light passing through the sample within this small wavelength interval. This voltage is plotted against wavelength with appropriate corrections. The machine has been described by French (1957).

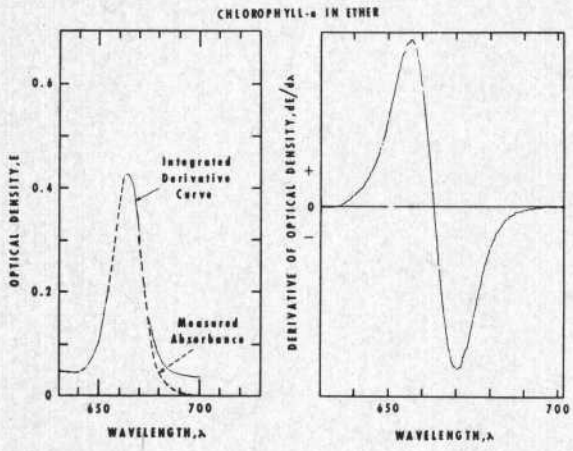

Figure 8. *Left dotted:* The absorption spectrum of the red band of pure chlorophyll *a* in ether. *Right:* The derivative absorption spectrum of the same sample of chlorophyll. (The full line in the left figure is the integrated derivative curve.)

A view of the equipment being operated by Mr. Harper who helped to build it is shown in figure 7. The sample is placed directly in front of a tube painted white on the inside with an opal glass plate at one end and the photocell at the other. Having the opal glass diffusing surface close to the sample makes the scattered light reach the photocell equally well regardless of the direction in which it emerges from the sample. This effect is very important for reasons discussed later.

Derivative Spectra of Live Plants

Derivative spectra look different from absorbance curves. They may, however, be integrated to compare with the usual form of data presentation. To start with a famliiar substance, let us compare in figure 8 the absorption of a chlorophyll solution as measured by an ordinary recording

spectrophotometer and by the derivative spectrophotometer. The familiar curve on the left is the absorption spectrum of a pure solution of chlorophyll *a* in ether prepared by Smith and Benitez. On the right is the curve for the same solution measured in the derivative spectrophotometer. The derivative curve crosses the zero line at the absorption peak, 662 mμ. It is positive on the left and negative on the right of the peak position. The curve is nearly symmetrical.

A derivative spectrum and its integrated form for sea lettuce are given in figure 9. The double

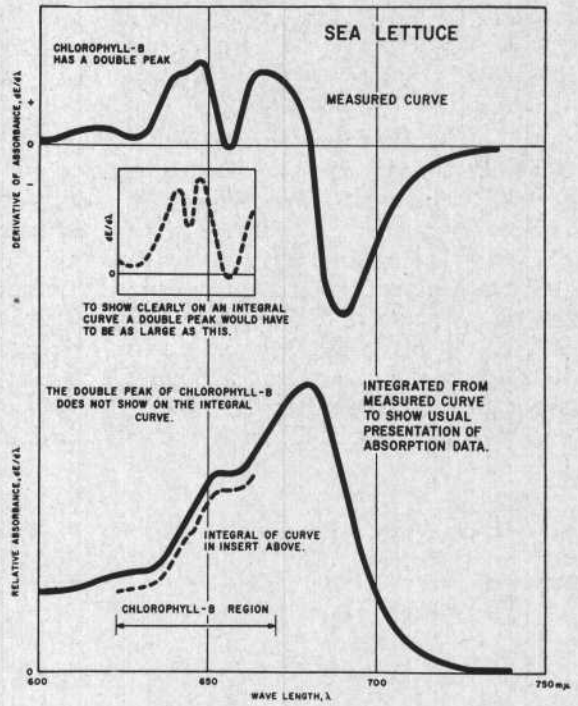

Figure 9. Derivative spectrum of *Ulva* showing a doubled chlorophyll-*b* peak. (In collaboration with Dr. Francis Haxo)

peak of chlorophyll *b* is evident in the derivative curve but is not detectable in the absorbance curve. The insert shows how large the double peak would have to be on a derivative curve to be detectable on an integral curve. This comparison gives some idea of the value of derivative as compared with integrated measurements. So far no other species than sea lettuce has been found to have the chlorophyll-*b* peak doubled. This measurement was made in collaboration with Dr. Francis Haxo.

Figure 10 shows some derivative spectra of live algae. *Nannochloris*, which does not contain chlorophyll *b*, was sent by Dr. Mary Belle Allen. The curve shows absorption only due to chlorophyll *a*. The middle curve for *Hematococcus* shows a peak due to chlorophyll *b* which is larger than that found in most green algae. The lower curve of figure 10, for *Gonyaulax*, measured in collaboration with Dr. Francis Haxo, shows the presence of chlorophyll *c* as well as a simple form of chlorophyll *a*. Other curves for various plants are shown in figure 11. Regardless of the complications caused by overlapping of chlorophyll *b* with the chlorophyll *a* spectra it is very clear that there is considerable diversity between plants in the chlorophyll *a* region of the spectrum.

It may be possible to take the differences between some of these curves and thereby obtain characteristic derivatives of absorbance curves for the individual components of the chlorophyll *a* absorption spectrum. Attempts to deduce the basic component curves for all the different forms of chlorophyll *a* have been somewhat disappointing for reasons that are not yet clear. Part of the

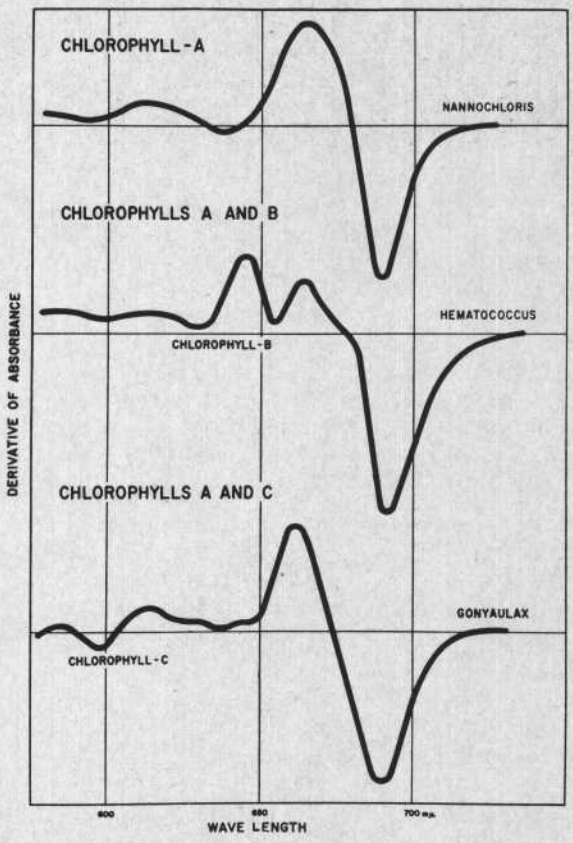

Figure 10. Derivative absorption spectra of algae containing chlorophyll *a*, *a*+*b*, and *a*+*c*. (*Nannochloris*—Dr. M. B. Allen, *Gonyaulax*, Dr. F. Haxo)

trouble seems to be the simultaneous presence of more than two components in most plants. Pairs of plants being compared may differ in the proportions of more than two of their components.

The bottom curve of figure 11 compares chlorophyll *a* in ether with chlorophyll *a* in *Botrydiopsis*. A comparison of the *Nannochloris* and *Botrydiopsis* curves indicates that there must be different forms of chlorophyll *a* and it is highly likely that both of these curves represent mixtures of several components.

The curve for *Chlorella* in figure 11 is probably the most striking one of all in showing the presence of two chlorophyll *a* components. *Chlorella* derivative spectra have a distinct shoulder. When measured at 5°C., the shoulder actually becomes an extra peak on the derivative curve.

Duckweed has a component absorbing at appreciably longer wavelengths than do the chlorophyll components of *Chlorella*. Here the conclusion that more than two forms of chlorophyll occur simultaneously appears inescapable. The two upper curves of figure 11 contrast a simple type of chlorophyll *a* spectrum with a complex type. Qualitatively the existence of several forms of chlorophyll *a* is clear in spite of our present difficulties in making quantitative deductions as to the actual shape of the separate curves for the individual components. We will discuss only two of these deduced curves for individual components.

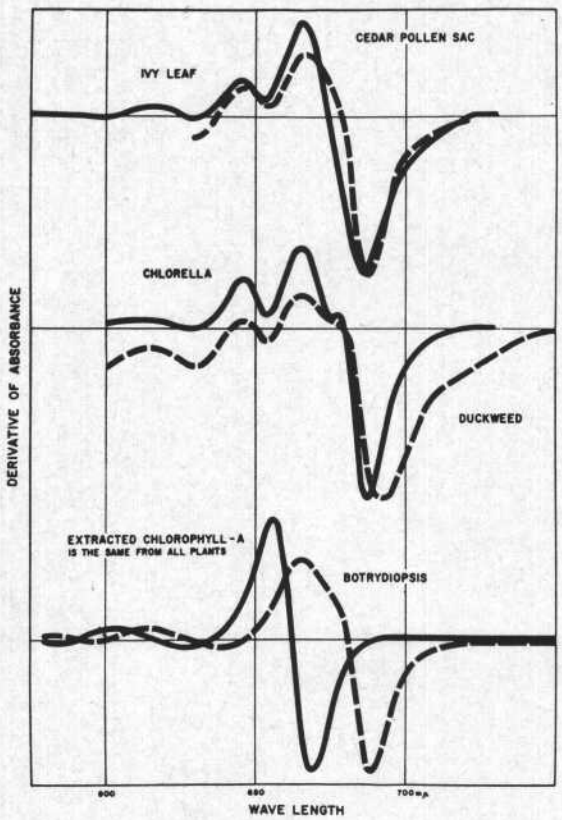

Figure 11. *Lower:* Derivative absorption spectrum of chlorophyll *a* in ether compared with chlorophyll *a* in an alga. *Middle and upper:* Derivative spectra of four plants with chlorophyll *b* that have chlorophyll-*a* bands of different shapes.

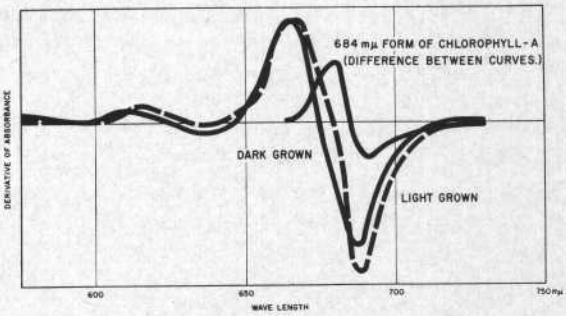

Figure 12. Derivative spectra of a *Chlorella* mutant lacking chlorophyll *b* grown in light and in dark. The difference between the curves may be the spectrum of a form of chlorophyll *a* that develops in light. (In collaboration with Dr. M. B. Allen)

Dr. Allen grew a *Chlorella* mutant that does not form chlorophyll *b*, figure 12. This mutant was particularly interesting because its spectra have different shapes for the cells grown in the light and in the dark. Measurements of the two spectra should be suitable for finding the characteristic curve for the extra chlorophyll *a* form produced in the light. The difference curve here shown may represent a form of chlorophyll *a* with a peak position of 684 mμ. This difference curve is more unsymmetrical than the spectra of chlorophyll *a* in ether or in the simpler spectra of algae and looks much like the spectrum of colloidal chlorophyll suspended in water except that it is at a longer wavelength.

Chlorophyll *a*, 695, in *Euglena*

Derivative Absorption Spectra

Mrs. Elliott and I have studied the spectra of many *Euglena* cultures. Young *Euglena* gives derivative absorption curves very much like those of any other green alga. The shoulder characteristic of *Chlorella* chlorophyll is, however, less pronounced in *Euglena* and the amount of chlorophyll *b* is always smaller than in typical green plants. However, an extra peak at 700 mμ is found in the derivative absorption spectrum of some *Euglena*

cultures. This extra peak, figure 13, is present only in old cultures. We have measured several cultures in which this peak exceeds the height of the 690 mμ derivative peak common to green algae.

To put this situation in more familiar terms, let's look at these data in integral form, figure 14. The broader curve corresponds to the old culture, the narrow curve to the young one. The difference between the two corresponds to the extra component. (The 700 mμ derivative peak corresponds to an absorption maximum at 695 mμ.) This difference curve is much more skewed than one would expect for a component of an absorption spectrum of chlorophyll based on an analogy with the way chlorophyll *a* looks in solution or the way a whole band looks in the organisms that have been investigated so far. It does, however, like the 684 component from *Chlorella,* have much the same shape as the spectrum of a colloidal suspension of chlorophyll *a* in water as discussed in the last section.

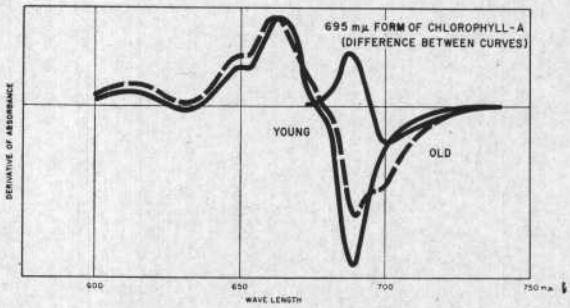

Figure 13. Derivative spectra of young and old cultures of *Euglena.* The difference between the two represents the spectrum of an *in vivo* form of chlorophyll *a* with an absorption peak at 695 mμ.

FLUORESCENCE SPECTRA

If this extra hump in the derivative absorption curve is a real pigment, we might be able to detect it in the live cells by fluorescence spectroscopy. Chlorophyll *a* in common green algae gives a fluorescence peak at about 685 mμ with a low shoulder in the 720-730 region. Some years ago we found an extra peak due to an unidentified pigment having a fluorescence peak at 705 mμ in the diatom *Nitzschia* (French 1955). We thought *Euglena* might show an extra fluorescence peak due to its absorption band at 695 mμ and that its fluorescence spectrum would probably look much like that of *Nitzschia.*

Figure 15 is a comparison of the fluorescence spectrum of *Chlorella* with that for a 7-day-old

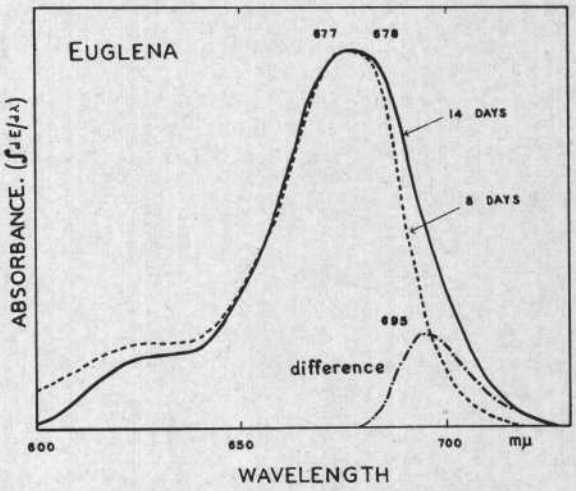

Figure 14. The data of figure 13 in integral form.

culture of *Euglena.* The *Euglena* flourescence is slightly higher in the region just beyond 700 mμ. Otherwise this young culture matches *Chlorella* quite closely. However, older *Euglena* cultures give very different fluorescence spectra as illustrated in figure 16. A 10-day culture of *Euglena* is higher in the region beyond 700 mμ than at 7 days. At 12 days this is more pronounced, and after 22 days the usual 685 mμ chlorophyll *a* peak shows only as a slight shoulder in the left part of the curve and the broad peak at about 705-710 mμ predominates. The extra peak is broader than that of *Nitzschia.* The derivative spectra of these cultures are shown in figure 17. The fluorescence spectra might be taken as evidence to indicate the reality of the new peak as a real pigment. If both

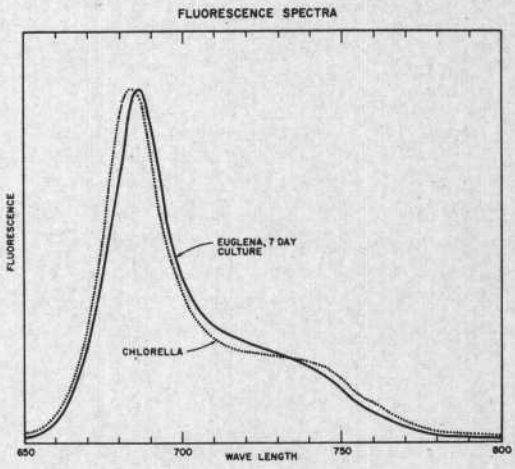

Figure 15. The fluorescence spectrum of a young *Euglena* culture nearly matches that of *Chlorella.*

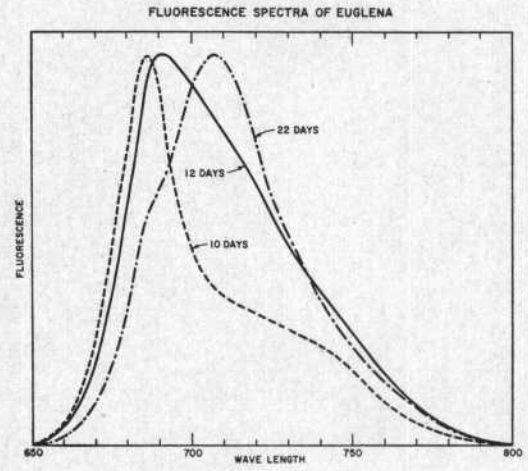

Figure 16. Fluorescence spectra of *Euglena* cultures of different ages showing the gradual appearance of a fluorescence peak at 706 mμ corresponding to the absorption peak of figure 17. (The spectra are not corrected for the change of sensitivity with wavelength of the photomultiplier tube, Du Mont #6911.)

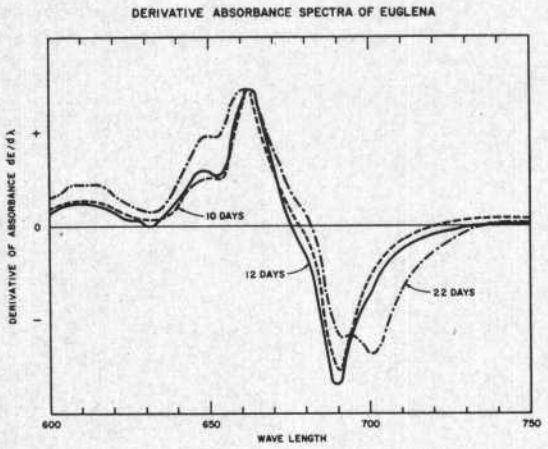

Figure 17. Derivative absorption spectra of the same *Euglena* cultures used for figure 16.

the derivative absorption and the fluorescence curves are due to an artifact rather than to a new pigment, it must be a very good artifact. Let us, therefore, examine some effects that could influence the shape of an absorption curve to make it look as though is were composed of more individual pigments than really exist.

Optical Effects That Complicate Pigment Detection

The Flattening Effect

One of the difficulties in studying the detailed shape of absorption spectra of living organisms is the fact that the pigment comes in small particles. If the absorption of light by a single particle is very high, then the absorption spectra for the suspensions are distorted. This phenomenon is known as the flattening effect. It has been investigated mathematically by Duysens. Briefly, what this flattening effect does is to squash down the top of the band, thereby correspondingly broadening the bottoms. However, this effect, since it depends only on the absorption coefficient for a particular wavelength for the particle concerned, cannot distort a curve on one side of the peak and not on the other. If the extra hump in our *Euglena* curves is an artifact, it cannot be due to the ordinary flattening effect because the extra absorption in *Euglena* appears only on the long wave side of the band.

Light Scattering by Colorless Components

One of the primary difficulties in measuring absorption spectra of whole cells is the fact that the cell walls and internal particles scatter light in various directions. Thus the length of the light path in the sample not only is unknown but also varies with wavelength. Furthermore the relative intensity coming out at various directions is wavelength dependent. Scattering lowers the height of the peaks in proportion to the valleys of the absorption curve. To get around this difficulty the simplest arrangement, in principle, is to put the material whose spectrum is to be measured inside a white sphere. The intensity at any point on the surface of this sphere is supposed, as a first approximation, to be an average of the light coming out of the sample in all directions. Thus the distorting effects of scattering are minimized by sphere measurements.

Dr. Shibata has devised a more practical method usable with ordinary recording spectrophotometers which instead of a sphere requires only a sheet of opal glass. The opal glass diffuses the light coming out one side of the sample. This arrangement acts more or less as a half sphere, for it gives an average of all the light from the sample on one side. A convenient device for holding leaves in contact with an opal glass plate for use in a recording spectrophotometer is described by Smith, Shibata, and Hart. With opal glass Shibata, Benson, and Calvin studied the absorption spectra of a number of plants including *Euglena*. Figure 18 compares the *in vivo* spectrum with that for alcohol extracts from the same quantity of cells. Without opal glass the spectrum for the living cells would appear much higher and

the peaks would be much broader. This is our reason for using an opal glass diffusing screen directly behind the sample in the derivative spectrophotometer.

The distortion of absorption spectra by scattering from cell walls can be greatly reduced also by Barer's method of suspending the sample in a protein solution with a refractive index equal to that of the cell walls. Another way of reducing scattering is to squeeze the cells through a needle valve under very high pressure, thus disrupting the cells completely (Milner, Lawrence, and French). The cell walls may be centrifuged out after such treatment, leaving a reasonably clear preparation. The needle valve homogenizer also breaks chloroplasts down to extremely small particles that give much less scattering. Suspensions of cells treated in this manner may be measured in an ordinary spectrophotometer to give a fairly good idea of the location and shape of their absorption peaks. While this method is rough on the structure nevertheless chloroplasts put through the needle valve retain much of their photochemical activity. The pigments therefore must still be in the same active form as in the intact cells.

If the new 695 mμ absorption band of *Euglena* is an artifact due to some optical effect dependent on particle size we should be able to eliminate the band by reducing particle size. We have measured the derivative spectra of old *Euglena* cultures put through the needle valve homogenizer. The extra absorption was somewhat reduced but not eliminated by this treatment. Strangely enough various centrifugal fractions of the homogenized *Euglena* all gave identical spectra, thus showing that the new absorption band is not dependent on particle size. The partial loss of the band by homogenization must therefore be due to some other factor than a decrease of particle size.

LIGHT SCATTERING BY PIGMENT PARTICLES

Dr. Latimer at the University of Illinois measured the light coming out at right angles to an incident beam from a suspension of *Chlorella*. These data gave the dotted curve shown in the middle of figure 19. The peak position of the scattered light is about 12 mμ to the long wavelength side of the true absorption peak shown by the bottom curve. This scattering peak is caused by the well known phenomenon of anomalous dispersion: the refractive index of the pigment changes greatly in the neighborhood of an absorption band. The refractive index difference between the pigment and its surroundings is largest

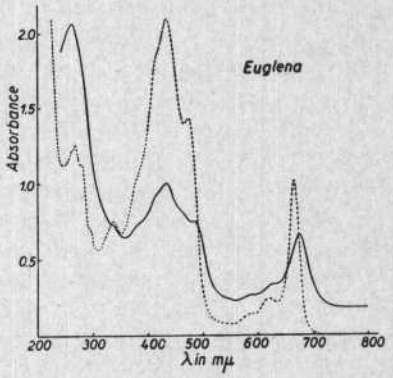

Figure 18. *Full line:* Absorption spectrum of a suspension of *Euglena*. *Dotted line:* An alcohol extract of the same suspension in the same volume. From Shibata, Benson, and Calvin.

on the long wavelength side of the band thereby greatly increasing the scattering in this wavelength region. The upper curve of figure 19 shows the absorbance of the same *Chlorella* suspension as the lower curve but measured without the opal glass plates. This curve looks much broader particularly on the long wavelength side. It is this effect, among others, that makes us worry about the possibility of *Euglena's* 695 mμ peak being an artifact rather than a real pigment.

Since the scattering varies with the angle at

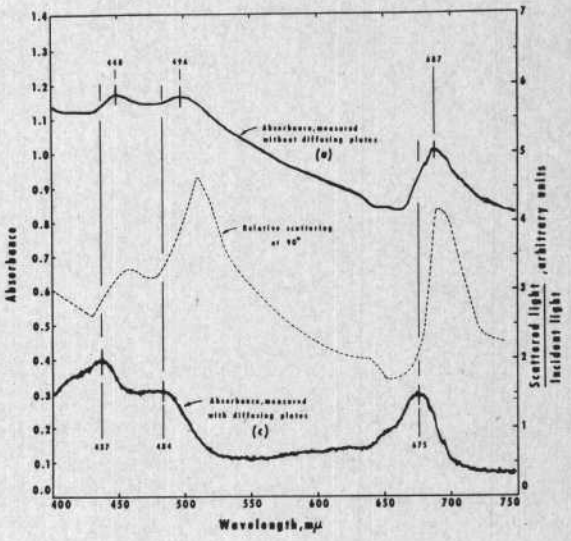

Figure 19. *Upper and lower curves:* The absorption spectrum of a *Chlorella* suspension measured with and without an opal glass diffusing plate. The bottom curve with the opal glass is closer to the correct absorption. *Middle curve:* The light scattered at 90° from a *Chlorella* suspension. The scattering peak is at a longer wavelength than the absorption peak. From Latimer.

which the light comes out of the sample, Dr. Latimer constructed an apparatus to measure the light from a cell suspension at a number of different angles. This was done by moving a doughnut-shaped aperture either close to the photocell or close to the suspension, thereby varying the angle through which the light was gathered. The bottom curve of figure 20 shows the absorption spectrum of a sample of *Chlorella* measured with a piece of opal glass directly behind it. The top part of figure 20 shows measurements made with the doughnut in various places. The right-hand curve labeled 0° for light coming straight through in a narrow beam gives a peak wavelength of 688 mμ. This is an unusually long wavelength for the absorption peak of chlorophyll *a* in *Chlorella*. The center curve shows the peak at 682 mμ. It was measured with light at about 3.4° from the center line. The curve on the left in figure 20 is for light emerging from the sample at an angle of about 32°. With this arrangement the peak position appeared to be at 668 mμ. Thus, by selecting the conditions of measurement it is possible to get an apparent absorption peak for the same sample that varies over a 20 mμ range. This experiment leads to extreme skepticism of detailed discussions of pigment identifications based on absorption spectra of intact cells. Small variations in the amount of scattering could well cause a great deal of uncertainty in the interpretation of spectra of whole plants. Thus we see that the broadening of an absorption may be rather poor evidence for the presence of an extra pigment. Not only the peak position but the entire shape of the spectrum depends on the angle in which the light is gathered. All the derivative spectra were measured with an opal glass diffuser behind the sample. Therefore scattering errors of the type discussed should not be present.

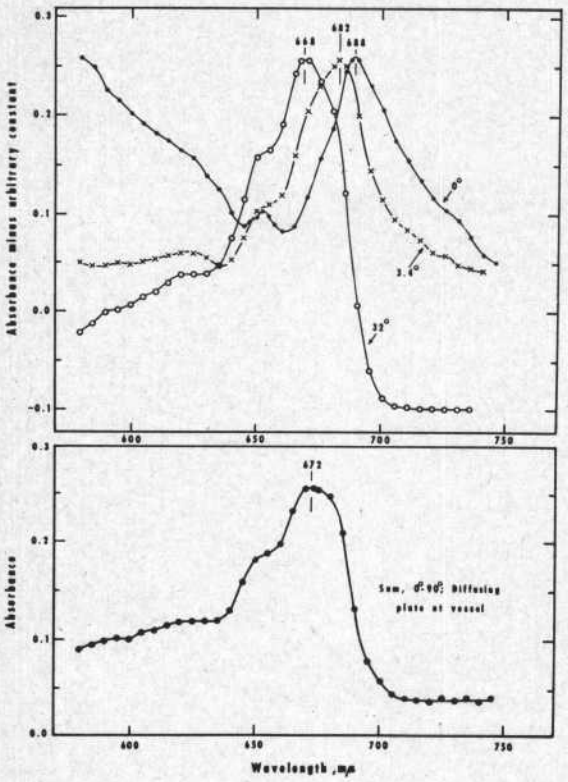

Figure 20. *Bottom curve:* The absorption spectrum of a *Chlorella* suspension measured with an opal glass plate to catch light at all angles. *Top curves:* The apparent absorption spectra of the same *Chlorella* suspension as measured with light from the suspension emerging at various angles. From Latimer.

Distortion of Fluorescence Spectra By Reabsorption

Because of the possible uncertainties discussed above, in the identification of pigments from absorption spectra of live cells the apparent confirmation of the actual presence of a new form of chlorophyll *a* in *Euglena* by fluorescence spectroscopy is of critical importance. There is, however, a somewhat similar source of confusion in pigment identification by fluorescence spectroscopy. This is the distortion of fluorescence spectra by reabsorption of fluorescent light within the sample. With dark green leaves or with dense suspensions of algae this distortion can be very serious. However, in dilute suspensions of algae such as were used for the *Euglena* experiments we have had no trouble previously from reabsorption. If reabsorption of the fluorescent light has had any influence on the shape of the curves in figure 16, it must be due to an extraordinarily high pigment concentration within the individual cells.

The distorted spectra of dark green leaves gives a broad false peak at about 740 mμ (French 1955) while the extra fluorescence peak of the *Euglena* (figure 16) is at 706 mμ so this band does not seem to be similar to the artifact caused by reabsorption of the fluorescence.

Unsymmetrical Chlorophyll Absorption Bands

A comparison of dissolved and colloidal chlorophyll is shown in figure 21. This figure compares chlorophyll *a* in alcohol solution with the same amount diluted in water to give a colloidal solution which was measured immediately and again after 30 minutes. The colloidal chlorophyll

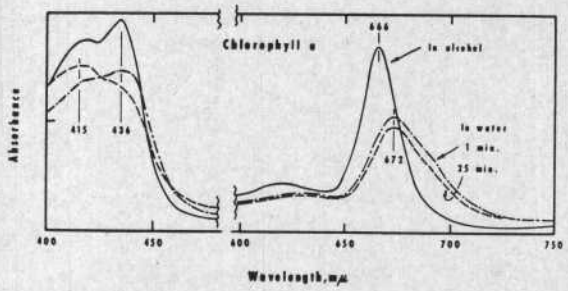

Figure 21. The absorption spectra of an alcohol solution of chlorophyll *a* compared to the same amount of chlorophyll in colloidal form suspended in water immediately after dilution from alcohol and after 30 minutes.

has a much broader absorption band than the solution. After standing for 25 minutes the spectrum of the colloidal material shows some interesting changes in shape with time. The ratio of the two blue peaks changed as the particles grew in size. Solid chlorophyll particles have broad peaks that are much less steep on the long wavelength side. The shape of the red band of colloidal chlorophyll is very much like that of the extra *Euglena* pigment obtained by difference (figure 14) and of the derivative spectrum of the difference between the *Chlorella* mutant grown in light and dark. While the shapes of these three curves are similar their peak wavelengths are very different: in colloidal chlorophyll 672, *Chlorella* extra pigment 684, and *Euglena* extra pigment 695 mμ.

Summary

Some of the evidence for the existence of two forms of *in vivo* chlorophyll *a* is discussed.

It is suggested that there are more than two forms of chlorophyll *a* in many plants.

Derivative spectra characterizing a 684 form of chlorophyll *a* in *Chlorella* and a 695 mμ form in old *Euglena* are given.

Acklowledgments

Mrs. Brian J. Elliott grew many algal cultures for this work and helped continuously with the experiments. Numerous algal cultures and freshly collected marine algae were contributed by Dr. Francis Haxo and Dr. Mary Belle Allen, to both of whom I am grateful for much valuable discussion. Mrs. Elliott, Mr. Richard W. Hart, and Miss Jennifer Wootton gave much time to the preparation of the figures.

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Energy Transfer in Photosynthesis

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Perhaps the feature in the process of photosynthesis which best distinguishes it from other photobiological reactions is its property of energy transfer. During this symposium we have heard of a number of biological photochemical reactions, and of course photosynthesis is one of these. However, photosynthesis is different from most of these other reactions in that during the process of photosynthesis a major part of the absorbed light energy is stored in the form of the bonds of chemical compounds.

I need hardly stress the point to biologists that all of the energy of living systems on this earth is dependent upon this one process which stores energy in the form of chemical bonds. All other biological systems degrade this energy and use it to carry out their life processes. Moreover, photosynthesis is a *very efficient* process for storing chemical energy. Just how efficient it is has been a subject of many protracted investigations. The actual efficiency still remains in controversy, but we need not concern ourselves too much with the exact figure because even the most pessimistic estimates of the efficiency of photosynthesis are still rather impressive.

In order that we may have a clear idea of what we are talking about in terms of energy transfer, I will indicate one of the equations which has frequently been used to express the process of photosynthesis. We commonly consider this process as a reaction, in which carbon dioxide and

$$1)\ CO_2 + H_2O \xrightarrow{\text{light}} (CH_2O) + O_2\ \triangle F = +116\ \text{Kcal}$$

water undergo an oxidation-reduction under the influence of light. The product of this reaction can be written as glucose, although actually photosynthesis leads to a great many other products besides glucose. The energy associated with this reaction is usually expressed by chemists in terms of the molar-free energy change, or $\triangle F$, and since energy is stored by this reaction, $\triangle F$ for the reaction as written is positive and is about 116 kilocalories of chemical energy per mole.

How does the plant get the energy to drive this reaction? This comes from the light, and in order to equate the light energy with stored chemical energy, it is necessary to make a transformation in which one uses the familiar principle that the energy of a light photon is equal to Plank's constant times the frequency of the light. This gives us the energy of one single photon which is a pretty small packet of energy. We want to think in chemical terms now, (i.e., moles, etc.) so we multiply by Avagadro's number, $N = 6 \times 10^{23}$, and this gives us the energy for one einstein of light, or one "mole" of light, $E = Nh\nu$. This comes out to be about 41.5 kilocalories in the case of light with a wave length of 6800 angstroms.

You will notice that I used light of the longest wave length that can be used efficiently in photosynthesis. Of course, photosynthesis can be driven with other light in the visible spectrum, but the shorter wave lengths have more energy per photon and there is good reason to believe that this excess energy is dissipated, and that the energy stored in excited chlorophyll is equivalent to 6800 angstroms or something like 42 kilocalories per einstein. There has been a controversy with respect to the efficiency of photosynthesis. Reported values have ranged from 4 or less quanta per molecule of O_2 evolved to 8 or 10 quanta. I'll not go into these differences in this discussion but instead will say that it is now possible to measure the quantum efficiency not only by means of manometric technique, which is always difficult when one has to work with two gases and differential manometry, but also by other types of measuring instruments which determine independently levels of oxygen and carbon dioxide. In one simple system we circulate gas through a suspension of algae and measure continuously the level of oxygen by means of an infra-red analyzer. When we do this, we obtain values that run around 6.5 to 7.5 quanta per molecule of oxygen evolved. Some idea of the overall efficiency of photosynthesis may be obtained if one accepts a value of 6 and remembers that 6 einsteins correspond to an energy of about 249 kilocalories. If this is compared with the 116 kilocalories per mole of CO_2 reduced to glucose in the reaction shown earlier, the efficiency is found to be around 47%.

One can make similar calculations for other quantum requirements. Thus photosynthesis must

be considered to be a very efficient process indeed. We shall see later that photosynthesis must involve 20 or 30 individual steps. It is not a single reaction as shown here, but rather a very complex one of which we know maybe 14 or 15 steps, and are reasonably certain that there are about that many more steps which remain as yet unknown. With as many as 25 steps, each of them on the average 97% efficient, there would be an overall efficiency of about 47%.

The second factor affecting efficiency is that photosynthesis is a very rapid process. The most efficient kind of process for energy transfer is an equilibrium process in which A + B (representing two chemicals) go to C + D. If these products and reactants are nearly at equilibrium with respect to conversion to each other, then the free energy change in transforming A + B to C + D is about zero. However, for processes in which there must be a rapid net conversion of these reactants to these products, it is necessary

$$2)\ A + B \xrightleftharpoons{\text{near equilibrium}} C + D \quad \Delta F \simeq O.$$

that the system be some distance from equilibrium, hence less efficient. In other words, photosynthesis is such an efficient process that the small average amount of free energy change available per step in the process must be very economically used in order that the process may operate at the observed rate.

We have concluded that photosynthesis is a very efficient process, also a very complex one. It is therefore reasonable to examine any mechanisms that are put forth as the pathway of energy conversion during photosynthesis to see if these mechanisms are consistent with the idea of an efficient process. Some of the biochemical pathways that have been proposed in the past have been very inefficient and could almost be dismissed on these grounds without further consideration. For example it has been proposed that the formation of one molecule of adenosine triphosphate, a high energy phosphate from adenosine diphosphate and inorganic phosphate might require the utilization of one light quantum. The formation of high energy phosphate in this way requires something of the order of 10 kilocalories per mole. If this formation used up 40 kilocalories (equivalent to one einstein) obviously it would be only a 25% efficient step and could not find a place in an overall process as efficient as photosynthesis.

Photosynthesis involves several stages, each of which can be subdivided into many smaller stages. One usually thinks of photosynthesis as beginning with the absorption of light energy by some pigment, which may be chlorophyll, or one of the accessory pigments that absorbs light and then transfers that energy to chlorophyll. The second step is a conversion of this excited state of chlorophyll, or the energy of this excited state, into some more stable form of physical or chemical energy. While not much is known about this step we hope to suggest some possibilities later on. The next step involves the utilization of this packet of physical or chemical energy in stripping an electron from the oxygen of water—in other words oxidizing water and transferring this electron to some electron acceptor which we visualize as being a coenzyme or cofactor such as triphosphopyridine nucleotide. This cofactor is thus converted to its reduced form and is available for further biochemical reactions. In addition to triphosphopyridine nucleotide, some adenosine triphosphate must be made and we usually postulate that this is made by an electron transport system which operates between reduced TPN and the intermediate in the oxygen evolution.

The final stage in the process is the utilization of these cofactors, this energy-rich adenosine triphosphate and the reduced coenzyme which we believe to be triphosphopyridine nucleotide, in the reduction of carbon dioxide in the carbon-reduction cycle of photosynthesis. Now, we know the most about the last step, carbon reduction, so I will start there. The reason so much is known about carbon reduction is that there is available a technique for following the pathway of carbon through this rather complex sequence of intermediate compounds. This technique involves using a tracer element, C^{14}, as a marker for labeling the CO_2 which enters the cycle. This labeled CO_2 is allowed to be taken up by the intermediates in the photosynthetic cycle and for varying periods of time is transported through this sequence so that various intermediate compounds become labeled. The number of labeled intermediates and the degree to which they are labeled will depend upon the time of exposure of the plant to the tracer element. $C^{14}O_2$ enters into Compound A, Compound B,

$$3)\ C^*O_2 \text{——} A^* \text{——} B^* \text{——} C, \text{ etc.}$$

Compound C, and so forth and each compound is labeled. After a few seconds Compound A will be labeled; at a longer time Compound B will be labeled; at a still longer time Compound C, and so forth. In theory, then, all one needs is some method of analyzing for these intermediate compounds to see where the label is at various lengths of time after the initial exposure to CO_2.

The appropriate method of analysis was found in the use of two-dimensional paper chromatography. In this method the plants were quickly killed after a short period of photosynthsis by running them into alcohol. Extracts of the plant material were prepared and placed on the corner of a large rectangular sheet of filter paper, after which the chromatogram was developed in two dimensions with suitable solvents. In this way the various soluble metabolites were separated from each other. The location of radioactive compounds on the chromatogram was determined by radioautography and the identity of these compounds was established by means of various chemical tests. A typical radioautograph obtained from a unicellular plant, *Chlorella*, which had been exposed to $C^{14}O_2$ for 30 seconds is shown in figure 1.

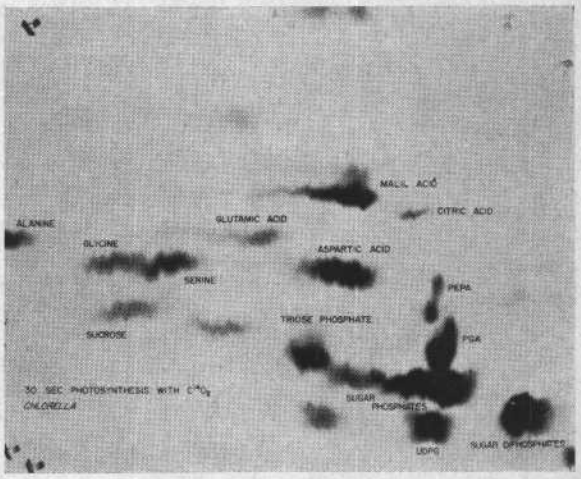

Figure 1.

The detailed development and application of these methods to the study of the path of carbon reduction in photosynthesis have been published (1). The more important types of study which were carried out by these methods include: 1) variation in carbon labeling of intermediate compounds as a function of time of exposure to $C^{14}O_2$, 2) location of carbon fourteen label within the molecules by chemical degradation of isolated compounds, and 3) changes in patterns of labeled compounds which accompanied changes in the environment of the photosynthesizing plant (i.e., light-dark transients, etc.).

As a result of the application of these methods the pathway of carbon reduction was worked out in most of its details and is shown as we now believe it to be, in figure 2.

The first step in this cycle is the carboxylation reaction in which CO_2 adds to ribulose-1-5-di-

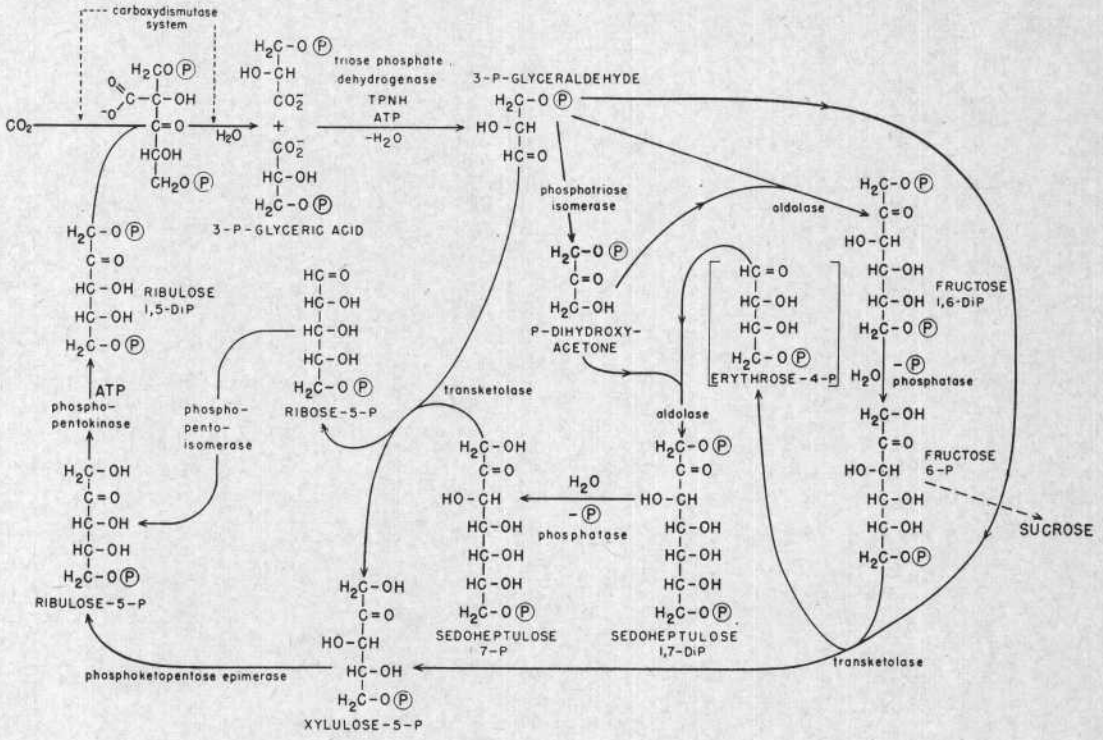

Figure 2.

phosphate. There is formed an intermediate compound in the carboxydismutase enzyme system shown between the dotted arrows, and this compound then splits with water to give two molecules of phosphoglyceric acid. These molecules of PGA are then reduced. The reduction requires both TPNH and ATP and leads to the formation of 3-phosphoglyceraldehyde which is a junction point for the divergence of pathways. Various sugar phosphate rearrangements then occur, and the ultimate result is that five molecules of triphosphopyridine are rearranged to give three molecules of pentose monophosphate. Another key reaction then occurs; this is the formation of the 1,5-ribulose diphosphate from ribulose 5 phosphate and this reaction requires adenosine triphosphate. We see, therefore, that the input of cofactors or energetic compounds to this cycle occurs at two places. In the reduction of phosphoglyceric acid with TPNH and ATP, one molecule of each of those cofactors is required for each molecule of phosphoglyceric acid reduced. So, for each molecule of CO_2 which enters the cycle, there is required at that point two molecules of TPNH and two of ATP. A third ATP is required for the formation of the ribulose diphosphate making the total requirement per CO_2 reduced equal to three molecules of ATP and two of TPNH.

We are now in a position to consider the energetics of the carbon reduction cycle. For each molecule of CO_2 reduced we may write the expenditure of energy as that provided by the hydrolysis of three molecules of ATP and the oxidation of two molecules of TPNH.

4) $3\ [ATP + H_2O \longrightarrow ADP + \text{inorganic phosphate}]\ \triangle F = -32$ Kcal.

5) $2\ [TPNH + \frac{1}{2}\ O_2 + H^+ \longrightarrow H_2O + TPN^+]\ \triangle F = -103$ Kcal.

The total energy expended by equations (4) and (5) is $\triangle F = -135$ Kcal., while you will recall from equation (1) that the total energy stored during the reduction of CO_2 to glucose was $\triangle F = +116$ Kcal. Therefore, there is something like 20 kilocalories actually expended in pushing

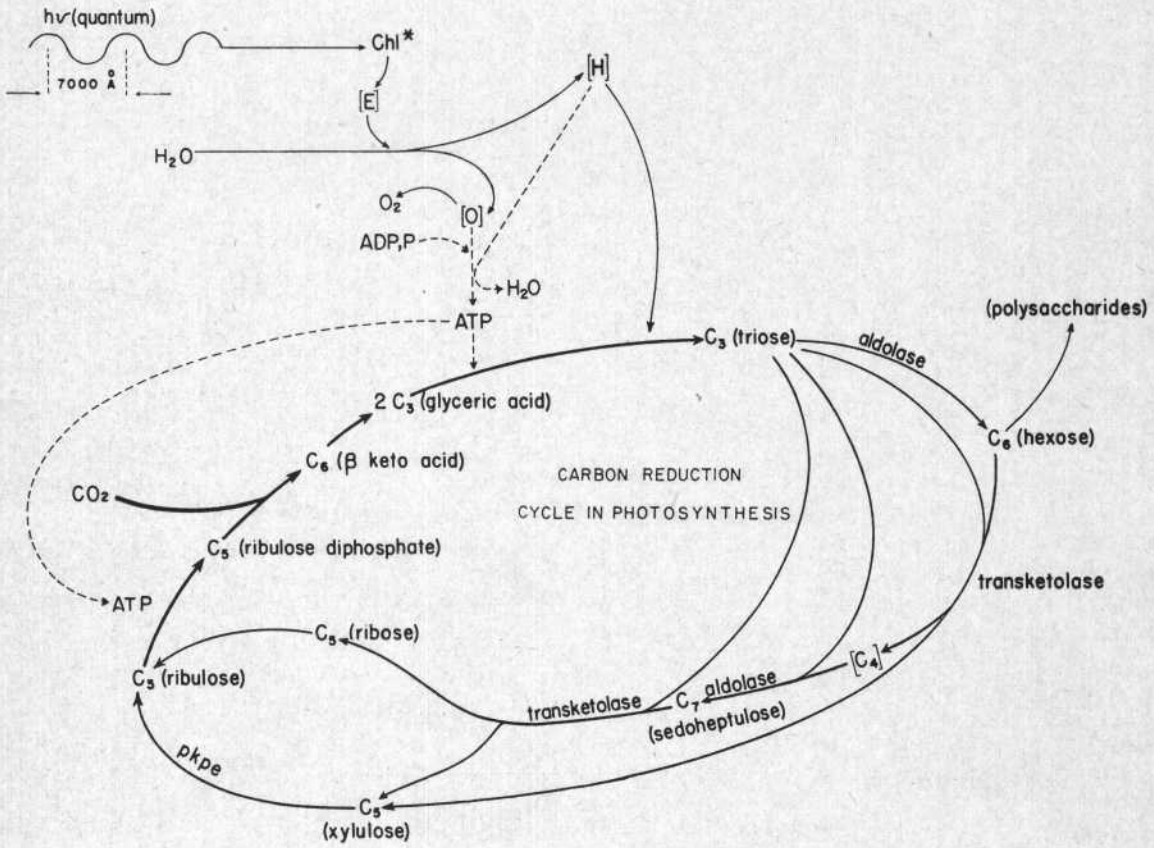

Figure 3.

the cycle around at a rapid rate. This is the energy that is used to drive the cycle. The rest of the energy, the 116 kilocalories, is all stored in the form of useful chemical energy. So we can say that the efficiency of the carbon reduction cycle is equal to $\frac{116}{135}$ or about 85%—truly a remarkable efficiency for such a complicated biochemical process.

Thus far the discussion has been taken up mostly with the carbon reduction cycle. As I said at the outset, we know the most about this aspect of photosynthesis. In figure 3 there is shown the relation between the primary photochemical reactions and the carbon reduction cycle. The carbon reduction cycle is shown in some detail because so much is known about it. The other stages, which are just as important, are shown as just a few dotted lines because so little is known about them. The first step in photosynthesis obviously is the absorption of light energy by chlorophyll and in some way this is used to produce chemical energy (E), which is then used to break an O-H bond of water and eventually to produce oxygen and hydrogen in some bound form. The bound oxygen may then undergo at least two fates. It may be liberated as oxygen gas or it may react with some of the hydrogen or reducing agent and this is a reaction which involves the liberation of energy. Light energy was used to separate oxygen and hydrogen. If they combine again there is liberated energy which can be employed with the formation of high energy phosphate from adenosine diphosphate and inorganic phosphate. The reducing agents plus ATP are then used to drive the carbon reduction cycle in the manner we have already indicated.

In the consideration of energy transfer in photosynthesis, I am going to pass over the intermediate stages entirely (the formation of ATP from TPNH, etc.), and discuss very briefly the possible mechanism of the primary process in photosynthesis; that is, the absorption of light energy and its conversion to some form of physical or chemical energy. In the past few years, it has become possible to bring some new techniques to bear on this problem. One of these was pioneered by Commoner (2), who used the electron paramagnetic resonance method to measure the spin of unpaired electrons and free radicals in biological materials. He found evidence that during the absorption of light by chloroplast preparations of one kind or another, there were created what appeared to be free radicals or unpaired electrons of some sort. The other new technique is the technique of studying delayed light emission or luminescence. This was pioneered amongst others by Strehler. (3) Dr. Sogo, in our laboratory is studying the electron spin resonance and free radicals (4) and Dr. Tollin is studying the luminescence. (5) Something about the nature of conversion of energy of excited chlorophyll may be indicated by the structure of the chloroplasts seen in figure 4. This is only one of many fine electron microscope pictures that have been produced by various workers. I simply show it here to recall to your mind the laminar structure of the chloroplast. This is a cross-section of the granum and you see here the layers. The interpretation of these layers is a matter of some discussion at this time. One sees many hypotheses in which there are shown rows of chlorophyll molecules, lipids, protein, etc. However, the interpretation of these pictures is not clear as yet. Nevertheless, one thing that appears certain is that there is a very highly organized structure at the molecular level. The distances between these layers is of the order of 50 or 60 angstroms and this is comparable to the size of chlorophyll molecules which are something like 15 angstroms on a side. We may speculate that these layers consist of proteins and lipoproteins with orderly arrays of chlorophyll molecules at the interfaces between the two different kinds

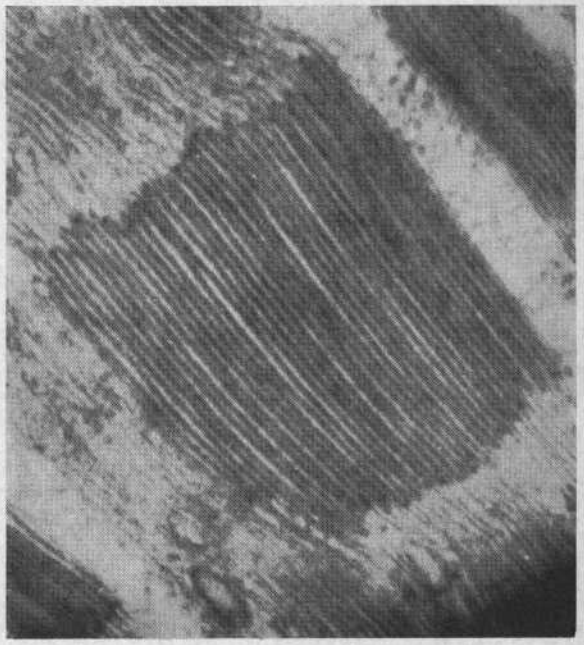

Figure 4. Granum (Maize), A. G. Vatter.

of biochemical substances. It seems reasonable that this ordered array has certain properties which do not exist for chlorophyll as individual molecules in solution. The absorption of light energy by the array of chlorophyll molecules may be followed by the transmission of light energy from one molecule to another. The array of molecules permits transfer of energy from one to another along one of these surfaces. Thus, the energy of excitation or the "exciton" as some people prefer to call it, migrates along this surface until it reaches some particular point at which (in our hypothesis) an ionization occurs. At some imperfection or different chemical species along the layer, ionization takes place, and the hole, which is simply the absence of the electron, moves one way and the electron the other.

This hypothesis is pictured schematically in figure 5. The lower left block represents the ground state of the energy levels of chlorophyll in which there are a number of energy levels very close to each other. The upper left block represents the state to which chlorophyll is excited upon absorbing a photon of light. Then several alternative things may happen. 1) The cholorophyll might lose the light energy as an emission, in which case there is a fluorescence. 2) The singlet energy state of chlorophyll might be transformed to another excited electronic state, most probably the cholorophyll triplet state, or 3) ionization may occur directly from the single state or from the triplet state. It is not known whether ionization from the singlet or the triplet state is more important. Once the ions are formed they may enter a conduction band. The excited states of these chlorophyll molecules are not completely filled with electrons as are the ground states. Consequently, whereas there is no conduction in the ground state, the excited states have many empty orbitals and it is possible for electrons and positive charges to move from one to another through this conduction band. In this way, perhaps, charges migrate along the two dimensional chlorophyll array until they reach some point at which they are trapped. We can think of the electron traps as some kind of oxidizing agent which becomes a reducing agent upon accepting an electron. The positive hole may be trapped in some way which permits it ultimately to react with water. Since it is a positive charge, it could remove an electron from water and form some intermediate in the evolution of oxygen. One cannot at present specify what that intermediate might be.

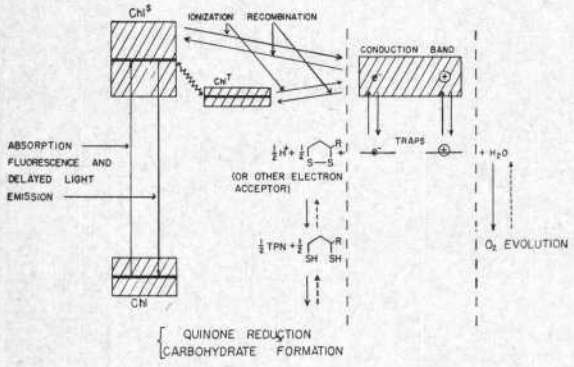

Figure 5. Proposed scheme for various photochemical processes in photosynthesis.

As for the nature of the primary reducing agent formed by the trapping of the electron, it has been proposed that this substance might be reduced to thioctic acid. (6) There has been no evidence to either dismiss or substantiate this hypothesis in the last three years. However, whether the electron reacts with thioctic acid or with TPN to produce a reduced cofactor doesn't make much difference energetically because these reduced cofactors have nearly the same energy carrying capacity.

In figure 6 is shown some of the experimental evidence which indicates the presence of unpaired electrons. This figure shows the electron spin resonance signals obtained upon shining light on chloroplasts prepared from spinach. If the scheme that we have just shown is correct, then on shining light on chloroplasts one should see the photochemical creation of some kind of signal indicating unpaired electrons. Here such a signal is seen as observed at 25° and at —150°. This figure is simply a plot of the absorption of energy as a function of a change in field strength. Now, it is possible to set the field strength to correspond to one of these peaks and then study the absorption of energy as a function of light, on and off. In figure 7 is shown the result of such a study. It can be seen that the signal increases rapidly both at 25° and at —150°. It is significant that when one turns the light off at 25°, the signal drops down immediately but at —150° the decay time is quite long. This indicates that whereas the rise of the signal is a photochemical process, more or less independent of temperature, the decay appears to be more in the nature of a chemical reaction which is affected by temperature. This data from the unpaired electron studies can be compared with luminescence studies in some respects.

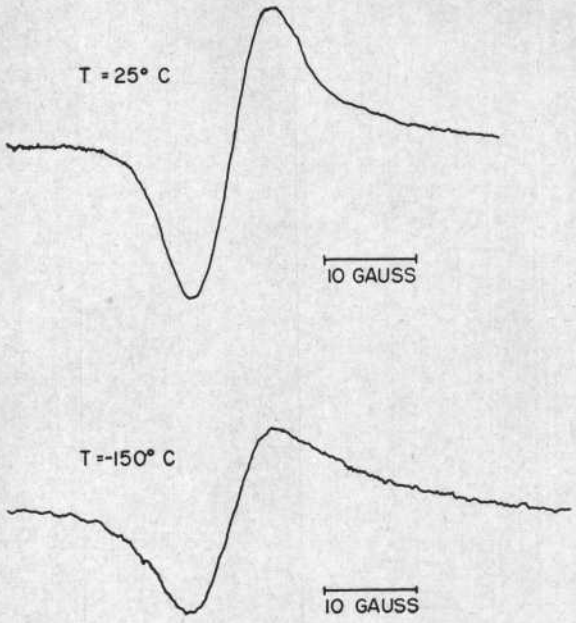

Figure 6. Light Signals from whole spinach chloroplasts.

The hypothetical scheme shown in figure 5 finds some support from the experiments on luminescence or delayed light emission from plants. In these experiments, plant material is subjected to brief flashes of intense light of selected wave lengths, after which the emission of light from the plant is followed as a function of both time and wave length. One of the findings from the experiment was that the emission spectrum corresponds to that of the chlorophyll singlet state rather than to the triplet state, so that the triplet state is not necessarily implicated as an intermediate in the energy transfer mechanism. This is an important conclusion from the standpoint of the efficiency of energy transfer in photosynthesis since, if ionization can be shown to take place directly from the singlet excited state or some state energetically close to the singlet state, then there need not be a loss of 5 to 10 kilocalories per einstein in the first stage of energy conversion, whereas such a loss would be experienced if the energy must be "passed through" the triplet excited state which is thought to be that much lower in stored energy than the singlet state.

Perhaps even more important to the hypothesis presented above is the experimental finding that some appreciable delayed light emission occurs at —70°C, once again indicating that energy may be stored in non-enzymatic chemical and physical processes (such as ionization) during the early stages of photosynthesis. It may be that this energy is stored in "trapped charges" as suggested in the hypothetical scheme and that only a small amount of thermal activation energy is required in order to reactivate these charges into the conduction band where they can recombine, leading to excited chlorophyll (singlet state) and luminescence. Besides supporting the hypothesis proposed for the early stages in energy transfer, this evidence speaks for energetically efficient transfer in these early stages.

If the picture we have presented is correct, the "traps" are probably not any deeper energetically than about 10 kilocalories. This means that of the some 42 kilocalories of energy that are absorbed per einstein of red light, no more than about 10 kilocalories are lost. In other words the process by which absorbed light energy is converted to the energy of these holes and electrons might be something like 75% efficient. With such an efficient process and with the efficient carbon reduction cycle discussed earlier, one can easily account for the overall efficiency of photosynthesis which is of the order of 50%, provided the conversion of reduced triphosphopyridine nucleotides to the oxidized forms which accompanies the formation of high energy phosphate is also a reasonably efficient process. If we may draw an analogy to the case of oxidative phosphorylation where it is possible to obtain at least three molecules of adenosine triphosphate for each molecule of TPNH oxidized, then we know that these intermediate processes are reasonably efficient and that the overall energy efficiency determined experimentally is consistent with the mechanism that we have presented.

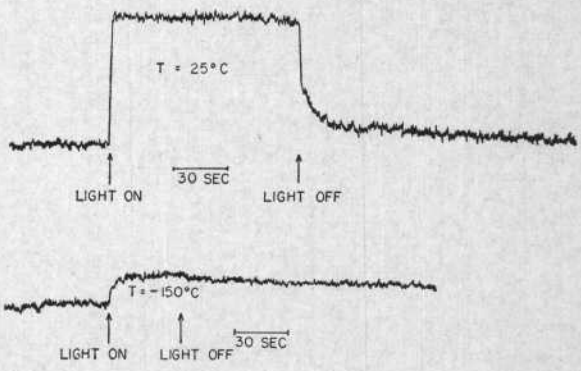

Figure 7.

DISCUSSION

Question: What about the strong label that you pick up in malic acid?

Dr. Bassham: Yes, well, we believe that the malic acid is, in fact, a product of carbon reduction in photosynthesis. It is not an intermediate in the carbon reduction cycle, which leads to carbohydrate but is most certainly an intermediate in the reduction of carbon dioxide to amino acids. The label, I think, is indicative of a second carboxylation, of probably pyruvic acid or phosphopyruvic acid which leads ultimately to malic acid. The formation of malic acid is greater in the light than in the dark so that some photochemical energy is employed there and this leads in turn to aspartic acid and to other various substances which can be made from the tricarboxylic acid cycle, such as glutamic acid. So, we definitely consider it to be an intermediate, although not an intermediate in the formation of sugars.

Question: Do you have any information concerning the relative amounts of light energy going to CO_2 reduction and to photophosphorylation in the normal plant?

Dr. Bassham: Well, we would suppose that the amount that goes to direct reduction, the amount of TPNH used up directly, as compared with that which is required for ATP formation, is simply relative to the amount required. Now, for the carbon reduction cycle which leads to sugars, we've already indicated that we need two TPNH's equivalent to 102 kilocalories and three ATP's to about 30 so it is about three to one in favor of the direct conversion of TPNH to reduced carbon. Certain other compounds such as amino acid and fats require different proportions of ATP and TPNH. You can calculate for each of these what this proportion might be.

Question: Why do you use aldolase in your scheme for the seven carbon sugar and not transaldolase?

Dr. Bassham: The answer to that is that there is really no strong preference for one or the other. I have seen no particular evidence that would indicate that it would have to be transaldolase. Actually, we originally proposed aldolase and we see no reason to abandon that proposal. There is the evidence that sedoheptulose diphosphate is formed and sedopheptulose diphosphate, of course, would be formed by the aldolase reaction. You could argue, though, that it is a side reaction which is not an actual intermediate. Also, it turns out that if you count up the number of steps in the carbon reduction cycle it is somewhat more economical to use the aldolase reaction rather than to make another hexose molecule and use the transaldolase. But these are all arguments which can be disputed. There is simply no way to decide at this time.

Question: Could you say anything concerning your intermediate compound and Kamen's unknown compound found in *rhodospirillum rubrum?*

Dr. Bassham: I am not sure what intermediate compounds the questioner had in mind, and I don't believe that I could answer this since I am not familiar with Kamen's compound. Does anyone here know more about this? If not, I'll just have to pass that one by.

Question: What is the relation of 3-PGA to 2-PGA of CO_2 fixation?

Dr. Bassham: Now, this is rather easy to answer. Three PGA is the carbonated product of CO_2 fixation, we believe, simply because of the mechanism of the carboxylation reaction shown, in which splitting of the molecules of the unstable keto acid intermediates would lead to two molecules of 3-PGA. Two PGA is, of course, formed directly from 3-PGA; not in the cycle leading to sugars, but in the path leading to 2-PGA, to phosphorpyruvate, and then via carboxylation to malic, aspartic, and other substances. It is also an intermediate in the formation of alanine, probably from 3-PGA.

Question: You said there was no evidence to show that thioctic acid is not involved in the light reaction. Surely arsonite doesn't inhibit the light reaction.

Dr. Bassham: Well, I might say about these inhibitation reactions that sometimes they are a bit misleading. Now, it has been reported, for example, that p-chloromercuro-benzoate which might be expected to inhibit SH compounds, did not inhibit certain phases of the Hill reaction. I believe Arnon and his workers mentioned this at one time. However, they found later on, I believe, if I am not misquoting them, that the stoichiometric formation of reduced triphosphopyridine nucleotide in the Hill reaction is inhibited by p-chloromercuro-benzoate. Now, it is sometimes possible to bypass these inhibitors. For instance, you may be able to get some type of Hill reaction which accepts electrons directly from the photochemical reaction without going through thioctic acid, perhaps, if that is an intermediate. I don't know whether it is or not. And as far as arsonite is concerned, I don't know what levels of arsonite

might be required to inhibit photosynthesis, but I wouldn't take such negative evidence as positive proof that thioctic acid is not an intermediate in photosynthesis, because I have been misled by these inhibitions before, particularly negative ones.

Question: I understand that this bacterial photosynthesis occurs at wave lengths of light out at 9000°A. and at that wave length there is only about three-fourths as much energy as that available to green plants, and maybe it is not enough energy to split water. Would you agree with that?

Dr. Bassham: Yes, absolutely, and the only reason you can set a potential limit on green plant photosynthesis is that you can recognize the two end products. You can recognize oxygen, you can recognize carbohydrate, and with those as end limits, you can set up energy requirements. However, you can't necessarily do that in the case of bacteria, because you don't know at what potential the oxidizing power is being utilized. This is one other point. The requirement for the oxidizable substrate can be visualized in two ways: one is just satisfying the photochemical OH, and the other is that the bacteria get energy by oxidizing the substrates with the photochemical OH. The bacteria will also take advantage of this reaction and will gain energy as when they oxidize the hydrogen sulfide in the case of the sulfur bacteria, or the organic hydrogen donors in the case of the athiorhodacciae.

Question: What do you use to determine your density and take into account . . .

Dr. Bassham: This is a function that the primary process involves, probably the transfer of one electron per photon absorbed. That is my first hypothesis. Of course, there is a total of four electrons transferred per CO_2 reduced. Then, secondly, we get the figure for the energy from the longest wave length of light that can be utilized in photosynthesis, which is something like 6800 angstroms, and this turns out to be about 42 kilocalories per mole or 1.8 electron volts per electron. Does that answer your question?

Question: Yes, but it sounds kind of loose to me, the whole thing does, because the method that you ordinarily use to measure quantum yield would be this: you would have to know the size of the quantum, the magnitude; then you would have to know the intensity of light; and then you would have to know the amount of light absorbed versus scattered and reflected.

Dr. Bassham: This is a very complex business, measuring quantum requirements. I have played around a little bit with this and other people have worked on it a lot more, but you do have to do exactly that. You have to may your light field, you have to know how much light is scattered, and you have to have a more or less monochromatic light source. In other words, you need to know the wave length of the light.

Dr. French: Can I put in a word on that subject?

I don't think that the difficulties are in the physical measurements; they are troublesome, but they have been solved. The difficulty is in the biological material; that is, it varies all over the place. I think one reason that these quantum yields vary so much is that the ratio of active to inactive chlorophyll is different in different plants and in the same plant at different times. That's a simple-minded explanation, but I think it will cover some of the discrepancies.

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Bacterial Photosynthesis

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I would like to use the time allotted to me today to present the general aspects of bacterial photosynthesis and to give some recent data concerning experiments conducted on the bacterial photosynthetic mechanism. In terms of both economic importance and research effort expended, the photosynthetic process in bacteria falls far short of plant photosynthesis. Since plant photosynthesis has been most extensively investigated, I would like to use it as a departure point in discussing the process that occurs in bacteria.

In a general sense, the photosynthetic process in plants can be divided into the following four phases:

(1) The primary photochemical act whereby light is absorbed by the chlorophyll contained in orderly form in the chloroplast. This absorbed energy is then expressed by the chloroplast as chemical energy in terms of an oxidizing component and a reducing component. These are conveniently expressed as [OH] and [H] respectively, representing a photolysis of water.

(2) Utilization of the oxidizing component to produce oxygen.

(3) Utilization of the reducing component to ultimately reduce carbon dioxide to sugars.

(4) A recombination of the reducing and the oxidizing components via an electron transport system contained in the chloroplasts. This process is accompanied by the incorporation of inorganic phosphate into adenosine triphosphate (ATP). This process is designated photophosphorylation.

The first reaction, the photochemical act, involves the absorption of light to produce excited chlorophyll molecules, and the resultant shifting of electrons results in the production of the oxidizing and the reducing powers characteristic of photosynthesis. The other three reactions are biochemical reactions not dependent upon light, and save for the reactions leading to the evolution of oxygen, the enzymes involved are not much different from the enzymes encountered in ordinary respiratory pathways.

In considering the photosynthetic process in bacteria, let us first consider the four general parts of the photosynthetic apparatus in plants for comparison purposes. To those acquainted with bacterial photosynthesis, it is obvious that there is considerable similarity in the two processes. This was recognized very early by van Niel, and from comparative biochemical reasoning he advanced a general scheme for bacterial photosynthesis (1). With the more recent information available about the process, I would submit that except for phase 2 above, the bacteria perform a photosynthesis that in its general outline is very similar to plant photosynthesis.

Figure 1. Schematic representation of bacterial photosynthesis.

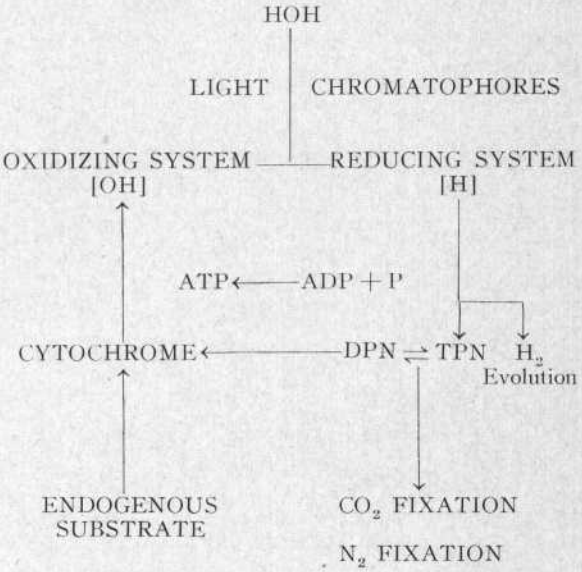

Figure 1 is a representation of the bacterial photosynthetic process. The bacterial chlorophyll system absorbs light, using the light to produce both an oxidizing component and a reducing component. The reducing component can be used for cellular reductions, including the reduction of carbon dioxide to sugar. The reducing component and the oxidizing component can be linked through a series of enzymatic reactions to yield ATP, through the process called photophosphorylation. In these reactions, the bacteria are similar to the

TABLE 1. CHARACTARISTICS OF PHOTOSYNTHETIC BACTERIA

Organism	Color	Chlorophyll type	Substrate required
Green bacteria (*Chlorobium*) ..	Green	Chlorobium chlorophyll	Inorganic sulfur compounds
Purple sulfur bacteria (*Chromatium*)	Purple to red	Bacterio-chlorophyll	Inorganic or organic compounds
Purple nonsulfur bacteria (*Rhodospirillum rubrum*) .... (*Rhodopseudomonas spheroides*)	Purple to red or brown	Bacterio-chlorophyll	Organic compounds plus vitamins

plants, but they do differ in their use of the oxidizing component. Whereas plants evolve oxygen, this reaction has never been observed in any photosynthetic bacterium. Consequently, the oxidizing power generated photochemically partially goes to accomplish oxidation of cellular components. This represents the main difference between plant and bacterial systems.

Table 1 presents some of the general characteristics of the three types of photosynthetic bacteria, the green sulfur, the purple sulfur, and the purple nonsulfur bacteria. The sulfur bacteria are autotrophic bacteria, since they can grow on inorganic media in the presence of light, while the nonsulfur bacteria are heterotrophic in the presence of light, requiring organic molecules for substrate and also a variety of vitamins for maximum growth.

I would like now to consider the four major divisions of the photosynthetic process separately, comparing bacterial and plant systems, and to present some new information concerning certain phases of bacterial photosynthesis.

The Primary Photochemical Act

Isotopic data obtained with plants, using water with oxygen-18, have led to the conclusion that the direct chemical result of the absorption of light quanta is a formal splitting of water to yield an oxidizing component and a reducing component. The oxidizing component is represented as [OH], because it would be the oxidation level of a peroxide, while the reducing component can be represented as [H]. There is some question as to the conclusiveness of the isotope experiments (2), but in general the splitting of water in the plant system is accepted. When one turns to the bacterial system, however, the tool used to examine the plant system is no longer available, since oxygen is not given off during bacterial photosynthesis. Consequently, there is no direct evidence to indicate that water is split during bacterial photosynthesis, and the inclusion of water splitting in schemes of bacterial photosynthesis is based on comparative grounds. It is evident that the bacterial system does generate an oxidizing power and a reducing power in a manner similar to that of green plants, and in terms of the ultimate fate of the oxidizing and the reducing powers, it is acceptable to think of the oxidizing and the reducing powers as being equivalent to the [OH] and [H] generated in plant photosynthesis.

Duysens *et al* (3) have examined the spectroscopic changes occurring in *R. rubrum* cells upon illumination, and have found reversible changes which can be correlated with an oxidation of the bacteriochlorophyll by light. This oxidation would concomitantly produce a reduced component, XH, and the oxidized chlorophyll could then interact with cytochromes to produce the photooxidations in the bacterial cell. This production of an oxidizing power and a reducing power by the chlorophyll could be due to the removal of an electron from a "conduction band" in the organized chlorophyll molecules, with the simultaneous production of a hole."

A summary comparison of the structure of the photosynthetic apparatus in both plants and bacteria reveals considerable similarity, and lends support to the hypothesis that the primary photochemical act in both cases is very similar. Table 2 compares plant and bacterial photosynthetic apparatus. In plants, only one of the chlorophylls found in the chloroplast, chlorophyll *a*, is active in joining the light energy to biochemical systems. In bacteria, from the information available, the cell may contain more than one bacteriochlorophyll, but only one is active in the transfer of light energy to biological systems (4). Thus, in *Rhodospirillum rubrum*, there are several forms

TABLE 2. COMPARISON OF PLANT AND BACTERIAL PHOTOCHEMICAL SYSTEMS

	Bacteria	Plants
Photosynthetically active pigment	Bacteriochlorophyll or Chlorobium chlorophyll	Chlorophyll *a*
Accessory pigments	Carotenoids	Cartenoids
Location of photosynthetic apparatus	Chromatophores, .03 to .06 microns in diameter	Chloroplasts 3 microns diameter
Quanta required to reduce CO_2 to level of carbohydrate	10 (*Chlorobium thiosulfatophilum*) 8.5 - 13.5 (*Chromatium*) 5 (*Rhodopseudomonas capsulatus*)	6 to 8

of bacteriochlorophyll present in the cell, as shown by spectroscopic data, but only one of these forms is active in linking the light system to the biochemical system. Species of the green sulfur bacteria contain a chlorophyll distinct from both bacteriocholophyll and chlorophyll *a*, for which the term *chlorobium* chlorophyll applies (5). The structure of *chlorobium* chlorophyll is not known, but the bacteriochlorophyll found in the purple bacteria is a tetrahydro porphyrin, containing two more hydrogens than does plant chlorophyll (1).

For both plants and bacteria, the photo synthetic apparatus is contained in a particle within the cell. The chloroplast of plant cells is relatively large, with an average diameter around 3 microns. The bacterial analogue is the chromatophore, which contains all the photosynthetically active pigment with associated enzymes, but is understandably much smaller than the chloroplast. The chromatophores of *R. rubrum* have been estimated at .06 microns in diameter (6). Contained in the plant chloroplast and the bacterial chromatophore are accessory pigments and carotenoids. In some fashion these accessories transfer absorbed light energy to the active chlorophyll form (4). The exact mechanism of transfer is not known, but a system of "inductive resonance" has been advanced as one possibility (4).

One point speaking highly for similarity of mechanism in both plant and bacterial photosynthesis is a general agreement in the number of quanta required in each system to reduce a carbon dioxide molecule. The reported quantum numbers for plant photosynthesis vary a great deal, but the majority fall between 6 and 8 quanta per carbon dioxide. Similar type experiments with bacteria yield an average quantum number of 10 for *Chlorobium thiosulfatophilum*, and this value is independent of the substrate used (7). Experiments with *Chromatium* gave values from 8.5 to 13.5 using thiosulfate as the substrate (8), while *Rhodopseudomonas capsulatus* gave a value of 5 (9). It should be pointed out that whereas plants liberate oxygen with the oxidizing power, bacteria oxidize substrate molecules such as sulfide, thiosulfate, pyruvate, etc. On a comparative basis, the bacteria are far less efficient in utilizing the absorbed light energy even though the quanta absorbed per carbon dioxide assimilated may be quite similar.

Photophosphorylation

It has been demonstrated that both *Chromatium* (10) and *R. rubrum* (11) chromatophores are capable of forming ATP from ADP and inorganic phosphate when they are illuminated. This further points up the similarity of plant and bacterial systems, since plant chloroplasts also carry out an active photophosphorylation. The incorporation of inorganic phosphate into ATP undoubtedly results from oxidation-reduction reactions occurring in an organized electron transport system in the chromatophore or chloroplast, with the initial reducing and oxidizing powers being supplied by the chlorophyll systems.

The photophosphorylating system in plants is stimulated by the addition of magnesium ions, riboflavin monophosphate, vitamin K, ascorbate, phenazine methosulfate, TPN, and extractable factors from the chloroplasts (12,13). Pyridine nucleotide reductase, the enzyme required to couple the photochemical system with TPN reduction, also stimulates the reaction since it is a component of the system. The above requirements are understandable in terms of the electron transfer system operative in mammalian respiration with the possible exception of ascorbate. There is some doubt whether ascorbate does have a physiological function in the photophosphorylation pro-

TABLE 3. PHOTOPHOSPHORYLATION IN PLANTS AND BACTERIA

Organism	Reference	Rate	Activators
R. rubrum	11	20 μmoles/hr/mg protein	DPNH succinate
Chromatium	27	70 μmoles/hr/mg chlorophyll	Phenazine methosulfate, ascorbate
Spinach chloroplasts	28	500 μmoles/hr/mg chlorophyll	FMN, ascorbate, phenazine methosulfate, soluble factors.

cess since it may be acting as a shunt in the isolated chloroplasts.

The photophosphorylation system in *R. rubrum* chromatophores is stimulated by the addition of DPNH, magnesium ion, and succinate (11). The system from *Chromatium* is stimulated markedly by the addition of phenazine methosulfate and to a less extent by ascorbate. Flavins inhibit the reaction (10), as does dinitrophenol and the couple of 2,6-dichlorophenolindophenol and ascorbate. Thus we see that the photophosphorylation requirements do differ in some respects between plants and bacteria. Table 3 compares some basic properties of the plant and bacterial systems regarding the rate of the phosphorylation reaction. If the data for *R. rubrum* were placed on the chlorophyll basis, the rates of photophosphorylation in bacteria would be comparable to the rates obtained with plant material. It would be very interesting to have the quantum yield of photophosphorylation determined, to see how it compares with the quantum requirements for carbon dioxide fixation.

Reducing System

Experiments conducted with whole cells show that the photosynthetic bacteria are capable of producing a reducing power under the influence of light. This is best exemplified by the photoproduction of hydrogen from *R. rubrum* cells, (14). Since *R. rubrum* requires the presence of an organic acid for hydrogen evolution, it is possible that the hydrogen comes from the organic acid, perhaps by the intermediate formation of formate which is decomposed by a hydrogenlyase system in the bacteria to yield hydrogen gas. However, investigations concerning the source of the liberated hydrogen have indicated that it probably does not arise from formate via the hydrogenlyase system, and most probably comes from the reductive system produced in photosynthesis. (14,15). The fact that light inhibits the formate decomposition into carbon dioxide and hydrogen by *R. rubrum* cells would support this assumption (15). To my knowledge, no definite isotope experiments to show the source of the evolved hydrogen have been done. If the experiments were performed with deuterium labeled substrate and no label appeared in the hydrogen evolved, it would show that the organic substrate molecules could not give rise to the hydrogen. If the label did appear, the experiment would not be decisive.

Although there is no direct proof that nitrogen is fixed in the photosynthetic bacteria by utilizing the reducing power produced photochemically, it appears probable that this is so since nitrogen gas serves to inhibit the photoproduction of hydrogen gas (15). This could easily arise by a competition of the hydrogenase and nitrogen-reducing systems for the photochemical [H]. More definite conclusions concerning both photoproduction of hydrogen and nitrogen fixation must await a reproduction of these processes in cell-free systems, a feat not yet accomplished.

In the case of plants, a demonstration of the photoreducing power is quite simple. Isolated chloroplasts dispose of the oxidizing power in the process of oxygen evolution, leaving behind in the chloroplasts a net reducing power, which is readily detected in terms of reduction of dyes, quinones, and pyridine nucleotides. For bacteria, however, such is not the case since the entirety of both the oxidative and reductive systems must be accomodated within the cell. Accordingly, repeated attempts over a number of years by myself and other investigators have not yielded any evidence of the photoreductive system in cell free systems of photosynthetic bacteria. To secure such evidence the photochemical oxidizing system should be satisfied and the possibility of a back reaction of the oxidative and reductive systems made as small as possible. Recent experiments with cell free extracts of *R. rubrum* by Dr. M. Kamen and myself (16) showed such extracts could oxidize ascorbic acid if the dye 2,6-dichlorophenolindophenol was also present. We offered as a possible

TABLE 4. DPN PHOTOREDUCTION COUPLED TO LACTATE FORMATION

No. of experiments	Average Lactate Content in μMoles			
	Zero time	Light	Dark	Light-Dark
5	.47	.72	.70	.02 (.01-.05)*

* These values represent the range covered.

mechanism for this reaction an oxidation of the ascorbate via the photochemical [OH] system. With this in mind, and borrowing some information and material from the plant photosynthetic process, an attempt was made to demonstrate a photoreduction of pyridine nucleotides by chromatophores of *R. rubrum* using the ascorbate-dye couple to react with the photochemical [OH].

The enzyme which transfers electrons (or hydrogens) from the cholorophyll system in chloroplasts to pyridine nucleotides has been shown to be operative in photophosphorylation of chloroplasts, and has been extensively purified by Dr. A. San Pietro. This enzyme was prepared from spinach leaves in our laboratory according to directions furnished by Dr. San Pietro, and was used to link the photoreductive system of chromatophores with pyridine nucleotides.

To demonstrate DPN photoreduction, a system was prepared containing *R. rubrum* chromatophores; the pyridine nucleotide reductase from spinach to couple the chlorophyll system with the pyridine nucleotides; pyruvate and crystalline lactic dehylrogenase to remove any DPNH as it was formed; ascorbic acid and 2,6-dichlorophenolindophenol to interact with the photochemical [OH] system; and buffer to keep the pH at 7.0. The results of an extended series of such experiments were disappointing since it was not possible to obtain more than an indication that any DPNH was being formed in the system as demonstrated by lactate formation. Results of a series of runs are given in table 4, showing that there was only slight preferential formation of lactate as a function of illumination, and it is questionable if any significance should be attributed to the slight differences obtained.

TPN was added to one experiment designed to detect DPN photoreduction, and it was observed that in this case there was a definite small amount of lactate formed. Accordingly, the possibility that the photochemical reducing system might be reducing TPN was investigated by substituting TPN for DPN in the test system, using oxidized glutathione and glutathione reductase to remove TPNH as it was formed. Thus, TPNH formation would be evidenced in terms of reduced glutathione formation as a function of illumination of the chromatophores. Results of a series of such experiments are given in table 5. From these results it is seen that TPN can indeed be photoreduced by chromatophores of *R. rubrum* and it illustrates the preference of the photoreducing system for TPN. It should be mentioned that in the experiments represented here the ascorbate-2,6-dichlorophenolindophenol couple was not included since it was shown that the extent of reduction was greater in their absence. This effect of ascorbate can be explained in terms of reoxidation of the reduced glutathione by dehydroascorbic acid via the enzyme dehydroascorbic acid re-

TABLE 5. COMPONENT STUDY FOR PHOTOREDUCTION OF TPN BY *R. rubrum* CHROMATOPHORES. THE EXPERIMENTAL SYSTEM CONTAINED 20 μMOLES PHOSPHATE BUFFER pH 7.2, .60 μMOLE TPN, 40 μMOLES OXIDIZED GSH, 4 mg. of GSH REDUCTASE, 4 mg. OF PYRIDINE NUCLEOTIDE REDUCTASE, AND *R. rubrum* CHROMATOPHORES WITH CHLOROPHYLL CONTENT AS DESIGNATED IN A FINAL VOLUME OF 3.2 ml. EXPOSURE TIME WAS TWO HOURS. FOR THE HEATED ENZYME PREPARATIONS, A TWO MINUTE PERIOD IN A BOILING WATER BATH WAS USED, EXCEPT FOR THE CHROMATOPHORE PREPARATION AS INDICATED.

Conditions	Micromoles GSH present		
	Light	Dark	Light -Dark
Experiment 1			
Complete system, .24 mg. chlorophyll	2.38	1.50	.82
Add Ascorbate (20 μmoles).....	1.25	1.08	.17
Experiment 2			
Complete system, .20 mg. chlorophyll	1.81	.85	.96
Minus TPN	.30	.33	−.03
Minus oxidized GSH	.13	.20	−.07
Heated chromatophores (2 mins.)	1.12	.55	.57
Heated chromatophores (30 mins.)	.46	.32	.14
Heated GSH reductase	.48	.53	−.05
Heated pyridine nucleotide reductase	.68	.72	−.04
Experiment 3			
Complete system, zero time .14 mg. chlorophyll	.25	.24	.01
Complete system after two-hour illumination	1.17	.61	.56

ductase. Thus, nothing was added to the system to accomodate the photochemical [OH] system and still the photoreduction of TPN proceeded. The question arises, if an equivalent oxidation occurred, what was being oxidized? The answer to this question is not known. However, it must be that some endogenous product contained in the chromatophores is being oxidized. To some degree this resembles the experiments of Vishniac (17) in which the chlorophyll from plants was recombined with a protein fraction to yield a protein-chlorophyll complex capable of photoreducing TPN in a coupled system with GSH and glutathione reductase. In Vishniac's experiments the nature of the oxidized product also is not known.

The experiments with *R. rubrum* chromatophores were performed in specially adapted Thunberg tubes under strictly anaerobic conditions because the photoreduction of TPN could not be observed in the presence of oxygen.

A series of experiments was performed to see if it might be possible to increase the TPN photoreduction rate by adding some hydrogen donor to the system to interact with and satiate the photochemical [OH] system. In this regard, ferrocyanide, hydroquinine, pyrogallol, epinephrin, paraphenylenediamine and DPNH were tested, but none of these compounds activated the system. Instead, these compounds inhibited photoreduction of TPN, with the inhibitions ranging from 50 to 100 percent.

A series of experiments was performed with the system geared for TPN photoreduction, to see if it might be possible to inhibit the recombination of the photochemical [H] and [OH] systems via the photophosphorylation system, and thus enhance the net production of TPNH. The inhibitors tested were amtimycin A and sodium amytal, known inhibitors of the electron transport system operative in oxidative phosphorylation. However, both compounds caused a decrease in the amount of TPN photoreduced.

Several compounds which stiumlate the process of photophosphorylation were tested in the present system. FMN, menadione, and phenazine methosulfate all caused a decrease in the amount of TPNH formed. However, in terms of their effect in stimulating a recombination of the [H] and [OH] system via photophosphorylation, these results were expected.

The observed photoreduction of TPN is the first direct evidence in a cell free system that the photosynthetic bacteria do create a reducing power under the influence of light. In view of other known biological reactions, the TPNH so produced could then go to reduce carbon dioxide, produce molecular hydrogen, or function in other cellular reductions such as nitrogen fixation. The fate of the TPNH in the whole cell, of course, would depend upon the metabolic condition of the cell. In the case of the photoautotrophes, the TPNH would largely go to reduce carbon dioxide to form cellular materials. For the photoheterotrophes such as *R. rubrum*, the TPNH could go for either carbon dioxide reduction in the presence of substrates of lower oxidation state than carbohydrate or for nitrogen fixation and hydrogen formation depending upon needs of the cell. It should be stressed that the preference for TPN in the bacterial system described here may not reflect the true picture in the chromatophore. The reaction mixture contained a pyridine nucleotide reductase prepared from spinach, and the TPN specificity may be imparted by this enzyme.

Oxidative System in Photosynthetic Bacteria

Relatively little is known about the fate of the oxidative system in photosynthetic bacteria. From the stoichiometry of substrate disappearance by illuminated bacteria, it is apparent that the substrate does undergo a net oxidation. This is best exemplified for the sulfur bacteria; the early paper of van Niel (1) gives data on sulfide disappearance and appearance of sulfate in the case of the sulfur purple bacteria. Under the influence of light the sulfide is completely oxidized to sulfate under anaerobic conditions, and the only obvious explanation for this phenomenon is an oxidation by the photochemical [OH] generated in the chromatophores.

Direct evidence for photochemical oxidations occurring in the bacterial cell comes from the investigations of Chance and Smith (18), who used the double beam spectrophotometer developed by Chance to measure the changes in absorption spectra of *R. rubrum* as a function of aerobicity and illumination. It was shown that under the influence of light, the cytochrome components within the bacterial cell became more oxidized. In this regard, the bacteria resemble algae, wherein the cytochrome system also becomes more oxidized under the influence of light. In the light of known biochemical reactions, the logical compounds to mediate in the cellular oxidations effected by light would be cytochromes, since they also act as terminal members of the regular respiratory pathway. The presence of cytochromes in photosynthetic bacteria, including those which live exclusively

under anaerobic conditions, has been previously shown (19, 20).

In the case of the cytochrome c from *R. rubrum*, exposure of chromatophores of this organism to the reduced cytochrome c results in a rapid photochemical oxidation of the reduced cytochrome (21). This would afford excellent evidence for the photochemical [OH] system if it were not for the fact that the reaction requires the presence of air. Under anaerobic conditions the reaction is stopped, and accordingly it is questionable if this can represent the photochemical oxidations taking place in the bacterial cell, since the photooxidations catalyzed by the cell can take place under anaerobic conditions. It should be remembered, however, that in this case also, one should only be capable of demonstrating a net oxidation if the photochemical reducing system were accommodated in some fashion to prevent its back reaction with the photochemical [OH] system. Since oxygen can react with the photochemical reducing system of plant chloroplasts (22), the oxygen requirement for the photochemical oxidation of cytochrome c by extracts of *R. rubrum* could be explained in terms of the oxygen interacting with the photochemical reducing system. However, it may well be that the oxygen serves in a photochemical oxidation by oxygen itself, which reaction could be catalyzed by the bacteriochlorophyll.

In conclusion, I would like to mention briefly some experiments designed to gain a little more information about the nature of the photochemical [OH] system. It is well known that in the case of photoheterotrophes capable of performing regular aerobic oxidations in the dark, illumination causes a marked decrease in the rate of oxygen uptake (1, 23). One obvious explanation of this phenomenon is that there is a competition between photochemical [OH] and molecular oxygen for the oxidizable substrate in the bacterial cell. Thus, in the presence of light the oxidations would be performed using the photochemical [OH] system, and oxygen would not be required. An alternative explanation for the phenomenon would involve a strict interdependence of oxidation and phosphorylation in the chromatophore.

It is well known that in the case of mammalian mitochondria, where phosphorylation and oxidation processes are intimately linked, the rate of oxidation is controlled by the phosphorylation system. In the absence of phosphate acceptors in this system neither phosphorylation nor oxidation will proceed. If the same condition existed in the case of chromatophores, and the rate of aerobic oxidation was controlled by the level of phosphate acceptors present, such as ADP, one can explain the light effect on oxygen uptake as being due to a depletion of phosphate acceptors as a result of photophosphorylation. Since the chromatophores of *R. rubrum* do carry out a rapid photophosphorylation and also have a moderately good DPNH oxidase activity, it was thought a series of experiments with *R. rubrum* chromatophores could test the latter hypothesis.

R. rubrum chromatophores were tested for their DPNH oxidase activity after illumination for a 5-minute period. It was found that in the majority of the preparations tested, there was a light-induced inhibition of the DPNH oxidase activity. Figure 2 shows the DPNH oxidase assay

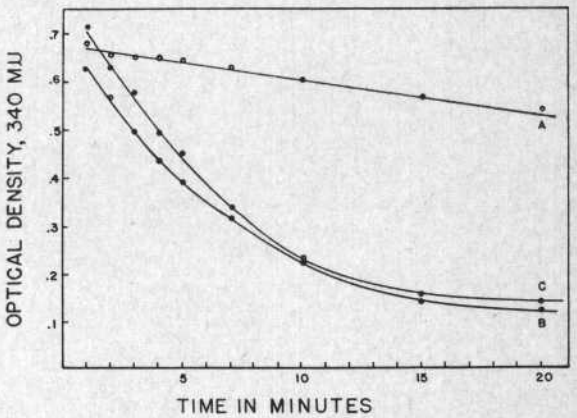

Figure 2. Effect of illumination on the DPNH oxidase activity of *R. rubrum* chromatophores. Curve A, illuminated chromatophores. Curve B, non-illuminated chromatophores. Curve C, illuminated system with chromatophores replaced by non-illuminated chromatophores.

for a preparation which showed good differential activity following illumination. In figure 2, curve A represents the illuminated system, curve B represents the non-illuminated system, while curve C represents a system which had been illuminated and had the chromatophores removed by centrifugation, after which fresh non-illuminated chromatophores were added. This showed there was no inhibitor produced in the medium by the illumination.

At first glance, the inhibition of the DPNH oxidase activity would indicate that light inhibition of oxygen uptake by whole, respiring *R. rubrum* cells could not be due to a competition between oxygen and photochemical [OH] for oxidizable substrate, since light should either have no effect

on the rate of DPNH oxidase activity, or, more probably, should increase the rate at which DPNH is oxidized. It was felt that this indicated that the light effect on whole cells, regarding their oxygen uptake, was the result of a depletion of available phosphate acceptors through the process of photophosphorylation. However, when this possibility was tested by adding back to the system various possible phosphate acceptors such as adenosine diphosphate, guanosine diphosphate, inosine diphosphate, uridine disphosphate, and cytidine diphosphate, these compounds failed to overcome the light-induced inhibition. Therefore, the explanation of the phenomenon in terms of lack of phosphate acceptor appears untenable.

Other compounds were tested with the light-inhibited *R. rubrum* chromatophores, to see if the inhibition could be overcome. It was found that the inhibition could be overcome, in order of decreasing effectiveness, by 2,6-dichlorophenol indophenol, FMN, cytochrome c and FAD. It was also observed that the inhibition by light could not be produced under anaerobic conditions. Likewise, when the illuminated chromatophores were tested for their cytochrome reductase activity, their DPNH disphorase activity, and their cytochrome oxidase activity, the first two activities were inhibited in a manner parallel to the DPNH oxidase activity, while the cytochrome oxidase activity was not effected.

In view of the above information, it seems logical that the light effect involves destruction of some essential component of the DPNH oxidase system, most likely at the flavin level. It may well be that the chromatophores, as prepared in the isolated state, have lost some of their structural integrity, thus allowing the chlorophyll to catalyze a photooxidative destruction of the flavin contained in the chromatophore. In plant chloroplasts, it appears that the carotenoids serve a protective function, in saving the chloroplast from oxidative destruction due to the photochemical [OH], and a similar situation exists in bacteria. Sistrom *et al* (24) have shown that when a mutant of *Rhodopseudomonas spheroids,* lacking carotenoids, is exposed to the light it will grow successfully anaerobically, but exposure to both light and oxygen results in death due to photooxidation processes. Likewie, Cohen-Bazire and Stanier (25, 26) have shown that carotenoid synthesis in *R. rubrum* can be prevented by addition of diphenylamine to the growth medium. Such cells are sensitive to light and oxygen, since illumination aerobically results in decreased growth of the culture and also a parallel decrease in chlorophyll content. In view of this information, the light-induced inhibition of DPNH oxidase activity appears to be due to a photooxidative destruction of the flavin component of the DPNH oxidase system.

Summary

The photosynthetic process in bacteria has been considered in terms of four general divisions of the process: (1) the primary photochemical act leading to production of oxidizing and reducing components, (2) the recombination of the photochemical oxidizing and reducing components to produce ATP by the process known as photophosphorylation, (3) the fate of the photochemical reducing power in terms of cellular reductions, and (4) the fate of the photochemical oxidizing power in terms of cellular oxidations.

Experiments are discussed showing that chromatophores of *R rubrum* are capable of performing a photoreductoin of TPN, as demonstrated by coupling TPNH formation with glutathione formation by the enzyme glutathione reductase in the presence of a pyridine nucleotide reductase from plants. A similar series of experiments designed to detect DPNH formation in the light were not successful in demonstrating DPNH formation. Thus it appears the system is preferential for TPN photoreduction, but this specificity may be due to the pyridine nucleotide reductase used in the experiments.

Illumination of *R. rubrum* chromatophores leads to inhibition of the DPNH oxidase activity of the chromatophores. This inhibition could not be overcome by addition of various nucleotide diphosphates (ADP, GDP, IDP, CDP, UDP) to the system, so it appears the inhibition was not due to lack of phosphate acceptors for oxidative phosphorylation. Such a lack might arise from photophosphorylation. Instead, the inhibition seemed due to a photooxidative destruction of some essential component of the DPNH oxidase system, most probably a flavin.

Discussion

Dr. Gunsalus: A man was sitting at a banquet table. His wife had a piece of paper folded and passed it down to him. When it got down to him he opened it; it said SBS. He looked at it, scratched his head, and looked at her and wrote on the other side, "Don't you mean SOS?" She shook her head and finally it dawned on him what this means: "Be seen. Be brief. Be seated." I think this probably applies to a discussant as well

as to after-dinner speakers. It is quite clear that I don't have an important function here. It has been very pleasant to be on your campus and to be here at a time when this Colloquium is in progress. You have excellent speakers, and it has been a wonderful Colloquium to listen to. You have Dr. Went down there, who has been working very hard all afternoon assembling the pieces to give you an excellent digest of the preceedings this evening. There is one thing that I would like to say, and then make a few comments about Dr. Vernon's very nice presentation. What I would like to say to any young graduate students or any other young people who happen to feel that they would like to work in this area, the things you have heard today don't mean that the situation is closed. The way it looks to me is a little bit like the fellow who tried to diagnose the situation in a house down the street by watching what groceries were brought to the front door and what garbage was put in the pail at the back door. The reason I say this is that in order to study the problem of photosynthesis, Drs. Calvin, Arnon, Warburg, Emerson, French, here, and many others have found it necessary to look at a lot of the auxiliary equipment which goes with this process. There were several attempts to solve the problem of photosynthesis before anything was known about, let's say, CO_2 as the hydrogen acceptor in the process. It is *the* important hydrogen acceptor in photosynthesis. In terms of the reactions and the very nice cycles which Calvin and his people have untangled, these reactions are no different from those occurring in many heterotropic organisms. It is true, that because of the energy that comes out of the light reaction, the direction in which the sequence of events is driven is different from what you ordinarily see, but the reactions are no different. They are now returning, as Dr. Bassham very excellently showed you, to the problem of how this light is trapped and how it gets to chemical bonds. I think you'll find that the physicists are having a considerable influence on the biologists, plant physiologists, biochemists, and physical chemists at this stage in the process. Any of you young fellows who want to jump in, I'll tell you the water is warm.

Now with respect to Dr. Vernon's comments, I think Dr. Crescitelli this morning had a series of comments that were important in reference to microorganisms. That is, when all else fails, you look for the microbe. Well, the function of the microbe in this situation is simply that many of these organisms that Dr. Vernon has been describing are certainly aerobic heterotropic bacteria. It does turn out that they have an inducible system which, when light is shined on them, causes the chromatophors to show up the sixty micron particles that have in them the apparatus for accepting light energy and converting it into chemical energy under the inducing effect of light. This process seems to take over in competition with the oxidative processes. It turns out that these organisms are found commonly both in salt and fresh water and especially below the surface, and any light that gets by those at the top as it comes down to these more deeply situated aquatic forms, finds they have light receptors also. These organisms are able to take up the light and are able, under the anaerobic conditions existing where they live to carry out photosynthesis. Now, it would be silly of them to commit suicide under situations where the light gets to them by producing oxygen since this also would stop photosynthesis. Any organisms which were this silly have disappeared from nature long since by the process of evolution, and this is a particular adaptation, apparently to an anaerobic situation. It is true that hydrogen donors must be present, and sulfur happens to be one of them, and there are many organic compounds which are others. It is perhaps not unique, although it was a long time getting discovered, that these organisms also fix nitrogen so that they can get along in the depth of a pond under circumstances where they need only CO_2, nitrogen, light which gets to them, and some extraneous hydrogen donor. I have been guilty several times of saying that at any one time there are not more than two or three nor outside of half a dozen important biochemical problems being studied, and I think I still argue that viewpoint with a good many folks. One of the problems that comes out of the photosynthetic problem is present in other metabolic biochemical problems also. It is the problem of the source of hydrogen, hydrogen gas, and the source of hydrogen in terms of electrons. The source of hydrogen gas in microbial fermentations or in any kind of fermentation, for that matter, is far from being solved. It is reasonably clear, I might say quite clear, that the hydrogen that comes from triosphosphate in the oxidative process of glycolosis doesn't end up in hydrogen gas. The hydrogen that comes from the cleavage of pyruvic acid and apparently, in some instances, though not so in those well documented, from ketogluterate cleavage does appear as hydrogen gas. Now, as Dr. Vernon points out, one situation which has been

tested repeatedly is that of the cleavage of formic acid. The reason one looks to this is that the hydrogenase reaction cleaves formate to give hydrogen gas and carbon dioxide. This gives hydrogen gas the proper potential, that is of the hydrogen electrode of pH 7, which is about -.4 volts and formic dehydrogenase which operates at about this level. It is one of the few systems that does. But there are a good many hydrogen gas producing organisms or preparations in which no trace of formic dehydrogenase or formic hydrogenase can be observed. So this is one of the pieces that really has to be looked at. I think the important situation is that the potential of whatever previous system existed must be slightly negative to compete well at -.4 volts. Thioctic acid can operate here even though there is no positive evidence and the reason for this thinking is that first we all felt that this was important as a carrier for carbon dioxide pieces or other acyl pieces in cleavage of keto acids. But it has also been observed that when this compound is put in the presence of almost any biological system it shows up very quickly in its reduced form, the dimercapto form, under situations where the keto acid cleavage reaction cannot possibly be the predominant reductive force. Now whether this has meaning once it is traced out is neither here nor there. I think Dr. Bassham's viewpoint is a good one. A postulate was made by Calvin and his people which had much to recommend it at the level of intellectual flavor and appreciation, but there is not enough evidence in the book yet to tell us whether it is a good idea or a bad one. It wasn't easy to get the evidence so it is on the table. Why change your mind until you have evidence? It is a good hypothesis.

One thing about the efficiency of the photosynthetic process: I think it is probably anything you want to call it regardless of the quanta yield if you calculate it as it has been calculated many times. If you take an eight quanta photosynthesis and feel that ATP is worth whatever you like, ten calories, I think seven calories is better, but if you take seven calories for your ATP bond this will cut your efficiency somewhat. I think the critical value would be eight times whatever you figure the value of the quantum is for whatever wave length you are dealing with. If you take Dr. Bassham's value of forty, roughly, you've got about 32 kilocalories and actually the CO_2 reduction reaction to the level of carbohydrate is only about 100 kilocalories; so the overall efficiency is not better than 30%. Now, if you take the system that he did where you get 3 ATP's and 2 TPN's to make one carbon dioxide reduction you can get a very high value. But no one is sure that this is a good value. I am reminded very much of a situation with the fatty acid synthesis where everyone knows that if you take a molecule of CoA and put this on any basis you like and per molecule of CoA you take hydrogen, you ought to be able to form fatty acid, yet Green came up with a soluble system which required in addition to CoA, 2 ATP's and two pairs of hydrogens; and the efficiency dropped. I am only saying that a good number of people, and I've been as guilty as anyone (Dr. Bassham was slightly guilty of this today), pick values which would be ideal values if the thing goes as the carbon cycle would indicate and if there are no other reactions. The carbohydrate part of it would be this high in efficiency. Glycolosis isn't nearly this high; it is something like thirty-five calories as the energy difference between lactate and glucose and 2 ATP's at seven kilocalories as fourteen. So 14 over some 30 doesn't give you a very good efficiency. Likewise, with oxidative reactions. No one yet has been brave enough to suggest more than 3 ATP's per pair of hydrogens moved from low potential substrates (DPN or TPN if you like) to oxygen. If you figure this at about 45 to 50 kilocalories depending on what your taste is and feel that ATP is devaluated to seven kilocalories, $3 \times 7 = 21$; so you would have something like 21 to 50. I think it is not clear yet that the efficiency of any of the heterotropic biological processes goes beyond the quantum reaction; that is the transformation of light energy to chemical energy.

Another thing, I think that there was still the question about the CO_2 fixation when Ochoa first came up with a model reaction in which a strong reducing potential to make TPN or DPN could act through malic enzymes to form malate. This seemed to be a fine model system. One of the real difficulties was that the equilibrium in this reaction was bad and the pCO_2 of this reaction was not at all what one would expect for the system to take out of the air carbon dioxide with a partial pressure of .03, and I think probably that if one looks carefully at the ribulose diphosphate system we have a similar difficulty in the pCO_2 of the system. Now, whether this means in addition there is a CO_2 collecting and concentrating system which some biologists would like to call a "pump" or whether actually there is an enzyme that operates in photosynthesis with this low pCO_2, is something that is debatable. If one had

to have a pump to collect CO_2 to get the pCO_2 high enough to operate the ribulose diphosphate system at the rate at which photosynthesis seems to operate, this would decrease the efficiency because any pump system presumably would require more energy.

This is perhaps more than I should have said, and I thank you. It has been a pleasure to be here.

Dr. Bassham: I would like to make one or two very brief comments on Dr. Gunsalus's comments. First of all, with respect to the ATP and energy relations: While it is true that the standard free energy of ATP has been depreciated, normal biological systems usually have concentrations of inorganic phosphates that are estimated to be at least 10^{-2} or 10^{-3}M, with a reasonable assumption that ATP is equal to ADP in concentration and correct for the inorganic phosphate concentration. If you take the best average value for ATP standard free energy of hydrolysis, you come back to the values of 10.5 kilocalories. I don't think it is worth arguing about, but I thought I would mention this. The other thing that I wanted to point out is that in the carboxylation reaction, we have calculated the actual free energy change for the carboxylation of ribulose diphosphate to PGA and it turns out that that particular reaction has a very large free energy change, that is, relatively large free energy change, about nine kilocalories per five, which we interpret tentatively as meaning that this much negative free energy change is necessary in order to allow the carboxylation reaction to go in very small concentrations of carbon dioxide. Energetically, this means that you don't have to put in any outside energy other than this, as I think Dr. Gunsalus implied, but there is enough energy right there, liberated by the reaction, to permit the reaction to go at a very low concentration of CO_2.

Question: How did H_2S and other sulfur compounds, hydrogen donors, fit into the photosynthetic scheme?

Dr. Vernon: Of course, they would qualify as being those oxidizable substrates which the photosynthetic bacteria require. In terms of the metabolism of the thiobacillus, the ordinary anaerobic thiobacillae, there has been some indication that the sulfur compounds can interact with cytochromes. In other words, there are particular cytochromes in the thiobacillae and so it wouldn't be hard to imagine that the H_2S and the other sulfur compounds could be oxidized directly by a cytochrome compound in the bacteria. Of course, this would imply that maybe you have particular cytochromes in the sulfur bacteria. Of course, from the investigation of Kamen and myself, it is apparent that all photosynthetic bacteria, whether they live aerobically or anaerobically, do have cytochromes. So, it wouldn't be hard, actually, to visualize the oxidation of these sulfur compounds by cytochromes contained within the cell.

Question: Concerning the stability of the chromatophors, at what temperatures are the chromatophors heated?

Dr. Vernon: The experimental procedure was to have a boiling water bath and then immerse the chromatophors in the boiling water bath. So, essentially it was the temperature of boiling water. It was remarkably stable, very stable, but rigidly; of course, you can heat these things and they change color when you heat them, and as you cool them back down, they go back to their original color. They are remarkably heat stable, at least in this regard.

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Perspectives in Photobiology: A Summary*

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Colloquium Leader

I think that all of us will agree that the five most important interests during the last two days have been (1) the hospitality of Oregon State College, (2) the excellence of the Corvallis weather, (3) the photosynthesis induced by so much sun, (4) such an array of pigments, and (5) photoperiodism.

Now, since no light effects are possible without absorption, consideration of pigments has ranked very high in our discussions. We heard of the visual pigments and the chlorophylls, and it is quite interesting to note the similarities and differences both in the discussions and in the pigments. Actually, I think that this colloquium, instead of revealing a very large number of pigments, disclosed a very small number. For instance, chlorophyll was discussed in several papers and it was nearly always the same chlorophyll; at least it was very hard to classify differences between them. I think that this is a most interesting aspect because from that we really must conclude that photosynthesis is an unique process, which in the course of evolution has developed only once. Immediately chlorophyll was tied in with the whole process, although I would feel that another pigment could do this if you could invent other pigments with a structure permitting it to transmit the light energy which was absorbed to other parts of the plant. Even though we find a few other pigments, in principle we are dealing with porphyrin, even in bacteria and in the higher plants. Therefore, it is actually inescapable but to conclude that photosynthesis by various organisms is one and the same process and the energy transfers which are involved are all of the same general nature, as was brought out through the talks this afternoon.

An interesting observation is also apparant from the discussions of energy transfer. Both of the last speakers indicated the remarkable efficiency of the photosynthetic process. If basic efficiency refers to the energy transfer of absorbed light to the reduction of CO_2, this means we are dealing with efficiencies which are somewhere between 30 and 50%. Therefore, I should say that the hardest part of this process to explain is this enormous efficiency.

What has evolution done further with this? It really hasn't done too well. When you grow algal cultures, up to 10% of the daylight energy which falls on them can be transformed into chemical energy which you can harvest in the form of algal cells. The same thing can be said for tomatoes. Under ideal conditions of the laboratory, it is possible to find 10% of the light energy transformed into the chemical energy of the plant, but not more. Thus, we have a discrepancy between what the chloroplast can do and what the whole cell or the whole organism can do under ideal conditions; but that is not the end of our story. The actual efficiency, the maximum efficiency, under most optimal conditions in a sugar cane field, a sugar beet field, or wheat or corn fields, is, I think, only about 2.25%. This means only 2.25% of all the light energy which falls during the growth period on that field is transformed into chemical energy. This is something which is very diffiicult for me to understand. These cells have the ability to use practically 30 to 50% of this light energy and yet under the best possible conditions, they apparently waste from 28 to 48% and there is just 2% which you can ultimately harvest. So, there is a curious discrepancy which may lie in all calculations. Maybe they are all wrong.

It might be that under really good natural conditions we have a much higher efficiency of light utilization. This is still something which really needs either investigation or development. This is, I think, what we really should think of in the future. The capturing of light energy will become more and more important as the population of the world increases, and even with the availability of atomic power, a much larger percentage of the light energy which comes will have to be used. At the moment we use only a small amount of this light energy. We could use far more, with the present knowledge and under present conditions; but there is still this margin of 2% actual utilization of light energy under actual conditions, against 30 to 50% theoretically possible. Although we don't have to worry too much about the im-

* Transcript of Dr. Went's concluding remarks, edited by Colloquium Committee.

mediate future, this is something which is a real challenge for some time in the future.

There was another interesting point raised here; in the eye pigments, the rhodopsin, it was quite clear that the important part is not just the pigment moiety. The important thing upon which everything depends for the differentiation which occurs between the different organisms lies in the protein.

Now, what about chlorophyll? It is quite obvious that chlorophyll only can be effective if it is combined with proteins and with two other constituents. Yet in all work on chlorophyll, we are dealing with just the pigment itself and not with the protein pigment complex. Now, maybe this really has been answered already by Dr. French when he said it was such a terrible mess, these chloroplasts, but I think that is really not enough of a reason. If you have a mess, you can get something clean out of it. I am still looking forward to the time when it will be possible to also get good preparations of chlorophyll-protein complexes; perhaps more lipoids will be found associated too. Something like that will come unless in principle it would be impossible because the process in which the chlorophyll takes part is so much more complicated than the process of vision, where not so many chemical transformations have to take place at the same time. Yet, I think it should be possible. After all, Dr. French has already isolated a chlorophyll protein complex from bacteria many times.

There was very much said this afternoon in a biochemical way. I really was scared until Dr. Gunsalus got up and discussed that part. The only thing I would say about the biochemical study which was brought out by Dr. Bassham is that it is perhaps one of the most remarkable scientific stories of this century. In an old "beat down" building, a few chemists have been building a story with an efficiency that is incredible; a story of the path of photosynthesis which I don't think is equal to any other in biochemistry. I think all research directors and research administrators really should examine this study very specifically, because here has been an efficiency which I don't think has ever been surpassed in scientific research. This is really a magnificant story. Although I can't follow all of it, I am deeply impressed, and each time I hear about it there is a new extension.

Let us now go to several fields about which I know something more, and that would be on the problem of photoperiodism about which we have had quite a number of talks. I don't know whether you noticed, but I tried to hold back a little in my first talk.

Let's first mention that this anti-etiolation about which I spoke in the beginning, a phenomenon in which small amounts of light are able to counteract this effect of growth in complete darkness, is not just a photoeffect. When we give all the light to reduce the growth in one fell swoop, let's say half an hour of light, then there is very little effect. On the other hand, if you give the same amount of light in a number of portions once a day, then the effect is perhaps ten times as large. So, actually we should in this case also talk about the photoperiodic effect. This is just the thing that Dr. Hamner discussed, at least for soy beans and other plants. You have to give your light and dark in portions of 24 hours for the photoperiodic stimulus. When you give, at least for plants, the light and darkness all at once or if you give it in portions which differ from 24 hours, then the effect either won't occur at all or is very, very different. Now, I think this is something we might put down as a sort of scheme. We know that the organism takes in stimuli from the outside. Those stimuli are periodic, at least, in case of this anti-etiolation effect. So here we have a stimulus which is basically periodic and if you don't give it periodically, then it is ineffective.

Why does this have to be periodic? Let me first quote a few experiments which may give another aspect to this. When we try to grow tomatoes in continuous light, the first few days they grow nicely; but then their growth rate decreases, their photosynthesis decreases very much, and the leaves which are newly formed become abnormal with white spots; that is, they become etiolated. The whole plant deteriorates. It is a curious thing that these plants, for normal development, cannot use continuous light. You may say, "Well, I've heard that tomatoes grow in Alaska in 24 hours of light"; that will be discussed in a moment. Basically these tomatoes grown in continuous artificial light and constant temperatures will decrease their growth to a point where people like Arthur and others have found that those plants will die but if you give these plants periods of darkness, they are all right (this has just been investigated or is under investigation by one of the students, Mr. Christopherson). When, during every 24 hours we give the plants just one hour of darkness, the plant still doesn't do well. The remarkable part of it is that at 6 or 8 hours of darkness each day,

those plants grow far better than when they get continuous light.

So, here we have one aspect—these tomatoes (not only tomatoes; other plants also have it) need a period of darkness between the light periods. This is not the only part. If you now give them their darknesses not on a 24 hours basis but, for instance, 8 hours darkness and 8 hours light over a 16 hour cycle, then the plants are just as poor as if they are in continuous light. When you give them a 36 hour period—18 hours of light and 18 hours of darkness—they also are extremely poor. Therefore, there are two aspects. One is that there is a period of light that has to alternate with a period of darkness and the other, these periods have to have an overall cycle of 24 hours. So, what we are dealing with is a stimulus which has to be periodic. Why, you will see later.

This, of course, is all that is directly applicable in a Colloquium on Photobiology. I should leave it at that. But, there is another curious fact, and that is that you can grow tomato plants perfectly well in continuous light provided you fluctuate temperature (this was first found by Hillman and is now under further investigation by Christopherson). The relationship between the lengths of the lower temperature or the higher temperature and the effect is just exactly the same as with light and dark only it doesn't drop off because it is continuous light. Temperature fluctuation is completely equivalent with light fluctuation, but still it has to be over a 24 hour period.

This, I think, shows that what we are dealing with at the moment is a phenomenon of photoperiodism but it is not just the photo part which has been stressed so much; particularly now at the meetings; but it is also the periodic part and that, of course, is a very interesting point that Dr. Hamner also started to bring out. In the last few years a complete change is coming over the investigations in photoperiodism—the *photo* side is not stressed so much any more, but the *periodic* side is being stressed. This is really so more remarkable because, when Garner and Allard started to work on this, they called this phenomenon photoperiodism and I am quite sure that they had in mind that this was the combination of light, and a periodic application of this light. We lost sight of the periodic aspect for a long time, but it is coming back again.

In animal and in plants we have now another set of conditions; the response of these organisms may also be completely periodic. An example is the leaf movements which Bünning has investigated. Leaves of practically all plants (not just beans and leguminous plants, but tomatoes, tobacco, and almost every other plant) are in different position at night than during the day. For example, the leaves of tomatoes move up and the tips drop down at night. In animals, the response in terms of their activity (or, for instance, in the pigments of crabs) is also completely periodic, and is approximately 24 hours.

Now this is perhaps one of the most amazing things. We know that there is a whole train of reactions between the original stimulus, and what is going on in the organism and the response. There may be, as Dr. Farner made clear, 4, 5, or 6 steps in his birds. There are in plants, as Dr. Hamner indicated, transmission phenomena because the stimulus is received in the leaf and the response in flowering is in the growing tip. Therefore, we know that in between there is a transmission of whatever is perceived in one place and whatever is responding at another. Again I perhaps should stress that this is certainly the case in plants—and to some extent in animals. That is to say, synchronism has to occur somewhere between the stimulus and the effect. For these plant responses, we must get an entrainment of the response mechanism by the stimulus mechanism, and this entrainment can occur only within a limited range of cycle length. If the difference between the two is too great, no entrainment is possible and the whole plant becomes unbalanced and we get those abnormalities. I mentioned that if the tomato gets the wrong cycle it behaves as if it doesn't get any cyclic stimulus at all. Therefore, there must be some way in which one cycle can get synchronized with the other cycle, which means that the processes that go in between—the intermediate processes—must carry with them something in the nature of a pulse. Where this entrainment occurs doesn't make any difference. I think I can make a good case that the response as such is periodic and that, since the stimulus is periodic, the transmission of the stimulus to the response has to become periodic. Also, it has to be in the nature of the pulse.

I would suggest that the reason why the attempt to get a flower forming hormone, a florigen, has failed is that we are looking here for a substance when we should look for a periodic thing. This pulse probably will have to be transmitted in the form of a substance, and this also should then be periodic. Just extracting something and applying it will not do any good. You could only expect an effect (assuming these considerations are cor-

rect, and the substance is at all involved) if you apply it periodically.

Now, I come to the other question raised about the periodicity in the response. For the periodic reaction for the flower response, we know that we have the growing parts which first form leaf primordia and we have a certain period in the development of new organs which are formed in succession. Now, under the influence of the changed photoperiod, we get a certain change in the whole response of this growing point. Cell divisions start to go in a completely different direction; a completely different rhythm, and therefore I think that we should look for a periodic response in the growing tip itself. We know also that the photoperiodic stimulus ultimately is affecting the growing part. Bünning reported that in the growing tip itself, there is a very clear cut period of 24 hours in cell divisions. Basically we are dealing with a periodic response and I think also we can expect an entrainment of such a periodic response by a periodic stimulus.

This brings up another question. We have very often tried to find in the growing point of the plant a hormone or something else which would cause a correlation between the different cells. When all these cells divide in perfect order as we can see in the phyllotaxy of a stem, the leaves are all placed in a magnificent, orderly fashion practically without any exceptions. There must be some sort of correlating mechanism. We have not been able to find that there were any hormones which were formed actually in the tip and then move down. I would suggest that actually the cells in different parts of the growing point are correlated not so much by their hormones, which we have thought, but are synchronized in their division by the periodicity of the stimulus. When this periodic stimulus doesn't come any more, e.g. in tomatoes which are grown in continuous light and in constant temperature, the growing point also goes "haywire" and we don't get normal development. That is what we find since the growing point is very strongly affected in those plants. This is even more strongly affected in, for instance, the African Violet. If African Violets are grown at too low a temperature the growing point becomes completely disorganized and no leaf primordia are formed any more—and the whole plant dies.

I want to mention that the cycle lengths in plants are slightly affected by temperature. This is different from animals. Where this has been investigated in animals, it has been found that the cycle lengths seem to be independent of temperature. You can actually keep animals at higher or lower temperatures and you will find that their activity for whatever is periodic in their behavior remains just at the same cycle length. Now, this may be partly because the experiments are carried out with birds or other warm-blooded animals.

In the periodic response to temperature, we are dealing with a process which apparently is not a chemical process, but a diffusion phenomenon—a process in which all molecules are equally activated by temperature rather than specific molecules, as in chemical reactions, where we have a very high Q_{10} of 2 or 3. The Q_{10} of the photoperiodic process is approximately 1.2 or 1.25. This shows that periodic mechanisms, or this response mechanism, is of the nature of a diffusion phenomenom or may be any other physical phenomenom in which we are dealing with the sum total of the molecules.

What implication does this have for normal development of plants? Let's take a plant which has a normal 24-hour cycle at 25° C. Such a plant will grow perfectly in a climate with an average temperature of about 25° C. When you try to grow such a plant in a cool climate, it will nearly always be bucking the external cycle, which remains 24 hours, against its changed internal cycle, which is lengthened. Those plants do very poorly. It is a remarkable phenomenon that many tropical plants, when they are grown in a temperate climate, do very poorly and may eventually die, even though the temperature never gets near the freezing point. That is definitely the case with an African Violet; when you try to grow it out of doors in the cool spring, those plants, in the course of a number of months, die. I tried to grow them in an artificial climate in the Earhart Laboratory where I kept them on a 24-hour cycle. At 10° those plants died. I think it took them four or five months, but they were dead after that time. On the other hand, when I grew them on a 32-hour cycle at 10°, they grew slowly but were perfectly healthy and didn't show a sign of dying. When the plants, which were in poor condition after they had been at 10° and a 24-hour cycle for two or three months, were transferred to a 32-hour cycle, they completely recovered. Those that did very well on a 32-hour cycle died or became very poor on a 24-hour cycle.

Similarly, I think we can explain on this basis alone why temperate region plants cannot grow in a tropical region. If plants which had a 24-hour cycle at a low temperature, were brought to a

high temperature, they would get out of phase. If plants in the tropics could be subjected to 18-hour days or if in the northern regions the sun could be made to go that much slower, then all of these plants would grow, perhaps not perfectly, but fairly well.

This is just one part of the story which I want to talk about. There is another point in photoperiodism, which I think holds both for animals and plants, that I would like to bring out. That is work which was done recently by Dr. R. Sachs. It was known that there are certain plants that are so-called long-short day plants. Those plants can be kept under one single photoperiod indefinitely and never flower, but if you first give them long days and afterwards short days, then they will flower. These had not been really studied in detail until Sachs worked with the plant *Cestrum nocturnum* and found that when he kept the plant continuously in long days, in short days, or in 12-hour photoperiods, it never formed any flowers.

This was really quite a blow for us in the beginning because I was fairly well convinced that the Earhart Laboratory was a fine place to grow plants, but the cestrum never flowered in Earhart; while in ordinary greenhouses it flowered very well. The difficulty was that we never gave the plant successively first long days, and then short days. It turned out that when plants were treated with first a series of eight long days and then two short days only it would immediately form flower primordia and then flower. During the eight long days, or any other number of long days, there was actually no change visible in the growing point. The change in the growing point came only after it also had had two short days.

Then Dr. Sachs investigated the plant in further detail and found that the long day response of these plants was typically like the long day response of any other long day plant. The light period could be interrupted and that would prevent flowering. It also turned out that the temperature during the light period determined how much response there would be.

The short day treatment behaved just like the short days of short day plants. That is, it was the temperature during the night which determined the degree of response. A light interruption during the night also controlled it. So we have, in this case of long day, short day plants, a typical long day reaction which has to be followed by a typical short day reaction before the plant flowers.

Sachs made the following suggestion: maybe all short day plants are really long day short day plants, only the long day requirement is always satisfied in them. As Dr. Hamner already explained, you can really give long day plants relatively short long days and they are still reactive. In the case of cocklebur, for instance, if you give them eight hours of light the long day reaction is still satisfied, but at six hours of light per day it is not. Therefore you cannot grow a cocklebur plant on a 6-hour day and have it flower. On the other hand, you could say that a long day plant was one in which the short day reaction was always satisfied. Now this was something which impressed me but I've now heard Dr. Farner say in his words, "The long day treatment does two different things. It stimulates the sexual phase and at the same time it also brings the birds in a refractory condition, and that refractory condition can only be removed by giving them short days."

I understand that the same thing is true for other birds like turkeys. Might it not be that the turkey and this sparrow are short-day long-day animals; that they first need a short day to become sensitive to the long day treatment afterwards? This is one of those things which was also mentioned this morning. Many of these responses are very useful to the animal. If, for instance, these birds would lay eggs as long as they have long days, then they would lay eggs from May until August or September, which of course would be contrary to the type of response which you need. Therefore, by having this double response, they respond only to long days when the days first were shorter. So, in this way, even though it is not the gradually changing lengths of day which would do it, we have an actual requirement first for long days, then for short days or just the reverse.

I think that these are all the remarks which I would like to make in connection with what has been said in the previous days. I have fully enjoyed being here with you. I think that just looknig at these different problems in relation to each other gives another new perspective on the problem as a whole. We have seen that all these responses which we have been discussing are not just purely academic problems, but these are problems with very wide economic and practical implications as well. I hope that you also have found it that way.