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PHOTOPHYSIOLOGY

CURRENT TOPICS IN PHOTOBIOLOGY
AND PHOTOCHEMISTRY

Edited by

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PREFACE

Photobiological studies continue to arouse interest in ever-extending circles as the widespread action of light, even of long wavelengths, on cells of organisms becomes more evident. Of importance are repair mechanisms for maintaining normalcy in cells exposed to injurious radiations. Current investigations on genetic recombination as a means of repair of radiation-induced injury further indicate the multiplicity of mechanisms for this purpose.

In this volume most of the chapters deal with relatively restricted areas of photobiology. With increasing specialization it is likely that future reviews will cover narrower fields but in greater depth, as befits the present more intensive study of the phenomena.

I wish to express my appreciation for the cooperation and scholarly reviews of the contributors. I am also indebted to the advisory board and to many colleagues who suggested reviewers and reviews, not all of which, however, materialized. Nonetheless, the contributions represent a fair cross section of the various lines of research in photobiology at the present time. Whatever unbalance exists will probably be balanced by topics covered in the next volume.

ARTHUR C. GIESE

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Abbreviations

A	Intersystem intermediate	CCCP	Ketomalononitrile 3-chloro-phenylhydrazone (carbonyl cyanide 3-chlorophenylhydrazone)
ADP	Adenosine diphosphate		
ATP	Adenosine triphosphate		

Chl	Chlorophyll	PSU	Photosynthetic unit
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethyl urea	Q	Quencher of fluorescence, also primary electron acceptor for system II
DCPIP	2,6-Dichlorophenol-indophenol	P680	Energy trap for system II having one of its absorption bands at about 680 nm
FCCP	Ketomalononitrile 4-trifluoromethoxyphenylhydrazone (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone)	P700	Energy trap for system I
NADP ⁺	Nicotinamide adenine dinucleotide phosphate	X	Primary electron acceptor for system I
PMS	Phenazine methosulfate	Z	Primary electron donor for system II
PQ	Plastoquinone		

1. Introduction

1.1 General

When a chlorophyll (Chl) molecule, excited by light, returns from its first singlet excited state to its ground state, there is light emission within a few nanoseconds. This emission is red in color, and is the fluorescence of Chl. Of the plant pigments *in vivo*, Chl a and the algal biliproteins (phycobilins) fluoresce weakly; other pigments of photosynthetic importance do not have any measurable fluorescence.* These, including Chl a and phycobilins dissipate their electronic excitation energy also by other processes, of which the most important is the transfer of energy to other Chl a molecules—ultimately to certain special Chl a molecules (the reaction centers) where the energy is used for photosynthesis. The fluorescence characteristics of a molecule, e.g., the lifetime of the excited state, the quantum yield, the degree of polarization of fluorescence, the excitation and the emission spectra, are governed both by its chemical nature and by interaction with its environment. Thus fluorescence of Chl a *in vivo* has been used as a powerful tool in the analysis of photosynthesis, particularly of the process of excitation energy transfer (Weber, 1960; Duysens, 1964; Butler, 1966a,b; Goedheer, 1966; Govindjee *et al.*, 1967; Hoch and Knox, 1968; Fork and Amesz, 1969).

Both Chl a fluorescence and photosynthesis draw on the excited Chl a population, and thus a change in the photosynthetic rate is reflected as a change in the yield of fluorescence. This chapter† will place emphasis on the dependence of the efficiency of Chl a fluorescence on processes such

* Some intermediates of photosynthesis, e.g., the reduced form of nicotinamide adenine dinucleotide phosphate, NADPH, do fluoresce, but this is not under discussion here.

† No attempt has been made to cite and discuss all the papers in the field. Moreover, the emphasis is on work with intact cells.

as the electron and ion transport, which are components of what is collectively referred to as photosynthesis. The reader's familiarity with the general outline of the present theories of photosynthesis is assumed. When necessary, reference can be made to particular topics in several recent reviews as those by Hind and Olson (1968) (electron transport), Avron and Neumann (1968) (phosphorylation), Rabinowitch and Govindjee (1969) (general mechanisms), Boardman (1970) (two pigment systems), Cheniae (1970) (O_2 evolution), Fork and Amesz (1970) (intermediates of photosynthesis), and Packer *et al.* (1970) (ion movements and structural changes in chloroplasts).

The present chapter will deal specifically with the change in the fluorescence yield of Chl *a* with the time of illumination (i.e., the fluorescence induction, the fluorescence transient, or the Kautsky effect) and its relation to the photosynthetic reactions. [For a review of the older work on fluorescence transients, see Wassink (1951) and Rabinowitch (1956).] If one takes a dark-adapted suspension of algae, or a leaf, and shines bright light on it, Chl *a* fluorescence yield changes in a characteristic way (see Section 1.4). These changes have been arbitrarily divided into two broad categories—*fast changes* that are over within a second or two (at moderate-to-high intensities), and *slow changes* that may last for several minutes. The fast fluorescence changes reflect the momentary oxidation-reduction state of the photochemical reaction center, the rates of reactions associated with it, and with the intermediates of the electron transport chain. The slow changes, on the other hand, reflect the physical state of the pigment systems and the associated ionic changes and the rate of photophosphorylation. We believe that the slow changes are also affected by the oxidation-reduction reactions and the fast changes by the physical state of the pigment systems, but the extent of these effects is not yet clear. It is because of these relationships that the study of fluorescence transients has the potential of providing information regarding the mechanism of photosynthesis.

We must recognize some basic properties of photosynthesis before we can embark on a discussion of the above relationships. The two major concepts to be considered are (1) the existence of *photosynthetic units*, i.e., groups of several hundred Chl molecules that somehow cooperate to evolve O_2 , and (2) the existence of *two pigment systems* and the operation of *two light reactions* (I and II) in photosynthesis.

1.2 Photosynthetic Unit; Function and Interaction

The ratio of Chl to the assimilated carbon dioxide (or liberated oxygen), under experimental conditions ensuring the optimal utilization of light for photosynthesis, was found by Emerson and Arnold (1932a,b) to be

about 2500:1. This off-balance stoichiometry was taken as evidence for the existence of photosynthetic units (PSU), i.e., groups of pigment molecules acting in concert to collect and utilize photons. A few years later Gaffron and Wohl (1936) arrived at the same conclusion on the basis of results reported by Warburg and Negelein (1925). In Warburg's experiment, each Chl molecule absorbed one photon every 12 minutes. Assuming that each photon will eventually cause a one-electron reduction, it would take a Chl a molecule about 50 minutes to collect four quanta to reduce one carbon dioxide molecule. (To collect eight quanta, it would take 100 minutes.) This unrealistic state of affairs (when O₂ can, in fact, be evolved in less than a second after illumination) prompted Gaffron and Wohl to propose a speeding up of photosynthesis by means of groups of cooperating Chl molecules.

The previous discussion suggests that the size of the PSU per molecule of oxygen evolved is 2500 Chl; its size per electron transferred is $2500/4 \simeq 600$ Chl as four electrons must be transferred from H₂O to CO₂ to evolve one O₂. Since each electron transfer requires two separate light reactions (see Section 1.3), the size of PSU per primary light reaction is $600/2 = 300$ Chl molecules. The above conclusion is based on the assumption that a minimum of eight quanta are required for the evolution of one O₂ molecule (see Emerson and Chalmers, 1955; R. Govindjee *et al.*, 1968). Thus 300 Chl molecules make up one PSU, although smaller units have been identified in higher-plant mutants (Schmidt and Gaffron, 1968, 1969; Wild, 1968, 1969). The discovery that photosynthesis proceeds by means of two sequential photoreactions (Section 1.3) necessitates the grouping of the units, in accordance with the photoreactions they perform, as PSU I and PSU II. The pigment systems contained in them are referred to as pigment systems I and II.

A photosynthetic unit has a light-gathering (or "antenna") part (to be referred also as the bulk pigments) to which all pigment molecules except one belong, and a reaction center which contains the remaining Chl a molecule. The latter is "distinguishable" because of its proximity with the oxidoreduction couple and possibly with an enzyme. The bulk pigments consist of Chl a and Chl b (higher plants and chlorophytes), Chl c (bacillariophytes and phaeophytes), phycobilins (cyanophytes and rhodophytes), and carotenoids (i.e., xanthophylls and carotenes, all plants). These pigments enrich the plants with wider absorption bands, and therefore with a more efficient photon-harvesting apparatus.

Both pigment systems I and II contain the accessory pigments as well as Chl a, but they differ in the relative abundance of these pigments in them (Fig. 1). Pigment system I contains a larger proportion of the long waveforms of Chl a (Chl a 678, Chl a 685-705, the numbers refer to

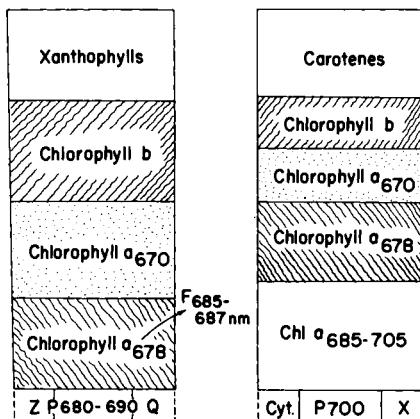


FIG. 1. Composition of the two pigment systems in photosynthesis.

their red absorption maxima), a smaller proportion of the short waveform of Chl a (Chl a 670) and of Chl b (or phycobilins, etc.), most of the carotenes, and all of the reaction center Chl a molecules (P700) having one of their absorption bands at 700 nm (for P700, see Kok, 1956, 1957a,b, 1959, 1961; Kok and Gott, 1960). This system does not have a fluorescence transient, and is weakly fluorescent at room temperature (Briantais, 1966; Brown, 1969). The pigment system II contains a larger proportion of Chl a 670, of Chl b (or phycobilins), most of the xanthophylls, a smaller proportion of the long waveforms of Chl a, and all of the reaction center Chl a molecules P680-690 (for P680-690, see Döring *et al.*, 1967, 1968, 1969). This system, relative to system I, is strongly fluorescent and shows the fluorescence transient (Briantais, 1966). The main fluorescence peak of plants at 685-687 nm originates in Chl a 678 of this system (Cho and Govindjee, 1970b). Most of the data on the time course of fluorescence yield reflect the changes in the system II.

The arrangement of the two pigment systems *in vivo* is open to speculation. There is evidence that they are present in separate membranes that are opposed to each other—system I on the outer side and system II on the inner side (Arntzen *et al.*, 1969; Briantais, 1969). There are two basic models for the arrangement of pigments in these membranes (see Robinson, 1966). There is the “lake” (the statistical or the multicentral) model in which reaction centers are embedded in a lake of pigments. A quantum, not trapped by one reaction center, has a probability of migrating to another reaction center. There is also the “isolated puddles” model in which each unit has its own reaction center. If the reaction center

is closed, the quantum is lost as fluorescence. In this model one lifetime of fluorescence is associated with the puddles of pigments that have open traps and another with those that have closed traps. The lake model for system II predicts a linear dependence of lifetime on the quantum yield of fluorescence, since most of the fluorescence comes from system II. Such a linear relationship is indeed found (Tumerman and Sorokin, 1967; Briantais *et al.*, 1970). Thus it seems that the lake model may be the favored one for system II. However an intermediate situation cannot be excluded.

When the excitation encounters a closed reaction center, it may be transferred to another unit. Evidence for the interunit energy transfer in system II was obtained by Joliot and Joliot (1964). These authors argued that if there was no such transfer, and each unit worked independently (as in the isolated puddles model), the rate of system II reaction (O_2 evolution) would be linearly proportional to the concentration of the open traps. However, if there was interunit transfer, the system II reaction rate would be higher than expected at times when all the traps were not open. This happens because the excitation quanta, not used by the unit they were absorbed in, enhance the probability for photochemistry by a factor of $1/(1 - e'p)$, where e' is the fraction of the open centers, and p is the probability of the interunit transfer. In such a situation, a nonlinear relationship for system II reaction versus concentration of open traps would be expected. Indeed such a relationship was observed by Joliot and Joliot (1964) who calculated the p to range from 0.45 to 0.55 (see also Delosme, 1967).

For photosystem I, there is evidence for both the "isolated puddles" and "lake" models. Probabilities of interunit transfer of the excitation energy in excess of 0.5 were reported for several algae by Fork and Amesz (1967). In isolated spinach chloroplasts, however, the rate of photoreaction I was found to be a linear function of the fraction of the open reaction center I (P. Joliot *et al.*, 1968) indicating an absence of interunit transfer in this system. It appears that there are variations from plant to plant, and even in the same plant under different conditions.

Let us now look at the intersystem excitation energy transfer. If the energy absorbed—but not utilized—by system II can be transferred to system I, it is called "spillover" (Myers, 1963). If the two systems are physically separated and the energy transfer from system II to system I is absent, we have the so-called "separate package" situation. In the nineteen sixties, it was a question of "spillover" versus "separate package." P. Joliot *et al.* (1968) found identical action spectra of photoreaction I whether the reaction center II was kept closed or open. If the spillover of energy occurred from system II to system I, one would expect

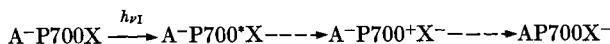
to see the additional participation of system II pigments in photoreaction I if the reaction center II were kept closed. This would happen because photons absorbed—but not used by photosystem II—would be turned over to and used by photosystem I. However if no spillover of energy occurs, the action spectra of photoreaction I would be unaffected whether the reaction center II were closed or open. Thus the experiments of Joliot *et al.* suggested that “separate package” hypothesis is the correct one. Contrary to this conclusion, Murata (1969b) and Murata *et al.* (1970) have presented evidence in support of the excitation energy spillover (see also Malkin, 1967; Avron and Ben-Hayyim, 1969). Obviously the photosynthetic system is a dynamic one. We expect to find or not find inter-unit or intersystem transfer depending upon the state of the pigment systems and the photosynthetic units. Duysens (1970) has recently suggested that a movement of membranes, containing the separate pigment systems I and II, away from or toward each other, could easily control the spillover of energy from system II to system I. Similarly the movement of photosynthetic units of the same type with respect to one another could control the interunit energy transfer. If the units are far apart, the “isolated puddles” situation exists; if they are close together, it is equivalent to a “lake.” In this picture, one can imagine many intermediate states. We hope that future efforts will be made to define the conditions for each state.

1.3 Two Light Reactions of Photosynthesis

An acceptable model of photosynthesis must make provision for the occurrence of two distinct pigment systems and photoreactions, the presence of a chain of electron transport intermediates (redox couples), and the presence of a phosphorylating mechanism converting adenosine diphosphate (ADP) to adenosine triphosphate (ATP). In addition, it must provide for the possibility of artificial electron transport in which only parts of the photosynthetic electron transport chain are used, and for the kinetics of the Chl a fluorescence yield. Indeed, since fluorescence is a measure of the photochemistry at the reaction centers of photosystem II, fluorescence kinetics may supply a criterion to test the merits of any model of photosynthesis. All models proposed, save one, invoke two (or more!) reaction centers communicating with each other by means of electron transport or a high-energy intermediate, e.g., ATP). The one exceptional model with a single reaction center capable of two distinct photoprocesses (J. Franck and Rosenberg, 1964) is now of historical importance only. It does not provide for the chain of electron transport carriers (experimentally found to exist) and of the two reaction centers P680–690 and P700, now known to exist.

The series model of Hill and Bendall (1960) is the most widely accepted model [cf. with scheme 7.V. of Rabinowitch (1945) and the scheme discussed by Emerson and Rabinowitch (1960)]. However several other models have been proposed in the last ten years. More recently, Govindjee *et al.* (1966), Arnold and Azzi (1968), and Knaff and Arnon (1969b) have proposed alternate models; these will not be discussed here but the references are given so that the reader can consult them to keep an open mind toward future development in this field. Experimental support for the series model was first provided by Duyssens *et al.* (1961), Kok and Hoch (1961), and by Witt *et al.* (1961). This model has since been elaborated to show a detailed electron transport path leading from water to nicotinamide adenine dinucleotide phosphate (NADP^+ ; see Hind and Olson, 1968). Given below is a simple version of the series model that does not include the names of most of the intermediates.

The pigment system I sensitizes light reaction I oxidizing P700 and reducing a low potential energy acceptor X that leads to the production of the strong reductant (X^-) and a weak oxidant (A) (Fig. 2):



the label A is applied to a pool of intermediates that includes two cytochromes, a plastoquinone, and plastocyanine. The strong reductant (X^-) ultimately reduces NADP^+ to NADPH. The pigment system II sensitizes

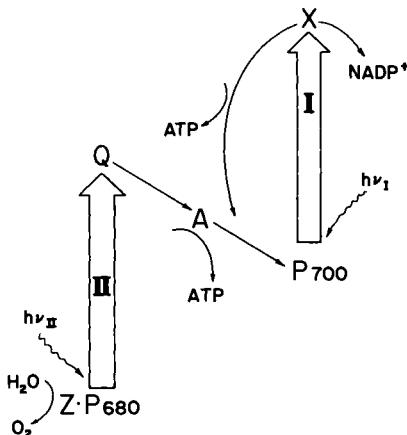
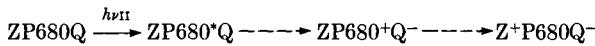


FIG. 2. Two light reactions in photosynthesis (simplified Hill and Bendall Scheme). Z, primary electron donor of light reaction II; P680, energy trap of pigment system II; Q, primary electron acceptor of system II—also a quencher of Chl a fluorescence; A, pool of intersystem intermediate; P700, energy trap of pigment system I; X, primary electron acceptor of system I; NADP^+ , nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate.

light reaction II that leads to the production of an oxidant (Z^+) and a weak reductant (Q^-):



(only by analogy to light reaction I). A strong oxidant (Z^{4+}) is formed only after four primary reactions (II), and then it reacts with water to evolve O_2 : $Z^{4+} + 2H_2O \longrightarrow Z + O_2 + 4H^+$; the weak reductant (Q^-) formed in each primary reaction II reacts with a member of the pool (A) to restore Q and A^- : $Q^- + A \longrightarrow A^- + Q$. During the latter exergonic reactions, molecules of ADP and P_i (inorganic phosphate) are esterified to ATP (*noncyclic phosphorylation*).

The reduced form of pyridine nucleotide NADPH (formed from X^-) then enters the carbon fixation cycle, or it returns its electrons, by one or several pathways, to an intermediate pool carrier. In the latter instance(s), electron transport traces a closed circuit passing through P700. This and the associated phosphorylation are referred to as the cyclic electron transport and the *cyclic phosphorylation*.

The series model offers unbiased explanations for the synergistic effect of the two photoreactions on the oxidoreduction states of electron carriers that are on the oxidizing (Z) or the reducing (X) end of the scheme, and their antagonistic effect on the intersystem pool carriers. It thus accounts for the enhancement of oxygen evolution (Emerson *et al.*, 1957; Emerson and Rabinowitch, 1960; Govindjee and Rabinowitch, 1960; Myers and French, 1960; Fork, 1963; Govindjee, 1963), and the reduction of $NADP^+$ (R. Govindjee *et al.*, 1962, 1964; P. Joliot *et al.*, 1968; Avron and Ben-Hayyim, 1969; Sun and Sauer, 1970). It also accounts for the reduction of cytochromes (components of pool A) by photosystem II and their oxidation by photosystem I (Duysens and Ames, 1962; Cramer and Butler, 1967); for reference to antagonistic effect on other intermediates, see the review of Vernon and Avron (1965).

The above picture of the two light reactions and the two pigment systems is consistent with fluorescence data. Kautsky *et al.* (1960) suggested two light reactions (but not two pigment systems) to explain their data on the time course of fluorescence. It is known that light absorbed by photosystem II causes the fluorescence yield of system II Chl a to increase, whereas light absorbed by photosystem I has the opposite (quenching) effect (Govindjee *et al.*, 1960; Butler, 1962; Duysens and Sweers, 1963; Munday and Govindjee, 1969b; Mohanty *et al.*, 1970). According to Duysens and Sweers (1963) light reaction II reduces a fluorescence quencher (Q), while light reaction I reoxidizes it by the intermediary of the pool A. Further ramifications of this theory and the newer relationships will be discussed later (see Sections 3.6 and 4.4).

1.4 The Fluorescence Induction

Light energy absorbed by the pigment systems has three possible fates: (a) to be used in photosynthesis (k_p), (b) to be radiated as fluorescence (k_f), and (c) to be lost in other processes including energy transfer to weakly fluorescent Chl a and heat (k_h). As a first approximation, the yield of Chl a fluorescence (ϕ_f) is an inverse measure of the efficiency of the photosynthetic process (ϕ_p).

$$\phi_f = k_f/(k_f + k_h + k_p), \quad \phi_p = k_p/(k_f + k_h + k_p)$$

This is why Chl a fluorescence has often been regarded as the inefficiency index of photosynthesis. Fluorescence simply competes with photosynthesis. This competition is seen from the increase in the fluorescence yield when (a) photosynthesis is poisoned (Kautsky and Zedlitz, 1941; Duysens and Sweers, 1963; U. Franck *et al.*, 1969), or (b) it is light-saturated (Shiau and Franck, 1947; J. Franck, 1949; Brugger, 1957; Krey and Govindjee, 1966; Bonaventura and Myers, 1969), or (c) the temperature is lowered (see U. Franck *et al.*, 1969). Similarly, it is evident from the antiparallel time course traced by the rates of O₂ evolution (or CO₂ uptake) and fluorescence during a large portion of the fast fluorescence transient (Kautsky and Hirsch, 1931; McAlister and Myers, 1940; Delosme *et al.*, 1959; P. Joliot, 1965b; Bannister and Rice, 1968). This competition between O₂ evolution and fluorescence is, however, not general. For example, parallel increase of both fluorescence and O₂ evolution are observed after prolonged illumination (Papageorgiou and Govindjee, 1968a,b; Bannister and Rice, 1968; Bonaventura and Myers, 1969). (Perhaps changes in k_h are responsible for this phenomenon.)

When dark-adapted photosynthetic organisms are subjected to continuous intense illumination, the following changes in the yield of Chl a fluorescence are observed (Fig. 3). At zero illumination time, the Chl a fluorescence yield rises instantly to the initial level O (origin), followed by an increase to an intermediate level I, a dip or a plateau D, and a high peak P. From peak P, there is a decline to a quasisteady state level S. The notation OIPS for the fluorescence transient was used by Lavorel (1959) and P. Joliot and Lavorel (1964). The label D for the first dip was used by Munday and Govindjee (1969a). Preparations of higher plant chloroplasts, in the absence of added oxidants, show the biphasic rise (OIDP) although no clear dip (D) is observed (see Forbush and Kok, 1968). The decay of P to S is extremely slow in such chloroplasts. However in the presence of oxidants or cofactors of the cyclic electron transport, Chl a fluorescence yield in chloroplasts decays

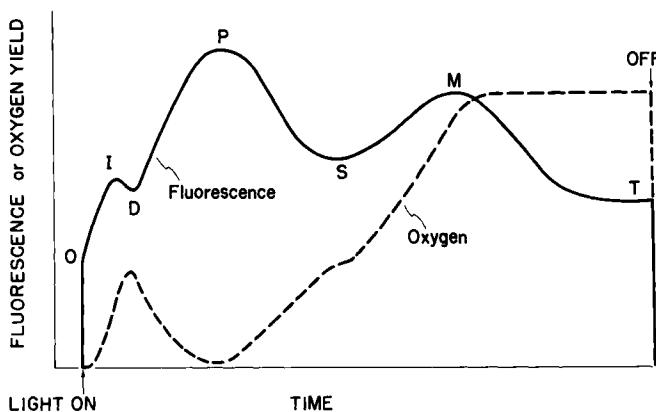


FIG. 3. Changes in the fluorescence yield of Chl a and of the yield of O_2 evolution, as a function of time of illumination (diagram, not drawn to scale).

to a low level (Malkin and Kok, 1966; Murata and Sugahara, 1969).

Both in the chloroplasts and in the algae, the intensity of fluorescence at the level O is referred to as "constant," and the difference in the fluorescence yield (ΔF) between P and O, i.e., P-O, as variable fluorescence. It is generally assumed that it is only the variable fluorescence that reflects changes in photochemistry (cf. Clayton, 1969); the yield of variable fluorescence increases with light intensity saturating at high intensity, but the yield of constant fluorescence is independent of intensity (Lavorel, 1963; Munday and Govindjee, 1969a; de Klerk *et al.*, 1969). The nature of fluorescence at O is not yet clear. Its yield remains constant when photochemistry changes; perhaps, it originates from the "bulk" chlorophylls of systems I and II before the energy is trapped at the reaction centers.

In whole cells, the OIDPS transient—the fast change—is over within 2 seconds (at medium intensities), and is known as the *first wave* of fluorescence induction.

Level S is not a real steady-state level, because in whole cells, e.g., of *Chlorella*, *Chlamydomonas*, *Porphyridium*, and *Cyanidium*, it is followed by a rise to a maximum M (or plateau) from where the yield declines to a terminal steady level T. In blue-green algae, e.g., *Anacystis*, *Plectonema*, and *Phormidium*, the decline M to T is extremely slow requiring several minutes (Papageorgiou and Govindjee, 1967). The SMT transient—the slow change—is also known as the *second wave* of fluorescence induction (see Papageorgiou and Govindjee, 1969).

Leaves of higher plants show a pronounced PSMT phase at elevated

carbon dioxide tensions (McAlister and Myers, 1940; J. Franck *et al.*, 1941). Under normal conditions one sees a slow decline from P to T with a shoulder for M. (For variations in fluorescence transients in different organisms, see U. Franck *et al.*, 1969.)

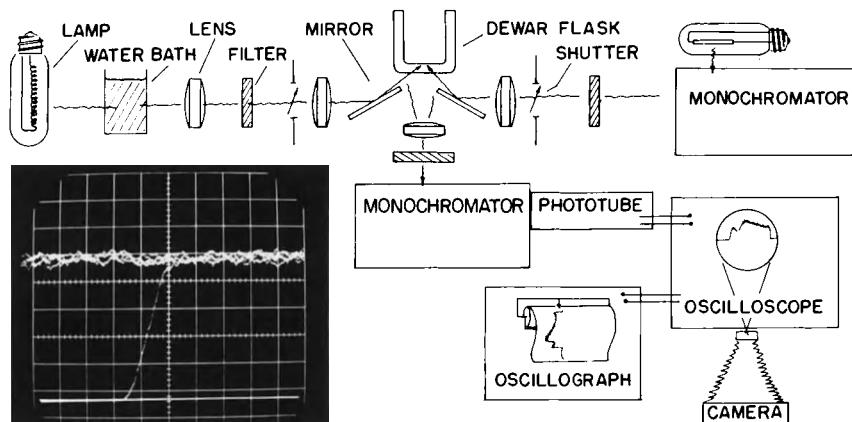
2. Techniques

The general techniques for measuring fluorescence have been discussed at length by Ellis (1966) and by Udenfriend (1962, 1969). In what follows, we will discuss briefly the instruments used for the measurement of the time course of Chl fluorescence *in vivo*.

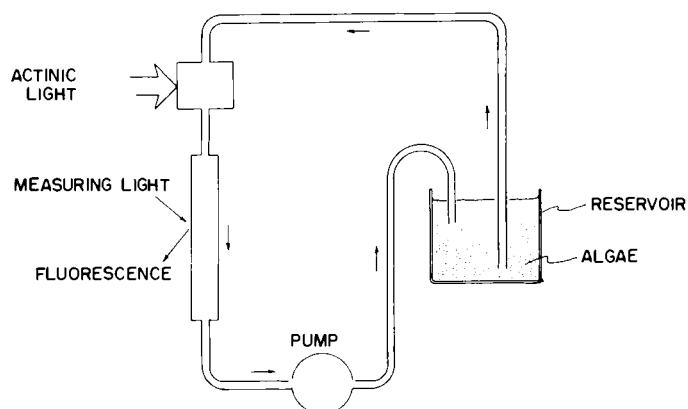
2.1 Stationary Method

The instruments in this category utilize samples which remain stationary in the sample holder. Changes in chlorophyll fluorescence yield with time are measured directly. In the simplest instrument, the sample is illuminated with a continuous (unmodulated) bright light of fixed intensity. This type of instrument has been used in all the older work on fluorescence induction [for recent use and modifications, see Delosme (1967) and Munday and Govindjee (1969a)]. Figure 4(a) shows the details of an instrument for the direct measurement of fluorescence induction in algae and in chloroplasts. At the intensity used in some experiments ($\sim 10^4$ ergs cm^{-2} sec $^{-1}$), transient changes of the fluorescence yield of Chl a are observed in a few milliseconds from the instant of illumination. Accordingly, photographic compur shutters are quite adequate for the sharp transition from darkness to light. These, with the narrowing of the iris diaphragm, provide opening times of about 1–2 msec. Much shorter opening times (a few microseconds) are needed if the intensity of excitation is increased to study the purely photochemical aspects of OI transition. This is achieved by shooting a metallic shutter out of the light path (Delosme, 1967). The fluorescence signal is either displayed and photographed on the screen of an oscilloscope or it can be recorded by a fast oscillographic recorder.

In a quite different instrument, one can assay the fluorescence yield with a weak modulated beam, the fluorescence yield changes are affected by other noninterrupted, i.e., continuous, bright actinic beams that cause specific photochemical change (e.g., the reduction of photosynthetic electron transport intermediates) (Duysens and Sweers, 1963). This instrument responds only to changes in the yield by the weak modulated beam because a tunable amplifier passes only the modulated signal. With this instrument, changes in the fluorescence yield can be amplified and measured precisely.



a



b

FIG. 4. (a) Diagram of a fluorometer for measuring fluorescence transients in algae and chloroplasts. It includes Bausch & Lomb monochromators, an EMI 9558B phototube, a Tektronix 502A oscilloscope, a Midwestern Instruments 801B oscillograph, and compur shutters from Burke and James; the vertically slanting curve in the insert shows the phototube signal during a shutter opening (horizontal scale: 1 msec/division). (After Munday and Govindjee, 1969c.) (b) Block diagram of the flow apparatus (redrawn from Lavorel, 1965).

2.2 Flow Method

Instead of having the fluorescing sample stationary, as in the methods described in the previous section, it can be made to flow at a regulated rate through a transparent capillary tube (Lavorel, 1962; 1965; Vreden-

berg and Duysens, 1965). The light exposure time of the sample, in this case, becomes a function of the flow rate and of the area illuminated. The fluorescence time course can be obtained by first flowing the sample, and then suddenly stopping the flow. Using different flow rates one can measure the yield of the different phases of the transient in a quasi-steady state.

Figure 4(b) shows a portion of the flow apparatus. The sample is drawn into the capillary tube by a syringe and a valve pump, and then returned to a reservoir. In the measuring compartment a strong measuring light assays the fluorescence yield at some point of the transient. When the flow is suddenly stopped, the changes in yield with time are recorded. Changes in fluorescence yield can also be made by an actinic illumination upstream of the flow of algae. The instrument of Vredenberg and Duysens (1965) is very similar to that of Lavorel (1965) except that their measuring beam is weak and is modulated; the changes in the yield are caused by continuous actinic beams.

To measure the emission spectra of variable and constant fluorescence, Lavorel's instrument is used as follows. The sample is subjected to repeated flow-rest cycles; the signal oscillates between a minimum (O) corresponding to the flow part of the cycle, and a maximum (P) corresponding to the rest part. These measurements are repeated at different wavelengths of observation to get fluorescence spectra. By adjusting the flow rate, fluorescence spectra can be obtained at any stage of the fluorescence induction. These spectra provide information regarding the pigment systems involved at various stages of the transient.

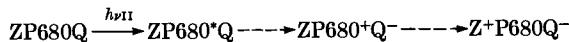
3. Fast Fluorescence Yield Changes and Electron Transport

3.1 Reactions at the Reaction Center II

The quantum yield of Chl *a* fluorescence depends on the rate of the primary photochemical reaction at the reaction center II. A knowledge of the mechanism of this reaction is, therefore, essential for the understanding of the relationship between Chl fluorescence and electron transport.

As noted in Section 1.3, the reaction center of system II is visualized as ZP680Q, where Z and Q are the unknown primary electron donor and acceptor of system II, and P680 is the energy trap of that system. Earlier suggestions of the existence of an energy trap in system II came from low-temperature fluorescence spectra of algae, chloroplasts and subchloroplast fragments (system II). An emission band in the region of 693–698 nm (F698) at 77°K, is preferentially excited by pigment system II, and originates in a Chl *a* species present in very small quanti-

ties; it has been postulated that this Chl a species fluorescing at 698 nm, and having an absorption band in 680–685 nm region, is the energy trap of system II (Bergeron, 1963; Brody and Brody, 1963; Govindjee, 1963, 1965; Broyde and Brody, 1966; Boardman *et al.*, 1966; Murata, 1968; Donze and Duysens, 1969; Cho and Govindjee, 1970a,b,c). However direct evidence for the existence of an active Chl a in system II (Chl a_{II} or P680–690) was first presented by Döring *et al.* (1967, 1968; 1969; also see Govindjee *et al.*, 1970; Floyd *et al.*, 1971) in spinach, Swiss chard and maize chloroplasts. Using a repetitive flash technique, Döring *et al.* discovered a light-induced absorbance change in pigment system II that decays, at room temperature, with a half-time of 0.2 msec (in contrast to 20 msec for P700). It is absent in system I particles, has peaks at 435, 640, and 682 nm, is abolished when DCMU ($10^{-6} M$) is added to the system, has the same dependence on intensity as O₂ evolution, and is present in a concentration of one per several hundred to a thousand Chl molecules. Floyd *et al.* (1971) have shown that the 680 nm absorbance change occurs also at 77°K, and at that temperature it recovers biphasically with half times of 30 μ sec and 4.5 msec (in contrast to P700 that recovers monophasingly with a half-time of 30 μ sec at 77°K). On the basis of the above observations, this absorbance change appears to be related to the reactions of system II. Döring and co-workers have shown that any condition that leads to the destruction of P680 always stops photosynthesis, but the reverse is not true. For example, if chloroplasts are heated to 50°C for 5 minutes, or washed with high concentrations of tris, or treated (and washed) with "wet" heptane, P680 change remains but electron transport ceases. We consider it likely that P680 is indeed the energy trap of system II. However it is still not clear how this trap operates. Döring *et al.* (1969) believe that it acts as a sensitizer without directly engaging in an oxidation-reduction reaction. But why, then, should it undergo absorbance change? Floyd *et al.* (1971) have expressed the possibility that P680 is oxidized in light and, under normal photosynthesis, recovers its electron indirectly from water. This could be represented as follows.



We consider this mechanism likely because (1) an excited molecule has a tendency to eject an electron rather than accept one; (2) the level of Chl fluorescence is determined by the abundance of reduced Q; hence oxidized Q quenches the Chl a excitation (Duysens and Sweers, 1963); this would suggest that $\text{ZP680}^*\text{Q} \longrightarrow \text{Z}^+\text{P680Q}^-$ is not possible because fluorescence rises within a few microseconds of illumination (Sybesma and Duysens, 1965; Delosme, 1967), and (3) oxidized Z can accumulate without a change in the fluorescence yield (P. Joliot, 1968).

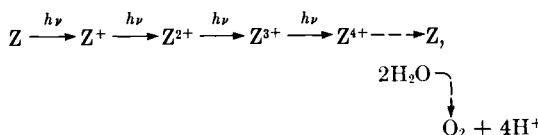
The nature of the primary electron acceptor (Q) is not clear. There is the possibility that it is a special minor fraction of quinones. Ames and Fork (1967) showed that $70 \mu M$ of certain oxidized quinones can quench 50% of the variable fluorescence rise. They have further demonstrated that this quenching was not due to the stimulation of the electron transport because it occurred even in the presence of the powerful inhibitor of electron transport 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). Kohl and Wood (1969) have shown that a light-induced electron-spin resonance (esr) signal of system II is absent in chloroplasts from which quinones have been extracted with heptane, and this signal is restored if certain plastoquinones are added back. Kohl and Wood suggested that this esr signal may be due to the plastochromanoxyl as well as plastosemiquinone free radicals formed from plastoquinone. If esr signal II indeed arises from the primary electron acceptor of system II, then it follows that Q may also be a type of plastoquinone (PQ). Stiehl and Witt (1969) suggested that two electron chains are arranged in parallel such that a pair of P680 are in contact with a pair of PQ-PQ. In this model, plastoquinone is reduced by P680 to PQ^- - PQ^- ; this is followed by a dismutation to PQ^{2-} and PQ; and lastly two single PQ^- molecules are formed in the plastoquinone pool by a redismutation reaction. Stiehl and Witt (1969) attributed to the postulated semiquinone PQ^- - PQ^- the absorbance change they discovered at 320 nm. R. Govindjee *et al.* (1970) found that extraction of quinones with "wet" heptane leads to an increase in the constant fluorescence (level O) and a decrease in the variable fluorescence; this is explained by assuming that heptane also extracts a part of " Q " that may be a quinone-type compound. Thus, there is only vague evidence that Q is a type of plastoquinone. In practice, Q is only recognized by its ability to quench fluorescence (Duygens and Sweers, 1963). When it is reduced to QH, fluorescence yield rises. Therefore one can measure the light reaction II by the initial rise in fluorescence with time (OI phase of fluorescence transient). However further reduction of Q eventually becomes limited by the size and the state of the pool A because Q^- reacts with A to restore Q and A^- .

Recent experiments by Erixon and Butler (1971) show that there is one-to-one correspondence between the redox changes of another compound C-550 (Knaff and Arnon, 1969a) and of Q . Thus, there is the likelihood that Q is identical to C-550.

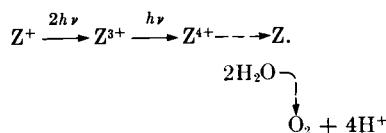
The chemical nature of Z is also unknown. It seems that oxidized Z decomposes water to oxygen in a process that shows a requirement for Mn ions. One may speculate therefore that Z contains Mn (cf. Cheniae, 1970). Although we know little of its chemical composition, we do know a great deal about the reactions of Z from the recent work of P. Joliot (1968), P. Joliot *et al.* (1969), Kok *et al.* (1970), and Forbush *et al.*

(1971) on O₂ evolution in flashing light (also see Kok and Cheniae, 1966). Let us briefly review the situation.

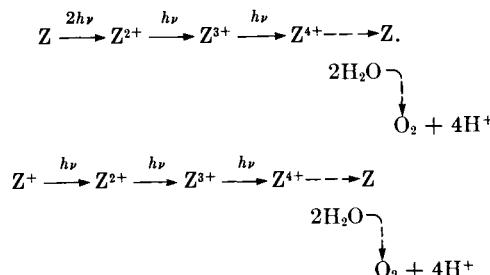
The reduction of Q is a one-electron process (Kautsky *et al.*, 1960; Delosme, 1967), but to evolve one O₂ molecule (Z response) four electrons must be transferred from H₂O to oxidized Z. P. Joliot *et al.* (1969) illuminated dark adapted photosynthetic specimens by a sequence of strong flashes and monitored the oxygen yield per flash under conditions that each photosynthetic unit received one quantum per flash and there was enough time for Q⁻ to return to Q between the flashes. The O₂ signal was largest at the third flash, other maxima were at the seventh and the eleventh flashes. There was a periodicity of four, but the oscillations damped off. Forbush *et al.* (1971) recently found that the detailed pattern of this result in chloroplasts is very different if the dark adaptation period is preceded by 1, 2, or 3 light flashes. They suggest, to explain these results, that in chloroplasts long lasting species of Z exist; in darkness there is 80% of Z⁺ and 20% Z. Kok's model for O₂ evolution could be formulated as follows (leaving aside the P680 and Q)



where solid arrows indicate reactions in single flashes and dotted arrow indicates a dark reaction. To explain the small amount of O₂ evolution in the second flash, Kok and associates assume a small probability of double hit, i.e., during one flash two quanta hit a photosynthetic unit, so that the following reaction is possible:



To explain the high yield in the third flash, the following reactions are suggested:



Kok's detailed model also incorporates the idea of some "misses," i.e., the unit receiving the quantum does not do anything. With these assumptions, all the kinetics of O_2 evolution are explained. However we wish to emphasize that there are alternate ways of explaining the kinetic data on O_2 evolution; Mar (1971) has evolved two new detailed models that also qualitatively explain all the results.

It is important to note that if Z is kept oxidized Q cannot be reduced by light, and the fluorescence rise with time cannot be expected. However if Q is present in its oxidized state, it should be possible to reduce it by first reducing Z by an external electron donor. Yamashita and Butler (1969) showed that washing chloroplasts with high concentration of tris (0.8 M; pH 8) stops the electron transport from H_2O to Z, and keeps the fluorescence at a low level. However if hydroquinone (or reduced phenylenediamine) is added, a light-induced fluorescence rise occurs. Q can also be reduced directly by dithionite ($Na_2S_2O_4$).

3.2 The OI Phase

When dark adapted cells are illuminated with light of moderate-to-low intensities, the rate of O_2 evolution is initially zero, and the fluorescence yield is low (all the traps are open as all molecules of Q are in the quenching form). Then, the fluorescence yield increases from O to I, but the rate of O_2 evolution remains zero for a while, accelerating slowly to a maximum. During this phase, fluorescence yield increases simultaneously (Delosme *et al.*, 1959; P. Joliot, 1965b, 1968; Bannister and Rice, 1968). At these intensities of illumination, the OI phase measured by several investigators (cf. Munday and Govindjee, 1969a) does not reflect a purely photochemical reaction as it includes the dark reaction of Q^- and A.

The lag in O_2 evolution—mentioned above—proportionally decreases as intensity of light is increased suggesting the reactions' photochemical nature. This lag, most clearly observed in very weak continuous light (P. Joliot, 1968), exists because two or four oxidizing equivalents must accumulate on Z in one photosynthetic unit before a molecule of O_2 will evolve (see Section 3.1). At time zero, the observed fluorescence is due to the slight inefficiency of the trapping process. During the lag period in O_2 evolution, fluorescence yield rises because of the reduction of Q to Q^- . With the intensity of light used, we deal with two processes—one that closes the traps because of the conversion of Q to Q^- , and the other that reopens the trap in a dark reaction with A: $Q^- + A \rightleftharpoons Q + A^-$. We imagine that the rate of this reaction is slow, i.e., the rate of closing of traps exceeds the rate of their reopening. Consequently fluorescence rises during this phase.

The oxygen begins to evolve as soon as some units have accumulated four oxidizing equivalents (if we assume Kok's model for O_2 evolution). The reopening of traps and O_2 evolution occurs because, as mentioned above, the weak photoreductant Q^- is restored to Q by a pool of A. However even during this phase, the closing of the trap exceeds their reopening, and fluorescence rises in parallel with O_2 (Fig. 5).

The purely photochemical O to I rise is observed only with very strong light (Delosme, 1967); almost all Q are reduced before Q^- interacts with A. If this is true, then no trap will be able to accumulate more than one oxidizing equivalent on Z, and no oxygen should evolve during this time (microsecond range), although fluorescence will rise to maximum. To our knowledge, parallel O_2 measurements at such high intensities are not available. Such measurements are not easy to obtain as we have to worry about the response time of the O_2 electrode. However in the presence of DCMU which is assumed to block the reaction of Q^- and A, the pure photochemical OI phase can be measured without any O_2 evolution.

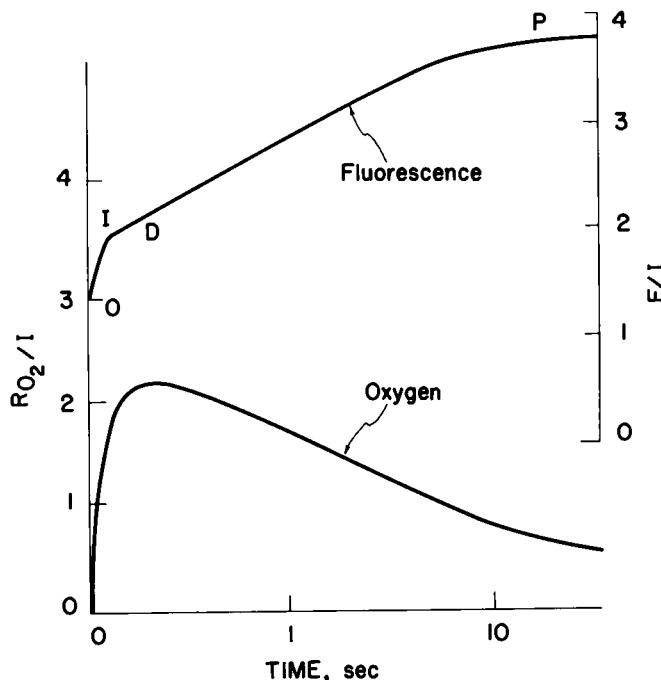


FIG. 5. Time course of the yield of fluorescence and of the rate of O_2 evolution as a function of time of illumination in the green alga *Chlorella*. Temperature, 5°C (redrawn from P. Joliot, 1968).

After the OI phase, a plateau or a decline ID is observed. Obviously a plateau will be observed if the fluorescence yield remains constant meaning thereby that the rates of formation and utilization of Q^- are equal. At such time, a more balanced overall reaction becomes possible, and a peak in O_2 evolution is observed.

The decline ID is observed more clearly in anaerobic cells although it is present in aerobic cells too (Kautsky and Franck, 1943; Munday and Govindjee, 1969a; U. Franck *et al.*, 1969) (Fig. 6). In anaerobic suspensions of *Chlorella*, Kautsky *et al.* (1960) found that if they replaced continuous illumination with periodic light flashes, the ID decline was delayed even when the total number of absorbed quanta remained the same. Thus the ID decline requires light. Munday and

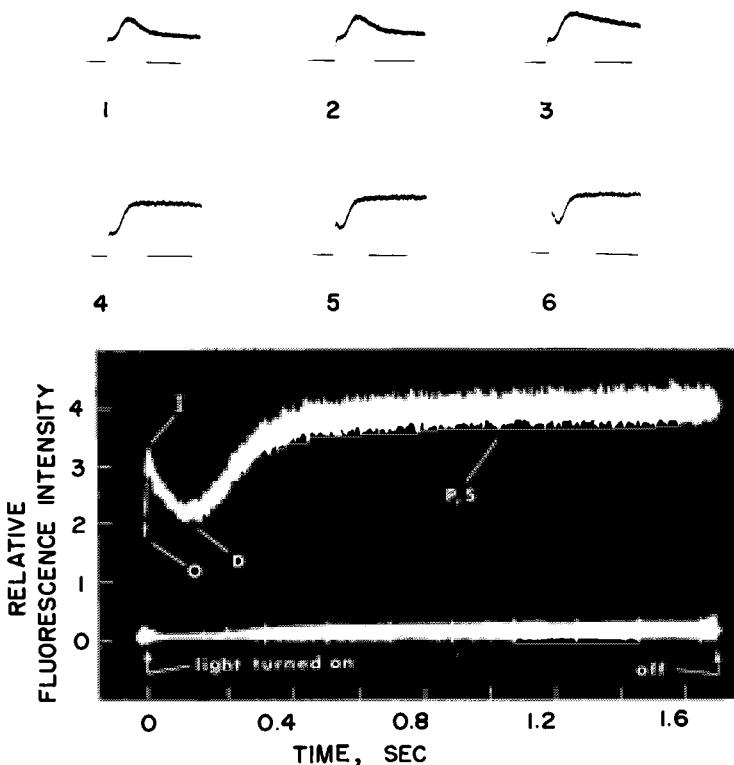


FIG. 6. Changes of the fluorescence transient in *Chlorella* during oxygen removal. The small photographs were taken at 4-minute intervals as 2.0% CO_2 in air was replaced by 2.6% CO_2 in argon. Replacement began a few seconds after photograph 1. The effect was complete by photograph 6, as subsequent transients were identical to that of number 6. The large photograph, from a different anaerobic experiment, shows the distinction between O, I, D, and P (after Munday and Govindjee, 1969a).

Govindjee (1969b) found that for the same number of absorbed quanta, the 705 nm (system I) background light gave only a slightly greater ($\sim 15\%$) effect in accelerating the ID decay than the 650 nm (system I + II) light. A reoxidation of Q^- by system I could account for the dip. However this interpretation needs to be modified because 650-nm light should have given a lower effect than what was observed. It appears that both systems I and II are almost equally effective in causing the dip.

What could this be due to? It appears that both pigment systems are involved in the ID decline as follows. A^- can be reoxidized in two ways: by system I, and by a reaction with O_2 molecules involving system II. Reoxidation of the pool A by O_2 (a Mehler type reaction; Mehler and Brown, 1952) has been suggested by Murata *et al.* (1966a) and Malkin (1968) as an explanation of the lower fluorescence yield at weak excitation intensities. This flow of electrons to O_2 is also suggested by the oxidation of photosynthetic carriers (Q^- , A^-) in darkness. (See also de Kouchkovsky and Joliot, 1967, for sites of action by O_2 .) Kok *et al.* (1966) suggested that a part of A pool is sensitive to O_2 . That molecular O_2 may quench fluorescence by a reaction with A^- can also be inferred from the higher fluorescence yields in anaerobic than in aerobic cells poisoned with DCMU. The intensification of fluorescence yield on the addition of sodium dithionite to DCMU poisoned *Chlorella*, observed by Homann (1968a,b), may also be due to the elimination of this O_2 effect. This, of course, implies that in an aerobic condition some Q are never converted to Q^- .

In apparent contradiction to the oxidation of A^- by O_2 as being the cause of the fluorescence dip (D) is the appearance of a prominent dip obtained with anaerobic algae. It must be borne in mind, however, that the O_2 evolution spike ("gush") is higher in anaerobic than in aerobic samples (Vidaver and Chandler, 1969). Moreover, the decline from the peak is more rapid in anaerobic than in aerobic cells as if O_2 produced is used up more quickly in the former case. This, along with the effective system I reaction, could explain the greater dip in anaerobic samples.

3.3 The DPS Phase

After the completion of the OID phase, the rates of oxygen evolution and fluorescence yield proceed in an antiparallel fashion (Delosme *et al.*, 1959; P. Joliot, 1968; Bannister and Rice, 1968) (Fig. 5). The rise in the fluorescence yield (DP) is associated in time with a decline in the rate of oxygen evolution, while the decline in fluorescence yield (PS) is simultaneous with an increase in O_2 evolution. The indication, therefore, is that the competition between photochemistry and fluores-

cence is an important determinant of the Chl *a* fluorescence yield at this stage. The kinetic pattern suggests that the sum of the quantum efficiencies of primary photochemistry (ϕ_p) and fluorescence (ϕ_f) may be constant. (The rise and fall of O₂ during the entire OP phase represents the well-known O₂ gush.) The notion of complementarity, however, is of only qualitative significance since the momentary magnitude of the rate of internal conversion cannot be assessed.

In general, the rise DP is attributed to the accumulation of reduced Q and A as a result of system II reaction (Kautsky *et al.*, 1960; Duysens and Sweers, 1963). The decline in O₂ evolution during this phase is due to the depletion of the oxidized pool A. When the oxidized pool A is almost empty, the O₂ evolution is at its minimum; this point in time coincides with the time at which P occurs. The O₂ gush is over.

This simple picture of DP rise may have to be slightly modified because Munday and Govindjee (1969c) found that if methyl viologen (1,1-dimethyl-4,4' dipyridinium chloride), which accepts electrons only from X⁻ the primary reductant produced by PSI (Kok *et al.*, 1965), is added to *Chlorella*, the DP rise is completely eliminated. Munday suggested that since system I has already begun to act near D (see Section 3.2), the cause of the accumulation of A⁻ and Q⁻ lies beyond A in the electron chain. It is the accumulation of X⁻. This is where the real "traffic jam" occurs evidently because the Calvin cycle is too slow to start functioning by the time of the peak P. This accumulation of X⁻ leads to the accumulation of A⁻, and thus, of Q⁻. When methyl viologen is provided, electrons are quickly drawn from X⁻. Thus there is no accumulation of Q⁻, and fluorescence rise DP is abolished. The simple explanation given in the previous paragraph is not complete, however, because it does not account for the fact that A⁻ could be reoxidized to A by system I light, making impossible the complete reduction of A and Q at the time of P. For this reason we believe that Munday's explanation may be closer to reality. His view is also in agreement with the fact that the "O₂ gush" is clearly seen in whole cells of algae, after a dark period where CO₂ reduction is delayed. This is also confirmed with chloroplasts in the absence of added oxidants; here an O₂ gush and an OIDP rise are observed without any CO₂ fixation.

Additional mechanisms must be invoked to explain a recent experiment by Duysens (1970) who found that a brief (microseconds) bright flash of system I light can instantly quench fluorescence yield at P. This experiment is difficult to explain by any of the above theories of DP rise that involve the accumulation of A⁻. System I light can only slowly reoxidize A⁻ to A and thus Q⁻ to Q causing quenching of fluorescence—this would be too slow to explain Duysens' experiment. Hence one has

to postulate a separate mechanism for a more direct quenching of system II fluorescence by bright system I light flashes. Obviously further work is needed to fully understand the DP transient.

The decay of the Chl a fluorescence yield along PS is the least understood part of the fluorescence transient. The fact that this decay is associated with a simultaneous rise in the rate of O₂ evolution points to the reoxidation of A⁻ as a possible cause of this fluorescence decline. Perhaps the Calvin cycle begins to operate then, and the "traffic jam" at X⁻ is removed allowing a balanced system I and system II reaction.

Whenever the net electron transport is at its maximum and is not limited by the oxidant in the system, there is no P. This is the situation with methyl viologen treated *Chlorella* cells, and with ferricyanide treated chloroplasts. When there is no P there is no P to S decline. However, in chloroplasts without added oxidants, there is DP rise, but no PS decline. This is so because all the intermediates (Q, A, X) are reduced during the DP rise, but there is no way to reoxidize them.

The above explanation of P to S, i.e., due to reoxidation of Q⁻ and A⁻, has difficulties. If light is turned off at the quasi-steady state S, where we imagine that most A and Q are in their oxidized states, and then turned on again, the OP rise cannot be observed as if something else has happened. Duysens and Sweers (1963) proposed that a quencher Q' (not capable of reduction by light) is formed. Recently, the concept of such a quencher has been abandoned in favor of another theory in which changes on and in the chloroplast membranes are suggested to occur (see Section 4.4.1). We believe that a comprehensive theory for P to S decline will probably include an interplay of such physical changes and of chemical changes in terms of the oxidation-reduction states of the intermediates Q, A and X; such a theory remains to be formulated.

3.4 Preillumination Effects

Preillumination of the photosynthetic tissue can alter the pattern of Chl a fluorescence kinetics in a manner that depends both on the light-induced shifts in the oxidation-reduction states of the intermediates, and on the slow physical changes (see Section 4.4.1). The picture becomes more complex when we consider that the pool A can interact with both the presystem II oxidants and the postsystem I reductants. Govindjee *et al.* (1966) and Munday and Govindjee (1969b) found that preillumination (or continuous background illumination) of *Chlorella* with weak system I light depresses the levels I and P, DP rise is delayed, PS is slowed down, and the level of S is raised. The decrease in I and P was ascribed to a shift of the A⁻/A equilibrium to a more oxidized

position, an argument supported by the greater effectiveness of far-red (system I) light in causing this effect. Vredenberg (1969) reported similar results with a red alga *Porphyra*; his interpretations were similar to those given above. These fluorescence transient changes are consistent with the data on O₂ evolution. For example, system I preillumination increases the O₂ evolution in system II light (French, 1963; Govindjee and Govindjee, 1965).

The increase in the "S" level, mentioned above, is possibly due to long term effects (see Section 4.4). These effects are also evident in the increased S level observed at higher intensities of illumination (Lavorel, 1959). Treatment with stronger light eliminates the minimum S, the fluorescence decay proceeds monotonously from P to the terminal level T (Bannister and Rice, 1968).

3.5 More about the Pool "A"

The pool A is not homogeneous. At least two kinetically distinct entities are thought to exist (A₁ and A₂). When Forbush and Kok (1968) plotted the amount of DCPIP reduced per flash of light as a function of the duration of the flash, they observed a biphasic rise. If an instantaneous equilibration of DCPIP with pool A is assumed, the amount of reduced DCPIP would be proportional to the amount of A reduced by the flash. The observed biphasic rise was interpreted as evidence for two subpools of A—a fast-reacting A₁ and a slower A₂. Malkin (1966) and Forbush and Kok (1968) obtained a good fit of their experimental data on fluorescence transients with their theoretical curves based on the assumption that two subpools of A exist.

How fast is the reaction between Q and A? P. Joliot (1965a) estimated the half-time constant ($t_{1/2}$) to be 3 msec at 5°C and 1 msec at 20°C. Forbush and Kok (1968) determined $t_{1/2}$ to be 0.6 msec from the measurements of the decay of fluorescence yield in isolated chloroplasts subjected to a saturating flash. It must be kept in mind that this half-time corresponds to a bimolecular rate; $t_{1/2} = 1/k_1 [A]_{total}$, where [A]_{total} represents all A in the oxidized form after darkness.

The half-time obtained from the kinetics of DCPIP reduction (as discussed above) is 4 msec for the fast-reacting component A₁. Since this half-time equals $1/k_1[Q]_{total}$, the ratio of [Q] to [A₁] was estimated (Forbush and Kok, 1968) as $[A_1]/[Q] = 4/0.6 = 7$. As $[A_1] = \frac{1}{3} [A]_{total}$, they obtained $[Q]/[A]$ to be 1/20. Similar values were earlier obtained by Malkin and Kok (1966) and P. Joliot (1965a) from measurements of area over the fluorescence rise curve (the OP transient) with and without DCMU, and from the measurements of the "O₂ gush" respectively.

Another approach, that has been used recently by Kok *et al.*, (1969), was to measure the relative number of electrons flowing through P700 as induced by either a short or long flash. The estimate of $[A]_{\text{total}}/[Q]$ by this method was 10. This ratio is somewhat smaller than that observed when reactions through system II alone were used for calculations. They explain this difference by assuming that photosystem I reacts only or mainly with one of the two subpools.

3.6 Discussion: Theories on Chl Fluorescence Kinetics

Earlier theories on the induction of Chl *a* fluorescence invoked processes which quenched the Chl *a* excitation and which were supposed to compete with photosynthesis. J. Franek and his co-workers (1941, 1945; Shiau and Franek, 1947) ascribed the fast fluorescence rise (O-P) to a chemical inactivation (narcotization) of Chl *a*. The narcotic substances were oxidized metabolites, whose later depletion reactivated Chl *a* and depressed fluorescence yield (P-S). To account for the same decay, Kautsky and Hormuth (1937) suggested quenching of Chl *a* excitation by oxygen. A fluorescence quencher, consumed by a photochemical reaction and regenerated by a subsequent thermal reaction was suggested by Ornstein *et al.* (1938) to account for the fast fluorescence transient (OPS).

The currently prevailing hypothesis for the fast change, discussed in Sections 3.2-3.4, is similar to the above suggestion of Ornstein *et al.* Kautsky *et al.* (1960) explained the fluorescence transient (OIPS) in terms of the two light reactions. In addition Duysens and Sweers (1963) explained the fluorescence transient within the framework of a two-pigment system-two light reactions hypothesis. The quantum yield of the system II Chl *a* fluorescence increases when the primary electron acceptor (*Q*) and the intermediate pool (*A*) are reduced by system II light. The yield decreases when they become oxidized (mainly) by system I light. In principle then, the magnitude of the variable Chl *a* fluorescence, i.e., the fluorescence level above the level 0, reflects the momentary proportion of reduced (closed) photosystem II reaction centers. Several kinetic treatments have been devised. Starting from a number of assumptions, several investigators have attempted to theoretically reproduce the course of the fast fluorescence transient (Malkin, 1966; Murata *et al.*, 1966b; Delosme, 1967; Munday, 1968; Munday and Govindjee, 1969a; Clement-Metral and Lavorel, 1969). The physical identity of each kinetic variable, that appears in the final expressions of these treatments, is not known. A further shortcoming of such treatments is the restricted applicability of the derived kinetic expressions to only the rise portion of the fast transient (OIP); some theories, in fact, do not even allow D. Because

of their present limited utility we will not discuss these kinetic theories here.

Finally, there are suggestions that there may be more than one quencher of fluorescence. Delosme (1967) proposed a second quencher and had called it R. Recently R. Govindjee *et al.* (1970) have suggested two quenchers Q_1 and Q_2 to explain their data on the fluorescence transient of heptane-extracted chloroplasts. Cramer and Butler (1969) obtained two midpoint potentials when they titrated Q. So in view of present evidence, the possible existence of two quenchers needs to be explored. Speculations have even been made that one of these quenchers may not be a real chemical, but simply a state of the matrix surrounding active Chl a_{II} .

4. Slow Fluorescence Yield Changes: The SMT Phase

4.1 General

The slow change in algae consists of a rise of the Chl a fluorescence yield from the level S to a broad maximum M and a subsequent slower decay to the terminal level T (Fig. 7). The decay MT is faster in the green and the red algae than in the blue-greens. (Adequate dark periods or preilluminations with far-red light are needed to repeat these transients.) During the SMT phase, the fluorescence yield of phycobilins (in

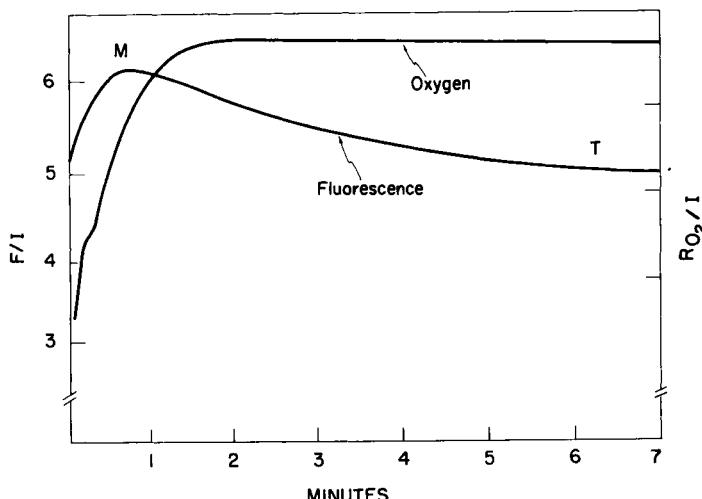


FIG. 7. Time course of the Chl a fluorescence yield and of the rate of oxygen evolution in *Chlorella*. Excitation λ , 480 nm; incident intensity, 3×10^3 ergs cm^{-2} sec^{-1} ; observation λ , 685 nm (after Papageorgiou and Govindjee, 1968b).

red and blue-green algae) does not change (cf. French and Young, 1952; Govindjee *et al.*, 1966). Figure 8 shows the fluorescence spectra of the blue-green alga *Anacystis nidulans* at S and M levels. The difference spectrum (M-S) shows only an increase in the Chl a fluorescence yield, but no decrease in the yield of phycocyanin fluorescence. This suggests that the increase in the yield of Chl a fluorescence is not due to an increase in energy transfer from phycobilins to Chl a. However, these data do not exclude the hypothesis that this increased Chl a yield is due to a decreased energy transfer (spillover) from Chl a of system II to Chl a of weakly fluorescent system I.

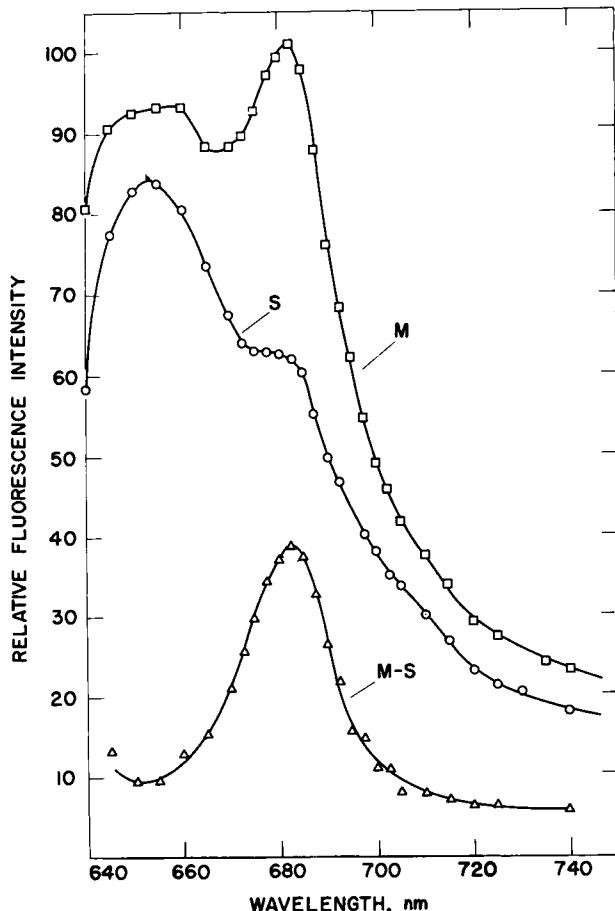


FIG. 8. Emission spectra of normal *Anacystis nidulans* at 3 seconds (level S) and 10 minutes (level M) of light exposure; M-S, the difference spectrum; excitation λ . 590 nm; observation λ , 685 nm (after Papageorgiou and Govindjee, 1968a).

Only the light absorbed by photosynthetic pigments can cause the slow fluorescence change—with green algae it is seen using light absorbed by Chl a or Chl b, while with the blue-green algae the slow change is seen using light absorbed mainly by phycocyanin. This emphasizes the photochemical character of the process and its possible relation to photosynthesis. A relationship with system I-sensitized cyclic electron transport is also inferred because DCMU-poisoned blue-green algae display a slow fluorescence change with light absorbed either by Chl a (system I) or phycocyanin (system II) (Govindjee *et al.*, 1966; Papageorgiou and Govindjee, 1967; Duysens and Talens, 1969).

As in any photochemical process, the SMT change depends upon the intensity of exciting light. At very light intensities, SMT change is absent. On increasing the intensity of excitation, the rates and amplitudes of the induction waves increase and saturate. The light intensity that saturates the yield at M is different than the intensities that saturate S and T (Papageorgiou, 1968). For the dependence of O₂, P, and S, in the fast transient, on light intensity, see Lavorel (1963), Govindjee *et al.* (1966), and Munday and Govindjee (1969a).

4.2 Electron Transport and the Slow Fluorescence Change

The complementarity between the yield of Chl a fluorescence and the rate of O₂ evolution (the rate of noncyclic electron transport), that characterizes the DPS phase of the fast transient, is absent in the SMT phase. During the SM portion, the rate of O₂ evolution rises together with the yield of Chl a fluorescence, and attains a constant level while the fluorescence decays along MT (Fig. 7). Whatever may be the relationship of the slow fluorescence to photosynthesis, it is not a competitive one. Therefore, the fraction of reduced photosystem II reaction centers is of secondary significance in determining the yield of the variable Chl a fluorescence at this stage.

Photosynthetic electron transport is, however, in some way a contributing factor for the slow change. Moreover cyclic electron flow is implicated in the SMT phase more directly than noncyclic. Although, DCMU-poisoned *Chlorella* does not exhibit the SMT change when exposed to light of moderate intensity (~ 10 kergs cm⁻² sec⁻¹), higher intensities of light (~ 50 kergs cm⁻² sec⁻¹) cause this slow fluorescence change (Bannister and Rice, 1968). The evidence for a cyclic flow of electrons *in vivo* is rather indirect (see Teichler-Zallen and Hoch, 1967; Rurainski *et al.*, 1970), but it is generally believed that in DCMU-poisoned cells only cyclic electron flow operates. It can be inferred that intense light supports cyclic electron transport of sufficient magnitude to somehow cause the fluorescence change. The contribution of the cyclic

electron transport also explains the slow fluorescence change of DCMU-poisoned *Anacystis nidulans* (Govindjee *et al.*, 1966) and of *Schizothrix calcicola* (Duysens and Talens, 1969).

Bannister and Rice (1968) were able to demonstrate the relationship of the cyclic electron flow to the slow fluorescence change from the following. They found that a mutant of *Chlamydomonas* having its non-cyclic—but not the cyclic—electron flow impaired was still capable of the slow fluorescence change. On the other hand, mutants missing both types of electron transport did not show the slow fluorescence change. A relationship with the cyclic electron flow (a system I reaction) does not, however, imply that we are now looking at system I fluorescence. It simply means that the system II fluorescence is somehow influenced by system I reactions.

4.3 Phosphorylation and the Slow Fluorescence Change

Strehler (1953) proposed a relationship between photophosphorylation and the fluorescence induction phenomena. He demonstrated a correlation between the time course of Chl a fluorescence yield and of the ATP content in *Chlorella*. Recently further evidence for the involvement of photophosphorylation has been acquired by the use of uncouplers of phosphorylation (Papageorgiou, 1968; Papageorgiou and Govindjee, 1968a,b). These uncouplers prevent the synthesis of ATP while permitting electron transport. In fact the electron transport is accelerated in chloroplasts performing Hill reactions since in the presence of uncouplers a rate limiting step is bypassed (Good *et al.*, 1966). In whole cells, however, the action of uncouplers is not so simple. A decrease in the concentration of photoproduced ATP could lead to a decrease in the rate of reactions of the Calvin cycle, and this indirectly decreases electron transport.

The powerful uncoupler of photophosphorylation FCCP (*p*-trifluoromethoxyphenyl hydrazone of ketomalonyl nitrile) at concentrations as low as 0.4 μM reduces the amplitude of the slow fluorescence change in *Schizothrix calcicola* by a factor of three (Duysens and Talens, 1969). At higher concentrations (1–10 μM), when it only partially inhibits the electron transport in whole cells, FCCP abolishes the MT phase in *Chlorella* and the rise SM in *Anacystis* (Papageorgiou, 1968; Papageorgiou and Govindjee, 1968a,b) (Fig. 9). FCCP not only abolishes the slow fluorescence induction of DCMU-poisoned *Chlamydomonas*, but also lowers the Chl a fluorescence yield, an observation for which no adequate explanation exists (Bannister, 1967; Bannister and Rice, 1968).

Murata and Sugahara (1969; also see Govindjee *et al.*, 1967) found that the fluorescence yield of DCMU-treated chloroplasts is slowly

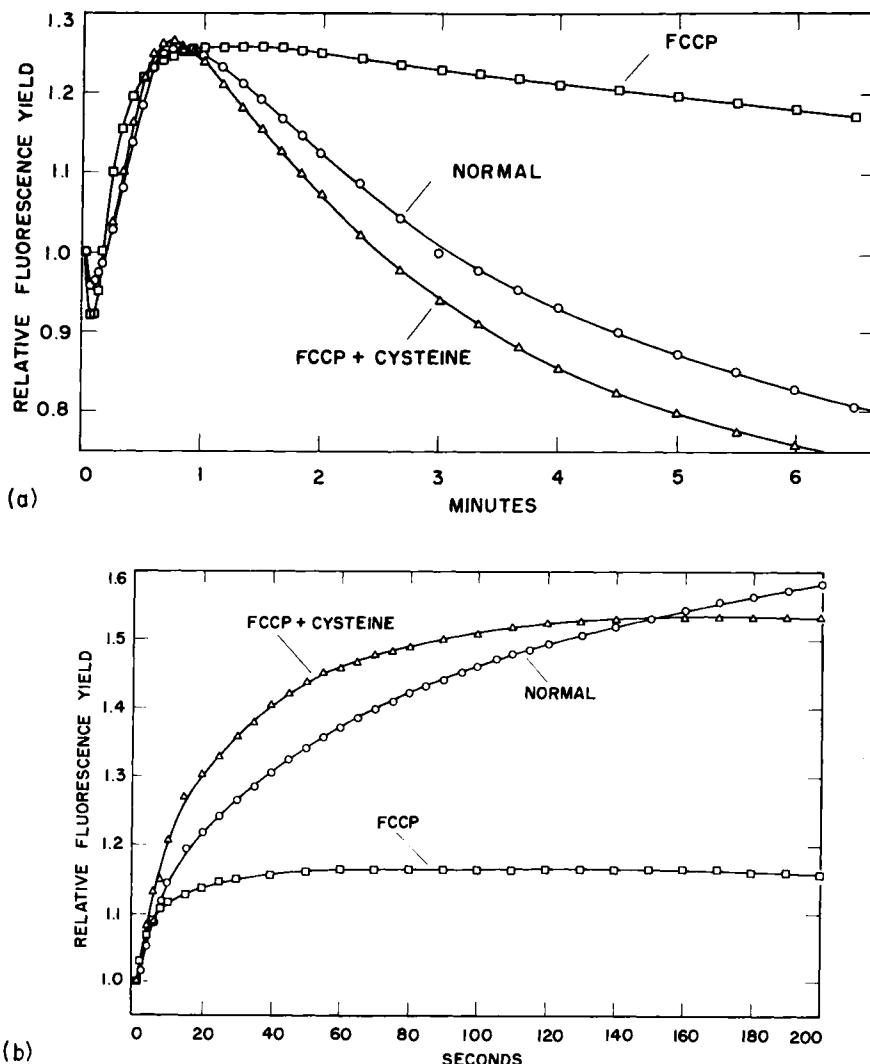


FIG. 9. (a) Time course of the relative fluorescence yield in *Chlorella pyrenoidosa* normalized at S. Control; with $3 \times 10^{-5} M$ FCCP; with $3 \times 10^{-5} M$ FCCP and $10^{-3} M$ cysteine; excitation λ , 480 nm; observation λ , 685 nm (after Papageorgiou and Govindjee, 1968b). (b) Time course of the relative fluorescence yield in *Anacystis nidulans* normalized at S. Control; with $3 \times 10^{-5} M$ FCCP; with $3 \times 10^{-5} M$ FCCP and $10^{-3} M$ cysteine; excitation λ , 590 nm; observation λ , 685 nm (after Papageorgiou and Govindjee, 1968a).

quenched by the addition of PMS. This depression of the fluorescence yield by PMS was reversed if an uncoupler of phosphorylation CCCP (*p*-chlorophenyl hydrazone of ketomalonyl nitrile) was added to the sample containing DCMU and PMS. The effect of CCCP, described above, seems opposite to that found by Bannister (1967). It is difficult to compare the two experiments because of the different systems and effective concentrations of uncouplers used. However this experiment supports the idea that uncouplers of phosphorylation influence the slow fluorescence changes.

The inhibition of the slow fluorescence change by uncouplers of phosphorylation is particularly pronounced at low intensities of light. At an incident intensity of $3.1 \text{ kerg cm}^{-2} \text{ sec}^{-1}$ ($\lambda = 480 \text{ nm}$) half-maximal inhibition in *Chlorella* is obtained at $4.5 \mu\text{M}$ of FCCP. This inhibition of the slow fluorescence change is reversed on adding cysteine hydrochloride (Papageorgiou, 1968; Papageorgiou and Govindjee, 1968a, 1969). These observations parallel those of increased uncoupling activity of FCCP at low light (Avron and Shavit, 1963) and the reversal of its activity by aminothiols (Heytler, 1963).

Another powerful uncoupler, atabrin, proved to be as effective as FCCP in suppressing the slow fluorescence change in algae and in isolated chloroplasts.

Park *et al.* (1966) have shown that phosphorylation and certain electron transport reactions that lead to CO_2 fixation do not occur if intact algae cells are fixed with glutaraldehyde. Such cells, however, are capable of DCPIP reduction and certain systems I reactions. A recent experiment by Papageorgiou and Mohanty (1969) on glutaraldehyde fixed *Porphyridium* cells show the complete absence of slow fluorescence change. This experiment confirms that electron transfer, involving system II only, is not enough per se to cause slow fluorescence changes (see Section 4.2). In addition, we can argue that since there was no phosphorylation in the fixed cells, there was no slow fluorescence change strengthening our view that phosphorylation and associated processes are somehow related to slow fluorescence changes in whole cells.

4.4 Discussion

4.4.1 GENERAL THEORIES

Wassink and Katz (1939) had recognized that several features of the slow fluorescence change were different from those of its fast counterpart. The slow change had proved to be insensitive to the oxygen content of the gas phase of the sample (Wassink and Katz, 1939; Kautsky and Eberlein, 1939). Carbon dioxide, on the other hand, appeared to play

some role since the "single-wave" kinetics in wheat and in *Hydrangea* were converted to a "double-wave" form at elevated carbon dioxide concentrations (McAlister and Myers, 1940; J. Franck *et al.*, 1941).

Any mechanism describing the events during the slow fluorescence change must take into account (1) the requirement of cyclic electron transport and (2) the apparent independence from the noncyclic electron transport. All hypotheses are essentially similar in the sense that they invoke a slow photoprocess which modifies the photosynthetic and emissive capacity of photosystem II units. This process is coupled to and is controlled by the cyclic electron transport and associated photophosphorylation.

According to Bannister and Rice (1968), the slow fluorescence rise SM is caused by some kind of "activation" of "inactive" photosynthetic units that leads to an increase in O₂ evolution as well as in fluorescence yield; this hypothesis gives no explanation for the slow fluorescence decay MT of the green algae.

Recently Murata (1969a,b) has provided evidence for changes in the spillover of energy from the strongly fluorescent system II to the weakly fluorescent system I—an increase in the fluorescence yield is due to a decrease in this transfer, and a decrease in the yield is due to an increase in the transfer. Thus the PS and MT decline would be interpreted as a consequence of an increase in this transfer and the SM rise to a decrease in this transfer. The experimental results supporting the existence of such a change in the spillover of energy are as follows. Murata (1969a) compared the 77°K fluorescence spectra of algae that were preilluminated with system II light with those that were kept in the dark. He found the fluorescence efficiency of Chl a of system II, which fluoresces mainly at 685 nm and 695 nm, to be lower and the fluorescence efficiency of Chl a of system I, which fluoresces mainly at 720 nm, to be higher in the preilluminated than in the nonpreilluminated sample. This suggests that preillumination with system II light causes an increase in the efficiency of energy transfer from system II to system I. A decrease in this transfer is caused by treating chloroplasts with Mg²⁺ (and other divalent ions). This is shown from the observed increase in the fluorescence yield of system II Chl a concomitant with a decrease in the fluorescence yield of system I Chl a (Murata, 1969b; Murata *et al.*, 1970; Mohanty, 1969). The decrease in transfer from system II to I is further confirmed by the observed increase in the rate of system II reaction (DCPIP reduction) and a decrease in the rate of system I reaction (NADP⁺ reduction with added DCPIPH₂, in the presence of DCMU).

The hypothesis of Murata (1969a) is consistent with that of

Bonaventura and Myers (1969) who proposed a variable distribution of the absorbed photons in the two photosystems. Bonaventura and Myers (1969) defined two states of the chloroplast: (1) *light state 1* in which quanta absorbed by system II remain mostly in system II and are not transferred effectively to system I; this state is created by illumination with system I light, or by prolonged darkness; and it has a high fluorescence yield, (2) *light state 2* in which quanta absorbed by system II are transferred to system I; this state is created by prolonged illumination with system II light; and it has a low fluorescence yield. To quantitate this variable distribution of quanta in the light states, Bonaventura and Myers defined a fraction α/α_{\max} , where α is the fraction of photons delivered to system II, and α_{\max} is the maximum value that α can attain. For state 2, this fraction is 0.9, and for state 1, it is 1.0. The difference is only 10%. Duyens (1970) has given a visual picture to these states (Fig. 10). He explains the transitions from one state to another as an energy dependent movement of pigment molecules of system II away from or closer to system I. When they are away from each other, state 1 exists, fluorescence yield is high, α is 1.0, and energy transfer is minimal. When they are close to each other, state 2 exists, the fluorescence yield is low, α is 0.9 and consequently energy transfer from system II to system I is maximal. Thus in addition to the control of fluorescence yield by the concentration of Q, we have to concern ourselves with the changes in the movement of chloroplast membranes.

The above hypotheses have been used to explain the changes in fluorescence yield in algae. A rapid quenching followed by an increase of the fluorescence yield of system II was observed upon addition of system I light in *Porphyridium* (Murata, 1969a). When the system I light was turned off, the yield increased further and then decayed slowly to the original level [confirmed by Bonaventura and Myers (1969) and Mohanty *et al.* (1970) in *Chlorella*]. Murata explained the increase in fluorescence yield after prolonged illumination of system I light by a decrease in energy transfer from system II to system I, that is, by the conversion of state 2 to state 1.

When Bonaventura and Myers (1969) replaced system II with sys-

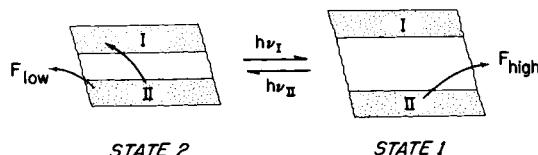


FIG. 10. A hypothetical picture of the two "states" based on the ideas of Duyens (1970), Bonaventura and Myers (1969), and Murata (1969b) (see text).

tem I light, a slow increase in the fluorescence yield—measured by a weak system II light—accompanied a parallel increase in the rate of O_2 evolution in *Chlorella*. This reminds us of the SM rise in *Chlorella* where too, both fluorescence and O_2 rise in parallel (Papageorgiou, 1968; Bannister and Rice, 1968; Papageorgiou and Govindjee, 1968a,b). This fluorescence rise is also explained as due to conversion of state 2 to state 1.

On the other hand, replacement of system I with system II light caused a slow increase in the fluorescence yield although the rate of O_2 evolution did not change significantly (Bonaventura and Myers, 1969). This kinetic pattern is almost identical to the MT phase in *Chlorella* when O_2 rate remains constant while fluorescence yield declines (Papageorgiou, 1968; Bannister and Rice, 1968; Papageorgiou and Govindjee, 1968b). This fluorescence decline is explained as due to the conversion of state 1 to state 2.

Now the PS decline in the fluorescence yield can be explained as resulting from conversion of highly fluorescent state 1 to weakly fluorescent state 2. In this picture, the Q' of Duysens and Sweers (1963) is equivalent to state 2. It must be converted to state 1 by a long dark period or far-red illumination before the OIDP rise can be observed again. This explains the inability to repeat OIDS without such a treatment.

There are some difficulties however, if PSMT changes are indeed entirely due to changes in the “states” of chloroplasts, i.e., P and M occur when chloroplasts are in state 1 and S and T when they are in state 2, then why do we obtain different types of changes in the rate of O_2 evolution during P to S (O_2 declines) as compared to the M-T phase (O_2 remains constant)—when both result from the conversion of state 1 to state 2. Also, why does the rate of O_2 evolution increase when state 2 is transformed into state 1 during the SM phase? Obviously other factors are involved and the slow fluorescence changes are not exclusively caused by the proposed changes in the states of the chloroplast membranes.

Addition of system I light at different points on the SMT transient showed different effects depending upon whether it was added on the SM or the MT phase, even when points having identical fluorescence yields were chosen, representing intermediate but identical “states” of the chloroplast (Fig. 11). On the SM rise part little, but on the MT decline part significant quenching was observed (Mohanty *et al.*, 1970). This observation suggests that identical “states” are not identical in their photochemical reactions. We believe that the SMT fluorescence transient

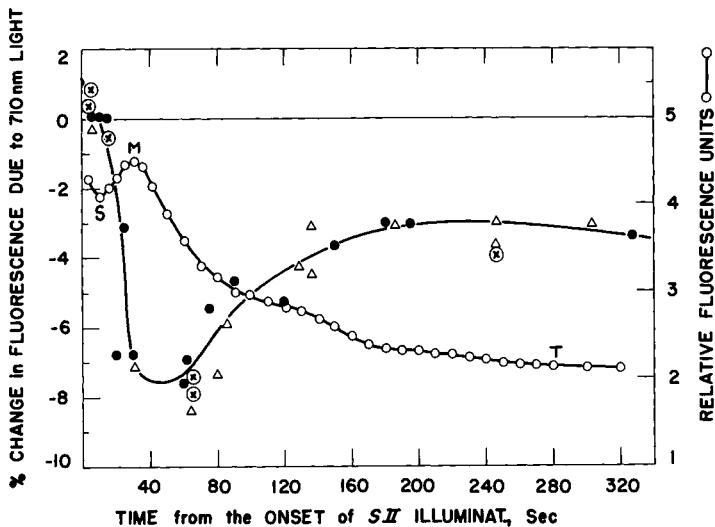


FIG. 11. Percent changes in the fluorescence yield of Chl a due to the addition of 710 nm light at various times during the fluorescence transient (exciting light, blue). Experiments with three different cultures (thick line with open triangles, solid circles, and crossed circles) are shown: the scale to the left. For comparison, the fluorescence transient for the cultures used in these experiments is represented by the curve with a thin line and small open circles: the scale to the right (after Mohanty *et al.*, 1970).

reflects both changes in oxidation-reduction intermediates as well as changes in "states" of the membrane although the exact pattern is not clear. (For the role of changes in ionic gradient, see Section 4.4.2.)

Papageorgiou and Govindjee (1968a,b) had earlier suggested that slow fluorescence changes are due to light-induced changes in the structure of the photosynthetic apparatus. A clue to this was the requirement of phosphorylating electron transport for the slow changes to occur. Phosphorylation is known to be accompanied by changes in the conformation of the thylakoids to a degree that depends upon the rate of electron transport and its phosphorylating capacity (Packer *et al.*, 1970). Whenever phosphorylation is absent, slow changes are absent. (The absence of slow changes in chloroplasts capable of "swelling" and "shrinkage" does not disprove the proposed relationship because the conformational changes may or may not be always related to the observed configurational changes, and vice versa.) It is proposed that the changes in the fluorescence yield need not be only due to changes in energy transfer from system II to system I. The structural changes

could lead to movement of Chl a molecule—within the pigment system II—away from each other leading to a decreased concentration quenching and thus an increase in the fluorescence yield. In this situation, the rate of internal conversion would decrease without affecting the trapping efficiency. On the other hand, a decrease in the fluorescence yield could be due to a movement of Chl a in system II toward each other causing an increased concentration quenching, i.e., an increase in the rate of internal conversion and a decreased fluorescence yield without affecting the trapping efficiency. This would explain the M to T decline without any change in the rate of O₂ evolution.

Whether one believes in the movement of system II and system I, or of Chl a within the system II, one expects light induced conformational changes of the membrane system to cause it. Suggestions for conformational changes have accumulated from several sources. Brody *et al.* (1966) observed spectral changes in fluorescence spectra of *Euglena* chloroplasts treated with concentrated salts [NaCl, MnCl₂, (NH₄)₂SO₄] and urea; they ascribed these changes to the conformational changes of the lipoprotein matrix. Murata (1969b) and Homann (1969) have demonstrated an increase of the steady-state fluorescence yield of Chl a in DCMU-poisoned chloroplasts by the addition of 3–5 mM Mg²⁺. Other cations also have some effect, but the effect is largest with Mg²⁺ (Murata *et al.*, 1970). Mg²⁺ also causes the largest volume changes in chloroplasts suspended in a low salt medium (Izawa and Good, 1966), and conformational changes are known to accompany volume changes. That chloroplast conformation regulates electron flow *in vivo* has received experimental support from the work of Heber (1969): at high light intensity, excessive photoshrinkage of chloroplasts occurring in a variety of leaves does suppress the rate of electron flow.

Light-induced changes in the chloroplast structure have been investigated by following light scattering changes and by electron microscopy (Murakami and Packer, 1970). Two types of structural changes have been distinguished—decrease in the membrane thickness (conformational change), which is thought to be brought about by proton uptake and a decrease in the spacing between the membranes that causes a flattening of the entire chloroplast (configurational change). (This flattening effect has been recently correlated with changes in the ATP level by Nobel *et al.*, 1969.) This only suggests that light-induced conformational changes occur in chloroplasts, but they cannot yet be related to the theories proposed above concerning fluorescence changes. Finer resolution in observing systems I and II containing membranes, and the ability to observe these changes as a function of time are needed before we can really attribute fluorescence changes to structural changes.

4.4.2 IONIC CONTROL OF EXCITATION TRAPPING

The smallest chloroplast substructure, capable of complete photosynthesis, is the thylakoid. The membranous thylakoid envelope (the lamella) serves two purposes. First, it supplies the matrix on which the photosynthetic pigments and the enzymes are organized. Second, the lamella functions as a selective osmotic barrier separating the thylakoid interior from the stroma. The permeability of this barrier is variable and it appears to be subject to regulation by the photosynthetic processes. These processes include electron (or H-atom) transport within the membrane, and H^+ transport from the outside to the inside of the thylakoid. In an elegant theory, Mitchell (1966) has proposed that electron transport alternating with H-atom transport leads to a net movement of H^+ from the outside to the inside of the thylakoid. This creates a chemical potential gradient on the membrane. A collapse of this gradient is responsible for the ATP production. The alternating electron and H-atom transfer is visualized as follows. In the thylakoid membrane, the O_2 evolving and the P700 oxidation sites are on the inner side of the membrane, whereas the plastoquinone and $NADP^+$ reduction sites are on the outer side of the membrane. In light reaction II, electrons are removed from Z to reduce Q. Water reduces oxidized Z by donating electrons, H^+ are deposited on the inside of the thylakoid membrane and O_2 is evolved. Plastoquinone is reduced by Q^- and a H^+ is picked up from the outside to complete this reaction as this reduction requires a H-atom. The next step is the reduction, by plastoquinone, of P700 which accepts electrons only, so a H^+ is again deposited on the inside of the membrane. As a result of light reaction I, P700 is oxidized, and X is reduced. The X^- reduces $NADP^+$ on the outer side of the membrane requiring H^+ that is picked up from the outside of the thylakoid. Thus, accompanied by electron (and H-atom) transfer from H_2O to $NADP^+$, there is a net accumulation of H^+ on the inside of the thylakoid. This not only causes a pH gradient (ΔpH) and an osmotic component, but an electrical field component ($\Delta\psi$) as well. A new detailed model of how all this comes about has recently been proposed by Kreutz (1970).

Witt and associates (Rumberg, 1964; Emrich *et al.*, 1969) have indirectly measured $\Delta\psi$ by following absorption changes that arise in certain pigments because of the Stark effect (or the electrochromic shift). A field strength of $10^5 V/cm$ has been calculated to exist (Schliephake *et al.*, 1968; Wolff *et al.*, 1969); this is sufficiently strong to cause changes in the pigments embedded in the thylakoid. Junge and Witt (1968) found that it takes one molecule of the antibiotic gramicidin D to obliterate the ionic and osmotic response of one thylakoid. Such a

"punctured" thylakoid exhibits only the very fast electrochromic absorption changes as the $\Delta\psi$ is dissipated rapidly due to the indiscriminate permeability of the membrane.

Further support for the Mitchell theory was also given by Uribe and Jagendorf (1967). Chloroplasts were first incubated in the dark at an acidic pH (4) and then rapidly transferred to an alkaline pH (8). This resulted in a net synthesis of ATP. This experiment provided a direct test of the hypothesis that the pH gradient is the driving force for the synthesis of ATP in chloroplasts. (It was, however, not clearly shown that a pH-induced electron flow was absent during the acid-base transition.) Formation of an ionic gradient has been shown to cause volume changes (swelling and shrinkage of chloroplasts) and changes in internal structure (see Itoh *et al.*, 1963; Packer, 1963; Izawa, 1965; Hind and Jagendorf, 1965; Dilley, 1966; Nobel, 1969). These changes are the consequence of the H^+ transport, and are thus consistent with the Mitchell theory. However reservations should be made before Mitchell theory is accepted in its present form (Slater, 1967).

We now consider the hypothesis that the rates of excitation trapping and of electron transport may be under the control of the ionic gradients. Rumberg and Siggel (1969) have demonstrated the control of the rate of electron flow through P700 by the extent of acidification of the chloroplast interior. In their experiment, the rate of P700 reduction (measured as the decay rate of the light-induced absorbance change at $\lambda \sim 700$ nm) was monitored at the end of an illumination period the duration of which determined the steepness of the pH gradient. The results indicated that a long illumination (i.e., a steeper H^+ gradient) caused a slower rate of P700 reduction. On the other hand, in the case of thylakoids that had been rendered leaky by the addition of gramicidin D, the rate of P700 reduction was found to be independent of the length of the preillumination period. These results show that a large H^+ ion gradient causes a reduction in the rate of electron transport.

Let us elaborate this idea of a control mechanism. Perhaps, the trapping of the quanta at the reaction center and the electron and ion transports are coupled and interdependent through a feedback control. Rapid trapping of the excitation causes an equally rapid electron transport and buildup of H^+ gradient. The latter is assumed to exert an inhibitory effect on the rate of the electron flow, the suppression of which results in an increase in the fraction of closed reaction centers causing, in turn, a reduction in the trapping rate. (This is supported by the experiments of Rumberg and Siggel described above.) However the collapse of the ionic gradient by the formation of the high energy inter-

mediate, and subsequent synthesis of ATP from it, will speed up the electron transfer.

We are now in a position to provide a description of the slow fluorescence and oxygen yield change on the basis of the above hypotheses. At P (Fig. 3), the ionic gradient will be assumed maximal, reaction centers closed, and the electron transport low (state 1; Section 4.4.1). During the P to S phase, the membrane potential collapses by the formation of the high-energy intermediate (cf. state 1 to state 2 transition). Thus, the inhibitory pressure of the H^+ gradient is removed. This causes an increase in the rate of electron transport as observed (O_2 evolution rate increases). We believe that the decline in the fluorescence yield from P to S is a result of two factors—(1) increase in the rate of electron transport, and (2) changes in the conformation of the membranes as the membrane potential collapses (Section 4.4.1).

The increase in the O_2 evolution during SM phase is due to the onset of CO_2 reduction that utilizes the pool of ATP present in cells. The H^+ transport, and thus the membrane potential again increases during this phase. This increase in potential causes the change in the conformation

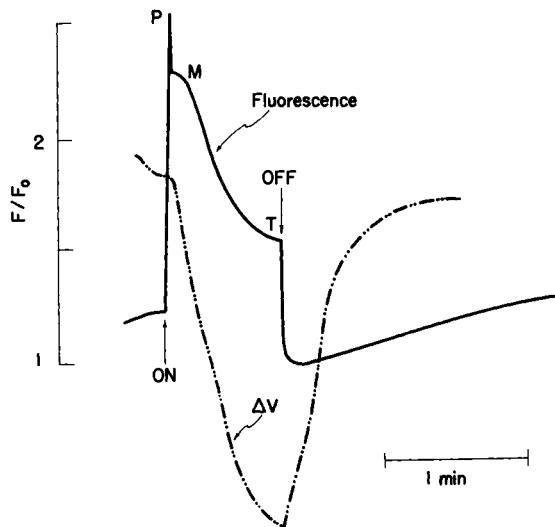


FIG. 12. Kinetics of light-induced changes in Chl a fluorescence yield and in potential (ΔV) across plasmalemma and tonoplast in *Nitella translucens*. Intensity of fluorescence exciting light was of the order of 10^{-11} nEinsteins $cm^{-2} sec^{-1}$; intensity of actinic light was approx. 3 nEinsteins $cm^{-2} sec^{-1}$; a downward movement of the potential recording means an increase in potential (less negative) (after Vredenberg, 1971).

of the membrane opposite to that during PS decay and the fluorescence yield rises. The inhibitory effect of the membrane potential becomes apparent again at M just as it does at P.

The fluorescence decline during MT is due to the same reasons as those causing PS except for one major difference. Although the potential gradient collapses and high-energy intermediate builds up just as in P to S, the rate of O_2 evolution remains invariable due to an enzymatic saturation in the CO_2 reduction cycle that does not permit a further rise in the rate of electron transport.

In recent years, evidence has accumulated for the existence of changes in the membrane potentials during the slow fluorescence transient. Vredenberg (1971) has made simultaneous measurements of membrane potentials across plasmalemma and tonoplast and the slow fluorescence transient PT. He found approximately parallel changes in the alga *Nitella translucens*—when the fluorescence yield declined, the membrane potential increased (less positive) (Fig. 12). If we assume that these changes reflect changes in the thylakoid membranes, Vredenberg's experiment may be a demonstration that in intact cells changes in light energy conversion—that lead to fluorescence yield changes—occur by a mechanism controlled by transport processes across the membranes.

5. Summary

Analyses of the fast fluorescence transient (discussed in Section 3) have yielded information regarding the excitation energy transfer, the working of the photosynthetic units, the primary photochemistry of pigment system II, the pool of intersystem intermediates, and the interaction of systems I and II.

The slow fluorescence induction, which remained a complex and enigmatic phenomena for many years, has begun to provide information concerning the changes in the physical status of the pigments *in vivo*. The notion that the dissipation of the Chl a excitation *in vivo* is subject to ionic and osmotic control, as well as to control by electron transport, has gained experimental support in recent years. In the future, we believe the Chl a fluorescence kinetics may be used for the elucidation of the processes that control the transport of excitation quanta, electrons and ions; Chl a is nature's "intrinsic" probe.

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Chapter 2

MECHANISM OF LIGHT-INDUCED CAROTENOID SYNTHESIS IN NONPHOTOSYNTHETIC PLANTS

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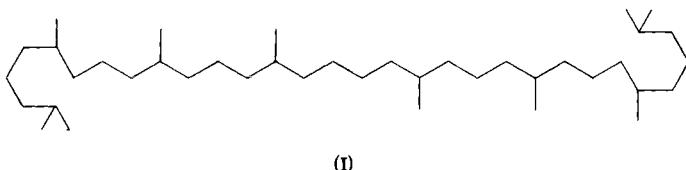
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1. Introduction

The carotenoids are probably the most widely distributed group of pigments present in all higher plants and in many protista, both photosynthetic and nonphotosynthetic. To a certain extent, they are also found in some animal groups, notably the marine animals. Whereas plants and protista have the metabolic machinery to synthesize these pigments *de novo*, the animals do not; the animal carotenoids are derived from these two sources. The comparative distribution of carotenoids in

various groups of organisms has been discussed in detail in a variety of reviews (see, e.g., Goodwin, 1965).

The carotenoids are tetraterpenes containing 8 isoprenoid units, with the following carbon-skeleton:



Although the commonly occurring carotenoids contain 40 carbon atoms as shown, a few with 50 carbons have been isolated from certain bacteria in recent years (Weeks and Garner, 1967). Most of the discussion that follows will be concerned with the 40-carbon carotenoids. By convention the carotenoids are classified into two groups: the carotenes which are hydrocarbons; and the xanthophylls which are oxygen-containing derivatives of carotenes.

The biosynthetic pathways of carotenoid formation have been reviewed by Goodwin (1965), Jensen (1965a), and Porter and Anderson (1967). For the purposes of this chapter a summary of the main features of the biosynthetic sequence of carotenoids is in order. Geranylgeranyl pyrophosphate is generally regarded as the immediate precursor in carotenoid biosynthesis. The formation of this terpenyl pyrophosphate occurs by the pathway shown in Fig. 1. In carotene biosynthesis (Fig. 2), two molecules of geranylgeranyl pyrophosphate by a tail-to-tail dimerization produce the first carotenoid, phytoene. Once phytoene is formed, it is converted to lycopene in a series of dehydrogenation steps. Each dehydrogenation step leads to the addition of two conjugated double bonds; thus phytoene has three conjugated double bonds, while phytofluene, ζ -carotene, neurosporene, and lycopene have 5, 7, 9, and 11 conjugated double bonds, respectively.

Evidence for the conversion of phytoene to lycopene has come from genetic studies, from *in vivo* experiments by the use of labeled precursors (Porter and Anderson, 1967) and, recently, from *in vitro* experiments (Kushwaha *et al.*, 1970). The last authors have shown that soluble extracts of tomato fruit plastids will convert phytoene- ^{14}C to lycopene- ^{14}C via the pathway in Fig. 2. Furthermore they have shown that the conversion of phytoene to phytofluene is dependent upon the presence of NADP, whereas the conversion of the latter compound to lycopene appears to require FAD and Mn^{2+} .

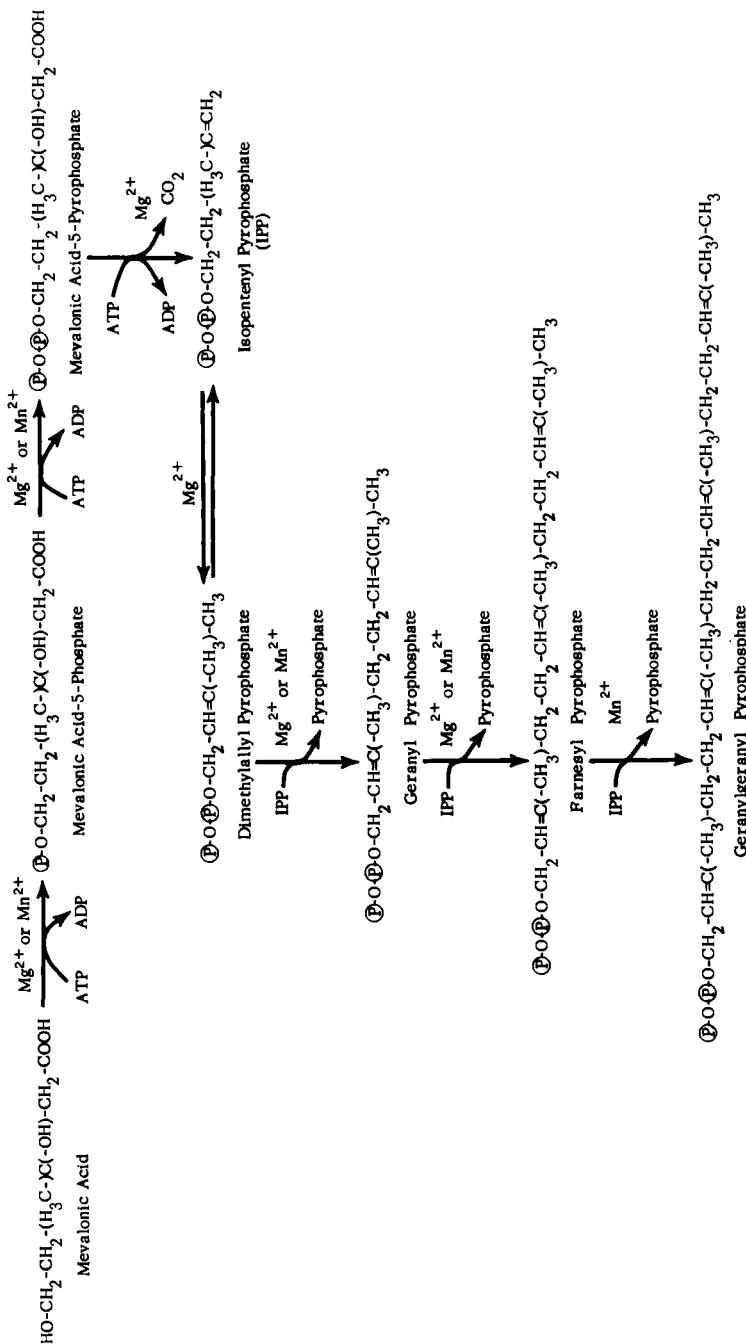


FIG. 1. Biosynthetic pathway for the conversion of mevalonic acid to geranylgeranyl pyrophosphate.

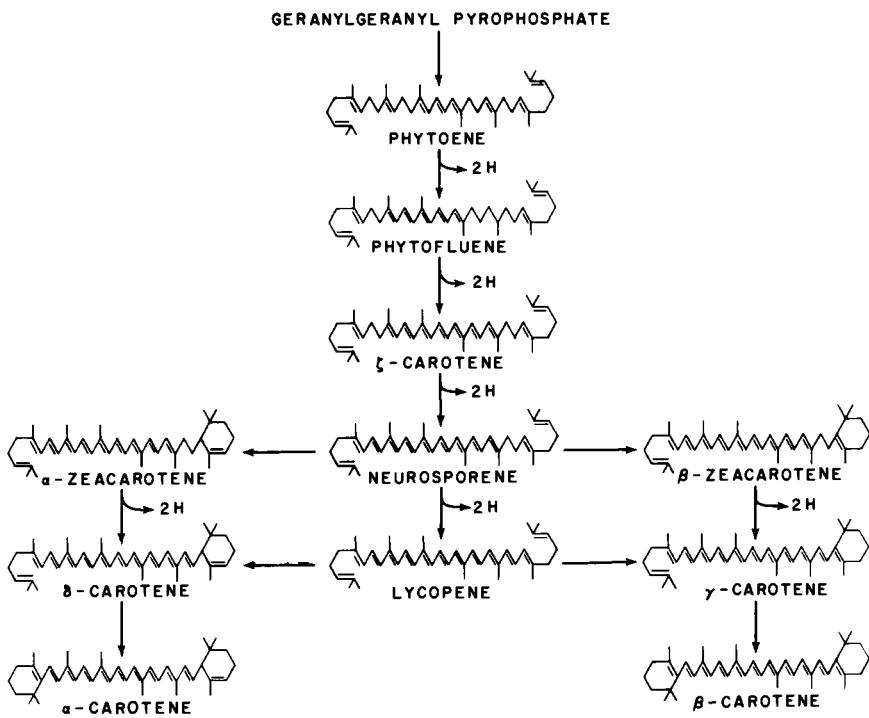


FIG. 2. Biosynthetic pathway for the formation of α - and β -carotenes from geranylgeranyl pyrophosphate.

The cyclic carotenes containing cyclohexylidene rings (such as β -carotene) are synthesized from the acyclic carotenes but the step at which cyclization occurs has been disputed. Both lycopene and neurosporene have been proposed as the substrates for the cyclization reaction (Fig. 2). Recent studies with soluble enzyme preparations (Kushwaha *et al.*, 1969) and with the intact organism (Howes and Batra, 1970b) have shown that lycopene is the substrate for cyclization. Although there is some evidence that neurosporene is also cyclized (Goodwin, 1965), direct proof is lacking. There is also evidence that α - and β -carotenes are formed by independent cyclization reactions rather than by an isomerization of β -carotene to α -carotene (Goodwin and Williams, 1965). Xanthophyll formation occurs when an oxygen atom(s) is inserted into a carotene molecule late in the biosynthetic sequence (Jensen, 1965a; Goodwin, 1965).

A number of factors are known to affect carotenoid biosynthesis in both photosynthetic and nonphotosynthetic plants. Of these factors, light has the most dramatic effect. In some plants it increases quantitatively

the level of synthesis of carotenoids, while in others it triggers carotenogenesis. The focus of this chapter is on the mechanism by which light triggers or induces the *de novo* synthesis of carotenoids in nonphotosynthetic plants.

2. Photoinduction of Carotenogenesis

A number of nonphotosynthetic plants (Table I) produce only traces of carotenoids when grown in the dark. However a brief exposure to light results in substantial carotenoid synthesis. Most of the studies, prior to 1954, have dealt with a description of this photoinduced carotenoid synthesis. It is only in recent years that considerable progress has been made in elucidating the biochemistry of this photoresponse. Studies conducted principally with *Mycobacterium* sp. (Rilling, 1962a, 1964), *Mycobacterium marinum* (Mathews, 1963; Batra and Rilling, 1964), *Neurospora crassa* (Zalokar, 1955; Rau, 1968; Harding *et al.*, 1969), and *Fusarium aquaeductuum* (Rau, 1967a,b) have shown that the photoinduced carotenoid synthesis consists of two steps: a photochemical reaction, which is followed by a series of dark reactions. The

TABLE I
SOME NONPHOTOSYNTHETIC PLANTS IN WHICH LIGHT TRIGGERS CAROTENOGENESIS

Plant	Reference(s)
Bacteria	
<i>Flavobacterium dehydrogenans</i>	Weeks and Garner (1967)
<i>Mycobacterium</i> sp.	Rilling (1962a)
<i>Mycobacterium balnei</i>	Wayne and Doubek (1968)
<i>Mycobacterium flavum</i> var. <i>methanicum</i>	Nikitina and Rabotnova (1969)
<i>Mycobacterium kansasii</i>	Wayne and Doubek (1964)
<i>Mycobacterium lacticolum</i>	Nikitina (1968)
<i>Mycobacterium marinum</i>	Mathews (1963); Batra and Rilling (1964)
<i>Mycobacterium rubrum</i>	Nikitina (1967)
<i>Myxococcus xanthus</i>	Burchard and Dworkin (1966)
<i>Myxococcus virescens</i>	Greene and Leadbetter (1963)
<i>Myxococcus flavus</i>	Greene and Leadbetter (1963)
Fungi	
<i>Cephalosporium diospyros</i>	Codner and Platt (1959)
<i>Dacryopinax spathularia</i>	Goldstrohm and Lily (1965)
<i>Fusarium aquaeductuum</i>	Rau (1962); Rau and Zehender (1959)
<i>Fusarium oxysporum</i>	Carlile (1956)
<i>Neurospora crassa</i>	Haxo (1949); Went (1901, 1904); Zalokar (1954)
<i>Pyronema confluens</i>	Carlile and Friend (1956)
<i>Syzygites megalocarpus</i>	Wenger and Lily (1966)

dark reactions eventually lead to substantial carotenoid production. A detailed discussion of each of these steps follows.

3. Photochemical Reaction

The photochemical reaction is temperature-independent and, in addition to light, requires O₂ (Rilling, 1962a; Batra and Rilling, 1964; Zalokar, 1955). The reaction is also irreversible, i.e., once exposed to light the organism is able to produce carotenoids after prolonged delay of as much as three months when stored at -15° (Rilling, 1962a). This suggests that the photoproduct is quite stable.

3.1 Light Dose

The amounts of carotenoids produced are linearly proportional to the light dose below saturation. The amount of light needed for maximal induction varies with the organism and the wavelength. For example, in *Mycobacterium* sp. (Rilling, 1962a), exposure of as little as 1 minute of 700 fc of light is sufficient to achieve saturation, while *M. marinum* (Batra and Rilling, 1964) requires an exposure of at least 15 minutes of the same light intensity for saturation. In terms of radiant energy requirement for half-saturation of response, *Mycobacterium* sp. requires 5.3×10^4 erg/cm² at 445 nm (Howes and Batra, 1970a), *M. marinum* requires about 3.6×10^5 erg/cm² at 404 nm (Batra, 1971), and *N. crassa* requires 10^5 erg/cm² at 465 nm (Zalokar, 1955).

The photoinduction reaction in *M. marinum* (Mathews, 1963; Batra and Rilling, 1964), *Mycobacterium* sp. (Rilling, 1964), *N. crassa* (Rau, 1968), and *F. aquaeductuum* (Rau, 1967a) follows the Roscoe-Bunsen reciprocity law. That is, a given dose of light leads to the same response independent of the time over which the dose is given. Zalokar (1955) reported that the reciprocity law is not followed in *N. crassa*; however a recent reinvestigation by Rau (1968), who used submerged cultures, has shown that the law is indeed followed in this organism as well. Whether the reciprocity law is followed in other organisms listed in Table I, remains to be determined.

3.2 Action Spectra

An important aspect of any photobiological response is the determination of the wavelengths that are most effective in bringing about the response (the action spectrum). Since light energy must be absorbed before it can produce a response, the determination of an action spectrum usually provides an indication of the chemical nature of the substance (photoreceptor) absorbing those wavelengths.

The action spectra of photoinduced carotenogenesis have been determined only for *N. crassa*, *F. aqueductuum*, *Mycobacterium* sp., *M. marinum*, and *Myxococcus xanthus*. These organisms show one of two types of action spectra depending upon the range of wavelengths effective in producing the response: (a) Flavin-like (or carotenoid-like) action spectrum, and (b) Porphyrin-like action spectrum. The flavin-like (or carotenoid-like) action spectrum is one whose maxima are similar to the absorption maxima of a flavin (or a carotenoid) and, thus, wavelengths longer than 520 nm are ineffective in inducing the response. The organisms that have this type of an action spectrum are *N. crassa*, *F. aqueductuum*, and *Mycobacterium* sp. The action spectra in this category are not sufficiently detailed to distinguish between flavins and carotenoids as receptors. The porphyrin-like action spectrum is one whose maxima are comparable to the absorption maxima of a porphyrin and, thus, wavelengths longer than 520 nm are effective in inducing carotenogenesis. The two organisms that have a porphyrin-like action spectrum are *M. marinum* and *Myxococcus xanthus*.

3.2.1 FLAVIN-LIKE (OR CAROTENOID-LIKE) ACTION SPECTRA

Zalokar (1955) determined an approximate action spectrum of carotenogenesis in *N. crassa* by placing the mycelia in the spectrum (400–500 nm) obtained from a spectrograph and exposing them to equal total energies of radiation. The quantity of carotenoids produced in the next 6 hours in the dark was determined. A plot of the amount of carotenoid synthesized vs wavelength resulted in the action spectrum. This action spectrum showed a plateau between 449 and 488 nm; wavelengths longer than 520 nm were ineffective. These results suggested that a flavin or β -carotene both of which have absorption maxima comparable to the maxima of the action spectrum could be the light-absorbing pigment. Since β -carotene could not be found in the mycelia, Zalokar concluded that a flavin (probably as a flavoprotein) must be the photoreceptor. However, the inability to detect β -carotene (which may be present in trace but sufficient amounts to act as the photoreceptor) in the mycelial pad cannot be considered as proof that it is not the photoreceptor. Therefore, the precise nature of the photoreceptor in *N. crassa* remains undetermined.

Using monochromatic wavelengths of equal total energies, Rau (1967a) has determined an accurate action spectrum of induction in *F. aqueductuum* (Fig. 3). The action spectrum has maxima at 375–380 nm and 450–455 nm, one shoulder at 430–440 nm and a further shoulder between 470 and 480 nm. Wavelengths longer than 520 nm were ineffective in inducing carotenoid synthesis. Because none of the caro-

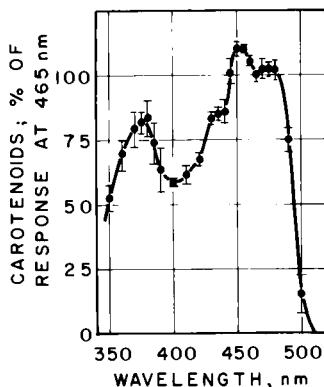


FIG. 3. Action spectrum of carotenogenesis in *F. aqueductuum* as determined with a constant flux of quanta (4.2×10^{-7} Einstein/cm 2). (From Rau, 1967a.)

noids isolated from *F. aqueductuum* absorbed at 375–380 nm, Rau has ruled out carotenoids as possible photoreceptors. He has concluded that a flavoprotein is the photoreceptor.

The action spectrum of induction in *Mycobacterium* sp. in the wavelength range of 310 to 600 nm has been reported by Batra and Rilling (1964). Dilute bacterial suspensions were exposed to known intensities of light from a monochromator for varying lengths of time, and the amounts of carotenoids determined after dark-incubation of 6 hours. For each wavelength a dose-response line was obtained. A plot of the slopes of these dose-response lines as a function of the wavelength resulted in the action spectrum. The action spectrum has a maximum at 365 nm and a plateau between 425 and 465 nm; however, no clearcut maxima in the wavelength range of 425–465 nm were discernible. Howes and Batra (1970a) have reinvestigated and extended this action spectrum into wavelengths shorter than 310 nm by using interference filters that transmitted monochromatic light of less than 10 nm bandwidth. This action spectrum (Fig. 4) has clear maxima at 280–285, 365–370, 410–415, 443–448, and 465–470 nm. This action spectrum of carotenogenesis has two important features which should be mentioned: (1) it has a distinct and large response peak at 280–285 nm not previously reported in any organism, and (2) the fine structure in the wavelength region longer than 400 nm is more clearly defined. Except for the 280–285 nm peak, the action spectrum for *Mycobacterium* sp. reported in Fig. 4 is, in general, similar to the action spectra in *N. crassa* (Zalokar, 1955) and *F. aqueductuum* (Fig. 3). Whether these organisms also show a response peak at 280–285 nm has not been reported.

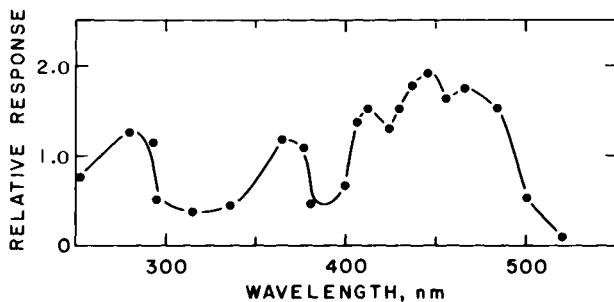


FIG. 4. Action spectrum of carotenogenesis in *Mycobacterium* sp. Bacterial suspensions (1%) were exposed to monochromatic light (obtained with interference filters bandwidth 10 nm or less; light source was 1000-W xenon-mercury lamp; light energy measurements were made with the Kettering microradiometer) at 0° in O₂ atm for various lengths of time. After the addition of 30 mM glucose and 2 mM (NH₄)₂SO₄, bacterial suspensions were incubated in the dark for 24 hours. A plot of the product of the light energy and time of illumination versus response (amount of carotenoids) gave a straight line (dose-response line) whose slope reflected the effectiveness of that wavelength of light to induce carotenoid synthesis. In this figure, the slopes of the dose-response lines are plotted against the wavelength to obtain the action spectrum. (From Howes and Batra, 1970a.)

To determine whether a carotenoid or a flavin is the photoreceptor in *Mycobacterium* sp., Howes and Batra isolated endogenous carotenoids and flavins from the dark-grown organism. The carotenoid fraction had absorption peaks at 327, 427, and 450 nm. Since these peaks varied considerably from those of the action spectrum and since no absorption peak could be found at 280–285 nm, it was concluded that carotenoid by itself could not act as the photoreceptor. The flavin fraction obtained from *Mycobacterium* sp., however, had absorption peaks at 290, 375, and 445 nm with shoulders at 420 and 470 nm. The agreement between these absorption maxima and the action spectrum maxima (Fig. 4) led to the conclusion that a flavin (probably as a flavoprotein) is the photoreceptor. However the possibility that a carotenoid–protein complex could be the photoreceptor cannot be discounted positively. In this case, the 280–285 nm action spectrum peak could be due to the absorption by the protein and subsequent transfer of energy to the carotenoid moiety. The shift in the near-UV and visible range could be due to the formation of a complex between the carotenoid and protein moieties.

In summary the photoreceptor in *Mycobacterium* sp. is either a flavin (possibly a flavoprotein), or a carotenoid–protein complex. To clarify this problem, one may use competitive inhibitors of flavins; if the photosensitivity of *Mycobacterium* sp. is decreased, a flavin must be the photoreceptor.

3.2.2 PORPHYRIN-LIKE ACTION SPECTRA

M. marinum and *Myxococcus xanthus* have the porphyrin-like action spectra. Mathews (1963) determined an approximate action spectrum by illuminating colonies of *M. marinum* growing on agar plates with monochromatic wavelengths and determining the amounts of carotenoids produced. Maximal carotenoid production occurred in the vicinity of 410–439 nm, with smaller maxima at 502, 528, and 562 nm. Since this action spectrum resembled in certain respects the absorption spectra of cytochromes, Mathews concluded that a cytochrome must be the photoreceptor. Batra and Rilling (1964) using dilute suspensions of *M. marinum* so as to obtain a more uniform illumination of cells obtained an action spectrum (Fig. 5) with a very strong maximum at 404 nm and two lesser maxima at 493 and 577 nm, suggesting that a porphyrin may be the photoreceptor. When *M. marinum* cells were disrupted a porphyrin fraction was extracted from the homogenate (Howes and Batra, 1970a). This fraction has absorption maxima (in 25% HCl) at 404, 548, and 592 nm with a shoulder at about 570 nm (Fig. 6). The porphyrin fraction has been identified as mesoporphyrin or coproporphyrin. If one compares the absorption spectrum of the porphyrin present in *M. marinum* with the action spectrum of photoinduction, it is found that the Soret peaks at 404 nm agree. However, the peaks toward the longer wavelengths at 493 and 577 nm of the action spectrum do not correspond precisely with the absorption maxima of the isolated porphyrin. This shift in the absorption maxima may be due to the solvent used to dissolve the

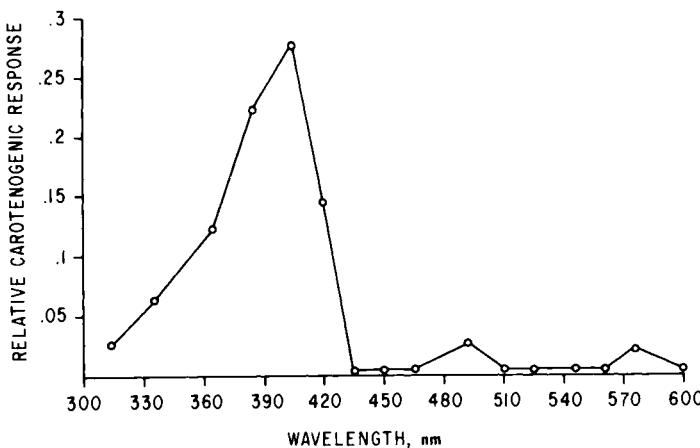


FIG. 5. Action spectrum of photoinduced carotenoid synthesis in *M. marinum*. The ordinate is the negative value of the slope of the dose-response line at any wavelength. (From Batra and Rilling, 1964.)

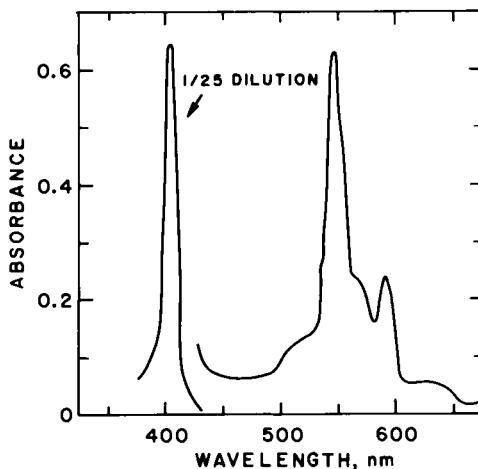


FIG. 6. Absorption spectrum of the porphyrin fraction obtained from *M. marinum*. The spectrum was determined in 25% HCl. (From Howes and Batra, 1970a.)

porphyrin. It is quite possible that, *in vivo*, the porphyrin may have absorption maxima that are in agreement with the action spectrum maxima. Therefore it appears likely that mesoporphyrin or coproporphyrin may act as the photoreceptor in *M. marinum*.

Myxococcus xanthus also has a porphyrin-like action spectrum slightly different from that of *M. marinum*. Burchard and Hendricks (1969) have measured an accurate action spectrum of photoinduction in *Myxococcus xanthus* and have found a principal peak at 405–410 nm and subsidiary peaks at 512, 533–548, 585, and 635 nm (Fig. 7, solid line). If the cells

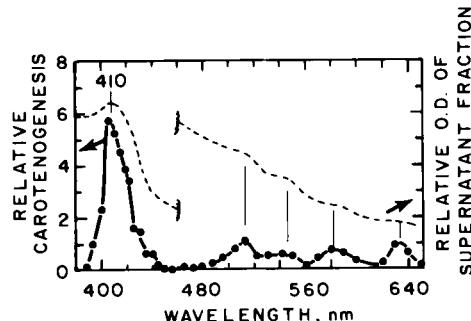


FIG. 7. Action spectrum (solid), expressed as carotenoid synthesis for equal incident quanta, in dark-grown, early stationary phase *Myxococcus xanthus*. The absorption spectrum (dotted) of the $10^4 \times g$ supernatant fraction of ultrasonically disrupted cells is also shown. (From Burchard and Hendricks, 1969.)

are disrupted ultrasonically and the homogenate is centrifuged ($10^4 \times g$), the resulting supernatant fraction has absorption maxima that correspond almost exactly with the maxima of the action spectrum (Fig. 7, broken line). Similarly, an acetone-extract of the cells has absorption maxima which are in agreement with those of the action spectrum. The compound in the extract serving as photoreceptor has been identified as protoporphyrin IX.

3.3 Oxygen Requirement

Oxygen required for the photochemical reaction (Rilling, 1962a; Howes *et al.*, 1969; Zalokar, 1955), could function (1) as an electron acceptor to keep certain redox compounds such as the flavins or the cytochromes in the proper oxidation state; or (2) as a direct molecular participant in the light-photoreceptor reaction. Three main observations suggest that the latter possibility is the case:

1. Ferricyanide and benzoquinone are satisfactory electron acceptors for *M. marinum* and *Mycobacterium* sp. But if these bacteria are illuminated under strict anaerobic conditions in the presence of any of these compounds they do not produce carotenoids (Rilling, 1964; Howes *et al.*, 1969).

2. Cyanide is an effective inhibitor of electron transport in these bacteria, yet it fails to inhibit photoinduction (Rilling, 1964).

3. If the dark-grown *Mycobacterium* sp. is preincubated with O_2 but then is illuminated in an atmosphere of N_2 , no induction occurs. However, induction does occur if the bacteria are preincubated with N_2 but are illuminated in the presence of O_2 . If O_2 were required only as an electron acceptor, the gas phase of the preincubation, rather than the gas immediately introduced before exposure, would be expected to determine the photoinducibility of the bacteria (Rilling, 1964). Thus it appears that O_2 plays a direct obligatory role in the photochemical reaction.

Attempts to measure the amount of light-induced O_2 uptake with a Warburg apparatus have been unsuccessful (Howes *et al.*, 1969). This may be due either to a lack of sensitivity of the Warburg apparatus to measure traces of O_2 consumed or that O_2 plays only a catalytic role in the photochemical reaction. Assuming that the first possibility is correct, one can speculate on the role of O_2 during illumination—that of photo-oxidizing an inhibitory substance or producing a photooxidized compound that may stimulate carotenoid production. (This is discussed in detail in Sections 3.7 and 4.5.) In this connection, it should be noted that light

is capable of producing singlet O₂ by photosensitization of certain redox substances (flavins and porphyrins!) as has been reported by Foote and Denny (1968). Singlet O₂ is possibly produced during this photochemical reaction and participates in the primary photooxidation process.

Rau (1969) has reported that when a suspension of dark-grown *F. aquaeductuum* or *N. crassa* was gassed with N₂ and then illuminated, the organisms produced 10%-75% carotenoids as compared to those that were illuminated in an O₂ atmosphere. This has led to the suggestion that O₂ does not directly participate in the primary photochemical reaction but functions as an electron acceptor. It is not clear why O₂ is required for the photochemical reaction in *Mycobacterium* sp. and *M. marinum* but not in *N. crassa* or *F. aquaeductuum*. It is rather unlikely that the mechanism of photoinduction is different. A more plausible explanation appears to be that strict anaerobic conditions during illumination were not obtained with *F. aquaeductuum* or with *N. crassa* (Howes *et al.*, 1969).

3.4 pH-Dependence

Photoinduction of the *Mycobacterium* sp. is markedly pH-dependent. If the dark-grown bacteria are preincubated at an acid pH at 0° for 5-10 minutes and then illuminated at the same pH, they are considerably less photosensitive than the bacteria that have also been preincubated at the acid pH for the same length of time but illuminated at pH 8.0 (Batra and Rilling, 1964). Although the effect of acid pH in diminishing the photoinducibility of *Mycobacterium* sp. is open to several interpretations, the following one has been preferred. The photoreceptive flavin could be associated with a protein as a complex, this complex being the active photoreceptor (Section 3.2.1). Flavoproteins dissociate at acid pH and in this case the dissociated components would be inert in the photoinductive reaction.

3.5 Inhibitors

Hydrosulfite inhibits the photochemical reaction in *Mycobacterium* sp. but not in *M. marinum* (Batra and Rilling, 1964). Since hydrosulfite is known to reduce flavins thereby eliminating their characteristic visible and near ultraviolet absorbance (Massey and Gibson, 1962), this observation provides indirect evidence that a flavin may be the photoreceptor in *Mycobacterium* sp. (Section 3.2.1).

Azide inhibits the photochemical reaction both in *M. marinum* and *Mycobacterium* sp. (Batra and Rilling, 1964). Since azide is known to complex transition metals (Chance, 1952), it is possible that a metal ion (probably also cytochromes) is involved in the photoinduction reaction.

3.6 Chemical Induction of Carotenogenesis

Two chemicals, a substituted mercuribenzoate and antimycin A, mimic the photochemical reaction in *F. aqueductuum* and *M. marinum*, respectively. Rau *et al.* (1967) has reported that *p*-chloromercuribenzoate (PCMB) and *p*-hydroxymercuribenzoate (PHMB) induce carotenoid production in the dark in the mycelium of *F. aqueductuum*; maximal production of carotenoids occurs at a concentration of 50–100 μM PCMB or PHMB (Fig. 8). Since these compounds are known to block SH-groups it was suggested that PCMB and PHMB act by blocking SH-groups on a specific compound. By extrapolation, therefore, the function of light and O_2 in the photochemical reaction would be a photooxidation of the SH-groups of the same compound. If this is the case, the addition of compounds containing SH-groups, such as cysteine, should reverse the

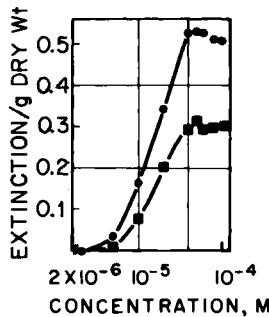
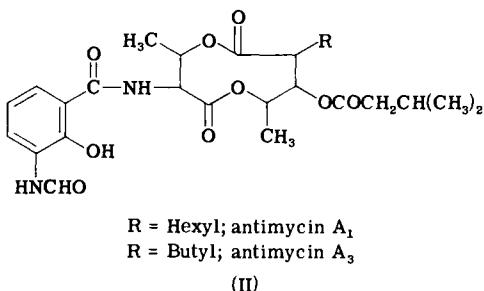


FIG. 8. Induction of carotenoid synthesis with *p*-chloromercuribenzoate (filled squares) and *p*-hydroxymercuribenzoate (filled circles) in *F. aqueductuum*. (From Rau, 1967b.)

light-mimicking effect of PCMB (or PHMB). This has not been reported. In this connection it should be mentioned also that other SH-group blocking compounds such as iodobenzoate, ethylmaleimide and iodoacetamide are ineffective in the dark-induction of carotenogenesis (Rau, 1967b), either because of lack of penetration of these compounds through the cell membrane, or that PCMB (or PHMB) induce carotenogenesis by a mechanism other than by blocking the SH-groups.

In contrast to their inductive effect in *F. aqueductuum*, PCMB and PHMB are ineffective in mycobacteria. However, antimycin A (II) has been found to induce carotenoid production in the absence of light in *M. marinum*; maximal production of carotenoids occurs at a concentration of 37.5 μM (Fig. 9) (Batra, 1967a,b). This figure also shows that saturating amounts of antimycin A are much more effective in the induction of carotenoid synthesis than saturating doses of light. For example, induction of bacteria with light alone led to the synthesis of 17 μg of carotenoids per 100 mg dry weight of bacteria, but if the bac-



teria were induced with saturating amounts of antimycin A, 37 μg of carotenoids were formed per 100 mg dry weight of bacteria.

It is also worth noting in Fig. 9 that the inductive effects of antimycin A and light are additive at every concentration of antimycin A used. An interpretation of these results would be that the sites of action of antimycin A and light are different in *M. marinum*. Similarly, PCMB (or PHMB) and light have different sites of action in *F. aquaeductuum* (Theimer and Rau, 1968). This has been observed in a mutant of *F. aquaeductuum* which produces carotenoids in response to PCMB (or PHMB) but not when exposed to light. However, since exactly the same types of carotenoids are synthesized regardless of whether carotenogenesis is induced by illumination or chemically (Table II), we must conclude that light and the chemicals induce carotenogenesis in an analogous manner but at different sites (Section 5).

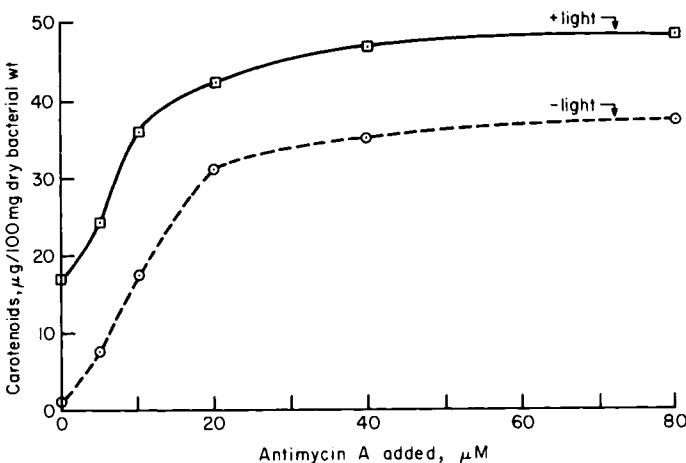


FIG. 9. Stimulation of carotenoid synthesis by antimycin A in *M. marinum* in the presence (open squares) and absence (open circles) of light. (From Batra, 1967b.)

TABLE II
CAROTENOID SYNTHESIZED BY *M. marinum* AFTER INDUCTION WITH LIGHT
AND ANTIMYCIN A^a

Fraction	Tentative identification	Absorption maxima (nm)	Carotenoid, $\mu\text{g/gm}$ bacteria, dry wt			
			Dark	Light	Antimycin A	Antimycin A + light
1	Phytoene	276, 284, 297	4.4	8.8	7.8	8.7
2	Phytofluene	330, 348, 367	Trace	1.3	1.2	2.8
3	α -Carotene	421, 444, 473	Trace	3.2	8.3	13.8
4	β -Carotene	424, 450, 484	0.5	38.7	120.9	166.2
5	Unidentified	422, 442, 468	0.6	2.7	3.9	6.0
6	Unidentified	427, 456, 492	—	1.0	1.1	1.3
7	Unidentified	425, 462, 490	—	1.2	1.9	2.0

^a From Batra (1967b).

The facts that antimycin A blocks respiratory electron flow at the cytochrome level in many organisms and that a porphyrin (which may be the prosthetic group of a cytochrome) is the photoreceptor for carotenogenesis in *M. marinum* (Section 3.2.2.) suggest the following hypothesis for the induction of carotenogenesis by light and antimycin A. When added to *M. marinum*, antimycin A initiates an alternate electron pathway by blocking the electron flow at the cytochrome level and shunting the electrons to the new pathway. Products of this new pathway lead to the enhanced synthesis of carotenoids. A similar role may also be assigned to light in the photoinduced carotenogenesis. When bacteria are illuminated, one of the cytochromes whose prosthetic group is the photoreceptor becomes activated by light and an alternate electron pathway becomes available. The products of this alternate electron pathway lead to the synthesis of carotenoids.

Although very attractive, this explanation that light and antimycin A induce carotenogenesis by interfering in the respiratory electron flow seems highly unlikely for three reasons.

1. 2-*N*-heptyl-4-hydroxyquinoline-*N*-oxide, which blocks electron flow at the same site as antimycin A (Chance, 1957), is unable to induce carotenogenesis.

2. Addition of 75 μM antimycin A (twice the amount needed for maximal carotenoid synthesis; Fig. 9) did not depress O_2 uptake by *M. marinum*.

3. The use of synthetic analogs and derivatives of antimycin A (II) has revealed that the reactive groups needed for the inhibition of electron flow and for the induction of carotenogenesis are different.

For inhibition of the electron flow, the substituted dilactone ring, the phenolic hydroxyl group, and the *N*-carbonyl group individually and collectively are indispensable (Rieske, 1967). On the other hand, only the phenolic hydroxyl group and the substituted dilactone ring, but not the *N*-carbonyl group are essential for the induction activity of antimycin A (Batra, 1971). Furthermore, replacement of the substituted dilactone ring of antimycin A (II) with a long alkyl straight chain results in its total loss of induction activity, but not in its inhibitory activity. These structure-activity relationship studies, thus, provide direct proof that antimycin A induces carotenogenesis by a mechanism other than through its effect on the electron transport system.

3.7 Role of Light: A Hypothesis

Since the electron transport system does not appear to be involved in the induction of carotenogenesis, the following alternate hypothesis concerning the mode of action of light and chemicals (antimycin A and the substituted mercuribenzoates) in such induction is suggested. The photochemical reaction, which requires light and O₂, results in the photooxidation of a specific compound. This specific compound may be a repressor (present in the dark-grown organism) which does not permit the synthesis of carotenogenic enzymes, or it may be a substance which is converted to an inducer molecule by photooxidation resulting in the synthesis of carotenogenic enzymes. Regardless of whether light and O₂ act by photooxidizing a repressor substance or by producing a photooxidized metabolite that acts as an inducer, the synthesis *de novo* of carotenogenic enzymes will be expected. We can also postulate that the chemicals (antimycin A and the substituted mercuribenzoates) act in an analogous fashion, i.e., they also induce the synthesis of carotenogenic enzymes and that they do so either by combining with a repressor or by acting as inducers.

4. Dark Reactions

As a result of the events initiated during the photochemical reaction, the organism produces large amounts of carotenoids on subsequent incubation in the dark. The parameters of this dark phase are discussed in this section.

4.1 Requirements

Carbon, nitrogen, and phosphorus sources are required for the dark reactions. In *M. marinum*, for instance, glycerol, (NH₄)₂SO₄ (or casein hydrolysate), and phosphate stimulate carotenogenesis considerably

(Batra *et al.*, 1969). Since nitrogen and phosphorus atoms are not present in the carotenoid molecule, they are believed to enhance carotenogenesis indirectly through their effect on the general metabolic activity of the organism.

Biotin and glycine also appear to be involved in carotenoid biosynthesis in *N. crassa* (Zalokar, 1957). Peptone suppresses carotenoid production and, interestingly, glycine addition overcomes this suppression. Trace metals have also been implicated in the formation of carotenoids in *Mycobacterium* sp. (Rilling, 1964). The mechanism of action of biotin, glycine, peptone, and trace metals remains unknown.

Requirement for O_2 in the dark reactions has been established (Rilling, 1962a; Batra *et al.*, 1969). In *Mycobacterium* sp., ferricyanide can replace O_2 as the electron acceptor in the dark phase to some extent (Rilling, 1962b).

4.2 Types of Carotenoids Synthesized

The types of carotenoids synthesized following the photochemical reaction, depend upon the organism. *Mycobacterium* sp. produces both carotenes and xanthophylls (Rilling, 1964). If the appearance of various carotenoids is studied as a function of time after the bacteria have been illuminated, one finds that the more saturated carotenes phytoene and phytofluene appear first. These are followed by the appearance of more unsaturated carotenes and xanthophylls (Rilling, 1964). Similar results (Harding *et al.*, 1969) are obtained when *N. crassa* is incubated in light in the presence of mevalonic acid- ^{14}C (an early precursor of carotenoids; Fig. 1) and the incorporation of radioactivity into various carotenoids is studied as a function of time. These time-course studies show that radioactivity appears first in phytoene. The relative order of synthesis of the remaining carotenoids is as follows: ζ -carotene before neurosporene; neurosporene before lycopene, 3,4-dehydrolycopene, γ -carotene and torulene; and the neutral pigments before neurosporaxanthin. These results, therefore, indicate that the various carotenoids are synthesized via the established biosynthetic pathway (Fig. 2) after photoinduction has occurred. As in other organisms, diphenylamine addition inhibits dehydrogenation of carotenes resulting in an accumulation of phytoene and phytofluene.

In contrast to *Mycobacterium* sp., *M. marinum* produces large amounts of β -carotene (95%) following induction with light or antimycin A; phytoene, phytofluene, ζ -carotene, and neurosporene are found only in trace amounts (Table II). This probably reflects the ability of *M. marinum* to convert rapidly the acyclic carotenes to β -carotene but its inability to oxygenate these carotenes to produce xanthophylls.

A recent observation (Howes and Batra, 1970b) has provided interesting information regarding the biosynthetic pathway of cyclic carotenes in *M. marinum* and *Mycobacterium* sp. If the light-induced or the antimycin A-induced *M. marinum* is incubated in the presence of the alkaloid nicotine, large amounts of lycopene, rather than β -carotene, accumulate (Table III). Accumulation of lycopene, but not of cyclic carotenes or xanthophylls, also occurs when light-induced *Mycobacterium* sp. is incubated in the presence of nicotine. Since lycopene is an acyclic carotene while β -carotene contains two cyclohexylidene rings (Fig. 2), it appears then that nicotine inhibits the cyclization reaction in cyclic carotene biosynthesis. The nicotine effect differs from that of diphenylamine in that the latter inhibits the dehydrogenation of carotenoids leading to the accumulation of phytoene and phytofluene. The optimal concentration of nicotine for the inhibition of cyclization is 5 mM. Below this concentration, a partial inhibition of cyclization occurs; at a concentration of 0.5 mM, nicotine causes the accumulation of lycopene (acyclic), γ -carotene (monocyclic) and β -carotene (dicyclic). Concentrations higher than 5 mM inhibit total carotenoid production. The observation that at a concentration of 0.5 mM nicotine γ -carotene can be detected in addition to lycopene and β -carotene, would suggest that (1) γ -carotene is an intermediate between lycopene and β -carotene (Fig. 2) and (2) the second cyclization reaction between γ -carotene and β -carotene may also be blocked by nicotine. The points of possible inhibition by nicotine are indicated in Fig. 10. Further studies have shown

TABLE III
EFFECT OF NICOTINE ON CAROTENOID BIOSYNTHESIS IN *M. marinum*^a

Flask ^b	Compound added during incubation			Un-identified fraction			
	During first incubation	During second incubation	Phytoene	Phyto- fluene	α - and β -carotenes	Lycopene	Total
	No.		(μ g/gm bacteria dry wt.)				
1			13.8	1.3	96.0	54.2	165.3
2	NIC	NIC	11.1	0.6		96.7	13.7
3	NIC		14.4	1.4	33.5	26.8	13.4
4			8.0	1.0			1.4
							10.4

^a From Howes and Batra (1970b).

^b Bacteria in flasks 1 to 3 were induced with antimycin A; bacteria in flask 4 were uninduced. Flask 1, no nicotine (NIC); Flask 2, nicotine present during the 48-hour incubation; and Flask 3, bacteria were incubated for 24 hours in the presence of nicotine which was then washed out and the bacteria were reincubated for the next 24 hours.

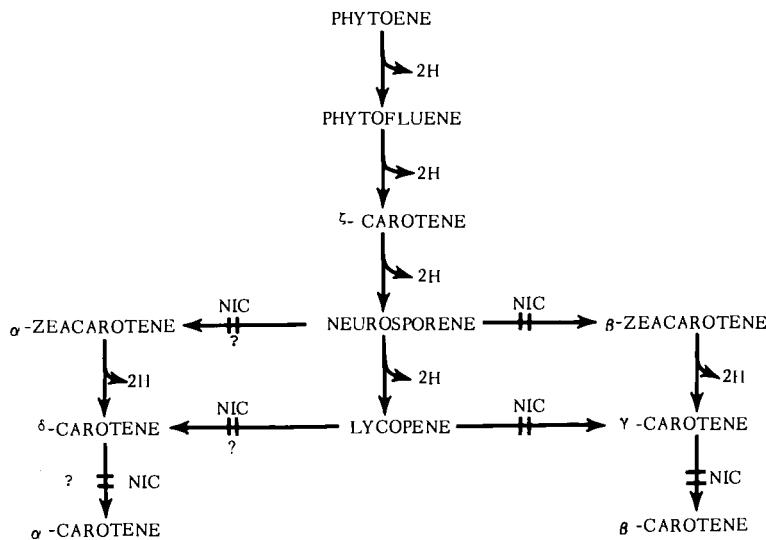


FIG. 10. Proposed points of nicotine (NIC) inhibition of carotenoid biosynthesis.

that if nicotine is removed by washing the cells with a phosphate buffer, the concentration of lycopene decreases while that of β -carotene correspondingly increases. Moreover, after nicotine is washed away γ -carotene, but not β -zeacarotene, can be detected in addition to β -carotene. These results therefore suggest that lycopene is cyclized to form β -carotene via γ -carotene in *M. marinum* and *Mycobacterium* sp. (Fig. 2). That lycopene can be cyclized to form β -carotene by spinach chloroplasts or tomato plastids (or their enzyme preparation) has been reported (Kushwaha *et al.*, 1969, 1971; Decker and Uehlke, 1961). The evidence against neurosporene being cyclized (which has been suggested by some investigators) is that (1) neurosporene is not accumulated when the cells are incubated in the presence of nicotine and (2) on nicotine removal, β -zeacarotene which would be the expected intermediate (Fig. 2) cannot be detected. However the inability to detect β -zeacarotene cannot be considered proof against neurosporene being cyclized. The resolution of the problem concerning neurosporene as the substrate for cyclization will have to await the development of appropriate cell-free systems.

N. crassa, after light-induction, produces phytoene, phytofluene, ζ -carotene, neurosporene, β -zeacarotene, lycopene, 3,4-dehydrolycopene, γ -carotene, torulene, and neurosporaxanthin (Jensen, 1965b; Harding *et al.*, 1969); β -carotene synthesis does not occur. *F. aqueductuum* produces mostly ζ -carotene, neurosporene, γ -carotene, lycopene, torulene and neurosporaxanthin (Rau, 1966); the kinetics of formation of these

carotenoids following induction with light or a substituted mercuribenzoate have not been reported.

4.3 Intracellular Location of Carotenoids

The carotenoid pigments in mycobacteria as well as in corynebacteria, micrococci and *Sarcina lutea* occur in easily sedimentable protein complexes (Saperstein and Starr, 1955; Jackson and Lawton, 1958; Mathews and Sistrom, 1959). The intracellular localization of these particles appears to be in the cell envelope (Mathews, 1963; Mathews and Sistrom, 1959), or perhaps in the cell wall-membrane complex, although the cell walls themselves appear to be devoid of carotenoids (Salton, 1956; Mathews and Sistrom, 1959). This association of the carotenoids with the cell membrane was established by the fact that upon ultrasonic disruption of the cells, the kinetics of the release of carotenoid pigments were in agreement with the kinetics of the release of cytochrome oxidase, phospholipid, NAD oxidase, and succinic dehydrogenase. Since the latter compounds are in association with the cell membrane (Mathews and Sistrom, 1959); Weibull, 1953; Storck and Wachsman, 1957; Mitchell and Moyle, 1956; Marr and Cota-Robles, 1957), it follows that the carotenoids must be localized in this intracellular structure (Mathews and Sistrom, 1959; Mathews, 1963).

One question that can be posed now is: Are the newly synthesized carotenoids (after photoinduction) deposited on the preformed cell membrane or are they added to a new membrane that is formed concurrently with the carotenoids? Mathews (1966) has conducted two experiments which show that the new carotenoids are added to the preformed membrane. (1) The electron microscopic studies show that the ultrastructure of the *M. marinum* cells remains unaltered after photoinduction and carotenoid synthesis, and (2) there is essentially no difference in the rates of ^{32}P -incorporation into phospholipid fractions of the illuminated and the unilluminated cells. Since ^{32}P -incorporation into the phospholipid fraction of the cell is a good indication of the formation of cell membrane material (Kolb *et al.*, 1963), it is concluded that no new cell membrane had formed following photoinduction.

4.4 Time Course of Carotenogenesis

In studies on carotenoid production as a function of time of dark-incubation following the photochemical reaction, there is always a definite lag period before any new carotenoids are formed. The lag period in *Mycobacterium* sp. and *N. crassa* is somewhere between 40 minutes to 1½ hours (Rilling, 1962a; Rau, 1968), while in *M. marinum* and *F. aquaeductuum* the lag period is approximately four hours (Batra, 1967b;

Rau, 1967b). The lag period is seen whether the induction is with light or with a chemical (antimycin A in *M. marinum* and PCMB or PHMB in *F. aquaeductuum*; Section 3.6). After this lag period, the rate of carotenoid production becomes maximal and then tapers off. The time period needed to reach this level of maximal carotenoid production depends upon the organism and the type of induction. For example, the light-induced *Mycobacterium* sp. on dark-incubation synthesizes carotenoids up to 14 hours (Rilling, 1962a), while under the same conditions, *M. marinum* produces carotenoids up to 24 hours (Batra, 1967b). However if *M. marinum* is incubated in light or if the incubation is carried out in the presence of antimycin A, carotenoid production does not stop after 24 hours but continues for more than twice this time period resulting, of course, in much larger carotenoid production (Fig. 11). Similarly, if *Mycobacterium* sp. is incubated in light carotenogenesis does not stop at 14 hours but continues for more than 48 hours (Howes and Batra, 1970a). The period during which carotenoid production occurs, can also be prolonged by giving saturating flashes of light to *Mycobacterium*

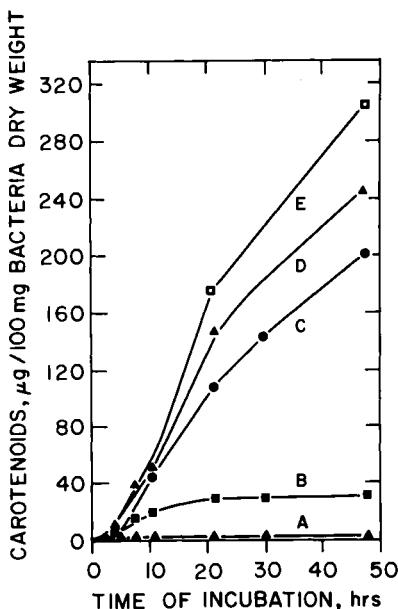


FIG. 11. Time course of carotenogenesis in *M. marinum* under various conditions. Curve A shows uninduced bacteria; B, bacteria given a saturating dose of light and then dark-incubated; C, bacteria incubated in continuous light; D, bacteria given antimycin A; and E, bacteria given light *plus* antimycin A. (From Batra *et al.*, 1969.)

sp. and *M. marinum* at regular intervals of 1 and 4 hours, respectively (Batra, 1967b; Howes and Batra, 1970a). The amounts of carotenoids produced under the flashing conditions are essentially the same as when the bacteria are incubated in continuous light. These results therefore suggest that continuous light (or flashing light) and antimycin A provide an uninterrupted stimulus to the organism to synthesize carotenoids. This stimulus may be in the form of a continuous derepression of the genetic sites resulting in the uninterrupted synthesis of carotenogenic enzymes.

4.5 Involvement of Protein Synthesis

When the induced organism is incubated, the lag period during which no new carotenoid formation occurs suggests the probable synthesis of carotenogenic enzymes *de novo* during this time. That this is indeed the case can be shown by the use of the inhibitors of protein synthesis. If chloramphenicol, puromycin, or cycloheximide is added immediately after induction—whether with light or with a chemical—synthesis of carotenoids is completely inhibited. When the addition is delayed, progressively more and more carotenoids are formed. This trend of inhibition by the protein biosynthetic inhibitors has been shown to occur in all organisms tested—*Mycobacterium* sp. (Rilling, 1962a, 1964) *M. marinum* (Batra, 1967b), *F. aqueductuum* (Rau, 1967b), and *N. crassa* (Rau, 1968; Harding and Mitchell, 1968).

The determination and the analysis of the kinetics of formation of carotenogenic enzymes have provided valuable insight into the mechanism of action of light and the chemicals (antimycin A and the substituted mercuribenzoates) in induction of carotenogenesis. The kinetics have been determined by adding protein biosynthetic inhibitors at various times during incubation of the induced organism. Thus dark-grown *M. marinum* which has been given a saturating dose of light and is subsequently dark-incubated, shows a sensitivity to the addition of chloramphenicol up to 4 hours insofar as carotenogenesis is concerned (Fig. 12). This, of course, is the lag period during which the synthesis of carotenogenic enzymes is expected (Section 4.4). Addition of chloramphenicol after four hours has no effect on carotenoid production under the conditions of initial illumination and then dark-incubation. However, when the incubation conditions are altered different kinetics are observed. If dark-grown *M. marinum* is incubated in the presence of antimycin A or is incubated in light, addition of chloramphenicol continues to have an effect on carotenoid production beyond the 4-hour period (Fig. 12).

These kinetic results can be explained as follows. When the dark-grown bacteria are illuminated, the derepression of the genetic sites

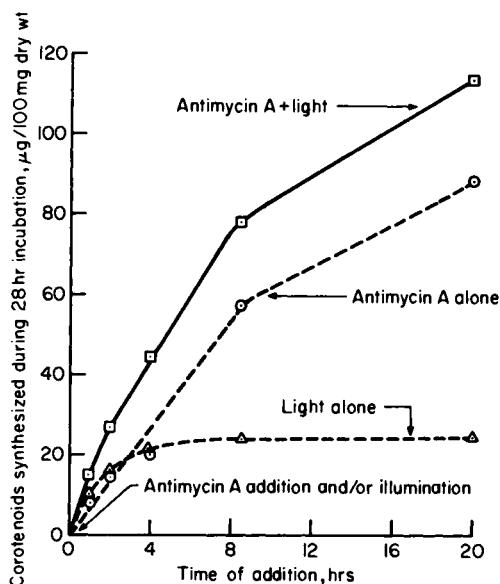


FIG. 12. Time of addition of chloramphenicol during dark-incubation (28 hours) of the induced *M. marinum*. (From Batra, 1967b.)

results. This derepression could be either the result of the production of a photooxidized metabolite *in situ* that acts as the inducer or to the photooxidation of the repressor substance present in the dark-grown organism (Section 3.7). In either case the derepression leads to the formation of carotenogenic enzymes. When the illuminated bacteria are incubated in the dark, a repression in further synthesis of carotenogenic enzymes sets in after 4 hours. This is reflected in the fact that the addition of chloramphenicol after this time has no effect. When the bacteria are incubated in the presence of antimycin A or when the incubation is carried out in light, the bacteria are kept in the derepressed state continuously. This continuous derepression results, of course, in the uninterrupted synthesis of carotenogenic enzymes as is reflected by the sensitivity of the bacteria to chloramphenicol addition beyond the 4-hour period.

It was mentioned above that the lack of a chloramphenicol effect after four hours of dark-incubation (Fig. 12) is probably due to the setting-in of repression in the synthesis of carotenogenic enzymes. If this reasoning is correct, then it follows that a second saturating-dose of light at the end of the 4-hour period should again make the bacteria sensitive to the chloramphenicol addition—that is, light should again derepress the genetic sites resulting in the formation of carotenogenic enzymes. This, in fact, is the case as shown in Fig. 13. Notice also that

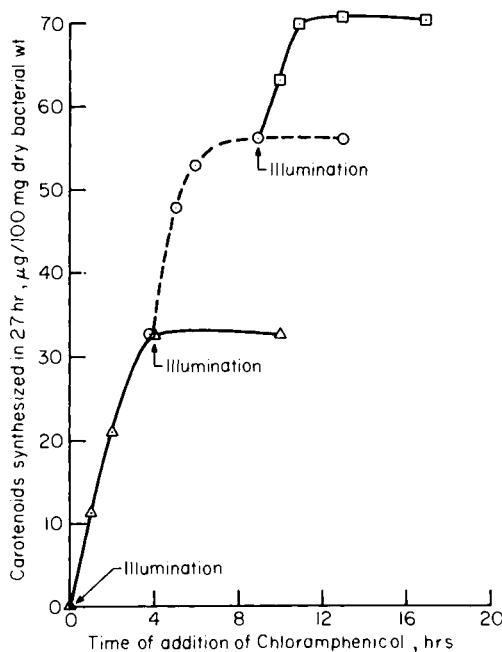


FIG. 13. Effect of periodic illumination on carotenoid synthesis in *M. marinum*. Aliquots of the bacterial suspensions were placed into a series of 25-ml Erlenmeyer flasks and illuminated for 20 minutes with 700 fc of light at 0° in air. After the addition of 0.2 ml of 2 M glycerol, the flasks were incubated in the dark. Chloramphenicol was added at various times during the first four hours of incubation. At this time all flasks were removed from the incubator and chilled. All flasks, except those to which chloramphenicol had been added, were illuminated for the second time and the incubation was started. During the next 4 hours, chloramphenicol was added at various times. All flasks were again chilled and those which did not have chloramphenicol were illuminated for the third time. Incubation was started and chloramphenicol was added at various times. At the end of 27 hours of incubations, amounts of carotenoids were measured. (From Batra, 1967b.)

saturating doses of light are needed at 4-hour intervals for the continued synthesis of carotenogenic enzymes and carotenoids.

One of the principal points of the explanation concerning the chloramphenicol effect is the possibility that light and antimycin A act at the DNA level. One piece of evidence that supports this concept is that proflavin, which is known to form complexes with DNA (Peacocke and Skerrett, 1956; Lerman, 1961) and thereby inhibit replication and transcription (Lerman, 1964; Nicholson and Peacocke, 1966), blocks carotenogenesis (Batra and Storms, 1968). A point that is worth emphasizing is that antimycin A induces carotenogenesis in *M. marinum* not through

its effect on the electron transport system (its usual mode of action; Section 3.6), but through its effect on the genetic sites resulting in derepression, and consequently protein synthesis. This novel mode of action of antimycin A has received further support from a recent report that this compound stimulates protein synthesis and cell division in *Tetrahymena pyroformis* (Elson *et al.*, 1970).

A reference was made previously to the work of Rau (1967b) who has shown that PCMB or PHMB can induce carotenogenesis in *F. aqueductuum*. In this case as well, the substituted mercuribenzoates act by inducing protein synthesis (carotenogenic enzymes) which can be demonstrated by the use of cycloheximide (Rau, 1967b).

5. Mechanism of Photoinduction

In Section 4, the mode of action of light and the chemicals in the induction of carotenogenesis was discussed in detail. At the risk of being repetitious, the following few statements and Fig. 14 summarize and place in proper perspective the mechanism of photoinduced carotenoid synthesis in nonphotosynthetic plants.

The dark-grown organism lacks the carotenogenic enzymes needed for the synthesis of carotenoids. The exposure to light and O₂ leads to the derepression of the genetic sites. In some organisms, the derepression can also be achieved by the addition of chemicals—antimycin A in *M. marinum* and PCMB or PHMB in *F. aqueductuum*; however, the sites of action of light and the chemicals are different (Section 3.6). Once derepressed, the organism produces carotenogenic enzymes via

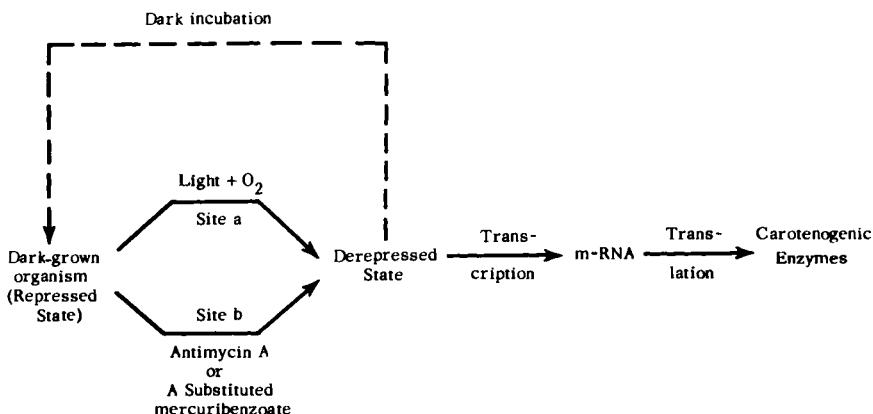


FIG. 14. Proposed mechanism of photoinduced carotenoid synthesis. (Modified from Batra and Storms, 1968.)

the classical scheme of transcription and translation. The requirement for a nitrogen source (Section 4.1), a lag period before any new carotenoids appear (Section 4.4) and the sensitivity of the organism to the biosynthetic inhibitors of proteins after induction (Section 4.5) all support the concept that protein synthesis occurs following induction with light or with a chemical.

Do the various carotenogenic enzymes appear in blocks or in sequence following induction? The first possibility implies that the structural genes for several enzymes are located on one operon. The induction of this operon would result in the synthesis of several enzymes and, as a result, to a group of carotenoids. The second possibility suggests that the induction leads to the synthesis of a carotenogenic enzyme not previously present in the dark-grown organism. The products of this new enzyme would then induce the synthesis of the next enzyme and this process will continue until all of the enzymes needed for carotenoid production have been synthesized. Although at the present time sufficient data are not available to locate the gene groupings, the observation that various carotenoids appear sequentially following induction would tend to support the second possibility (Rilling, 1964). In this connection, a comment concerning the location of the block in carotenogenesis in the dark-grown organism is in order. An analysis of the carotenoids present in various dark-grown organisms shows that phytoene is invariably present in relatively large amounts. Furthermore, when the unilluminated organism (such as *N. crassa*) is incubated in the dark in the presence of mevalonic acid-¹⁴C, a substantial amount of radioactivity is incorporated into phytoene but not into any of the other carotenoids (Harding *et al.*, 1969). These results therefore show that the biosynthetic reactions leading to the formation of phytoene from mevalonic acid (Figs. 1 and 2) are not blocked in the dark-grown cells and that the block is in the conversion of phytoene to phytofluene perhaps due to the absence of the enzyme phytoene dehydrogenase. This is therefore likely to be the first enzyme to appear after induction has occurred.

6. Relationship to Photodynamic Action

A comparison of the parameters of the photochemical reactions in photoinduced carotenogenesis and photodynamic action (Spikes, 1968; Krinsky, 1968; Wright and Rilling, 1963) reveals striking parallels between these two. Both are temperature-independent, require O₂, and utilize visible light. Thus, both appear to involve a photosensitized oxidation of certain cell constituents. Photodynamic action, however, leads to detrimental effects resulting in the death of the organism (Krinsky,

1968), while in the second case carotenoid production is stimulated. The quantity of light needed for photodynamic action is much larger (about tenfold) than that needed for the stimulation of carotenogenesis (Wright and Rilling, 1963). The same chemical probably acts as the photoreceptor for both the photoresponses (Burchard and Dworkin, 1966; Burchard and Hendricks, 1969).

Keeping the apparent similarities between photoinduction of carotenogenesis and photodynamic action in focus, one can speculate that the production of carotenoids on exposure to lower light intensities is an adaptive mechanism which many nonphotosynthetic microorganisms have evolved as protection against potentially lethal photodynamic conditions of higher intensities of visible radiation (sunlight). [The role of carotenoid pigments in the protection of the organism against photodynamic action has been discussed in detail by Krinsky (1968).] Supporting this speculation is the fact that generally most nonphotosynthetic microorganisms which live in the open and are constantly exposed to sunlight either always contain colored carotenoids or synthesize these pigments on exposure to relatively low intensities of visible light.

Singlet oxygen, which may be produced as a result of photosensitization of certain redox compounds such as flavins and cytochromes, is implicated in photodynamic action (Foote and Denny, 1968). It is conceivable that the singlet O_2 may also be involved in photoinduced carotenoid synthesis. Future studies should provide information concerning this point and may tie the two photoresponses together.

ACKNOWLEDGMENTS

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Chapter 3

PHOTOSENSITIZATION BY NATURAL PIGMENTS*

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1. Introduction

Some organisms have natural pigments which absorb certain wavelengths of light [visible and near-ultraviolet (UV)], thus sensitizing

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them to these wavelengths in sunlight. Such photosensitization may lead to serious damage or death to the cells containing the pigment, even though the same wavelengths of light are usually innocuous to organisms lacking such pigments in their cells.

When the pigment sensitizes the cells or organisms in which it is contained it is spoken of as an *endogenous photosensitizer*; when it sensitizes cells of other organisms which it reaches after ingestion or by surface contact, it is spoken of as an *exogenous photosensitizer*. Initially it was thought that a very large number of natural pigments were involved in photosensitization. However, it is now apparent that there are only four main classes of natural photosensitizers: hypericins, chlorophylls, porphyrins, and furocoumarins (Table I). Possibly other natural photosensitizing pigments also occur but proof is at present inadequate.

TABLE I
CLASSES OF NATURAL PHOTOSENSITIZERS

Class	Found in group of organism	Example	Citations
Hypericins	Flowering plants ^a	<i>Hypericum</i> sp.	Brockmann, 1952, 1957;
		<i>Polygonum</i> sp.	Blum, 1941, 1964
	Fungi	<i>Penicilliosis</i>	Brockmann and Neeff, 1951
Chlorophylls	Protozoa ^b	<i>Blepharisma</i> sp.	Giese and Grainger, 1970
	Mutant photosynthetic bacteria ^b	<i>Stentor coeruleus</i>	Møller, 1962
	Mutant algae ^b	<i>Rhodopseudomonas</i> sp.	Krinsky, 1968
Porphyrins	Mutant flowering plants ^b	<i>Chlorella</i>	Krinsky, 1968
		<i>Chlamydomonas</i>	
		<i>Zea mays-maize</i>	Krinsky, 1968
Psoralens	Some bacteria ^b	<i>Helianthus</i> , sunflower	Krinsky, 1968
	Some protozoans ^b	<i>Myxococcus</i>	Burchard and Dworkin, 1966
	Some worms	<i>Tetrahymena</i>	Rudzinska and Granick, 1953
	Some stock animals ^b	Sheep, cattle, etc.	Lambert, 1970 pers. comm.
	Man: some genetic strains; also in liver disease ^b	Man	Clare, 1956
	Man (after contact or ingestion of some plants) ^a	Man	Rimington <i>et al.</i> , 1967
			Pathak, 1969
			Musajo and Rodighiero, 1962

^a Exogenous to animals ingesting or in contact with plants.

^b Endogenous.

Quinones, found in all cells, are probably mediators of growth inhibition in cells, through absorption of visible and near UV light. They will not be considered here since they will be dealt with in a chapter by John Jagger to appear in a future volume of this work. Induction of mutations by visible and near UV will be dealt with in a chapter by Robert B. Webb, in a volume. Discussion is also omitted of photosensitization by antibiotics injected into man or animals, although these are natural products of cells, because such sensitization does not occur in nature (see Pathak, 1969). Nor is discussion here included of pigments which make nerve cells, including those in the central nervous system, sensitive to light (Kluver, 1944; Wettenberg *et al.*, 1970). These especially as they appertain to visual receptors, are summarized in other reviews (Arvanitaki and Chalazonitis, 1961; Kennedy, 1964; Hubbard and Kropf, 1967; Millott, 1968; Wald, 1968); in this function protein-carotenoid conjugates are usually involved.

No attempt will be made here to discuss the physical chemical mechanisms by which photosensitization occurs since this is the subject of several recent reviews (Spikes and Ghiron, 1964; Spikes and Livingston, 1969; Santamaria and Primo, 1964; Foote, 1968; Ogryzlo, 1970; Wilson and Hastings, 1970) to which the reader is referred. A consideration of the basic principles and mechanism of photosensitization is briefly considered by Pathak (1969).

Most natural photosensitizers are synthesized during the normal metabolism of organisms but some occur only during certain metabolic derangements. It is not the intent to dwell here upon pathological photosensitizations except in passing (see Clare, 1956; Shelley, 1962; Ippen, 1969; Urbach, 1969), nor will photoallergies be considered (see Kestin and Slatkin, 1953; Harber and Baer, 1969). Rather, attention will be focused largely upon photosensitization which is observed in normal wild-type and mutant cells or organisms as a consequence of exposure to certain wavelengths of light in the presence of the natural sensitizer.

The literature on each of these four main classes of natural pigments is quite large and citations cannot be given to all of it. Many of the older studies have been omitted even though some are historical. However a more complete account of the older literature may be found in some previous reviews and monographs to which reference is made. Some of the more recent literature, consisting of case studies on a few human patients, has also been omitted, largely because these studies have been, or will be, included in more comprehensive reviews. Some studies on stock animals have also been omitted because they added more documentation without at the same time seeming to extend the theoretical aspects of the subject, even though they do not appear to have been reviewed recently.

Natural photosensitizers are found in many types of organisms: bacteria, fungi, higher plants, protozoans, invertebrates, and vertebrates—at least in some parts of the organism and under some circumstances. It is the aim of the present chapter to gather the widely scattered information which is available for one or another of the four principle classes of natural photosensitizers, but not brought together in one place for all four of them.

Photosensitization implies that in the absence of an absorbing pigment an organism is incapable of reacting to light of certain wavelengths because the light is not absorbed. It has been shown that colorless cells are relatively insensitive to light of wavelengths longer than 320 nm unless a sensitizer is added. Man also is relatively insensitive to these wavelengths of light, sunburn resulting from absorption of sunlight (or artificial light) of wavelengths shorter than 320 nm.

Photosensitization does not imply need for oxygen or any other special conditions. Some of the photosensitizations occur only in the presence of oxygen and these are considered examples of photosensitized oxidations or *photodynamic action* (Raab, 1900; Blum, 1941, 1964; Santamaria and Primo, 1964). Such is the case with photosensitization of man to light by hematoporphyrin, that of range animals by hypericin, and that of mutants of photosynthetic bacteria by chlorophyll (Blum, 1941, 1964). However sensitization of human skin to light by psoralens (one isomer of the furocoumarins) also occurs at wavelengths longer than 320 nm but oxygen is not required (Musajo and Rodighiero, 1962). It is perhaps best for the present to speak of photosensitization in the generic sense and to designate in each case whether oxygen is or is not required.

A few conditions must be satisfied before a disease may be attributed to photosensitization.

1. The symptoms must be evoked after exposure to wavelengths of light which do not produce sunburn, e.g., sunlight through window glass (longer than 320 nm).

2. The suspected photosensitizing pigment must be isolated from the organism (preferably in pure form) and when applied to or injected into organisms and upon exposure to light, will produce the symptoms designated in No. 1 above.

3. The action spectrum of the photosensitization must to a degree correspond to the absorption spectrum of the purified sensitizer (Table IV) in No. 2 above (Blum, 1941).

2. Photosensitization by Hypericins

That sunlight provoked itching, irritation, and endematous eruptions sometimes followed by necrosis in unpigmented areas of the skin of range

animals has been known for a long time. In some parts of Arabia white horses were not used and even unpigmented patches on colored horses were darkened with henna or tobacco juice. In some parts of Italy, e.g., the Tarentine fields, only black sheep were grown because white ones grew ill on exposure to sunlight. It was shown that in both cases certain plants (species of *Hypericum*) eaten by the animals were the cause of their light sensitivity. As early as 1787 *Hypericum* was suspected by Cirillo who called the plant the quick poison of sheep, and in 1833 Hertwig stressed the connection between the disease and exposure to sunlight. In the field, observations have been made on horses, cattle, sheep, and sometimes goats; in the laboratory experiments have been performed on mice, rats, guinea pigs and rabbits. However, it was some time later that the relationship between *Hypericum crispum* in the diet and subsequent exposure to light was determined and an attempt made to isolate the causative substance (Ray, 1914; see Blum, 1941, 1964). No studies of the effect of hypericin upon bacteria or protozoans appear to have been made.

2.1 Hypericism

According to Blum (1941, 1964) there are over 200 species of *Hypericum* in the northern hemisphere, many of them used as decorative plants, and several other species in the southern hemisphere, but only six or seven species have been implicated in natural sensitization to light. In the Mediterranean area *Hypericum crispum* causes photosensitivity; in the United States and Australia it is mainly *Hypericum perforatum* (Klamath weed or St.-John's-wort). In Greece and North Africa both *H. perforatum* and *H. crispum* are present. Among other photosensitizing species are: *H. ethiopicum*, *H. hirsutum*, *H. leucopterychoides*, *H. pulchrum*, and *H. maculatum*.

Hypericum was introduced into the United States and Australia as an ornamental and soon spread as a weed, crowding out much more desirable range plants because of its extensive roots. It has become a pest in the range lands of Australia and in the ranges of Washington, Oregon, California, Idaho, Utah, and Montana in the United States, where it was at one time of considerable economic importance. White sheep are especially vulnerable. The ears and the skin around the eyes, ears and mouth become inflamed, causing itching, ulceration, and infection until the animal loses interest in food and even water. In severe cases the disease may be accompanied by sensory disturbances, and an initial rise in temperature followed by a fall. Extreme cases may end in convulsions and death. If the skin ulcers heal necrosis follows after a few days. Sheep fed on *Hypericum* may be sensitive to light for several weeks, but they become most sensitive after being shorn; the wool grown on such sheep

is inferior in quality and reduced in quantity. Pace (1942) described the etiology of the disease in white rabbits and showed that the action spectrum as determined by filters corresponded roughly with the absorption spectrum by partially purified extracts of *Hypericum perforatum*. He was able to induce the symptoms of the disease by injecting the extract into white rabbits, then exposing them to light. Wavelengths longer than 610 nm were ineffective in inducing the symptoms.

Hypericin is present in glandular spots on the petals, sepals, leaves and stems, the spots appearing blackish but yielding a reddish-colored juice when the flowers and other organs are macerated and expressed. It is interesting that the expressed red juice of *Hypericum* is said to have been recommended by Galen for therapeutic purposes and as late as 1907 the U. S. Dispensary states that *oleum hyperici* is useful for treatment of bruises (Blum, 1941). The distribution of hypericin and similar compounds in various species of plants and some animal cells is shown in Table II.

The pigment in the plant is highly soluble in methanol and ethanol.

TABLE II
ORGANISMS PRODUCING HYPERICIN-LIKE SUBSTANCES

Group	Genus and species	Family	Location	Citations
Flowering plants	<i>Hypericum^a perforatum</i>	Hypericaceae	U. S., Australia, world-wide	Brockmann, 1952, 1957
	<i>H. crispum</i>		Mediterranean	
	<i>H. pulchrum</i>		Europe	
	<i>H. maculatum</i>		Europe	
	<i>H. ethiopicum</i>		Ethiopia	
	<i>H. hirsutum</i>		Europe	
	<i>H. leucoptychoides</i>		—	
Fungi	<i>Polygonum esculentum</i>	Polygonaceae	Europe, U. S.	Brockmann, 1957
	<i>P. tartaricum</i>		Asia	
	<i>Penicillioopsis clavariaeformis</i>		Ubiquitous	Brockmann, 1957
Protozoans	<i>Blepharisma undulans</i>	Spirostomidae	Widespread	Giese and Grainger, 1971
	<i>B. americanum</i>		U. S.	
	<i>B. japonicum</i>		Japan	
	<i>B. intermedium</i>		India	
	<i>Stentor coeruleus</i>	Stentoridae	Widespread	Møller, 1962
	<i>Fabrea salina</i>	Stentoridae	Salt pools	Møller, 1962

^a In this 1957 review Brockmann cites 15 species of *Hypericum* as having hypericin-like pigments.

Chlorophyll and other substances are first removed by ether, then the hypericin is removed with methanol or ethanol; addition of HCl causes precipitation of crude hypericin (Brockmann, 1952). Pace and McKinney (1939, 1941) purified hypericin and studied its absorption spectrum; Brockmann (1952, 1957) by organic analytical procedures demonstrated it to be a naphthodianthrone—namely, hexahydroxy-naphthodianthrone with the empirical formula $C_{30}H_{16}O_8$ and the structural formula shown in Fig. 1. He presents reasons to believe that it is synthesized by dehydration of anthrones or anthranoles as shown in Fig. 1 (A)–(E).

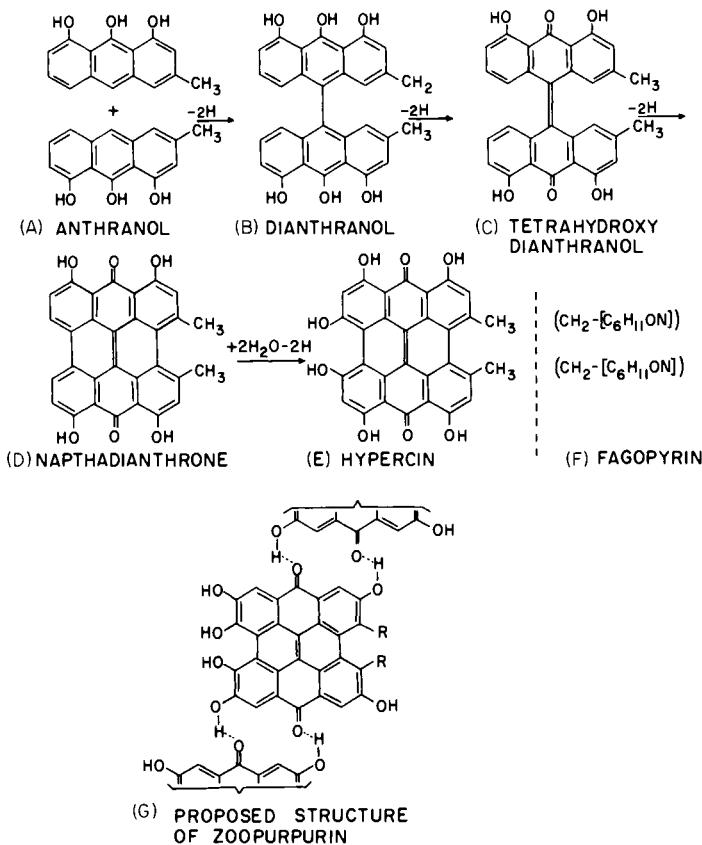


FIG. 1. A, B, C, and D are steps in the laboratory synthesis of hypericin, E. Such precursors are also found in *Hypericum perforatum* (St.-John's-wort), suggesting a similar biosynthetic pathway. In fagopyrin (F) from buckwheat the radicals shown in parentheses are substituted for H-atoms on the methyl groups of hypericin (after Brockmann, 1952, 1957). G, proposed structure of zoopurpurin from *Blepharisma* (after Sevenants, 1965).

Brockmann (1957) tested 22 species of *Hypericum*, 15 of which yielded a red fluorescent substance. He found that exposure to light of some extracts causes a shift of about 15 nm toward the blue end of the spectrum. This he demonstrated to be the result of formation of another form of the pigment called pseudohypericin. Plants contain a mixture of hypericins differing primarily in the radicals substituted for, or replacing hydrogens in, the CH_3 groups [Fig. 1(E)].

Hypericism is evoked only in the presence of oxygen and does not occur in ischemic skin. It is therefore an example of oxidative photosensitization.

2.2 Fagopyrism

Fagopyrism is similar to hypericism and follows feeding by stock animals (including swine and chickens) upon certain species of the genus *Fagopyrum* (*Polygonum*) or buckwheat, most commonly *F. esculentum*, and then exposing them to light. Several species of this genus are grown as grains here and abroad because they thrive on poor soil. In 1843 Fuchs recognized some relation between eating of buckwheat and the effects of light. Busck (1905) did also and attempted to isolate the substance responsible. Ohmke in 1908 obtained a fluorescent extract which, given by mouth, effectively induced photosensitivity in guinea pigs and rabbits. Details of these and other experiments, historically interesting, are given by Blum (1941). Chick and Ellinger (1941) point out that the pigment is present chiefly in the flowers and husks, the kernels containing little. Young flowers contain more than old ones. Neither urine nor feces of animals which have eaten the plants contained the active principal. Once affected, the animal remained sensitive to light for 5 weeks or more. The wavelengths absorbed by the extracts and those active in the photosensitization are in the range 540–610 nm, as in the case of hypericism. Buckwheat greens are no longer used as fodder because of the disease induced in stock animals (Clare, 1956).

Brockmann (1957) isolated enough of the fagopyrin from buckwheat flowers to analyze it by organic chemical techniques and assigned the empirical formula $\text{C}_{42}\text{H}_{36}\text{O}_{10}\text{N}_2$ to it [Fig. 1(F)]. He showed that fagopyrin can then be degraded hydrolytically to hypericin by removal of two aliquots of $\text{C}_6\text{H}_{11}\text{ON}$ from it, showing that one hydrogen atom in each of the two CH_3 radicals in hypericin is replaced by one colorless $\text{C}_6\text{H}_{11}\text{ON}$ radical. The plant contains two other forms of the pigment known as protofagopyrin and protohypericin. The pathway or synthesis of fagopyrin is probably the same as of hypericin, the addition of the colorless groups occurring after the synthesis of the hexahydroxynaphthodianthrone in the plant.

Buckwheat was apparently introduced into Europe from Asia, as the French name for it (sarrasin) suggests; it is still grown in Asia. The whole plant, either green or dried, was used for forage and the seeds are used by man and fed to stock animals. There are six species of buckwheat under cultivation, but besides *F. esculentum*, only *F. tartarium*, a more cold-resistant form grown in northern Asia, is in wide cultivation. Eating buckwheat cakes is unlikely to cause fagopyrism in man because the kernels contain little if any of the causative pigment. Wild plants related to buckwheat do not appear to contain photosensitizing substances.

2.3 Penicilliopsin

A pigment called penicilliopsin was extracted from the fungus *Penicilliopsis clavariaeformis* by Reinke in 1887. Brockmann and Neeff (1951) showed that the pigment is not a porphyrin as suspected by earlier workers and that it appears to be identical with hypericin. Neither photosensitizing properties nor its possible function in the fungus have been studied.

2.4 Zoopurpurin, a Hypericin-like Pigment of *Blepharisma*

That *Blepharisma* contains a red, fluorescent pigment has long been known, but its photosensitizing property was studied only recently. Because the pigment is an endogenous photosensitizer, it offers some unique opportunities for study (Giese, 1946).

Blepharisma is a large (ca. 300 μ), single-celled spirotrich ciliate. There are over a dozen species found in many parts of the world, differing not only in size, shape, and nature of the macronuclei, but also in content of pigment (Hirshfield *et al.*, 1965). Thus *B. japonicum* is vividly red, *B. intermedium* (from India) much the same, while *B. americanum* is much paler. Even an albino strain of *B. intermedium* has been isolated (Chunosoff *et al.*, 1965), and a blue strain of *Blepharisma*, *B. coeruleum*, has been observed in Lake Baikal, Siberia (Gajewskaja, 1927).

The pigment in *Blepharisma* occurs in 5 to 10 arrays of symmetrically arranged granules which, with the higher powers of the light microscope (1000 \times), can be readily seen just under the pellicle between each two rows of cilia (Giese and Grainger, 1971) (Fig. 2). Deeply pigmented blepharismas, e.g., *B. japonicum*, have finely particulate pigment granules. Each granule is about 0.5 μ in thickness and is bounded by a distinct membrane approximately 0.05 μ in thickness (Utsumi, 1953; Utsumi and Yoshizawa, 1957; Kennedy, 1965). The albino strain shows only occasional compact, irregular, pigment granules (Inaba *et al.*, 1958), which

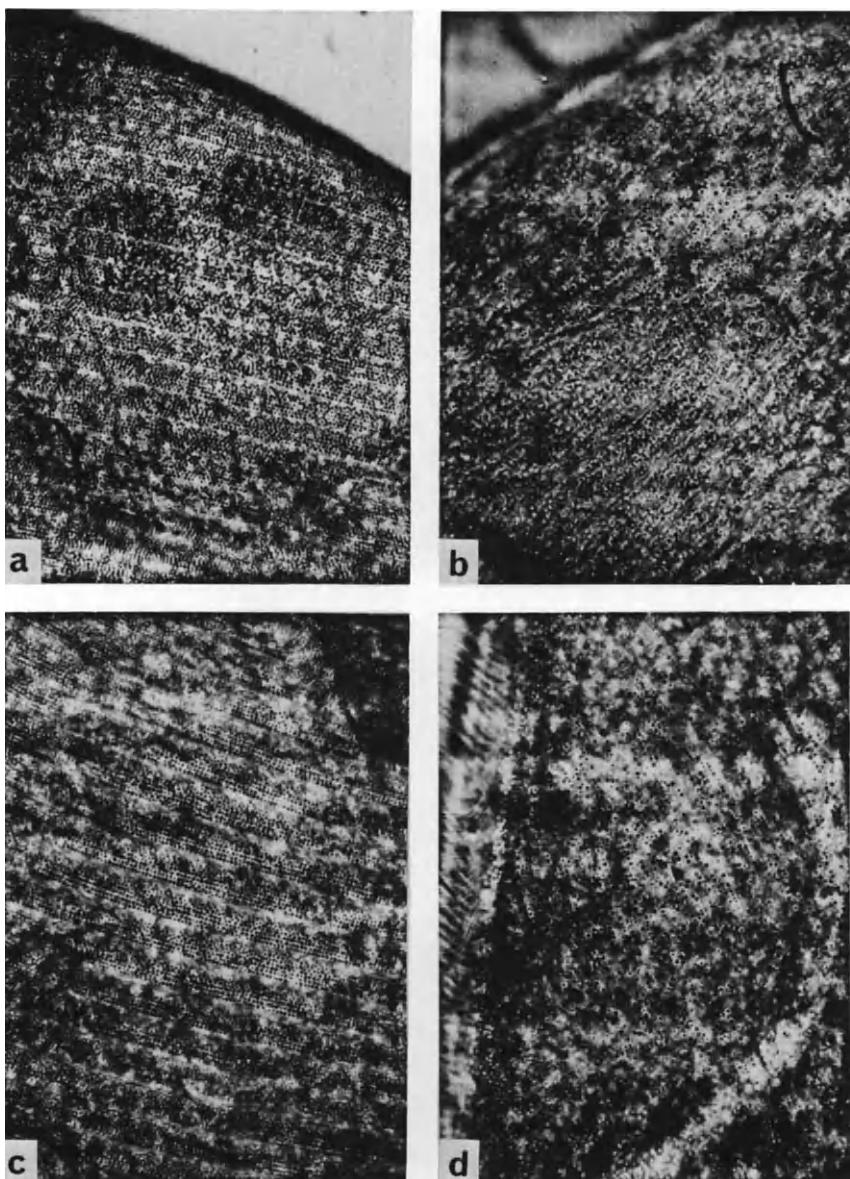


FIG. 2. Arrays of pigment granules in *Blepharisma intermedium*. (a) Red form of pigment; (b) red form, diminished array of granules after cold extrusion; (c) blue form of pigment; (d) blue form, diminished array after extrusion. (All $\times 1000$.)

accounts for the small amount of pigment extractable from albino *Blepharisma* (Chunsoff *et al.*, 1965).

Blepharismas grown in darkness or in red or yellow light accumulate pigment and are readily killed when exposed, in the presence of oxygen, to intense visible light (approx. 2700 fc), since only light of wavelengths shorter than 550 nm is effective. In the absence of oxygen, however, pigmented *Blepharismas* are not affected by an exposure to the same light (Giese, 1946). When *Blepharisma* in a Cartesian diver is exposed to intense visible light oxygen is rapidly absorbed, indicating oxidative photosensitization. The photooxidation ceases when illumination is interrupted (Giese and Zeuthen, 1949) (Fig. 3). Similar results were also obtained with the extruded pigment.

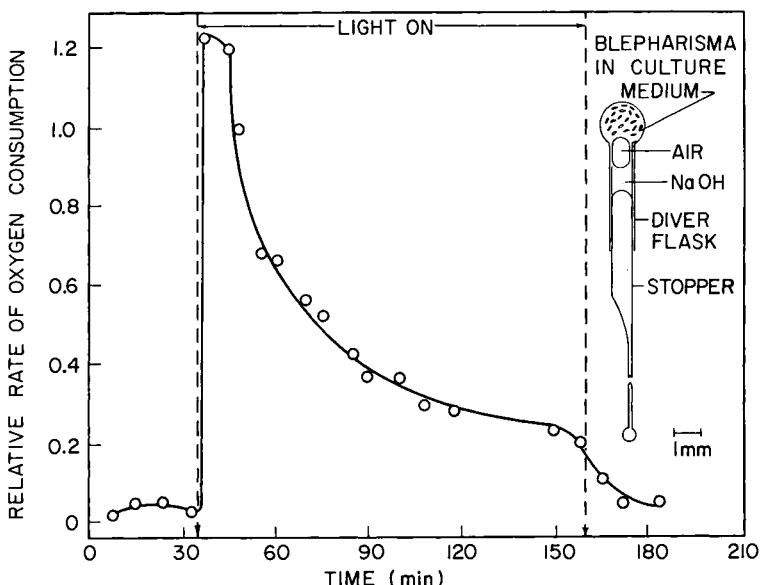


FIG. 3. Burst of oxygen consumption upon illumination of red *Blepharisma americanum* in a Cartesian diver (after Giese and Zeuthen, 1949).

2.4.1 CONVERSION OF ZOOPURPURIN TO A BLUE FORM BY LIGHT

Blepharisma grows and regenerates somewhat more slowly in dim visible light (150 fc) than in the dark, but the cells multiply, carry on normal nutrition, and even conjugate in the dim light under otherwise appropriate conditions. Under prolonged exposure to dim visible light the red pigment is altered ("bleached") to a grayish-blue color in *B. americanum* which normally contains relatively little pigment (Giese,

1938). In deep red species such as *B. intermedium* and *B. japonicum* the color change from the red to blue is striking. The red pigment reappears within 48 hours, if the blepharismas are kept in the dark and in a good state of nutrition.

The absorption spectrum of the red blepharismas peaks at wavelengths 242–250, 285, 330, 485, 540, and 572 nm (Fig. 4). In contrast, the absorption spectrum of the blue form of *B. americanum* has only two definite peaks in the visible: 560 and 600 nm, the peak at 485 of the red *Blepharisma* having almost disappeared. The peak at 330 nm and those at 245 and 280 nm remain much as in the red blepharismas.

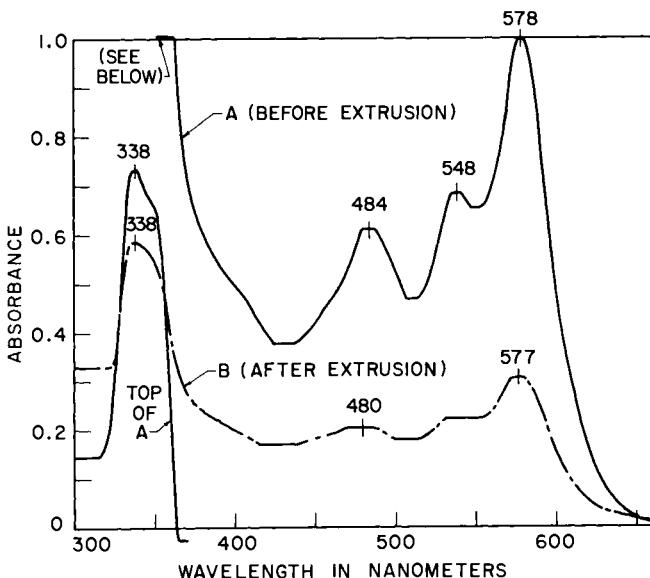


FIG. 4. Absorption spectrum of red *Blepharisma intermedium* before extrusion of pigment at A and after cold-induced extrusion at B.

It is important to note that although the height of the blue peaks is lower than that of the red peaks they are both of the same order of magnitude, indicating that light is absorbed by the blue pigment to a considerable degree (Giese and Grainger, 1970). One might therefore expect blue blepharismas to be photosensitive. However the blue forms in all three species of *Blepharisma* tested—*B. americanum*, *B. intermedium* and *B. japonicum*—were not sensitive to intense visible light. Furthermore, when the blue form was exposed to intense visible light in a Cartesian diver, there was no increase in oxygen consumption (Giese and Zeuthen, 1949). Apparently exposure to dim light had induced a change

in the pigment of *Blepharisma* such that it no longer acts as a photosensitizer.

2.4.2 PROPERTIES OF BLEPHARISMA PIGMENT

It was possible to shed the pigment from the granules of *Blepharisma* into the culture medium if the culture was suddenly chilled to 0°C for 30–120 seconds. If the cells were warmed to room temperature immediately afterwards, only a very few died, although prolonged exposure to 0°C killed them. Moreover, if such cells were transferred to bacterized nutrient medium and kept in the dark, they regenerated the pigment to the previous level in 48–72 hours; they failed to do so in balanced salt solutions without nutrient. The pigment can thus be “milked” by alternate growth in the dark and extraction by cold. The blue form of the pigment can also be extruded by the same method.

Interestingly, recovery of another crop of red pigment by blue cells is quicker with than without extrusion. This suggests that the cell must remove the blue pigment before replacing it with red pigment and when the blue pigment is extruded physically the cell is saved the labor of removing it chemically. Presumably the red pigment is being continually formed but the rate of formation (22–48 hours for the crop) is rather slow, compared to the conversion of red to blue by dim light (4–8 hours).

Pigment, red or blue, can also be extracted with alkaloids (Nadler, 1929), heat (Giese, 1953), as well as salts, e.g., NaCl, 0.9% (Prahabkara Rao, 1963), but these methods appear to be much more damaging than cold.

The *Blepharisma* pigment (red or blue) is readily soluble in various alcohols and other polar solvents. Møller (1962) extracted the pigment in methanol and studied its properties spectroscopically and chemically, and suggested its similarity to hypericin. His work was extended by Sevenants (1965) who extracted the pigment with acetone and, after purifying it, chromatographed it and compared it with hypericin with respect to chromatography, visible absorption spectra, fluorescence spectra, infrared absorption spectra, and chemical tests for various bond groupings in the molecule. He concluded that while there were some differences between zoopurpurin and hypericin, the basic structure of zoopurpurin, shown in Fig. 1(G), is very similar to that of hypericin [Fig. 1(E)]. The amount of pigment available was too small for organic chemical analysis; therefore, the formula for zoopurpurin must be considered tentative.

Absorption by the purified red form of the *B. intermedium* pigment dissolved in ethanol, peaks in the visible at 575, 537, and 485 nm (Fig. 4). There is a high peak in the near UV at 330 nm and peaks or

shoulders in the far UV at about 280 and 240. It should be noted that the absorption peaks are approximately the same whether they were determined on the live organisms in suspension or on extruded pigment obtained by cold treatment or on ethanol extracts of the pigment (in acetone the peaks are shifted slightly) (Giese and Grainger, 1971).

Absorption by the blue form of the *Blepharisma* pigment (extracted in ethanol from cells treated for 8 hours with dim visible light) is different from that of the red form, there being only two distinct peaks in the visible at 590 and 545 and one in the near UV at 330. Absorption by blue pigment in the far UV is similar to absorption by the red pigment (Giese and Grainger, 1970).

The similarity of the absorption spectra in all species of *Blepharisma* tested argues for a single type of pigment in all of them. The differences between the absorption spectra of *B. japonicum* reported by Utsumi (1953) and *B. intermedium* by Seshacher *et al.* (1957) from that of *B. americanum* are probably the result of differences in methodology and handling.

The pigment (presumably of *B. americanum* although given as *B. undulans*) studied by Sevenants (1965) was probably mostly in the blue form, judging from his published absorption peaks. In our laboratory all of the pigments, harvested in ethanol and stored for long periods in relative darkness were in the blue form, regardless of species. Apparently, a change had been induced by photooxidation during storage or possibly even by dark oxidation.

Experiments demonstrated that, whether the pigment is in the cell, or in aqueous solution, or in ethanol, its illumination in the presence of oxygen results in a shift from the red to the blue form, suggesting that the shift is the product of a photooxidation. As expected, when oxygen was carefully excluded from the ethanol extract of the pigment, illumination through a filter with a cutoff at 310 nm (2700 fc) did not change the absorption spectrum. Oxidation of the red pigment in alcohol extract can occur also in the dark in the presence of ozone, although it did not occur after saturation with weaker oxidizing agents such as oxygen, or treatment with either H_2O_2 or potassium chlorate. The spectral shift in this case was similar to that brought about by photo-oxidation. It has not been possible to convert the blue to the red pigment by any of the reducing agents tested such as sodium hydrosulfite, hydrogen sulfide, and hydrogen in the presence of zinc or platinum (Giese and Grainger, 1970).

A change in absorption by the pigment after illumination was noted by Utsumi (1953) who also repeated an experiment (Emerson, 1930) with pH on the absorption. He thought that the blue shift by alkali (and

pink by acid) was the same as the shift by illumination. However, in *B. intermedium* the "blue" (green) form obtained in alkaline solution (NaHCO_3) has a different absorption spectrum from the photooxidized blue pigment (Fig. 5), indicating a change different from that caused by photooxidation; furthermore, the change is reversible with acid while the photochemical change is not (Giese and Grainger, 1970).

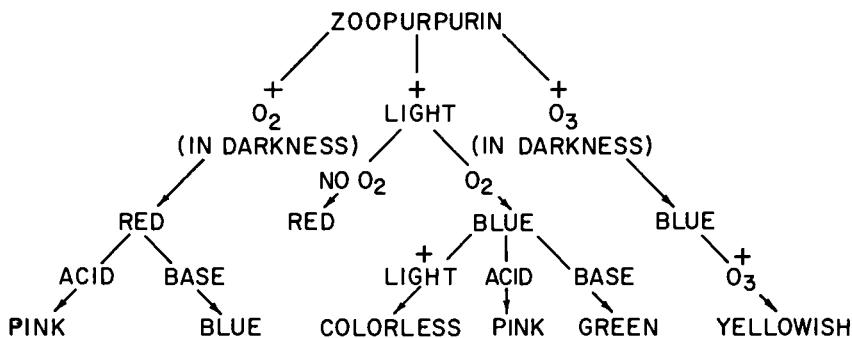


FIG. 5. Changes in color of zoopurpurin, the pigment of *Blepharisma*, after various types of treatment discussed in the text.

2.4.3 EXOGENOUS PHOTOSENSITIZING ACTION OF THE *Blepharisma* PIGMENT

The pigment of *Blepharisma*, as a true photooxidative sensitizer, in the presence of oxygen acts as an exogenous photosensitizer for various types of colorless cells which are then killed by strong visible light (Giese, 1946). In the presence of the *Blepharisma* pigment the excitability properties of nerve fibers were altered, the threshold of the neuron rose and the spike height declined rapidly after illumination, although the spike duration and the refractory period for crab and bullfrog nerve fibers did not change (Giese, 1957).

The shorter wavelengths of visible light are more effective in photosensitization of colorless cells as shown for *P. multimicronucleatum* (Giese and Grainger, 1970). Exogenous sensitization of colorless cells by the *Blepharisma* pigment thus appears to be very similar to its endogenous action in *Blepharisma*.

Evidence points to surface activity of the photosensitizing pigment of *Blepharisma*. When blepharismas were exposed to very intense visible light they were killed by what appears to be disturbance of water balance. Following enlargement, the contractile vacuole may hang behind the main part of the cell on a peduncle which may later burst. If the posterior end heals a new contractile vacuole soon forms, it may enlarge

considerably and burst, the process repeating itself, ultimately ripping the cell to shreds. Photosensitizing dyes generally appear to act on protozoans at the surface as shown by interference with water balance, vesiculation and cytolysis (Giese, 1966).

It is interesting that when blepharismas which had been adapted to $40\times$ the osmolal concentration of the culture medium were exposed to intense visible light they proved much more resistant than controls, being killed only after a lapse of about two hours. They showed little enlargement of the contractile vacuole following irradiation, perhaps because of little influx of water (Giese, 1970).

2.4.4 POSSIBLE ROLE OF THE PIGMENT OF *Blepharisma*

Møller (1962) has suggested that perhaps the role of pigments such as zoopurpurin in *Blepharisma* is orientation to light but there is little evidence to back this hypothesis for *Blepharisma*.

It has been shown that the albino strain of *B. intermedium* (containing only a very minute amount of pigment) is much less resistant to short UV radiation than the pigmented wild (red) type (Giese, 1965). Since *Blepharisma* pigments (red or blue) absorb the short UV radiation strongly (Giese and Grainger, 1970) and are superficial in the cell, absorption of incoming UV radiation in sunlight could protect the internal structures of the cell. Perhaps the strongest argument for a possible protective role of the pigment is the low degree of photoreactivation (reversal of UV damage) observed in *Blepharisma*. Whereas many of the colorless protozoans show close to 90% photoreactivation of division delay (Giese, 1966), red *Blepharisma* shows maximally only about 30% photoreactivation, even in absence of oxygen (Giese and Lusignan, 1961), as does the albino as well (Smith, 1970). In any case, the disadvantage of the sensitizing action of the pigment may be compensated for by the UV-protective function. That the colorless *Blepharisma* has not been found in nature strengthens the argument.

2.5 Stentorin, the Hypericin-like Pigment of *Stentor coeruleus*

Comparatively few studies have been made with stentorin, the pigment of *Stentor coeruleus*. This blue endogenous pigment is present in granules just under the pellicle, between ciliary rows, much as in *Blepharisma*. Møller (1962), on the basis of extensive studies of its chemical reactions and spectrophotometric properties, decided that the pigment was similar to hypericin although no direct chemical analysis has been made because of the small quantity of pigment available.

Møller found that *Stentor* occurred in two strains, one which showed

fluorescence under appropriate conditions and another which did not. Only the fluorescent strains were photosensitive and only from these was it possible to extract the pigment with ethanol and other polar solvents.

The pigment of nonfluorescent stocks of *Stentor* is not only insoluble in most solvents, but it is resistant to various hydrolyzing agents, possibly because of its highly polymerized state. Presumably, according to Møller, in the polymerized state it fails as a photosensitizer because it cannot produce the excited states necessary for reaction. No further work appears to have been published on this interesting pigment.

3. Chlorophyll

3.1 Photosensitization by Chlorophyll in Mutant Strains of Photosynthetic Bacteria

Griffiths *et al.* demonstrated in 1955 that when the wild-type purple bacterium, *Rhodopseudomonas sphaeroides*, was exposed to bright visible light in presence of oxygen, bacteriochlorophyll synthesis stopped, although it was resumed as soon as the cells were placed under anaerobic conditions even in the same light. However, in the blue-green mutant of this species the colored carotenoids characteristic of the wild type are replaced by the colorless polyene, phytoene, with only three conjugated double bands (see Fig. 7). In this mutant growth and bacteriochlorophyll synthesis occur in light in the absence of oxygen and growth occurs in darkness even in the presence of oxygen. Oxygen alone inhibits neither growth nor pigment synthesis, nor does light in the absence of oxygen, but both growth and bacteriochlorophyll synthesis cease when the cells are exposed to light in the presence of oxygen. In fact under these conditions most of the cells are killed, as shown by viability tests (Fig. 6) (Sistrom *et al.*, 1956).

The action spectra for killing of the blue-green mutant and the inhibition of bacteriochlorophyll synthesis in wild-type *R. sphaeroides* on exposure to light are essentially like the absorption spectrum of bacteriochlorophyll (Griffiths *et al.*, 1955), indicating that the bacteriochlorophyll is the endogenous photosensitizer. This conclusion is corroborated by decreased photosensitivity of the blue-green mutant grown for a long time in darkness. Thus growth on organic nutrients in the dark partitions the store of bacteriochlorophyll between progeny, thereby diluting the initial supply to the vanishing point (no bacteriochlorophyll synthesis occurs in darkness) and in absence of this endogenous photosensitizer the cells are not injured by light even in the presence of oxygen (Griffiths and Stanier, 1956).

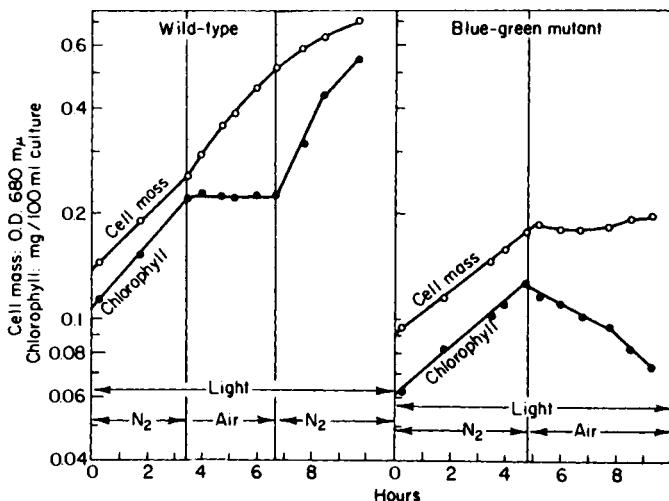


FIG. 6. The effect of oxygen (in air) in presence of light on growth and pigment synthesis in the blue-green mutant and the photosynthetic bacterium, wild-type *Rhodopseudomonas sphaeroides* (atmosphere: 95% N₂-5% CO₂, or 95% air-5% CO₂ as shown on the graphs; temperature 30°C). (From Sistrom *et al.*, 1956.)

3.2 Protective Action of Carotenoids

Since under similar conditions wild-type *Rhodopseudomonas* cells containing bacteriochlorophyll and colored carotenoids are not killed as are blue-green mutant cells containing bacteriochlorophyll but no colored carotenoids (Fig. 7), the colored carotenoids appeared to be the likely protective agents (Sistrom *et al.*, 1956). The experiments recorded below largely corroborated this suggestion. A green mutant in which carotenoids up to neurosporene with nine conjugated double bands are present, is shown to be more sensitive to light than the wild-type but not nearly as sensitive as the blue-green mutants.

A phenotype of the green mutant of *R. sphaeroides* was produced in the related species *R. rubrum* by inhibiting synthesis of colored carotenoids with diphenylamine (DPA), in the presence of which only colorless carotenoids are synthesized (Cohen-Bazire and Stanier, 1957). Such DPA-treated cells are sensitive to bright visible light in the presence of oxygen, although photokilling is considerably less rapid than in the blue-green mutant of *R. sphaeroides* and photodestruction of bacteriochlorophyll occurs even more slowly.

Dworkin (1959) argued that if colored carotenoids protect bacteriochlorophyll and are selectively photooxidized, then inhibition of the carotenoid reductions by exposure to low temperatures, which slows

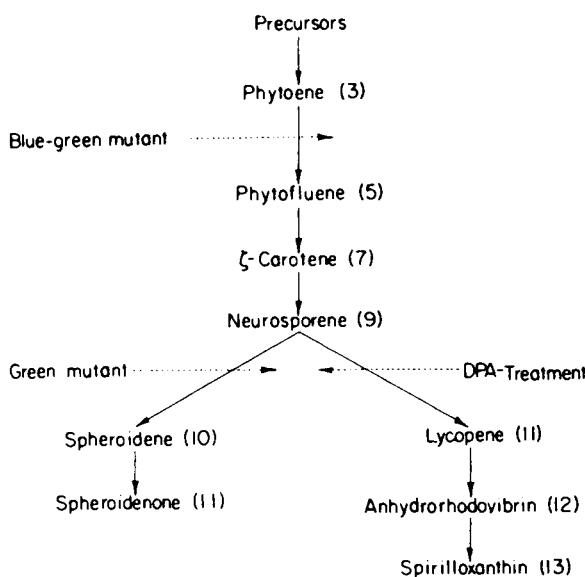
Rhodopseudomonas sphaeroidesRhodospirillum rubrum

FIG. 7. The pathway of carotenoid biosynthesis in wild-type and mutant strains of the photosynthetic bacteria, *R. sphaeroides* and *R. rubrum*. Blocks of synthesis, as a result of mutation, or diphenylamine (DPA)-treatment of wild type, are shown by dotted lines. The numbers in parentheses indicate the number of conjugate double bonds. (From Krinsky, 1968, p. 132.)

enzymatic reactions, should make wild-type cells sensitive to light. He illuminated cold-treated cultures of both *R. rubrum* and *R. sphaeroides*, and found enhanced killing at 1°C, confirming this hypothesis.

Since the colorless carotenoids with few conjugate double bonds do not protect the blue-green mutant while the colored ones, with many conjugate double bonds, protect the wild type, the relation between the number of unsaturated double bonds and the protective value of the carotenoid shown in Fig. 7 is of interest. It will be noticed that the blue-green mutant synthesized only carotenoids of a lower degree of saturation than the green mutant and is therefore much more sensitive to light in presence of oxygen. So is the diphenylamine (DPA) phenotype of the green mutant. In a study of six other mutants of *R. sphaeroides* with blocks at different points in the pathway of carotenoid biosynthesis, Crounse and Sistrom (1963) and Crounse *et al.* (1963) found that only

neurosporene (with 9 double bonds) is capable of some degree of protection against photosensitization in presence of oxygen.

Stanier and Cohen-Bazire (1957) and Claes and Nakayama (1959) showed that in the *in vitro* mixtures of chlorophyll and carotenoids containing 3, 5, 7, 9, or 11 conjugated double bonds, protection against photooxidation of chlorophyll in visible light occurred only when the number of such bonds was greater than seven (Fig. 8). Increasing the ratio of carotenoids to chlorophyll here also increases the degree of protection, the "maximal" value ("saturation") being at about 65% protection. Oxidative photosensitization is thus a relative phenomenon both *in vivo* and *in vitro* and dependent upon the degree of unsatura-

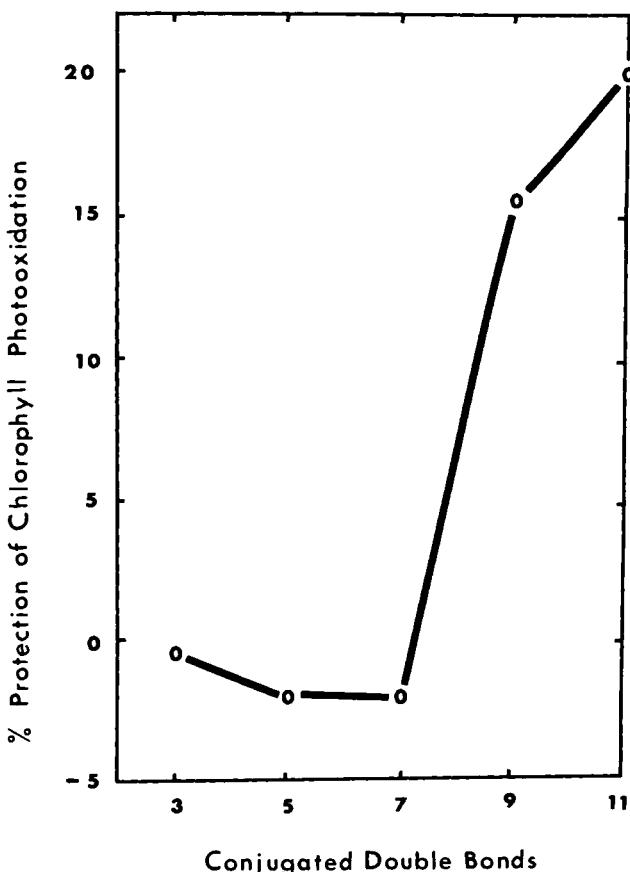


FIG. 8. The protective effect of carotenes, containing 3 to 11 conjugated double bonds, on the photooxidative destruction of Chl a in petroleum ether after 6 hours' illumination with red light. (From Krinsky, 1968, p. 175.)

tion of the carotenoid present (see Stanier, 1959). That factors other than presence of unsaturated carotenoids, possibly their organization in the cell membrane, are also important is indicated by the finding that some cells possessing the same unsaturated carotenoids are differentially sensitive to the same dose of light: thus the DPA-treated *R. rubrum* is killed while the green mutant of *S. sphaeroides* is not. Feldman and Lindstrom (1964) concluded that the carotenoids of *R. sphaeroides* act as biological buffers for excess oxidizing power.

That photosensitization in mutant strains of photosynthetic bacteria is not the result of peroxide formation is shown by studies with double mutants, which are blue-green but high in catalase activity (Clayton, 1961; Clayton and Smith, 1960). Such mutants were produced from mutant *Rhodopseudomonas* high in catalase and growing in hydrogen peroxide-containing media, by inducing mutations with UV irradiation and selecting blue-green mutants which were double mutants (blue-green, high catalase). The double mutants were no more resistant to visible light in presence of oxygen than the blue-green single mutant strain low in catalase.

3.3 Nature of Reactions Occurring during Photosensitization

The Q_{10} for killing of the blue-green mutant by visible light in air is approximately 1 over the span 4° to 40°C , suggesting a simple photochemical reaction. The Q_{10} for photodestruction of bacteriochlorophyll is 3.7 between 20° and 30°C , but photokilling will not occur at 6°C , for example, suggesting an energy transfer from bacteriochlorophyll to an adjacent acceptor molecule (Krinsky, 1968).

Permeability changes have been observed in photosensitized blue-green bacteria, as indicated by 260 nm-absorbing substances leaking from photosensitized cells after illumination (Matthews and Sistrom, 1960). Permeability change has also been measured with the dye, sodium 8-anilino-1-naphthalene sulfonate (ANSA) which fluoresces only when bound to a protein. Fluorescence was observed neither in the dye-containing medium around cells nor inside the cells before exposure to visible light, but was observed inside the cells after illumination, indicating entry of the dye into the cell and binding to its proteins. The increase in fluorescence of *Sarcina lutea* has a Q_{10} of 1.08 over the temperature range 4° – 22°C , indicating that the rate-limiting reaction (entry of dye or reaction with protein) is photochemical. However some experiments cast doubt that the membrane is the site of the photooxidation since, when the antibiotic polymixin (which disrupts cell membranes) is added at various times during light treatment it has effects on the

permeability of the cell to the dye 4 to 5 times the magnitude of the photosensitization alone. Either the photochemical effect causing change in permeability and/or structure of the membrane is slight compared with that of the antibiotic, or the cell membrane is continually repaired during light treatment alone. The polymyxin-treated (ruptured?) membrane may be fully open to the dye (Matthews and Sistrom, 1960).

Interestingly, desensitization to aerobic photosensitivity takes place in *R. rubrum* cells which have been grown in air in the dark. Since the cells grown in darkness have less pigment than those grown in light, their decreased photosensitivity could have been due in part to their attenuated bacteriochlorophyll content, but a quantitative relation between bacteriochlorophyll content and sensitivity could not be demonstrated. Furthermore, photodesensitized cells retained their greater resistance to light when tested with an exogenous synthetic photosensitizer (toluidine blue) in presence of oxygen. This suggests some change, not ascertained, possibly in the composition or structure (or both) of the chromophores and cell membranes (Dworkin, 1958, 1960).

Considerable evidence has accumulated for both photosynthetic and nonphotosynthetic bacteria to indicate that the photosensitizing effect may be localized in the cell membrane. In photosynthetic species both bacteriochlorophyll and carotenoids are in the membrane-associated chromatophores (Dworkin, 1958). In several of the nonphotosynthetic bacteria, subject to natural photosensitization, carotenoids have been localized in the cell membrane. In mutant cells colorless carotenoids synthesized in the cell membrane may well take the place of colored carotenoids. Krinsky (1968), in concluding an excellent account of the role of carotenoids in photosensitization, states that a number of alternate ways exist by which protection against endogenous photosensitizers in bacteria might occur. The carotenoids might (1) serve as a filter system to remove the light; (2) interact and quench the photosensitizer triplet states; (3) serve as the preferred substrate for photosensitizer oxidations; and (4) stabilize membranes or repair damaged ones. Some of these possibilities are subject to test but the appropriate experiments do not seem to have been performed.

3.4 Photosensitization in Algae

From X-rayed *Chlorella vulgaris* Claes (1954) isolated several mutants which lacked highly unsaturated carotenoids such as β -carotene present in the wild type. One of these mutants produced only phytoene and no xanthophylls, another phytoene plus phytofluene and ζ -carotene and a third mutant produced these three compounds plus β -zeacarotene. All three mutant strains grew in glucose, in the dark, but were killed by exposure to visible light in presence of oxygen.

Pigment destruction, accompanied by oxygen consumption, occurred in a carotenoid-deficient mutant of *C. vulgaris* on exposure to light of high intensity (100,000 lux), blue light being more effective than red. Metabolism was also affected. In the absence of a nitrogen source in the medium neither effect was observed (Kandler and Schötz, 1956). After 24 hours of dark adaptation, when the same mutant was subsequently exposed to high-intensity light (100,000 lux) two phases of bleaching were observed—(1) an induction period during which little bleaching occurred and which was more rapid in pure O₂, and (2) a period of rapid destruction of pigment in either air or O₂, the more reduced chlorophyll and carotenoids being more readily destroyed. These effects were prevented in a nitrogen atmosphere (Sironval and Kandler, 1958). In presence of CO₂ photosynthetic rate was depressed after 10 minutes, but oxygen evolution continued until all the chlorophyll was bleached.

A number of UV-induced mutants in *Chlorella pyrenoidosa* isolated by Allen (1939) and Bendix and Allen (1962) show a variety of photo-sensitizations. Some strains which were photosensitive lost their property when glucose was added to the medium. Others were photosensitive at some temperatures, not at others. Some were photosensitive when in highly aerated cultures not on slants. Some were sensitive only at high light intensity. Most of them lacked colored carotenoids, but even some containing colored carotenoids were photosensitive, perhaps because of altered localizations of the colored carotenoids in the membranes of the cells. Exposure to light of *C. pyrenoidosa* grown on a N-deficient medium, bleached both carotenoids and chlorophylls, the latter faster than the former, Chl a before Chl b, carotenes before xanthophylls (Aach, 1953).

Sager and Zalokar (1958) isolated a photosensitive pale green mutant of *Chlamydomonas reinhardi* which contained only α and β -carotene but no xanthophylls. Photosensitivity was shown only in presence of oxygen but was not studied in detail (Gross and Duggar, 1969).

Wild-type *Euglena gracilis* showed photosensitivity to red light (> 610 nm) when vigorously aerated, presumably via chlorophyll in the cell (Leff and Krinsky, 1967). *Euglena gracilis* wild type showed aerobic photosensitivity to low intensity of white light in the presence of streptomycin, not in its absence (Grainger and Giese, 1970). In this case the euglenas formed a pattern of a grill interposed between the light source and a plate culture seeded to form a lawn. The region of most intense light was essentially devoid of color. Part of this was the result of induced migration of the euglenas out of the light, part to bleaching of chlorophyll. A streptomycin-resistant mutant, however, proved insensitive to the light, showing no such pattern and no migration.

In a dark-adapted wild-type *E. gracilis*, carotenoids were destroyed on exposure to bright light. This occurred even in a chlorophyll-free mutant, indicating some photosensitizer other than chlorophyll. A crude action spectrum measurement suggested a porphyrin, possibly a precursor of chlorophyll (Wolken and Mellon, 1956).

Chlorophyll and carotenoids were destroyed in *Laminaria digitata* and *L. saccharina* by exposure to visible light in the long wavelength end of the spectrum, the carotenoids more rapidly than the chlorophylls, and carotene and fucoxanthin more rapidly than the xanthophyll fraction of the pigments (Montfort *et al.*, 1952).

3.5 Photosensitization in Higher Plants

In the albino mutant seedling of *Zea mays* (Indian corn) protochlorophyll was converted to chlorophyll in dim visible light. Continued illumination destroyed the chlorophyll but did not affect the reactions which produced more protochlorophyll in the dark (Koski and Smith, 1951). The white mutant contained no detectable carotenoids. In a series of 15 mutant strains ranging in carotenoid content from none in some mutants to almost the amount present in the wild type in others, photodestruction of chlorophyll was shown in all, indicating that the chlorophyll-carotenoid relationship was not as simple as in the photosynthetic bacteria (Smith *et al.*, 1959).

Isolation of the carotenoids from corn by chromatography in the wild type showed three carotenoid spots in an albino mutant, only one with an absorption spectrum resembling that of ζ -carotene (Faludi *et al.*, 1960). The carotenoid content of the leaves decreased with illumination in presence of oxygen, not otherwise.

Anderson and Robertson (1960, 1961) showed that the albino mutant of corn (white-3) accumulates the colorless carotenoid instead of the colored carotenoids of the wild type. Destruction of chlorophyll (of which there is little) occurs only in the presence of O_2 , not in N_2 . When white-3 was grown in low-intensity visible light (0.5 fc) it was bluish-green and contained small amounts of ζ -carotene, phytofluene and phytoene. The carotene and phytofluene were destroyed by exposure to more intense light along with the chlorophyll, but phytoene was not. They observed that the porphyrin enzyme catalase was also destroyed by light exposure and considered that possibly cytochrome, another porphyrin enzyme, might also be destroyed, although this was not ascertained. Catalase appeared to be in diminished quantity when chlorophyll was deficient (Eyster, 1950), although this has been questioned by Appleman (1952) who found that catalase was initially present in high concentration in etiolated plant seedlings (maize, wheat, barley, rye,

oats) and decreased on illumination at 25°C but did not decrease at 1°C. This suggests that the photochemical reaction is followed by secondary thermal reactions. However, crystalline catalase added to albino mutants was more readily destroyed than when added to wild-type strain, and only when O₂ was present (Mitchell and Anderson, 1965).

Somewhat similar results have been obtained with albino and yellow mutants of the giant sunflower *Helianthus annuus* (Wallace *et al.*, 1948). At very low light intensity some greening occurred, optimally at 0.24–0.5 fc. At 10 fc the plants (albinos) were white. The white mutant contained no carotenoids while the yellow one had xanthophylls (Wallace and Haberman, 1959). The mutants did not grow beyond the size made possible by the food supply contained in the cotyledons.

The distribution of chlorophyll-induced photosensitizations is summarized in Table I. It is evident that photosensitive mutants probably occur wherever plants with chlorophyll have been studied. Perhaps the term solarization or damage by sunlight, indicates its generality, even in the wild type when the light is very intense. No reports have been made of photosensitivity in *Bryophytes* or *Pteridophytes*—perhaps because they have not been subjected to such study. Chlorophylls are not absorbed from the gut of animals because of the side chains (phytyl especially) on the molecule which decrease its permeation to the vanishing point; therefore, chlorophyll sensitization in animals after feeding on green plants is quite unlikely. Phylloerythrin, a product of microbial action on chlorophyll lacking the side chains which would prevent permeation, is under certain circumstances an important animal photosensitizer (see below).

4. Porphyrins

A porphyrin (Fig. 9) is a cyclic compound composed of four pyrrole units [Fig. 9(B)] linked by methyne (—CH=) bridges. A porphyrin may be considered a derivative of porphin [Fig. 9(A)], which is a cyclic pyrrole but without substitutions in the side chain. It is these substitutions in the side chains which characterize a porphyrin, as illustrated for a few of them in Fig. 9(E)–(N). Heme is a porphyrin with iron bound to the nitrogen atoms of the pyrroles [Fig. 9(E)]. Chlorophyll (Fig. 9L, M) is a porphyrin with magnesium bound to the nitrogen atoms of the pyrroles and with a cyclopentanone ring on one pyrrol and a phytol hydrocarbon on another (Canterow and Schepartz, 1967).

Porphyrins are universal in aerobic organisms inasmuch as such cells possess the hemes, cytochrome and cytochrome oxidase. Hemoglobin (heme united to globin) occurs not only in the vertebrates but also in a

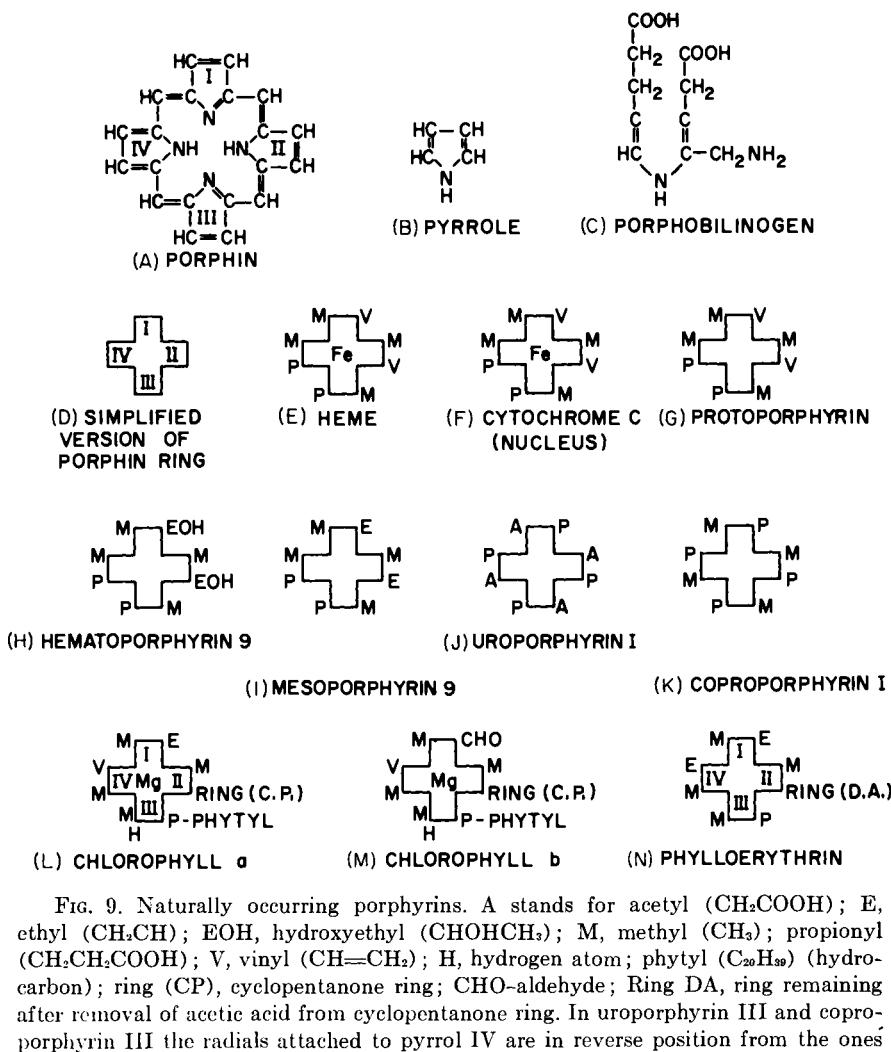


FIG. 9. Naturally occurring porphyrins. A stands for acetyl (CH_2COOH); E, ethyl (CH_2CH_3); EOH, hydroxyethyl (CHOHCH_3); M, methyl (CH_3); propionyl ($\text{CH}_2\text{CH}_2\text{COOH}$); V, vinyl ($\text{CH}=\text{CH}_2$); H, hydrogen atom; phytol ($\text{C}_{20}\text{H}_{39}$) (hydrocarbon); ring (CP), cyclopentanone ring; CHO-aldehyde; Ring DA, ring remaining after removal of acetic acid from cyclopentanone ring. In uroporphyrin III and coproporphyrin III the radials attached to pyrrol IV are in reverse position from the ones shown in uroporphyrin I and coproporphyrin I. Note 1: Phosphobilinogen is the monopyrrol precursor of porphyrin. Note 2: The porphyrins in the cell with catalytic activity have a metal atom attached to the pyrrol nitrogen, e.g., iron in cytochrome and heme, Mg in chlorophyll. The porphyrins acting as photosensitizers lack the metal component. (After several sources; largely after Canterow and Schepartz, 1967.)

wide variety of invertebrates (annelids, gephyrean worms, some echinoderms, arthropods, mollusks, flatworms, nematodes), and in *Paramelania* and legume root nodules (Prosser and Brown, 1961). Chlorocruorin, a heme pigment akin to hemoglobin, is found in a few species of annelid

worms. Chlorophyll is found in all photosynthetic bacteria and plants.

Because of the wide distribution of porphyrins, any species of aerobic life is a candidate for photosensitization since porphyrins are known to photosensitize a wide variety of cells. Chlorophyll photosensitization has already been discussed in Section 3. In this section other reported cases of porphyrin photosensitization in organisms will be considered. It is likely that even more cases of photosensitization by porphyrins will subsequently be documented. The distribution of reported cases of porphyrin sensitization in organisms is shown in Table I.

4.1 Phylloerythrin Photosensitization in Stock Animals

Phylloerythrin [Fig. 9(N)] is a porphyrin derivative of chlorophyll (Fig. 9L) formed during digestion of green plants in the gut, through conversion of chlorophyll by microorganisms. Normally phylloerythrin is voided from the animal by way of the bile into the feces. Under some conditions it enters the bloodstream and thus it gets to the skin where it can absorb light and serve as a photosensitizer. Quin and Rimington in 1935 described "geeldikkop" or "yellow-thickhead," a disease of sheep in the Karoo-veldt of South Africa, resulting from phylloerythrin photosensitization (see Clare, 1956). The name of the disease refers to its two major characteristics—yellow color from jaundice (icterus) induced by substances in ingested plants and swelling of the skin of the head resulting from edema induced by photosensitization with phylloerythrin. The disease is a sequel to feeding on plants of only certain species, especially when they are wilted during drought following the lush growth of the rainy season, at which time the wilting plants contain jaundice-producing substances (*icterogens*). Among the accused species of plants are several members of the genus *Tribolus* (hence the disease is often called tribolism), e.g., *T. terrestris* and *T. ovis* (family Zygomillaceae). Some species of *Lippia* (*L. rhemannii* and *L. pretoriensis*, family Verbenaceae), as well as species of the grass *Panicum* (*P. laevifolium* and *P. coloratum*, family Graminae), have also been considered icterogenic. The icterogen causes constipation of the large bowel and stops the discharge of bile. Under these conditions the phylloerythrin is retained in the gall bladder, reabsorbed into the bloodstream, and distributed to the skin.

Phylloerythrin was isolated from the blood of sick animals by Rimington and Quin (1934) and obtained in crystalline form, which when injected into the bloodstream of sheep not fed on green plants (controls) induced skin oedema after exposure to light, but jaundice was absent.

Jaundice (icterus) followed by photosensitization is induced by eating

the plants aforementioned, but if the jaundiced sheep are then given food without chlorophyll (e.g., grain), they remain temporarily jaundiced but are no longer photosensitive. No phylloerythrin appears in the bile or in the blood serum. As soon as feeding of greens is resumed, however, both the bile and serum disclose phylloerythrin and the sheep become photosensitive.

Icterus can be induced in sheep without icterogens by simply ligating the bile duct. Such sheep develop jaundice but are not photosensitive while on a chlorophyll-free diet. Placed on a green diet, on the other hand, they soon develop photosensitivity, even in absence of icterogens in the ingested plants (Rimington and Quin, 1934). It has been shown that the phylloerythrin absorbed into the blood sensitizes the cells of the skin to sunlight, even to wavelengths transmitted by window glass (Bourne, 1953). The ensuing photosensitization is an exaggerated form of that described for hypericism and fagopyrism. Thus, unprotected parts of the skin (on the face and the ears) swell enormously, the coronets at the base of the horns become purple-red, the skin turns hard and brown, a high fever develops and even a measure of shock, and affected sheep cannot drink or eat and starve to death; postmortem, they show marked jaundice in all tissues. Goats are sometimes affected in like manner.

A photosensitivity disease is shown in purebred Southdown sheep in New Zealand when the lambs first start eating grass. The causative agent is a phylloerythrin. Susceptibility appears to behave as a Mendelian recessive and is accompanied by some liver dysfunction (see Clare, 1956).

Although individual cases of geeldikkop continue to be documented, search of the abstracts discloses no recent advances in basic knowledge of phylloerythrin diseases. Little also has been done on the photophysiology of phylloerythrin photosensitization. An action spectrum determined with filters shows fair correspondence with absorption by phylloerythrin (Riemerschmid and Quin, 1941).

Another decomposition product of chlorophyll, pyropheophorbide *a* (not a porphyrin), has been found to photosensitize man and other animals. It was extracted from the digestive gland of several species of abalone (*Haliotis*) occurring on the Japanese coast (Hashimoto and Tsutsumi, 1961; Tsutsumi and Hashimoto, 1964).

4.2 Porphyria in Man and Stock Animals

Hematoporphyrin (Fig. 9H), a derivative of heme, serves as a very effective photosensitizer. It has also been used to sensitize protozoans (Lassen, 1927), bacteria, red blood cells, and mammals (Dankemeyer,

1930), including man. A heroic experiment is the "selbsversuch" performed by Meyer-Betz, who in 1913 gave himself an intravenous injection of hematoporphyrin (Fig. 10). He remained sensitive to light for several months afterwards, developing erythema, swelling, and pigmentation with each subsequent exposure to light. However hematoporphyrin has in no case been demonstrated to occur as a natural photosensitizer in man or animals (Laurens, 1933; Blum, 1941, 1964).

On the basis of the Meyer-Betz experiment it was thought that individuals with porphyria (urine burgundy-colored from porphyrins in some porphyrias) would likely be highly photosensitive because the pigment might be carried to the skin. However, the results of early experiments were inconclusive and porphyrias were therefore not necessarily considered photosensitizing (Blum, 1941). Considerable evidence has since accumulated to indicate skin photosensitizations in some porphyrias, not in others (Rimington *et al.*, 1967).

Perhaps skin photosensitization in some porphyrias was not recognized in the past because the commonest porphyria is the acute intermittent form in which no photosensitivity is observed. Considerable confusion exists in attempting to correlate the information about porphyrias from account to account. It would appear from the literature that each

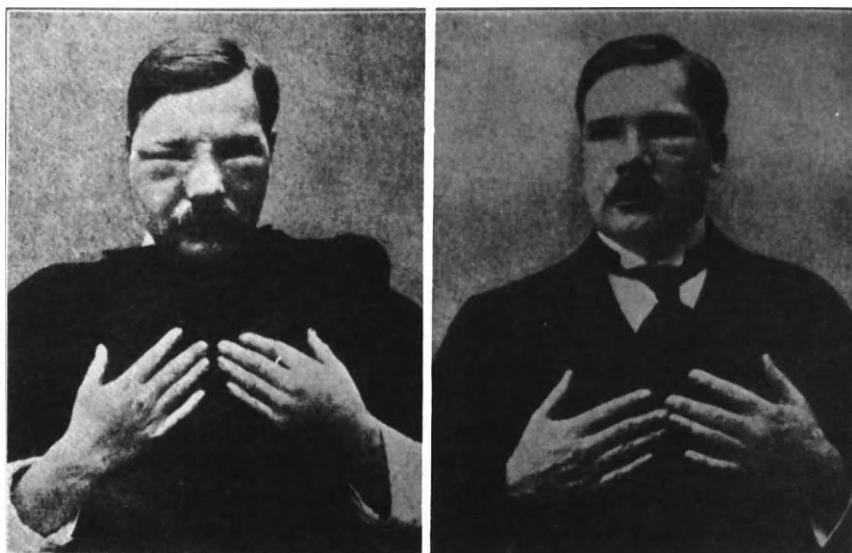


FIG. 10. Swelling observed on exposure to light after intravenous injection of 0.2 gm hematoporphyrin. Meyer-Betz, who performed this "Selbsversuch" on Oct. 14, 1912, was still sensitive to light at the beginning of December. By spring he was completely desensitized. (From Laurens, 1933, p. 496.)

author classifies the porphyrias as seems best for the material to be presented in his report. The many difficulties in classification, largely because of a lack of basic information, have been considered by Schmid (1966). Burnham (1969) has attempted to classify the porphyrias in the following manner.

-
1. Erythropoietic porphyrias
 - Congenital erythropoietic protoporphyrria
 - Congenital erythropoietic porphyria
 2. Hepatic porphyrias
 - Acquired
 - Toxic hepatic porphyria
 - Chemical porphyria (experimental)
 3. Congenital
 - Cutaneous hepatic porphyria
 - Intermittent acute porphyria
-

Several types of porphyria have been well documented. These are congenital erythropoietic protoporphyrria, congenital erythropoietic porphyria (coproporphyrina), congenital hepatic porphyria (porphyria cutanea tarda), toxic (symptomatic) hepatic porphyria, and variegate porphyria (Rimington *et al.*, 1967). Some of the porphyrias are seen in stock animals as well as man.

In congenital *erythropoietic protoporphyrinia* red cell protoporphyrin [type 9, see Fig. 9(G)] increases markedly (between 5–30 \times normal), sometimes coproporphyrin [Fig. 9(K)] as well. Protoporphyrin may also increase in the plasma. The urine is normal but stool porphyrin may be increased.

In vitro, red cells of individuals with erythropoietic protoporphyrinia are much more sensitive to light of wavelengths absorbed by porphyrins than those from normal individuals (Harber *et al.*, 1964; Fleischer *et al.*, 1966). However, little hemolysis appears to occur *in vivo* and significant anemia does not accompany the disease (Peterka *et al.*, 1965). The accumulation of porphyrins in the red cells can be detected readily by fluorescence microscopy and is a useful diagnostic test, the porphyrins having a typical fluorescence, different from that of other compounds likely to be found in the red cells. Hematopoietic tissue probably is not the only source of the excess protoporphyrins. Labeling experiments (glycine-¹⁵N) suggest origin in other tissues as well (Burnham, 1969). While it was considered possible that the increase in protoporphyrin in the body was due to failure of the enzyme ferrochelatase to unite iron

with porphyrins to form heme in the bone marrow, experiments (both *in vivo* and *in vitro*) demonstrate iron incorporation to be normal. At present, it is not known why in protoporphyrria protoporphyrins are produced in great excess (Burnham, 1969).

Patients suffering from this hereditary disease (dominant character), which begins in the first or second year of life, experience a marked stinging and burning of the skin on exposure to light (even in nonsunburn spectrum), and may be sensitive enough to react to light passing through thin clothes. Symptoms which may start at once or after a lapse of time following light exposure, and may last for several hours, days, or even weeks in extreme cases, are erythema, skin swelling followed by scabbing and peeling, and accompanied by itching. In some cases purpura may develop, especially in children. The areas most commonly affected are the front of the nose, the cheeks, and backs of the hands, between the thumb and index finger, and dorsum of joints. In severe cases scarring may occur. Less commonly, urticaria and wheals may develop on the exposed parts. Many cases are cited by Peterka *et al.* (1965), and a number are analyzed by Rimington *et al.* (1967).

Congenital erythropoietic porphyria is sometimes called *erythropoietic coproporphyrinia* (see Heilmeyer and Clotten, 1964; Rimington *et al.*, 1967). Because the classification of porphyria is difficult the details of the various types of metabolic derangement are still incompletely understood.

Erythropoietic congenital porphyria differs from erythropoietic protoporphyrinia inasmuch as uroporphyrin [Fig. 9(J)] and coproporphyrin [Fig. 9(K)] (the former more than the latter) accumulate in the urine (in protoporphyrinia the urine is essentially normal) and hemolytic activity is increased, although anemia is not usually observed. As in protoporphyrinia the skin develops photosensitivity and shows the symptoms described for protoporphyrinia. The genetic metabolic lesion appears to be at the level of uroporphyrinogen formation and studies on man indicate it to be an autosomal recessive (Burnham, 1969).

The genetics of this type of porphyria has been studied especially in cattle, where the disease ("pink tooth" of cattle, the teeth being colored by the porphyrin) is of special interest to stockmen. In one case on record a heterozygote bull was used in 100,000 artificial insemination matings before he was recognized as an erythropoietic porphyria carrier (Burnham, 1969).

Hepatic porphyria, also called by Rimington *et al.* (1967) *porphyria cutanea tarda* may be subdivided into two categories—one acquired or symptomatic (*porphyria cutanea tarda symptomatica*) and one congenital (*porphyria cutanea tarda hereditaria*). The symptoms in both

types of this disease develop late in life, hence the appellation, tarda. The symptomatic form of the disease results from liver damage (most commonly from alcoholism). Intake of excess iron may add to the difficulties in this disease (Rimington *et al.*, 1967). In both forms of this porphyria, symptomatic and hereditary, urinary uroporphyrin [Fig. 9(J)] is increased, coproporphyrin also to a lesser extent. Increase of porphyrins in urine is greater in the symptomatic form. The erythrocyte porphyrin is normal, but both protoporphyrin [Fig. 9(G)] and coproporphyrin [Fig. 9(K)] are raised in the stools. Plasma porphyrin levels also rise. Consequently, cutaneous photosensitivity is developed, but not to the extent found in erythropoietic protoporphyrina and porphyria (Rimington *et al.*, 1967; Burnham, 1969).

Variegate porphyria (Rimington *et al.*, 1967) is common among white people in South Africa and is a hereditary dominant character which develops at puberty. Cutaneous symptoms are somewhat similar to those for *porphyria cutanea tarda*, but in addition there may be symptoms of neuropathy, psychoses and abdominal symptoms much like those found in acute intermittent porphyria. Attacks appear to be precipitated by use of barbiturates. The red cell porphyrins are normal in this disease also, but the levels of urinary porphyrins, porphobilinogens and Δ -aminolaevulic acid levels (precursors or products of porphyrins) are markedly increased. Porphyrins are increased in stools and in the plasma. The latter leads to cutaneous photosensitivity. Some of the symptoms of *variegate porphyria* resemble those of acute intermittent porphyria, discussed below.

Congenital acute intermittent porphyria is characterized by a lack of photosensitivity; therefore, detailed discussion of it is not relevant to the present report. It is of interest, however, as

"... the most common form of abnormal porphyrin metabolism. It is characterized, as the name implies, by acute attacks, lasting days or months, and periods of remission of varying duration during which the symptoms all but disappear. The attacks are characterized by abdominal pain, variable neurological symptoms, and the excretion of large amounts of porphyrin precursors in the urine. . . . Attacks may be precipitated by drugs, most commonly barbiturates, sulfonamides, and estrogens. There is as yet no explanation for the abdominal pains or neurological disorders associated with acute attacks." (Burnham, 1969, pp. 486-487)

Some types of porphyria can now be induced in experimental animals, e.g., rabbits, by drugs such as Sulfone, Trional, and barbiturates, the disease being latent. This permits control of the conditions of induction and development making possible systematic biochemical and other studies which are impossible with human patients. These techniques may make possible resolution of some of the many difficulties in understand-

ing the various porphyrias which occur in stock animals and man (Burnham, 1969).

The photobiological aspects of the porphyrias displaying photosensitivity are relatively similar. The porphyrins have a strong band of absorption peaking at 400 nm and lesser peaks clustering about 500 and 600 nm. These wavelengths also cause photosensitization in skin in porphyrias, the action spectrum resembling the absorption spectrum of porphyrins (light of 400 nm being most effective, the 500–600 nm band somewhat effective) in all three types of porphyria: *porphyria cutanea tarda* (Wiskemann and Wulf, 1959; Magnus *et al.*, 1959), *erythropoietic porphyria* (Magnus *et al.*, 1961; Holti *et al.*, 1963) and *variegate porphyria* (Gordon, 1964).

Alison and Young (1964) observed the uptake of porphyrin by the lysosomes of cells of the monkey kidney and Alison *et al.* (1966) found that the action spectrum for injury to cells in tissue culture was similar to that for skin photosensitization in patients with protoporphyrinia. Slater and Riley (1966) showed that free radical scavengers protected lysosomes in illuminated rat epidermal cells sensitized by porphyrins by removal of the oxidizing free radicals that formed during illumination. It is interesting to speculate upon a similarity in sequence of events which may take place during action of sunburn radiations (wavelengths 290–320 nm) on normal skin, and of longer wavelengths (400–600) on skin containing porphyrins in patients with porphyria. In both it is probable that oxidizing free radicals are produced by absorption of the radiations, these in turn damage the lysosomes, their disruption in turn liberates hydrolytic and other enzymes which in turn damage the cells and evoke erythema by causing engorgement of small dermal vessels with blood. Materials diffusing from the ruptured lysosomes may also stimulate the melanocytes to deposit additional melanin in epidermal cells during both sunburn and in porphyrias. The burning and stinging sensation in porphyrin photosensitization may be a consequence of a photosensitizing effect on the local pain receptors (Rimington *et al.*, 1967).

A synergistic effect of infrared and visible radiation in photosensitization of skin in human cutaneous porphyria was pointed out by Runge and Watson (1962). In certain cases where monochromatic violet light (405 nm) failed to evoke skin photosensitization, simultaneous, or subsequent, exposure to infrared radiation (2600 nm) resulted in a significant reaction with erythema and vesiculobullous response. This interesting finding does not seem to have been exploited in subsequent studies.

4.3 Porphyrin Photosensitization in Invertebrates

Although porphyrins are present in representatives of the entire span of invertebrates sampled (Fox, 1953), there is little evidence for photo-

sensitization of such animals by the porphyrins. True, it is often stated that certain invertebrates are killed by exposure to the sun, but this could be a result of temperature or deficiency of oxygen as the water heats up. Without action spectra for the injury or response and identification of extracted pigment, or both, it is impossible to draw conclusions as to presence of a porphyrin as photosensitizer. Such evidence is of prime necessity because pigments other than porphyrins may serve as photosensitizers, especially the flavins which are as widely distributed in the animal kingdom as the porphyrins (Fox, 1953). A case in point is the necessity of light to induce spawning in some marine organisms. In the hydroid *Pennaria tiarella*, specimens kept in the laboratory in the dark for 10–12 hours and then exposed briefly to white light, will spawn after a latent period (Baker, 1936; Ballard, 1942). The hydroid *Hydractinea echinata* spawns only if exposed to light, kept in the dark, and again exposed to white light (Ballard, 1942). The ascidian *Styela partita* is somewhat similar in behavior (Rose, 1939). In the hydroid *Hydractinea epichoncha* ripening of the gonophores occurs in light; therefore, exposure to darkness after light is necessary for spawning (Yoshida, 1952). In the only case the hydrozoan *Hydractinea echinata* investigated by action spectroscopy preliminary evidence suggests a porphyrin (Hendricks and Borthwick, 1954).

A protoporphyrin was found in cultures of *Tetrahymena pyriformis* W, grown on an agar medium in the dark (Rudzinska and Granick, 1953; Kneuse and Short, 1966). This pigment, contained in granules in the cell, is extruded, possibly through the cytophyge, and colors the medium also. If the pigmented cells are illuminated in the presence of oxygen, they cytolize, although the pigment does not change color. Hematoporphyrin has long been known as a photooxidative photosensitizer to animals (Muir, 1954), including protozoans (Lassen, 1927). If accumulations of endogenous porphyrins occur in other protozoans they have not been reported.

4.4 Porphyrin Photosensitization in Bacteria

Although all cells possess porphyrins (cytochromes and cytochrome oxidase), in only a few species of bacteria photosensitization by porphyrins have been suspected and in a very few has it been observed.

Sarcina lutea, wild type, proved resistant to wavelengths shorter than 500 nm, but a mutant of this species lacking colored carotenoids was killed when illuminated by light of the same wavelengths in the presence of oxygen. In nitrogen the colorless mutant also proved resistant to the same wavelengths, indicating that killing was the consequence of photosensitized oxidation (Matthews and Sistrom, 1959). However,

a definitive action spectrum has not been determined, and though porphyrin sensitization has been suspected no pigment has been isolated.

In *Halobacterium salinarum* (Dundas and Larsen, 1963; Larsen, 1962) growth of the wild type was not affected by visible light in the presence of oxygen but in a UV-induced mutant of this species, synthesizing only colorless carotenoids, growth was decreased by exposure to visible light, though the cells were not killed (12,000 fc). The mutant also proved much more photosensitive than the wild type to exogenous photosensitization, e.g., in the presence of phenosafranine. It was shown that the carotenoids, localized in the cell membrane envelope, do not stabilize the membrane against osmotic stress resulting from dilution of the growth medium (a brine) with distilled water; they are apparently stabilizers only against aerobic photosensitization. Dependence on porphyrins, though suspected, has not been demonstrated. It is possible that instead, quinones are involved, as in many other cases of growth inhibition (Jagger, 1970). However growth in light may be affected by inhibition of cytochrome function as recently found in the alga *Prototrichia zopfii* (Epel and Butler, 1969).

Some bacteria, spoken of as *chromogenic bacteria*, develop pigments only during exposure to visible light, e.g., *Mycobacterium* (Baker, 1938). In *Mycobacterium marinum* both oxygen and visible light are necessary for the induced synthesis of carotenoids. The action spectrum for such synthesis suggests that an endogenous porphyrin sensitizer has control over the formation of the carotenoid. If dark-grown cells, containing little carotenoid, are suddenly exposed to very bright light in the presence of oxygen, they are killed. Wright and Rilling (1963) suggest that the carotenoids may protect by merely shading the true photosensitizer, but experiments to test this were not performed, nor was a porphyrin isolated.

Myxococcus xanthus is also a photochromogenic bacterium (Burchard and Dworkin, 1966). Dark-grown cells exposed to light at 18,000 fc in the presence of oxygen were killed (photolyzed). Photosensitized killing is temperature-independent, requires monovalent ions in the medium, and obeys the reciprocity law. From cells of *Myxococcus* they isolated a porphyrin-like compound with properties akin to protoporphyrin a [Fig. 9(G)], presumably responsible for the photosensitization. Addition of this compound to other cells in presence of oxygen photosensitized them to light. The porphyrin is accumulated during the stationary phase of growth of the culture. Cells from an exponentially growing culture possess only 1/16 as much porphyrin as cells from the stationary phase. As expected, cells from an exponentially growing culture are much more light-resistant than stationary phase cells (Burchard and Dworkin, 1966).

Carotenoids have recently been shown to protect against lethal photosensitization by porphyrins in animals (Matthews, 1964).

5. The Furocoumarins—Psoralens

The furocoumarins are tricyclic compounds composed of conjoined coumarin and furan rings (Fig. 11) (whence the name furocoumarin). Furan and coumarin can be condensed in 12 different ways but only two of these isomeric forms occur in nature, namely the *psoralens* and the *isopsoralens* (*angelicins*) in which a variety of substitutions are possible, many of them occurring in nature. Some of the commonest are shown in Fig. 11.

While in the literature the terms psoralen and furocoumarin are often used interchangeably, strictly speaking, psoralen and isopsoralen designate only two of the 12 isomeric forms of furocoumarins. A further confusion arises from the fact that many of the psoralens and isopsoralens

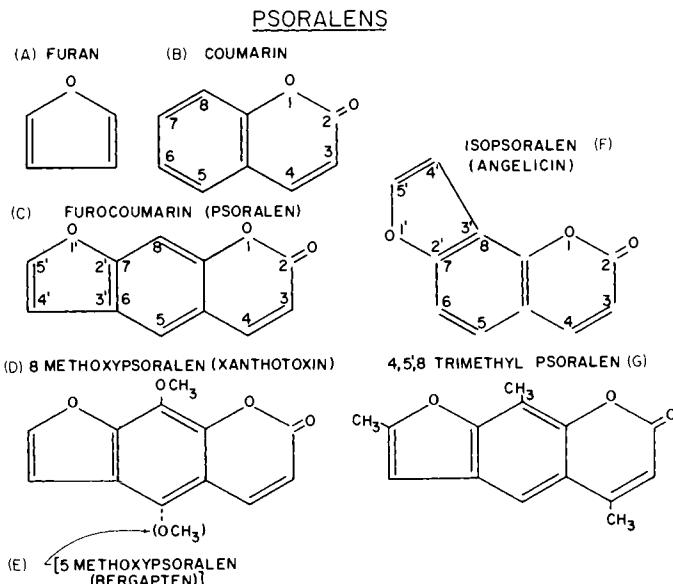


FIG. 11. Some furocoumarins. Furocoumarin is formed by combining furan (A) and coumarin (B) rings, neither of which separately has photosensitizing action on the skin or cell suspensions. Of the 12 possible isomeric forms of furocoumarin only two are found in plants: psoralen (C), the linear form, and isopsoralen (*angelicin*) (F), one of the several possible angular forms. Many substituted psoralens and isopsoralens occur in nature. Only a few are shown: 8-methoxysoralen (D); 5-methoxysoralen (E); angelicin (F); 4,5',8 trimethylpsoralen (G). Some psoralens have several synonyms. (For details, see Pathak *et al.*, 1962.)

were isolated and tested for their effect on the skin long before their chemical nature was known; therefore, names were assigned to them often based on the name of the plant of origin. Also, when the compound was isolated at different times, in different places, and each individual assigned a name to it, several synonyms would be found.

Many effects of the psoralens were recognized long ago. Thus leucoderma or vitiligo (an albino patch of the skin) was treated in India (about 1400 B.C.) with extracts of the black seeds of the leguminous plant *Psoralea corylifera* (containing psoralens), in the Nile Valley (about 1000 B.C.), and in China during the Sung Period (about 970 A.D.) with similar preparations (Hoernale, 1912; Fitzpatrick and Pathak, 1959).

As early as 1834 Kalbrunner had isolated bergapten (5-methoxy-psoralen) from bergamot oil, but it was almost a century later that this was shown to be the cause of berlock dermatitis induced by exposure to sunlight after coating the skin with bergamot oil. Also, it has been known for a long time that celery workers are subject to photosensitization (Henry, 1933, 1938; Birmingham *et al.*, 1961). Kuske (1940) first attempted to unify the scattered information about these natural photosensitizers and concluded that probably all were psoralens. To Kuske the data implicated psoralens in perfume dermatitis, berlock dermatitis,

TABLE III
FAMILIES OF PLANTS CAUSING PHYTOPHOTODERMATITIS^a

Family	Common name	Examples (species)
Psoralens extracted		
Umbelliferae	Parsley family	Garden parsley (<i>Petroselium sativum</i>) Angelica (<i>Angelica archangelica</i>) Bishop's weed (<i>Ammi majus</i>) Bergamot (<i>Citrus bergamea</i>) Rue (<i>Ruta graveoleus</i>) Lemon (<i>Citrus limonum</i>)
Rutaceae	Rue family	Fig (<i>Ficus carica</i>) Babachi (<i>Psoralea corylifera</i>)
Moraceae	Fig family	
Leguminosae	Legume	
Photodermatitis observed		
Ranunculaceae	Buttercup family	Buttercup (<i>Ranunculus</i> sp.)
Cruciferae	Mustard family	Mustard (<i>Brassica</i> sp.)
Convolvulaceae	Morning glory family	Bindweed (<i>Convolvulus arvensis</i>)
Rosaceae	Rose family	Agrimony (<i>Agrimonia eupatoria</i>)
Compositae	Sunflower family	Yarrow (<i>Achillea millefolium</i>)
Chenopodiaceae	Saltbrush family	Goosefoot (<i>Chenopodium</i> sp.)

^a Mostly from Pathak *et al.*, 1962. Mention in some articles, without documentation, has also been made of some members of the Mimosae and Hypericaceae as plants causing phytophotodermatitis.

meadow grass dermatitis, fig dermatitis, and other types of human phytophotodermatitis. Fahmy, in 1947-48, isolated three psoralens from a weed used by herb doctors in Egypt; psoralen, 5-methoxysoralen and 8-methoxysoralen (Fitzpatrick and Pathak, 1959).

At present plants of quite a few families have been shown to cause phytophotodermatitis (Table III), and psoralens have been extracted from plants in four of the families: Umbelliferae (parsley family), Leguminosae (legume family), Rutaceae (rue family, including citrus) and Moraceae (fig family) (Pathak *et al.*, 1962).

5.1 Psoralen Photosensitization

The etiology of psoralen action is much like that of severe sunburn (Fitzpatrick *et al.*, 1955). After ingestion of plants containing psoralens, contact with such plants, or application of extracts from such plants to the skin, subsequent exposure to light results in erythema. With prolonged exposure and larger doses of psoralens, or both, blistering occurs, followed by itching and scaling. However, in contrast to sunburn the psoralen reaction can be induced by visible and nonerythemic near-UV radiation (340-440 nm) (Clark, 1961). The resemblance between sunburn and psoralen photosensitization is not only superficial since in both, lysosomes break down. Erythema is probably a result of photoreaction products diffusing into the dermal region, inducing engorgement of the blood vessels, whence the red color. Pigmentation (melanization) occurs in both cases. The disorder following psoralen photosensitization is often called "contact phytophotodermatitis," although it may result from ingestion of the psoralens and their subsequent uptake into the bloodstream and thence to the skin. After ingestion of such psoralens and light-exposure, photosensitization may result not only in skin reactions but also in gastric irritation, nausea, nervousness, insomnia and even depression, symptoms not found in sunburn.

The psoralens and isopsoralens in plants are readily extracted in chloroform, ethanol, acetone, and acetic acid (Fowlks, 1959b). The two common parental furocoumarins extracted from plants are the linear substitution compounds derived from psoralen and the angular-noded substitution compounds derived from isopsoralen (angelicin) (Fig. 11). From these, many substitutions in turn are possible, some of which occur in nature, others only in the laboratory. In all, some 80 natural and synthetic furocoumarins as well as some 50 coumarins and coumarin derivatives have been tested as photosensitizers on human and guinea pig skin (Fowlks, 1959a; Pathak and Fitzpatrick, 1959; Pathak *et al.*, 1960, 1967; Musajo and Rodighiero, 1962, 1969). Much of the literature pertaining to these tests has been recently summarized (Pathak, 1969).

The general conclusions from a comparison of their activity as skin photosensitizers are as follows.

1. The coumarins and their derivatives are completely ineffective as photosensitizers, regardless of modifications and substitutions.
2. Psoralen, the parent compound, is very effective but a few methyl-substituted derivatives may be even more potent than psoralen (Musajo, 1969).
3. The linear structure is more effective as a photosensitizer than the angular, e.g., psoralen is more active than isopsoralen (angelicin), and bergapten (5-methoxypsoralen) than isobergapten (5-methoxyangelicin).
4. Introduction of an OH radical at the 8 position (see Fig. 11) or at the 3', 4', 4' and 5' positions in the molecule removes the photosensitizing activity; thus xanthotoxol (8-hydroxypsoralen) is inactive while xanthotoxin (8-methoxypsoralen) is active; and bergaptol (5-hydroxypsoralen) is inactive while bergapten (5-methoxypsoralen) is active. However methylation of the OH in xanthotoxol and bergaptol restores their photosensitizing activity.
5. Photosensitizing activity is removed also by introduction of nitro, amino or acetyl amino radicals at the 5 or 8 positions; or by substitution of the *n*-propyloxy, *n*-butyloxy, isoamylloxy or benzyloxy radicals in the 5 position; or by substitution of the carboxylic radical as well as 4'-phenyl 4-methyl radical at the 8 position.
6. Substitution of N(CH₃)₂, Cl, CN or NH₂ radicals in the 8 position and substitution of Cl, ethoxy, or isopropoxy radicals in the 5 position does not increase activity and may decrease it.
7. Substitution of methyl radicals at the 3, 4, 4', 3 and 4', 4 and 4', 4 and 5' positions decreases activity somewhat.
8. Similar substitutions into isopsoralen (angelicin) have similar effects.
9. Dimers of active compounds are inactive.

10. The 3-4 bond of psoralens plays an important role, certain substitutions at this point decreasing or destroying activity. It may, in fact, be the point at which union with the photosensitized molecule is achieved (Pathak *et al.*, 1967; Khadzhai and Kuznetsova, 1965).

Some studies have demonstrated that enzymes in the skin may be damaged by psoralen photosensitization, e.g., succinic dehydrogenase was inactivated by photosensitization in presence of psoralen (Pathak and Fowlks, 1961). The initial reaction appears to be photochemical inasmuch as the dose of illumination may be fractionated without altering the effect, indicating obedience to the reciprocity law (Fowlks *et al.*, 1958). However the temperature coefficient is higher than for a photochemical reaction, suggesting that secondary thermochemical reactions become

limiting (Fowlks, 1959a). Oxygen is not necessary for psoralen photosensitization (Musajo and Rodighiero, 1962; Krauch *et al.*, 1965), a matter of great interest inasmuch as photosensitizations by so many other compounds are photooxidative (Blum, 1941). For psoralen-photosensitized killing of bacteria and inactivation of phage, as well as in reactions between psoralens and DNA, oxygen may even interfere with photosensitization, competing for the sensitizer (Matthews, 1963).

Psoralens absorb in the far UV (220–280 nm) and near UV (340–380). They fluoresce at 420–460 nm and display an action spectrum in the near UV (e.g., 340–380 for 7-methoxypsoralen; Pathak and Fellman, 1960; Pathak, 1961). Studies with a monochromator indicate that agreement between absorption and action spectra is incomplete (Buck *et al.*, 1960; Freeman and Troll, 1969). The following list shows the degree of penetration of various bands of light in the skin:

400–1400 nm	to coreum, causes hyperemia
320–400 nm	to Malpighian layer and basal cell layer
280–320 nm	to Malpighian layer and blood vessel network, causes sunburn
260–280 nm	to <i>stratum germinativum</i>
200–260 nm	through horny layer (corneum) only

It is evident that various wavebands of light are differentially screened and that the far UV is largely eliminated before it reaches cells in which photosensitized reactions occur; in that case one would hardly expect them to be represented in the action spectrum (Fitzpatrick *et al.*, 1963).

Because of the complexity of the skin and therefore the difficulty of determining the action of psoralens in photosensitized reactions, studies have also been made of their photosensitization effects on simpler systems: phage, bacteria, erythrocytes, and tissue cultures, where it was hoped the analysis of the fundamental reactions might be more direct. Fowlks *et al.* (1958), using wavelength 366 nm, investigated the psoralen photosensitization of three gram-negative (*Escherichia coli*, *Proteus vulgare* and *Pseudomonas aeruginosa*) and three gram-positive (*Bacillus subtilis*, *Staphylococcus aureus* and *S. fecalis*) bacteria. The gram-positive bacteria proved more susceptible than the gram-negative. Of the 13 fluorescent compounds, 8, mainly psoralens, isolated from plants proved damaging to bacteria. The substituted psoralens, which had no effect on human skin also had no effect on bacteria. Coumarins, as might have been expected, were also ineffective, as incidentally were three other types of compound, e.g., flavones, isolated from plants. Oginsky *et al.* (1960) found that *E. coli* and *S. aureus* were killed in visible light in the presence of psoralens; also, that in *E. coli* the radiation-resistant strain B/r is much more resistant to psoralen photosensitization than strain B,

although the two are equally sensitive to heat and to photooxidative photodynamic dye. Matthews (1963) made a comparative study of lethal photosensitization of *Sarcina lutea* with 8-methoxysoralen (not requiring oxygen) and toluidine blue, a typical photodynamic dye (photo-oxidative), and observed a striking difference in action between the two. Thus toluidine blue affected the cell membrane, increasing the outward movement of some compounds (e.g., nucleic acid precursors showing high absorption at 260 nm), and inactivated cell enzymes (possibly in the cell membrane) such as pyruvic oxidase and adenosine deaminase. On the other hand, with 8-methoxysoralen and light no permeability change was observed, but among the survivors were found many penicillin-resistant mutants, five orders greater in number than among those treated with toluidine blue, suggesting that DNA is perhaps affected (Matthews, 1963). Animal viruses were affected (mutated?) also by psoralen photosensitization and in mammalian tissue culture giant cells were formed (Colombo *et al.*, 1965).

5.2 Photosensitized Reactions between Psoralen and Nucleic Acids

Many studies have been made on the photosensitization of DNA by psoralens. Musajo *et al.* (1965, 1966, 1967b) demonstrated by the use of $[-O^{14}CH_3]$ -bergapten that the photosensitizer united with DNA at the pyrimidine moiety, not the purine, to form an addition product (adduct) which they were able to isolate. Dell'Acqua and Rodighiero (1966b) found that little binding occurred in the dark (and high salt concentration reduced such binding as occurred), indicating that the bonding between the photosensitizer and the DNA is apparently the result of a photochemical reaction. They also demonstrated an increase in the melting temperature (T_m) of the adduct from bonding DNA with the psoralen photosensitizer, indicating more than a temporary association between the two molecules (Dall'Acqua and Rodighiero, 1966a,b). That there is chemical bonding between DNA and psoralen photosensitizer after light exposure is also indicated by changes in fluorescence properties of the psoralens in the psoralen-DNA adduct (Musajo *et al.*, 1965). Psoralen photosensitization also alters the template properties of the DNA involved in the reaction as shown by Chandra and Wacker (1966).

Krauch *et al.* (1965, 1967) demonstrated that psoralens bind not only to DNA (at the thymine moiety) but also to a lesser extent to RNA (at the uracil moiety). The photoproduct with thymine was isolated by paper chromatography and split again into the initial compounds. Musajo *et al.* (1967a) showed that the psoralen is covalently bound to thymine by a cyclobutane type of linkage (Fig. 12). The pyrimidine

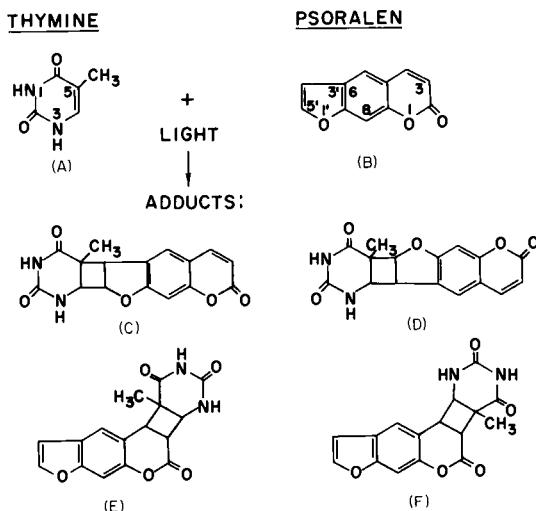


FIG. 12. Possible photoinduced psoralen adducts with thymine. Adducts C and D have a violet fluorescence when viewed in near UV; adducts E and F do not. (After Musajo, 1969, p. 377.)

bases always photoreact at their 4,5 double bond, whereas the psoralens react at their 4', 5' or their 3,4 double bonds (Fig. 12). This leads to the formation of four possible photoproducts between psoralen and thymine molecules as shown in Fig. 12. It is considered that thymine in DNA reacts with psoralen in an analogous manner. Isolation of two fluorescent thymine-psoralen adducts (4', 5' photoproducts) and one nonfluorescent (3,4 photoproduct), from DNA irradiated in the presence of psoralen, is evidence that such a reaction occurs. The fluorescent compounds were present in three times the abundance of the nonfluorescent one (Musajo, 1969).

That the same type of reaction between psoralen and DNA as obtained in the laboratory occurs in living cells is somewhat more difficult to prove, but the following evidence has accumulated showing comparable effects *in vitro* and *in vivo*.

1. The action spectra for the photoreactions between DNA and 8-methoxysoralen and DNA and 5-methoxysoralen are similar to the action spectra for the induction of erythema in human and guinea pig skin, the near-UV being most effective in all four.

2. The relative effect of a series of psoralens, including their potent methyl-derivatives, on the formation of adducts with DNA parallel those on induction of erythema in guinea pig skin, those psoralens

which are most effective on skin being most effective in photochemical reaction.

3. DNA extracted from the skin of guinea pigs photosensitized with tritiated psoralen showed radioactivity, while no trace of the radioactivity could be isolated from protein fractions (unpublished experiments of Pathak *et al.*, quoted from Musajo, 1969). Seemingly, the bonding reactions between DNA and psoralens occur *in vivo* as well as *in vitro*.

4. Similarly, mouse ascites tumor cells treated with psoralens and exposed to visible light lost their tumor-inducing potency. When tritiated psoralen was used, it could be demonstrated that the DNA extracted from the mouse cells was radioactive. Furthermore, from the DNA was isolated a fluorescent adduct with thymine, identical to one isolated *in vitro* (Musajo, 1969).

Psoralens also photoreact with RNA and adducts with uracil in the RNA can be demonstrated. Furthermore, when skin is treated with tritiated psoralens and illuminated with near UV, it takes up the label. RNA extracted from such skin is radioactive, showing a bonding reaction *in vivo* similar to the one *in vitro*. Effects of psoralen photosensitization on cells may be, in part, attributable to such effects on RNA, but are probably of lesser consequence than similar effects on DNA because RNA is present in greater abundance, and any destroyed can be replaced so long as the DNA is intact. It is not possible to say at present how important the role of the RNA-psoralen reaction on overall cell injury may be, but the genetic effects of psoralen photoreactions are DNA-localized (Freeman, 1968; Freeman and Troll, 1969).

The physical-chemical basis of psoralen photosensitization on skin and the basic mechanism involved have been the basis of a number of studies (see Yeargers and Augenstein, 1965, 1968; Pathak *et al.*, 1961; Pathak, 1969). As indicated in the Introduction, this subject will not be considered here.

5.3 Psoralens in Cancer Research

Psoralens have been used for treating vitiligo since ancient times (Fitzpatrick *et al.*, 1955, 1966; Fitzpatrick and Pathak, 1959), and are used at the present time, on occasion, to get a quick suntan (Elliott, 1959; Stegmeier, 1959). Therefore, the production of mutations in bacteria and alterations (mutations?) in DNA phages during psoralen photosensitization is of special concern inasmuch as psoralens in vitiligo treatment or suntan preparations might result in genic changes of a cancerous nature in the human skin exposed to strong sunlight. Increased cancer incidence following psoralen applications to the skin of mice and guinea

pigs has been reported and cataracts were found in the eyes (Musajo, 1955; Griffin *et al.*, 1958; Cloud *et al.*, 1960, 1961; Clark, 1961; Hakim *et al.*, 1961). The morphological changes in the eye have been studied and described. The results, however, are not unequivocal. Fitzpatrick *et al.* (1966) in a summary of human cases treated with psoralens for vitiligo, in some cases for 12 years, points out that no increased cutaneous cancer incidence was observed. Thus in two series of experiments with middle-aged individuals who had shown histological evidence of cutaneous cancer, in Texas on the one hand (MacDonald *et al.*, 1963), and in Australia on the other (Hopkins *et al.*, 1963), the individuals after two years' treatment with 8-methoxysoralen and radiation showed no greater incidence of cancers than controls treated with placebos. Also, no cataracts were reported. It is possible that the skin of mice and guinea pigs is more sensitive than that of man, permitting quicker induction of cancer by psoralen photosensitization, or that the much larger doses used on the animals or a greater area relative to the volume of the animal, may account for the difference. Judgment on carcinogenesis by psoralens must be withheld pending additional studies.

6. Photosensitization of Human Skin by Miscellaneous Phototoxic Substances

Digalol trioleate (tanbark oleate) is a photosensitizer as are the coal tars and pitch (Crow *et al.*, 1961). Also, riboflavin, quinine, arsphenamine, barbiturates, estrone, ethylstilbestrol and triethylene melamine, as well as the antibiotics Declomycin, Aureomycin, Griseofulvin, Tetra-chlorosalicylanilide, sulfa drugs, paraaminobenzoic acid, sulfonylurea, chlorothiazides, phenothiazines, serve as photosensitizers (Pathak, 1969). After some virus infections, e.g., after vaccinia vaccination, and in the disease lymphogranuloma venereum, photosensitization to visible light may appear. Perhaps in these cases the reactions should be called *phototoxic reactions* since the effects are damaging (Ippen, 1969).

Ippen (1969) has suggested a useful classification of photosensitized reactions, whether the photosensitizer is natural or synthetic.

1. *Photoautoreaction* in which a sensitizer, however applied—topically or internally—simply initiates the normal photochemical reactions of the skin, similar to what is found after exposure of the normal skin to the sun. The phytophotodermatoses induced by psoralens are of this type. *Xeroderma pigmentosum* is a greatly exaggerated form of photodermatosis in individuals who lack the gene for repair of UV injury. In such individuals sunlight affects the skin profoundly, aging it unduly fast and

senile skin is induced even at an early age. In pellagra a somewhat similar but less marked reaction is found (Ippen, 1969).

2. Quite different are the *photoheteroreactions* in which the cutaneous photoreaction causing eruption results from a product derived after the original substance has undergone a photochemical conversion into a toxic form. This can best be demonstrated by an example. If a photoallergic person is treated with chlorpromazine, and afterwards is exposed to light, he develops an acute eczema in the exposed area. If the chlorpromazine is exposed to light first, and then applied to the photoallergic person in the dark, he develops the symptoms. If the untreated

TABLE IV
ILLUSTRATIVE ABSORPTION SPECTRA AND ACTION SPECTRA OF
NATURAL PHOTOSENSITIZERS^a

Class of compound	Absorption spectra (nm)	Action spectra (nm)	Citation
Hypericins			
Hypericin	610-540	Approximately the same	Pace and McKinney, 1941
Zoopurpurin			
Red	575, 540, 485, 330	550-330	Giese and Grainger, 1970
Blue	600, 560, 330	Inactive	
Chlorophylls			
a	662(618, 575, 532) 430	660-430	Krinsky, 1968
b	648, 460		
Porphyrins			
Protoporphyrin	410-398 (Soret bd.) lesser abs. 620- 400, and 400-300	370-340 ^b (300-400)	Magnus <i>et al.</i> , 1959, 1961
Phylloerythrin	Soret band, etc. Details not determined.	Approximately the same. Details not determined.	Blum, 1941, 1964
Furocoumarins			
8-Methoxysoralen	303, 248, 217 ^c	360-320	Pathak, 1969
5-Methoxysoralen	313, 267, 221	360-320	
Psoralen	329, 295, 245, 212	360-320	

^a The data are illustrative. Various discrepancies are found, but no one has examined and evaluated the data in many cases.

^b Slight peaks in some cases at 600, 500, 460, 441, 420, 410. The action spectrum depends upon the criterion taken—whether erythema, whealing, etc.

^c The discrepancy between absorption and action spectra of psoralens is unresolved. Pathak (1969) suggests that the absorption by the psoralens bound to the cellular material may be quite different from their absorption in pure solutions. If this could be measured, perhaps agreement between absorption and action spectra might be found.

chlorpromazine is applied to the patient in the dark he shows no symptoms. It is evident that something has been produced from chlorpromazine by illumination (dimeric free radicals) either in the skin or in the test tube and is a consequence of the photochemical reaction—an allergen (hapten) has been produced which in turn causes the eczema. Similarly, illumination of sulfanilamide produces hydroxylamine which serves as an allergen. In the group of photo-hetero-reactions are the conditions known as photoallergic reactions (e.g., light and sulfanilamide or chlorpromazine), light urticaria, certain porphyrias (not by hematoporphyrin or erythropoietic porphyrias), hydroa vacciniforme, chronic polymorphic light exanthema and similar eruptions (Ippen, 1969). In certain of these reactions, e.g., hydroa vacciniforme and some porphyrias, no primary allergen is found but something later develops which causes damage to blood vessels, connective tissue and even some internal organs such as the liver (Urbach, 1969).

These interesting problems are the result of ingestion of drugs, or application of synthetics and are therefore beyond the confines of this chapter.

7. Summary and Conclusions

1. Although there may be many kinds of natural pigments which act as photosensitizers, there appear to be four main groups—the hypericins, chlorophylls, porphyrins, and psoralens.
2. All of these pigments mediate their effects in the non-sunburn part of the sun's spectrum (wavelengths longer than 320 nm).
3. Three of the groups of natural photosensitizers are photooxidative—hypericins, chlorophylls, and porphyrins no damage to the cell being observed in the absence of atmospheric oxygen.
4. Only the psoralens act in the absence of oxygen; in fact, oxygen may interfere with the photosensitizing action of psoralens.
5. Hypericins and chlorophylls appear to act primarily as photosensitizers on cell membranes. There is some evidence that porphyrins might do so also, at least in some cases, but the subject has been little studied. Skin reactions to porphyrins result from deeper effects on cells—lysosomes being disrupted. The psoralens form photoadducts with DNA and to a lesser extent with RNA. As a consequence they cause mutations.
6. Each of the pigments shows characteristic absorption bands. In all cases excited states are produced by absorption of radiation.
7. Action spectra of the natural photosensitizer show some correspondence to absorption spectra though some bands which are absorbed by pigments, e.g., in short UV, do not appear in the action spectra, probably

because they are filtered out before reaching the particular cells in the skin (Table IV).

8. When endogenous pigments can be extracted, they are effective as exogenous photosensitizers to colorless cells.

9. Some of the natural pigments appear to have no function in the organisms in which they occur, e.g., the *hypericins*, though a possible UV-screening function is suggested for the pigment of the protozoan *Blepharisma* which absorbs far UV strongly. The *chlorophylls* have an obvious function in photosynthesis and it is only derangement in the organization of pigmentary systems in membranes of mutant cells which leads to photosensitization by chlorophyll. The *porphyrins* are building blocks of important cellular pigments and enzyme systems. It is only under certain metabolic derangements and in mutants that the porphyrins become photosensitizers. The *psoralens* act as fungicides, protecting plants in which they occur from invasion of fungi.

10. Thus, photosensitization by natural pigments generally appears to be the result of accidental juxtaposition of colorless cell and sensitizing pigment, serving no immediate function in the physiology of the organism.

11. Studies of photosensitization by natural pigments show their importance to veterinary and human medicine, and to understanding some interesting features of photobiology.

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Chapter 4

SOLAR UV IRRADIATION AND THE GROWTH AND DEVELOPMENT OF HIGHER PLANTS

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1. Introduction

Ultraviolet radiation has long been known to be quite important in biological photochemical reactions. Interest in the possible effects of ultraviolet radiation on plant growth and development was initiated in the late 1800's and early 1900's. During this period, ultraviolet radiation was demonstrated as the principal agent responsible for the reaction of human skin to solar radiation. The classic work of Finsen, which culminated in his Nobel Prize in 1903, demonstrated that skin tuberculosis could be cured with UV radiation. His discovery led to the establishment of a great number of therapeutical institutions and sanitariums throughout the European continent. Sunburn and carcinogenesis of untanned Caucasian skin were also demonstrated as responses to ultraviolet radiation during these early years.

Botanists responded with a series of pioneering investigations on the effect of artificial and solar ultraviolet radiation upon plant

growth and development. A great variety of physiological and morphological plant responses to UV radiation have been subsequently demonstrated over the past 75 years. Most of these experiments, however, have employed ultraviolet lamps which usually emit radiation quite unlike the radiation present in the normal terrestrial solar spectrum.

Since most biological responses induced by UV radiation are highly wavelength-dependent, a reasonably exact interpretation of the composition of UV radiation used in various artificial irradiation experiments must be taken into account. Otherwise, the relevance of such experiments remains in some doubt when the natural solar ultraviolet radiation regime is considered. Since most of what is known concerning plant response to UV radiation is the result of laboratory experiments using artificial sources of UV radiation, this review will first consider the range of plant reactions which have been demonstrated when plants have been exposed to various UV wavelengths. Earlier comprehensive reviews by Lockhart and Brodführer Franzgrote (1961) and Dubrov (1963) have already reviewed most of the plant UV radiation research before 1960 in some detail. A shorter and more recent review by Füchtbauer (1969) discusses some of the later work. There is little need to repeat the coverage of these reviews other than to summarize the sundry of plant responses to UV radiation in the context of the effective UV wavelengths.

Of particular importance in this discussion will be the action spectra of plant UV responses in relation to the available solar UV radiation in natural environments. Since most measurements of solar UV radiation intensities have been taken without consideration for the biological effectiveness of various UV wavelengths, the interpretation of many of these measurements remains rather nebulous. The importance of solar angle, atmospheric turbidity, elevation above sea level, cloud cover, total atmospheric ozone concentration, and the UV albedo of the earth's surface with respect to the total UV irradiation intensity and wavelength composition will be considered in some detail.

Laboratory experiments using artificial UV radiation and measurements of solar UV irradiation have led to a great deal of speculation as to the importance of solar UV irradiation in nature, particularly in areas where plants are subject to reasonably high UV irradiation intensities. A limited amount of experimentation has been carried out in special glass houses or gardens using various filters to selectively remove or transmit solar UV radiation. The results of these various filter studies have been quite variable. Some investigators have demonstrated substantial changes in plant growth and phenology under various types of UV filters (Schanz, 1920; Brodführer, 1955). Other

studies (Caldwell, 1968; Moore, 1970) have not found such striking changes in plant growth form. Since there are a number of factors which might contribute to the differences in these results, this review will discuss these experiments in some detail.

Finally, the biological defense mechanisms against solar UV radiation will be considered. Reflectance or absorption of UV radiation in the outer tissue layers may provide a certain amount of protection against UV irradiation. Reversal of UV-induced damage by concomitant or subsequent high-intensity irradiation of longer wavelengths known as photoreactivation, may be of great importance for plants in natural environments. If photoreactivation is indeed effective in nature, the interpretation of much of the artificial UV irradiation research is further confused when the natural solar UV environment is considered.

The commonly used definitions of UV spectral bands originally proposed by Coblenz (Meyer and Seitz, 1942) will be used in this review.*

2. Plant Reactions to Artificial UV Irradiation

Much of the experimentation using artificial sources of UV radiation has been carried out using common germicidal UV lamps. These lamps are usually low-pressure mercury vapor radiation sources which emit essentially monochromatic radiation at 2537 Å. A few of the other spectral lines characteristic of the emission of mercury vapor are also present in the radiant output from a germicidal lamp; however, these are in very small proportion in relation to the dominant 2537 Å peak. Germicidal irradiation is indeed biologically effective principally because 2537 Å irradiation is readily absorbed by proteins and nucleic acids (Giese, 1964). In fact the effectiveness of germicidal irradiation is apparently quite universal throughout the plant and animal kingdoms.

Since the terrestrial solar spectrum is effectively depleted below approximately 2950 Å, UV radiation from a germicidal lamp bears little relation to what might be expected from solar irradiation in nature. As is well known, these shorter wavelengths are filtered out by ozone in the upper atmosphere.

Higher-pressure mercury vapor lamps emit radiation at the 2537 Å wavelength, but in addition, a substantial proportion of the radiant emission occurs at the longer wavelengths characteristic of mercury vapor. Since many of the studies have not specified the exact type of mercury vapor lamp employed, it is impossible to know the pressure of the mercury vapor in the lamp and therefore the exact spectral

* UV-A: 3150-4000 Å; UV-B: 2800-3150 Å; UV-C: less than 2800 Å.

TABLE I
PLANT RESPONSES TO UV-B AND UV-C IRRADIATION
FROM UV LAMPS IN THE LABORATORY^a

Plant response	Wavelengths (Å)	Reference
Reduced virus susceptibility of <i>Phaseolus vulgaris</i>	2537	Benda (1955)
Increased calcium content of tomato, radish, lupine, corn, sunflower, and soybean	2900-3100	Benedict (1934)
Inhibition of pollen germination in <i>Aloe vera</i> , <i>Antirrhinum majus</i> , and <i>Ananas comosus</i>	2537	Brewbaker <i>et al.</i> (1965)
*Cell division and elongation inhibition in <i>Iasione montana</i>	~2000-3800	Burström and Gabrielsson (1964)
*Chloroplast disappearance in guard cells of bean leaves	2537	Chessin (1961)
Prevention of N.A.A.-induced epinasty in pea plants	2537	de Zeeuw and Leopold (1957)
Inactivation of auxins in <i>Hydrangea</i> <i>opuloides</i> and acceleration of flowering in <i>Linum usitatissimum</i> and <i>Statice bonduelli</i>	2000-3800 max at 2537	von Denffer and Schlitt (1951)
Nuclear microvacuolization, nuclear pyknosis, linear contraction of chromosomes in roots of <i>Allium cepa</i>	~2537	Drets (1959)
Inhibition of cytochrome oxidase and oxygen uptake in <i>Xanthium</i> and inhibition of catalase and pyruvate kinase in <i>Allium</i>	2537	El-Mansy and Salisbury (1971)
Stimulation of vegetative growth of <i>Begonia</i>	2900-3100	Gilles (1939)
Promotion of petiole abscission in <i>Gossypium hirsutum</i>	2537	Hall and Liverman (1956)
Closing of stomata in <i>Begonia</i> <i>semperflorens</i>	2537	Hercik (1964)
*Mutation of maize endosperm	2537	Ikenaga and Mabuchi (1966)
Stimulation of germination in cucumber and tomato	2537	Kiyanitsy and Nichkevicha (1957)
*Promotion of petiolar abscission in <i>Coleus</i> plants	2537	Klein (1967)
Inhibition of stem elongation in sunflower and bean	2537	Lockhart (1958)
Increase in chlorogenic acid and rutin in <i>Nicotiana tabacum</i>	2000-3800 max at 2537	Lott (1960)

TABLE I (*Continued*)

Plant response	Wavelengths (Å)	Reference
Inhibition of vegetative growth in barley seedlings	2537	Natarajan and Nirula (1963)
Inhibition of leaf lesions in potato, enhanced pigmentation in apples and potato leaves	3000-3150	Nilsen (1969)
*Respiration stimulation in tobacco leaves	2537	Owen (1957)
Tumor induction in gametophytes of <i>Pteridium aquilinum</i>	2537	Partanen and Nelson (1961)
Decrease in free amino acids in pea plants	2537	Payne <i>et al.</i> (1954)
Increase of nitrogen and phosphorus content in <i>Stellaria media</i> , <i>Epilobium collinum</i> , <i>E. alpinum</i> , <i>Silene inflata</i>	2800-3200	Pirschle (1941)
*Extrachromosomal mutation in yeast	~2537	Pittman and Loker (1961)
Reduced auxin content in seedlings of <i>Brassica rapa</i>	2000-3800 max at 2537	Popp and McIlvaine (1937)
Repression of germination of <i>Phacelia tanacetifolia</i>	2537	Schulz <i>et al.</i> (1963)
Increased ash, calcium and phosphorus content in <i>Nicotiana tabacum</i> , <i>Salvia splendens</i> , <i>Lycopersicon esculentum</i> , <i>Datura stramonium</i>	2000-3800 max at 2537	Stewart and Arthur (1934)
*Alteration of the absorptive abilities of the cytoplasm, change in isoelectric points of the nucleus and cytoplasm, and loss of ability to undergo plasmolysis in <i>Allium</i> bulb epidermis	2000-3800	Tageeva and Dubrov (1961)
Increased absorption of rubidium by excised roots of <i>Phaseolus aureus</i>	2537	Tanada (1955)

* Plant responses which have been demonstrated to be photoreactivable are indicated by an asterisk.

composition of the irradiation. In most cases, however, the principal and probably most effective wavelength was 2537 Å.

The most common plant response to mercury vapor lamp irradiation is some form of visible lesion, which often does not develop until several hours after the irradiation. This may be simply a chlorosis (Tanada and Hendricks, 1953), but more commonly appears as a glazing

and browning of the tissues attributed to the presence of oxidized, polymerized, phenolic compounds followed by a subsequent collapse and further degradation of the cellular structure in the area of the lesion (Cline and Salisbury, 1966a). This damage can be caused by UV radiation at most any wavelength below 3150 Å in the UV-B and UV-C, although the effectiveness of the longer wavelengths approaching 3150 Å is much less than for wavelengths within the UV-C spectrum (Caldwell, 1968). The active chromatophores for this photochemical tissue destruction are probably nucleic acids as will be discussed later.

The UV-B and UV-C portions of the spectrum have also been shown to effect a great number of other photochemical plant responses in addition to visible lesions and damage (see Table I). Since most of the plant responses to UV-B and UV-C are qualitatively similar and for the most part distinct from those plant responses to UV-A radiation, this review will consider UV-B and UV-C effects separately from UV-A effects.

2.1 Responses to UV-B and UV-C Irradiation

Table I indicates the wide range of plant responses which has been demonstrated to be the result of artificial UV-C and UV-B irradiation. While this review concentrates on the response of higher plants to UV irradiation, a few examples of lower plant response to UV radiation have been included to indicate the broad spectrum of physiological phenomena which has been reported in the literature. The wavelengths which have been employed in these various experiments are also indicated in Table I. As was suggested earlier, it is often difficult to determine the exact spectral composition of the lamps used in many of these experiments, which necessitates reporting a range of wavelengths which would include those employed. In a number of cases, medium pressure mercury vapor lamps may have been used in which case the UV radiant emission would range from 2000 to 3800 Å. However, the maximal output is still at 2537 Å and most of the photochemical response would probably be attributable to this wavelength. Those physiological phenomena in Table I marked with an asterisk have been demonstrated as photoreactivable, i.e., the damage is reversible if the plant is irradiated with high intensity visible or UV-A radiation immediately following the exposure to UV-B or UV-C irradiation.

Although there is a great variety of physiological and morphological phenomena which has been demonstrated to be the result of UV-B and UV-C radiation in the literature, the principal chromatophores for this multifarious group of plant responses may be similar. Giese (1964) has

pointed out that action spectra for the response of animal cells to UV irradiation commonly conform to either the absorption spectra of proteins or nucleic acids. Figure 1 shows the generalized nature of the action spectra when protein and nucleic acids are alleged as the active chromatophores for the response to UV irradiation. Naturally, the absorption spectra for proteins and nucleic acids would be expected to correspond reasonably closely to the respective action spectra curves as Giese has illustrated. Although protein chromatophores are particularly affected at approximately 2800 Å and nucleic acid chromatophores at 2600 Å, the action spectra of both groups of chromatophores are nearly the same above 2900 Å, which is of principal interest in this review which is considering the effects of solar UV irradiation.

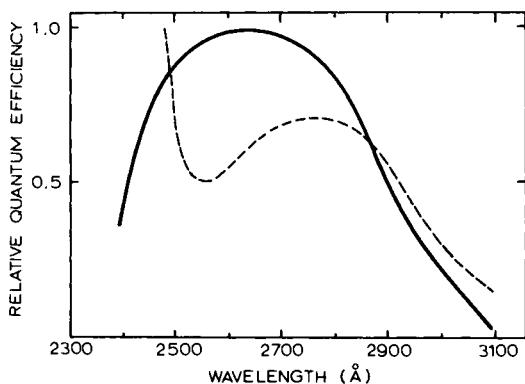


FIG. 1. Generalized action spectra for biological responses to UV irradiation involving protein (broken line) and nucleic acid (solid line) chromatophores. Adapted from Giese (1964).

Action spectra for various plant responses to UV radiation are shown in Fig. 2. These action spectra data have all been adjusted to show a relative effectiveness of 1.0 at 2800 Å. These values have been represented in terms of either the relative effect per number of quanta $\text{cm}^{-2} \text{ Å}^{-1}$ (relative quantum effectiveness), or as the reciprocal of the number of quanta cm^{-2} required to produce a given response at a particular wavelength (relative quantum sensitivity). The intermittent lines in each graph in Fig. 2 represent the spectral sensitivity of a UV phototube chosen to approximate the biological action spectra for UV effects on plant cells above 2800 Å in an earlier study (Caldwell, 1968). Nucleic acids or proteins are probably the responsible chromatophores in these action spectra since these spectra conform rather closely to the action spectra and correlative absorption spectra for nucleic acids and proteins

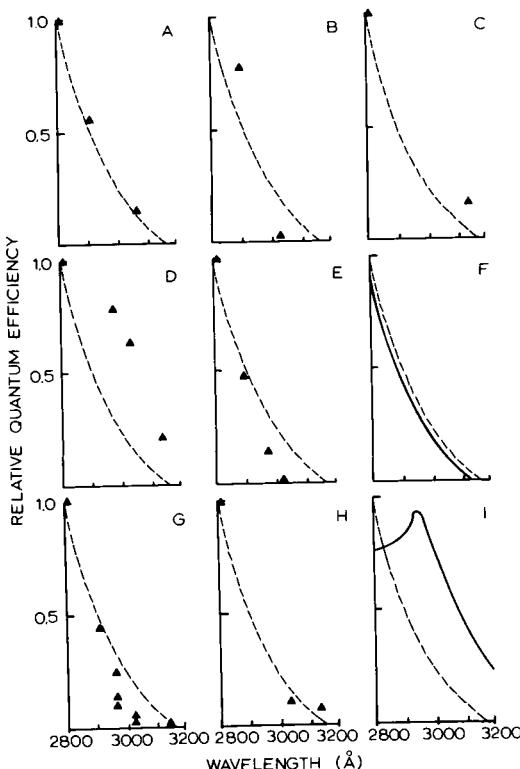


FIG. 2. Plant UV-B action spectra relative to 2800 Å. A, Mutation of liverwort spores, *Sphaerocarpus donnellii* (Knapp *et al.*, 1939); B, mutation in the fungus *Trichophyton mentagrophytes* (Hollaender and Emmons, 1941); C, inhibition of photosynthesis in *Chlorella pyrenoidosa* (Bell and Merinova, 1961); D, cessation of cytoplasmic streaming in epidermal cells of *Allium cepa* (Glubrecht, 1953); E, frequency of "endosperm deficiencies" in corn due to chromosomal deletion or mutation (Stadler and Uber, 1942); F, germicidal action spectrum (Luckiesh, 1946); G, epidermal cell damage in *Oxyria digyna* leaves (Caldwell, 1968); H, induction of chromosomal aberrations in *Tradescantia paludosa* pollen (Kirby-Smith and Craig, 1957); I, base curvature of *Avena coleoptiles* (Curry *et al.*, 1956). Intermittent lines represent the spectral sensitivity of a UV phototube chosen to approximate the biological action spectra for UV-B effects on plant cells (from Caldwell, 1968). All of the action spectra have been expressed in terms of relative quantum efficiency which denotes either relative quantum effectiveness (spectra A, B, E, F, H, and I) or relative quantum sensitivity (spectra C, D, and G).

represented by Giese (1964). An exception is the curve relating to the base curvature of *Avena coleoptiles* (Fig. 2I). Here the active chromophore was suspected to be indoleacetic acid (Curry *et al.*, 1956).

All of the action spectra, except that of Stadler and Uber (1942),

have been based on the number of quanta cm^{-2} impinging on the organism. Thus these spectra are the result of wavelength-dependent absorption, refraction, and reflection by the cell wall, cytoplasm, etc. as well as the absorption characteristics of the chromatophores (Giese, 1968). Due to careful transmission measurements by Uber (1939), Stadler and Uber (1942) were able to base their action spectrum for mutation (Fig. 2E) on the number of photons actually striking the nucleus of the pollen.

Biological effects attributable to nucleic acid absorption of UV are now considered to often involve the formation of cyclobutane pyrimidine dimers in the nucleic acids (Setlow, 1966; Saito and Werbin, 1969). These dimers apparently render the nucleic acids ineffective in controlling cell activities, e.g., protein synthesis, etc. Dimers can be readily monomerized by high intensity radiation between 3100 and 5500 Å providing the proper enzymes exist in the cell (Jagger, 1958; Setlow, 1966; Saito and Werbin, 1969). The physiological manifestation of the UV irradiation is correspondingly repressed. This apparently universal phenomenon known as photoreactivation has been demonstrated only to effect the breakage of dimers (Hanawalt, 1969). Therefore any lesion or effect of UV which is photoreactivable is usually attributed to dimer formation (Setlow, 1966). When protein chromatophores are involved in the response to UV radiation, some form of cytoplasmic alternation occurs which is not photoreactivable (Vladimirov *et al.*, 1970).

Although there are relatively few plant action spectra which have been demonstrated for the effects of UV-B irradiation, many of the plant responses demonstrated for UV-C irradiation may have UV-B action spectra quite similar to those shown in Fig. 2. Since photoreactivable phenomena are thought to be the ultimate result of pyrimidine dimer formation and dimer formation is the direct result of UV absorption by nucleic acids, photoreactivable UV-C effects can be considered as possessing UV-B action spectra approximately the same as the absorption spectra for nucleic acids. Those phenomena marked with an asterisk in Table I, which denotes that photoreactivability of these phenomena has been demonstrated, may be considered to possess UV-B action spectra characteristic for nucleic acid chromatophores. Furthermore, many of the other phenomena which are the result of UV-B and UV-C irradiation which have not yet been demonstrated as photoreactivable may indeed be photoreactivable or may involve protein chromatophores which would also possess similar UV-B action spectra.

Because of the similarity of UV-B action spectra for many plant responses to irradiation (see Fig. 2) and the probability that many other phenomena where protein and nucleic acid chromatophores are involved may also conform to these generalized action spectra, Caldwell

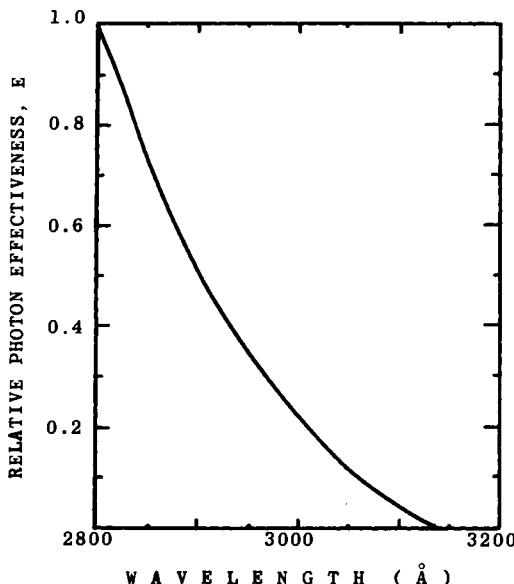


FIG. 3. A generalized curve to represent the relative photon effectiveness of UV-B irradiation to induce biological response when protein or nucleic acid chromatophores are involved. From Caldwell (1968).

(1968) has suggested that a single curve for the relative photon effectiveness for plant UV-B phenomena involving nucleic acid and protein chromatophores might be adopted. Since a single curve would be generally applicable to many effects of UV-B irradiation on both plant and animal cells, such a curve would be extremely useful in defining the biological effectiveness of UV-B irradiation. This curve is shown in Fig. 3. Based on this curve, biologically effective irradiance can be defined for the UV-B portion of the spectrum. The biologically effective photon irradiance of monochromatic UV-B irradiation, $EPI(\text{UV-B})_\lambda$, can then be represented as the product of the relative photon effectiveness at that wavelength, E_λ , as defined by Fig. 3, and the number of quanta $\text{cm}^{-2} \text{ sec}^{-1} \text{ Å}^{-1}$ at that wavelength, Q_λ .

$$EPI(\text{UV-B})_\lambda = Q_\lambda \cdot E_\lambda \quad (1)$$

For polychromatic radiation such as would be received from the sun or various artificial irradiation sources, the effective UV-B photon irradiance would be

$$EPI(\text{UV-B}) = \int_{2800}^{3150} Q_\lambda E_\lambda d\lambda \quad (2)$$

This defines an absolute unit of measurement which takes into account the biological effectiveness of UV-B irradiation when protein and nucleic acid chromatophores are involved. If quantitative comparisons of various sources of UV-B irradiation including the sun are to be taken in the context of biological effectiveness, such a definition is essential. Although nucleic acid and protein chromatophores may be involved in a particular biological response, the apparent UV-B action spectra for effects on underlying tissues in many organisms may be altered due to wavelength-dependent absorption, refraction and reflection by outer tissues (Giese, 1968). Nevertheless a standard unit would be generally applicable and should be adopted.

Unfortunately plant biologists have been slow to adopt photometric standards which would allow careful comparisons between irradiation sources. Definitions of biological photometric units within the UV-B and UV-C have been adopted for erythema of human skin (Luckiesh, 1946). The unit of measurement is termed the E-viton, which is analogous to the lumen (Luckiesh, 1946). The E-viton has been standardized by the International Commission on Illumination as relative to $10 \mu\text{W}$ at 2967 \AA . A G-viton has also been proposed as relating to germicidal effectiveness of UV-B and UV-C. The unit has hardly been utilized, however, since nearly all germicidal work has dealt with monochromatic radiation at 2537 \AA (Luckiesh, 1946). As is the case with the lumen, the E-viton and G-viton unfortunately have been defined on the basis of energy efficiency rather than quantum efficiency per wavelength. Since the absorption of UV and visible radiations in the photochemical reactions of biological systems occurs in terms of whole quanta, the proper, and increasingly accepted, representation of irradiance in biology is the number of quanta $\text{area}^{-1} \text{ time}^{-1} \text{ wavelength}^{-1}$ (Seliger and McElroy, 1965).

Although the relative energy efficiency per wavelength of the proposed G-viton unit if adjusted to quantum effectiveness coincides closely in the UV-B with the relative photon effectiveness curve proposed by Caldwell (1968) (see Fig. 3), it is suggested that the EPI(UV-B) unit be adopted rather than to adjust the unused G-viton unit for the UV-B portion of the spectrum.

In order to discuss the capacity of terrestrial solar UV-B irradiation to cause the many phenomena demonstrated when plants have been subjected to artificial sources of UV-B and UV-C irradiation, it is necessary to define the effective photon irradiance used in these various experiments. If the spectral output of the lamps used is not known precisely, or if UV-C monochromatic radiation as would come from germicidal lamps was employed, it is extremely difficult to predict the possible

effectiveness of solar irradiation in causing these same phenomena. Where monochromatic UV-B irradiation has been used or precisely defined polychromatic sources of UV-B irradiation, such as in the action spectra represented in Fig. 2, it is quite possible to consider these in the context of solar UV intensities. Table II contains the reported total doses and dose rates in biologically effective photon units for the action spectra in Fig. 2. Since the biologically effective photon doses and dose rates were not always the same at each wavelength in these studies, the wavelength of the reported doses and dose rates is also given in Table II. Total doses and dose rates employed by Stadler and Uber (1942) are well within the range of solar UV-B irradiation intensities which might occur in nature (see Figs. 9 and 10). The dose rates

TABLE II
UV IRRADIATION DOSES AND DOSE RATES EMPLOYED IN THE
DETERMINATION OF UV-B ACTION SPECTRA

Action spectrum (from Fig. 2)	Dose rate [EPI(UV-B)]	Total dose [EP(UV-B)]
A	—	4.4×10^{15} (at 3032 Å)
B	—	4.6×10^{11} (at 2967 Å)
C	2.79×10^{14} (at 3120 Å)	4.2×10^{17} (at 3120 Å)
D	1.1×10^{15} (at 3050 Å)	1.5×10^{17} (at 3032 Å)
E	5.7×10^{10} (at 2967 Å)	9.1×10^{11} (at 2967 Å)
F	—	—
G	1.1×10^{14} (at 2967 Å)	4.3×10^{17} (at 2967 Å)
H		2.2×10^{18} (at 3020 Å)
I		4.2×10^{14} (at 2800 Å)

used in the studies of Glubrecht (1953), Bell and Merinova (1961), and Caldwell (1968) are much greater than those normally occurring in nature. However, the total doses employed in these action spectra determinations and in the studies of Hollaender and Emmons (1941) and Curry *et al.* (1956), who did not report dose rates, could occur in the course of a single day's irradiation in the field. The total dose employed by Kirby-Smith and Craig (1957) would far exceed the total daily UV-B irradiation which could be expected under the most extreme situation in nature. Of course estimates of the potential effectiveness of solar UV-B irradiation in producing these plant responses in nature are based solely on the measured solar EPI(UV-B) intensities and the UV intensities used in the laboratory experiments. Other concomitant environmental variables such as temperature or accompanying visible irradiation

cannot be taken so easily into account and may influence the potential effectiveness of solar UV irradiation.

Although there is a very limited amount of research dealing with the effect of artificial UV-B irradiation on plants where wavelengths within the normal terrestrial solar spectrum have been employed, the potential effectiveness of this portion of the spectrum has been amply demonstrated. Furthermore, because of the apparent universality of action spectra where protein and nucleic acids are the principal chromatophores, there is good reason to assume that many of the phenomena which have been demonstrated in the preponderance of research dealing with UV-C irradiation might also be effected by wavelengths within the normal solar UV-B spectrum. A recognition of the exponentially increasing effectiveness of shorter wavelengths within the UV-B spectrum in causing biological responses will be particularly important in a consideration of the spectral composition of normal solar UV irradiation in nature.

2.2 Responses to UV-A Irradiation

Although UV-A radiation has not generally been considered to be as effective in inducing photochemical responses in biological systems as UV-B radiation, a variety of plant responses have been demonstrated using artificial sources of UV-A irradiation. Table III contains a number of plant responses which have been demonstrated as a result of UV-A irradiation. The approximate wavelength range of the effective irradiation is also included in Table III. As with many of the experiments utilizing UV-C and UV-B irradiation, the exact spectral composition of UV-A irradiation used in these experiments is often difficult to ascertain. As is the case for Table I, the list of phenomena in Table III is by no means an attempt to compile a complete digest of the literature. Klein and Edsall (1967) allude to a variety of additional plant responses to UV-A not included in Table III.

Klein and Edsall (1967) also suggest that UV-A radiation from fluorescent lamps commonly used in growth chambers may be one of the prime reasons for the poor growth exhibited by many plants in such chambers.

Action spectra for several plant responses to UV-A irradiation are shown in Fig. 4. There is little apparent correspondence between these various action spectra which suggests that UV-A plant responses cannot be resolved to a few possible common chromatophores as is the case for UV-B and UV-C-induced phenomena. Several chromatophores for UV-A absorption in plant tissues have been proposed. Carotenoids and

TABLE III
PLANT RESPONSES TO UV-A IRRADIATION FROM UV LAMPS IN THE LABORATORY

Plant response	Wavelengths (Å)	Reference
Change in the osmotic concentration in epidermal cells of <i>Allium cepa</i>	3600-3800	Biebl (1942)
Increased alkaloid content of <i>Datura stramonium</i> ; decrease in volatile oils of <i>Thymus vulgaris</i> and <i>Mentha crispa</i>	3660	von Braun (1939)
Stimulation of photosynthesis in wheat	3650	Burns (1942)
Respiration stimulation in <i>Elodea densa</i> and <i>Potamogeton densus</i>	3500-3800	Gessner (1938)
Enhancement of carotenoid and chlorophyll development in <i>Allium</i>	3650	Godnev <i>et al.</i> (1959)
<i>Trans-to-cis</i> conversion of β -D-glucosyl- α -hydroxycinnamic acid in <i>Melilotus alba</i>	3200-4000 max at 3600	Haskins <i>et al.</i> (1964)
Decrease in fresh weight of <i>Tagetes erecta</i>	3550-3800 max at 3650	Klein <i>et al.</i> (1965)
Repression of liquid cell cultures of <i>Ginkgo</i> pollen	3400-3800 max at 3650	Klein and Edsall (1967)
Reduction in phytotoxicity of 2,4-dinitrophenol in <i>Phaseolus vulgaris</i>	3400-3900 max at 3600	Mellor and Salisbury (1965)
Decrease in total shoot length of <i>Stellaria media</i> , <i>Epilobium collinum</i> and <i>E. alpinum</i>	3200-4100	Pirschle (1941)
Repression of germination of <i>Phacelia tanacetifolia</i>	3200-3850 max at 3600	Schulz <i>et al.</i> (1963)
Interference of mitosis in onion root tip meristems	3400-3800 max at 3650	Wolff <i>et al.</i> (1967)
Increased tocopherol content of vegetation	3200-4000 max at 3600	Zolotnitskaya and Akopyan (1960)

flavins have been postulated as the chromatophores for the tip bending of *Avena* coleoptiles (Curry and Thimann, 1961). Cytochromes and quinones have also been implicated as chromatophores in UV-A-induced impairment of respiration (Klein and Edsall, 1967). Since both forms of phytochrome absorb in the UV-A portion of the spectrum (Butler *et al.*, 1964), many of the phenomena resulting from exposure to UV-A radiation may be the ultimate result of radiation absorption by phytochrome.

Because of the lack of uniformity in the UV-A action spectra, the usefulness of a biological effectiveness curve such as was defined for UV-B phenomena is obviously questionable. Fortunately the need for such a biological effectiveness curve and corresponding units of biologically effective irradiation for the UV-A spectrum is not as critical as

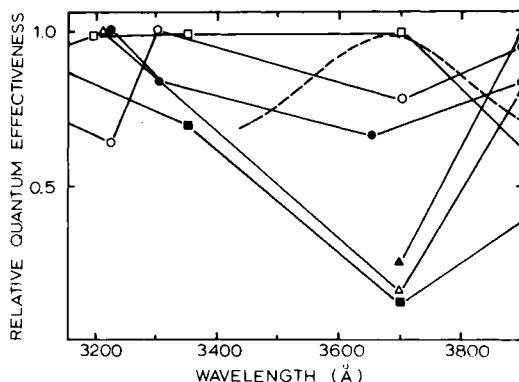


FIG. 4. Plant UV-A action spectra. Intermittent line is the phototropic tip curvature of *Avena* coleoptiles; approximate minimally effective dose at 3650 Å was 9.2×10^{-11} quanta cm^{-2} (Curry and Thimann, 1961). Open circles represent the induction of lettuce seed germination; constant dose of 4×10^{13} quanta cm^{-2} was applied at all wavelengths (Wagné, 1966). Solid circles represent unfolding of wheat leaves; constant dose of 4×10^{13} quanta cm^{-2} was applied at all wavelengths (Wagné, 1966). Open triangles represent repression of growth in crown gall tissue cultures of *Parthenocissus tricuspidatus*; a continuous intensity of $50 \mu\text{W}/\text{cm}^2$ was applied at all wavelengths (at 3600 Å, 9.1×10^{13} quanta $\text{cm}^{-2} \text{ sec}^{-1}$) (Klein, 1964). Solid triangles represent induction of protoplasmic streaming in *Avena* coleoptiles; doses of 5.0×10^{12} quanta cm^{-2} were applied (Bottelier, 1934). Solid squares represent stimulation of respiration in disks of potato tissue, seeds of pumpkin, and white leaves of *Acer negundo*; doses not given (Montfort and Rosenstock, 1950). Open squares represent induction of chloroplast movement in *Mougeotia*; doses not reported (Haupt, 1959).

for the UV-B spectrum. The principal justification for this statement is that unlike the highly variable spectral composition of UV-B solar and sky irradiation, the UV-A spectral composition in nature is not influenced by changes in solar angle, cloudiness, and atmospheric ozone (Herrmann, 1947; Bener, 1960). Therefore a determination of UV-A irradiation in nature with most any sensor which has a reasonably broad spectral sensitivity in the UV-A spectrum would at least give measurements of UV-A radiation which would always be proportional to the biologically effective photon irradiance for a particular physiological response.

For the UV-A action spectra studies in which absolute intensities of irradiation were reported, doses and dose rates utilized in these experiments do occur in nature. However, all the biological phenomena for which UV-A action spectra are reported in Fig. 4, as well as most of the plant reactions in Table III, also occur in response to visible or UV-B irradiation. Furthermore the response to UV-B or visible irradia-

ation is more pronounced than the response to UV-A irradiation. This naturally reduces the importance of UV-A radiation as a unique environmental factor. In contrast, most of the UV-B phenomena are effected exclusively by wavelengths in the UV-B and UV-C portions of the spectrum.

3. Terrestrial Solar Ultraviolet Irradiation

3.1 Spectral Composition of Solar UV Irradiation

The nature of solar UV irradiation to which plants are actually exposed in the field is of particular interest in UV photobiology. Although laboratory experiments using artificial sources of UV irradiation have demonstrated a great variety of physiological and morphological changes in plants following irradiation, the implication of these experiments for plants growing in nature remains in some doubt.

Solar ultraviolet radiation reaching the earth's surface consists entirely of UV-A and UV-B. The rather arbitrary upper wavelength limit of 4000 Å is the lower limit of visible radiation as defined by the standard luminosity curve (Seliger and McElroy, 1965). Ultraviolet radiation represents less than 5% of the total energy striking the ground. The spectral composition of UV radiation reaching the earth's surface is represented in Fig. 5. This curve is based on measurements by Bener (1960) of total global irradiation, i.e., that which is coming directly from the sun (direct solar) plus that which has been scattered in the atmosphere (sky radiation). With increasing wavelength the intensity of global UV irradiation increases exponentially. In contrast, the biological action spectra for UV-B radiation exhibit an exponential increase in the efficacy of decreasing wavelengths (see Fig. 3). Therefore the exact spectral composition of solar UV-B irradiation must be taken into account if this irradiation is to be measured in a biological context.

The lower wavelength limit of solar UV radiation reaching the earth's surface is generally considered to be approximately 2900 Å (Koller, 1965). Atmospheric ozone is the principal agent of absorption of UV-C and UV-B radiation although the total amount of ozone in the atmosphere would be equivalent to a layer only 3 mm thick at standard pressure and temperature (Koller, 1965). As shown in Fig. 6 the transmissivity to UV radiation decreases logarithmically below 3100 Å. Under certain circumstances, wavelengths below 2900 Å may indeed reach the earth's surface. Photographic plates exposed in spectrographs at high elevations in the Alps have demonstrated the presence of spectral lines at 2896 Å, 2881 Å (Tousey, 1966), and 2863 Å (Koller, 1965). These photographic records of wavelengths below 2900 Å were

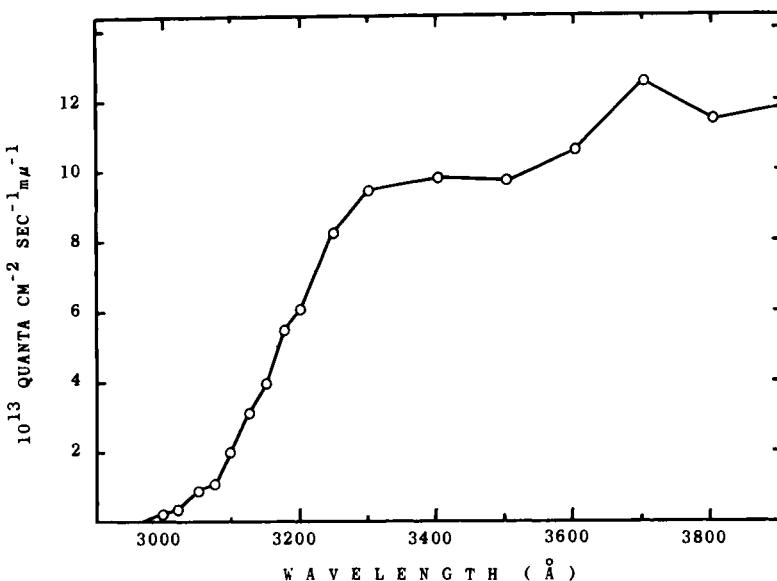


FIG. 5. Average July noontime global UV irradiance at Davos, Switzerland, 1590-m elevation. (The air mass, M , equals 1.1.) From measurements reported by Bener (1963).

obtained by extremely long exposures under ideal atmospheric conditions. In a recent elaborate theoretical discussion, Barker (1968) suggests that a photon flux intensity of approximately 10^{16} photons cm^{-2} month $^{-1}$ might be expected at wavelengths below 2900 Å. He argues that long-term exposures at these very low flux densities might be important in the photodegradation of certain synthetic polymers. He presents the rather exciting possibility that during statistically infrequent days when the atmospheric ozone concentration at a particular location might be less than 0.14 atm·cm (the thickness of the ozone layer at STP), the

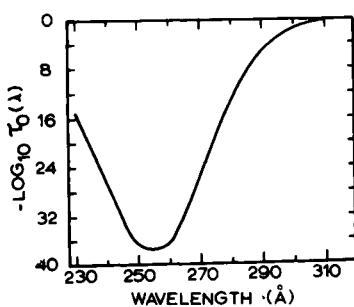


FIG. 6. Logarithm of the transmittance for 0.280 atm·cm of atmospheric ozone at various wavelengths. Adapted from Barker (1968).

photon flux at wavelengths as short as 2700 Å might be greater in a period of a few hours than over a period of several years under normal ozone conditions. Although there are no measurements to support Barker's hypothesis, if there is an appreciable photon flux below 2900 Å on a long-term basis or infrequent short bursts of irradiation below 2900 Å when the ozone concentration is low, the possibility exists that this irradiation below 2900 Å might be of importance for biological phenomena since these wavelengths are so effectively absorbed by proteins and nucleic acids (see Fig. 1).

The spectral composition of UV-B solar irradiation is much more variable than the composition of solar UV-A irradiation. Because of the pronounced wavelength specificity of biological phenomena in the UV-B, these changes in the spectral composition of solar UV-B irradiation will be discussed. With increasing solar angles from the zenith there is an especially strong attenuation of UV-B radiation, particularly at the shorter wavelengths (Schulze, 1956; Robinson, 1966; Bener, 1960). The measurable lower spectral limit may increase to wavelengths as high as 3150 Å when the angle from the zenith is 80°. Atmospheric turbidity due to haze, dust, or air pollution can also cause large changes in UV-B spectral composition. As might be inferred from the transmissivity curve of ozone (Fig. 6), changes in atmospheric ozone concentration would be much more important at the shorter UV-B wavelengths. This has been verified by measurements of Bener (1960). At higher elevations above sea level the atmosphere is thinner and therefore more transparent. Changes in spectral composition of UV-B may well be influenced by changes in elevation above sea level although this has not been experimentally verified.

In addition to these changes in the spectral composition of UV-B, there are also pronounced changes in the proportion of total global UV-B radiation coming as direct solar or scattered sky radiation. Molecular scattering in the atmosphere known as Rayleigh scattering occurs as an inverse fourth-power function of the wavelength. Therefore, with decreasing wavelength, an increased component of the global radiation will be sky radiation. This is particularly pronounced in the UV-B portion of the spectrum. For global UV-B irradiation, the ratio of direct solar radiation to sky radiation, I/S , is also influenced by the solar angle from the zenith, z . With large zenith angles, the ratio I/S for UV-B becomes substantially less than 0.5 at 1590 m elevation (Bener, 1963). Changes in atmospheric ozone concentration can also alter the proportion I/S to some extent (Bener, 1963). Furthermore, at higher elevations, there is a thinner atmosphere which not only results in less absorption of UV radiation but also less scattering. Consequently there is a pro-

nounced increase in I/S as a function of altitude, A (Pettit, 1932; Caldwell, 1968).

Solar UV-A irradiation represents in excess of 90% of the total energy in the solar UV spectrum (Robinson, 1966). In contrast to UV-B radiation, the spectral distribution, except under conditions of very large zenith angles, is virtually constant (Herrmann, 1947; Bener, 1960). Variations in atmospheric ozone concentration have essentially no effect on wavelengths greater than 3300 Å, and very little effect in the region from 3150 to 3300 Å (Bener, 1960). Absorption by ozone thus becomes of negligible importance for the UV-A spectrum. The proportion I/S for UV-A varies with zenith angle and wavelength in much the same manner as for UV-B radiation. The values I/S are greater, however, due to the λ^{-4} function of Rayleigh scattering (Bener, 1963).

3.2 Intensity of Global UV Irradiation

3.2.1 MEASUREMENTS OF BIOLOGICALLY EFFECTIVE UV IRRADIATION

The total global radiation intensity is primarily dependent on the solar angle, elevation above sea level, total atmospheric ozone concentration, atmospheric turbidity and air pollution, and finally on the degree of cloud cover. In addition to these primary factors, the distance to the sun causes an annual variation in radiation intensity of approximately 4% for the total solar energy reaching the earth, and minor solar fluctuations may cause changes on the order of 1% (Koller, 1965; Barker, 1968).

Unfortunately, it is often difficult to make direct comparisons between the UV irradiation intensity measurements of different investigators. Unless monochromatic determinations are made at each wavelength in the UV spectrum such has been done by Coblenz and Stair (1936) and Bener (1960, 1963), the spectral sensitivity of receivers which are sensitive to polychromatic radiation is of extreme importance and unfortunately oftentimes not specified. The difficulty of making monochromatic determinations particularly in the UV-B portion of the spectrum is due primarily to the great change in magnitude of energy with decreasing wavelengths. Fabry and Buisson (1921) point out with early measurements that the radiant energy at 2900 Å is one millionth of that at 3150 Å. Furthermore, measurements which take into account only direct solar UV-B irradiation with no measurement of sky radiation may neglect in excess of 50% of the total global UV-B irradiation falling on a horizontal plane. The detailed spectral measurements of global radiation by Bener (1960, 1963) contribute some of the best

information on solar UV irradiation. Unfortunately, his measurements were carried out at only one location.

Measurements of polychromatic irradiation are difficult to interpret unless the specific spectral response of the meter used in each case is specified. Even if the spectral sensitivity of the radiation sensor is specified, the interpretation of the measurements in the context of biological effectiveness is difficult. For example, meteorologists often report measurements in the UV-B spectrum as total energy below a specific wavelength.

Since UV-B irradiation at 2900 Å is 50 times as biologically effective as UV irradiation at 3130 Å, a measurement of total energy below a given wavelength such as 3150 Å can be very misleading since there may be easily 40 times the number of quanta $\text{cm}^{-2} \text{ sec}^{-1}$ at 3130 Å as at 3000 Å under clear sky conditions. If the UV-B portion of the solar spectrum were nearly always constant in spectral composition this would not be so serious because a determination of total energy below a given wavelength would be at least always proportionate to the total biologically effective photon flux. As has been discussed, however, this short-wave portion of the terrestrial solar spectrum is highly variable and the lower wavelength limit may vary from approximately 2900 Å to above 3200 Å. If monochromatic measurements of the spectral composition cannot be made, the best alternative is to use a sensor with a spectral sensitivity closely approximating the biological action spectra for UV-induced effects. This has been done occasionally when UV irradiation was measured in the context of erythema of human skin. Caldwell (1968) employed a sensor with a spectral sensitivity as shown in Fig. 3 to approximate the biological action spectra of UV-B-induced effects where proteins and nucleic acids are the principal chromatophores.

The following discussion will attempt to compare some of the measurements of UV irradiation in relation to the principal factors which might alter the UV radiation reception at the earth's surface. The first of these are solar angle and elevation above sea level.

3.2.2 AIR MASS AND ALTITUDE ABOVE SEA LEVEL

Air mass is a term commonly employed to represent the distance that solar radiation must travel through the earth's atmosphere. The prime factor which alters this distance is, of course, solar angle. Thus changes in solar angle whether the result of time-of-day, time-of-year or latitude should result in the same change in air mass. In most cases air mass is defined as the secant of the angle of the sun from the zenith. Therefore, when the sun is directly overhead the air mass is unity and increases with greater zenith angles. In this review air mass will be

assumed to be always equal to the secant of the zenith angle. Sometimes corrections are applied for very large zenith angles to take into account refraction of the atmosphere and curvature of the earth when the sun is near the horizon (Koller, 1965).

Occasionally a correction in the air mass term is also employed to incorporate the effects of elevation above sea level (Pfleiderer, 1950). Initially this may appear as a reasonably good idea since the air mass is meant to represent the total optical path length through the earth's atmosphere and elevation does certainly diminish this optical path length for any given solar angle. Such a representation, however, assumes erroneously that a stable homogeneous atmosphere exists in terms of UV extinction. However, this is not the case. As was suggested earlier, the principal UV absorbing component of the atmosphere is ozone and the distribution of ozone (see Fig. 7) is concentrated substantially above 10-km elevation (28 mb). The very small amount of ozone in the lower atmosphere constitutes a minor proportion of the total.

Although most of the UV absorption takes place above 10 km elevation where nearly all of the total atmospheric ozone is located, in high mountain areas there is a greater penetration of UV radiation due to the decreased concentration of aerosols, haze, and the normal molecular constituents of the atmosphere, O₂ and N₂, all of which contribute to

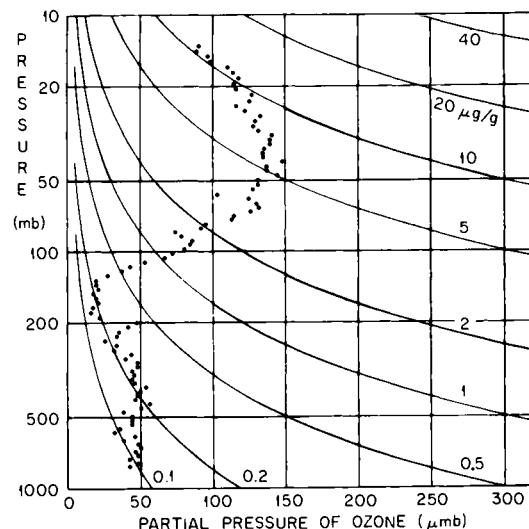


FIG. 7. Vertical ozone distribution in the atmosphere at Ft. Collins, Colorado, July 15, 1964. Altitude is represented in millibars atmospheric pressure. Adapted from Hering and Borden (1965).

some extent to the extinction of UV irradiation. The magnitude of change in global UV irradiation with change in solar angle or elevation above sea level is still, however, not a matter of total agreement. Figure 8 shows the diurnal variation of global UV irradiation at several wavelengths from the measurements of Bener (1963) at 1590-m elevation. As can be seen in Fig. 8, irradiation at shorter UV wavelengths is much more depleted as solar angle from the zenith increases as compared to longer wavelengths. This results in a continually changing spectral composition for the global UV-B spectrum throughout the day, particularly at shorter wavelengths. The time of year also causes corresponding differences in intensity at these wavelengths as would be expected due to changes in air mass. Measurements of total UV radiation at wavelengths less than 3132 Å by Coblenz and Stair (1936) indicate that this UV region of the spectrum changes much more with solar angle than the total solar short-wave irradiation. For example, between 9 and 12 a.m. total solar irradiation might increase by 7%, while UV irradiation at wavelengths less than 3132 Å will experience a twofold increase.

In order to account for the change in UV irradiation intensities with elevation, both the direct solar and diffuse sky components of global irradiation must be taken into account since the ratio I/S increases in

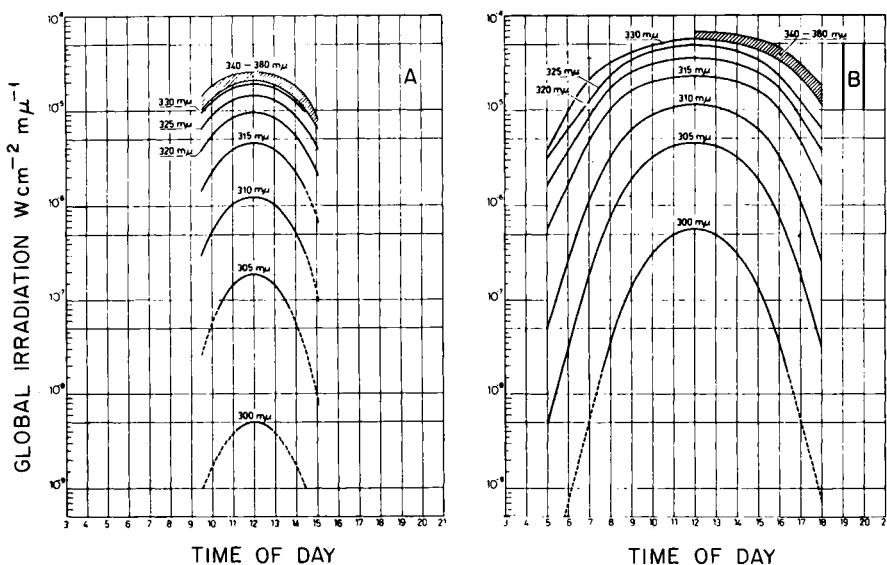


FIG. 8. The diurnal variation in global UV irradiation at several wavelengths as measured at Davos, Switzerland, in December (A), and in June (B). From Bener (1963).

a pronounced manner with increasing altitude. On the basis of earlier measurements Meyer and Seitz (1942) suggested that direct solar UV-B irradiation increases sharply with altitude but the sky component remains constant at least up to 3000 m. For the sky component, this would suggest that there is an equivalent compensation of reduced UV absorption at high altitudes by a reduction in the amount of Rayleigh scattering. Monochromatic radiation measurements at 3200 Å by Pettit (1932) demonstrated an increase in direct solar UV irradiation of 45% between 257- and 1740-m elevation. However, the sky UV component decreased 45% which resulted in a net global irradiation (at 3200 Å) increase of only 6% between 257 and 1740 m.

Compiling data from several authors, Sauberer (1955) estimated a 34% increase for global UV-B irradiation from sea level to 3000 meters. He recognized no net change in sky UV-B irradiation with altitude and therefore suggested that the global irradiation increase was a strict function of the increase in the direct solar component. Gates and Janke (1966) calculated the increase in direct solar UV (total irradiation less than 3200 Å) from sea level to alpine elevations at a latitude of 40° N. Due to the lack of measurement of sky UV irradiation, they applied values of I/S obtained by Bener (1960) at 1590 m with some adjustments for alpine conditions at 3650 m. They estimated that at an air mass of 1.05 there would be a net increase in global UV irradiation of 50% from sea level to 3650 m. At an air mass of 2.00, they predicted a 120% increase from sea level to 3650 m. Caldwell (1968) measured biologically effective UV-B irradiance at several elevations from 125 to 4350 m over a range of air mass values, total atmospheric ozone concentrations, and various atmospheric turbidity conditions. The change in the direct solar and sky components of global biologically effective UV-B irradiation are shown as a function of elevation above sea level at two different air mass values in Fig. 9. These curves were generated from multiple regression equations which have taken into account variations in total atmospheric ozone and atmospheric turbidity. These regression equations accounted for 96% of the variability in the data. When the sun is high in the sky (air mass = 1.1 in Fig. 9A) there is no change in total global UV irradiation from sea level to 2000 m and only a 16% increase from 2000- to 4300-m elevation. From 2000 to 4300 m there is a 36% increase in the direct solar component, however, this is compensated by an absolute decrease in the sky component to cause the resultant change in total global irradiation of only 16%. This absolute decrease in the sky UV component is much more pronounced at greater angles from the zenith. In Fig. 9B where air mass = 2.00, the direct solar component of biologically effective UV-B irradiation increases 390% from sea level to

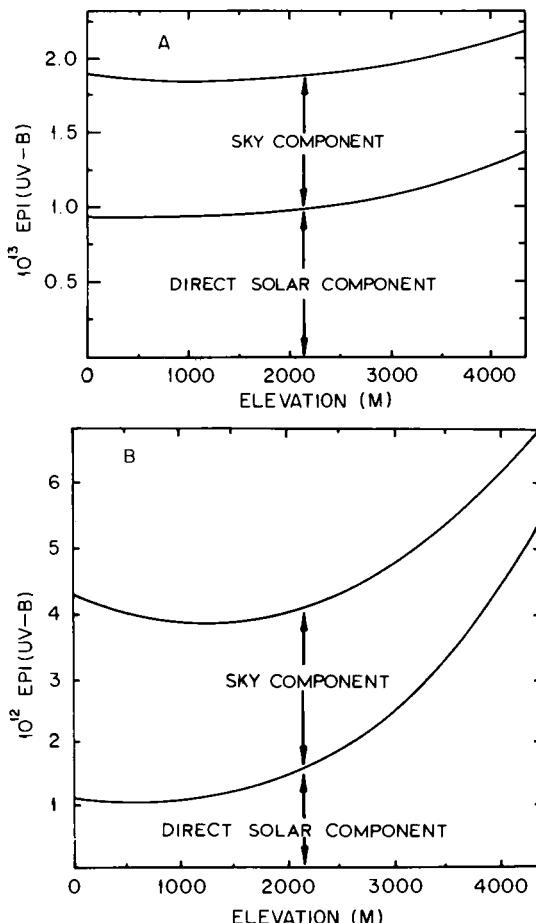


FIG. 9. Direct solar and sky UV-B irradiance as a function of elevation for cloudless conditions with an atmospheric ozone concentration of 0.301 atm·cm and an atmospheric turbidity coefficient of 0.04 (A) at an air mass of 1.10; (B) at an air mass of 2.00. From Caldwell (1968).

4300 m. However, because of the substantial decrease in sky UV-B irradiation there is only a 66% increase in the total global UV-B irradiation. Total biologically effective global UV-B irradiation is integrated for a complete day under cloudless conditions under equivalent conditions of atmospheric ozone and atmospheric turbidity for several elevations above sea level in Fig. 10. Under these conditions, for a complete day, the biologically effective UV-B irradiation at 4350-m elevation is only 26% greater than at 1670-m elevation.

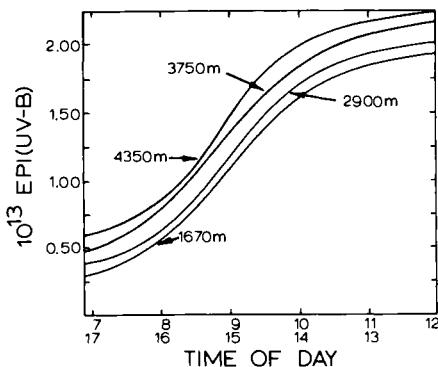


FIG. 10. Global UV-B irradiance for a cloudless day in late July. Total irradiation for a 10.2-hour day at 1670-m elevation is 4.386×10^{17} biologically effective photons per cm^2 , EP; at 2900 m, 4.6644×10^{17} EP; at 3750 m, 5.1216×10^{17} EP; and at 4350 m, 5.5578×10^{17} EP. These integrated values are plotted for an atmospheric ozone concentration of 0.301 atm·cm and an atmospheric turbidity coefficient of 0.04. From Caldwell (1968).

It is extremely difficult to reconcile the differences in the various calculations and measurements reported in the literature for solar UV-B irradiation at various elevations above sea level. There are two primary reasons for the lack of agreement—(1) The spectral composition of the UV-B irradiation reported in these various calculations and measurements is often quite different. As was discussed earlier, the variability of the spectral composition of UV-B irradiation with air mass and elevation might easily account for some of these differences. (2) Unless careful measurements of the sky component of total global UV-B irradiation were carried out, it is extremely difficult to predict the total change in global UV-B irradiation with elevation above sea level. Many of the predictions of UV-B irradiation change with elevation are based on inappropriate ratios of *I/S*.

Because of the relatively constant UV-A spectral composition and virtual independence of UV-A from ozone absorption, reasonable predictability of global UV-A irradiation for given values of zenith angle, atmospheric turbidity, and elevation has been achieved (Schulze, 1960). Conformity of absolute energy values calculated theoretically and detailed spectral measurements of global UV-A irradiation by Bener (1962), however, are not particularly good. Measured values were often systematically as much as 10% lower than theoretical values. However, for relative differences, Schulze (1960) found good agreement of theoretical and measured values of UV-A. He found a linear 10% increase of global UV-A irradiation per 2000-m change in elevation. Using pig-

mentation (tanning) of the human skin as a biological indicator of UV-A irradiation intensity he further verified such a 10% increase.

In summary, with increasing air mass there is a more pronounced depletion of global UV irradiation as compared with total solar irradiation. This is particularly enhanced in the short wavelengths of the UV-B portion of the spectrum. With increasing elevation above sea level, global UV-A irradiation increases linearly in a predictable fashion. The change in UV-B global irradiation, however, is less predictable. Although there is apparently a very steep increase in direct solar UV-B irradiation there is a concomitant absolute decrease in the magnitude of sky UV-B irradiation resulting in only a modest increase in total global UV-B irradiation with increasing elevation above sea level. Other factors at high elevation such as the increased reflection from snow and rocks may contribute to an increased global UV-B irradiation regime at higher elevations, as will be discussed later.

3.2.3 ATMOSPHERIC OZONE

Because of the overwhelming importance of atmospheric ozone as the principal cause of attenuation of the UV-B portion of the UV spectrum, fluctuations in total ozone concentration are of particular interest when the global UV-B irradiation regime is being considered.

The total atmospheric ozone is not a constant but varies considerably with latitude and time of year. As a general rule, total ozone concentration is higher at greater latitudes in both the northern and southern hemispheres. In the late winter and early spring, total ozone concentrations are maximal. At the same time there is a maximum gradient of ozone with latitude. In the late summer and autumn, ozone concentrations at all latitudes are minimal and the gradient with latitude is also at a minimum. Although this trend is roughly similar in both northern and southern hemispheres, there are a number of differences related to differences in the general circulation system in the two hemispheres. Total ozone concentration is apparently independent of longitude except for day-to-day variations associated with advection and vertical movement in the lower stratosphere (Griggs, 1966). Measurements of total atmospheric ozone during the International Geophysical Year (MacDowall, 1960) revealed that between the equator and 20°N. latitude, total ozone concentrations were around 0.250 atm·cm with very little annual variation. During the spring when the latitudinal gradient in total ozone is greatest, concentrations at 60 to 80°N. latitude in March were as high as 0.480 atm·cm. In the temperate and polar latitudes the annual variation in total ozone can easily be on the order of 0.100 atm·cm (MacDowall, 1960; Bener, 1960). Day-to-day fluctuations in total ozone

concentration at any one location can be on the order of magnitude of at least $0.025 \text{ atm} \cdot \text{cm}$ (Bener, 1960). These day-to-day fluctuations are a result of the variation in the local pressure patterns and storm conditions. Usually on a diurnal basis, the total ozone concentration tends to be higher in the morning hours than in the afternoon (Koller, 1965; Griggs, 1966).

As would be expected from the transmissivity curve of ozone (see Fig. 6), changes in total atmospheric ozone are much more important for shorter wavelengths in the solar UV-B spectrum. Measurements by Bener (1960) at Davos, Switzerland over a range of ozone concentrations between 0.197 and $0.373 \text{ atm} \cdot \text{cm}$ indicated that there was virtually no effect of total ozone concentration on UV wavelengths above 3300 \AA . At wavelength 3200 \AA , a change in the amount of atmospheric ozone from 0.200 to $0.300 \text{ atm} \cdot \text{cm}$ decreases the global irradiation by a factor of only 1.2 to 1.6 depending on the solar angle. At 3100 \AA there is a corresponding decrease by a factor of 2.3 to 6.0, while at 3000 \AA the decrease in intensity is by factors of 8 to 16. Therefore at the shorter, and more biologically effective, UV-B wavelengths the total atmospheric ozone concentration is a particularly important factor in terms of global UV irradiation. Barker (1968) suggests that under conditions of low total atmospheric ozone that an appreciable photon flux below wavelength 2900 \AA may be received on the earth's surface as was discussed earlier.

In the temperate and polar latitudes where the annual variation in ozone concentration is substantial, a reasonably large effect on biologically effective UV-B irradiation might be expected. This was verified by measurements of Bener (1963) and Coblenz and Stair (1936, 1944), which demonstrated substantially higher values of UV-B irradiation in the fall as compared with the spring at equivalent solar angles. This is particularly true at the shorter wavelengths. Barker (1968) estimates that the integrated monthly UV irradiation for all wavelengths less than 3132 \AA is substantially higher in the second half of the year from June 21 to December 21 than in the first half of the year because of the lower ozone concentrations. The "excess" UV-B irradiation for the second half-year amounts to 11.6% of the total yearly UV-B flux. If biologically effective UV-B radiation were taken into account here instead of total UV irradiation below 3132 \AA , these differences in spring and fall UV irradiation should be even greater.

Although the daylight may be continuous at polar latitudes, solar angles from the zenith are always large. Daniels (1962, 1967) has calculated total solar irradiation between 2900 and 3130 \AA for various times of year at various latitudes. He concluded that at 80°N . latitude

there would still be sufficient UV-B irradiation for erythema to occur within 30 min during midday in the summer months. He neglected, however, to take into account in his calculation the increasing ozone concentrations at these higher latitudes. Measurements of Coblenz *et al.* (1942) at latitudes between 60 and 78°N. of total UV-B irradiation less than 3132 Å indicated no substantial difference in irradiation as compared to values from Washington, D. C. at equivalent air mass values. Recent measurements of biologically effective UV-B irradiation (Caldwell, 1972) at 71°N. indicate lower global UV-B irradiation intensities than at temperate latitudes for equivalent air mass values. The greater ozone concentrations at 71°N. are probably responsible for the reduced intensities of these biologically effective UV-B wavelengths.

By way of summary, seasonal and latitudinal variations in atmospheric ozone concentration do exert substantial effects on biologically effective UV-B irradiation as has been borne out by radiation measurements.

3.2.4 ATMOSPHERIC TURBIDITY AND AIR POLLUTION

The presence of haze or other particulate matter in the atmosphere can effect an appreciable attenuation of total solar irradiation. Under a highly turbid atmosphere, such as would be found in industrial areas, an appreciable attenuation of all wavelengths in the terrestrial solar spectrum should be expected. For global UV irradiation measurements at 1590-m elevation where atmospheric turbidity levels are quite low, no relationship was found between either global or sky UV irradiation and atmospheric turbidity (Bener, 1960). Caldwell (1968) found a small significant contribution of atmospheric turbidity in regression equations for measurements between 125 and 4350-m elevation for direct solar biologically effective UV-B irradiation and for sky UV-B irradiation. With increasing atmospheric turbidity, there was a small decrease in direct solar UV-B and a small increase in sky UV-B irradiation probably due to increased atmospheric scattering. For measurements of global biologically effective UV-B irradiation, however, there was no significant contribution by atmospheric turbidity. These measurements, however, were taken under conditions of reasonably low levels of turbidity (decadic atmospheric turbidity coefficients for 5000 Å per unit air mass ranged between 0.003 and 0.0099). Apparently increasing atmospheric turbidity may alter the proportion I/S for this portion of the UV spectrum; however, there is little influence on the total global UV-B irradiation under these conditions.

In areas of significant air pollution, a decrease in biologically effective UV-B irradiation might be reasonably appreciable. Not only would high

levels of atmospheric turbidity be effective in depressing global UV-B irradiation but many of the gases associated with air pollution absorb appreciably in the UV spectrum below 3100 Å (Barker, 1968).

3.2.5 ALBEDO OF THE EARTH'S SURFACE

It is evident that reflection of UB irradiation from a snow surface might increase the total UV irradiation load on an organism if irradiation from all angles is being taken into account. However, even for downward irradiation falling on a horizontal plane the increased albedo of the ground surface will increase the downward flux due to scattering in the atmosphere. The measurements of Bener (1960) do show this increased downward flux of irradiation for wavelengths above 3100 Å. Below this wavelength, the importance of ground reflection could not be detected because of the overwhelming influence of atmospheric ozone on intensities at these shorter wavelengths. For UV-A irradiation between 3300 and 3600 Å where ozone absorption is negligible, Bener (1960) found that global irradiation during the winter when snow cover was present was about 30% higher than corresponding intensities during the summer at comparable air mass values. He was not able to predict the influence of increased ground reflection on UV-B wavelengths. Undoubtedly the increased UV irradiation regime at higher elevations is magnified by ground reflection when snow or ice is present for both UV-A and UV-B irradiation.

3.2.6 CLOUD COVER

Although most UV irradiation measurements have been taken when the sky is nearly cloudless, the presence of clouds would certainly be expected to cause some alternation in UV intensities as it does for total solar irradiation. Caldwell (1968) investigated the effects of scattered cumulus clouds which did not directly obstruct the sun on total global biologically effective UV-B irradiation. The presence of such clouds caused a small positive increase in UV-B irradiance in the regression equations derived from measurement data. Global and sky UV-B irradiation intensities were increased approximately 10% by the presence of the average cumulus cloud pattern. This increase in irradiation intensity is probably the result of increased scattering and reflection. Nevertheless these measurements did not include other types of cloud cover or situations when the sun image was obstructed by cloud cover which would obviously create a great reduction in total global UV irradiation intensities. Bener (1964) investigated the effect of different types of cloud cover on sky UV irradiation at 3300 and 3700 Å. These measurements were taken when the sky was completely overcast

and the solar image was not detectable. The average ratio of sky UV irradiation for overcast skies to sky UV irradiation for clear skies varied with solar angle from the zenith and the type of cloud cover as well as the time of year. For low clouds, the average value of this ratio at all solar angles from the zenith varied between 0.65 and 1.75. For high clouds this ratio ranged between 0.9 and 1.47. At smaller angles from the zenith these ratios tended to be higher. For longer wavelengths, i.e., 3700 Å and for visible irradiation, the ratio of sky irradiation for overcast skies/sky radiation for clear skies was even greater. Although sky UV irradiation may not be greatly reduced by cloud cover, and in some cases enhanced, the direct solar component of global radiation is absent. Therefore the net result is usually a decrease in global UV irradiation. Bener's research dealt only with UV-A wavelengths. At shorter wavelengths the reduction caused by cloud cover may be even greater.

3.2.7 CORRELATION OF UV IRRADIATION WITH TOTAL SOLAR IRRADIATION

Although the UV-B portion of the terrestrial solar spectrum varies substantially in intensity and spectral composition, particularly at the shorter wavelengths, reasonably good correlations of total solar irradiation with global UV-B irradiation have been made. Coblenz (1949) established average ratios of UV-B (total irradiation below 3132 Å) to total solar irradiation for an eight-year period. He proposed ratios for each month of the year which would show a reasonably good relationship between total solar irradiation reception and UV-B irradiation. These ratios were empirically derived. Caldwell (1968) established an empirical regression equation which would predict global biologically effective UV-B irradiation from total global irradiation. This equation was based on measurements in August and September from altitudes of 1600 to 4350-m elevation under various degrees of cloudiness. Although this regression equation has a reasonably good coefficient of determination, r^2 , of 0.83, the equation may not apply during other months of the year or at different elevations, etc. Empirical ratios and correlations would be needed for each particular elevation and time of year, etc.

4. Response of Higher Plants to Filtered Solar Irradiation

Although a great variety of plant responses have been demonstrated as a result of exposure to artificial sources of UV irradiation and appreciable quantities of biologically effective solar irradiation are avail-

able in nature, the importance of solar UV irradiation as an ecological factor for higher plants is still not altogether clear. A number of experiments have been carried out in special greenhouses and gardens covered with UV-absorbing and transmitting filters which may lend insight to the question of the effectiveness of UV irradiation in nature.

During the early part of this century when the interest in the medical effects of UV irradiation stimulated botanists to conduct a variety of experiments using artificial sources of UV on plants, a few individuals undertook experiments in glasshouses or gardens using window glass to filter out UV radiation. An example of this work is that of Schanz (1920) at 760-m elevation. He used window glass to filter out UV-B and some UV-A irradiation for plants growing in a garden at 760-m elevation. He compared the morphology of plants growing under the glass with plants exposed to the direct sunlight. He found great alterations in plant growth form and reported a marked enhancement of shoot growth with exclusion of solar UV irradiation by the glass. With the development of glasses which were transparent to most of the UV spectrum more controlled experiments could be performed. An example is the work of Pfeiffer (1928) which compared plants growing in a greenhouse covered with a special UV-absorbing glass (transmitting 3900 to 7200 Å) to plants growing under a UV-transparent glass (transmitting 2900 to 7200 Å). He concluded that the exclusion of UV irradiation by the UV-absorbing glass produced longer shoots, thinner leaves and inhibited root development. Jacobi (1928) conducted similar experiments with glass filters. His results in agreement with Schanz (1920) and Pfeiffer (1928) demonstrated an increased height and spindliness of plants grown without UV radiation.

Popp and Brown (1936) reviewed much of the literature concerning the effect of solar or artificial irradiation on plant growth and development. They were very critical of many of the early experiments for lack of sufficient sample sizes and the failure to specifically isolate UV irradiation as the single contributing factor causing the alleged differences in plant growth, flowering, fruiting, and chemical composition. In their own carefully controlled greenhouse experiments (at approximately 200-m elevation) they found no effect on rate of plant elongation, fresh and dry weight yield, amount of flowering and fruiting, or nitrogen, carbohydrate and pigment content due to the removal of solar UV irradiation in special glasshouses. They concluded that at sea level the exclusion of solar UV radiation could probably have little effect upon higher plants. They did concede, however, that at higher elevations, solar UV irradiation may be somewhat effective. In terms of artificial irradiation experiments, they stated that the only plant responses to

UV irradiation which could be clearly demonstrated were the distinctly harmful effects due to wavelengths less than 2900 Å.

Following the review of Popp and Brown (1936) little work was done using filtered sunlight to investigate the effect of UV irradiation on plant growth. Finally Brodführer (1955) published a major work dealing with the influence of solar UV irradiation on plant growth. This often-cited work was conducted primarily at Davos, Switzerland (1575-m elevation) and involved a garden of transplants and seedlings native to several localities at various elevations above sea level. Normal window glass, absorbing principally below 3200 Å, was used to exclude solar UV irradiation from the garden. Window screening served as a control filter and a series of alternating strips of window glass and screening were employed to yield an intermediate dose of UV irradiation. Brodführer used these three filters to yield respective relative UV doses of 2%, 100%, and 33%. Parameters used to measure growth involved all shoot dimensions as well as fresh and dry weight yield. The time required for flowering and fruiting was also considered.

Depending on the native locality of the species or ecotype, different amounts of UV irradiation either inhibited or stimulated plant growth and flower development. Plants from high altitudes grew optimally under a high UV regime, and correspondingly, plants originally from low elevations performed best under low UV conditions. Brodführer concluded that plant growth and development could either be stimulated or inhibited by different UV doses. The response of the particular plant was usually considered a function of the location of the population from which it was derived. This complemented the earlier work of Eltinge (1928) who, utilizing artificial UV of wavelengths greater than 2900 Å, found stimulation or slight inhibition of growth and flower development depending on the dose applied. Stimulation of plant growth following artificially produced UV irradiation (2900 to 3100 Å) was also discovered in a number of common garden vegetables by Gilles (1939) and Benedict (1934). Repression of growth by artificial UV-A was recently reported in *Tagetes erecta* L., *Impatiens balsamina* L., *Lycopersicon esculentum* Mill. (Klein *et al.*, 1965). Dose-dependent acceleration or inhibition of flowering in *Linum usitatissimum* L. was achieved by artificial 3660 Å irradiation (von Braun, 1939) and by mercury vapor lamp irradiation (~2000 to 3800 Å, max. 2537 Å) (von Denffer and Schlitt, 1951). Eltinge (1928) concluded from her own work that each plant has its own particular UV requirement for optimal growth and development. Brodführer's results might give an ecological basis for this apparent individual plant UV requirement. Unfortunately, however, there exists a strong possibility that plant temperatures under the

window glass in her experiments could have been substantially higher than under the window screening which would naturally confound the experiment. Caldwell (1968) replicated Brodführer's window glass and window screen filters and found that while air temperatures were not significantly different under the two filters as Brodführer had claimed, leaf temperatures were substantially higher under the glass filters. If even slight differences in plant temperatures existed for long periods of time in Brodführer's experiments, these striking differences in plant growth which she claimed were a function of UV irradiation might then be explained at least in part as a response to temperature. This would coincide with the reports that high altitude plants appeared to perform better under high UV regimes. Perhaps the high altitude plants were performing better because they were growing at somewhat lower temperatures beneath the screen filters. Furthermore, stimulation or inhibition of a particular plant by a given amount of UV irradiation was dependent upon the prevailing temperature conditions at Davos, according to Brodführer. This would certainly occur if differences in plant growth and development under glass and screen filters were largely a function of plant temperatures.

Caldwell (1968) conducted experiments at 3750-m elevation in Colorado in which various filters were used to exclude solar UV irradiation in undisturbed plant communities of the alpine tundra. Control filters were selected which transmitted UV irradiation and possessed similar transmission characteristics in the visible and infrared portions of the spectrum. The energy balance beneath both the UV-absorbing and UV-transmitting filters was apparently the same because soil and plant temperatures were always equivalent under the two types of filters. Five native alpine species were selected for detailed phenological measurements. Although there was a slightly greater degree of stem elongation and an increase in aerial growth and yield of plants when UV irradiation was excluded which is in general agreement with the laboratory of Klein *et al.* (1965) and the early solar UV-exclusion experiments of Schanz (1920) and Pfeiffer (1928), these effects were not statistically significant nor as dramatic as these earlier accounts suggested. The only statistically significant effect of UV exclusion was an enhanced flowering of one species (*Trifolium dasypyllum*). Other than the enhanced flowering of this one species there was no evidence at all to suggest that high elevation plants might be stimulated by UV irradiation as Brodführer (1955) and Eltinge (1928) have suggested. Perhaps if plants from low elevation were grown under UV-transparent and absorbing filters at high elevations a greater effect might be realized.

Several horticulturists have claimed that solar UV irradiation is

responsible for various types of lesions and alterations in growth form. Foley (1963) working with cucumber plants at 670-m elevation in Idaho claimed that solar UV irradiation was responsible for smaller leaves which tended to be shiny, thick, and leathery in texture and oftentimes exhibited small white patches resembling salt deposits. He compared the performance of plants grown under window glass, under polyethylene (which he claimed removed 66% of the solar UV irradiation) and in the open. Plants cultured under polyethylene possessed leaves which were normal in character and somewhat longer internodes as compared with plants grown in the open. Plants cultured under the greenhouse glass, where all irradiation below 3300 Å was eliminated, performed even better. Unfortunately no information was given on the differences in microenvironment for each cultural situation. Therefore, it might be difficult to attribute these effects solely to UV irradiation. Campbell (1962) claimed that brown blotches on the surface of honeydew melons exposed to solar irradiation for prolonged periods could be duplicated by the use of a germicidal UV lamp (2537 Å) in the laboratory. He did not, however, establish the fact that solar UV irradiation was the cause of these lesions in the field. More recent and controlled experiments by Moore (1970) in the San Luis Valley of Colorado (2338-m elevation) demonstrated a slight enhancement of leaf elongation of onion due to exposure to solar UV-B irradiation. Moore's experiments involved the use of UV-absorbing and transmitting plastic films. Although leaf temperatures were not measured, the microenvironments under the two types of plastic films were comparable. The presence or absence of solar UV-B irradiation apparently made no significant difference in the vine length of potatoes grown in the same situation. Nilsen (1969) reported that the exclusion of solar UV irradiation greatly enhanced the vegetative growth and tuber yield of a strain of potatoes at 692-m elevation in Washington. He based this conclusion on the fact that plants grown under irradiation filtered through different types of plastic films were much more vigorous than plants grown in the direct unfiltered solar irradiation. There was little apparent difference, however, in the plants grown under DuPont Company mylar plastic film which filters out UV-B irradiation as compared to plants grown under polyethylene and polyvinyl chloride films both of which transmit at least part of the UV-B spectrum. Therefore it is difficult to assume that the striking increase in vegetative growth under plastic filters is really due to solar UV irradiation. It is more likely a result of either reduced total solar irradiation or an alteration in the energy balance beneath the filters with subsequent changes in plant temperatures. These same plant responses, although not nearly so dramatic, were also reported when this experiment was repeated in a growth chamber equipped with

fluorescent lights with reasonably high output in the red and far-red portion of the spectrum (Sylvania Gro-Lux Wide Spectrum lamps). Klein *et al.* (1965) filtered out all irradiance below 3850 Å from fluorescent (cool white) and incandescent light sources in a normal growth chamber and found that there was a slightly enhanced vegetative growth of several plants such as *Tagetes erecta*, common tomato, and corn. Because of the experimental design used by Nilsen (1969) it is difficult to determine if the plant response in his experiments was due, in fact, to the removal of certain UV wavelengths as was apparently the case with the work of Klein *et al.* (1965). Nevertheless, the type of response in each case appeared similar.

The efficacy of solar UV irradiation in effecting the same specific biological response as artificially produced UV irradiation has been reported by several investigators. An example is the work of Zimmerman and Hitchcock (1939) who demonstrated that either solar UV irradiation or artificial UV irradiation would cause a change from the inactive *trans* form to the active *cis* form of cinnamic acid thereby resulting in epinasty and curvature of tomato plants. Cinnamic acid was implicated as the true chromatophore in this case because irradiation of this chemical with artificial UV irradiation and subsequent application to plants which had not been irradiated did cause the same type of response, namely epinasty and stem curvature.

Although a great number of plant responses have been demonstrated as the result of UV lamp irradiation and biologically effective global UV irradiation is often of comparable intensities in nature, sound evidence of the efficacy of solar UV irradiation as a significant factor altering the growth and development of higher plants is still very much lacking. The popular notion that the short stature of high elevation vegetation is the result of solar UV irradiation is still widespread. The real impact of solar UV irradiation in nature still awaits further experimental verification.

5. Defense Mechanisms of Higher Plants against Solar UV Irradiation

Though not all the plant responses demonstrated as the result of UV irradiation are considered as damaging or disadvantageous for the plant, the majority of evidence indicates that UV irradiation is usually detrimental, particularly UV-B irradiation. The argument that a certain amount of UV irradiation is stimulative or even required by certain types of plants (Eltinge, 1928; Brodführer, 1955) still requires substantiation.

Although the biologically effective UV-B irradiance used in demonstrating many of the plant responses to artificial sources of UV-B (see Table II) are within the range of values received in nature (see Figs. 9 and 10), solar UV-B irradiation still has not been demonstrated as a particularly deleterious factor of the environment. Plant defense mechanisms may be responsible for protecting plants in areas of high exposure.

5.1 Optical Properties of Plants

5.1.1 UV REFLECTANCE

The most easily envisaged protective mechanism against UV irradiation is that of optical screening of UV irradiation by outer plant tissues. Although the corollas of many flowering plants possess high reflectivity in the UV-A portion of the spectrum, presumably to attract pollinators (Eisner *et al.*, 1969), most reports indicate that the UV-A reflectivity of most leaves is consistently quite low, i.e., less than 10% (Shul'gin *et al.*, 1960; Barth, 1957; Kozlovka, 1957; Caldwell, 1968). Cappelletti (1930) indicated that many alpine leaves had very high UV-A albedos; however, he presented no quantitative data. Reports by Kozlovka (1957) and Caldwell (1968) indicated that the UV-A reflectivities of leaves of high altitude alpine plants were no greater than those of plants from lower elevations. Tomentose leaves such as are found on *Eriogonum jamesii* or blue-tinted tomentose leaves as occur in the genus *Artemisia* do possess a somewhat greater UV-A reflectance (Caldwell, 1968; Kozlovka, 1957; Barth, 1957); however, the UV-A albedo is still less than 10% in all cases. Unfortunately reports of UV-B reflectance of plant leaves have not been found for this review.

5.1.2 UV ABSORPTION BY OUTER TISSUE LAYERS

Although most evidence suggests that UV-A reflectance by plant leaves is quite low, a strong UV absorbance by the outer tissue layers of leaves and stems has been demonstrated.

Shul'gin *et al.* (1960) have demonstrated very high UV-A absorption values, 88%-92%, and very low transmission values, less than 2%, for intact leaves of a number of species. Penetration of UV radiation through the leaf epidermis has been studied extensively by Lautenschlager-Fleury (1955) and Caldwell (1968). Lautenschlager-Fleury measured dried pieces of epidermis in a spectrophotometer. (She estimated a consistent 3%-4% increase in UV transmission could be attributed to the drying of the epidermal strips before measurement.) Caldwell (1968) used a slightly different technique in which fresh

epidermal strips were measured for UV-A transmission immediately after being removed from the plant. In all measurements from both studies, the percentage UV transmission through the leaf epidermis was consistently quite low (usually less than 10%). Portions of plants exposed to greater irradiation intensities consistently possessed lower epidermal UV transmission values. Epidermal transmission values of the upper epidermis in planophilic leaves were consistently greater than those of the lower epidermis, and similarly, portions of stem tissue exposed to direct irradiation were much lower in UV transmission of the epidermis than portions of the stem which were shaded. Plants growing in shaded habitats possessed generally higher transmission values than plants growing in exposed habitats.

Lautenschlager-Fleury (1955) took water extracts of intact epidermal strips from *Vicia faba*, which did not possess a thick cuticle, and found that the strong and variable UV absorption component of the epidermis was water soluble. The remaining cell components, though apparently intact structurally, exhibited only a small UV absorbance. Caldwell (1968) also found that most of the absorption of UV in epidermal strips could be accounted for by material which was soluble in methanol and methanol-water-HCl solvents (see Fig. 11). These solvents were chosen to be particularly effective in extracting flavonoids and related phenolic compounds which are especially strong UV absorbers in the plant (Stafford, 1965; Geissman, 1955; Block *et al.*, 1958). In addition to flavonoids, some xanthophylls, cuticular waxes and other lipids probably occurred in the extract. Shibata (1915) found that the epidermal and

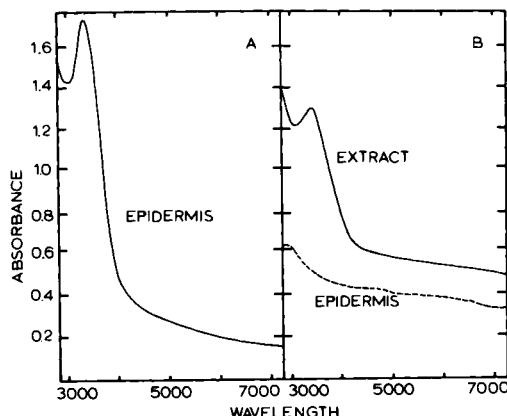


FIG. 11. Epidermal UV transmission of an intact strip of *Oxyria digyna* before (A), and after (B) extraction with methanol and methanol-water-HCl. From Caldwell (1968).

underlying mesophyll cells of most plants contained large quantities of UV-absorbing flavone derivatives. Usually the upper epidermis of leaves contained greater amounts of these flavonoids than the lower epidermis.

In plants with a thick cuticle, Lautenschlager-Fleury (1955) ascribed an appreciable UV absorbance below 3200 Å to these waxes. Similarly Caldwell (1968) found a high UV absorbance around 3000 Å in ether extracts of epidermal tissue which had already been extracted with methanol-water-HCl to remove flavonoids. Cuticular waxes were presumably the chief components in these ether extracts. Metzner (1930) used microphotographic techniques to investigate the absorption of various intact cell components between 3500 and 4000 Å. He attributed very little UV attenuation to cellulose, hemicellulose and silicates. The cuticle absorbed to some extent, but was not considered as the protective agent against UV-A irradiation. Attention was called by Metzner to the tannins and flavonoids as particularly important in UV absorption by the intact cell. Evaluation of UV absorption by the wax components of epidermal cells by Wuhrmann-Meyer and Wuhrmann-Meyer (1941) indicated a maximum absorbance occurred in the region of 2900 to 3000 Å. Although wax extinction coefficients in this UV region varied a great deal among species, UV absorption was generally considered as quite important in UV-B protection. Cuticular UV transmittance measurements by Cappelletti (1961) also emphasized the importance of UV-B absorption by waxes.

To demonstrate the importance of UV epidermal absorption, Caldwell (1968) exposed alpine leaves of several species to UV-B irradiation. If the UV epidermal transmission of the upper and lower epidermis differed significantly there was a corresponding difference in sensitivity to tissue destruction by UV-B irradiation. Toth (1949) also found excellent correlation between resistance to UV-B damage and the thickness of the outer cell wall for epidermal cells of *Allium cepa* bulbs.

Oftentimes epidermal tissue with a high content of anthocyanins also possesses a much reduced UV epidermal transmission (Caldwell, 1968). In bulbs of *Allium cepa*, Biebl (1943) found evidence that UV-B destruction of epidermal cells was an inverse function of the anthocyanin content. This was also found to be the case for several alpine plants (Caldwell, 1968). On the other hand, Nystrom *et al.* (1955) found photosynthesis inhibition by 2537 Å UV irradiation was not diminished by the presence of anthocyanin pigments in *Coleus*. The UV epidermal transmission at 2537 Å was, however, not measured in this study. Although anthocyanins may often enhance the UV filtration capacity of the outer tissues of many plants, tissues without apparent anthocyanins

per se may still possess very low UV transmission values in outer tissues due to the UV absorbance by colorless flavonoids and waxes. Cline and Salisbury (1966a,b) surveyed over 70 species for susceptibility to germicidal UV irradiation (2537 Å) and irradiation from an unfiltered xenon lamp designed to simulate the extraterrestrial solar spectrum including the UV-C portion of the spectrum. Generally, the most resistant species were conifers and succulents. Although no studies were made of the extinction coefficients by flavonoid compounds in the outer tissues or UV transmission through the epidermis, the authors attributed the resistance of conifers to several layers of subepidermal cells. *Opuntia* contained an extensive network of globular crystals in the epidermis which the authors suggested may be important in reflecting and absorbing much of the incoming radiation.

From available evidence, it appears that UV absorption in outer plant tissues does confer protection from UV-induced damage of plant tissues. Flavonoids and related compounds including the anthocyanin group are probably some of the most important components in the extinction of UV radiation in epidermal and subepidermal cells. Depending on the species, cuticular waxes may also be important in UV-B attenuation. Other compounds and structures may also contribute to the absorption of UV radiation in various plant species.

Since those plants and portions of plants which are most exposed to the solar irradiation in nature apparently have greater UV absorption capacities in the outer tissue layers as was discussed earlier, it might be expected that plants growing at higher elevations would have lower UV epidermal transmission values. Lautenschlager-Fleury (1955) did demonstrate a decrease in epidermal UV transmission with increasing elevation in the Alps from sea level to 2500 m. Similarly, Shibata and Kishida (1915) reported extensive measurements of flavonoid concentrations in plants from Japan and Switzerland. They found that the flavonoid content of alpine plant organs was as much as ten times greater than that of plants from lower elevations.

Measurements of UV epidermal transmission and the UV absorbance of flavonoid leaf extracts by Caldwell (1968) did not show a statistically significant difference between plants from different elevations above sea level. This contradiction could result from the fact that slightly different parameters were measured in these three studies.

With sudden changes in the irradiation regime, the UV epidermal transmission of plants may change quite suddenly. Caldwell (1968) investigated the UV absorbance in outer plant tissues for alpine plants emerging from spring snowbanks. As UV and total irradiation increased suddenly with the melting of the snowbank, there was a dramatic and

concomitant increase in the UV absorbance of the epidermis of leaf and stem tissues. Plastic filters which absorbed either total solar irradiation or merely the UV portion of solar irradiation were also shown to delay the formation of anthocyanin and other flavonoid pigments which are particularly responsible for UV absorption in the epidermis. Upon removal of these filters and exposure to direct insolation, the plants developed a capacity to absorb UV radiation in epidermal and other plant tissues within a half-day.

5.2 Reversal of UV Damage

Although absorption of UV irradiation in the outer tissues of plants is undoubtedly of great importance, the reversal of UV damage by various mechanisms is probably of equal significance in nature.

If the biological response to UV-B or UV-C irradiation has been decreased or completely reversed by a concomitant or subsequent irradiation (by nonionizing radiation), photoreactivation has taken place (Jagger and Stafford, 1962). Photoreactivating radiation must usually be of very high intensity and in the wavelength range of 3130 to 5490 Å (Jagger, 1958). Some of the principal photoproducts as a result of the irradiation of nucleic acids with UV-B and UV-C irradiation both *in vitro* and *in vivo* are pyrimidine polymers. The most prevalent type are cyclobutane dimers of thymine (Setlow, 1966). Normal photoreactivation has been shown rather clearly to be simply the cleavage or monomerization of these pyrimidine dimers. Photoreactivation, PR, has been discovered in a variety of microorganisms including viruses and mycoplasma, protozoa, algae, higher plants, reptiles, certain fish and amphibians, and even marsupials. Most of the demonstrated photoreactivable effects of UV have been due to inactivating UV irradiation of wavelengths outside the solar spectrum, e.g., 2537 Å. Nearly all of the detailed studies on the nature of PR have been conducted with simple biological systems such as bacteria. The importance of photoreactivation for higher plants growing in nature where UV damage would be due to wavelengths above 2900 Å has been scarcely investigated.

Photoreactivation has been amply demonstrated in higher plants in which the inactivating irradiation was primarily UV-C (Bawden and Kleczkowski, 1952; Benda, 1955; Tanada and Hendricks, 1953; Cline and Salisbury, 1966a,b; Cline *et al.*, 1969). Photoreactivation has also been demonstrated for higher plants when the UV inactivating radiation was composed of UV-B wavelengths normally within the solar spectrum (Caldwell, 1968). Although the nature of the photoproducts of UV irradiation are probably similar when the inactivating radiation is either UV-C or UV-B, the proportion of various types of photoproducts

may differ with the wavelength of the inactivating irradiation. If this is the case, the efficacy of photoreactivation might also be different as Jagger (1958) has reported from microorganisms. In his review on photoreactivation, Jagger reported that in microorganisms that many effects caused by inactivating UV irradiation between 2180 and 3130 Å have been shown to be photoreactivable. He suggests that in microorganisms, photoreactivation occurs to about the same extent for effects caused by UV between 2500 and 2900 Å, but outside this range photoreactivation of UV-B and UV-C responses is less effective. Therefore for organisms exposed to terrestrial solar UV irradiation above 2900 Å, photoreactivation might be expected to be less effective. However, this has never been tested for higher plants. Although it is mere speculation at this point, there may be a sound photochemical reason for a reduced effectiveness of PR for damage caused by wavelengths above 2900 Å. Although thymine dimers have been shown to be the major photoproduct of UV irradiation in all photoreactivable effects, other photoproducts of UV irradiation are also known to exist. Dimers of other pyrimidines can occur. Cytosine dimers and mixed dimers of thymine and cytosine as well as dimers containing uracil have been reported (Hanawalt, 1969). Recently a thymine trimer was described from *in vitro* irradiation experiments (Flippen *et al.*, 1970). Hydration products of cytosine, covalent cross-linking of DNA to protein, and other nucleic acid photoproducts have also been demonstrated (Hanawalt, 1969). As with pyrimidine dimers, presumably these other photoproducts result in incapacitation of the nucleic acids to perform their normal functions of directing metabolic processes in the cell including protein synthesis. Of the various photoproducts due to UV irradiation of nucleic acid, only the pyrimidine dimers have been demonstrated to undergo photoreactivation (Hanawalt, 1969). The newly discovered thymine trimer has also been shown to undergo photoreversal *in vitro* (Flippen *et al.*, 1970). The other photoproducts of nucleic acids as well as photochemical products of amino acid residues and proteins and their subsequent lesions are not generally considered as photoreactivable. Although thymine dimers are generally considered as the major photoproduct of UV-B and UV-C irradiation, the proportion of pyrimidine dimers to other photoproducts could be somewhat smaller as a result of UV-B irradiation by wavelengths within the solar spectrum as compared to that resulting from irradiation by 2537 Å or other UV-C wavelengths. In this case the efficacy of photoreactivation could indeed be expected to be smaller as Jagger (1958) has suggested for microorganisms.

The importance of photoreactivation for plants growing in a normal solar irradiation regime has been implied by Caldwell (1968). Leaves

of several alpine plants were exposed to 2967 and 3023 Å irradiation over a 9.5-hour period with intensities controlled to closely simulate biologically effective UV-B irradiation on a summer day at alpine elevations. During and following this UV irradiation, the leaves were not exposed to any other wavelengths. The UV epidermal transmission values were quite low and in the same range of values as would be expected in nature. A few of these leaves did exhibit severe lesions indicating UV damage which has never been observed in the field. The absence of such lesions in nature might suggest the importance of photoreactivation as a protective mechanism in alpine plants. When solar UV-B radiation intensities are high in nature, intensities of other wavelengths in the UV-A and visible portions of the solar spectrum are also high. Therefore concomitant and subsequent irradiation by these longer wavelengths could be responsible for photoreactivation in nature. In recent experiments by Cline *et al.* (1969), leaf discs of *Xanthium* were exposed to predominantly 2537 Å UV irradiation and the efficiency of simultaneous versus subsequent photoreactivating irradiation was compared. They found that while simultaneous photoreactivation was indeed effective, it was not nearly as efficient in reactivation of the UV damage as was a postirradiation treatment. They concluded for plants exposed to solar irradiation outside the earth's atmosphere, that concomitant photoreactivation would not be of great importance. This would probably bear little relation to plants growing on the earth's surface, however, since the UV-C irradiation doses used in these experiments were reasonably massive in a biologically effective irradiation context as compared with the biologically effective UV-B irradiation normally impinging on the earth's surface. Furthermore since the highest intensities of biologically effective UV-B radiation are limited to the middle hours of the day when the air mass is reasonably small, plants growing in nature would usually be exposed to a significant amount of visible and UV-A irradiation following the primary onslaught of UV-B irradiation during the middle hours of the day.

The importance of photoreactivation in nature has also been implicated by the experiments of Gurskii *et al.* (1961). Working in the field, they supplemented the normal solar UV regime in the high mountains of Russia with high intensities of artificial UV-B and UV-C irradiation which would normally be sufficient to cause severe photochemical damage. Plants growing under this supplemented irradiation situation showed no signs of tissue damage. This same absence of tissue destruction occurred when this experiment was carried out at a lower elevation in western Russia. They suggested that the ability of these plants to withstand these high intensities of supplemental UV-B and UV-C irradiation was probably due to photoreactivation.

In addition to photoreactivation other mechanisms of protection or repair of UV-induced damage may also occur in higher plants. Photo-protection is defined as protection of a biological system against UV irradiation damage by a previous exposure to radiation of longer wavelengths. This phenomenon requires very high intensities of radiation and is known to occur only in a few microorganisms. There is no evidence for the occurrence of this phenomenon in higher plants (Jagger, 1964; Jagger and Stafford, 1962). Evidence of mechanisms of dark repair of UV-induced damage has also been reported for various microorganisms through the use of mutant strains which lack the normal photoreactivating enzymes (Hanawalt, 1969). Although dark repair processes may also be quite important in higher plants, again there is no direct evidence of their existence.

In addition to the presence or absence of photoreactivating irradiation, other environmental conditions during or following UV irradiation have also been shown to be an important influence on the degree of biological response to UV irradiation. El-Mansy and Salisbury (1971) have shown that UV-C irradiation of higher plants is much less effective in producing damage at 10° as compared to 20° and 30°C. They reason that since the immediate photochemical reactions should be reasonably temperature-independent, it is the subsequent dark metabolic reactions that result in nucleic acid or protein alteration that are probably temperature-dependent. They demonstrated changes in the activity of several enzyme systems and pigments as a result of UV irradiation. In microorganisms there is evidence that the temperature of the biological system during UV irradiation can alter the nature of the immediate photoproducts. At lower temperatures, fewer pyrimidine dimers are formed and absorption of UV by proteins is implicated (Hanawalt, 1969). Environmental conditions following UV irradiation which would result in the inhibition or delay of DNA replication and therefore afford an opportunity for dark repair mechanisms to take place could also result in reduced UV damage to the biological system. This has been demonstrated for microorganisms when cyanide and other chemicals have been applied to inhibit metabolism following the UV irradiation (Giese, 1968). Mutant strains of *Escherichia coli* which lack the normal photoreactivating enzymes will exhibit a reduced damage from UV-C irradiation if this radiation treatment is followed by UV-A irradiation (Jagger *et al.*, 1969). Although dimers are not being monomerized in this mutant strain, the subsequent UV-A irradiation probably results in an inhibition of metabolic reactions preventing DNA replication and therefore allowing dark repair mechanisms to operate (Jagger *et al.*, 1969). This phenomenon is known as "indirect photoreactivation." Presumably low temperatures or other environmental stresses applied dur-

ing and following UV irradiation might likewise act to delay metabolism, prevent DNA replication, and allow dark repair to take place. As far as higher plants are concerned, this is all mere speculation at this point. Nevertheless the interaction of other factors of the environment with biologically effective UV-B irradiation must always be taken into consideration.

Although relatively few higher plants have been actually demonstrated as possessing the ability to photoreactivate UV-B damage, it would not be unreasonable to assume that most higher plants do, in fact, possess this potential. The apparent widespread occurrence of the ability to photoreactivate UV-C-induced damage in microorganisms and higher plants gives weight to the idea that most higher plants are probably able to photoreactivate damage caused by solar UV-B irradiation. The apparent universality of photoreactivation in biological systems lends credence to the importance of the photoreactivation phenomenon in nature. Although this photoreactivation ability may be a mere evolutionary accident or a holdover from periods when the earth did not possess its protective ozone layer, it is unlikely that the photoreactivation system which involves several specific enzymes would be retained in so many biological groups if it were not still of selective advantage.

In addition to photoreactivation in higher plants other metabolic mechanisms of repair or protection can not be neglected in future research.

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Chapter 5

PHOTOBIOLOGY OF PLANT VIRUSES

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1. Introduction

1.1 Nature of Virus Inactivation

Tobacco mosaic virus (TMV) has been investigated photobiologically and photochemically more than any other virus. Potato virus X (PVX) and tobacco necrosis virus (TNV) are the only other plant viruses that have been investigated in any detail as regards effects of UV radiation. Other plant viruses were used very little in photobiological work of any kind. Consequently, this review is concerned mainly with TMV, PVX, and TNV. For previous reviews, see Kleczkowski (1957, 1960) and McLaren and Shugar (1964). Infective free ribonucleic acids isolated from these viruses will be designated TMV-RNA, PVX-RNA, and TNV-RNA, respectively.

To appreciate the problems discussed below it is necessary to remember that a particle of each of these viruses consists of a single molecule of RNA surrounded by a protein coat composed of many molecules (sub-units) arranged in some orderly fashion. It is still an open question as

* Deceased.

to whether the protein molecules of any one virus are identical, or whether there are two or more different kinds of molecules differing from each other in some feature, e.g., in the way the polypeptide chain is folded. Table I shows some features of particles of the three viruses. The numbers given in the table have been roughly compiled from data available at the present moment.

Changes resulting from exposure to UV of a virus or of the nucleic acid isolated from a virus, are caused by radiation energy absorbed by them. By contrast, changes resulting from their exposure to visible light through the effect of a photodynamically active dye (such as, for example, acridine orange, proflavine, methylene blue, neural red, and so on) are caused by energy absorbed by molecules of the dye that have formed a complex with a virus particle or with the nucleic acid of the virus. The excited complex reacts with free oxygen and this results in loss of infectivity. Photodynamic inactivation can, therefore, be considered as dye-sensitized photooxidation of a substrate. When infectivity of a virus is inactivated photodynamically, there is the problem of penetration of a dye through the protein coat of the virus, the problem of interaction of the dye with the nucleic acid or with the protein of the virus and the problem of supply and diffusion of oxygen. As these problems do not exist when infectivity is inactivated by UV, photodynamic inactivation appears to be more complicated than inactivation by UV.

A basic fact about the inactivation by UV of infectivity of the three viruses shown in Table I, or of the nucleic acids isolated from them, is that it proceeds according to first-order kinetics at least approximately. This is the most documented fact observed also with many other viruses,

TABLE I
SOME FEATURES OF TMV, PVX, AND TNV VIRUSES^a

Virus par- ticle	Features				Mass of RNA molecules (daltons)	Mass of a protein molecule (daltons)	No. of protein molecules per par- ticle
	Shape	Mass (daltons)	Dimensions (nm) ^b	RNA content			
TMV	Rigid rod	40×10^6	300 (l) 15 (w)	5 %	2×10^6	18×10^3	2100
PVX	Flexible filament	35×10^6	520 (l) 10.5 (w)	6 %	2×10^6	22×10^3	1500
TNV	Sphere	8×10^6	27 (d)	19 %	1.5×10^6	33×10^3	200

^a All numbers are approximate. TMV is tobacco mosaic virus; PVX, potato virus; and TNV, tobacco necrosis virus.

^b l = length; w = width; d = diameter.

although there are exceptions. Photodynamic inactivation of the RNA isolated from a virus also follows first-order kinetics (Sastry and Gordon, 1966a; Singer and Fraenkel-Conrat, 1966; Ito *et al.*, 1967; Orlob, 1967), although the rate of inactivation may decrease after a few minutes of irradiation possibly because of decomposition of the dye (Singer and Fraenkel-Conrat, 1966). The kinetics of photodynamic inactivation of intact TMV is affected by the failure of the dye to sensitize all the virus particles (Sastry and Gordon, 1966a), but inactivation may, nevertheless, follow first order kinetics in its early stages (Oster and McLaren, 1950). Rates of inactivation of other viruses follow various survival curves, some corresponding to first order kinetics and some others deviating from it in various ways. The deviations can often be ascribed to such causes as a progressive increase in sensitization, decomposition of the sensitizing dye, depletion of oxygen, etc. (see Hiatt, 1967).

The fact that inactivation follows first order kinetics means that the proportion of the original infectivity (p) remaining after irradiation is

$$p = e^{-kD} \quad (1)$$

where D is the dose of supplied radiation energy and k is a constant whose magnitude depends on the units of measuring radiation energy and also on the conditions of irradiation. Thus k is a constant only in a given set of circumstances.

The fulfillment of Eq. (1) is usually interpreted to mean that inactivation of a virus particle is a "single-hit" process caused by a single quantum of absorbed energy. Many quanta are absorbed, but only an occasional one causes inactivation.

The probability that an absorbed quantum will inactivate is called the *quantum yield* for inactivation. For a more detailed treatment of this problem, see McLaren and Shugar (1964) or Kleczkowski (1968a). A quantum yield has to be qualified as it may refer to UV radiation energy absorbed by a whole virus particle or by its nucleic acid only. In photodynamic inactivation, i.e., when light energy is absorbed by a dye, a quantum yield may refer to energy absorbed by a complex formed by the dye with a virus particle or with a molecule of nucleic acid of the virus, or to energy absorbed by a molecule of the dye combined with the nucleic acid. Known quantum yields for inactivation of infectivity of plant viruses or of their free nucleic acids, are within rather wide limits of $\sim 5 \times 10^{-3}$ and 10^{-5} .

Another basic fact concerning inactivation of a virus by UV radiation is that the association between the nucleic acid and the protein in a virus may alter results of absorption of radiation of energy by the nucleic acid. In other words, when the nucleic acid is inside the virus, its response to

absorbed radiation energy may differ from that of free nucleic acid. This will be referred to as a *photochemical interaction* between the nucleic acid and the protein. Some changes may be prevented completely, e.g., formation of photoreversible pyrimidine dimers in the RNA or TMV is prevented when the RNA is inside the virus (Tao *et al.*, 1969; Carpenter and Kleczkowski, 1969), and the rate of others may be altered, e.g., in certain conditions the quantum yield for inactivation of the RNA or TMV when it is inside the virus is about 1/20th of that of the free RNA (Kleczkowski and McLaren, 1967). Thus photochemical interaction between the nucleic acid and the protein inside a virus may protect the nucleic acid from damage caused by UV radiation, or it may make it more sensitive. The result of the interaction depends on the species of the virus and may also depend on the wavelength of inactivating radiation and on some other conditions.

As visible light is not absorbed by nucleic acids, there can be no question of a similarly defined photochemical interaction between the nucleic acid and the protein during photodynamic inactivation of a virus. There may be an interaction in the sense of a modification of an effect of energy absorbed by a complex formed between the nucleic acid and a dye because the complex is inside a virus particle. This seems not to have been investigated, but it would not be easy, if at all possible, to overcome the difficulty of determining how much dye is actually complexed with the nucleic acid inside a virus particle. The nucleic acid can, however, be protected from photodynamic effects of light in some other ways, for example by impermeability or by poor permeability for dyes of the coat protein of a virus [good examples of this are the enteroviruses (Crowther and Melnick, 1961; Schaffer, 1962)].

As regards chemical aspects of inactivation or viral infectivity by irradiation, UV radiation appears to cause loss of infectivity by damaging pyrimidine residues of nucleic acids, purines being incomparably more resistant to destruction by UV than pyrimidines (see McLaren and Shugar, 1964). By contrast, photodynamic inactivation appears to result from damage to guanine residues (Singer and Fraenkel-Conrat, 1966; Sastry and Gordon, 1966b; Waskell *et al.*, 1966).

1.2 Repair of Radiation Damage

Some kinds of radiation damage in virus nucleic acid can be repaired by the host plant and the repair is usually referred to as reactivation. The form of repair of UV damage that relies on exposure of the infected host plant to visible light, which is called *photoreactivation*, has been much investigated (Bawden and Kleczkowski, 1953, 1955, 1959; Rushizky *et al.*, 1960; Merriam and Gordon, 1965; Small and Gordon, 1967;

Hidalgo-Salvatierra and McLaren, 1969; Evans and McLaren, 1969a,b; Tao *et al.*, 1969; Carpenter and Kleczkowski, 1969; Kleczkowski and Govier, 1969; Govier and Kleczkowski, 1970). It appears to be a phenomenon specific for damage caused in nucleic acids by UV radiation. It has been noted to occur neither with photodynamically inactivated plant viruses (or their free nucleic acids) nor with a photodynamically inactivated *coli* bacteriophage (Welsh and Adams, 1954).

Dark repair or dark reactivation, not requiring light, has been extensively investigated with UV-irradiated bacteria and bacteriophages, but on only three occasions with plant viruses (Werbin *et al.*, 1966; Kleczkowski, 1968b; Govier and Kleczkowski, 1969). Only with one system has some evidence been obtained, namely with UV-irradiated TNV inoculated on *Chenopodium amaranticolor* (Kleczkowski, 1968b). The kind of damage in this virus that was repaired by photoreactivation in other plants (such as tobacco or French bean), was apparently repaired in *Chenopodium* in darkness, although some uncertainty remains and the fact of dark repair of this virus cannot be considered established. Whether or not photodynamically inactivated plant viruses are susceptible to dark reactivation has not been investigated, but some positive evidence was obtained with a bacteriophage (Harm, 1968).

Multiplicity reactivation, i.e., regaining of infectivity by collaboration of several particles each of which is noninfective by itself, could not be demonstrated with UV-inactivated TMV, TNV, and tomato bushy stunt virus (Bawden and Kleczkowski, 1953), but was evidenced in UV-irradiated free RNA from TMV (Takahashi *et al.*, 1968). Whether or not photodynamically inactivated plant viruses are capable of multiplicity reactivation has not been investigated, but a *coli* bacteriophage, which is capable of it after UV inactivation, is not after photodynamic inactivation (Welsh and Adams, 1954).

In contrast to much work on effect of radiations on infectivity of viruses and of their free nucleic acids, very little has been done about their effects on virus protein. Some changes known to occur in the protein of UV-irradiated TMV are quite drastic, resulting in loss of antigenicity (Kleczkowski, 1962) and of the ability to conform to the structure of virus particles, eventually causing their disintegration (McLaren and Kleczkowski, 1967). The quantum yields for these changes are, however, so small that infectivity of the virus is lost long before they become appreciable. Photodynamic effects on the isolated proteins of TMV and of bromegrass mosaic virus are also known to be drastic, resulting in loss of antigenicity and of the ability to reconstitute the virus (Bell and Orlob, 1970), but there is no information concerning the relationship between the rates of these changes and of inactivation of infectivity.

All the above-mentioned effects of radiations on plant viruses or on their free nucleic acids, are reviewed and discussed in the following sections. As regards the effects of exposing the host plant to UV radiation on the virus inside the plant, and also on the plant itself, there have been no new developments in this subject since it was last reviewed (Kleczkowski, 1960).

2. Loss of Infectivity of Irradiated Virus: Causes

When virus protein plays an active part in the mechanism of infection, e.g., in the tail of a bacteriophage or, possibly a layer of protein molecules of vaccinia virus (see Joklik, 1964), there is a possibility that not only damage in virus nucleic acid, but also in virus protein can result in loss of infectivity. There is no indication that protein of any known plant virus plays an active part in the mechanism of infection, but damage in the protein could still result in inactivation of infectivity if it interfered with "uncoating" of the nucleic acid. That this may be so has been suggested to explain a much greater susceptibility of TMV to inactivation by irradiation at wavelengths around 230 nm than at longer wavelengths. At the shorter wavelengths a virus particle may be inactivated by a quantum of radiation energy absorbed either by its RNA or by its protein, whereas at longer wavelengths only energy absorbed by the RNA can inactivate (Siegel and Norman, 1958; McLaren and Moring-Claesson, 1961). The fact quoted in support of this suggestion is that the ratio of the amount of radiation energy absorbed by the protein to that absorbed by the nucleic acid increases rapidly as the wavelength decreases below 240 nm.

If energy absorbed by virus protein can indeed inactivate infectivity, there are two alternatives—(1) Energy absorbed by protein causes damage in the protein, and a virus particle with damaged protein may be noninfective even though its RNA may be undamaged. (2) Damage in virus protein is not relevant to inactivation of infectivity, but some energy absorbed by the protein may be transferred to the RNA, thereby inactivating infectivity.

Siegel and Norman (1958) were not explicit as regards the two alternatives, but McLaren and Moring-Claesson (1961) definitely had in mind alternative 1, the possibility which they considered as being: "The host plant can not extract active RNA from a coat of protein which has become denatured by UV light, and therefore the quantum yield is higher than would be expected if the RNA was equally available to the plant after irradiation at any wavelength."

Alternative (1) was disproved by Kleczkowski and McLaren (1967)

who showed that relative infectivity of nucleic acid isolated from TMV irradiated at 230 or at 280 nm was the same as that of a sample of the irradiated virus from which the nucleic acid had been isolated. Thus the removal of protein coat from the nucleic acid did not affect the relative infectivity, and so any damage caused by the radiation in the protein coat is irrelevant, irrespective of whether the wavelength is about 230 nm or longer. There is also evidence that UV-irradiated TMV is "uncoated" in the host plant at the same rate as the nonirradiated virus (Hayashi *et al.*, 1969). The conclusion is that loss of infectivity of the virus caused by UV irradiation is a result of damage only in the nucleic acid of the virus. So far this has been experimentally demonstrated with only one plant virus, namely TMV, but throughout this article this will be assumed to apply also to other plant viruses, such as TNV and PVX.

No comparable information is available concerning photodynamic inactivation of infectivity of plant viruses, but that of poliovirus has been shown to result from damage only in its RNA (Wilson and Cooper, 1965). By contrast, photodynamic inactivation of a poxvirus is probably caused mainly by damage in its protein which is believed to function actively in the mechanism of infection as the so called "viral inducer protein" (Joklik, 1964; Turner and Kaplan, 1966).

Alternative 2 has no experimental support at the moment, but it is a possibility. The fact that when TMV is irradiated with UV, the fluorescence of its protein is neither increased nor quenched by its RNA (Shore and Pardee, 1965), shows there is no direct transfer of singlet state excitation energy between the RNA and the protein. This, however, does not exclude the possibility of a transfer of energy in another form.

3. Inactivation of Free RNA

Nucleic acids isolated from only a few plant viruses have been investigated photobiologically, and the investigations have not been extensive. Any generalizations are therefore premature, especially because the problem is complicated by the fact that quantum yields for inactivation vary with environmental conditions as, for example, salt concentration in the medium. Comparisons between the behaviors of different RNAs can therefore be made properly only when based on results obtained in identical conditions, and such results do not yet exist. Table II shows quantum yields for UV inactivation of infectivity at $\lambda = 254$ nm (without photoreactivation) of RNAs isolated from TMV, PVX, and TNV. The quantum yield for each RNA increased as the concentration of a buffer decreased, although a great difference in the concentration resulted

TABLE II
QUANTUM YIELDS ($\times 10^3$) FOR INACTIVATION (WITHOUT PHOTOREACTIVATION) OF
INFECTIVITY OF FREE RNAs^a

Source of RNA	Phosphate buffer pH 7.0 molarity				Borate buffer pH 7.5 molarity	
	0.1	0.07	0.001	0	0.1	0.01
TMV ^b	1.2	—	2.3	3.2	—	—
PVX ^c	—	—	—	—	2.0	3.0
TNV ^d	—	0.7	—	—	—	—

^a From three viruses at $\lambda = 254$ nm in buffers of different concentrations.

^b Evans and McLaren (1969a).

^c Govier and Kleczkowski (1970).

^d Kassanis and Kleczkowski (1965).

in a relatively small difference in the quantum yield. PVX-RNA was more susceptible to inactivation by UV than TMV-RNA (higher quantum yield) when irradiated in buffers of the same molarity, but the buffers were different, namely borate and phosphate. Electric conductivity of the borate buffer is much smaller than that of the phosphate buffer of the same molarity, and comparisons between the quantum yields and conductivities suggest that similar quantum yields would be obtained with both RNAs if they were irradiated in the different buffers but at concentrations at which they have the same conductivity. It is possible, therefore, that both RNAs are intrinsically similar as regards their susceptibility to inactivation by UV, and that quantum yields for inactivation in identical conditions may be similar, but this still remains to be ascertained experimentally. TNV-RNA seems to be intrinsically somewhat more resistant than the other two RNAs.

There is at present no information about any effects of environmental conditions on photodynamic inactivation of free nucleic acids of plant viruses, but only indications that both the rate and the presence or absence of photodynamic inactivation (with neutral red) of free RNA of poliovirus depend on environmental conditions such as salt concentration in the medium (Wilson and Cooper, 1965).

Changes in salt concentration may affect quantum yields for inactivation of infectivity of the free nucleic acid of a virus (by UV or by visible light through photodynamic effect of a dye) by affecting physical structure of the nucleic acid, some kinds of lethal changes being prevented by some kinds of structure. There is, for example, evidence that salts can affect the secondary structure of RNA based on formation of double helical regions (Doty *et al.*, 1959; McMullen *et al.*, 1967). Photodynamic

effect of a dye can also be affected by changes in salt concentration and/or in the pH of the medium, because the ability of the nucleic acid to complex with the dye may be affected.

Quantum yields for inactivation of free nucleic acids of viruses by UV can also vary with the wavelength of inactivating radiation. Figure 1 shows such a variation when PVX-RNA is irradiated in 0.01 M borate buffer at pH 7.5. A variation in the wavelength between 230 and 280 nm was not found to have an appreciable effect on the quantum yield for inactivation of TMV-RNA when irradiated in water (Rushizky *et al.*, 1960) or in 0.017 phosphate buffer at pH 7.0 (Kleczkowski, 1963), or of TNV-RNA in 0.067 M phosphate buffer at pH 7.0 (Kassanis and Kleczkowski, 1965). There may be interactions between the wavelength of the inactivating radiation and salt concentration and/or the pH of the medium, but this has not been investigated.

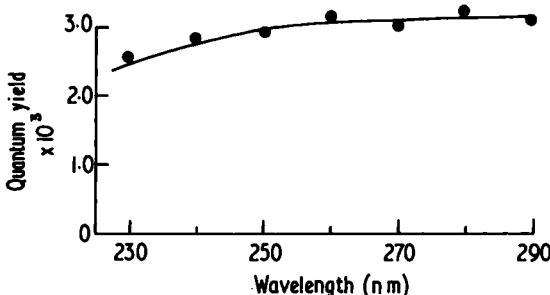


FIG. 1. Dependence of quantum yields for UV inactivation of infectivity of PVX-RNA (obtained without photoreactivation) on the wavelength of inactivating radiation (according to Govier and Kleczkowski, 1970).

So far we have considered inactivation of free RNAs in nonphotoreactivating conditions. Whether this means inactivation due to total damage by UV, or to damage that remains unrepaired by a process that operates irrespective of exposure of a test plant to light, i.e., by "dark reactivation," still remains unknown. By contrast, the repair of irradiated free RNAs by photoreactivation has been well established and investigated in some detail, as discussed below. All possible kinds of damage can thus be divided at least into two kinds, those that can, and those that cannot, be repaired by photoreactivation.

Quantum yields for photodynamic inactivation by monochromatic visible light of TMV-RNA sensitized with acridine orange were obtained by Ito *et al.* (1967) at wavelengths between 40° and 520 nm. The average value of the quantum yield, with respect to energy absorbed by the complex formed between the dye and the molecules of the RNA, was

2.2×10^{-4} . The quantum yield varied somewhat from one wavelength to another, the extreme values being 2.8×10^{-4} and 1.8×10^{-4} , but there was no consistent trend.

These results were obtained by irradiating a mixture of TMV-RNA and the dye in 0.6 M NaCl buffered in 0.05 M phosphate buffer at pH 6.7. As the quantum yield for inactivation of an RNA by UV radiation depends on the environmental conditions, such as salt concentration in the medium, it is possible that the quantum yield for photodynamic inactivation also depends on such conditions. Thus any comparison between quantum yields applies only to specified sets of conditions. Comparisons between shapes and positions of action spectra may, however, depend much less on the conditions in which they have been obtained, mainly because each can be plotted on an arbitrary scale, provided that each particular action spectrum was obtained in conditions that were constant and otherwise suitable for obtaining an action spectrum for a material that is so diluted that it is almost transparent to the radiation (see Kleczkowski, 1970).

Figure 2 shows action spectra for inactivation by UV (without photo-reactivation) of RNAs from different viruses and by visible light of

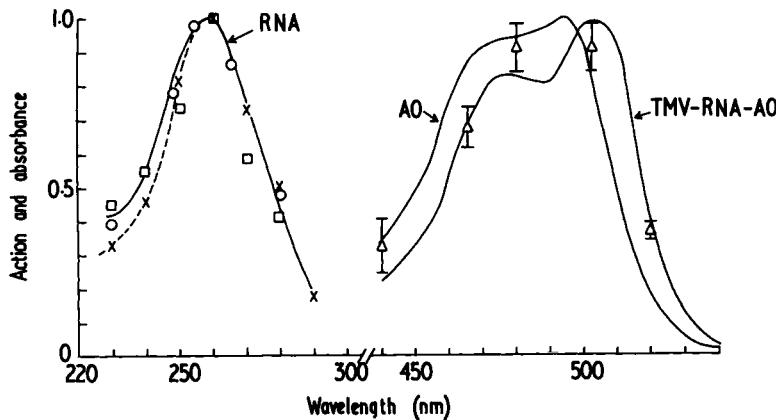


FIG. 2. Points of action spectra for UV-inactivation of free nucleic acids of TMV (○) (according to McLaren and Moring-Claesson, 1961), of TNV (□) (according to Kassanis and Kleczkowski, 1965) and PVX (X) (according to Govier and Kleczkowski, 1971), and for photodynamic inactivation of free nucleic acid of TMV with acridine orange (Δ) (according to Ito *et al.*, 1967). The lengths of the perpendicular lines show the standard errors of the corresponding estimates. The curves marked "RNA," "AO," and "TMV-RNA-AO" are absorption spectra of the free RNA of PVX, of acridine orange and of the complex between the nucleic acid and acridine orange, respectively. Ordinates of the points and the curves are in arbitrary units such that the maximal points correspond to the value of 1.0.

TMV-RNA sensitized with acridine orange. The points of action spectra are compared with the curves of absorption spectra of PVX-RNA (RNA), of acridine orange (AO) and of the complex formed between TMV-RNA and the dye (TMV-RNA-AO). (Absorption spectra of PVX-RNA, TMV-RNA, and of TNV-RNA are so similar that it does not matter which is used for the purpose of comparison.) The points of action spectra (action) and absorption spectra (absorbance) are all plotted in arbitrary units. Action spectrum was obtained by plotting values proportional to the values of k of Eq. (1) obtained at different wavelengths, against the wavelengths.

A deviation of an action spectrum for inactivation of infectivity from parallelism with the absorption spectrum of a material can be interpreted as a result of a variation (from one wavelength to another) of the quantum yield of energy absorbed by the material. The points of action spectra for inactivation by UV of TMV-RNA and of TNV-RNA follow quite closely the UV absorption spectrum of the RNA, whereas those for inactivation of PVX-RNA deviate at wavelengths below 250 nm. However, the deviations are relatively small, and we can conclude that the action spectra for inactivation by UV of free RNAs of the three viruses are roughly parallel to the UV absorption spectrum of the RNA.

The action spectrum for the photodynamic inactivation of TMV-RNA is roughly parallel to the absorption spectrum of the dye (AO) or of the complex formed between the RNA and the dye (TMV-RNA-AO), but the rather large standard errors make it difficult to decide which of the two absorption spectra is approximated nearer.

4. Inactivation of Whole Viruses

4.1 Inactivation by UV

As discussed in Chapter 1, inactivation by UV of infectivity of a whole plant virus, such as TMV, TNV, and PVX, can be considered to be a result of damage in its RNA. Therefore if inactivation of infectivity of a whole virus, with respect to energy absorbed by its RNA, proceeds in any way differently from that of its free RNA, the difference is a result of some kind of photochemical interaction between the RNA and the protein of the virus. For example, when intact or reconstituted TMV is irradiated by UV, a kind of damage in the RNA that can be repaired by photoreactivation, does not occur at all, although it does when free TMA-RNA is irradiated (Bawden and Kleczkowski, 1959; Rushizky *et al.*, 1960). Thus the interaction between the RNA and the protein in TMV results in a complete protection of the RNA from photoreactivable kind of damage, so that only nonphotoreactivable kind of damage can

occur. Another expression of the interaction is an alteration in the quantum yield for inactivation, as will be discussed in the following, the phenomenon of photoreactivation being dealt with specifically in Section 5.

A feature of the interaction is the extent to which it varies from one virus to another, from having no demonstrable effect in TNV (Kassanis and Kleczkowski, 1965), through causing a relatively slight protection, i.e., a relatively small decrease in the quantum yield for inactivation, in PVX (Govier and Kleczkowski, 1970) to causing a large extent of protection in TMV (Kleczkowski and McLaren, 1967).

It is convenient to have a quantitative estimate of the degree of protection from UV, and we shall use for this purpose the formula

$$R = 1 - \Phi_v/\Phi_N \quad (2)$$

where Φ_v and Φ_N are quantum yields for inactivation of infectivity of a viral RNA when it is inside the virus and when free, respectively. Thus the value of R is 0 when there is no protection, and 1 when protection is complete.

Effects of the interaction can depend on the wavelength of inactivating radiation and on the environmental conditions such as, for example, salt concentration in the medium. The dependence on the wavelength of the interaction in TMV is shown in Table III. At the wavelength of 280 nm the interaction results in a considerable protection of the RNA, the quantum yield for its inactivation inside the virus being only about 5% of that of the free RNA, so that the value of R is about 0.95. The protection disappears completely as the wavelength decreases from 254 to 230 nm when the quantum yield for inactivation of the RNA inside the virus becomes about the same as that of the free RNA without

TABLE III
DEPENDENCE OF PHOTOCHEMICAL INTERACTION BETWEEN RNA AND THE PROTEIN
IN TMV ON THE WAVELENGTH OF INACTIVATING RADIATION^a

Irradiated material	Wavelength of inactivating radiation (nm)	Quantum yield for inactivation of the RNA ($\times 10^3$)
RNA in TMV	280	0.05
	254	0.10
	230	1.1
Free TMV-RNA	230-280	1.0

^a Computed from results obtained by Kleczkowski and McLaren (1967) and Bawden and Kleczkowski (1959) for materials irradiated in 0.067 M phosphate buffer at pH 7.0. The numbers are only approximate. The quantum yield for inactivation of the free RNA was obtained without photoreactivation.

photoreactivation. As the latter is the sum of the quantum yields for inactivation due to photoreactivable and nonphotoreactivable kind of damage, whereas only nonphotoreactivable kind of damage occurs in the RNA that is inside the virus, the quantum yield for inactivation due to the nonphotoreactivable kind of damage at the wavelength of 230 nm seems to be even greater in the RNA inside the virus than in the free RNA.

The dependence of the degree of protection on the wavelength of inactivating radiation was investigated in greater detail with PVX, and the results are shown in Fig. 3, separately for photoreactivable and for nonphotoreactivable kind of damage (curves PR and NPR, respectively). It will be seen that in this virus the RNA is protected, though only partially, from both kinds of damage and that, although the degree of protection varies with the wavelength, it is present at all wavelengths from 230 to 280 nm. The ratio of the degree of protection against photoreactivable damage to that against nonphotoreactivable damage also varies with the wavelength of inactivating radiation.

The results of irradiation of the RNA of TNV were identical irrespective of whether it was inside the virus or free, and they did not depend on the wavelength to any detectable extent (Kassanis and Kleczkowski, 1965), so that there is no evidence for any photochemical interaction between the RNA and the protein in this virus.

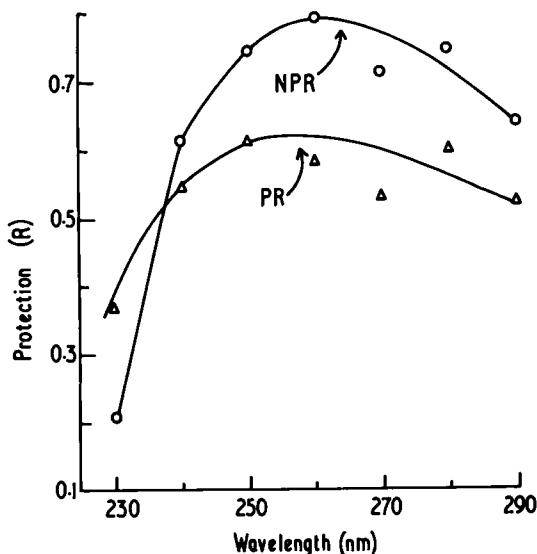


FIG. 3. Part-protection (R) of the RNA inside PVX by the virus protein against photoreactivable (PR) and nonphotoreactivable (NPR) kind of damage.

The extent, or even the presence or absence, of an interaction between the nucleic acid and the protein in a virus may depend on environmental conditions, such as, for example, salt concentration or the pH of the medium. Table II shows that the quantum yield for inactivation of free PVX-RNA at $\lambda = 254$ nm in 0.1 M borate buffer differs from that in 0.01 M borate buffer. By contrast, the quantum yield for inactivation of the RNA inside the virus is the same at both molarities (about 10^{-3}). Consequently, the degree of protection of the RNA by the protein in 0.1 M buffer differs from that in 0.01 M buffer [the values of R being 0.45 and 0.65, respectively (Govier and Kleczkowski, 1970)].

Variations from one virus to another in the dependence on the wavelength of the photochemical interaction between the nucleic acid and the protein, reflect in differences between the action spectra for their inactivation. The action spectra to be considered presently are those that apply to irradiation of dilute materials that are nearly transparent to UV (see Kleczkowski, 1968a, 1970). As in TNV there is no evidence for any interaction at any wavelength, the degree of protection (R) being 0 at all the wavelengths tested, the action spectrum for inactivation of the virus is the same as that for inactivation of the RNA isolated from the virus (see Fig. 2), both being closely parallel to the absorption spectrum of the RNA (Kassanis and Kleczkowski, 1965).

The action spectrum for inactivation of PVX, shown in Fig. 4, deviates quite distinctly from parallelism with the absorption spectrum of the RNA. Although the action spectrum for inactivation of the free RNA

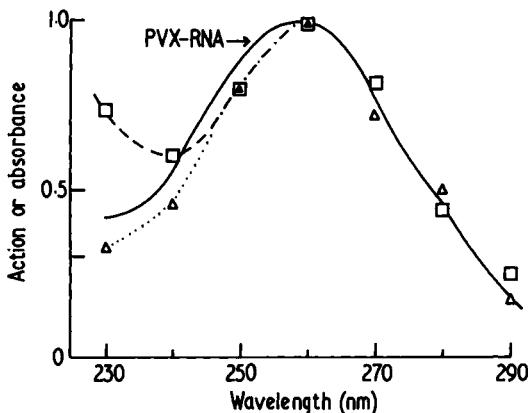


FIG. 4. Points of action spectra for UV inactivation of PVX (□) (according to Kleczkowski and Govier, 1969) and of PVX-RNA (△) (according to Govier and Kleczkowski, 1971). The continuous curve is an absorption spectrum of PVX-RNA. Ordinates of the points and of the curve are in arbitrary units such that the maximal points correspond to the value of 1.0.

of the virus also deviates slightly from parallelism with the absorption spectrum of the RNA, it deviates in the opposite direction. Thus the action spectra for inactivation of PVX and of free PVX-RNA are not parallel to each other, although their shapes do resemble each other, as they also resemble the shape of the absorption spectrum of the RNA.

By contrast, the action spectrum for inactivation of TMV, shown in Fig. 5, diverges so far from the absorption spectrum of the RNA and, consequently, from the action spectrum for inactivation of free TMV-RNA, that there is no similarity between them. This is a result of a great variation from one wavelength to another in the degree of photochemical interaction between the RNA and the protein of the virus.

To attain a graphic illustration of a photochemical interaction between the nucleic acid and the protein of a virus, a comparison should be made between the action spectra for inactivation of the virus and of the free nucleic acid of the virus. Usually, however, the purpose of obtaining an action spectrum is identification of the absorber of the radiation energy that causes inactivation, and this is done by comparing the action spectrum with the absorption spectrum of the substance that may be expected to be the absorber, i.e., with the absorption spectrum of the nucleic acid of the virus. Parallelism between the action spectrum and the absorption spectrum will be expected if the quantum yield for inactivation is independent of the wavelength and if there is no wavelength-dependent photochemical interaction between the substance that

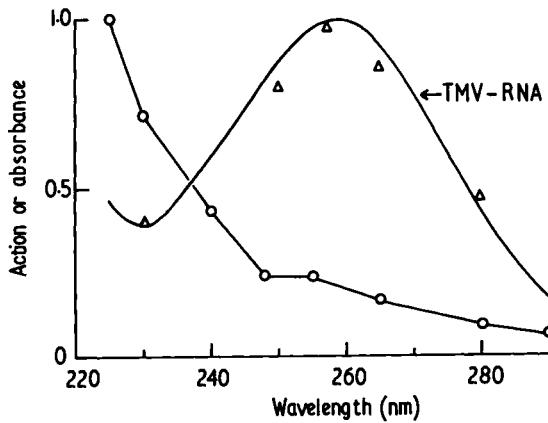


FIG. 5. Points of action spectra for UV inactivation of TMV (○) (according to Hollaender and Duggar, 1936) and of TMV-RNA (△) (according to McLaren and Moring-Claesson, 1961). The curve marked TMV-RNA is an absorption spectrum of TMV-RNA. Ordinates of the points and of the curve are in arbitrary units such that the maximal points correspond to the value of 1.0.

is directly involved in the loss of infectivity (i.e., the nucleic acid) and some other substance, i.e., the virus protein.

When making a comparison between the action spectrum for inactivation of a virus and the absorption spectrum of the nucleic acid of the virus, the problem may arise whether or not the combination between the nucleic acid and the protein of the virus affects the absorption of radiation energy by either of them. There is evidence that the free TMV-RNA may be so folded that short double helical regions may exist, caused by hydrogen bonding between pairs of corresponding bases (Doty *et al.*, 1959; McMullen *et al.*, 1967). This would imply that there may be hypochromicity in free TMV-RNA, but not in the RNA inside the virus. However, any difference between UV absorption by free components of TMV and by the same components when combined to form the virus, cannot be appreciable because absorption by the free RNA and free protein of the virus, taken in the proportions in which they occur in the virus, and the amount of scatter that would be expected to occur, add up approximately to the absorption by the whole virus at any particular wavelength, so that the absorption spectrum of the virus is approximately reproduced (McLaren and Takahashi, 1959).

The nature of what is here called the photochemical interaction between the nucleic acid and the protein in a virus is still unknown. The explanation based on the assumption of an alteration of the absorption coefficient of the nucleic acid resulting from the combination with the virus protein can be excluded. To explain a decrease in susceptibility of the nucleic acid when it is inside the virus to inactivation by UV, a decrease in its absorption coefficient would have to be assumed, whereas, as discussed above, the absorption coefficient of the nucleic acid which is inside the virus, if at all different appreciably from that of the free nucleic acid, can only be greater.

Three possible explanations of the interaction have been suggested: (1) When the nucleic acid is inside the virus a physical structure is imposed on it that precludes some photochemical changes. (2) A reaction between parts of the nucleic acid is prevented by a steric hindrance resulting from combination with the protein of the virus. (3) There is a transfer of energy between the nucleic acid and the protein. These possibilities were discussed by Govier and Kleczkowski (1970).

Whatever the mechanism of the interaction, one of its features appears to be its dependence on some properties of the protein. For example, "hybrid" viruses obtained by cross-reconstitution from the RNA of one and the protein of another strain of TMV were found to be identical in susceptibility to inactivation by UV with the original strain that supplied the protein coat (Streeter and Gordon, 1967). A "hybrid" virus composed

of the RNA from PVX and the protein from TMV was much closer to TMV than to PVX in its susceptibility to inactivation by UV (Breck and Gordon, 1970).

4.2 Photodynamic Inactivation

Photodynamic inactivation of only three plant viruses and of their free nucleic acids has so far been studied, namely of TMV, alfalfa mosaic and cucumber mosaic viruses (Oster and McLaren, 1950; Sastry and Gordon, 1966a; Orlob, 1967). All these viruses are much less susceptible to photodynamic inactivation than their free nucleic acids in the sense that their inactivation proceeded at a slower rate and at least in some virus-dye systems, e.g., that of TMV and acridine orange, only a proportion of infectivity of a virus may be susceptible to inactivation, whereas free RNA of the virus can be inactivated completely (Sastry and Gordon, 1966a). Nevertheless, the rate of inactivation of the viruses follows the first-order kinetics at least in early stages.

Thus the nucleic acids of the viruses are protected by their proteins from photodynamic inactivation. A numerical assessment of the degree of protection may be more complicated than that against inactivation by UV. When inactivation of a virus proceeds at first and then stops, an assessment can be made simply by stating the proportion of the virus that failed to become sensitized by a given dye in a given set of conditions. However, when inactivation follows first order kinetics at least initially, and when irradiated preparations transmit most of incident light (say, about 90% or more), another kind of assessment is given by the formula

$$F = 1 - (\log p_v)/(\log p_N) \quad (3)$$

when p_v and p_N are the proportions of the original infectivities of the virus and of the free nucleic acid of the virus, respectively, that remain after identical exposure to visible light in the same environmental conditions. The value of $F = 1$ means complete protection and $F = 0$ means no protection.

Three not mutually exclusive explanations of the protective effect are possible—(1) The dye cannot easily penetrate through the protein coat of the virus; (2) combination between the nucleic acid and the protein of the virus prevents complexing between the nucleic acid and the dye; or (3) when the nucleic acid is inside the virus, a configuration is imposed on it that prevents a reaction with the dye, or precludes a certain kind of damage.

Possibility 1 is supported by the following consideration. Viruses are known to differ as widely in their susceptibility to photodynamic

inactivation with any one photodynamically active dye, as different dyes differ in their efficiency to photosensitize the same virus (Hiatt, 1960, 1967). Poliovirus, which is completely resistant to photodynamic inactivation at pH values of about 7 or less, becomes susceptible when grown in a cell culture to which a photodynamically active dye, such as neutral red or proflavine, has been added. The dye is then incorporated into the structure of virus particles which can then be inactivated by exposure to visible light (Crowther and Melnick, 1961; Schaffer, 1962).

Photodynamic effect of dyes may also depend on the pH of the medium. For example, acridine orange or toluidine blue have almost no photodynamic effect on TMV at pH values below 7, but the effect appears when the pH is increased, the optimal being about 9 (Orlob, 1967). Wallis and Melnick (1963) showed previously that poliovirus, which had been considered completely resistant to photodynamic inactivation, can be made susceptible when it is specially purified so that it is freed from extraneous organic materials, and then treated with a dye and exposed to light in an alkaline medium. Optimal pH for neutral red seems to be about 8. Organic buffers (tris or glycine) prevent photosensitization. The dependence of photodynamic inactivation of viruses on the pH of the medium does not necessarily support possibility 1, but the dyes may penetrate protein coats of some viruses better at some pH values than at others, and this may affect photodynamic effects of dyes. Similar reasoning can also be applied to salt concentration in the medium, which has been shown to affect photodynamic inactivation of TMV (Orlob, 1967).

The rate of photodynamic inactivation of infectivity of viruses or of their free nucleic acids depends on the concentration of the dye in the medium, and when the concentration exceeds a certain limit, infectivity is inactivated at an appreciable rate even in darkness (Chessin, 1960; Orlob, 1967). Infectivity of some virus preparations can also be inactivated by visible light without any added dye. This was noticed in preparations of cucumber mosaic virus (Orlob, 1967) and also in those of some animal viruses such as measles virus and Semliki Forest virus (Cutchins and Dayhuff, 1962; Appleyard, 1967). As the rate of inactivation appears to depend on the presence and concentration of free oxygen in the medium, it seems probable that inactivation is a result of photodynamic effect of some naturally occurring pigment.

5. Photoreactivation

When the infectivity that remains in a preparation of a plant virus (or of the nucleic acid isolated from it) after an exposure to UV radiation is assayed by the local lesion method, it may be found greater when

the assay plants are exposed to visible light *after* inoculation with the irradiated preparation than when they are kept for some time in darkness (Bawden and Kleczkowski, 1953, 1955, 1959; Rushizky *et al.*, 1960; Kassanis and Kleczkowski, 1965; Govier and Kleczkowski, 1970). The phenomenon is called *photoreactivation*. Exposure of a virus preparation to visible light before or after UV irradiation, but before inoculating it to an assay plant, or of the assay plant before inoculating it with the irradiated preparation, do not increase the remaining infectivity of the preparation. Thus no plant virus yet tested has shown anything comparable to the phenomenon called *photoprotection*, which is a partial protection of an organism from lethal effects of UV radiation by exposing the organism to visible light *before* subjecting it to UV radiation (Jagger, 1960).

Photoreaction was found to occur with most plant viruses that had been tested (Bawden and Kleczkowski, 1955; Cadman and Harrison, 1959). When it was found not to occur with a virus (such as TMV or tobacco rattle virus), it was found to occur with the RNA isolated from the virus, provided the RNA was first isolated and then irradiated (Bawden and Kleczkowski, 1959; Harrison and Nixon, 1959). In other words, UV-irradiated nucleic acids of all plant viruses so far tested showed the phenomenon of photoreactivation, some irrespective or whether inside the virus or free, and some only when free.

Photoreactivation of UV-irradiated plant viruses follows the *dose reduction principle* which was formulated by Kelner (1949) for photoreactivation of UV-irradiated microorganisms. This means that photoreactivation is equivalent to a decrease of the dose of irradiation by a *dose reduction factor* (f) whose value is between 0 and 1.0 and is constant for a given system in a given set of circumstances. Thus when the fraction of surviving infectivity of an irradiated virus preparation in the absence of photoreactivation is $p_d = \exp(-kD)$ [see Eq. (1)], in the presence of photoreactivation it is $p_i = \exp(-fkD)$, when f is the dose reduction factor. A convenient measure of the extent of photoreactivation is the *photoreactivable sector*

$$S = 1 - f \quad (4)$$

When $S = 0$ there is no photoreactivation, and when $S = 1.0$, photoreactivation is complete, i.e., all the infectivity lost as a result of UV irradiation is restored by photoreactivation. The value of S is therefore the proportion of lethal lesions caused in virus nucleic acid by UV irradiation that are photoreactivated in a given set of conditions, and f is the proportion of those that are not. The extent of photoreactivation is sometimes given as "percent of photoreactivation," and this equals $100S$.

Kinetics of photoreactivation of UV-inactivated plant viruses has

not received much attention, but that of a *coli* bacteriophage has been studied in detail (Bowen, 1953). The progress of photoreactivation was found to depend on two separate reactions, only one of which is caused by photoreactivating light (light reaction), whereas the other can occur in darkness (dark reaction), both being first-order reactions. The former is independent of temperature, whereas the latter increased with the increasing temperature between 0° and 45°C. Bowen suggests that the dark reaction may be to provide the light absorbing pigment that is used in the light reaction.

There is evidence that nucleic acid of an UV-irradiated virus must be "uncoated" in the host plant before it can be photoreactivated, and if it is then not photoreactivated within a relatively short time, it is decomposed. This can be concluded from two facts—(1) A period of about 30 min after inoculation is needed for most UV-inactivated (at $\lambda = 254$ nm) particles of PVX that are capable of photoreactivation, to become ready for photoreactivation. (This does not require light.) Then about a 15-min exposure of the host plant to daylight is sufficient to photoreactivate most of them, but if the plant is kept for another $\frac{1}{2}$ –1 hr in darkness, there is almost no photoreactivation (Bawden and Kleczkowski, 1955). (2) UV-irradiated (at $\lambda = 254$ nm) free RNA of TMV is ready for photoreactivation immediately after inoculation to the host plant, and ceases to be capable of photoreactivation if the inoculated plant is kept in darkness for $\frac{1}{2}$ –1 hr (Bawden and Kleczkowski, 1960).

Photoreactivation of DNA is based on photoenzymatic repair of damage caused by UV radiation, and not on any "by-pass" mechanism. This was shown by demonstrating that photoreactivation of UV-irradiated transforming DNA can be obtained *in vitro* by exposure to visible light in the presence of a photoreactivating enzyme (Rupert, 1960). Until photoreactivation of a UV-irradiated plant virus, or the RNA isolated from it, can also be achieved *in vitro*, evidence that it is also a result of photoenzymatic repair of damage in its RNA can only be based on analogy. Evidence is strengthened by the fact that the range of wavelengths of light that causes photoreactivation of the transforming DNA *in vitro* is between 300 and 500 nm (J. K. Setlow and Boling, 1963), which is the same as that for photoreactivation of PVX and of TMV-RNA inside the host plant (Chessin, 1958; Hidalgo-Salvatierra and McLaren, 1969). This seems to be the range of wavelengths of photoreactivating light generally. Photoreactivation of UV-irradiated DNA bacteriophages, and of lethal effect of UV on microorganisms and on cells of higher plants, is also caused by light of wavelengths between 300 and 500 nm (Dulbecco, 1950; Kelner, 1951; Jagger and Latarjet, 1956;

Chessin, 1958). The similarity between the damaging and restoring agents is therefore quite striking. A photon carrying somewhat more than 4 eV of energy causes damage, and that carrying somewhat less causes photoreactivation.

There is direct evidence that photoreactivation of plant viruses is not a result of photosynthetic activity of the host plant. This can be concluded from two facts. (1) Photoreactivation does not occur when the plant inoculated with UV-irradiated PVX or TMV-RNA is exposed to light of wavelengths longer than 500 nm (Chessin, 1958; Hidalgo-Salvaterra and McLaren, 1969), whereas photosynthesis does; (2) photoreactivation occurs in white leaves of a variegated variety of *Xanthi* tobacco, whereas photosynthesis does not (McLaren *et al.*, 1970).

The extent of photoreactivation, i.e., the magnitude of S of Eq. (4), may depend on conditions of the medium in which a material is irradiated and/or on the wavelength of the radiation. Only TMV-RNA has yet been used so far to test for a dependence of the value of S on the ionic strength of the medium, and the value was found to increase from 0.23 to 0.38 as the molarity of phosphate buffer (pH 7.0) changed from 0.001 to 0.1 (Evans and McLaren, 1969a).

Dependence of the value of S for photoreactivation of some UV-irradiated plant viruses and/or of free RNAs isolated from them, on the wavelength of inactivating radiation is shown in Fig. 6. The values of

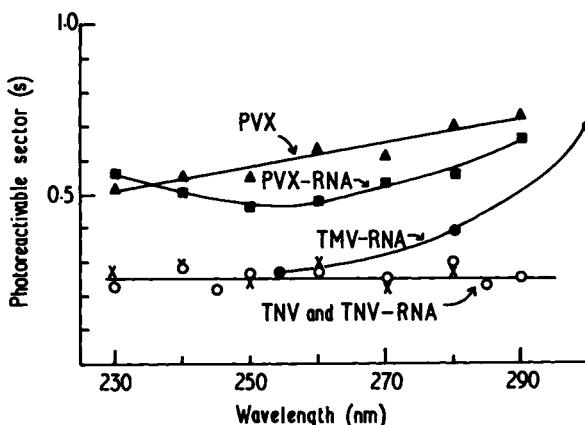


FIG. 6. Dependence of photoreactivable sectors on the wavelength of inactivating radiation. The sectors of PVX and of PVX-RNA were obtained by irradiation in 0.01 M borate buffer at pH 7.5 (Kleczkowski and Govier, 1969; Govier and Kleczkowski, 1971), those of TMV-RNA in water (Merriam and Gordon, 1965), and those of TNV (○) and of TNV-RNA (X) in 0.067 M phosphate buffer at pH 7.0 (Kassanis and Kleczkowski, 1965).

S for PVX, PVX-RNA and TMV-RNA do vary with the wavelength, whereas those for TNV and TNV-RNA do not. It should, however, be taken into account that, as the ionic strength of the media in which the different materials were irradiated varied from one material to another, some of differences between the lines shown in Fig. 6 (especially in the magnitude of the ordinates) may have resulted from differences in the ionic strength or in the quality of salt in the medium.

Merriam and Gordon (1965) found that there are at least two kinds of photoreactivable lesions in the UV-irradiated TMV-RNA. One kind is formed only when the wavelength is increased in the range from about 280 toward 300 nm, whereas the other is formed at all wavelengths between 254 and 300 nm. (The former can be photoreactivated until about 5 hr after inoculation of UV-irradiated TMV-RNA, whereas the latter in less than 3 hr.) The fact that the value of S for TNV or TNV-RNA appears constant within the whole range of tested wavelengths, whereas those of the other materials increased as the wavelength increased toward 300 nm, may be explained by assuming that in the RNA of TNV only lesions of the second kind are formed.

Also illustrated in Fig. 6 is the fact that whereas the value of S for TNV is the same as that for TNV-RNA, those for PVX differ from those for PVX-RNA. This can be explained by assuming that the RNA and the protein in PVX are so combined that there is an interaction between them which affect the value of S , whereas there is no such interaction in TNV. This interaction in TMV goes further than in PVX, resulting in the value of S being 0.

At wavelengths longer than 240 nm the values of S for PVX are greater than those for PVX-RNA. This suggests that the interaction between the RNA and the protein in PVX enhances the rate of formation of photoreactivable kind of damage. Actually the rate is decreased, but that of nonphotoreactivable kind of damage is decreased even more (see Fig. 3), hence the increase in the value of S .

Small and Gordon (1967) found that photoreactivation of UV-irradiated TMV-RNA can be prevented by recombining it with the virus protein, i.e., by "reconstituting" the virus from the irradiated free RNA and the nonirradiated protein. The nucleic acid isolated again from the "reconstituted" virus can be photoreactivated. The recombination with the protein does not, therefore, either repair the photoreactivable kind of damage in the RNA or change it into some irreparable form. By contrast, the RNA isolated from the irradiated virus is not photoreactivable, so that the photoreactivable kind of damage in the RNA is completely prevented when the RNA is irradiated inside the virus. There is also evidence that when UV-inactivated TMV is inoculated to a plant, its RNA is "uncoated" in the plant just as it is when nonirradiated virus is

inoculated (Hayashi *et al.*, 1969). The reason for the absence of photoreactivation of the UV-irradiated free RNA that has been subsequently recombined with the virus protein, is unknown. It seems possible that when an RNA molecule has a photoreactivable kind of lesion, it may not combine with the protein in the normal way, but in a way that prevents it from being separated again by the "uncoating" mechanism of the plant. This may be so because a different manner of combination resulted in a different way of aggregation of the protein particles. As the normal mode of combination between the RNA and the protein does not allow photoreactivable lesion to be formed in the RNA, it does seem possible that the presence of a photoreactivable lesion in the RNA may not allow the combination to occur in the normal way.

As regards the chemical nature of the photoreactivable lesion, it is possible that photoreversible dimerization of adjacent pyrimidine residues may be at least one kind of such lesion. That this may be so can be concluded first from analogy with bacterial transforming DNA with which there is much evidence in favor of such dimers being involved (Wacker, 1961; R. B. Setlow and Setlow, 1962; J. K. Setlow and Setlow, 1963; Wulf and Rupert, 1962; J. K. Setlow *et al.*, 1965; R. B. Setlow, 1966), and second from the fact that such dimers were found in the UV-irradiated TMV-RNA (where photoreactivable lesions are present), but not in the RNA irradiated inside the virus (where such lesions are absent) (Tao *et al.*, 1969; Carpenter and Kleczkowski, 1969). However, there is also evidence that seems to contradict the involvement of the dimers. This has been discussed by Carpenter and Kleczkowski (1969) who concluded that more of relevant evidence is needed to resolve the conflict, and that it is possible that not all the photoreactivable lesions are the dimers and not all the dimers are necessarily lethal or photoreactivable.

Another candidate which has been considered for the role of at least one kind of photoreactivable lethal damage is hydration of pyrimidine. That this may be so was concluded from effects of deuterium oxide and of HCN on the rate and/or extent of photoreactivation (Evans and McLaren, 1969b; Evans *et al.*, 1969). Although hydration of pyrimidine may be a kind of lethal damage, the fact that it occurs in the RNA of TMV when the RNA is irradiated inside the virus, i.e., when photoreactivable damage does not occur (Tao *et al.*, 1969), suggests that it is not a photoreactivable kind of damage.

6. Multiplicity Reactivation

Multiplicity reactivation was first reported to occur with some coli bacteriophages (Luria and Dulbecco, 1949). When the concentration

of inactivated phage particles was sufficiently increased, infectivity of the irradiated phage preparation increased more than would correspond to the concentration of remaining active phage particles. The conclusion was that some phage particles that are inactive singly, may cause infection when acting together in the same cell, provided that between them they have a full complement of undamaged genes.

Tests with UV-irradiated preparations of TMV, TNV, and tomato bushy stunt virus gave no evidence that particles inactive singly could cause infection when acting jointly (Bawden and Kleczkowski, 1953). All the irradiated preparations gave dilution curves of the same form as those given by control virus preparations, until the concentration of virus in irradiated preparations reached about 0.1%. Then, instead of getting a steeper curve to be expected with multiplicity reactivation, the irradiated preparations gave flatter curves than the non-irradiated control preparations, because at this level the inactivated particles began to prevent infection with the active ones.

Although UV-inactivated TMV thus appears incapable of multiplicity reactivation, it has been demonstrated in UV-irradiated free RNA isolated from TMV, or in free RNA isolated from irradiated TMV (Takahashi *et al.*, 1968). This shows that the kinds of damage that can be overcome by multiplicity reactivation are formed in free RNA as well as in the RNA inside the virus. It has also been shown that UV-inactivated particles of TMV are "uncoated" in the inoculated plant just as active particles are (Hayashi *et al.*, 1969). Therefore the only factor that still remains to be considered as the reason for the absence of multiplicity reactivation when UV-irradiated TMV is inoculated, is some effect of the presence of virus protein inside the cell of the host plant. The protein may, for example, cause the phenomenon of exclusion, so that not more than one virus particle may enter the cell. This view is supported by the fact that UV-inactivated virus particles can interfere with infection by active particles (Bawden and Kleczkowski, 1953).

7. Effects of Irradiation on Virus Protein

Effects of UV radiation on the protein of TMV were investigated both when the protein was irradiated after it had been isolated, and when it was a part of a virus particle. Irradiation of a solution of isolated protein in 0.067 M phosphate buffer at pH 7.0 resulted in aggregation of the protein, some aggregates being amorphous and some rod-shaped, the rods being of about the same width as the virus particles but considerably shorter. The shape of the rods differed from that of the virus particles in that they were less perfectly formed and the walls of many of them

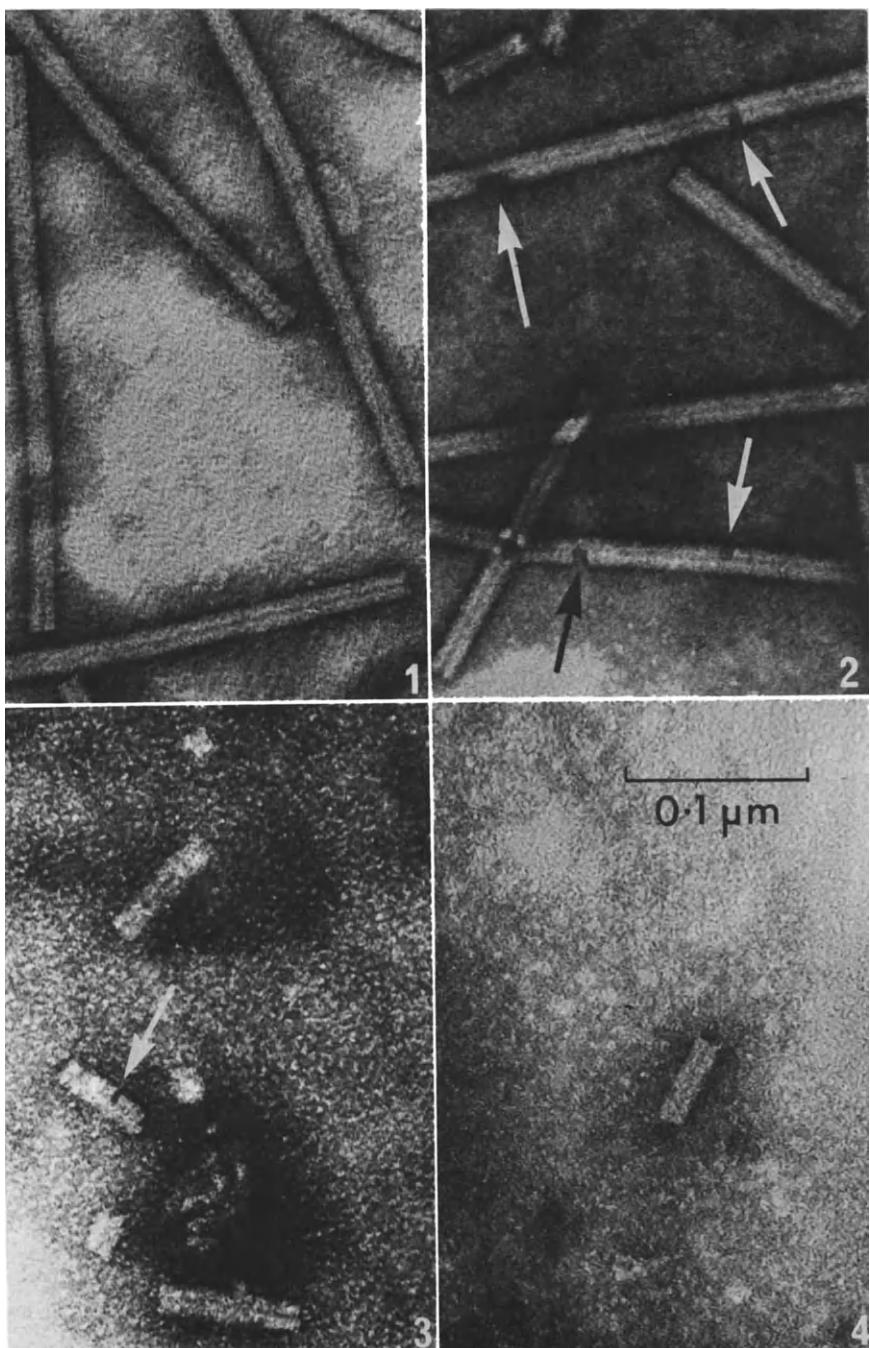
were lacerated. The aggregation was accompanied by an increase in electrophoretic mobility to about twice that of the original protein (whose particles consist of three molecules), so that it became almost equal to that of the virus. The aggregation therefore occurred in an orderly fashion, the molecules arranging themselves in such a way that the parts of their surface with a higher charge density than the average, remained exposed to the environment, as they are in the virus particles. About half of the protein became aggregated when 4.7 J of radiation energy (at $\lambda = 254$ nm) were absorbed by each mg of the protein (Kleczkowski, 1959).

As regards effects of UV radiation on the protein of TMV when it is a part of the virus particle, i.e., when intact TMV is irradiated, two effects have been studied—the loss of antigenicity and of the ability to conform to structural requirements of the virus particle. Antigenicity is understood here as the ability to combine with antibody to TMV. About 4.7 J of radiation energy (at $\lambda = 254$ nm) were absorbed by each mg of the protein when this ability was reduced by one-half (Kleczkowski, 1962). As it is also the amount of energy that must be absorbed by isolated protein of TMV when half of it became aggregated, both changes may result from the same structural alteration caused by UV.

At least initially the loss of antigenicity is approximately a first order reaction. The quantum yield for loss of antigenicity, computed on the assumption of first order kinetics, (taking the molecular weight of the protein as 18,000 daltons), was about 0.004 (at $\lambda = 254$ nm). This is close to 0.0047, which was computed by McLaren and Hidalgo-Salvatierra (1964) for "inactivation" of the protein of TMV from amino acid composition and relevant photochemical information, on the assumption that destruction of any energy absorbing residue is necessary and sufficient to "inactivate" a molecule, and on some other simplifying assumptions.

Loss of antigenicity of irradiated TMV need not coincide with loss of immunogenicity, but this has not been investigated.

The ability of protein molecule to conform to structural requirements of a virus particle is here understood as the ability to be a part of the particle, so that the loss of the ability means that the molecule falls out of a virus particle. If the molecules that are thus altered are distributed in a virus particle at random, as their number increases the probability will increase that some will occur near to one another, and these could cause any still "native" molecules between them to fall out also. This will result in gaps on the walls of virus particles large enough to be seen in an electron micrograph [Fig. 7 (2) and (3)]. Virus particles presumably break into shorter segments when such gaps break their continuity, and



this eventually leads to complete disintegration of the particles [Fig. 7 (4)]. The rate of formation of thus altered molecules follows first-order kinetic at least initially, and the quantum yield (at $\lambda = 254$ nm) for the formation was found to be about 0.0003 (McLaren and Kleczkowski, 1967) which is smaller by an order of magnitude than for the loss of antigenicity.

However, in spite of this, the usual experience is that as soon as no antigenicity can be detected in an irradiated preparation of TMV, electron-microscopic examination shows that virus particles have disintegrated, so that only a few small segments can still be found (Kleczkowski, 1962). This is probably so because the rate at which protein molecules fall out of the particles increases rapidly as irradiation progresses (McLaren and Kleczkowski, 1967), and this is so probably because increasing numbers of still "native" molecules fall out together with those that have lost the ability to conform to the structure of virus particles.

It may be of interest to compare susceptibility of TMV to inactivation of infectivity by UV at $\lambda = 254$ nm with susceptibility of its protein to the changes that have just been considered. Using current information concerning the relevant quantum yields, molecular weights, relative amounts and absorbances of the RNA and of the protein (and assuming that they have roughly the same absorbances when they are parts of the virus as when free), it can be computed that when, for example, 99.9% of infectivity is inactivated, only 5% of antigenicity is destroyed, and only 0.3% of protein molecules are made unable to remain in the virus particle. Thus, when TMV is irradiated at $\lambda = 254$ nm, its infectivity is much more susceptible to inactivation than its protein to the loss of antigenicity or of the ability to form part of the virus particle.

The loss of the ability of UV-irradiated virus protein to reconstitute the virus has not been studied. It is possible that the alteration which would cause the loss of this ability is less drastic than that which makes the protein molecule fall out of the virus particle, and would therefore be expected to have a higher quantum yield.

Exposure to visible light in the presence of methylene blue of the proteins isolated from TMV and from brome grass mosaic virus resulted in the loss of their antigenicity and of the ability to reconstitute the viruses (Bell and Orlob, 1970). This occurred in spite of the apparent

FIG. 7. Electron micrographs of a TMV preparation (stained with 1% potassium phosphotungstate) after irradiation at 254 nm (as a layer 0.1 cm thick at a concentration of 0.1% in 0.067 M phosphate buffer at pH 7.0) with the following doses (in einsteins/cm²)—(1) none; (2) 2×10^{-5} ; (3) 3.2×10^{-5} ; (4) 4.7×10^{-5} . Gaps in the virus particles are indicated by arrows.

absence of binding between the dye and the proteins. There was also destruction of some of tyrosine, tryptophan, methionine, and histidine in the protein of brome grass mosaic virus and of the first two in the protein of TMV which does not contain the other two. It still remains unknown whether the proteins in intact viruses can be similarly sensitized to visible light, and how the rates of the photodynamic effects on the proteins compare with those of inactivation of infectivity.

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Chapter 6

THE ROLES OF GENETIC RECOMBINATION AND DNA POLYMERASE IN THE REPAIR OF DAMAGED DNA

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1. Introduction

1.1 Discovery of the Repair of Radiation Damage

The first indication that cells might have the capacity to recover from radiation damage was the observation that minor modifications in the handling of the cells, e.g., growth media, temperature, etc., had a marked effect upon the ultimate viability of irradiated cells. Thus in 1937, Hollaender and Claus found that higher survival levels of UV-irradiated fungal spores could be obtained if they were allowed to remain in water or salt solution for a period of time before plating on nutrient agar. Roberts and Aldous (1949) extended these observations by showing that the shapes of the UV survival curves for *E. coli* B could be changed quite drastically simply by holding the irradiated cells in media devoid of an energy source for various times before plating on nutrient agar (Fig. 1). This phenomenon, known as *liquid*

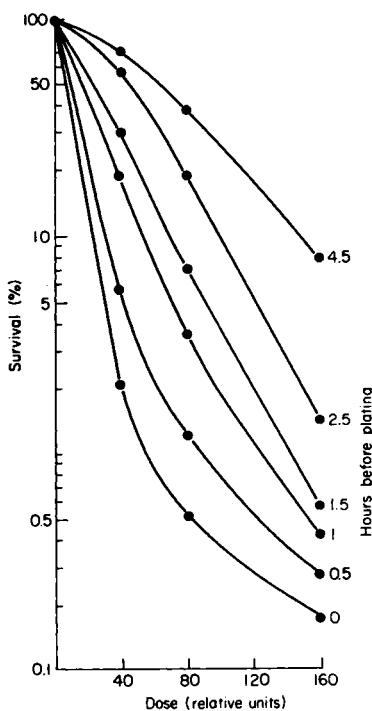


FIG. 1. UV radiation survival curves for *E. coli* B. After irradiation the cells were suspended in a liquid medium without an energy source for the times indicated before being plated on nutrient agar. It is evident that both the slopes and the shapes of the survival curves can be altered by the postirradiation treatment of the cells. (From Roberts and Aldous, 1949.)

holding recovery, has been shown to require intact *uvr* genes (Ganesan and Smith, 1969), the genes that control the first step in excision repair (Howard-Flanders *et al.*, 1966). Thus holding *E. coli* B (Rupert and Harm, 1966) and certain recombination deficient (*rec*) strains of *E. coli* K-12 (Ganesan and Smith, 1968a) in nonnutritive media appears to improve the efficiency of the excision repair process.

Another method for studying or detecting the presence of repair systems in cells is the split-dose technique used by Elkind (1967) and Elkind and Sinclair (1965). The rationale for this type of experiment is that if there is no repair of radiation damage, the ultimate survival of cells should be little affected, whether the total radiation dose is given at one time, or whether only part of the dose is given at one time and the remainder is given later. However, if the survival of the cells

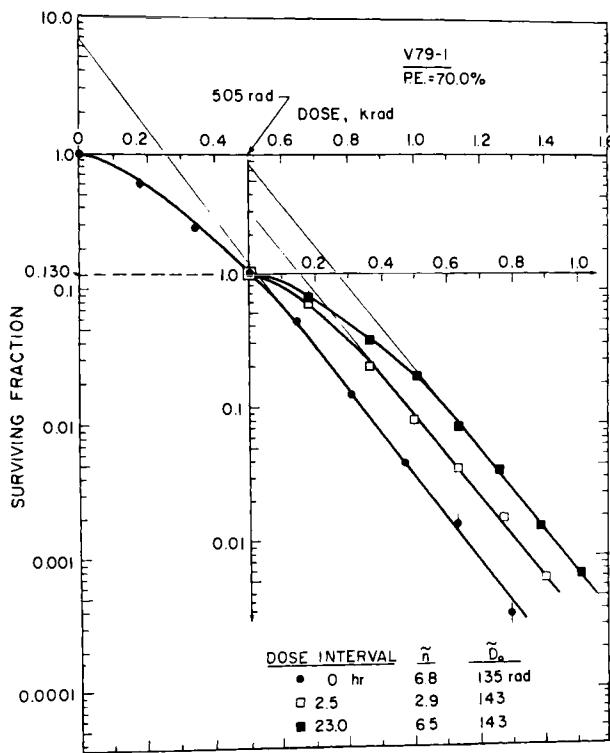


FIG. 2. X-ray survival curves for Chinese hamster cells (V79-1) using fractionated doses. When there were either 2.5 or 23.0 hours between the first dose of 505 rads and the subsequent doses of X rays, the cells were more resistant than if there was no fractionation of the dose. This shift in resistance suggests that the cells have repaired part of the damage produced by the first dose of radiation. (From Elkind, 1967.)

receiving a split dose of radiation is greater than that of cells receiving the same total dose delivered at one time, it seems reasonable to conclude that during the interval between irradiations the cells were able to repair a portion of the first dose of radiation (Fig. 2). This split-dose technique has been most widely used with mammalian cells in tissue culture, although some work has been done with microorganisms (Kiefer, 1970; Harm, 1968).

A reinterpretation of the meaning of the diverse shapes of radiation survival curves has also led to the hypothesis that certain cells can repair radiation damage. According to classical target theory, a shoulder on a survival curve should indicate multiple targets (or multiple hits on targets). However, closely related mutants of *E. coli* are not expected to have markedly different numbers or types of targets, yet their survival characteristics are markedly different (Fig. 3). The shoulders on the survival curves of the more resistant strains have been reinterpreted as implicating the capacity of these cells to repair radiation damage (Haynes, 1964b). The shoulder of a survival curve thus represents the dose range within which the cells can cope with

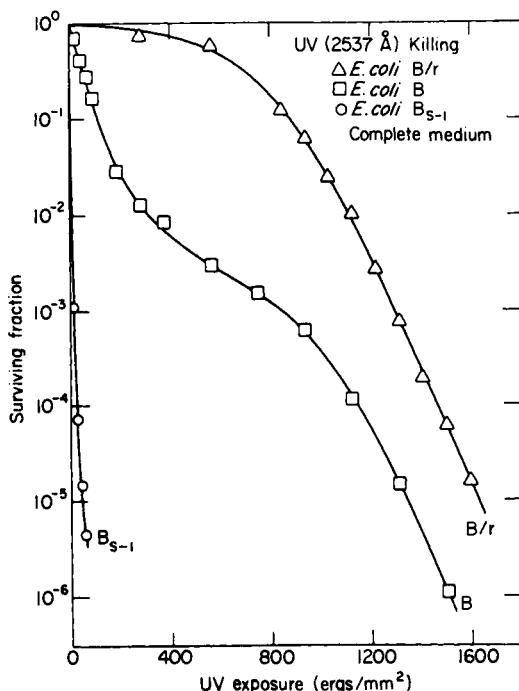


FIG. 3. UV radiation survival curves of different mutants of *E. coli* B. (From Haynes, 1964a.)

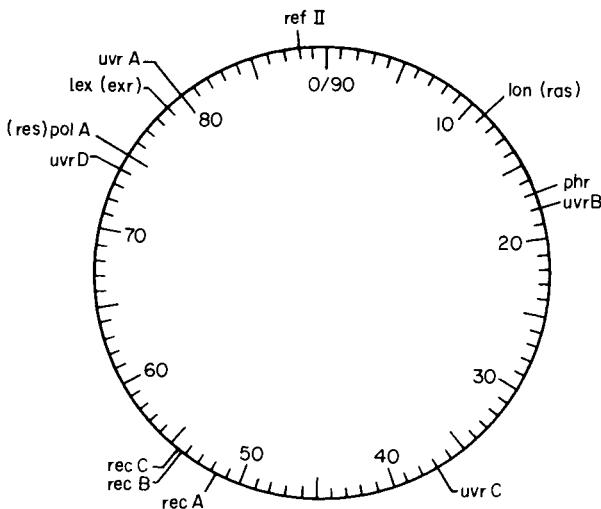


FIG. 4. Mutations in *E. coli* (see following tabulation) affecting radiation sensitivity. (Modified from Taylor, 1970.)

Mutant designation	Map position	Deficiency or sensitivity	Reference
<i>exr</i> (<i>lex</i>)	(79)	UV; X rays	Howard-Flanders and Boyce (1966) Mattern <i>et al.</i> (1966)
<i>lon</i>	11	Filament formation	Howard-Flanders <i>et al.</i> (1964)
<i>phr</i> <i>polA1</i>	(17) 75	Photoreactivation DNA polymerase; X rays	Van de Putte <i>et al.</i> (1965) de Lucia and Cairns (1969) Gross and Gross (1969)
<i>ras</i>	(11)	UV	Walker (1969, 1970)
<i>recA</i>	51.7	Recombination, UV, X ray	Willets <i>et al.</i> (1969)
<i>recB</i>	54.2	Recombination, UV, X ray	Emmerson (1968a); Willets and Mount (1969)
<i>recC</i>	54.5	Recombination, UV, X ray	Emmerson (1968a); Willets and Mount (1969)
<i>ref II</i>	(88)	UV-sensitive, colicin E2-resistant, recombination (?)	Holland and Threlfall (1969)
<i>res</i> <i>uvrA</i>	(75) 80	UV; X ray; DNA polymerase UV	Kato (1970) Howard-Flanders <i>et al.</i> (1966)
<i>uvrB</i>	18	UV	Howard-Flanders <i>et al.</i> (1966)
<i>uvrC</i>	37	UV	Howard-Flanders <i>et al.</i> (1966)
<i>uvrD</i>	74	UV	Ogawa <i>et al.</i> (1968)

the damage produced. At higher doses where the survival curve becomes steep, the repair systems themselves may either have become inactivated by the radiation or the number of lesions in the DNA may exceed the capacity of the repair system to cope with this damage.

With the mapping of several genes that affect the radiation sensitivity of cells (Fig. 4) and the determination of the biochemical deficiencies of several of these mutants (for reviews see Smith and Hanawalt, 1969; Witkin, 1968; Strauss, 1968; Howard-Flanders, 1968), both the presence and importance of enzymatic mechanisms for the repair of radiation damage have been firmly established.

1.2 Mechanisms for the Repair of Radiation Damage

Three modes of repair have been documented.

1. *The damaged part of the molecule is restored to its functional state in situ.* This may result from the spontaneous "decay" of the damage to an innocuous form, e.g., dehydration of pyrimidine photohydrates or the recombination of X-ray-induced radicals, or may be accomplished by some enzymatic mechanism, e.g., photoreactivation. Photoreactivation, the enzymatic splitting of cyclobutane-type pyrimidine dimers *in situ* mediated by exposure to visible light, has recently been reviewed (Harm *et al.*, this volume) and will not be further described here.

2. *The damaged section of a DNA strand is removed and replaced with undamaged nucleotides to restore the normal function of the DNA.* This mechanism is the basis of the excision-repair system which is known colloquially as "cut and patch" (for recent reviews see Hanawalt, 1968, 1969; Howard-Flanders, 1968; Smith and Hanawalt, 1969). This system constituted the first discovery of a mechanism of "dark repair" (to distinguish it from photoreactivation which is mediated by visible light, see above). It has been shown to repair a variety of radiation and chemically induced structural defects in DNA but was originally observed as a mechanism for the repair of UV-induced cyclobutane-type pyrimidine dimers. A schematic representation of the steps postulated to be involved in excision repair is shown in Fig. 5.

The first steps in this repair system are the recognition of damage and the introduction of a break in the DNA chain near the lesion (incision step). This is followed by the complete removal of the lesion from the DNA (excision step) and possibly a further widening of the excised region. The excision of pyrimidine dimers is under the control of the *uvr* genes (Boyce and Howard-Flanders, 1964; Howard-Flanders *et al.*, 1966).

After the excision step, the resulting gap is thought to be filled by

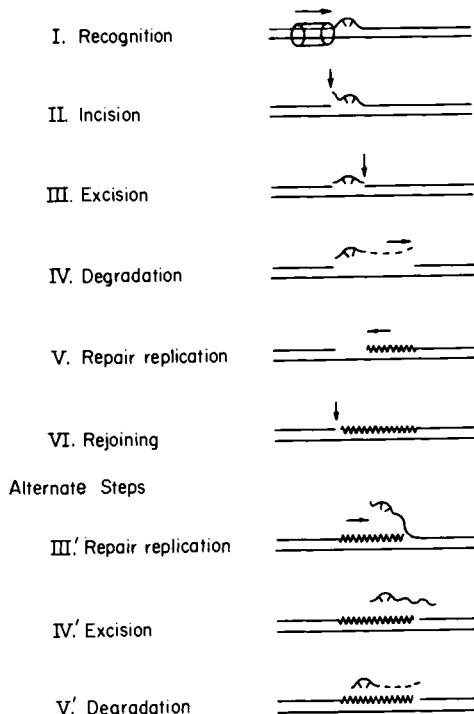


FIG. 5. Schematic representation of the postulated steps in the excision repair of damaged DNA. Steps I through VI illustrate the "cut and patch" sequence. An initial incision in the damaged strand is followed by local degradation before resynthesis of the region has begun. In the alternative "patch and cut" model, resynthesis step III' begins immediately after incision step II and the excision of the damaged region occurs when repair replication is complete. In either model the final step (VI) involves a rejoining of the repaired section to the contiguous DNA of the original parental strand. (From Smith and Hanawalt, 1969.)

the action of DNA polymerase (repair replication) using the opposite strand of DNA as the template. The *uvr* mutants do not perform repair replication (Cooper and Hanawalt, 1971). There is evidence that the *E. coli* DNA polymerase can perform both the excision step (but not the incision step) and the polymerase function (Kelly *et al.*, 1969). A polymerase-deficient mutant of *E. coli* has been isolated (de Lucia and Cairns, 1969) which shows a slightly enhanced sensitivity to UV irradiation but a marked sensitivity to X irradiation (Town *et al.*, 1971b). This and similar mutants will be further discussed in Section 7.

Finally, when the excised region is filled with undamaged nucleotides the single-strand interruption is closed enzymatically (probably by polynucleotide ligase; Olivera and Lehman, 1967) yielding repaired

DNA. In support of this argument, a ligase-deficient mutant has been isolated and shown to be sensitive to UV radiation (Pauling and Hamm, 1968) and to X radiation (Dean and Pauling, 1970).

A study of excision repair in mammalian cells has led to the observation of an apparent correlation between carcinogenesis and the defective repair of DNA (Cleaver, 1968). Fibroblasts from patients with *Xeroderma pigmentosum* were found to exhibit much-reduced levels of repair replication after UV irradiation. It was suggested that the failure of DNA repair might be related to the fatal skin cancers that patients with this hereditary disease develop upon exposure to sunlight.

Since bacterial strains that are deficient in the excision repair of UV-induced damage are not more sensitive than wild-type cells to killing by aerobic X irradiation (Howard-Flanders and Boyce, 1966; Kapp and Smith, 1970a), it may be hypothesized that either the excision-repair system does not play a major role in the repair of X-ray-induced damage in the DNA of bacteria or that the incision step is not required for the repair of ionizing radiation damage since X rays themselves produce breaks in the DNA backbone. The repair of X-ray-induced damage in DNA will be discussed in more detail in conjunction with a dark repair system that is controlled by the genes governing genetic recombination (Sections 5 and 6.3.3).

3. The damage, while not being directly repaired, is either ignored or bypassed, and the missing genetic information is supplied by redundant information within the cell. Several lines of evidence have suggested that the excision mode of repair is not the only mechanism by which cells can repair radiation damage to their DNA in the dark. The first indication was that bacterial cells deficient both in excision repair (*uvr*) and in genetic recombination (*rec*) (see Section 2) were much more sensitive to killing by UV than were cells carrying either mutation alone (Fig. 6). This suggested (Howard-Flanders and Boyce, 1966) that certain steps in genetic recombination might be important in the repair of radiation damage.* Second, the fact that *uvr* cells show a large recovery of viability when plated on minimal medium as compared to plating on complex medium (i.e., minimal medium recovery; see Section 4.2) suggests that excision-deficient cells are still able to

* From the similarity of the survival curves for *uvr* cells and for *recA* cells (Fig. 6), one may presume that the excision-repair system and the recombinational repair system(s) (see Sections 3-5) are of about equal importance to the survival of UV-irradiated cells of *E. coli* K-12. A refinement of this statement is that at low doses of UV radiation the recombinational repair system is most important, but at higher doses (less than 10^{-3} survival; Fig. 6) the excision-repair system plays a greater role (see also Radman *et al.*, 1970).

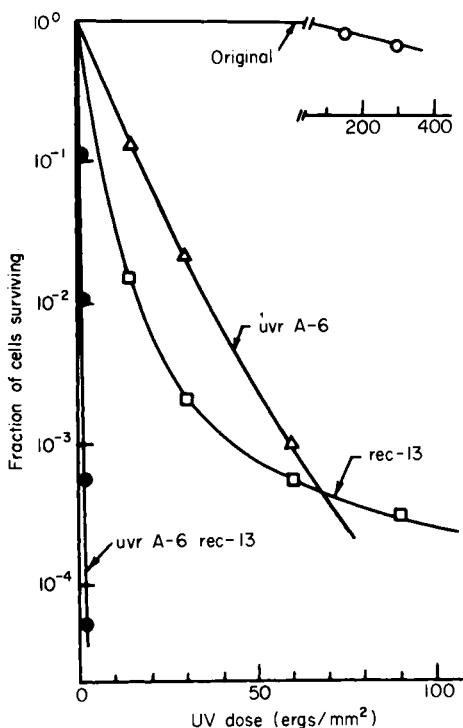


FIG. 6. Ultraviolet radiation survival curves of mutants of *E. coli* K-12. (From Howard-Flanders and Boyce, 1966.)

repair radiation damage (Ganesan and Smith, 1968b). Third, it has been demonstrated that photoproducts such as pyrimidine dimers do *not* permanently stop DNA synthesis in cells that are deficient in the excision mode of repair (Rupp and Howard-Flanders, 1968; Smith, 1969). Fourth, the DNA that is synthesized immediately after UV irradiation in excision-deficient cells of *E. coli* K-12 has discontinuities when assayed in alkaline sucrose gradients. The mean length of newly synthesized DNA approximates the distance between pyrimidine dimers in the parental strand. With further incubation of the cells, however, these discontinuities disappear and the DNA approximates the molecular size of that from unirradiated control cells (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968). A postreplication repair mode is thus indicated that appears to be mediated by some of the enzymes involved in genetic recombination (Ganesan and Smith, 1968b; Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968). A discuss-

sion of this *recombinational repair system* constitutes the major emphasis of the present report. In addition, the role of DNA polymerase in repair (especially of X-ray-induced damage) is discussed.

2. Genetic Recombination

2.1 Definition and General Considerations

One of the more important landmarks in the history of bacteriological sciences was the discovery by Lederberg and Tatum (1946a,b) of genetic recombination in bacteria (for reviews see Hayes, 1968, page 650; Curtiss, 1969). Certain strains of bacteria, e.g., *E. coli* K-12, show sexuality and undergo conjugation. There is a one-way transfer of genetic material from donor to recipient bacteria. On the average, 10-

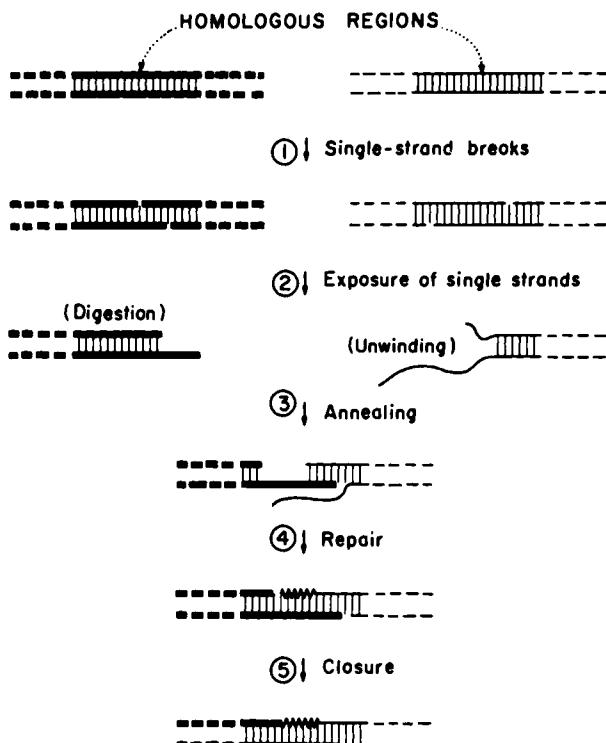


FIG. 7. Hypothetical scheme for recombination by a break-reunion mechanism (see text). Newly synthesized DNA is indicated by the zigzag line segment. (From Drake, 1970, page 32.)

20% of the donor chromosome is transferred. With a certain probability, recombination can occur by the breakage and rejoicing of homologous chromosomes to yield progeny carrying genetic information derived from both parents.

Although it is the central phenomenon of genetics and the principal tool of genetic analysis, the molecular basis of recombination is not known. However, simple generalizations at the molecular level can be made (Drake, 1970, page 20) to explain the observed biological consequences of recombination (Fig. 7) (see also Clark, 1971a; Davern, 1971; Haynes *et al.*, 1968).

1. Single-strand breaks are produced in the two DNA molecules.
2. Single-stranded regions are generated at these breaks by nuclease digestion or by unwinding.
3. Annealing occurs between complementary single strands from the different DNA molecules.
4. Repair processes convert the hybrid molecule to a normal two-stranded helical structure without single-strand interruptions.

A mechanism to explain the absence of reciprocal recombination in bacteriophage has been proposed by Boon and Zinder (1969). In their model only three DNA chains are broken in the single recombinational event that yields one recombinant and one parental genome (Fig. 8).

Both of these models require extensive repair replication which appears to require DNA polymerase. However, the observation that a DNA polymerase deficient mutant is not deficient in genetic recombination (see Section 7) raises the question as to the nature of the DNA synthesizing enzyme that is involved in genetic recombination.

It has generally been assumed that the many manifestations of recombination, e.g., transformation, transduction, conjugation, etc., are various aspects of a single molecular mechanism. Clark (1971a,b), however, has eloquently pointed out that there may be numerous mechanisms of recombination. He redefines recombination as "*a set of pathways* by which elements of nucleic acid interact with a resultant change either in base sequence or in linkage of genes or parts of genes."

The recent isolation and partial characterization of mutants defective in recombination, e.g., Clark, 1967, and the discovery of enzymes determined by the recombination genes (Buttin and Wright, 1968; Oishi, 1969; Barbour and Clark, 1970; Goldmark and Linn, 1970) allows, even demands, that the terms of enzymology and intermediary metabolism now be applied to recombination. Thus the concepts of branched, alternate or cyclic pathways and of control at all steps of these pathways should replace the unitary mechanism adhered to in the past. The precise molecular mechanism involved in each type of

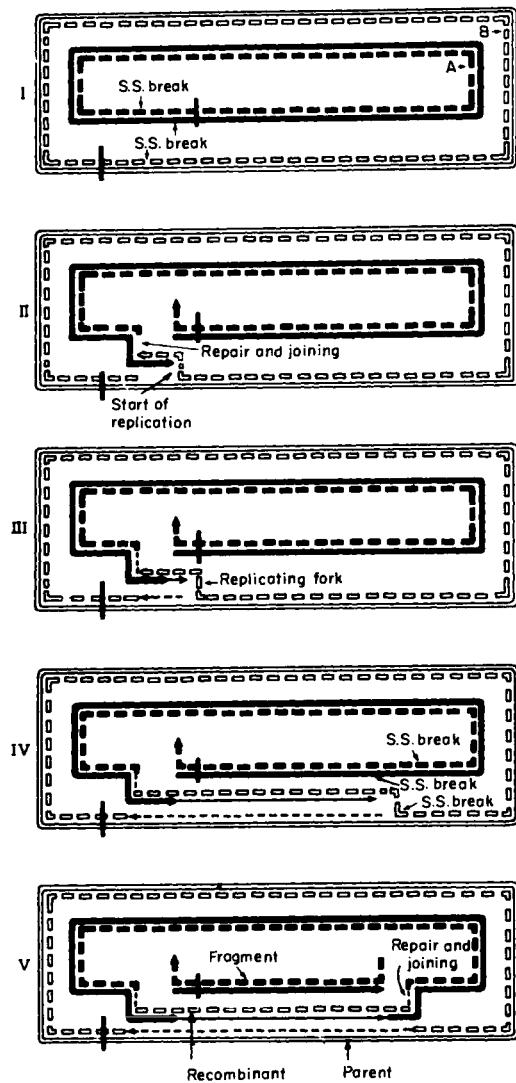


FIG. 8. Successive stages in a recombination event that yields one recombinant and one parental genome. The two parental DNA molecules are labeled A (thick solid lines) and B (thick hollow lines). They are drawn with their genetically homologous regions aligned. The thick intact lines represent plus (+) strands while the thick broken lines represent minus (-) strands. New synthesis is represented by the corresponding thin lines. All + strands have the same 5' to 3' direction (given here as counterclockwise), so do the minus strands (clockwise). Arrows indicate the 3' ends at free ends and at boundaries between strands of different origin. The vertical bars position two mutations. The designations "SS (single-strand) break," "repair and joining," and "start of replication" are topological not chronological. (Modified from Boon and Zinder, 1969.)

recombination phenomenon must be determined even though they appear to have a common physiological feature.

2.2 Properties of Recombination-Defective Mutants

Genetic studies of *E. coli* have revealed the presence of at least three cistrons in which mutations produce a drastic reduction in the number of recombinants resulting from conjugation (or transduction). The *recA*, *recB*, and *recC* loci lie within a 5% segment of the *E. coli* chromosome map (Fig. 4).

The *recB* and *recC* genes govern the synthesis of an ATP-dependent nuclease which has been partially purified. Both an apparently double-strand exonucleolytic activity (Buttin and Wright, 1968; Oishi, 1969; Barbour and Clark, 1970) and a single-strand endonucleolytic activity (Goldmark and Linn, 1970) have been observed. Mutations at either *recB* or *recC* eliminate these activities.

The *recA* gene product, on the other hand, seems to control or inhibit the *recB* *recC* nuclease activity *in vivo*. The *recA* cells degrade very much more of their DNA after UV irradiation than do *rec⁺* cells. Mutations at *recB* or *recC* prevent this degradation in both *recA⁺* and *recA⁻* cells (Willets and Clark, 1969). It appears that in the absence of a functional *recA* product the *recB* *recC* nuclease is freed of control and thus degrades more DNA than in the presence of a functional *recA* gene product.

Recombination frequencies (conjugation or transduction) in *recA* mutants are about 10^{-3} to 10^{-5} compared to a value of 1 for *rec⁺* cells (Willetts and Mount, 1969). The *recA* gene product thus appears to be virtually indispensable for genetic recombination. The *recB* and *recC* mutants show an intermediate level of recombination, about 1–2% of that of *rec⁺* cells (Willetts and Mount, 1969).

Since the *recB* or *recC* mutants have the same amount of residual recombination ability as the double mutant, *recB recC*, it seems unlikely that this residual activity is due to the presence of 1–2% of the ATP-dependent nuclease. A more plausible explanation is that the residual activity represents a pathway of recombination alternative to that involving the *recB recC* enzyme. Some success has already been achieved in selecting mutants that may be deficient in these alternate pathways (Barbour *et al.*, 1970; Clark, 1971a,b).

F lac⁺ donor strains carrying *recB* or *recC* can mobilize their chromosome with efficiencies ranging from about 10% to more than 50% of that of the wild-type value, depending on the age of the male cell line. In contrast, the level of chromosome transfer from donors carrying *recA* is reduced to less than 10^{-4} of normal (Wilkins, 1969).

The *recA* strains cannot be mutated by ultraviolet light (a frequency

of $<2 \times 10^{-3}$ compared to 1 for $recA^+$), while $recC22$ derivatives show an intermediate mutability ($\sim 10^{-1}$) compared to rec^+ cells (Witkin, 1969a).

The $recA$ mutants show a low rate of spontaneous production of phage λ , while $recB$ and $recC$ mutants show essentially a normal rate (Willets *et al.*, 1969).

Mutations at $recA$ suppress UV-induced filament formation in *lon* strains without affecting capsular polysaccharide production (Green *et al.*, 1969).

The $recB21$ mutant is sensitive to thymineless death (Cummings and Mondale, 1967), whereas $recA1$ is not (Cummings and Taylor, 1966). Both $recA$ and $recB$ strains are sensitive to killing by nalidixic acid and 5-fluorodeoxyuracil, however (Green *et al.*, 1970).

The frequency of lethal sectoring, i.e., not all daughter cells survive, for wild-type K-12 or *uvr*⁻ cells is about 0.005; for $recA$, it is about 0.063; for $recB$, about 0.077, and for $recA13\ uvrA6$ it is about 0.112 (Haefner, 1968). The viability of stationary phase cells when assayed on nutrient broth is 89% for rec^+ cells, 74% for $recA$ cells and 39% for $recB$ cells (Haefner, 1968).

Inactivation of any one of the three genes, $recA$, $recB$, or $recC$ prevents *uvr*⁻ cells from showing Minimal Medium Recovery (higher survival after UV irradiation when plated on minimal medium as against complex medium plates) (Ganesan and Smith, 1970) (see Section 4.2).

Liquid Holding Recovery (LHR), the recovery of UV-irradiated bacteria when held in nonnutritive medium prior to plating on nutrient medium, is an expression of the excision repair system and depends upon the presence of intact *uvr* genes (Ganesan and Smith, 1969). LHR has been observed in *E. coli* K-12 only in $recA$ mutants (Ganesan and Smith, 1968a). The absence of repair functions mediated by the $recA$ gene (see Section 3) is thought to serve as a window through which the excision-repair system can be more easily viewed. Mutations at $recB$ and $recC$ do not provide such a window, i.e., LHR is not observed in these mutants (see Section 3).

The $recA$ mutants are the most sensitive of the *rec* mutants to UV and X irradiation. The $recB$ and $recC$ mutants show intermediate sensitivities to radiation killing. The D_{37} values for exponentially growing *E. coli* K-12 rec^+ , $recA13$, $recB21$, and $recC22$, and *uvrB5* are 5.7, 1.1, 1.7, 1.7, and 5.7 krads, respectively (Kapp and Smith, 1970a). The UV dose (254 nm) to kill exponentially growing *E. coli* K-12 cells to 10^{-2} (grown and plated on minimal medium) is 950, 50, 370, 400, and 155 ergs/mm² for rec^+ , $recA13$, $recB21$, $recC22$, and *uvrB5*, respectively (K. C. Smith and M. E. O'Leary, unpublished data).

The *recA* mutants can't perform postreplication repair after UV irradiation, whereas *recB* and *recC* cells show no apparent deficiency in this response (Smith and Meun, 1970) (see Section 3).

The *recA* mutants are deficient in their ability to repair X-ray-induced single-chain breaks in DNA, while *recB* and *recC* cells show an intermediate ability in this regard (Kapp and Smith, 1970a) (see Section 5).

3. Recombinational Repair of UV-Induced Damage

3.1 Introduction

The current model for a recombinational repair system (postreplication repair system) is based on the observation that UV-induced lesions in DNA that cannot or are not repaired by the excision-repair system are bypassed during normal replication leaving gaps in the newly synthesized daughter strands opposite the radiation-induced lesions in the parental strands. These gaps in the daughter strands are subsequently repaired (in recombinational repair proficient strains) yielding DNA that appears normal in the ultracentrifuge. Thus the original UV-induced lesions are not directly repaired but are bypassed. The lesions that are repaired are the gaps produced in the daughter strands opposite to the UV-induced lesions. The evidence that led to this model and our current knowledge concerning its genetic control and the biochemical steps involved are summarized below.

3.2 DNA Synthesis Can Occur on UV-Damaged Templates

The effect of UV radiation on the kinetics of DNA synthesis has recently been reinvestigated. It has been shown conclusively that pyrimidine dimers do *not* permanently inhibit DNA synthesis in cells that are deficient in the excision repair of pyrimidine dimers (Smith, 1969; Rupp and Howard-Flanders, 1968). It is therefore possible to investigate the replication of bacterial DNA in excision-defective mutants containing a "known" number of damaged bases. Rupp and Howard-Flanders (1968) have measured the molecular weight of the DNA synthesized upon damaged templates in UV-irradiated bacteria, using the technique of McGrath and Williams (1966). In this technique, bacterial spheroplasts containing radioactive DNA are gently lysed on top of an alkaline sucrose gradient and then sedimented in an ultracentrifuge. The alkaline pH converts the double-stranded DNA to single-stranded DNA. The distance that the single-stranded pieces of DNA move in the gradient in a given time is proportional to their molecular weight.

The DNA from excision deficient cells (*uvr*⁻) that had been labeled

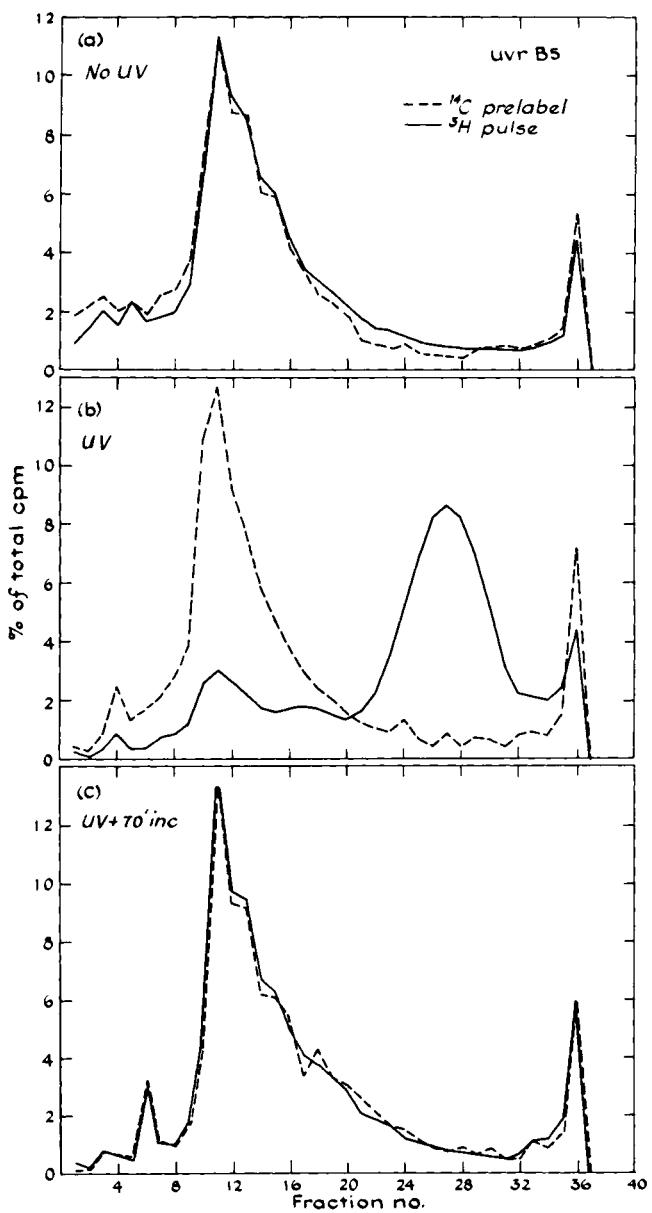


FIG. 9

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with ^{14}C -thymine prior to UV irradiation exhibited essentially the same sedimentation characteristics as the DNA of unirradiated cells even if they were allowed to incubate in growth medium for 10 or 70 minutes before banding in the ultracentrifuge (Fig. 9). This indicated that the parental DNA in these excision-deficient strains was not broken down nor were a large number of single-chain breaks introduced into the parental DNA during the incubation period. The UV-damaged parental DNA of excision-defective mutants therefore appeared to be a stable template upon which DNA could be synthesized (Rupp and Howard-Flanders, 1968).

3.3 Characteristics of the DNA Synthesized on UV-Damaged Templates

Although the DNA synthesized during a 10-minute labeling period in the unirradiated cells sedimented nearly as fast as the parental DNA (Fig. 9a), the DNA synthesized in 10 minutes by UV-irradiated *uvr* cells (Fig. 9b) sedimented less rapidly and therefore was of much lower molecular weight (Rupp and Howard-Flanders, 1968).

The factors presently known to affect the sedimentation characteristics of the DNA synthesized after UV irradiation are UV dose, photoreactivation, and excision repair.

3.3.1 UV Dose

The molecular weight of the DNA synthesized after UV irradiation is a function of the dose of UV (Fig. 10). The higher the dose of UV, the slower the sedimentation rate, and therefore the smaller the molecular weight of the DNA synthesized after UV irradiation (Rupp and Howard-Flanders, 1968).

Knowing the number of pyrimidine dimers produced per erg of UV radiation (Rupp and Howard-Flanders, 1968; but contrast Smith, 1969, page 490) and the molecular weight of the *E. coli* chromosome (Cairns,

FIG. 9. Sedimentation in alkaline sucrose gradients of DNA labeled before and after UV irradiation. *E. coli* K-12 (*uvrB5*) were grown for several generations on ^{14}C -thymine and the ^{14}C -thymine was removed from the medium. Control and irradiated cells (63 ergs/mm 2 at 254 nm) were then grown with ^3H -thymidine for 10 minutes. Aliquots were stored in ice, while another sample of the irradiated cells was incubated for an additional 70 minutes in nonradioactive medium. The cells were converted to spheroplasts with lysozyme and EDTA (Rupp and Howard-Flanders, 1968) and lysed on top of an alkaline sucrose gradient and spun in rotor SW 50.1 at 30,000 rpm for 105 min at 20°C in a Spinco L2-65B centrifuge. After centrifugation the tubes were punctured and about 40 5-drop fractions were collected on paper discs. The discs were dried, washed in acid, dried and counted for radioactivity. (K. C. Smith, 1969, unpublished results.)

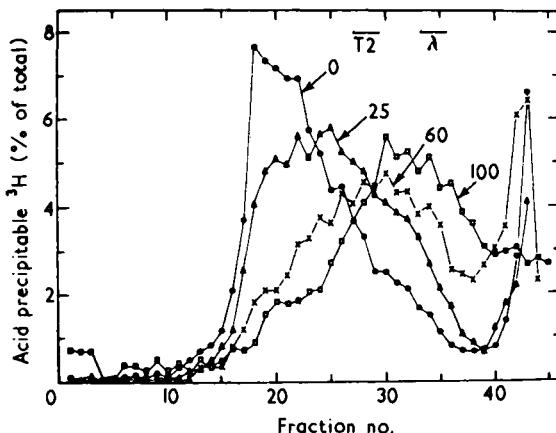


FIG. 10. The sedimentation characteristics of the DNA synthesized by *E. coli* K-12 AB2500 *uvrA6* after several doses of UV irradiation. The conditions were similar to those described in Fig. 9, except that the DNA was not prelabeled and all samples were assayed immediately after labeling. Centrifugation was in a SW 50 rotor for 120 minutes at 30,000 rpm at 20°C in a Spinco L2 centrifuge. The positions of intact strands of phages T2 and λ are indicated. The numbers by the arrows refer to dose of UV (254 nm) in ergs/mm². (From Rupp and Howard-Flanders, 1968.)

1963), the mean distance between pyrimidine dimers in the *E. coli* chromosome for a given dose of UV can be calculated. The average molecular weight of the DNA synthesized after UV irradiation was found to be in close agreement with the average molecular weight of the pieces of parental DNA that were present between the pyrimidine dimers. Therefore the obvious conclusion was that the DNA was synthesized along the undamaged section of the parental DNA but the polymerase skipped the section containing the pyrimidine dimers, thus leaving a gap in the daughter-strand DNA (Rupp and Howard-Flanders, 1968).

3.3.2 PHOTOREACTIVATION

When UV-irradiated cells were exposed to visible light under conditions that favor photoreactivation and then pulsed with radioactive thymidine, it was found (Fig. 11) that the pieces of DNA were much larger than those synthesized by cells prior to the photoreactivation treatment (Rupp, 1968; Smith and Meun, 1970). Since photoreactivation is known to repair pyrimidine dimers *in situ* (for a review see Harm *et al.*, this volume), it follows that pyrimidine dimers are important in determining the length of the pieces of DNA that are synthesized after

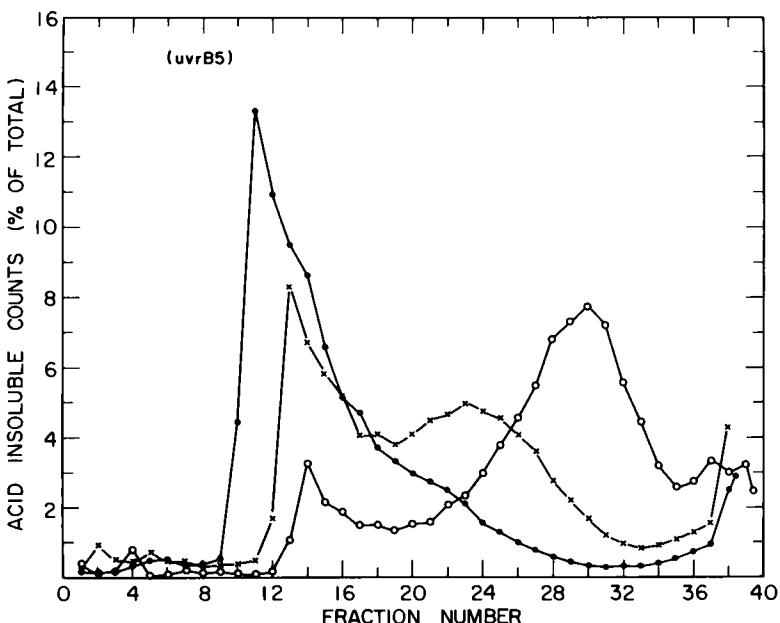


FIG. 11. The effect of photoreactivation upon the sedimentation of DNA subsequently synthesized by UV-irradiated cells of *E. coli* K-12 AB2499 (*rec⁺ uvrB5*). Immediately after irradiation (63 ergs/mm²), one sample of irradiated cells and one sample of unirradiated cells were exposed to the light from two Westinghouse black light bulbs (F15T8) for 20 minutes under conditions previously described (Smith and O'Leary, 1967). The irradiated sample which was not photoreactivated was shaken on a rotating platform for 20 minutes in the dark. The samples were then labeled for 10 minutes with ³H-thymidine and centrifuged as described in Fig. 9. The amount of photoreactivation was estimated by plating the cells on a complex medium to minimize dark recovery (Ganesan and Smith, 1968b). In the photoreactivated sample 81% of the cells formed colonies on complex medium, while only 2% did so in the unphotoreactivated sample. Unirradiated control (—●—●—); irradiated but not photoreactivated (—○—○—); irradiated and photoreactivated (—×—×—). (From Smith and Meun, 1970.)

UV irradiation. However, it should be noted that the photoreactivating treatment did not completely eliminate the synthesis of short pieces of DNA (Fig. 11). This residual synthesis of intermediate sedimenting DNA could reflect the presence of unrepaired pyrimidine dimers or indicate the importance of other types of photochemical lesions in causing the synthesis of short sections of DNA. In support of the latter hypothesis, there is evidence that UV-induced DNA-protein cross-links can cause the synthesis of slowly sedimenting DNA (K. C. Smith, 1970, unpublished observations).

3.3.3 EXCISION REPAIR

If cells that are capable of excision repair, i.e., *uvr⁺*, are UV irradiated and then pulsed for 10 minutes with radioactive thymidine, small pieces of DNA are synthesized much as they are in cells that are excision deficient (Rupp, 1968; Smith and Meun, 1970). Since the sedimentation characteristics of the DNA synthesized immediately after UV irradiation are approximately the same for *uvr⁺* cells (Fig. 12a) as for *uvr⁻* cells (Fig. 12b), the excision repair system apparently does not markedly interfere with the postreplication repair process, at least not during the first 10 minutes after irradiation.

This brings up the interesting question of how these two repair systems function without interfering with each other. The data in Fig. 12a indicate that although the excision-repair system does not markedly affect the portion of the parental DNA lying 10 minutes of growth (after 63 ergs/mm²) in front of the growing point (referring to its position at the time of irradiation) it does affect, i.e., repairs, the parental DNA lying between 10 to 20 minutes of growth in front of the growing point.

If excision-proficient cells (*uvr⁺*) are irradiated with UV and, then allowed to grow for 40 to 60 minutes before adding the radioactive thymidine, the molecular weight of the newly synthesized radioactive DNA is comparable to that synthesized in the unirradiated control cells (Fig. 12a). This suggests that excision-proficient cells can repair, in about 40 minutes, the majority of the damage that causes DNA to be synthesized in short pieces after UV irradiation.

On the other hand, if excision-minus cells (*uvr⁻*) are grown for about 90 minutes after UV irradiation before adding the radioactive thymidine, there is little difference between the molecular weight of the newly synthesized DNA whether labeled immediately after UV or after 90 minutes of growth after UV (Fig. 12b). This indicates that in the absence of the excision repair system, at least the majority of the lesions leading to the synthesis of small pieces of DNA after UV irradiation are *not* repaired.

The implication from these facts is that the DNA synthesized after UV irradiation contains gaps that are opposite the pyrimidine dimers (and other lesions) present in the parental template strands of the DNA (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1971). Since these experiments were performed in strong alkali, it could be argued that the newly synthesized DNA contains some kind of alkali labile bond opposite each pyrimidine dimer which is cleaved when the DNA is placed in alkaline sucrose gradients. Howard-Flanders and co-workers

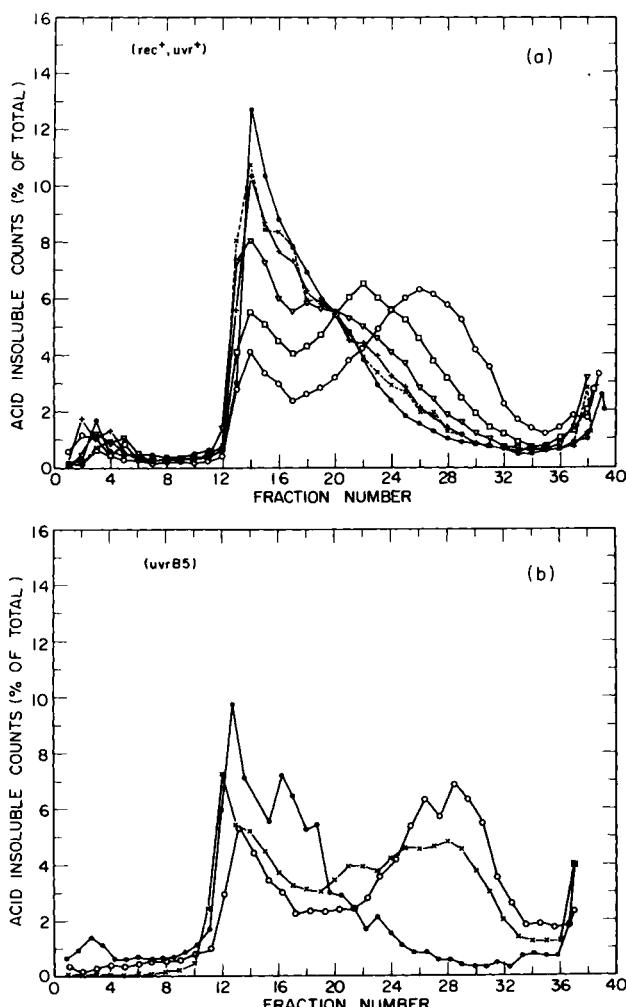


FIG. 12. The effect of excision repair on the sedimentation of DNA synthesized by UV-irradiated cells. Samples of the irradiated cells were incubated in the dark for various periods of time before being labeled with ^3H -thymidine and centrifuged as described in Fig. 9. (a) Cells of an excision proficient strain (AB2497 rec^+ uvr^+). Unirradiated control (—●—●—); irradiated but not incubated before labeling (—○—○—); irradiated, incubated for 10 minutes before labeling (—□—□—); irradiated, incubated for 20 minutes before labeling (—▽—▽—); irradiated, incubated for 40 minutes before labeling (—+—+—); irradiated, incubated for 60 minutes before labeling (—×—×—). (b) Cells of an excision-deficient mutant (AB2499 rec^+ uvrB5). Unirradiated control (—●—●—); irradiated, but not incubated before labeling (—○—○—); irradiated, incubated 90 minutes before labeling (—×—×—). (From Smith and Meun, 1970.)

(1968) have performed an experiment in the absence of alkali to test this hypothesis. Pulse-labeled DNA was isolated from control and irradiated cells with phenol. The DNA was denatured by heating for 5 minutes at 100°C and then centrifuged in a *neutral* sucrose gradient. The pulse-labeled heat-denatured DNA from the UV-irradiated cells sedimented more slowly in a neutral sucrose gradient than did the DNA from the control cells. It thus appears unlikely that the low molecular weight DNA chains synthesized upon the damaged template are joined by alkali labile bonds.

More recent proof that the DNA synthesized after UV irradiation contains gaps comes from experiments designed to measure the size of the gaps. Using calibrated benzoylated naphthoylated DEAE cellulose chromatography columns that show a selectivity for single-stranded DNA, Iyer and Rupp (1971) have shown that each gap in the newly synthesized daughter strands is about 1000 nucleotides long, or about the size of an "Okazaki fragment" (Okazaki *et al.*, 1968).

For the Okazaki fragments to be reasonably uniform in length one may postulate that there must be a code for starting and stopping the synthesis of these fragments. To explain the large size of the gaps in the daughter strands opposite the lesions in the parental strands, one may further postulate that the presence of a pyrimidine dimer causes the polymerase to skip to the next initiation point for an Okazaki fragment. One might also imagine that it is these partially completed Okazaki fragments that are degraded to explain the observation that the newly synthesized DNA is preferentially degraded by UV-irradiated cells (Horii and Suzuki, 1968, 1970; Hanawalt and Bremelis, 1968).

3.4 Repair of Defects in the Daughter Strands of DNA

If instead of running gradients on the UV-irradiated cells immediately after labeling them for 10 minutes with ^3H -thymidine they are further incubated in the presence of nonradioactive thymidine for various periods of time before sedimentation in alkaline sucrose, the lifetime of the low molecular weight DNA synthesized on the damaged template can be investigated. During such an incubation the slowly sedimenting material is converted to a fast sedimenting form that is comparable in its sedimentation with that of the control DNA (Fig. 9c).

This increase in molecular weight cannot be due to degradation of the pulse labeled DNA and a reutilization of the label for the subsequent synthesis of high molecular weight DNA. If this were true, the radioactivity in the small molecular weight piece should simply disappear and radioactivity should appear directly in the high molecular

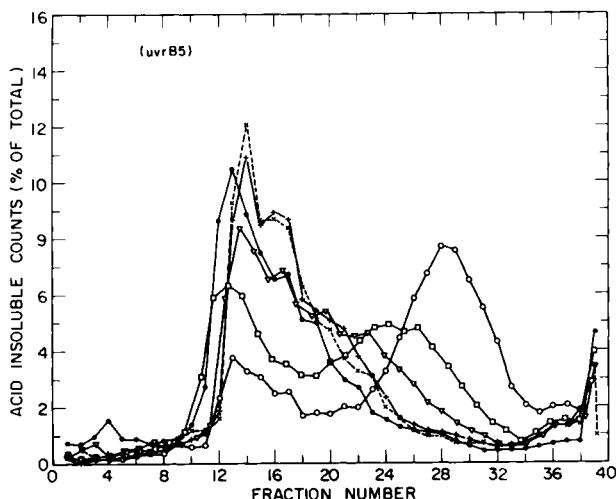


FIG. 13. The effect of different reincubation times on the sedimentation of DNA synthesized by UV-irradiated *E. coli* K-12 AB2499 (*rec⁺ uvrB5*). Samples of the irradiated cells were reincubated in nonradioactive medium for various times after labeling with ^3H -thymidine and were centrifuged as described in Fig. 9. Survival was not determined in this experiment, but in other experiments under the same conditions the survival of this strain on minimal medium was 80%. Unirradiated control (—●—●—); irradiated, but not reincubated (—○—○—); irradiated, reincubated for 10 minutes (—□—□—); irradiated, reincubated for 20 minutes (—▽—▽—); irradiated, reincubated for 40 minutes (—+—+—); irradiated, reincubated for 60 minutes (—×—×—). (From Smith and Meun, 1970.)

weight fraction. What is observed is that there is a progressive shift in the molecular weight of the pulse labeled material with time towards the molecular weight of the unirradiated DNA (Fig. 13). This suggests that during incubation the lower molecular weight material is joined together to form high molecular weight DNA.

3.5 The Role of Parental DNA in Filling the Gaps in the Daughter Strands

If genetic information was not available during the time of DNA synthesis to fill in these gaps then one may ask where the information (or the material) to fill the gaps comes from subsequently. Currently, the most plausible explanation is that the gaps are filled with undamaged material from the parental strands by some mechanism of genetic recombination as shown schematically in Fig. 14. However, this process would leave gaps in the parental DNA and it appears from prelabeling experiments (Fig. 9) that the parental DNA is not broken down into small fragments, nor are there an appreciable number of chain breaks

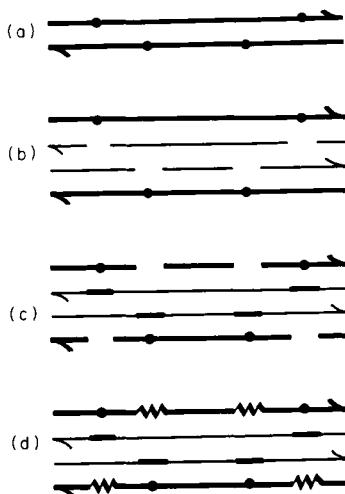


FIG. 14. A model for postreplication repair of UV-damaged DNA. (a) Dots indicate base damage produced in the DNA; (b) DNA synthesis proceeds past the lesions in the parental strands leaving gaps in the daughter strands; (c) filling of the gaps in the daughter strands with material from the parental strands by a recombinational process; (d) repair of the gaps in the parental strands by repair replication.

observed in *uvr*⁻ cells during the time that the discontinuities in the daughter strand DNA are being repaired. This suggests either that the parental DNA is not being utilized for filling the gaps in the daughter strands or that simultaneously with the transfer of parental material to the daughter strand the interruptions in the parental strands are repaired by repair replication. Even so, one would expect to find a slight increase in the number of single strand breaks present in the parental DNA and as yet this has not been observed.

In another type of experiment to test the involvement of parental DNA in postreplication repair, excision defective cells were density labeled for several generations in a ¹³C-¹⁵N medium containing ¹⁴C-thymine. The cells were then transferred to light medium without radioactive label for 30 minutes so that the growing point would be incorporating only light label at the time of irradiation. The cells were then exposed to 0, 20, or 50 ergs/mm² (254 nm) and incubated in the presence of ³H-thymidine in light medium for 30 minutes. The DNA was isolated, heat denatured, and the resulting single-stranded DNA was centrifuged to equilibrium in a cesium chloride gradient. In the unirradiated cells the heavy ¹⁴C-peak and a light ³H-peak were sym-

metrical and well separated from one another. After irradiation the ^{14}C -peak remained heavy and quite symmetrical whereas the ^3H -peak became skewed (as a function of dose) toward the heavy side of the gradient (Fig. 15). This indicates that in the UV-irradiated cells the newly synthesized DNA has become associated in the same strand with dense label that was synthesized 30 minutes before irradiation. Rupp and Howard-Flanders (1969) have interpreted these results as suggesting that the intermediate density material was produced by recombinational exchanges between sister duplexes, and offer this as a method by which the gaps are repaired in the DNA that is synthesized after UV irradiation.

From a consideration of the amount of radioactivity appearing in the intermediate density region in the above experiment and the number of pyrimidine dimers produced by the dose of radiation used, Howard-Flanders *et al.* (1971) have calculated that there is one sister strand exchange for each 1.3 dimers bypassed during replication, i.e., 1.3 gaps produced.

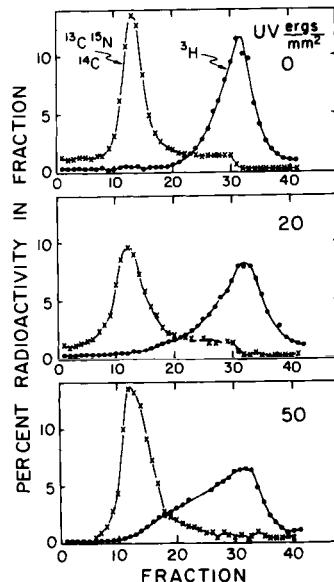


FIG. 15. Evidence for sister strand exchanges in UV irradiated but not in unirradiated *E. coli* K-12. Cells were prelabeled with ^{13}C , ^{15}N , and ^{14}C -thymine; grown 30 minutes in ^{12}C , ^{14}N , nonradioactive medium; UV-irradiated and then grown in ^3H -thymidine for 30 minutes; DNA isolated and denatured and banded in CsCl gradients (Rupp *et al.*, 1970).

3.6 Genetic Control of Postreplication Repair

From a consideration of survival curves (Section 1.2 and Fig. 6) it was inferred that certain steps in genetic recombination might be important in the repair of radiation damage. If the postreplication repair of UV-radiation damage is mediated by some of the enzymes involved in genetic recombination (Ganesan and Smith, 1968b; Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968) then recombination deficient mutants should be unable to repair the discontinuities in the DNA synthesized after irradiation.

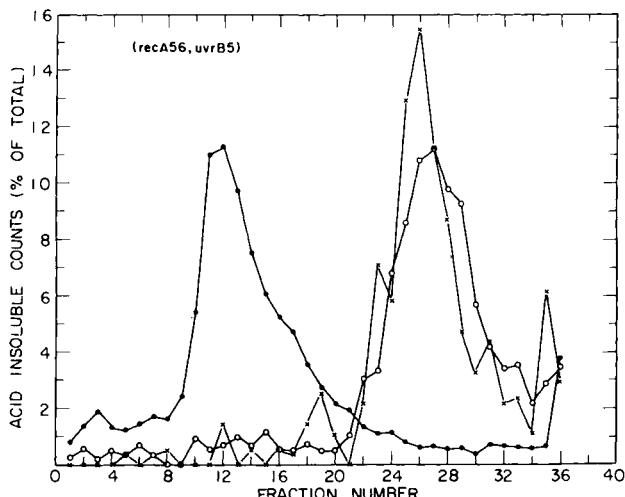


Fig. 16. Sedimentation in alkaline sucrose of DNA from UV-irradiated cells of *E. coli* K-12 SR72 (*recA56 uvrB5*). The procedure used was similar to that described in Fig. 9, except that the DNA was not prelabeled. Of the irradiated cells < 0.1% formed colonies on minimal medium agar. Unirradiated control (—●—●—); irradiated, but not reincubated after labeling (—○—○—); irradiated, reincubated for 70 minutes after labeling (—×—×—). (From Smith and Meun, 1970.)

To test this hypothesis, three *recA* derivatives, AB2487 (*recA13 uvr⁺*), SR74 (*recA56 uvr⁺*) and SR72 (*recA56 uvrB5*), were tested for postreplication repair. All three behaved in essentially the same way. Figure 16 shows the results obtained with SR72. Labeled DNA synthesized in irradiated cells sedimented more slowly than that formed in unirradiated controls. However, reincubation of the irradiated cells after labeling did not result in any detectable increase in the rate of sedimentation.

tation of the labeled DNA, in contrast with the results for the *rec⁺* strains. Under these conditions the *recA* mutants did not appear to undergo postreplication repair.*

However, much of the labeled DNA was degraded into acid-soluble material during the reincubation period, reducing the sensitivity with which repair could be detected. This occurred in both *uvr⁺* and *uvr⁻* derivatives of *recA* mutants.

The degradation of DNA synthesized by *recA* cells before exposure to UV could be inhibited by starving the cells for amino acids required for growth (Ganesan and Smith, 1968b; Horii and Suzuki, 1968). Therefore, to minimize degradation in *recA* mutants and permit labeling of DNA after irradiation, a new procedure was devised. Glucose, as well as amino acids, was removed for 90 minutes before irradiating the cells. After irradiation, glucose was added together with ^3H -thymidine for the 10-minute labeling period. The ^3H -thymidine was removed by filtering and the cells, resuspended in complete medium minus amino acids, were either chilled immediately or reincubated before analysis on alkaline sucrose gradients. After submitting the cells to this starvation procedure, very little of the radioactivity incorporated into DNA was lost during reincubation in the absence of amino acids. Control experiments indicated that repair did occur in *rec⁺* cells under these conditions (Fig. 17a), but even in the absence of degradation there was no evidence for repair after 63 ergs/mm² in the *recA* mutant examined (Fig. 17b). The absence of repair in *recA13* thus appears to be due to a deficiency in repair function rather than to the inhibition of repair by DNA breakdown (Smith and Meun, 1970). A similar conclusion has been reached concerning the deficiency of *recA* mutants for repairing X-ray induced single-chain breaks in DNA (Kapp and Smith, 1970a; see Section 5).

One *recB* and one *recC* mutant have also been examined for their ability to perform postreplication repair. Figure 18 presents the results obtained with the *recB* mutant. The *recC* strain behaved similarly. In both, DNA synthesized by irradiated cells sedimented more slowly than

* However, after only 15 ergs/mm² ($\sim 5\%$ survival) *recA13* (AB2487) shows no deficiency in its ability to perform postreplication repair. Similarly, *E. coli* B_{s-1} thy shows only a partial ability to perform postreplication repair after 63 ergs/mm², i.e., the slowly sedimenting pieces of newly synthesized DNA move part way toward the position of control DNA during 70 minutes of incubation, but after 30 ergs/mm² (survival was not determined after 30 ergs/mm², but after 10 ergs/mm² the surviving fraction was 2.6×10^{-3} ; Smith, 1969) *E. coli* B_{s-1} thy shows "complete" repair in about 50 minutes. These results suggest that these cells are not totally deficient in the gap-filling step of postreplication repair (K. C. Smith, unpublished observations).

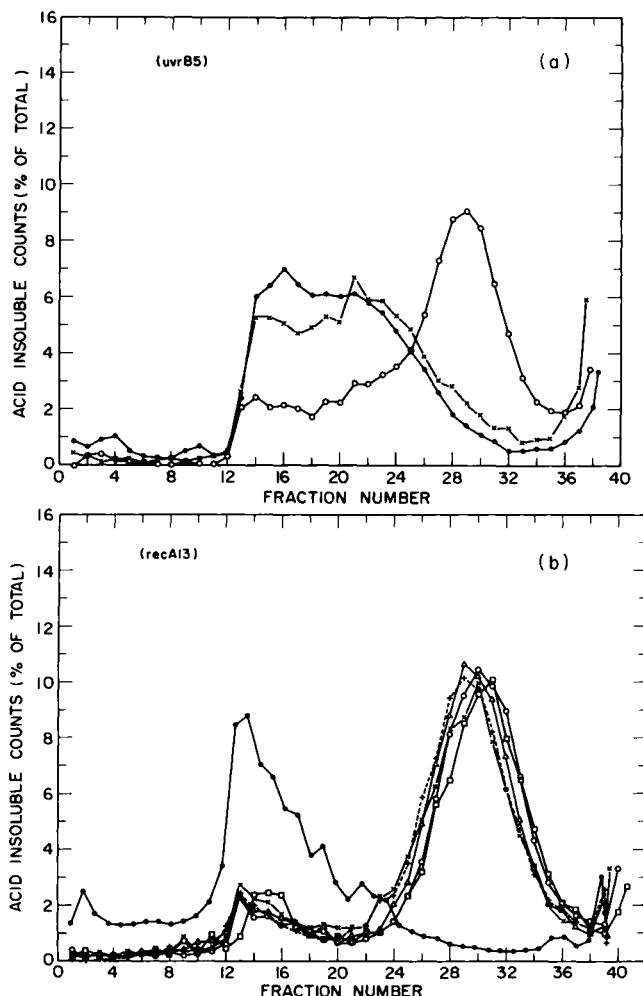


FIG. 17. Sedimentation in alkaline sucrose of DNA synthesized after UV irradiation by *E. coli* K-12 cells starved for glucose and amino acids for 90 minutes before irradiation. Glucose, but not amino acids, was added after irradiation. The cultures were incubated in the presence of ^3H -thymidine for 10 minutes and then processed as described in Fig. 9. (a) AB2499 (*rec*⁺ *uvrB5*). Unirradiated control (—●—●—); irradiated, but not reincubated after labeling (—○—○—) (when plated on minimal agar, 63% of the irradiated cells survived); irradiated, reincubated for 60 minutes after labeling (—×—×—). (b) AB2487 (*recA13* *uvr*⁺). Unirradiated control (—●—●—); irradiated, but not reincubated after labeling (—○—○—) (when plated on minimal agar, 0.4% of the irradiated cells formed colonies); irradiated, reincubated for 10 minutes after labeling (—△—△—); irradiated, reincubated for 20 minutes after labeling (—+—+—); irradiated, reincubated for 30 minutes after labeling (—□—□—); irradiated, reincubated for 60 minutes after labeling (—×—×—). (From Smith and Meun, 1970.)

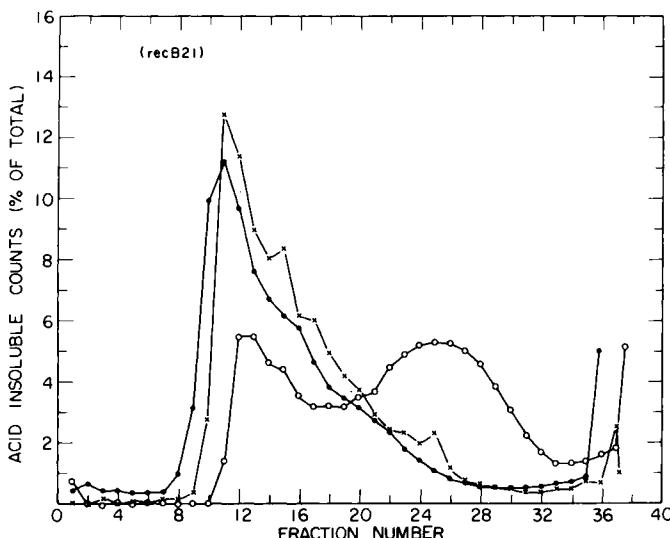


FIG. 18. Sedimentation in alkaline sucrose of DNA from irradiated cells of *E. coli* K-12 SR78 (*recB21 uvr^r*). The conditions were similar to those described in Fig. 9, except that the DNA was not prelabeled. Survival was not determined in this experiment, but in other experiments under the same conditions, the survival of this strain on minimal medium was 28%. Unirradiated control (—●—●—); irradiated, but not reincubated after labeling (—○—○—); irradiated, reincubated for 70 minutes after labeling (—×—×—). (From Smith and Meun, 1970.)

that made under the same conditions by unirradiated cells. In both, reincubation after labeling led to an increase in sedimentation rate of the labeled DNA. It appeared from this that *recB* and *recC* cells, unlike *recA* cells, were able to repair the majority of the defects formed in DNA synthesized after exposure to 63 ergs/mm² of UV radiation (Smith and Meun, 1970; Howard-Flanders *et al.*, 1971). The same appeared to be true for the *recC* mutant when examined after exposure to 120 and 180 ergs/mm² of UV radiation (Smith and Meun, 1970).

Since the *recB* and *recC* mutants appear to show no deficiency in the postreplication repair of gaps in daughter-strand DNA it suggests that the ATP-dependent nuclease coded by the *recB* and *recC* genes, while not being necessary for the gap-filling step, may either be necessary for some subsequent step in postreplication repair (Howard-Flanders *et al.*, 1971) or may be involved in a second type of recombinational repair (see Section 4).

RecB and *recC* mutants show a partial deficiency in the repair of X-ray-induced single-chain breaks in DNA (Kapp and Smith, 1970a; see Section 5).

4. New Recombinational Repair Systems?

It may be somewhat premature to speak of new recombinational repair systems when we are still uncertain of many features of the postreplication repair system discovered by Rupp and Howard-Flanders (1968). Nevertheless, there appears to be sufficient data available to suggest the presence of additional pathways for recombinational repair. This section presents several working hypotheses.

The *recA* mutants are "reckless" in the sense that they excessively degrade their DNA after irradiation, while *recB* and *recC* mutants are "cautious" and show less than normal degradation after irradiation (Howard-Flanders and Boyce, 1966). The *recA recB* and *recA recC* double mutants are "cautious" (Willetts and Clark, 1969). These data suggest that the *recB recC* nuclease is under the control of the *recA* gene product. In the absence of this control the *recB recC* nuclease functions in a "reckless" manner.

However, when the genetic control of postreplication repair was evaluated it was found that *recA* mutants could not perform the gap-filling step but *recB* and *recC* mutants could (Section 3). This suggests that functions other than that of the *recB recC* nuclease are also under the control of the *recA* gene.

If there is really only one recombinational repair mode then it must be postulated that the *recB recC* nuclease is involved in some step subsequent to the gap-filling step (Howard-Flanders *et al.*, 1971). If the *recB recC* gene function is only responsible for some step along the same pathway but subsequent to the gap-filling step of *rec* repair, then one would expect *recB recC* mutants to be just as sensitive to UV irradiation as are *recA* mutants. The *recB recC* mutants, in fact, are more resistant to killing by UV irradiation than are *recA* mutants. It seems reasonable, therefore, that the *recB* and *recC* genes may control a repair system that is at least partially distinct from the gap-filling repair system.

Assuming that there may be separate recombinational repair systems one may obtain a crude estimate of their relative importance by comparing the relative sensitivities of *rec⁺ uvr⁻*, *recA uvr⁻*, *recB uvr⁻* and *recC uvr⁻* mutants to UV irradiation. The doses of UV radiation required to kill these mutants to 10^{-3} survival (assayed on complex medium) are 120, 3, 33, and 33 ergs/mm², respectively (Ganesan and Smith, 1970; unpublished observations). The systems controlled by the *recA* gene repair 120 minus 3 or 117 ergs of damage. The system(s) controlled by the *recB* and *recC* genes repair 120 minus 33 or 87 ergs of damage, and 33 minus 3 or 30 ergs of damage are repaired by the *recA* system

in the absence of the *recB recC* system. Thus, about 75% of the total repair controlled by *recA* depends upon the *recB recC* nuclease. Rather than reflecting the efficiencies of two different processes for the repair of one type of lesion these results may reflect the relative proportion of two distinct types of lesions present in UV-irradiated DNA that are repaired with different efficiencies by the same process.

4.1 Predictions on the Molecular Basis of *recB recC* Repair

4.1.1 THE GAP-FILLING STEP IN *recB* AND *recC* MUTANTS MAY BE ABNORMAL

Since the gap-filling step of postreplication repair appears to proceed efficiently in *recB* and *recC* mutants (Section 3) but does not result in a marked increase in viability or a rapid return of normal DNA synthesis kinetics, it has been suggested that "The template strands constructed after UV-irradiation do not consist of normal continuous material able to form twin helical DNA free from branching or other structural errors" (Howard-Flanders *et al.*, 1971).

If branched chains of DNA are produced as an intermediate in recombinational repair, the branches would have to be removed by nuclease action. Therefore, in accordance with this hypothesis, the above data on viability would suggest that 25% of the gap-filling requires no cleaning action by the *recB recC* nuclease but 75% of the filled gaps do require this action.

4.1.2 THE *recB recC* NUCLEASE MAY BE REQUIRED TO REPAIR PARENTIAL STRAND BREAKS INDUCED BY THE DIRECT ACTION OF UV RADIATION

The radiation damage that is produced in DNA can be separated into two categories—base damage, and chain breaks. UV irradiation produces base damage primarily and very little is known about the production of chain breaks by the direct action of UV radiation. The opposite picture is true for X rays, i.e., chain breaks appear to be the lesions of major biological importance and little is known about the production of base damage *in vivo*.

In vitro studies on the production of single-chain breaks in DNA by the direct action of UV radiation (254 nm) have suggested that these breaks are produced with a low efficiency (Marmur *et al.*, 1961; Freifelder and Davison, 1963; Hutchinson and Hales, 1970). It is known, however, that the environment of the DNA has a marked effect upon its photochemical reactivity (Smith, 1967; Rahn, 1970). The fact that chain breaks are produced in low yield *in vitro*, therefore, in no way precludes their formation in higher (or lower) yield *in vivo*. Correspond-

ing studies *in vivo* apparently have not been performed. In order to demonstrate the production of single-strand breaks *in vivo* by the direct action of UV radiation, it may be necessary to inhibit their repair chemically or to use appropriate repair deficient mutants.

Since the *recB recC* nuclease appears to be required for the repair of some of the single-chain breaks produced in parental DNA by X irradiation (Section 5), it is suggested that this nuclease may also be involved in the repair of single-chain breaks produced in parental DNA by the direct action of UV radiation (hypothesis I).

Quinacrine potentiates the killing of UV-irradiated *rec⁺* cells but not of *recA* or *recB* cells (Section 6). This drug does not irreversibly inhibit the gap-filling step of postreplication repair in either *rec⁺* or *recB* cells. These results suggest that quinacrine may inhibit the *recB* repair system. If UV-irradiated *rec⁺* cells (1000 ergs/mm²; 0.5% survival) are incubated in growth medium for various times before incubation with quinacrine there is a progressive loss in sensitivity to quinacrine. After 90 minutes quinacrine no longer affects the viability of these cells suggesting that it may take 90 minutes to complete the *recB recC* repair (Z. Fuks and K. C. Smith, unpublished observations).

At least three approaches to testing hypothesis I may be suggested.

1. Look for the production of DNA chain breaks induced by the direct action of UV in *recB uvr⁻* mutants compared to *rec⁺ uvr⁻* mutants (measured immediately after irradiation).
2. Look for the production of DNA chain breaks induced by the direct action of UV in *pol⁻ uvr⁻* mutants compared to *pol⁺ uvr⁻* mutants (see Section 7).
3. Compare the yield of primary UV-induced chain breaks in *rec⁺ uvr⁻* cells with and without the immediate postirradiation addition of quinacrine.

4.1.3 THE *recB recC* NUCLEASE MAY BE REQUIRED TO MEND "REPAIR-INDUCED" BREAKS IN PARENTAL DNA

The *recB recC* nuclease appears to have several activities, an exonucleolytic function against single- and double-stranded DNA that requires ATP and an endonucleolytic function against single-stranded (but not double-stranded) phage fd circles (Section 2). Depending upon the isolation procedure the latter activity shows a variable requirement for ATP (S. Linn, personal communication). Assigning only one kind of repair to the *recB recC* system thus appears to be too restrictive.

One mechanism that has been postulated for the gap-filling step of postreplication repair is the insertion of parental material into the daughter-strand gap (Howard-Flanders *et al.*, 1971). Since this would produce gaps in the parental strands (Fig. 14) the *recB recC* gene

product may be important in the repair of these parental-strand gaps (hypothesis II).

The repair of the parental strands after the gap-filling step, however, may not be absolutely essential for survival (assuming that at least one daughter strand is completely repaired) but it may greatly improve the probability of successful repair by maintaining the continuity of the parental strands even though they may still contain the initial UV-induced lesions.

Hypothesis II can be tested in *recB uvr⁻* mutants by looking for enhanced fragmentation of the parental strands (compared to *rec⁺ uvr⁻* cells) during the postreplication gap-filling process. The effect of quinacrine on this process in *rec⁺ uvr⁻* cells should also be examined. Since this type of repair would be in response to the presence of pyrimidine dimers in DNA, photoreactivation should greatly diminish the necessity for this type of *recB recC* repair (in contrast with hypothesis I).

4.2 Minimal Medium Recovery (MMR)

4.2.1 DISCOVERY AND GENERAL PROPERTIES OF MMR

The extreme UV sensitivity of *rec* mutants of *E. coli* K-12 suggested that certain enzymatic steps in genetic recombination might also be common to a system for the repair of radiation damage to DNA (Howard-Flanders and Boyce, 1966). The first direct evidence that *rec⁺* cells had a radiation recovery system was the observation that after UV irradiation *rec⁺ uvr⁻* cells showed a higher survival on minimal growth medium than they did on complex growth medium. For example, after exposure to UV-radiation (254 nm), *E. coli* K-12 *uvrB5* showed a survival of 1.4×10^{-3} when plated on complex medium and a survival of 3.7×10^{-1} when assayed on minimal medium plates. Presumably some element of the complex medium inhibited the repair of radiation damage in these cells. This phenomenon has been called "minimal medium recovery," or MMR (Ganesan and Smith, 1968b). Since MMR is characteristic of excision-defective mutants, it evidently reflects a type of repair independent of the excision-repair process.*

* Complex medium has also been shown to adversely affect the excision repair system when studied in *rec⁻ uvr⁺* mutants (Ganesan and Smith, 1968a,b; 1969). Complex medium does not affect the survival of UV-irradiated *rec⁻ uvr⁻* cells or of *rec⁺ uvr⁺* cells. The absence of an effect on *rec⁻ uvr⁻* mutants may be explained on the basis that they possess no repair systems to be inhibited. The absence of an effect on the *rec⁺ uvr⁺* strains may be explained (albeit weakly) on the basis of either (1) the inhibition observed in the *rec⁻ uvr⁺* and *rec⁺ uvr⁻* strains somehow prevented in the *rec⁺ uvr⁺* strain, or (2) the partial inhibition of excision repair and MMR by complex medium is compensated for by the action of the postreplication repair system which is not inhibited by complex medium.

The MMR characteristic of excision-defective cells depends upon the presence of functional *rec⁺* genes. This conclusion is based upon the observation that excision-defective cells that are also mutant at *recA18*, *recA56*, *recB21*, or *recC22* failed to show MMR (representative data for *recB21 uvrB5* are shown in Fig. 19). Further proof came from the observation that *rec⁺ uvr⁻* derivatives obtained from *rec⁻ uvr⁻* strains by transduction or by reversion regained the capacity for MMR. Thus inactivation of any one of the three genes, *recA*, *recB*, or *recC* prevents *uvr⁻* cells from showing MMR (Ganesan and Smith, 1970).

The inhibition of recovery by complex medium is at least partially irreversible. When incubated in complex medium after UV irradiation, *rec⁺ uvr⁻* cells do not recover and gradually lose the capacity to recover when switched to minimal medium (Fig. 20).

The inhibition of MMR by complex medium does not appear to be complete since survival of UV-irradiated *rec⁺ uvr⁻* cells on complex medium is always higher than the survival of irradiated *rec⁻ uvr⁻* cells on minimal or complex medium (Fig. 19).

The discrepancy between the survival of UV-irradiated *E. coli* K-12 (*rec⁺ uvr⁻*) cells when plated on minimal or complex medium is not

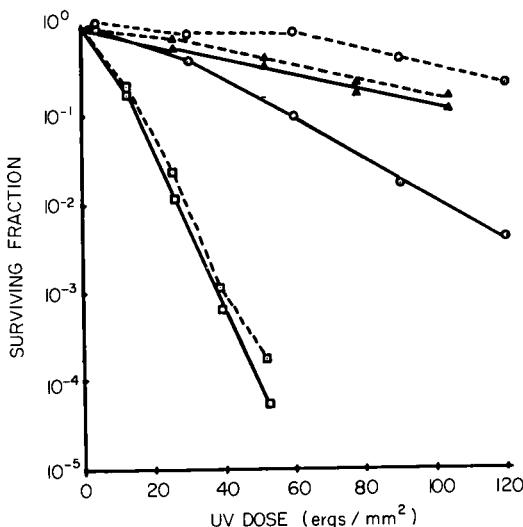


FIG. 19. Effect of plating medium on the survival of *E. coli* K-12 SR87 (*recB21 uvrB5*) compared to its parents, AB2499 (*rec⁺ uvrB5*) and JC5743 (*recB21 uvr⁺*). Cultures growing exponentially in minimal medium were UV-irradiated (254 nm) and plated on yeast extract-nutrient agar (solid lines) or minimal medium (dotted lines). \odot represents AB2499; Δ , JC5743; \square , SR87. (From Ganesan and Smith, 1970.)

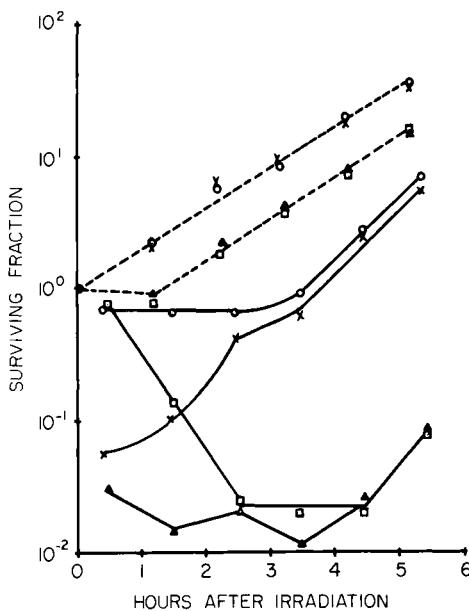


FIG. 20. Effect of liquid media on the recovery of *E. coli* K-12 AB2499 (*rec*⁺ *uvrB5*). Cultures growing exponentially in minimal medium were irradiated (72 ergs/mm²), and then incubated in liquid minimal medium or yeast extract-nutrient broth (YENB) liquid. At intervals, samples were plated on minimal medium or YENB agar. Solid lines indicate irradiated cultures, broken lines indicate unirradiated controls. O, Incubated in minimal medium, plated on minimal medium agar; X, incubated in minimal medium, plated on YENB agar; Δ, incubated in YENB liquid, plated on YENB agar; □, incubated in YENB liquid, plated on minimal medium agar. The increase in colony-forming units among the irradiated cells after 2.5 hours in minimal medium, and after 3.5 hours in YENB liquid probably reflects growth. (From Ganesan and Smith, 1970.)

greatly modified by whether they are grown prior to irradiation in complex or minimal medium (A. K. Ganesan, unpublished observations).

Recombination frequencies are greatly reduced if, after mating, the cells are plated on minimal medium versus supplemented medium (Riley and Pardee, 1962; Gross, 1963). This appears contradictory to the observation that another function controlled by the *rec* genes, the survival of *uvr*⁻ mutants after UV irradiation as exemplified in MMR, is markedly depressed in the presence of complex medium.

It appears that the induction of a mutation by UV irradiation requires a recombinational event. UV mutability is either eliminated or significantly reduced in strains carrying a *recA1* or *recC22* mutation (Witkin, 1969a). It is therefore of interest that the yield of UV-

induced mutations is increased if cells are grown on complex medium instead of minimal medium after UV irradiation (Witkin, 1961, 1966; Cheung and Bockrath, 1970; Hill, 1968; Bridges *et al.*, 1967; Doudney, 1968).

The depression of the viability of UV-irradiated cells by complex medium (MMR) is controlled by the *recA*, *recB*, and *recC* genes. We have assumed that the *recB* and *recC* gene functions lie on a repair pathway separate from the gap-filling step of postreplication repair and that it is this former pathway that is inhibited by complex medium. This is consistent with the hypothesis (Witkin, 1969b; Bridges *et al.*, 1968) that the gap-filling step of *rec* repair (which is not inhibited by complex growth medium; see below) is the "error-prone" step. Conversely, these results may be interpreted to suggest that the *rec* repair system inhibited by complex medium can efficiently and accurately repair premutational UV damage.

Complex growth medium does not appear to affect the yield of X-ray-induced mutations (Munson and Bridges, 1966). When grown on minimal medium and plated on minimal or complex medium, *uvrB5* strains (but not *uvrA6* or *uvrC34* strains) show MMR after X irradiation under air or nitrogen (S. Phinney and K. C. Smith, unpublished observations).

4.2.2 Is MMR THE SAME AS POSTREPLICATION REPAIR?

The discovery of MMR is excision-defective cells (Ganesan and Smith, 1968a,b) came at about the same time as the report by Rupp and Howard-Flanders (1968) describing discontinuities in the DNA synthesized in excision-defective cells after UV irradiation. It was hypothesized in this laboratory that the recovery in viability seen in MMR might be due to the repair of the discontinuities in the daughter strands of DNA in UV-irradiated *uvr-* mutants. If this were true then switching *rec⁺ uvr-* cells to complex medium after UV irradiation should inhibit the repair of these discontinuities.

To test this hypothesis, *E. coli* K-12 *uvrB5* cells were irradiated (63 ergs/mm² at 254 nm), grown for 10 minutes in the presence of ³H-thymidine to label the newly synthesized DNA, and then were switched either to nonradioactive minimal growth medium or to complex growth medium (0.75% Difco yeast extract, 2.3% Difco nutrient broth) and incubated for 70 minutes. The cells were then lysed and the DNA sedimented in alkaline sucrose gradients as described in Fig. 9. The gradient data (not shown) indicated that there was no difference in the amount of repair in the two types of culture media, that is, the complex medium did not appear to inhibit the repair of the discontinuous strands of newly synthesized DNA (K. C. Smith, 1968, unpublished observations). Thus, to express its adverse effect on viability, complex medium

must adversely affect some step in this postreplication repair system other than the gap-filling step or may affect some new repair mechanism.

4.2.3 PREDICTIONS ON THE MOLECULAR BASIS OF MMR

A mutation of *recA*, *recB*, or *recC* inactivates MMR. The repair system underlying MMR thus appears to be a *rec* repair system. Since complex medium inhibits MMR but not the gap-filling step of postreplication *rec* repair, MMR may therefore be an expression of *recB recC* repair.

Since MMR, as defined, is only observed in *rec⁺ uvr⁻* cells it suggests that the repair system underlying MMR may respond to the presence of pyrimidine dimers in DNA. This is confirmed by the observation that photoreactivation eliminates MMR in *rec⁺ uvr⁻* cells (A. K. Ganesan and K. C. Smith, unpublished observations). These results suggest that MMR may be an expression of the type of *recB recC* repair designated as hypothesis II (Section 4.1.3).

4.3 Possible Pathways of Recombinational Repair

Figure 21 attempts to summarize schematically the hypotheses set forth in this section, the assumption being that UV radiation produces two types of damage in DNA, base damage and chain breaks. Base damage is bypassed during postirradiation DNA synthesis leaving gaps in the daughter strands (Fig. 14). The *recA* mutants cannot repair these daughter-strand gaps but *recB* and *recC* mutants can. These gaps are filled with parental material, thus producing gaps in the parental strands. The *recB recC* pathway repairs these "repair-induced" gaps in the parental strands (hypothesis II). In addition the *recB recC* pathway may repair parental strand breaks produced by the direct action of UV radiation (hypothesis I).

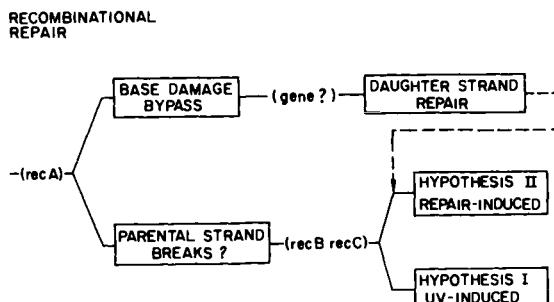


FIG. 21. Proposed pathways for the recombinational repair of UV-damaged DNA. See Section 4.3 for a description of these pathways. Those segments having a question mark or labeled hypothesis are not proven.

5. Recombinational Repair of X-Ray-Induced Damage

The major lesion induced in DNA by aerobic X irradiation appears to be the chain break. Single-chain breaks have been shown to be repaired in a resistant (*E. coli* B/r) but not a sensitive (*E. coli* B_{s-1}) strain of bacteria (McGrath and Williams, 1966). Double-chain breaks do not appear to be repaired in *E. coli* and the production of double-chain breaks has been correlated with the killing of wild-type *E. coli* by X irradiation (Kaplan, 1966). However, it has been suggested that single-strand breaks in DNA may be lethal to those cells unable to repair these lesions (McGrath and Williams, 1966; Kaplan, 1966). Cells that are deficient in the excision repair of UV damage (*uvrB5*) show little or no increased sensitivity to killing by aerobic X irradiation compared with wild-type cells. However, cells that are deficient in genetic recombination are very easily killed by X rays (Fig. 22). This suggests that the genes that control genetic recombination also control a system for the repair of X-ray damage.

These two suggestions—that the *rec* genes control a repair system for X-ray damage and that unrepaired single-chain breaks in DNA can

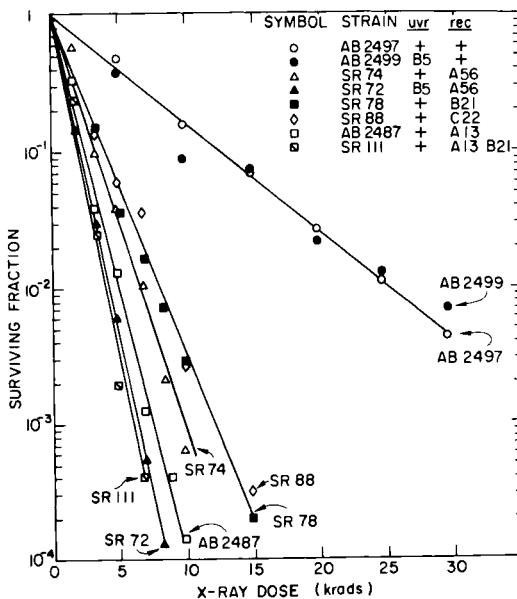


FIG. 22. The X-ray sensitivities of *uvr* and *rec* mutants of *E. coli* K-12. Cells in exponential growth were irradiated in minimal medium supplemented with required metabolites (SMM), diluted in double-distilled water, plated on SMM-agar, and incubated from 24–72 hours at 37°C. (From Kapp and Smith, 1970a.)

be lethal—have been tested and found to be correct (Kapp and Smith, 1970a). The analytical procedure used was essentially that described by McGrath and Williams (1966). Bacterial cells were *prelabeled* in their DNA by growth in radioactive thymine or thymidine. After irradiation the cells were either reincubated in nonradioactive growth medium for different periods of time or were immediately converted to spheroplasts by treatment with lysozyme and EDTA and then floated on a layer of 0.5 N NaOH on top of a linear sucrose gradient (5–20%) in 0.1 N NaOH. The spheroplasts ($\sim 10^6$) were very gently mixed into the 0.5 N NaOH with a pin and the gradients were spun in an ultracentrifuge under appropriate conditions. The bottoms of the plastic gradient tubes were punctured and drops were collected and assayed for radioactivity content to obtain the distribution of the DNA in the gradient. The strong NaOH used in this procedure denatures the DNA into single strands. Since the position of the DNA in the gradient is proportional to its molecular weight, the production and repair of single-chain breaks in DNA can be easily followed. Although the number of single-chain breaks present in bacterial DNA following irradiation (22 krads) was essentially the same for all strains tested (however, see Section 7), there was a considerable variation in the ability of the various *rec* mutants to repair these chain breaks (Kapp and Smith, 1970a). A typical experiment for wild-type *E. coli* K-12 is shown in Fig. 23.

By comparing the sedimentation profile of DNA from unirradiated *rec⁺* cells (Fig. 23, curve A) with that from irradiated cells (Fig. 23, curve B) it can be seen that X-irradiation produced a decrease in the molecular weight of the bacterial DNA. A comparison of the sedimentation profiles obtained after 20, 30, and 60 minutes postirradiation incubation (Fig. 23, curves C–E) shows that reincubation resulted in a progressive shift in the sedimentation profiles toward that of the unirradiated cells. This restoration (which was complete in 40–60 minutes) reflects the functioning of a repair process that rejoins broken pieces of parental DNA with bonds stable in alkali.

The extent of repair that occurs in the *uvrB5 rec⁺* mutant cannot be distinguished from that which occurs in the *uvr⁺ rec⁺* strain (Kapp and Smith, 1970a). A mutation at *uvrB5* therefore has little effect upon the repair of X-ray-induced single-strand breaks in DNA or upon survival after aerobic X irradiation (Fig. 22).

In contrast, the *recA56* mutant appears unable to rejoin X-ray-induced single-strand breaks to any great extent even after a post-irradiation incubation period of 70 minutes (Fig. 24). Similar findings have been reported for another *recA* derivative of *E. coli* K-12 (JC1569b) after a lower dose of irradiation (7.5 krads) (Morimyo *et al.*, 1968).

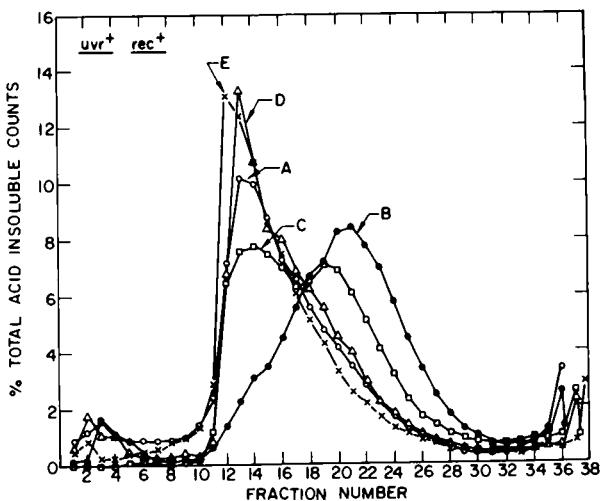


FIG. 23. Sedimentation patterns of DNA from X-irradiated *E. coli* K-12 AB2497 (*uvr*⁺ *rec*⁺). A, Unirradiated; B, 22 krads, no reincubation; C, 22 krads, 20-minute reincubation; D, 22 krads, 30-minute reincubation; E, 22 krads, 60-minute reincubation. Cells whose DNA was labeled with ^3H -thymidine were converted to spheroplasts and lysed on top of alkaline sucrose gradients (5–20% sucrose in 0.1 N NaOH). Sedimentation was performed in an SW 50.1 rotor for 105 minutes at 30,000 rpm at 20°C. The direction of sedimentation is from right to left. (From Kapp and Smith, 1970a.)

The gradient results of *recA* mutants are complicated by a decrease in the total number of acid insoluble counts with increasing incubation time (from 2.63×10^4 to 6.77×10^3 counts/min in 70 minutes) and the appearance of an additional low molecular weight peak near the meniscus. These complications are a consequence of the extensive DNA degradation that occurs in *recA* derivatives following both UV and X irradiation (Clark *et al.*, 1966; Morimyo *et al.*, 1968). To circumvent this problem of degradation, a *recA13 recB21* double mutant was employed. This double mutant shows the low level of DNA degradation following UV irradiation characteristic of strains carrying *recB21* alone (Willetts and Clark, 1969). A similar low level of degradation of DNA by this double mutant was found following X irradiation. The sedimentation profiles obtained (Fig. 25) show that like the *recA56* mutant, this *recA13 recB21* derivative exhibits only a small capacity for the repair of X-ray-induced single-strand breaks. The absence of repair by the *recA* mutants thus appears to be due to a deficiency in repair function rather than to the inhibition of repair by DNA breakdown (Kapp and Smith, 1970a).

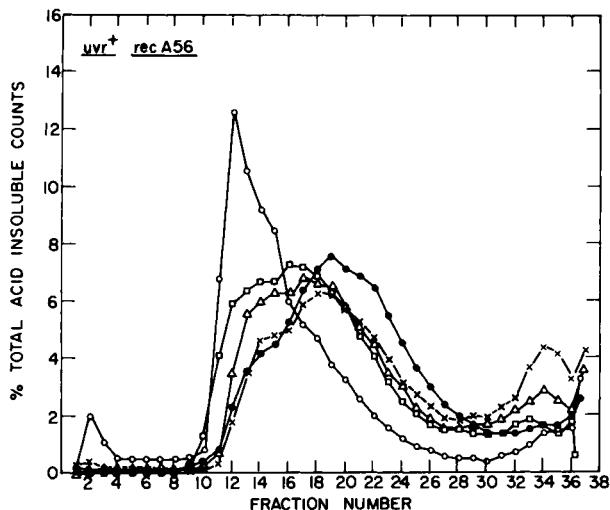


FIG. 24. Sedimentation patterns of DNA from X-irradiated *E. coli* K-12 SR74 (*uvr*⁺ *recA56*). ○, Unirradiated; ●, 22 krads, no reincubation; □, 22 krads, 20-minute reincubation; △, 22 krads, 40-minute reincubation; ×, 22 krads, 70-minute reincubation. Lysis and sedimentation were performed as in Fig. 23. (From Kapp and Smith, 1970a.)

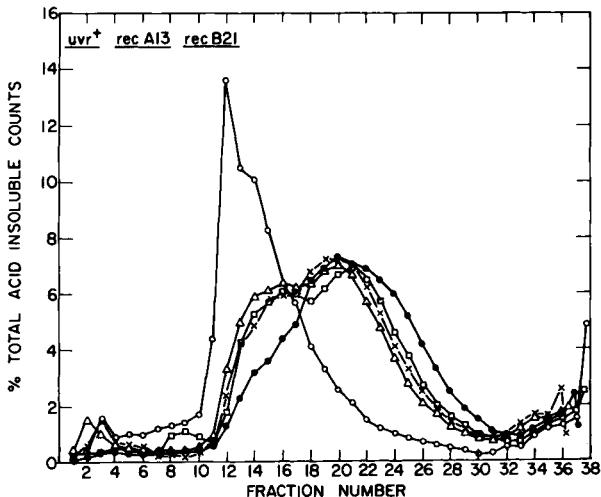


FIG. 25. Sedimentation patterns of DNA from X-irradiated *E. coli* K-12 SR111 (*uvr*⁺ *recA13* *recB21*). ○, Unirradiated; ●, 22 krads, no reincubation; □, 22 krads, 20-minute reincubation; △, 22 krads, 40-minute reincubation; and ×, 22 krads, 60-minute reincubation. Lysis and sedimentation were performed as in Fig. 23. (From Kapp and Smith, 1970a.)

The sedimentation profiles of the DNA from the *recC22* derivative showed that some rejoining of single-strand breaks did occur (Fig. 26). However, the shift in the sedimentation patterns for the reincubated cells was not as substantial as that for the *rec⁺* cells and an appreciable amount of the DNA remained in the nonrepaired low molecular weight DNA peak. The rejoining that did occur was accomplished during the first 30 minutes of incubation. A similar result was obtained for the *recB21* mutant (Kapp and Smith, 1970a).

X radiation can produce DNA chain breaks by several different mechanisms (Fig. 27). This results in the formation of chains having termini of different chemical composition (Kapp and Smith, 1970b). It has been suggested that nucleases may be necessary to 'clean' the termini before repair can occur (Kapp and Smith, 1968). The results shown in Fig. 26 indicate that only a portion of the X-ray-induced chain breaks can be repaired in the absence of the *recB recC* nuclease. When the specificity of this nuclease is better understood we may then hope to know the types of X-ray-induced DNA chain breaks that can and cannot be repaired in cells deficient in this enzyme.

Thus *rec⁺* cells (whether *uvr⁺* or *uvrB5*) are resistant to killing by aerobic X irradiation and have the capacity to repair single-chain

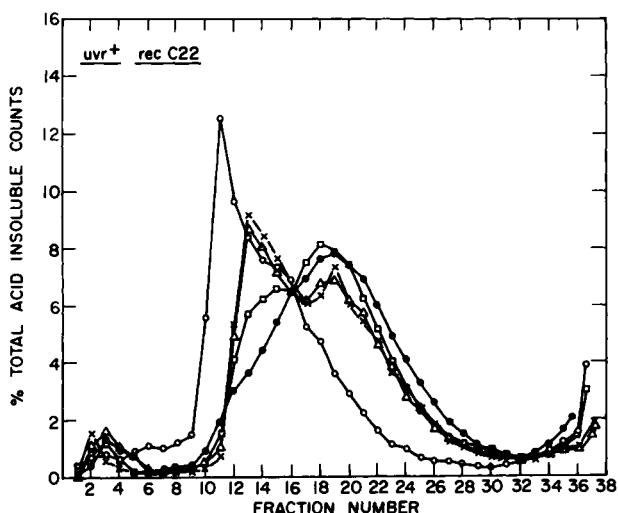


FIG. 26. Sedimentation patterns of DNA from X-irradiated *E. coli* K-12 SR88 (*uvr⁺ recC22*). ○, Unirradiated; ●, 22 krads, no reincubation; □, 22 krads, 10-minute reincubation; △, 22 krads, 30-minute reincubation; ×, 22 krads, 60-minute reincubation. Lysis and sedimentation were performed as in Fig. 23. (From Kapp and Smith, 1970a.)

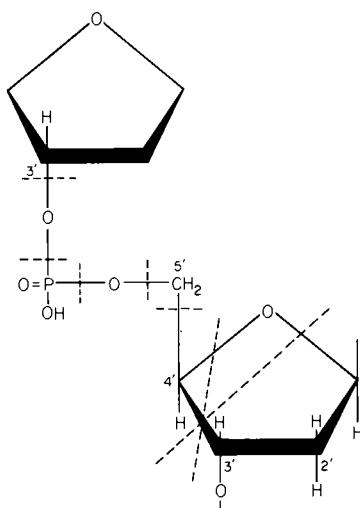


FIG. 27. Possible mechanisms for the formation of single-strand breaks in DNA. (From Kapp, 1970.)

breaks in DNA. The *recA* strains are deficient in genetic recombination, are sensitive to killing by X irradiation and show little capacity to repair X-ray-induced single-chain breaks in DNA (however, see Section 7). This is true even under conditions where the postirradiation degradation of DNA characteristic of *recA* strains is prevented (Fig. 25). The *recB* and *recC* mutants show an intermediate deficiency in genetic recombination, an intermediate sensitivity to killing by X irradiation and an intermediate ability to repair single-chain breaks in DNA. There is a correlation, therefore, between the inability to repair single-strand breaks and the radiosensitivity of the *rec*⁻ mutants of *E. coli* K-12.

This correlation between the inability to repair single-chain breaks and the radiosensitivity of bacteria is further documented by studies using drugs that appear to selectively inhibit (in *rec*⁺ strains) the recombinational repair of X-ray-induced single-chain breaks in DNA (see Section 6).

Since the repair of X-ray-induced single-chain breaks is mediated in part (see Section 7) by the genes controlling genetic recombination, one may speculate as to the biochemical mechanism by which these single-chain breaks are repaired. After UV irradiation, the gaps that need to be repaired are in the newly synthesized daughter strands (Fig. 14). At this stage these cells contain at least four strands of DNA that can be used for recombinational processes to give at least one un-

damaged genome. In the case for X-rays, however, immediately after irradiation the breaks are in the parental strands. One may then ask where the extra DNA required for recombinational events can come from. This may come from additional nuclei within the cells since it has been shown that diploid yeast cells are more resistant to X-ray inactivation than are haploid cells (Mortimer, 1958). However, the complete sequence of recombinational events may not be required for the repair of X-ray-induced single-chain breaks. It is possible that only a few of the enzymes normally required for genetic recombination are used in the repair of X-ray-induced single-chain breaks, e.g., specific nucleases to clean the termini of the broken chains. Moreover, it is not known whether the repair of X-ray-induced chain breaks takes place before, after or during DNA replication.

6. Drugs and Physiological Factors Affecting Recombinational Repair

6.1 Introduction

A basic technique in biology for gaining information about a particular system is to perturb the system and see how it responds. This technique has been used to study repair systems. One approach has been to grow bacteria under various conditions, e.g., minimal or rich medium, before and/or after irradiation and to see what effect this has on the survival of the irradiated cells. The phenomenon of minimal medium recovery has already been discussed in Section 4.2, but other modifications of growth medium that modify repair will be presented in this section.

Another approach has been to use metabolic inhibitors to determine, for example, whether DNA, RNA, or protein synthesis is required for a particular repair system to function. A practical application of the chemical inhibition of repair is the potential use of drugs in conjunction with radiation therapy for the more effective treatment of cancer. This point will be further discussed below.

6.2 Physiological Factors Affecting Repair

Previous reviews have dealt with growth conditions that affect the viability of irradiated bacteria (Rupert and Harm, 1966; Stapleton, 1960). The material to be presented here will be largely restricted to those conditions that have been shown to directly affect repair processes or *not* to affect the viability of specific mutants (thus implying an effect on a particular type of repair process in nonmutant strains).

6.2.1 LOG VS. STATIONARY PHASE

The X-ray sensitivity of *E. coli* B/r varies with its position in the growth cycle (Stapleton, 1955). The effect of growth phase on the production and repair of X-ray-induced single-chain breaks in DNA has been investigated by Town *et al.* (1971a). Late log phase *E. coli* B/r cells are 1.6 times more sensitive to killing by X rays than are stationary phase cells when grown in Brain Heart Infusion (BHI) + glucose. The number of single-chain breaks formed per krad is the same for log and stationary phase cells (see also Ginsberg and Webster, 1969). Stationary phase cells show a somewhat greater ability to repair single-chain breaks (especially after high doses of X rays) than do log phase cells. The rapidity and extent of DNA degradation after X irradiation by log phase cells is greater than for stationary phase cells. The enhanced viability exhibited by stationary phase cells thus appears to correlate both with enhanced single-chain break repair and the reduced degradation of DNA (Town *et al.*, 1971a).

The UV sensitivity of *E. coli* B/r has been found to be greater in log phase than in stationary phase growth (Morton and Haynes, 1969). Similar results have been obtained for *E. coli* 15 TAU (Ginsberg and Jagger, 1965a).

A repair-deficient strain, *E. coli* B_{s-1}, does not show this growth phase dependent variation in UV sensitivity (Morton and Haynes, 1969), suggesting that the response depends upon the presence of repair functions. *E. coli* B_{s-1}, however, is mutant at three loci (Fil, Exr, Her) that affect radiation sensitivity (Witkin, 1968). It would therefore be of interest to use mutants each deficient in only one repair system and thus to determine if the excision (*uvr*) or the *rec* repair system (or both) are responsible for this phenomenon.

There is negative evidence available to suggest that the *rec* repair system may be of major importance in determining the resistance to UV of stationary phase cells. Starving log phase cells for required amino acids and/or uracil prior to irradiation yields cells that have radiation sensitivities similar to stationary phase cells (Ginsberg and Jagger, 1965b). This type of treatment does not appear to enhance excision repair (Sedliakova *et al.*, 1970).

6.2.2 STATIONARY PHASE ± GLUCOSE

E. coli B/r grown to stationary phase in complex medium (peptone) are more sensitive to killing by X rays than are cells grown to stationary phase in complex medium to which glucose has been added (Stapleton and Engel, 1960; Stapleton and Fisher, 1967). The effect of these growth

conditions on the production and repair of X-ray-induced single-chain breaks in DNA has been investigated (Town *et al.*, 1971a).

E. coli B/r cells grown to stationary phase in peptone medium (PO cells) are 3.4 times more sensitive to killing by X rays than cells grown to stationary phase in peptone medium supplemented with glucose and phosphate buffer (PG cells). The yield of single-strand breaks is the same for both types of cells (but the absolute yield is higher than in the cells grown in BHI + glucose). The kinetics for the repair of single-chain breaks are the same for both types of cells for about 30 minutes. After this time period further repair ceases in the PO cells but continues in the PG cells provided that glucose is present in the medium. Postirradiation DNA degradation is both more rapid and more extensive in PO cells than in PG cells whether incubated in their preirradiation medium or in reciprocal medium. The survival of stationary phase *E. coli* B/r grown in PO or PG medium is likewise unaffected by the presence of glucose in the plating medium, and thus correlates better with the lower DNA degradation seen in the PG cells than with the increased strand rejoining, since this latter process requires the presence of glucose. An alternative explanation is that these differences in degradation may reflect a difference in the number of un-repaired single-chain breaks, which was too small to be detected by sedimentation analysis after low doses of X rays, but is nevertheless the true determinant of the difference in viability (Town *et al.*, 1971a).*

Although the glucose effect is shown by *E. coli* B/r it is not shown by the radiation sensitive strain B_{s-1} (Stapleton and Fisher, 1967). In *E. coli* K-12 the integrity of the *rec* genes (Friesen *et al.*, 1970) and the *lon* gene (Adler and Hardigree, 1964) are required for the glucose effect to be expressed. Although as sensitive as the *rec* strains to X irradiation, the DNA polymerase deficient strain *polA1* still shows the glucose effect (unpublished observations cited in Town *et al.*, 1971a). Since mutations in the *rec* genes of *E. coli* K-12 appear to affect both the rejoining of DNA single-strand breaks (Kapp and Smith, 1970a) and the glucose effect on stationary phase cells (Friesen *et al.*, 1970), a fully functional rejoining system (and/or a normally controlled degradation system) appears to be required for the expression of the glucose effect on survival after X irradiation.

*There appears to be no simple explanation for the extensive breakdown of DNA observed after X irradiation in bacterial cells (for a review see Pollard, 1970). Does DNA breakdown prevent repair or is it a normal part of repair, e.g., excision repair? Is DNA breakdown the reflection of the inability to repair, i.e., unrepairs chain breaks acting as nuclease attachment sites? The degradation seen in X-irradiated cells is probably some combination of all these factors.

6.2.3 AMINO ACID STARVATION

Starvation of cells for essential amino acids inhibits protein (and RNA) synthesis but allows DNA synthesis to go to completion, i.e., chromosomes in the process of replication are completed but new rounds of replication are not initiated. This type of starvation makes repair proficient strains of *E. coli* more resistant to UV irradiation (Hanawalt, 1966; Smith *et al.*, 1966), but does not affect the sensitivity of a repair deficient strain (*E. coli* B_{s-1}) to UV (Hanawalt, 1966). This resistance to UV irradiation does not appear to be due to an enhancement of the excision-repair process (Sedliakova *et al.*, 1970) (however, see Billen and Bruns, 1970). An obvious alternative, therefore, is that this treatment enhances the efficiency of the *rec* repair system. The starving of *rec*⁺ *uvr*⁻ cells for required amino acids for two hours prior to UV irradiation markedly enhances minimal medium recovery, a phenomenon that depends upon functional *rec* genes (Ganesan and Smith, 1970) (see Section 4). The effect of prior amino acid starvation on the gap-filling process of *rec* repair, however, has not been reported.

Amino acid (and/or uracil) starvation prior to irradiation also makes bacteria more resistant to killing by X rays (Ginsberg and Jagger, 1965b; Billen and Bruns, 1970), again suggesting that *rec* repair may be involved.

6.3 Drugs that Irreversibly Inhibit Repair

This section does not attempt to be an exhaustive review of drugs that affect the survival of irradiated bacteria but is meant to indicate a systematic approach to the problem of explaining how these drugs act. This is now facilitated by the fact that several new systems for the repair of damage to DNA have been described. We will not be concerned here with the trivial inhibition of repair, i.e., by compounds that inhibit repair by inhibiting metabolism but whose effect is completely reversible and therefore has no additional effect on the viability of irradiated cells. The key word, therefore, is irreversibility.

6.3.1 AN IMPURITY IN HYDROXYUREA

The impact of describing a new biological effect is somewhat lessened if the structure of the compound producing the effect is unknown. Nevertheless, the observations on the ability of an impurity in certain commercial preparations of hydroxyurea (HU) to inhibit the repair of radiation damage serve as a model for the screening of other inhibitors of repair and therefore will be described here.

While investigating the possible effects of DNA synthesis inhibitors

on the repair of X-ray-induced single chain breaks in DNA it was observed that certain commercial preparations of hydroxyurea (which later proved to be chromatographically impure) inhibited this repair. Figure 28 demonstrates that irradiated cells of *E. coli* K-12 reincubated in growth medium were able to repair single-chain breaks in their DNA, those incubated in the presence of impure HU were not.

To investigate the reversibility of the impure HU-induced blockage

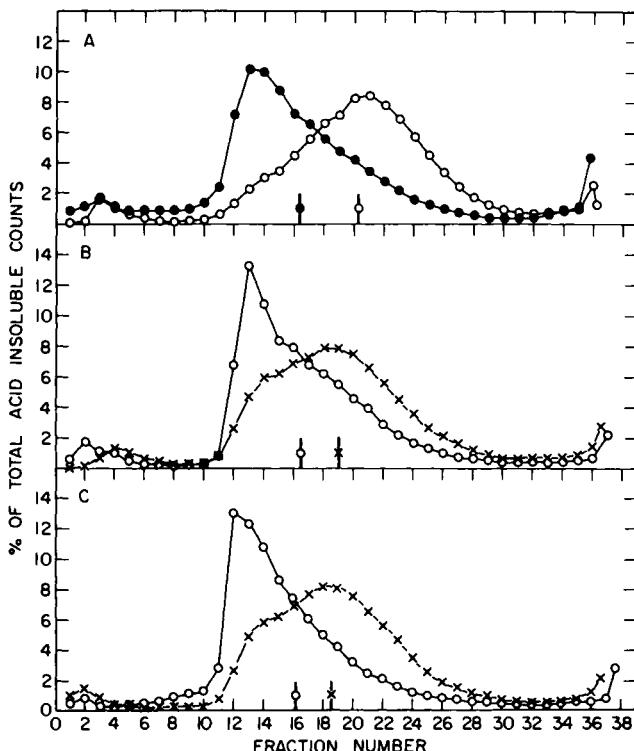


FIG. 28. Effect of chromatographically impure HU (Nutritional Biochemicals Corp.) on the repair of single-strand breaks in *E. coli* K-12 *rec*⁺ (AB2497) cells. Cells in exponential growth labelled with ³H-thymine were irradiated, converted to spheroplasts and lysed on top of alkaline sucrose gradients (5–20% sucrose in 0.1 N NaOH). Sedimentation was performed in a SW 50.1 rotor for 105 minutes at 30,000 rpm at 20°C. The direction of sedimentation is from right to left. The short vertical bars under the peaks indicate the first moments of the distribution of radioactivity. Less than 50% of the DNA was degraded during the 60-minute reincubation and HU treatment had no effect on the amount of degradation. A, No incubation: ●, Unirradiated. ○, 22 krads; B, 30 minutes postirradiation incubation in growth medium with or without 0.1 M HU: ○, 22 krads; no HU; ×, 22 krads, with HU; C, 60 minutes incubation: ○, 22 krads, no HU; ×, 22 krads, with HU. (From Kapp and Smith, 1971.)

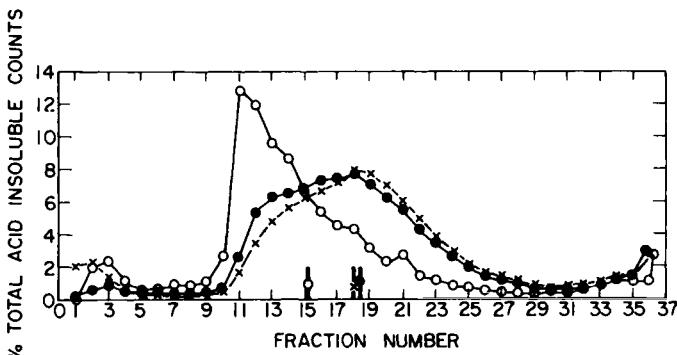


FIG. 29. Lack of repair of single-chain breaks in X-irradiated *E. coli* K12 *rec*⁺ (AB2497) cells during incubation in impure HU (Nutritional Biochemicals Corp.) or following removal of HU. Lysis and sedimentation were performed as in Fig. 28. ○, 22 krads incubated 60 minutes in growth medium without HU; X, 22 krads, incubated 60 minutes in growth medium containing 0.1 M HU; ●, 22 krads, incubated 60 minutes in growth medium containing 0.1 M HU and then for an additional 60 minutes in growth medium without HU. (From Kapp and Smith, 1971.)

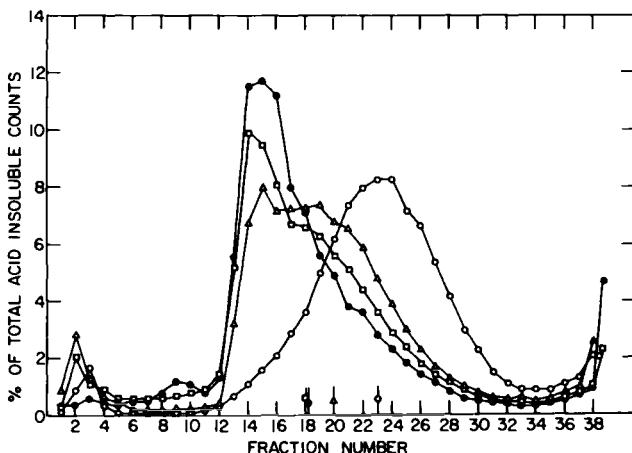


FIG. 30. Reversal of the partial inhibition of repair of X-ray-induced single-chain breaks in *E. coli* K-12 *rec*⁺ (AB2497) cells following the removal of chromatographically pure HU (Pierce Chemical Company). Lysis and sedimentation were performed as in Fig. 28. ●, Unirradiated cells, no incubation; ○, 22 krads, no incubation; △, 22 krads, incubated 60 minutes in growth medium containing 0.1 M HU; □, 22 krads, incubated 60 minutes in growth medium containing 0.1 M HU and then washed and incubated for 60 additional minutes in growth medium without HU. (From Kapp and Smith, 1971.)

of repair of single-strand breaks, prelabeled *rec⁺* cells in exponential growth were irradiated and reincubated in growth medium containing 0.1 M HU for 60 minutes. The HU was removed by filtration and the cells were reincubated for an additional 60 minutes in growth medium. The samples were then sedimented in alkaline sucrose gradients. The rejoining of single-strand breaks was inhibited during the 60-minute incubation in the presence of impure HU and continued to be inhibited after the removal of the HU and subsequent reincubation in growth medium for 60 minutes (Fig. 29). Therefore the chromatographically impure samples of HU irreversibly inhibited the repair of X-ray-induced single-strand breaks. In contrast, chromatographically pure samples of HU (Pierce Chemical Company) produced only a partial blockage of single-strand break repair which was reversible upon removal of the pure HU (Fig. 30).

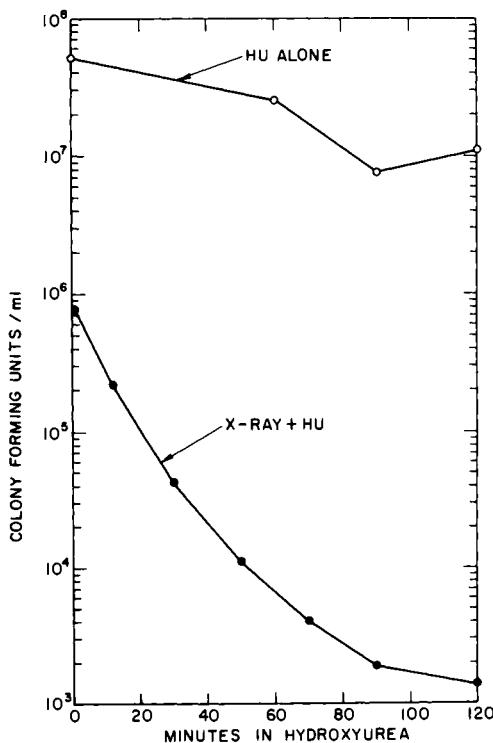


FIG. 31. Postirradiation treatment of *E. coli* K-12 *rec⁺* (AB2497) cells with 0.1 M impure HU (Nutritional Biochemicals Corp.) in growth medium at 37°C. The number of viable cells is plotted as a function of time in 0.1 M HU subsequent to X irradiation. ○, HU alone; ●, 17.6 krads irradiation then HU treatment. (From Kapp and Smith, 1971.)

Since the repair of X-ray-induced single-chain breaks is important for the survival of irradiated *rec⁺* cells (Kapp and Smith, 1970a), treatment of irradiated cells with an agent that inhibits the repair of single-chain breaks should sensitize *rec⁺* cells to X-ray-induced killing, but should have little effect on *recA* mutants which are deficient in the ability to repair single-strand breaks. To test this hypothesis, irradiated cells were treated with impure HU for various periods of time before plating for viability. Figure 31 shows that impure HU markedly potentiated the killing of X-irradiated *rec⁺* cells. In contrast to the results for the *rec⁺* strain, there appears to be no potentiation of killing of X-irradiated *recA56* cells by impure HU at either dose of radiation employed (Fig. 32).

Chromatographically pure samples of HU (Pierce Chemical Co. and

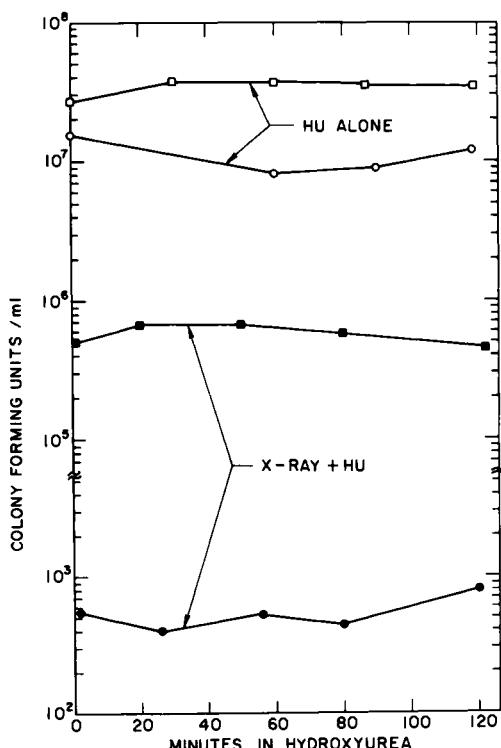


FIG. 32. Postirradiation treatment of *E. coli* K-12 *recA56* (SR74) cells with 0.1 M impure HU (Nutritional Biochemicals Corp.) in growth medium at 37°C. The number of viable cells is plotted as a function of time in 0.1 M HU subsequent to X irradiation. ○, HU alone; ●, 11.7 krads irradiation then HU treatment; □, 5.1 krads irradiation then HU treatment. (From Kapp and Smith, 1971.)

K&K Labs.) were also tested for their ability to potentiate X irradiation induced killing in *rec⁺* cells. Table I demonstrates that only the chromatographically impure HU (Nutritional Biochemicals Corporation) potentiated killing.

In a second type of experiment, the cells were allowed to repair the X-ray-induced single-strand breaks for varying periods of time prior to treatment with impure HU for 60 minutes. Figure 33 illustrates the response of the *rec⁺* strain and the *recA56* mutant. The irradiated *rec⁺* cells showed a dramatic loss of sensitivity to HU during incubation in growth medium. By 40 minutes they had almost completely returned to preirradiation levels of sensitivity to HU. The time course for the loss of sensitivity to HU following irradiation was similar to that for

TABLE I
EFFECT OF HU FROM DIFFERENT MANUFACTURERS ON VIABILITY^a

Irradiation (krad)	HU Employed		Incubation time (min)	Viability (col- ony forming units/ml)
	Manufacturer	Lot number		
None	None added	—	0	1.1×10^7
None	K&K Laboratory	87331F	90	5.8×10^7
None	Pierce Chemical Company	10018-1	90	3.4×10^7
None	Nutritional Biochemicals Corporation	2247	90	1.3×10^7
24.6	None added	—	0	1.7×10^5
24.6	K&K Laboratory	87331F	90	1.9×10^5
24.6	Pierce Chemical Company	10018-1	90	3.1×10^5
24.6	Nutritional Biochemicals Corporation	2247	90	6.8×10^2

^a *E. coli* *rec⁺* cells (AB2497) in logarithmic growth phase were incubated at 37°C in supplemented minimal medium containing 0.1 M HU. Samples were taken after 90 minutes, diluted, and plated on SMM-agar. (From Kapp and Smith, 1971.)

the rejoicing of single-strand breaks in the *rec⁺* strain (Kapp and Smith, 1970a). In contrast, no significant enhancement of survival of the *recA56* cells appeared to be produced by incubation in growth medium prior to treatment with HU at either dose of X-radiation employed, consistent with the observation that *recA* mutants are deficient in their ability to repair single-chain breaks (Morimyo *et al.*, 1968; Kapp and Smith, 1970a).

These results demonstrate that certain samples of chromatographically *impure* HU, but not pure samples, have the properties associated with a new class of *postirradiation* radiosensitizers. The impure samples

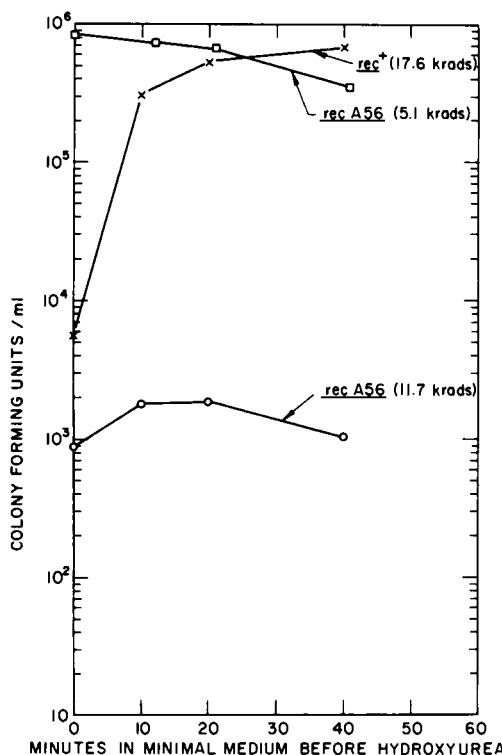


FIG. 33. Effect of time of postirradiation incubation of *E. coli* K-12 on their subsequent sensitivity to impure HU (Nutritional Biochemicals Corporation). Cells were irradiated in minimal medium without glucose and incubated at 37°C in supplemented minimal medium. At the times indicated HU was added to the cultures (final conc. 0.1 M) and allowed to act for 60 minutes. The number of surviving cells is plotted as a function of time of incubation prior to HU treatment. The *rec*⁺ cells received 17.6 krads and the number of surviving cells after irradiation alone was 1.5×10^6 cells/ml (surviving fraction (S.F.) = 1.4×10^{-2}). The *recA56* cells received either 11.7 or 5.1 krads and the number of survivors after irradiation alone was 1.8×10^3 cells/ml (S.F. = 1.3×10^{-4}) or 8.2×10^5 cells/ml (S.F. = 3.7×10^{-2}), respectively. Control studies (not shown here) indicate that less than a two-fold increase in viable cells results when the irradiated (17.6 krads) *rec*⁺ cells are held in supplemented minimal medium for periods of up to 40 minutes prior to plating. (From Kapp and Smith, 1971.)

of HU irreversibly prevented the repair of X-ray-induced single-strand breaks in the DNA of *rec*⁺ cells of *E. coli* K-12; they greatly reduced the viability of these irradiated *rec*⁺ cells, yet they produced no detectable sensitization of irradiated repair deficient *recA* cells. If *rec*⁺ cells were first allowed to repair their X-ray-induced single-strand breaks,

they were then not sensitive to the subsequent addition of impure samples of HU.

It should be stressed that for an agent to produce radiosensitization by the inhibition of single-strand break repair, this inhibition must be irreversible. If the inhibition is reversible, the cells will repair their DNA chain breaks once they are plated in the absence of the agent and little if any effect on viability would result. This probably explains the lack of sensitization of *rec⁺* cells by pure samples of HU.*

The employment of the *rec⁺* strain and the *recA56* mutant of *E. coli* K-12, as described, provides a rapid screening technique for additional compounds which may sensitize cells to radiation by irreversibly blocking *rec* repair.†

6.3.2 QUINACRINE

Quinacrine (Atabrine) (a derivative of 9-amino acridine) markedly potentiates (100-fold at 0.15 mM) the killing of X-irradiated cells of wild-type *E. coli* K-12 cells by X-rays but has no effect on the survival of X-irradiated *E. coli* K-12 *recA13* (cells deficient in the slow repair of DNA chain-breaks). Moreover, it irreversibly inhibits the repair of X-ray-induced single-chain breaks in wild-type *E. coli* K-12 (Fuks and Smith, 1971). These results suggest that the mechanism by which this drug potentiates the killing of X-irradiated cells is by inhibiting the *rec* repair of single-chain breaks in DNA. The sensitivity to the effects of quinacrine disappears after 40 minutes of growth of X-irradiated cells. This is the time it takes for the slow repair (*rec* repair) of single-chain breaks in *E. coli* K-12. (Kapp and Smith, 1970a.)

Quinacrine also potentiates the killing of UV-irradiated wild-type *E. coli* K-12. This drug has no effect on the survival of UV-irradiated *recB* cells and does not irreversibly inhibit the gap-filling step of the postreplication repair system in *rec⁺* or *recB* cells (after 200 ergs/mm²). These results suggest that quinacrine may potentiate the killing of UV-irradiated cells by inhibiting the repair system mediated by the *recB* gene. After a UV dose of 100 ergs/mm² (0.5% survival) it takes about

* Moroson and Tenney (1968) found no sensitization of *E. coli* B/r by HU (purity and manufacturer not specified) when the cells were X irradiated in the presence of HU and oxygen but did find sensitization when they were irradiated in the presence of HU in the absence of oxygen.

† Chromatographically impure HU markedly potentiates the killing of UV-irradiated *rec⁺* *uvr⁺* cells of *E. coli* K-12 and appears to have no effect upon the postreplication gap-filling process (K. C. Smith, unpublished observations). The molecular basis of action of impure HU to potentiate the killing of UV-irradiated cells remains to be determined.

90 minutes of incubation in growth medium before wild-type *E. coli* K-12 lose their sensitivity to quinacrine. This presumably is the time it takes to complete the *recB recC* repair (Z. Fuks and K. C. Smith, unpublished observations).

6.3.3 ACRIFLAVINE

The effects of acriflavine on the lethal and mutagenic actions of UV-radiation have recently been reviewed (Doudney, 1968; Witkin 1969b). Acriflavine appears to affect the excision-repair system but its action cannot be exclusively by this mode since it causes considerable enhancement of the lethal effect of UV radiation in excision-defective (*Hcr*⁻) strains (Harm, 1967). Witkin (1969b, page 544) has postulated that "inhibition of recombinational repair may account for most of the lethal enhancement exerted by acriflavine even in *Hcr*⁺ strains having normal excision." There are many similarities in the effectiveness of acriflavine and of caffeine as repair inhibitors (Witkin, 1969b). Mutational synergism between UV radiation and caffeine or acriflavine is most pronounced at low doses, diminishes with increasing dose, and is not observed at doses above 500 or 1000 ergs/mm² (see references in Witkin, 1969b).

Acriflavine has also been shown to affect the survival of bacteria irradiated with 8 MeV electrons (Alper, 1963). Since under the conditions of these experiments UV-irradiated cells were more affected by acriflavine than were cells exposed to ionizing radiation, Alper (1963) concluded that "DNA is more heavily involved in killing by ultraviolet than by ionizing radiation." These differences in response to acriflavine may be reinterpreted in the light of more recent information. Wild-type bacterial cells appear to have at least two mechanisms for repairing UV damage and acriflavine appears to inhibit both (Witkin, 1969b). Wild-type bacterial cells also appear to have at least two mechanisms for the repair of X-ray damage, a slow *rec* repair system (Section 5) and a fast *pol* repair system (Section 7). Since most of the X-ray-induced single-chain breaks in DNA are repaired in a few minutes in buffer in *E. coli* by the *pol* repair system (Section 7), repair by this system would be finished and therefore would not be inhibited when the cells were plated in the presence of acriflavine. Only the *rec* repair would be left to be inhibited on the plates. Acriflavine has been shown to inhibit the *rec* repair of single-strand breaks after X irradiation and this inhibition is at least partially irreversible (Kapp, 1970).

Alper (1963) showed that acriflavine had a greater potentiating effect on cells exposed to ionizing radiation in the absence of oxygen. To be consistent with the above speculations, this suggests that more damage

of the type that is repairable by *rec* repair is produced by X rays under anoxia.

The response of bacteria to UV damage is more similar to their response to anoxic X-ray damage than it is to their response to aerobic X-ray damage (Alper, 1965). One possible interpretation of this may be that base damage becomes more important relative to chain breaks when cells are exposed to ionizing radiation in the absence of oxygen. This is consistent with the observation that although excision-defective cells appear to have the same sensitivity to aerobic X irradiation as do wild-type cells (Fig. 22), *uvrA6* cells are more sensitive to anoxic X irradiation (Rupp *et al.*, 1970).

In *uvr⁻* cells, damaged bases would presumably be bypassed during replication and the resultant gaps in the daughter strands would be repaired by the *rec* repair system as discussed in Section 3 for the bypass of UV-induced damage. This hypothesis is consistent with the observation that *E. coli* K-12 *recC22* cells show a larger than average oxygen enhancement ratio, i.e., they show a larger than average protection from ionizing radiation in the absence of oxygen (Emmerson, 1968b). We have seen in Section 3 (and discussed in Section 4) that *recB recC* mutants show no apparent deficiency in the bypass-repair system presumed to operate on base damage.

6.3.4 CHLORAMPHENICOL

Chloramphenicol (5 µg/ml) potentiates the killing of both UV- and X-irradiated *E. coli* B/r (Forage and Gillies, 1969). For populations of cells killed to the same extent (~20% survival) this drug appears to have a greater potentiating effect after UV irradiation than after X irradiation.

Chloramphenicol (15 µg/ml) inhibits the slow repair (but not the fast repair) of X-ray-induced chain breaks in the DNA of *Micrococcus radiodurans* (Dean *et al.*, 1970).

Chloramphenicol (40 µg/ml) inhibits the recovery of *E. coli* K-12 from UV or X irradiation if they are *rec⁺* (*uvr⁺* or *uvr⁻*), but not if they are *recA13* (*uvr⁺* or *uvr⁻*). This drug does not irreversibly inhibit the gap-filling step of the postreplication repair system for UV damage. Chloramphenicol partially inhibits the slow *rec* repair of X-ray-induced single-strand breaks in wild-type *E. coli* K-12 and this inhibition is irreversible. The sensitivity of wild-type *E. coli* K-12 to chloramphenicol disappears quickly (30–60 minutes) when X-irradiated cells are incubated in growth medium. This disappearance of sensitivity occurs much more slowly in UV-irradiated cells (Ganesan and Smith, 1971).

6.3.5 CHLOROQUINE

Chloroquine (a derivative of 4-amino quinoline) potentiates the UV-induced killing of *E. coli* B (Yielding *et al.*, 1970) and of wild-type *E. coli* K-12 and *recA18* (Smith and O'Leary, 1971). Chloroquine does not potentiate the UV-induced killing of *E. coli* K-12 *uvrB5* or the aerobic X-ray-induced killing of wild-type *E. coli* K-12 (Smith and O'Leary, 1971). It may be recalled that *uvrB5* cells are not more sensitive than wild-type cells to aerobic X irradiation (Fig. 22).

These observations suggest that chloroquine potentiates the killing of UV-irradiated cells by inhibiting the excision-repair system. Its apparent lack of effect on the *rec* repair system seems sufficient to explain its absence of effect on the viability of aerobic X-irradiated wild-type *E. coli* K-12.

Chloroquine in the presence of caffeine has been shown to potentiate the effectiveness of the X-ray-induced regression of tumors (plasmacytomas) in golden Syrian hamsters (Gaudin and Yielding, 1969).

6.3.6 THE POSSIBLE IMPORTANCE OF REPAIR INHIBITORS AS ADJUNCTS TO THE TREATMENT OF CANCER BY RADIATION THERAPY

The radiation sensitivity of cells depends upon three parameters.

1. The presence of dose-modifying factors during irradiation, e.g., O_2 , sulfhydryl compounds, photosensitizing compounds, etc. (for reviews see, Emmerson, 1971; Bridges, 1969; Smith and Hanawalt, 1969, pp. 65, 179, 203).

2. The intrinsic sensitivity of the radiation target, i.e., DNA. The factors affecting this parameter are ploidy, the size of the DNA, whether it is single- or double-stranded (Kaplan and Moses, 1964), and the base composition of DNA (Haynes, 1964b; Kaplan and Zavarine, 1962).

3. The ability of the cell to repair radiation damage.

Of these three parameters the ability to repair appears to be the most important in determining the radiation sensitivity of a cell. Clearly if a cell has no ability to repair radiation damage then techniques affecting parameters 1 and 2 would be the major methods of modifying the radiation sensitivity of these cells. To go to the other extreme, if a cell had an infinite capacity to repair radiation damage then radiation sensitivity could not be modified by parameters 1 and 2.

It is now well established that radiation produces several different types of damage and cells have several types of repair systems. The different types of radiation damage may well be repaired with different efficiencies and accuracies. Parameters 1 and 2 may function to shift the relative proportions of the damage produced from easily repairable

to difficultly repairable damage. The relative repairability of any one class of DNA damage will of course be a characteristic feature of each type of cell.

Since it appears that the radiation sensitivity of a cell is determined largely by its ability to repair radiation damage, then it is not surprising that drugs that specifically inhibit this repair (see above) are powerful "radiation potentiators" (or alternatively, "postirradiation sensitizers").

Radiation therapists have tried for many years and with limited success to modify the radiation sensitivity of tumors by techniques designed to affect parameters 1 and 2 above. Now that it has been shown that repair systems can be selectively inhibited by drugs that are added subsequent to irradiation it is hoped that these inhibitors can soon be successfully used as adjuncts to the treatment of cancer by radiation therapy.

7. The Role of DNA Polymerase in Repair

7.1 General Properties of Polymerase-Deficient Mutants

Although the *E. coli* DNA polymerase has characteristics expected of a repair enzyme (Kelly *et al.*, 1969) the recent isolation of a mutant defective in polymerase activity (de Lucia and Cairns, 1969; Gross and Gross, 1969) has considerably advanced our understanding of the role of DNA polymerase in repair.

This mutant (*polA1*) shows an increased sensitivity to UV irradiation (Gross and Gross, 1969; Kanner and Hanawalt, 1970; Boyle *et al.*, 1970) but is more resistant than an excision-defective mutant, *uvrA6* (Kanner and Hanawalt, 1970). Although UV sensitive itself, *polA1* shows no deficiency in the host cell reactivation of UV-irradiated phage T7. Thus the mutant is *hcr⁺* (de Lucia and Cairns, 1969).

The *polA1* mutant is very sensitive to killing by methylmethane-sulphonate (MMS).* The parent strain forms colonies normally in the presence of 0.04% MMS, whereas the mutant plates with an efficiency of about 10⁻⁷ (de Lucia and Cairns, 1969).

The *polA1* mutant is also very sensitive to killing by X irradiation. In fact, it is as sensitive to X irradiation as is *recA* (Town *et al.*, 1971b).

The frequency of genetic recombination is reduced by a factor of

*The biological effects of MMS are in many ways similar to those produced by X rays. Strauss *et al.* (1971) have recently reviewed the subject of the production and repair of MMS-induced damage in bacterial and mammalian cells.

two in the *polA1* mutant but so is the efficiency of DNA transfer compared to the parental strain (Gross and Gross, 1969).

7.2 The Deficiency of *polA1* in the Repair of UV Damage

Preliminary experiments indicated that the amount of "repair replication" in UV-irradiated *polA1* was similar to that in the parent strain (Kanner and Hanawalt, 1970). Actually the amount of "repair replication" is 2-3 times greater in *polA1* than in the parent strain after the same dose of UV (dose to kill 90% of parental strain) (P. Cooper and P. C. Hanawalt, personal communication).

The *polA1* mutant degrades more of its DNA after low doses of UV radiation than does the parental strain. This nuclease activity appears to be exonucleolytic (Boyle *et al.*, 1970).

The *polA1* mutant cells are about 5 times more UV sensitive and *uvrA6* cells are about 20 times more UV sensitive than wild-type cells. A *polA1 uvrA6* double mutant is only slightly more UV sensitive than the isogenic *uvrA6* single mutant. This suggests that the UV sensitivity associated with *polA1* is primarily the result of a reduction in the efficiency of the excision repair pathway (Gross, 1971).

Since the rate and extent of excision of pyrimidine dimers is only slightly reduced in *polA1* (Boyle *et al.*, 1970) the defect in *polA1* presumably reduces the efficiency of repair after excision. This is confirmed by the observation that the DNA of *polA1* cells contain more single-strand breaks in their DNA than *pol⁺* when incubated for the same time after UV irradiation (Kanner and Hanawalt, 1970; Boyle *et al.*, 1970).

If functional polymerase is truly absent *in vivo* as it is *in vitro*, i.e., less than 1-2% residual activity, then one may ask how the gaps in the DNA produced by excision are repaired in *polA1*. Since the *recA* repair system efficiently repairs gaps in DNA in both daughter (Section 3) and parental strands (Section 5) the possibility exists that the *recA* repair system substitutes for DNA polymerase in repairing the gaps left after excision in *polA1*. The increased UV sensitivity of *polA1* cells would thus be explained by assuming that the *recA* system is slightly less efficient in repairing the gaps produced by excision than is DNA polymerase (Gross, 1971).

It is of interest, therefore, that attempts to construct a *recA polA* double mutant have been unsuccessful (Witkin, personal communication; Gross, 1971). This suggests that such a mutant is inviable. Presumably, gaps in DNA that are formed during normal growth may be repaired either by DNA polymerase or the *recA* repair system. Evidence to support this concept comes from a different *polA* mutation, *polA4*. Extracts

of *polA4* cells contain no detectable polymerase activity, however, the strain has almost wild-type resistance to MMS at 37°C but is fairly sensitive at 30°C or below. The polymerase in *polA4* thus appears to be cold-sensitive. A *recA56 polA4* double mutant has been constructed at 37°C. It grows well at 37°C but shows little viability at 26°C (Gross, 1971).

7.3 The Deficiency of *polA1* in the Repair of X-Ray Damage

Single-chain breaks constitute one of the more important types of lesions produced in DNA by X irradiation. Currently there is a dichotomy in the literature concerning the energy required to produce a single-chain break in DNA. While many authors favor ~60 eV/break (see summary in Dean *et al.*, 1969), those who have worked with *E. coli* have all generated data similar to those of McGrath and Williams (1966) which yields a value of 300–600 eV/break (Town *et al.*, 1971a; Ginsberg and Webster, 1969; Achey and Whitfield, 1968; Kaplan, 1966; Kapp and Smith, 1970a).

Alexander *et al.* (1970) have shown that in *Micrococcus radiodurans*, approximately 90% of the breaks produced by aerobic X irradiation are rapidly (minutes) repaired in buffer at 30°C but not at 0°C. The remainder of the breaks not repaired in buffer are restituted slowly (hours) when the cells are incubated in growth medium at 30°C. These observations have led to the suggestion that, in *E. coli*, many of the single-strand breaks in DNA are rapidly repaired before the samples can be analyzed by sedimentation. Such a situation would be consistent with the apparent requirement of about 500 eV to produce a DNA chain break in *E. coli*. No further breaks were revealed, however, when *E. coli* were held at 0°C and pH 7 during irradiation (McGrath and Williams, 1966; Achey and Whitfield, 1968; C. D. Town, unpublished observations).

The extreme X-ray sensitivity of *polA1* (compared with its moderate sensitivity to UV radiation) prompted an investigation of the ability of this mutant to repair X-ray-induced single-chain breaks in DNA. These studies revealed an unexpectedly high yield of breaks per dose of radiation compared with *pol^t* (Fig. 34).* These results led to the speculation that *polA1* might be defective in a *rapid repair* system for

* Kato (1970) has observed an increased number of single-chain breaks per dose of X rays in a radiation sensitive mutant *E. coli* R15 (*res*) that has turned out to be polymerase deficient. Upon incubation to allow the repair of single-chain breaks, however, this mutant shows degradation of the DNA rather than repair. The X-ray sensitivity of this mutant is comparable to a *recA recB* mutant and its UV sensitivity is comparable to a *uvrA* mutant. This enhanced sensitivity to UV and the extensive degradation after X irradiation indicates that the *res* mutation is distinct from *polA1*, although both are deficient in DNA polymerase.

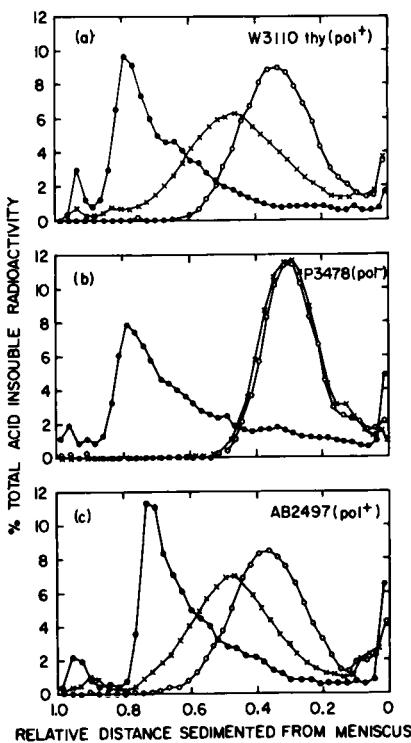


FIG. 34. Sedimentation of DNA from *E. coli* K-12 *pol*⁺ (W3110 *thy*), *polA1* (P3478) and *pol*⁺ (AB2497) after X-irradiation with 16.3 krad followed by immediate lysis on a 0.5 N NaOH, 0.5% Sarkosyl (Geigy NL30) cap on an alkaline sucrose gradient (5–20% sucrose in 0.1 N NaOH). (a) *pol*⁺ (W3110 *thy*); (b) *polA1* (P3478); and (c) *pol*⁺ (AB2497). The cells were irradiated (16.3 krads) in 0.05 M phosphate buffer at pH 6.9 and 25°C (X) or at pH 8.0 and 0°C (○). Unirradiated control (●). Note that the centrifugation time in (c) was reduced from 120 minutes to 105 minutes. Calculations indicate an apparent energy requirement in W3110 *thy* of 80 eV/break after irradiation at 0° and pH 8.0 and 266 eV/break at 25° and pH 6.9. The corresponding values for AB2497 are 141 and 386, while those for P3478 are 59 under both conditions. When repair was irreversibly inhibited in SR108 by 0.1 M EDTA a value of 75 eV/break was obtained. EDTA does not affect the results for *polA1*. (From Town *et al.*, 1971b.)

chain breaks which had not previously been detected in *E. coli*. This has been confirmed by finding conditions which inhibit this process in *pol*⁺ (Fig. 34). In *polA1* and "completely" inhibited *pol*⁺ the energy required to produce single-chain breaks is ~75 eV/break (Town *et al.*, 1971b).

7.4 Summary

The Kornberg polymerase (at least in the form that can be assayed in extracts *in vitro*) appears not to be involved in normal DNA replica-

tion but is involved in the repair of DNA. In this case repair is taken to mean the reconstruction of sections of DNA damaged by normal cellular processes as well as by radiation or chemicals. DNA polymerase has the characteristics, *in vitro* (Kelly *et al.*, 1969), of the excision and repolymerizing enzymes postulated to catalyze the second step in excision repair. The ability to excise pyrimidine dimers, however, is only slightly reduced in *polA1* cells (Boyle *et al.*, 1970). In the absence of a functional polymerase it is expected that gaps produced in UV-irradiated DNA by the incision enzyme(s) are filled by the recombinational repair system, which appears somewhat less efficient than polymerase in this function (Clark, 1971). Mutants deficient both in repair polymerase and recombination function appear to be inviable (Witkin, personal communication; Clark, 1971).

There appear to be two major systems for the repair of X-ray induced chain breaks in DNA. One requires DNA polymerase and repairs ~85% of the chain breaks in a few minutes. The second requires functional *rec* genes and repairs chain breaks slowly (20–40 minutes). It is not known if these two systems overlap or if they are specific for different types of chain breaks. X rays form chain breaks having different chemical characteristics (Kapp and Smith, 1970b). These have sometimes been classified as *clean breaks*, e.g., a phosphate ester break, or *dirty breaks*, e.g., sugar damage and/or base loss. It is presumed that the clean breaks can be more quickly repaired than dirty breaks.

8. Conclusions

8.1 General Considerations

The radiation sensitivity of a cell depends upon three parameters—(1) the presence of dose modifying factors during irradiation; (2) the intrinsic sensitivity of the radiation target, i.e., DNA; and (3) the ability of the cell to repair radiation damage. Of these three parameters the ability to repair radiation damage appears to be the most important.

Radiation produces two major classes of damage to DNA—(1) base damage, and (2) chain breaks. Many types of base damage can be formed by UV and X radiation (see review by Smith and Hanawalt, 1969, pp. 57, 193). Many types of chain breaks can also be formed (Fig. 27).

The relative yield of these different kinds of damage is markedly affected by parameters 1 and 2 above. Whether the cells are dry or wet, spore or vegetative cell, frozen, etc., i.e., the physical state of the DNA, also affects the types of radiation damage produced (Smith, 1967). Some

of this damage can be easily repaired, i.e., requires only one enzymatic step, e.g., photoreactivating enzyme; ligase, while others may require several enzymatic steps, e.g., nuclease, polymerase, ligase, or the *rec* repair system.

Although there appear to be different repair systems for different types of damage, these repair systems can often repair the same types of lesions. For example, pyrimidine dimers can be photoreactivated (*in situ* repair); excised ("cut and patch") or bypassed (*rec* repair). Each of these repair systems probably has a different set of parameters (growth conditions or chemical inhibitors) that affect its function. Each repair system may also have a different intrinsic accuracy for repair and thus have a different effect on viability and on mutation production.

The different repair mechanisms may well have enzymatic steps in common, e.g., ligase. It is also quite possible that some processes now called *rec* repair may not include all the steps involved in genetic recombination but may only require one or a few of the same enzymes. The execution of one type of repair process may necessitate the involvement of a second type of repair, e.g., the parental strand gaps created during the *rec* repair of daughter strand gaps might be filled by the repair replication steps of excision repair. Thus the terms of enzymology and intermediary metabolism should be used in describing repair processes, including the concepts of branched, alternate, and cyclic pathways.

Another concept to keep in mind is that these repair systems may not all have evolved primarily for the purpose of repairing radiation damage to DNA but may be necessary for normal metabolic processes. This concept is strengthened by the fact that a *polA1 recA* double mutant appears to be nonviable.

8.2 Repair of UV-Induced Damage

Figure 35 broadly outlines three mechanisms for the repair of UV-induced damage. Photoreaction is covered in the chapter by Harm *et al.* (this volume) and will not be discussed here.

Figure 35 suggests that the polymerase (+ ligase) pathway may be the major route for repairing the parental strand gaps resulting from the excision of damaged bases. It has been suggested (Gross, 1971) that in the absence of a functional DNA polymerase the *rec* system may be able to fill these gaps. The fact that *polA1* is moderately sensitive to UV suggests that the proposed *rec* repair of excision gaps in this strain is less efficient than is repair by DNA polymerase (+ ligase).

It should be kept in mind that similar experimental techniques have been used to support the concepts of repair replication (polymerase repair of excision gaps) (Pettijohn and Hanawalt, 1964) and recombi-

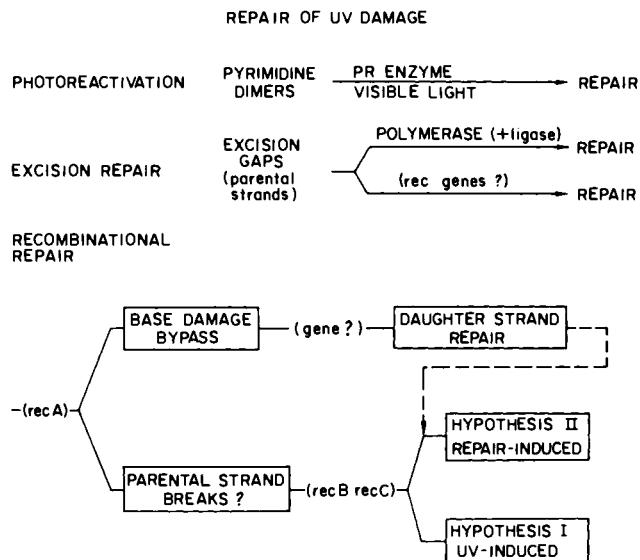


FIG. 35. Possible pathways for the repair of UV-damaged DNA. See Section 8 for a description of these pathways.

nation repair (parental strand involvement in the repair of daughter-strand gaps) (Rupp *et al.*, 1970; Fig. 15). The incorporation of density label under the peak of normal DNA has been used to suggest repair replication while the presence of DNA at intermediate densities has suggested recombinational repair. It is possible that repair replication experiments, e.g., after thymine starvation, that show a large amount of DNA of intermediate density, e.g., Pauling and Hanawalt, 1965, may reflect recombinational repair rather than excision repair.

The arguments for the possible presence of separate recombinational repair systems have been presented in Section 4. It has been assumed that UV radiation produces both base damage (major lesion) and chain breaks (minor lesion). Base damage is bypassed during DNA synthesis leaving gaps in the daughter strands. The *recA* mutants cannot repair daughter-strand gaps but *recB* and *recC* mutants can (Section 3). It has been suggested that these gaps are filled with parental material. This would then produce gaps in the parental strands. The *recB recC* pathway has been postulated to be involved in the repair of these gaps in the parental strands (hypothesis II). In addition the *recB recC* pathway may repair parental strand breaks produced by the direct action of UV radiation (hypothesis I).

Several inhibitors have been found that depress the viability of

UV-irradiated cells but thus far none has been shown to irreversibly inhibit the daughter-strand gap-filling step of *rec* repair. The action of certain of these inhibitors has been shown to require the presence of functional *rec* genes, e.g., quinacrine; complex growth medium on *uvr* cells. Since these agents do not inhibit the gap-filling step of *rec* repair it has been suggested that they may be inhibiting a second type of *rec* repair.

Section 4 outlines several experiments that may prove the presence of additional pathways of *rec* repair. When additional recombination deficient mutants become available it will be of interest to test them for their ability to perform the gap-filling repair step and the two additional *rec* repair steps hypothesized in Fig. 35 (Section 4).

8.3 Repair of X-Ray-Induced Damage

Very little is known about the chemistry or biological importance of DNA base damage produced *in vivo* by X radiation. The discussion in Section 6.3.3 suggests that base damage (or at least damage repaired in a manner similar to UV-induced damage) may be of major importance in cells irradiated in the absence of oxygen.

For cells irradiated in the presence of oxygen, it seems clear that the lesion of major importance is the DNA chain break, both single and double. Although there is evidence to suggest that both single- and double-chain breaks are repaired in the very radiation resistant bacterium, *Micrococcus radiodurans* (Kitayama and Matsuyama, 1968; Dean *et al.*, 1966), double-chain breaks do not appear to be repaired in wild-type *E. coli* and the radiation sensitivity of *E. coli* has been correlated with the production of this lesion (Kaplan, 1966). There are mutants of *E. coli* that are unable to repair X-ray-induced single-chain breaks in their DNA. The enhanced X-ray sensitivity of these mutants appears to correlate with their *inability* to repair single-chain breaks (Kapp and Smith, 1970a; Town *et al.*, 1971b).

Most of our knowledge about the repair of X-ray induced damage is concerned with the repair of single-chain breaks. There are at least two separate mechanisms for the repair of X-ray-induced single-chain breaks based upon the speed of the reactions. One is fast (a few minutes in buffer) the second is slow (\sim 40 minutes in growth medium) (Fig. 36).

The system controlled by *polA* is responsible for the rapid repair of \sim 85% of the X-ray-induced single-chain breaks (Section 7). The "ligase only" pathway is postulated on the basis that a few percent of "clean-breaks" are probably formed.

The slow repair of X-ray-induced single-chain breaks is controlled by the *rec* genes (Section 5). The *recA* cells are unable to perform this

REPAIR OF X-RAY DAMAGE

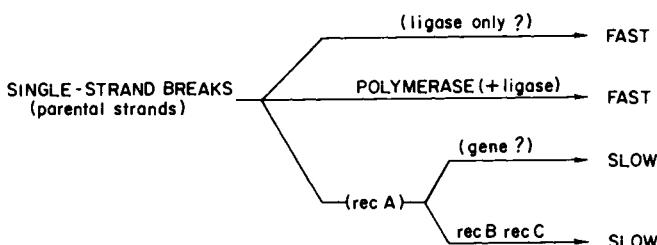


FIG. 36. Possible pathways for the repair of DNA damaged by X rays. See Section 8 for a description of these pathways.

type of repair, while *recB* and *recC* cells show a partial ability to repair chain breaks (Section 5).

The nature or possible extent of the interaction between these two repair systems (*pol* and *rec*) is not known. Preliminary data indicate that the *pol⁺ rec⁺* cells can repair more chain breaks than the sum of the efforts of *rec⁺pol⁻* and *rec⁻pol⁺* cells, which suggests that the two systems may have some interdependency (C. D. Town, unpublished observations).

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Chapter 7

THE STUDY OF PHOTOCHEMICAL REPAIR OF UV LESIONS IN DNA BY FLASH PHOTOLYSIS

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1. Introduction

The general phenomenon called "photoreactivation" can be defined in a broad sense as "the reduction in response to far ultraviolet irradiation

of a biological system resulting from a concomitant or post-treatment with nonionizing radiation," as proposed by Jagger and Stafford (1965). Such an effect was described in the alga *Fucus furcatus* by Whitaker (1941) and was possibly seen even earlier (Prát, 1936), but recognition of photoreactivation as a general phenomenon stems from its rediscovery by Kelner (1949a,b) in bacteria and—almost simultaneously—by Dulbecco (1949, 1950) in bacteriophage. Since then, PR effects have been shown to range very far indeed through the biological world.

The definition of the term "photoreactivation" given above is purely operational and can therefore comprise effects based on widely differing mechanisms. Several phenomena discovered in the last decade stress this point, e.g., *indirect photoreactivation* resulting from more extensive dark repair (Jagger and Stafford, 1965) and *direct photochemical monomerization of pyrimidine dimers* at wavelengths around 240 nm (R. B. Setlow and Setlow, 1962). However, the most important and widespread mechanism is *photoenzymatic repair* (PER) which will be treated exclusively in this article. In this case, UV lesions are repaired by an enzyme ("photoreactivating enzyme") which requires light energy in the near-UV and violet-blue spectral range for its action. In general, wavelengths ranging from about 310 to 480 nm are effective, with the maximally efficient wavelengths depending on the particular organism.

Indications of the involvement of enzymatic processes in photoreactivation were first obtained when Dulbecco (1949, 1950) observed that photoreactivation of phage occurs only if the light is applied to intracellular phage, while the inactivating UV radiation can be applied to either the extracellular or the intracellular phage. No photoreactivation was observed if the phage alone, or the host cells alone, or both independently were illuminated. This suggested that, besides the light, a cellular factor is required. Investigation of the photoreactivation kinetics in phage by Dulbecco (1950) and G. H. Bowen (1953a,b) further indicated the involvement of a temperature-dependent dark reaction, suspected to be enzymatic in nature.

Real evidence for the requirement of an enzyme in the photoreactivation process was provided by *in vitro* studies, using UV-irradiated transforming DNA of *Haemophilus influenzae* and extracts of *E. coli* cells (Goodgal *et al.*, 1957; Rupert *et al.*, 1958). If such mixtures were exposed to photoreactivating light, the "UV survival" of transforming activity was increased. Exposure to photoreactivating light of either the DNA alone, or the extract alone, or both separately, did not increase the transforming activity. Likewise, extracts of *Haemophilus influenzae* (which is itself a nonphotoreactivable species) proved to be ineffective in promoting photoreactivation.

Elaboration of this *in vitro* PR system, using extracts of yeast (*Saccharomyces cerevisiae*), which contain less DNase than extracts of *E. coli*, and quantitative study of the photoreactivation effects under varied experimental conditions (Rupert, 1960, 1961, 1962a,b) demonstrated the existence of a *photoreactivating enzyme* (PRE). These investigations have led to the following reaction scheme.



According to this scheme the photoreactivating enzyme E combines with the substrate S (a photorepairable UV lesion) to form an enzyme-substrate complex ("ES complex"). Absorption of light energy photolyses the complex, and in this process the enzyme is released, and the substrate is converted into the repair product P. All later work has been found compatible with this scheme.

Photochemical investigations showed that cyclobutyl pyrimidine dimers in UV-irradiated DNA are the photorepairable lesions (for reference, see R. B. Setlow, 1966), which constitute the major fraction of all lethal lesions. Illumination of UV-irradiated DNA in the presence of PRE causes these dimers to disappear (Wulff and Rupert, 1962; J. K. Setlow, 1964); their monomerization under these conditions was later directly demonstrated (Cook, 1967). Thus the reaction scheme represents formation of a complex of PRE with a pyrimidine dimer, and its subsequent photoenzymatic splitting.

Our knowledge of PER and photoreactivation in general has been reviewed at various times (Dulbecco, 1955; Jagger, 1958; Rupert, 1964; Rupert and Harm, 1966; J. K. Setlow, 1966; Cook, 1970). The present article summarizes recent work which provides more detailed insights into the PER process and the properties of the photoreactivating enzyme through the use of short intense light flashes (H. Harm and Rupert, 1968, 1970a,b; H. Harm, 1969; W. Harm *et al.*, 1968; W. Harm, 1969, 1970a,b).

2. The Use of Light Flashes in the Analysis of Photoenzymatic Repair

2.1 General

In the past, studies on PER have been mainly carried out with continuous illumination, either with polychromatic, short-wavelength visible light, or with monochromatic radiation of the proper wavelengths. While this has led to many important insights, a detailed quantitative analysis of the component steps of the whole process meets the difficulty

that PRE molecules, liberated through the light-dependent repair step, can enter the reaction again and cause more repair. A clear experimental separation of the left side of the reaction scheme, "complex formation," from the right side, "photolysis of the complex," is highly desirable. This can be achieved by using a short intense flash of light, since only those UV lesions* can be repaired which are complexed at the moment of the flash. The number of these at any time can be determined from the biological effect and their rate of change can be observed.

The technique of "flash photolysis" was originally developed in photochemistry for the study of light-dependent processes closely associated with others occurring in the dark. By delivering a large dose of light within a time much shorter than that required for subsequent dark reactions, the photoprocess can be completed before these dark reactions have progressed significantly. Typically, flash illumination creates excited molecules which then decay or react over time intervals of nanoseconds to milliseconds, as followed optically by their absorption or emission of light (see e.g., Porter, 1968; Grossweiner, 1966). Studies of this kind have contributed greatly to the understanding of photochemical mechanisms.

The same principle of separating light-dependent and light-independent reactions by applying the light in the form of a short flash has been used in our photorepair experiments. The difference from conventional flash-photolysis methods is that we are dealing with dark processes lasting seconds or minutes, thus placing only very gentle requirements on the maximum permissible flash duration. The dark processes precede (and are thus independent of) the light-dependent process, and lead to the formation of the light-sensitive compound (the ES complex), whose amount can be determined at any time by flash-dissociating it into a stable product, i.e., the "repaired" UV lesion. While it may eventually be feasible to study the light-dependent reaction of the complex by conventional flash-photolysis methods in order to investigate its detailed steps, in the present instance we are simply concerned with separating the "complex formation" step from the "photolytic repair" step.

The first attempt at using intermittent light to elucidate the kinetics of photoreactivation was made by G. H. Bowen (1953a,b, summarized by Dulbecco, 1955), working with bacteriophage T2. Although the intensities available to him were no greater than those employed in continuous illumination, and the usual "flash" durations were long compared to those employed in our work, ingenious use of this technique

* We use in the following pages the term "UV lesion" or "lesion" to mean any potentially lethal UV photoproduct in DNA.

enabled him to outline the basic sequence of dark and light-dependent reactions which we now consider correct. However, the state of knowledge at that time did not permit these results to be understood in terms of the underlying enzymatic mechanism.

The experimental conditions employed in our present work have two basic requirements. (1) The biological systems used must show appreciable photoreactivation from a single flash. (2) One must be able to correlate quantitatively the observed effect with the number of complexed UV photoproducts present. To satisfy requirement 1, the concentration of PRE molecules *versus* the concentration of substrate molecules, i.e., pyrimidine dimers in irradiated DNA, must be such that a considerable fraction of the substrate is in complexed form at the moment of the flash. In addition, the flash must have sufficient strength to photolyse a reasonably large fraction of the ES complexes present. Knowing exactly what this fraction is, and knowing—from the biological effect obtained—the number of substrate molecules that have been converted to the repair product, permits calculating the number of ES complexes present, and thus satisfies requirement 2.

Most of our results have been obtained with a rather simple flash device. It consists of either 4 or 6 electronic flash units designed for photographic use, mounted symmetrically and facing a sample located in the center of the array (Fig. 1). The light is filtered through blue glass, absorbing strongly at wavelengths shorter than 320 nm, to avoid possible damage to the biological material. Usually all units of the 4-unit device are discharged simultaneously, producing a flash of about 1 msec duration. For some experiments single units were discharged sequentially, in which case the 6-unit device was used (either alone or together with the 4-unit device), to allow longer recharging time between successive uses of the same unit. It will be shown in Section 2.3 that even a single-unit discharge is sufficient to photolyse virtually all of the ES complexes present.

The different biological systems chosen for our study enabled us to obtain sizable repair effects with a single flash and to evaluate comparatively the results obtained *in vitro* and *in vivo*. All our UV irradiations were made with low-pressure mercury lamps, emitting chiefly the wavelength 254 nm.

2.2 *In Vitro* and *in Vivo* Studies

2.2.1 *In Vitro* STUDIES

The biological system employed here was described earlier (Rupert, 1960) and has since been used by various workers. In essence, it consists



FIG. 1. 4-unit and 6-unit flash devices as employed in these studies. Yashica PRO 50 flash units were used in most cases, but several other commercial brands of similar shape gave essentially the same results. The Yashica units were powered by a 6-V automobile battery instead of the four 1.5-V dry cells normally used in each unit.

of transforming DNA from *Haemophilus influenzae* (usually carrying a high-level streptomycin-resistance marker), mixed with a PRE-containing extract from yeast cells. The transforming activity is defined by the number of transformants obtained per unit volume of this mixture when it is added to competent wild-type *Haemophilus* cells. In this manner, comparison of the transforming activity of UV-irradiated DNA with that of unirradiated DNA determines the "marker survival." Exposure of the UV-irradiated DNA to light after mixing it with the PRE-containing extracts results in an *increased marker survival due to PER*. Under our standard conditions the reaction components were suspended at room temperature in phosphate-buffered saline of pH 6.8 and an ionic strength of 0.14. Details of the experimental method and manipulations are given in the relevant papers (H. Harm and Rupert, 1968, 1970a,b; H. Harm, 1969).

The great advantage of this *in vitro* system is that for a given UV dose and DNA concentration the percentage of UV photoproducts complexed with PRE can be controlled by varying the concentration of the PRE-containing extract. This enables one to adjust the conditions so that they are suitable for the particular experimental purpose. Also

the influence of experimental parameters like pH and ionic environment can be readily investigated.

2.2.2 *In Vivo* STUDIES

In most cases stationary phase cells of the *E. coli* strain B_{s-1} (Hill, 1958) or its mutant B_{s-1}-160 (see Section 3.3) were UV irradiated and their inactivation and PER measured by colony formation after plating on suitable nutrient media. In some instances PER or UV-irradiated bacteriophage T1 infecting logarithmically growing B_{s-1} or B_{s-1}-160 host cells was investigated. These systems are very UV-sensitive, i.e., an appreciable survival decrease results from the presence of relatively few pyrimidine dimers; consequently a corresponding survival increase can be achieved by PER of a small number of dimers. Only under such circumstances can one expect to see a measurable effect from flash photolysis, because the number of PRE molecules in *E. coli* cells is rather low (cf. Section 3.2). In more UV-resistant systems (such as wild-type *E. coli*) the ratio between PRE molecules and pyrimidine dimers would, at any survival level, be too small to cause a recognizable flash effect. Methodological details can be found in the relevant papers (W. Harm *et al.*, 1968; W. Harm, 1969, 1970a,b).

2.3 Photolytic Efficiency of a Single Flash

A single flash from a 4-unit discharge photolyses virtually all of the enzyme-substrate complexes present, thus causing repair of the corresponding number of UV lesions. Evidence for this has been obtained for both the *in vitro* and the *in vivo* system in two ways.

1. It can be shown that the repair effect obtained with a single flash is not limited by the amount of light provided by a 4-unit discharge. If only 3, 2, or 1 units are discharged simultaneously, the magnitude of the repair effect is the same as with a 4-unit discharge, as seen in Fig. 2. Only if the amount of light received by the sample decreases further, e.g., by exposing it to a 1-unit flash at a greater distance, does the PER effect diminish.

2. Under conditions where the enzyme molecules are in sufficient excess over the repairable UV photoproducts, so that essentially all lesions are complexed, PER obtained with a single flash is maximal, i.e., it is the same as the highest level obtainable with continuous illumination. This could not possibly be the case if the probability of an existing ES complex being photolyzed by a single flash were less than unity. The necessary excess of PRE over substrate can be easily realized *in vitro*, while *in vivo* it can only be achieved with mutants, such as

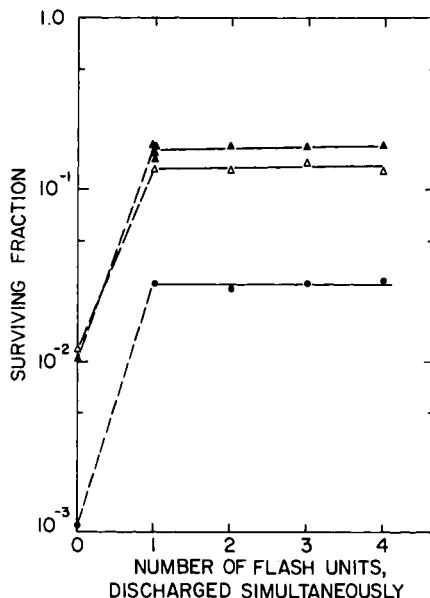


FIG. 2. Survival increase resulting from photolysis by a single flash of varying strength. *Lower curve*, T1 phage (irradiated with 280 erg mm⁻²) in B_{s-1} cells. *Upper curves*, Haemophilus DNA *in vitro*, irradiated with 2000 erg mm⁻²; the solid and open triangles refer to two experiments with different PRE/DNA ratios. (Redrawn from H. Harm and Rupert, 1968; W. Harm *et al.*, 1968.)

B_{s-1}-160, which contain an increased number of PRE molecules (cf. right panel of Fig. 5).

2.4 Determination of the Number of ES Complexes from the Observed Flash Repair Effect

Since a single flash photolyses all the ES complexes present, their number should equal the number of pyrimidine dimers monomerized by such a flash. We determine this number from the biological photo-reactivation obtained, as outlined below.

In the theoretical example illustrated in Fig. 3, a UV dose D causes a certain survival in the dark. As a result of photoenzymatic repair the survival increases to the level marked "PR," which is the same as the dark survival obtained with a (smaller) UV dose D' . We shall call the difference $D-D' (= \Delta D)$ the "formal dose decrement," which is the portion of the UV dose whose inactivating effect is annulled by the photoreactivating treatment. To relate ΔD with the number of pyrimidine dimers monomerized by PER, the following considerations apply.

For the DNA's used in our study, the number of pyrimidine dimers

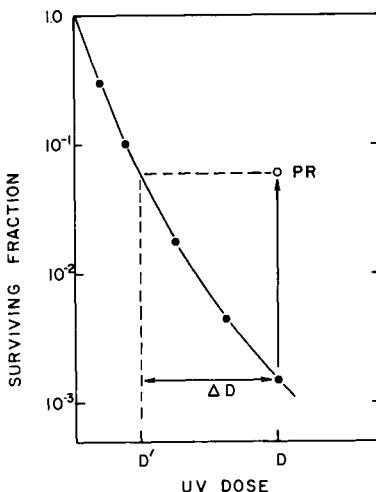


FIG. 3. Quantitative characterization of the PER effect by the dose decrement ΔD , as described in the text.

produced by a unit UV dose per unit amount of DNA is known. We can reasonably assume that the number of dimers monomerized, expressed by a dose decrement ΔD , resembles the number of dimers produced by a corresponding dose increment ΔD . Only a minor uncertainty comes from the fact that a small fraction of the total UV lesions (about 15% in *E. coli* and 10% in *Haemophilus* DNA) are not photorepairable. These could be either pyrimidine dimers that are incapable of repair (on account of the nature of their nearest-neighbor bases, or for some other reason), or they could represent a different type of UV photoproduct. For various reasons (including the low precision of dimer measurements) it is at present not known which of the two alternatives is correct. Thus, for simplicity, we have in all our calculations made the assumption (implicitly or explicitly) that both photorepairable and nonphotorepairable lesions are dimers. However, our essential results would hardly be affected if the alternative assumption were the correct one, since the fraction of nonphotorepairable lesions is small.

Our kind of analysis is based on the fairly well-established fact that UV radiation, within the range of doses applied in our experiments, produces pyrimidine dimers in proportion to the dose (e.g., R. B. Setlow and Carrier, 1966). It is further based on the reasonable assumption that all photorepairable inactivating photoproducts are equivalent, in the sense that they contribute with equal probability to the decrease in survival and that their photorepair contributes with equal probability to the survival increase.

ΔD can range from zero up to some maximum value ΔD_{\max} , depending on the initial UV dose D and on the particular biological system under study. For some purposes it is practical to express the observed PER effects in either terms of $\Delta D/\Delta D_{\max}$ (equaling the fraction of total photorepairable lesions that are photorepaired) or in terms of $\Delta D/D$ (equaling the fraction of total lethal lesions that are photorepaired). The latter is also called the "photoreactivated sector" (PRS).* It should be pointed out that this analysis applies for PER produced by either flash or continuous illumination. However, only in the case of flash photolysis can the calculated number of dimers monomerized be equated with the number of ES complexes present at a given time.

Consider first our *in vivo* systems. A UV dose of 1 erg mm⁻² of 254-nm UV radiation produces about 6.5 pyrimidine dimers in an *E. coli* chromosome (Rupp and Howard-Flanders, 1968). In *B_{s-1}* cells $\Delta D_{\max}/D$ is about 0.85, i.e., about 85% of the lethal UV photoproducts are photorepairable and thus constitute substrate for the PRE. Assuming that all lethal lesions are pyrimidine dimers, the number of "substrate molecules" in a stationary-phase cell would be $0.85 \times 6.5 \times D$, where D is measured in erg mm⁻², and a dose decrement of ΔD erg mm⁻² corresponds to repair of $6.5 \times \Delta D$ substrate molecules. DNA of phage T1 is very similar to that of *E. coli* in base composition and nearest-neighbor frequencies, but its mass is only 0.011 as great. Thus for T1 the above expressions would have to be multiplied by 0.011.

For *Haemophilus* transforming DNA *in vitro*, an incident dose of D erg mm⁻² (254 nm) causes in W μ g of DNA the formation of about $2.2 \times 10^9 \times D \times W$ pyrimidine dimers (for reference, see H. Harm and Rupert, 1968, p. 366). Assuming that all of the lethal lesions are dimers, the initial substrate concentration under these conditions would be $2.2 \times 10^9 \times D \times W \times 0.9$, where 0.9 is the fraction of total lesions which are photorepairable in this system.

It should be pointed out that in quite a number of our experiments only the relative amount of photorepair, i.e., $\Delta D/\Delta D_{\max}$, rather than the absolute amount, i.e., ΔD , is the relevant quantity. In such cases any uncertainties about the amount of DNA involved, the number of dimers produced per unit UV dose, or the fraction of these which are photorepairable will have little if any effect on the conclusions. This will become evident in later sections of this chapter.

Pyrimidine dimers are substrate not only for PER, but also for

*This quantity is not to be confused with the photoreactivable sector, often referred to in the literature, which is defined as the fraction of the UV dose that can be removed by maximal photoreactivation. The photoreactivable sector, or PRS_{max}, is thus $\Delta D_{\max}/D$.

enzymatic dark-repair processes, and the actions of these two types of repair therefore overlap greatly. Since in our experiments PER precedes dark repair, the latter would not affect the calculated amounts of substrate present at the beginning of PER. However, dark repair could bias the observed extent of PER and thus affect the correspondence between photorepaired lesions and ΔD . For example, in phage T4, v^+ gene repair reduces $\Delta D_{\max}/D$ to approximately 0.30, from a value of 0.65 found in the repair-deficient v^- strain and in phage T2 (W. Harm, 1963). It is obvious that the observed ΔD would become zero if all the photorepaired lesions were those which would otherwise be dark-repaired.

The fact that our *in vivo* systems are greatly deficient in dark repair makes it *a priori* likely that the observed ΔD correctly expresses the extent of PER. In the *Haemophilus* DNA system the influence of dark repair was tested by using the same DNA samples (unirradiated, irradiated at various UV doses, and flash-photoreactivated) for transformation of dark-repair proficient and dark-repair deficient cells. Although the UV survival of the transforming activity is considerably higher in the proficient cells, the photorepaired fraction of either the inactivating lesions (expressed by $\Delta D/D$) or of the photoreactivable lesions ($\Delta D/\Delta D_{\max}$) was found essentially the same as in the repair-deficient cells (H. Harm and Rupert, 1970c). J. K. Setlow *et al.* (1968) found only a slight increase in $\Delta D/\Delta D_{\max}$ when using a repair-deficient strain. Thus, in any case, dark repair does not seem to affect the data obtained through this kind of analysis.

3. Determination of the Number of Photoreactivating Enzyme Molecules

3.1 "Titration" of Enzyme Molecules *in Vitro*

Knowing the number of pyrimidine dimers in DNA and the fraction of these complexed with PR enzyme (as expressed by the magnitude of $\Delta D/\Delta D_{\max}$ under flash illumination), it is possible to titrate enzyme molecules against pyrimidine dimers in DNA *in vitro*, provided we work under conditions where essentially all the enzyme present is complexed. For a simple binary equilibrium in the dark, we can write from reaction scheme (1)

$$\frac{[ES]_{eq}}{([E]_0 - [ES]_{eq})([S]_0 - [ES]_{eq})} = \frac{k_1}{k_2} = K \quad (2)$$

where $[E]_0$ and $[S]_0$ are the total enzyme and substrate concentrations, respectively, and $[ES]_{eq}$ is the concentration of the complexes at equilibrium.

The fraction $P = [ES]_{eq}/[S]_0$ of the total substrate complexed with the enzyme can be calculated as a function of $[S]_0/[E]_0$ for any value of the parameter $[E]_0K$ by substituting $P[S]_0$ for $[ES]_{eq}$ in Eq. (2) and suitably rearranging. Examples are shown in the left panel of Fig. 4, where $\log P$ is plotted versus $\log([S]_0/[E]_0)$ with various values of $[E]_0K$ as parameters. For a fixed $[E]_0$ it is possible—regardless of the magnitude of K —to find a range of $[S]_0$, where the slope of the curve is 45° . In this range P is inversely proportional to $[S]_0$, i.e., all of the enzyme is complexed. The 45° portion extrapolates to $P = 1$ at the value $[S]_0/[E]_0 = 1$, i.e., where the concentration of pyrimidine dimers equals the concentration of enzyme molecules.

In practice, it is necessary to use conditions where the 45° slope is obtained at reasonably high P values, since P corresponds to $\Delta D/\Delta D_{max}$, which below 0.1 can be measured with only a low degree of accuracy. The results of actual experiments, obtained with a constant concentration of PRE and varying concentrations of irradiated DNA (corresponding to a varying concentration of substrate) are shown in the right hand panel of Fig. 4. The observed $\Delta D/\Delta D_{max}$, resulting from a single flash illumination, gives curves strongly resembling those in the left panel. At a sufficiently low DNA concentration, this fraction reaches a plateau close to 1.0, as might be expected if $[E]_0K \gg 1$, while at high DNA concentrations the decrease of $\Delta D/\Delta D_{max}$ becomes inversely proportional to

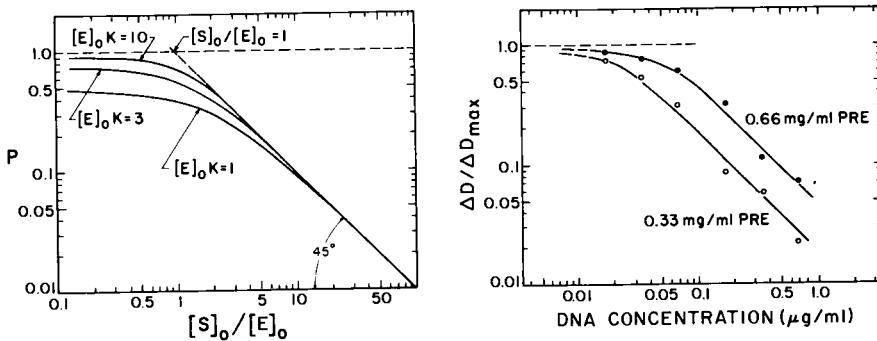


FIG. 4. Determination of the number of PRE molecules *in vitro*. Left panel, Theoretical curves showing the fraction of substrate complexed with PRE as a function of the initial substrate/enzyme ratio, for various values of the parameter $[E]_0K$. Right panel, Experimental curves showing the fraction of substrate complexed (expressed by $\Delta D/\Delta D_{max}$) vs. concentration of DNA, irradiated at 1920 erg mm^{-2} , for two different PRE concentrations. The existence of a 45° slope permits calculating the number of PRE molecules present if the number of substrate molecules per unit amount DNA is known, as described in the text. (Right panel redrawn from H. Harm and Rupert, 1968.)

the DNA concentration. In this range, the number of photoproducts repaired equals the number of PRE molecules in the mixture.

It is evident that the two PRE concentrations used, which differ by a factor of 2, give curves that differ also by about a factor of 2. Calculations have shown that, for instance, 0.33 mg protein extract from yeast cells (about 5- to 10-fold enriched for the PRE), contains about 7×10^{10} PRE molecules or about 1 PRE molecule per 3×10^9 daltons protein (H. Harm and Rupert, 1968). Assuming that the molecular weight of the PRE does not exceed 10^5 daltons,* these figures indicate that the PRE constitutes less than 10^{-5} of the total protein mass in unpurified yeast extract.

3.2 The Number of Enzyme Molecules in *E. coli* Cells

We have been able to determine the number of PRE molecules per stationary phase *E. coli* cell from the results shown in the left panel of Fig. 5. A survival curve for B_{s-1} was established, keeping the cells either dark, or applying a single light flash about 10 minutes after UV irradiation.[†] The dose decrement obtained with a single light flash at each UV dose was about 3.0 erg mm^{-2} for all doses above 6.4 erg mm^{-2} . A ΔD of 3 erg mm^{-2} corresponds to about 20 pyrimidine dimers repaired per cell, indicating that 20 ES complexes is the maximal number which can be present at any given time. As this number is not exceeded even though the substrate concentration increases further with increasing UV dose, it must be limited by the number of PRE molecules in the cell, this number being about 20. Assuming a total protein content per *E. coli* cell of roughly 10^{-13} gm, or 6×10^{10} daltons (Luria, 1960), we have about 1 PRE molecule per 3×10^9 daltons protein. Thus the fraction of PRE among the total cell protein is probably of the order of 10^{-5} .

The relatively low number of PRE molecules in B_{s-1} cells seems to be typical for *E. coli*. Flash PER of phage T4 $v-x$, infecting comparatively B_{s-1} and B/r cells, showed that these two strains, which differ greatly in their UV sensitivities, have about the same PRE content (W. Harm *et al.*, 1968). By the same criteria, derivatives of

* Preliminary results by Muhammed (1966) suggested an average molecular weight of about 3×10^4 for the protein of a 3000-fold purified yeast PRE preparation. However, our work shows that such purity would mean an enzyme content of less than a few percent, and thus makes the relevance of this figure uncertain. For the PRE of a blue-green alga, *Anacystis nidulans*, Saito and Werbin (1970) found a molecular weight close to 10^5 .

† It will be shown in Section 4.1 that the ES complex formation in these cells is relatively slow, so that it takes about 5 minutes until the equilibrium of complex formation is attained.

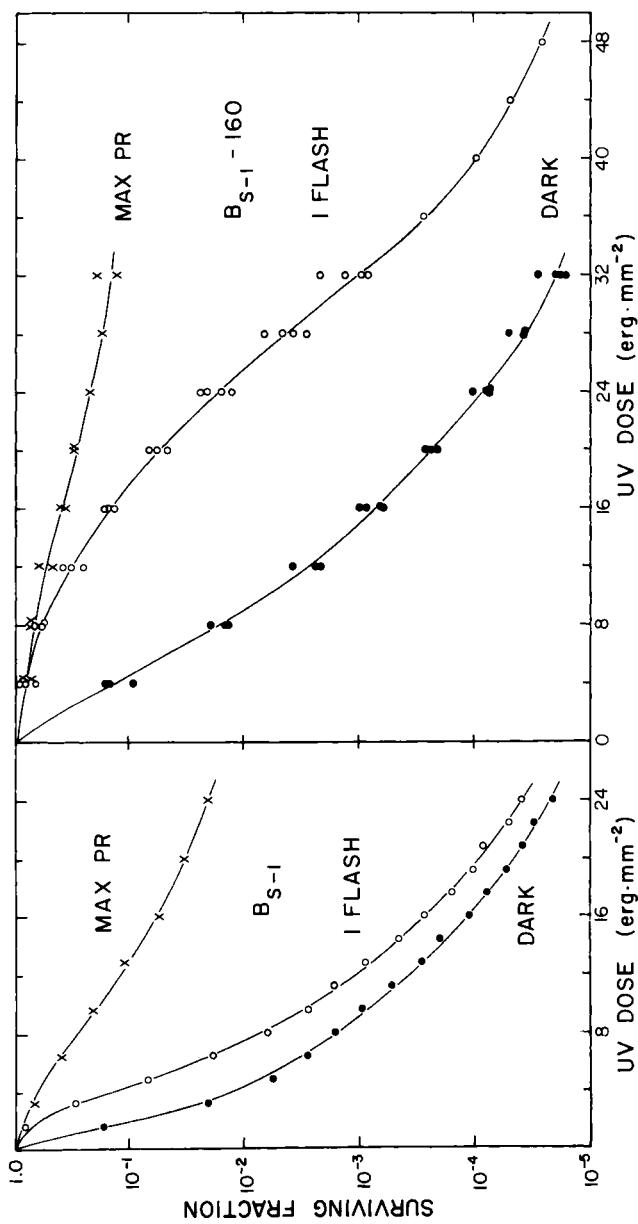


FIG. 5. Survival increase of irradiated *E. coli* resulting from PER by either a single flash (○) or continuous illumination until maximum repair is obtained (X). ● corresponds to survival without PER. *Left panel*, strain B_{s-1} ; *right panel*, strain $B_{s-1}-160$, which has increased PRE content. (From W. Harm *et al.*, 1968; W. Harm, 1969.)

E. coli strain K12, i.e., AB 2437 and AB 2480, displayed an even smaller number of PRE molecules: about 12 to 15 per cell.

3.3 Mutants with Increased Enzyme Content

In view of the low PRE content of *E. coli* cells we expected to find mutations causing increased production of PRE. Three such mutant strains were isolated from mutagen-treated cultures of B_{s-1} cells (W. Harm, 1969), and one of them, called $B_{s-1}-160$, has proved very useful for some of the work summarized here. The isolation procedure was simply based on the expectation that a mutant with higher PRE content should be recognizable by its more extensive PER after a single flash.

The right panel of Fig. 5 shows that at a UV dose of 16 erg mm^{-2} a single flash increases the survival by a factor of 200 in $B_{s-1}-160$ cells, compared to a factor of 2–3 in B_{s-1} cells. At doses below 8 erg mm^{-2} , the repair accomplished by a single flash in these cells is virtually the same as the maximum PER obtained with continuous illumination. As pointed out in Section 2.3, this provides additional evidence that a single flash repairs essentially all lesions complexed with PRE.

The number of PRE molecules per cell in strains $B_{s-1}-160$, as well as in the two other mutant strains isolated, has been determined by the maximum dose decrement to be obtained with a single flash, in much the same way as it has been done with B_{s-1} cells. The results are shown in Fig. 6. The maximum level of ΔD corresponds

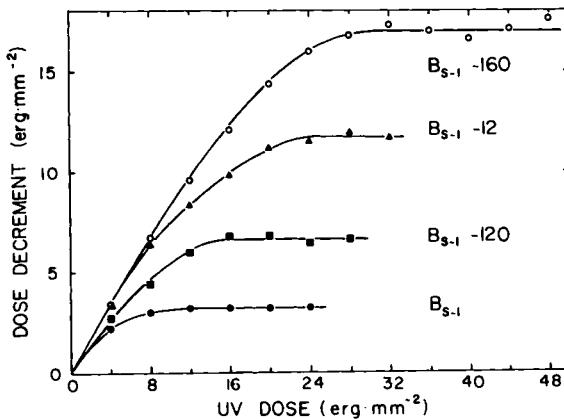


FIG. 6. Dependence of the dose decrement obtained with a single flash on the irradiation dose for various *E. coli* strains. The plateau level reached at sufficiently high irradiation doses permits calculation of the number of PRE molecules per cell of the respective strain, as described in the text. (From W. Harm, 1969.)

to about 110 PRE molecules per cell for B_{s-1} -160, and 43 and 75 for strains B_{s-1} -120 and B_{s-1} -12, respectively.

Mutants with increased PRE content not only show the greater ΔD by a single flash, but also show—as expected—a much faster photo-repair under continuous illumination than B_{s-1} cells (W. Harm, 1969).

4. The Complex-Formation Step

4.1 The Overall Reaction

According to the reaction scheme [Eq. (1)], the time course for the formation of *ES* complexes in the dark should follow the differential equation

$$(d[ES]/dt)_{\text{dark}} = k_1[E][S] - k_2[ES] \quad (3)$$

where $[ES]$, $[E]$, and $[S]$ are the concentrations of complex, free enzyme, and free substrate, respectively. k_1 is the rate constant for formation of complexes, and k_2 is the rate constant for dissociation of complexes in the dark. If no further increase with time is observed, i.e., if $d[ES]/dt = 0$, the reaction has attained equilibrium and

$$[ES]_{eq} = k_1[E][S]/k_2 \quad (3a)$$

which is simply another form of Eq. (2).

4.1.1 *In Vitro*

Complex formation *in vitro* can be experimentally observed as follows. The reaction begins when PRE and irradiated transforming DNA are mixed, at which time $[ES]$ is zero. The increase of $[ES]$ in approaching equilibrium is measured by withdrawing samples at various times after mixing, applying a single flash and determining the number of lesions repaired (as described in Section 2.4). The example given in Fig. 7 shows the increase of $\Delta D/\Delta D_{\max}$ with the time period allowed for the dark reaction. This increase is of course identical with the relative increase in the number of *ES* complexes formed. One sees that under conditions commonly applied for PER *in vitro*, complex formation is a relatively slow process. For the more concentrated mixture it takes at room temperature at least 2 minutes to attain the dark equilibrium; for the 5-fold diluted mixture it takes roughly 30 minutes. After complexes in an equilibrium mixture are photolyzed by a flash, the formation of new complexes can be observed by applying a second flash at still later times. The figure shows that the second equilibrium is reached much more rapidly than the first. The explanation for this difference is given in Section 4.2.

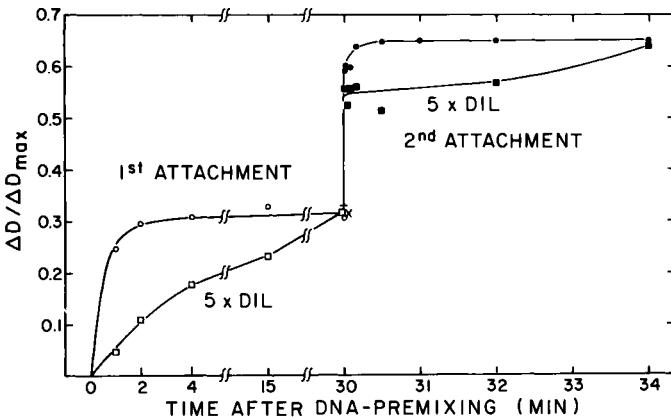


FIG. 7. Complex formation *in vitro* as a function of time. PRE and irradiated *Haemophilus* DNA were mixed at time 0, and the fraction of substrate complexed (expressed by $\Delta D / \Delta D_{max}$) was determined by applying a single flash at the time shown on the abscissa (open symbols). Samples having received a 2-unit flash at 30 minutes, marked by (+) and (X), obtained a second 2-unit flash at the times indicated (closed symbols). The reaction mixture contained either 0.17 $\mu\text{g}/\text{ml}$ DNA, UV-irradiated with 2400 erg mm^{-2} , and 1.33 mg/ml PRE-containing extract (○, ●), or 1/5 these concentrations (□, ■). (Redrawn from H. Harm and Rupert, 1968.)

4.1.2 *In Vivo*

In the *E. coli* cell, complex formation begins at the moment when the substrate is created by UV irradiation, since the enzyme is already present. In the very UV-sensitive strain *B_{s-1}*, where an adequate radiation dose can be applied in about one second, the beginning of complex formation is fairly precisely defined. Its progress can then be followed by applying a single light flash to samples at fixed times and determining cell survival, as shown in Fig. 8. At room temperature it takes about 5 minutes until complex formation has reached the equilibrium. Liberation of PRE from complexes by flash photolysis after reaching the dark equilibrium starts a second "round of complex formation." In contrast to the *in vitro* results, the kinetics of the two "rounds" are similar.

4.2 Local versus Average Enzyme Concentration

The difference seen *in vitro* between the first and the second "round of complex formation," as contrasted to the similarity between the first and second round in *E. coli* cells, can be explained as follows. In the reaction mixture *in vitro* the distribution of the substrate is not random. At the UV dose applied, each of the DNA fragments (of an

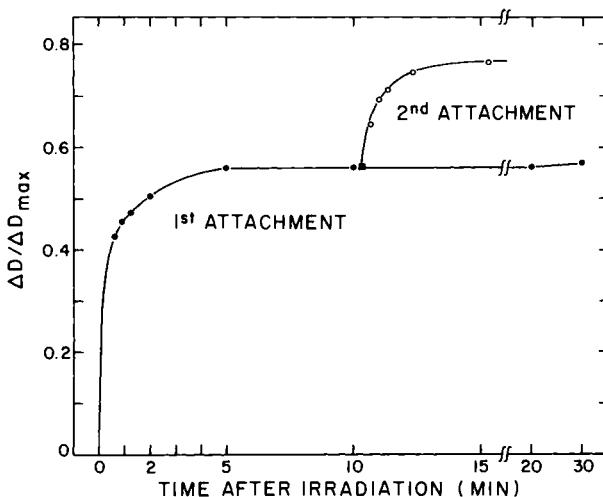


FIG. 8. Complex formation *in vivo* as a function of time. B_{s-1} cells were irradiated with 4.8 erg mm^{-2} at time zero, and the fraction of substrate complexed (expressed by $\Delta D/\Delta D_{max}$) determined by applying a single flash at the time shown on the abscissa (●). The sample having received a 2-unit flash at 10 minutes and 20 seconds (■) received a second 2-unit flash at various times afterwards (○), thus indicating the second "round of attachment." (Redrawn from W. Harm *et al.*, 1968.)

average size of about 2×10^7 daltons) contains about 150 dimers. Under the conditions of the experiment, such DNA fragments are separated from each other by average distances which are larger than the dimensions of each double-stranded coil. The dimers are consequently clustered in the solution.

In contrast, the distribution of PRE molecules is random at the moment of addition to the DNA, but becomes nonrandom as they attach to this clustered substrate. Thus after flash photolysis the average distance between a PRE molecule and the nearest remaining UV lesions should be considerably smaller than when the mixture is first assembled. The difference between the first attachment and the second attachment kinetics should therefore reflect essentially the difference in the diffusion time required for a PRE molecule to encounter a lesion in these two situations.

This explanation is consistent with the observation that *in vivo* no appreciable difference was found between first and second rounds of complex formation. In an *E. coli* cell the DNA ($5 \times 10^{-9} \mu\text{g}$) extends over most of the cell volume (roughly 10^{-12} ml), corresponding to a 1000-fold higher concentration than in an *in vitro* reaction with $5 \mu\text{g}$

DNA/ml. Under these conditions the distribution of substrate should be much closer to random, so that PRE molecules liberated by photolysis would have little advantage compared to nonreacted PRE.

The kinetics of both the first and second rounds of complex formation *in vitro* presumably obey Eq. (3) with the same k_1 and k_2 . The difference is mainly in $[E]$, which in the case of first attachment is the *average* enzyme concentration, i.e., the number of PRE molecules/total volume of the reaction mixture, but in the second attachment is the *local* concentration, i.e., the number of PRE molecules per unit volume around a DNA molecule). It is difficult to define just what this volume is, although it is clear that the local concentration must be considerably higher than the well-defined average concentration. For this reason, determination of k_1 should be based only on the first attachment kinetics. Even there, the nonrandom distribution of $[S]$ makes such a determination only approximate, since Eq. (3) implies random distribution of both $[E]$ and $[S]$.

4.3 Determination of the Reaction Rate Constant k_1

According to Eq. (3), the initial slope of the attachment kinetics in the dark should specify k_1 . However, this would lead to only a fairly rough estimate. The following experimental set-up was designed for a more accurate measurement of k_1 *in vivo* and *in vitro*. From the beginning of the reaction, light flashes were applied in rapid sequence (1 to 2 flashes per second) for various time periods. This ensured that the enzyme is always released very shortly after formation of an ES complex so that the concentration of free enzyme throughout the whole period is close to $[E]_0$, the concentration of free PRE when no substrate is present. Furthermore, since by this procedure $[ES]$ is kept small, and since k_2 is not large (as will be shown in Section 4.4), the second term of Eq. (3) can be neglected, thus giving

$$(d[ES]/dt)_{\text{dark}} \approx k_1[E]_0[S] \quad (3b)$$

As the formation of an ES complex is immediately followed by repair under these conditions, $d[ES]$ in Eq. (3b) can be replaced by $-d[S]$, so that we obtain by integration

$$\ln([S]_t/[S]_0) \approx -k_1[E]_0 t \quad (4)$$

In other words, the relative concentration of substrate remaining should be an exponential function of the time t . Since $[S]_t/[S]_0$ can be determined experimentally (equaling $1 - \Delta D/\Delta D_{\text{max}}$) and $[E]_0$ is known, k_1 can be calculated.

4.3.1 In Vivo

Experimental results obtained with B_{s-1} and $B_{s-1}-160$ cells are shown in Fig. 9. Evidently the curves reflect some heterogeneity with respect to k_1 , since they are not straight lines as expected from Eq. (4). The values below are obtained from the initial slopes of the curves, and thus correspond to a weighted average for k_1 . As mentioned earlier, $[E]_0$ for stationary phase B_{s-1} cells is about 20, and for $B_{s-1}-160$ about 110 molecules/cell volume. Therefore, the slopes in Fig. 9 correspond to $k_1 = 1.8 \times 10^{-3}$ cell volumes·molecules⁻¹ sec⁻¹ in the case of B_{s-1} , and 1.5×10^{-3} in the case of $B_{s-1}-160$ (W. Harm, 1970b). Probably this difference is within the limits of accuracy, and one can assume that the value obtained for B_{s-1} is closer to the correct value, since the longer times involved make the approximation better. In units conventionally used for enzyme kinetics this corresponds to about 1×10^6 liter·mole⁻¹ sec⁻¹, assuming a cell volume of 10^{-15} liter (cf. Luria, 1960).

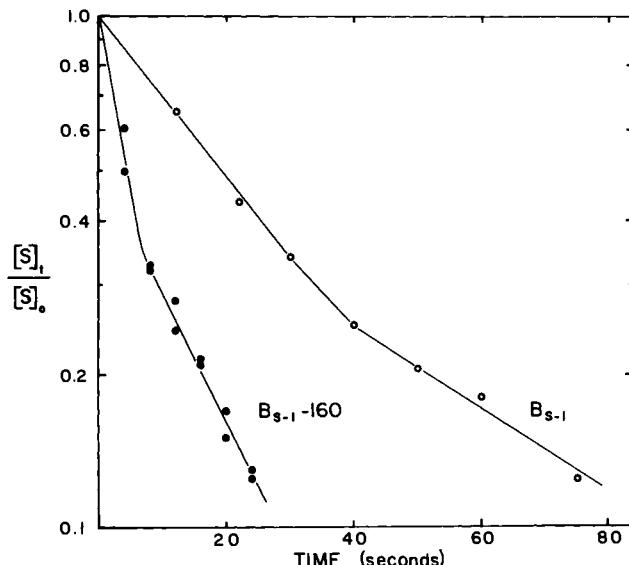


FIG. 9. Determination of the reaction rate constant k_1 in B_{s-1} and $B_{s-1}-160$ cells, irradiated with 8 erg mm^{-2} . The concentration of $[S]_t$ of photorepairable lesions remaining unrepaired after time t , relative to the concentration $[S]_0$ of those originally present, is plotted as a function of time. During this time light flashes were given in rapid sequence to ensure repair (and thus liberation of the PRE molecules) very soon after formation of an ES complex. (From W. Harm, 1970a.)

4.3.2 *In Vitro*

The same type of experiment has been carried out with the *Haemophilus* DNA system. Here, of course, a greater choice of experimental parameters is available than for the cellular system, and the influence of some of them, viz. pH and ionic strength, will be discussed in Section 4.6. Under our "standard" conditions (pH 6.8, ionic strength 0.14, and room temperature $\sim 23^\circ\text{C}$), k_1 was found to be 2.6×10^7 liter mole $^{-1}$ sec $^{-1}$. This value is about 25 times higher than the k_1 found for *E. coli* cells (H. Harm and Rupert, 1970a).

In view of the great similarities of the *E. coli* and yeast PRE this difference might seem surprising, but it is indeed expected. k_1 expresses the probability that a PRE molecule will collide with a UV lesion times the probability for complexing to occur upon collision. The first probability is a function of the diffusion rate, which is inversely proportional to the viscosity of the medium, and hence k_1 should be much higher in the aqueous *in vitro* mixture than in the highly viscous interior of a cell. We have tested this point *in vitro* by adding sucrose or glycerol to reaction mixtures in amounts which increase the viscosity by factors of 2 and 5, and found the values of k_1 were correspondingly decreased in each case. Hence, from the published diffusion rates of macromolecules (including proteins) in cell extrudates of *E. coli* (Lehman and Pollard, 1965), a factor of 25 increase for k_1 does not seem unrealistic.

4.4 Determination of the Reaction Rate Constant k_2

In principle, k_2 could be determined independently of k_1 , if one were able to create experimental conditions under which formation of new complexes is completely precluded, so that any dark dissociation of existing complexes registers as a decrease in [ES]. Under such circumstances the product $k_1[E][S]$ in Eq. (3) could be neglected and the expected decrease of complexes in the dark should essentially follow the equation

$$(d[\text{ES}]/dt)_{\text{dark}} \approx -k_2[\text{ES}] \quad (5)$$

or

$$\ln([\text{ES}]_t/[\text{ES}]_0) \approx -k_2 t \quad (5a)$$

where $[\text{ES}]_0$ is the concentration of complexes at the equilibrium, and $[\text{ES}]_t$ is the concentration after the time period t during which formation of new complexes is precluded. Such conditions can be approximated in two ways: (a) by introducing excessive amounts of competing substrate to which dissociating PRE molecules could attach;

(b) by using a PRE inhibitor which would block most of the non-complexed UV lesions. Neither of these two approaches has proved to be fully satisfactory, but they have provided at least a rough estimate of k_2 .

4.4.1 COMPETING SUBSTRATE METHOD

For the *in vitro* system, competing substrate can easily be added to the transformation mixture after equilibrium complex formation in the form of highly UV-irradiated unmarked, i.e., wild-type *Haemophilus* DNA, or calf thymus DNA, while the corresponding control mixtures obtain an equal amount of unirradiated DNA. The disappearance of complexes according to Eq. (5a) can then be followed by withdrawing samples at various times and determining $[ES]_t/[ES]_0$ from the photo-repair effect obtained with a single flash given at time t . Unless the excess of competing substrate is very large, $[ES]_t$ will attain a lower, but finite value, corresponding to a new equilibrium. It is necessary therefore to adjust $[ES]_t$ and $[ES]_0$ by subtracting the value for $[ES]_\infty$, which is the concentration of complexes remaining in the original transforming DNA after the new equilibrium is reached.

The results of such experiments are shown in Fig. 10. One realizes that the adjusted experimental values of $[ES]_t/[ES]_0$ do not follow a

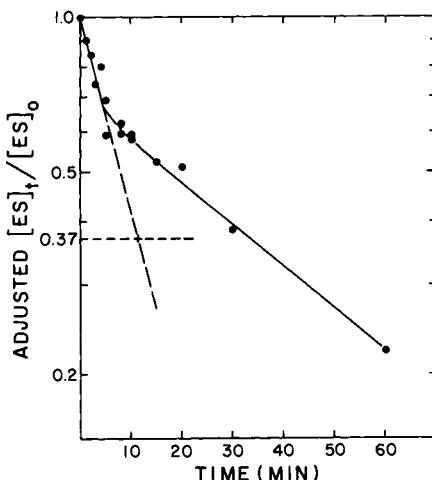


FIG. 10. Determination of the reaction rate constant k_2 *in vitro*. The decrease in the relative concentration of complexes (adjusted as described in the text) is shown as a function of time elapsed after adding competing substrate to the reaction mixture. (Redrawn from H. Harm and Rupert, 1970a.)

straight line in semilog plot, as would be expected from Eq. (5a) if all complexes behaved the same. The curve rather exhibits some inhomogeneity with respect to the stability of the existing complexes. From the initial slope we obtain a weighted average k_2 of $1.5 \times 10^{-3} \text{ sec}^{-1}$.

Employing the competing substrate for cellular conditions is more difficult, but has been achieved in the following manner. UV-irradiated T1 phage were used to infect $B_{s-1}-160$ cells in the presence of chloramphenicol to preclude intracellular phage development. After equilibrium complex formation between PRE and the UV lesions in the phage DNA, the infected cells were subject to a UV dose sufficient to create a large excess of substrate in the form of irradiated bacterial DNA. The latter UV dose need not be large, since the *E. coli* chromosome contains roughly 100 times as much DNA as the T1 chromosome, so that the concomitant additional T1 inactivation can be kept small. The relative decrease of ES complexes in phage DNA was then followed as a function of time, by photoreactivating the phages with a single flash and determining their survival.

The results, shown in the left panel of Fig. 11, resemble those obtained *in vitro* in that they indicate a heterogeneity with respect to k_2 , with the weighted average value amounting to $1.9 \times 10^{-3} \text{ sec}^{-1}$ at room temperature.

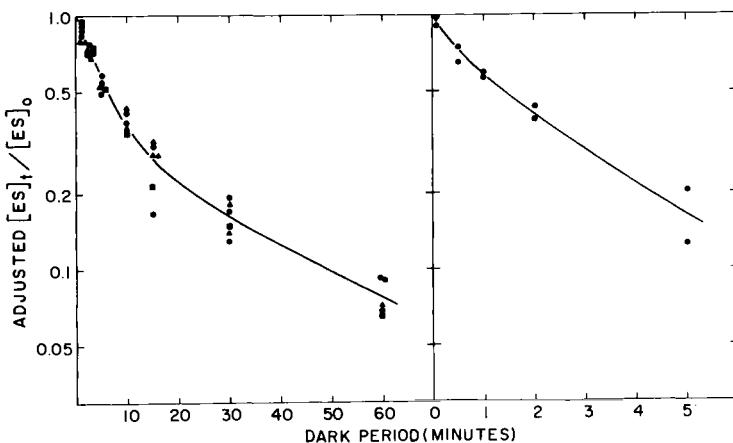


FIG. 11. Determination of the reaction-rate constant k_2 *in vivo*. *Left panel*, The decrease in the relative concentration of complexes in phage DNA (adjusted as described in the text) is shown as a function of time elapsed after creating competing substrate in the form of irradiated bacterial DNA. The *right panel* shows the corresponding decrease of ES complexes in irradiated bacterial DNA after addition of caffeine (16 mg/ml) at time 0. (Redrawn from W. Harm, 1970a.)

4.4.2 DISSOCIATION OF COMPLEXES IN THE PRESENCE OF AN INHIBITOR

It will be shown in Section 6 that caffeine acts as an inhibitor for the formation of ES complexes, but does not affect their photolysis. Although this substance does not prevent complex formation completely, it slows it down considerably so that the equilibrium concentration of [ES] is lower than in the absence of caffeine. Therefore, adding caffeine to cells in which [ES] is at equilibrium should lead to a time-dependent loss of [ES] until the new equilibrium is reached. This permits determining k_2 in a manner analogous to the competing substrate method, except that lesions in bacterial DNA can be studied directly, and the phage can be omitted. The results in the right panel of Fig. 11 show that the k_2 obtained with the caffeine method is about 7 times higher (1.3×10^{-2}) than that obtained with the competing substrate method. The values obtained *in vitro* with caffeine inhibition are also higher (4 to $7 \times 10^{-3} \text{ sec}^{-1}$) than those obtained with competing substrate (see right panel of Fig. 12).

4.4.3 CALCULATION OF k_2 FROM THE EQUILIBRIUM CONCENTRATION OF COMPLEXES

If the correct value of k_1 is known, one should be able to calculate k_2 from the equilibrium concentration of ES complexes. According to Eq. (2), at equilibrium in the dark

$$k_2 = \frac{k_1([E]_0 - [ES]_{eq})([S]_0 - [ES]_{eq})}{[ES]_{eq}} \quad (6)$$

With k_1 known, $[E]_0$ and $[S]_0$ established for the experimental conditions, and $[ES]_{eq}$ determined from the flash PER effect, k_2 can be calculated. For obtaining reliable estimates, conditions are required under which $[ES]_{eq}$ is reasonably large but not too close to either $[E]_0$ or $[S]_0$ in value. Calculations gave k_2 values around $5 \times 10^{-3} \text{ sec}^{-1}$ for our *in vivo* system and about $1.9 \times 10^{-3} \text{ sec}^{-1}$ *in vitro*. In both cases these values are intermediate between those obtained with the caffeine and competing substrate methods.

4.4.4 COMPARISON OF k_2 MEASUREMENT METHODS

k_2 can be defined as the probability for transition in the dark of an ES complex to the dissociated state. The experimental distinction between the two states is based on the criterion that the complex can be photolysed (and thus repaired) by a single light flash. However, for the alternative nonphotolysable state various possible situations can

be envisaged, all of which would qualify—according to this criterion—for the dissociated state. Thus, in view of the non-random distribution of enzyme and substrate at dark equilibrium, it is not surprising that k_2 values obtained with various methods differ from each other, simply because these methods imply different definitions and criteria for the dissociated state.

1. *Competing substrate method.* Shortly after a complex has lost its photorepairability, the likelihood that the liberated enzyme will reattach to the same or neighboring lesions in the same DNA region should be greater than the probability it will attach to competing substrate, if the latter is more distant. This is probably the case both *in vitro* (where the substrate is “clustered”; cf. Section 4.2), and *in vivo* (where the complexes are in phage DNA and the competing substrate is in bacterial DNA). Thus k_2 values obtained with the competing substrate method correspond essentially to those dissociations where the detached enzyme molecule diffuses far enough to be likely to react with a competing lesion. Accordingly, we would expect this method to give k_2 values lower than those obtained with the two other methods, in agreement with our data.

2. *Equilibrium method.* Calculation of k_2 from the level of dark equilibrium according to Eq. (6) also probably leads to an underestimate of k_2 (if k_2 is defined as the probability for a photorepairable complex to become nonphotorepairable). The reason is that in Eq. (6)—for lack of better knowledge—we use for $[E]_0$ the *average* total enzyme concentration, instead of the (higher) *local* enzyme concentration (cf. Section 4.2), which would logically apply for the equilibrium condition.

3. *Caffeine method.* The highest k_2 values (obtained with caffeine as an inhibitor) might be closest to the “correct” value, if k_2 is defined as mentioned in (2). Since caffeine is used at rather high concentration (about 0.08 M), it is likely to interfere immediately after dark dissociation of a complex with its possible reformation. On the other hand, we cannot rule out the possibility that in some cases caffeine “shoulders” its way into a fully functional complex, thus actually facilitating its dissociation. The result would be an overestimation of k_2 by this method.

We can be confident that the physical association between a UV lesion and a PRE molecule (in the sense that there is no room for the association of the same lesion with another PRE molecule) is amenable to photorepair at essentially all times. Were it not, maximum photorepair could not be obtained with a single light flash. Therefore the definition of k_2 as the dark transition of a UV lesion from the photorepairable to a nonphotorepairable state is probably most adequate for purposes

of enzyme kinetics. In this respect our k_2 values determined with the competing substrate method and calculated from the dark equilibrium are probably too low. However, the choice of method does not depend so much on which one gives the "correct" k_2 value, as on how one intends to use the value obtained.

4.5 Temperature Dependence of k_1 and k_2

The methods described in Sections 4.3 and 4.4 have also been used to determine the dark reaction rate constants at temperatures higher or lower than ambient. The results are presented in the conventional form of "Arrhenius plots," where the logarithm of a rate constant is plotted *versus* the reciprocal of the absolute temperature. The left panel of Fig. 12 shows data obtained with the *E. coli* system; the center and right panels summarize those obtained with *Haemophilus* DNA *in vitro*.

The straight lines obtained in such a plot can be expressed by

$$\ln k = -E_A/RT + \text{constant}$$

where R is the gas constant ($=1.99 \times 10^{-3}$ kcal·mole $^{-1} \cdot ^\circ K^{-1}$), and T is the absolute temperature. Thus the slope defines the activation energy E_A , i.e., the excess over the average energy which a molecule must temporarily assume in order to react. The slopes obtained with the *E. coli* system correspond to an activation energy of about 11 kcal·mole $^{-1}$ for complex formation (k_1), and to about 4.5 kcal·mole $^{-1}$ for the dark dissociation (k_2). The respective values for the *in vitro* system are 9.3 kcal·mole $^{-1}$ and 5.1 to 5.7 kcal·mole $^{-1}$.

These values permit some conclusions about the energetics of the dark reactions. As noted in Section 4.3, complex formation involves at least two events in sequence—a "rendezvous" of the enzyme with its substrate through random diffusion, and a subsequent coupling. The rate of the "rendezvous" step is proportional (among other things) to the reciprocal of the solution viscosity, which depends on the temperature in the same general fashion as the number of activated molecules does. Since the observed effect of viscosity on k_1 (Section 4.3) shows that the rendezvous rate strongly affects the rate of complex formation, the change of viscosity with temperature must make a contribution to the apparent activation energy. If every encounter between reactants led promptly to coupling, this contribution would, in the case of the *in vitro* system, amount to about 3.7 kcal·mole $^{-1}$ (as determined from tabulated data for the viscosity of water), and would constitute the whole effect of temperature on reaction rate over the range studied. Clearly this is not the case, since the measured value is larger (9.3 kcal). Under these

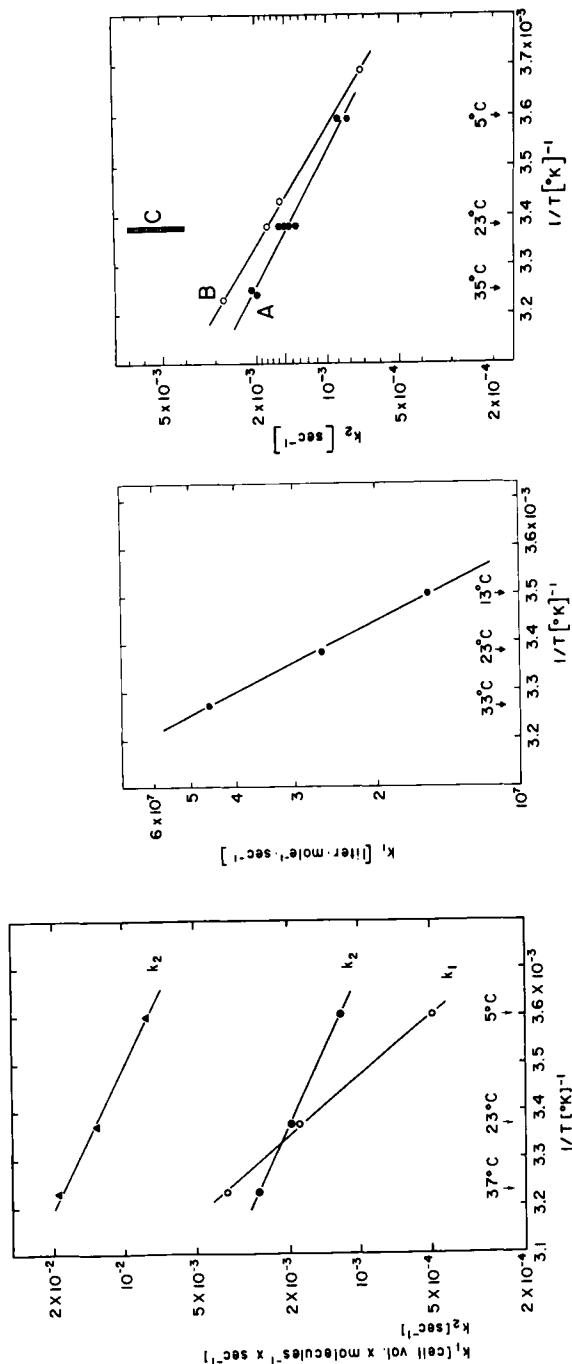


Fig. 12. Temperature dependence of rate constants k_1 for complex formation, and k_2 for complex dissociation, presented as Arrhenius plots. *Left panel*, Dependence of both constants in *E. coli* cells, showing for k_2 the results of measurements by the caffeine method (\blacktriangle) and the competing substrate method (\bullet). *Center panel*, Dependence of constant k_1 for yeast PRE *in vitro*. *Right panel*, Dependence of constant k_2 for yeast PRE *in vitro*, showing results of measurements by both the competing substrate method (curve A), and the dark equilibrium method (curve B). The range of values obtained by the caffeine method at 23°C (labeled C) is shown for comparison. (Left panel from W. Harm, 1970a; center and right panels from H. Harm and Rupert, 1970a.)

circumstances it is not obvious what part of the 3.7 kcal can legitimately be subtracted in order to estimate an activation energy for the coupling of rendezvoused reactants, but it is evident, that subtracting any value between 0 and 3.7 kcal·mole⁻¹ from 9.3 kcal, leaves this activation energy at least equal to, and possibly larger than that for dark dissociation of the complex (5.1 to 5.7 kcal). Consequently the energy of the system before coupling is either about the same or smaller than after coupling. On the other hand, the complexes have a very high stability, as shown by the large equilibrium constant $K = k_1/k_2$, amounting to $\approx 10^{10}$ to 10^{11} liter·mole⁻¹ under the various experimental conditions employed. Thermodynamic analysis shows this high stability is due entirely to a large entropy increase during passage to the complexed state, as follows.

The "standard" free energy change for complexing may be calculated from the equilibrium constant, using the relation $\Delta F^\circ = -RT \ln K$. K *in vitro* is known as a function of temperature from the data in the center and right panels of Fig. 12, permitting ΔF° to be resolved into enthalpy (ΔH°) and entropy (ΔS°) terms: $\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ$. This results in $\Delta H^\circ \approx 3600$ to 4200 cal·mole⁻¹ and $\Delta S^\circ \approx 58$ to 61 cal·mole⁻¹·°K⁻¹. Thus at room temperature the term $T\Delta S^\circ$ contributes a free energy change of 17 to 18 kcal·mole⁻¹ in favor of the binding, while the change in enthalpy (essentially the energy change) actually *opposes* binding by roughly 4 kcal·mole⁻¹. The same is true *in vivo*, for which the

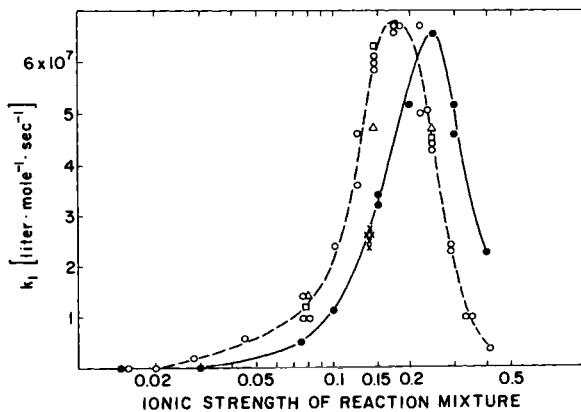


FIG. 13. Dependence on ionic strength of rate constant k_1 for complex formation by yeast PRE *in vitro*. Open symbols: reaction mixtures with fixed phosphate buffer and varying NaCl concentrations. (□, ○, and △ represent data taken with relative substrate concentrations of 0.4, 1 and 2, respectively.) ●, reaction mixtures with phosphate and NaCl concentrations in equimolar proportions. Crosses represent data from the "standard" reaction mixtures described in the original paper. (From H. Harm and Rupert, 1970a.)

data in the left-hand panel of Fig. 12 yield $\Delta H^\circ \approx 6500 \text{ cal} \cdot \text{mole}^{-1}$ and $\Delta S^\circ \approx 58 \text{ to } 62 \text{ cal} \cdot \text{mole}^{-1} \cdot {}^\circ\text{K}^{-1}$.

It is interesting that the binding of RNA polymerase to DNA also seems to be a matter of an entropy increase, rather than an enthalpy decrease (P. Witonsky, personal communication). The same has been found for binding of isoleucyl-tRNA synthetase to isoleucine-tRNA (Yarus and Berg, 1970). These examples suggest a general pattern, which may also hold for specific binding of other enzymes to nucleic acids.

4.6 Dependence of k_1 and k_2 on Ionic Strength and pH

The processes of complex formation and dissociation are critically dependent on the ionic make-up of the reaction mixture. This is seen by varying this make-up in two different ways—(1) changing the monovalent salt concentration while keeping a constant low concentration of buffer, or (2) varying the salt and buffer together in fixed proportions. Because the findings described in the next section show that

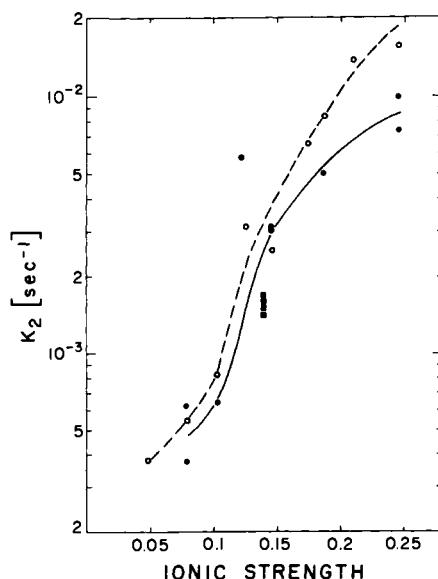


FIG. 14. Dependence on ionic strength of rate constant k_2 for dissociation of ES complexes *in vitro*. Reaction mixtures had fixed phosphate buffer and varying NaCl concentrations. \circ , measurements by the dark equilibrium method; \bullet , measurements by the competing substrate method. Solid squares represent data from the "standard" reaction mixtures described in the original paper. (From H. Harm and Rupert, 1970a.)

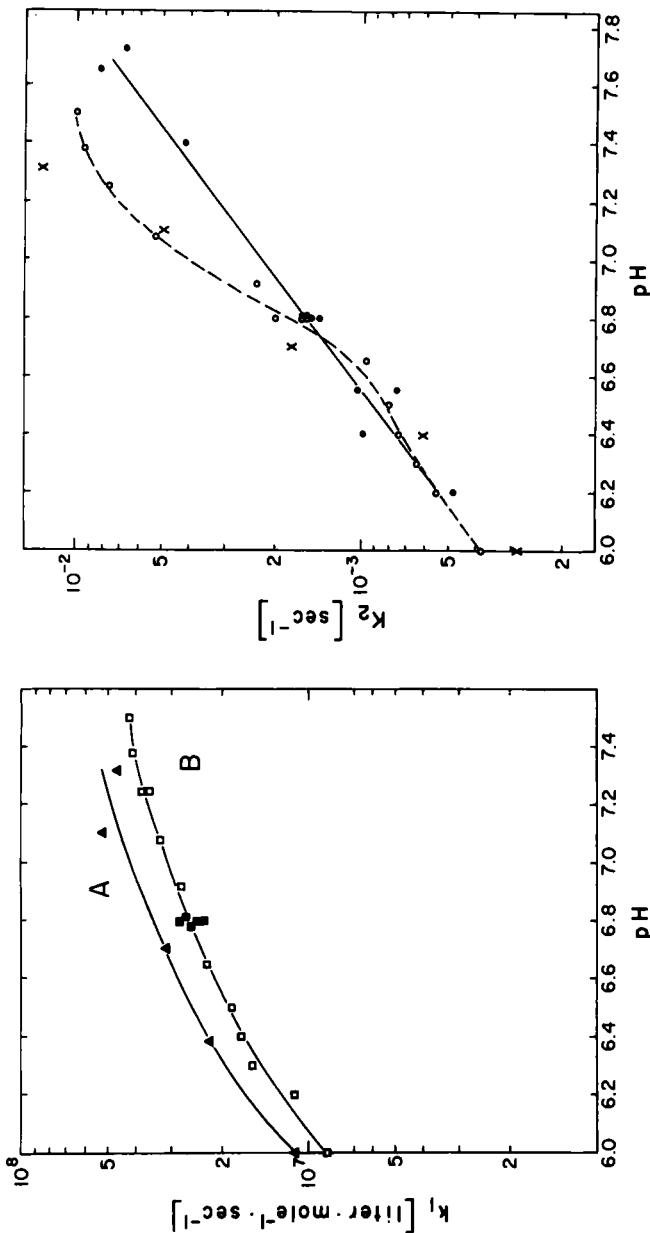


Fig. 15. Dependence on pH of rate constants k_1 for enzyme-substrate complex formation by yeast PRE *in vitro*, and k_2 for complex dissociation. *Left panel*, Dependence of k_1 , when pH varied by changing the phosphate buffer composition at fixed concentration (curve A), and by titrating a higher concentration buffer with unneutralized molar tris or HCl (curve B). *Right panel*, Dependence of k_2 . Dashed curve gives measurements using the equilibrium method with pH varied either by changing the phosphate buffer composition (X), or by titrating higher concentration buffer with Tris or HCl (O). Solid curve shows measurements using the competing substrate method, with pH varied by titrating the buffer (●). (From H. Harm and Rupert, 1970a.)

the photolytic reaction is independent of ionic strength, the same measurement method for k_1 (as described in Section 4.3) can be used. As illustrated in Fig. 13, these two series of mixtures give similar, though not identical, dependence of k_1 on salt concentration when the latter is expressed in terms of the ionic strength μ . This rate constant decreases by an order of magnitude for a 3-fold change in μ above or below the value corresponding to the maximum. Although k_1 is known to vary with μ for other enzymatic reactions (Kistiakowsky *et al.*, 1952), such marked dependence is unusual, and the theoretical reason for it is not known. The experimental points corresponding to the dashed curve of Fig. 13 are well described by a simple empirical expression: $1/k_1 = 1/k_{1a} + 1/k_{1b}$, where $k_{1a} = 1.1 \times 10^5 \exp(16.4\sqrt{\mu})$ and $k_{1b} = 1.7 \times 10^{11} \exp(-16.4\sqrt{\mu})$ liter·mole⁻¹ sec⁻¹ (H. Harm and Rupert, 1970a).

k_2 also varies with ionic strength in a somewhat related way, as shown in Fig. 14. It is difficult to measure this rate constant satisfactorily when the ratio k_1/k_2 becomes very small, i.e., at higher ionic strengths, since the initial concentration of complexes becomes too low. However, at lower ionic strengths the curve is reasonably well represented by $k_2 = 3.5 \times 10^{-6} \exp(16.4\sqrt{\mu})$ sec⁻¹. The similarities between the empirical expressions for k_1 and k_2 suggest some regularities in the details of complex formation and dissociation which are not yet understood.

The reaction rates of most enzymes vary with pH, an effect which has in some cases been elaborately analyzed (see, e.g., Alberty, 1954; Laidler, 1958). In the case of PRE the dependence has been followed only descriptively and over a limited range, as shown in Fig. 15. Varying the pH in two ways: viz., changing the composition of phosphate buffers at a fixed total concentration, or titrating phosphate-buffered reaction mixtures with unneutralized tris or HCl, showed essentially the same dependence. Both k_1 and k_2 increase with increasing pH, but the equilibrium constant k_1/k_2 is appreciably higher at lower pH.

5. The Photolytic Repair Step

5.1 Determination of the Photolytic Constant

The reaction scheme for PER [Eq. (1)] resembles the Michaelis-Menten scheme for other enzymatic reactions, except that the step $ES \rightarrow E + P$ is absolutely dependent on light energy. Hence the rate constant k_3 , which has the dimension of reciprocal time, is defined only when the light intensity I during the reaction is specified. In the simplest case possible, absorption of a single photon by the complex would lead with a constant probability to its photolysis. In this case

one would expect k_3 to be directly proportional to the intensity: $k_3 = k_p I$. The *photolytic constant* k_p (having dimensions of a reciprocal light dose) should be dependent only on the wavelength of illumination, and independent of intensity.

For examination of these points it is important to observe the photo-repair under steady monochromatic illumination with virtually all substrate in the complexed form. Incomplete complexing (which is likely under many conditions) would lead to underestimates of k_p , since photons potentially effective for repairing a complex would be wasted if some of the substrate were not combined with PRE. In this case the results would also depend on k_1 and k_2 . *In vitro*, a high PRE/substrate ratio, together with a sufficiently high concentration of all reactants, provides the required maximum level of complex formation. *In vivo* irradiation of B_{s-1}-160 cells with a moderate UV dose (8 erg mm⁻²) permits complexing of lesions to the full extent (see right panel of Fig. 5). In any case, test illumination with a single flash guarantees that the necessary conditions are met, if it causes the maximum photo-reactivation possible with the system under study.

For the 1-photon reaction case, the concentration of complexes $[ES]_L$ remaining unrepaired after illumination with the light dose L , when the total number originally present was $[ES]_0$, should follow the function

$$\ln([ES]_L/[ES]_0) = -k_p L \quad (7)$$

provided that the complexes are homogeneous in all having the same photolytic constant. Figure 16 shows for B_{s-1}-160 cells the decrease of $[ES]_L/[ES]_0$ as a function of L , at three wavelengths in the spectral region of maximal PER efficiency. As found for the dark reaction rate constants, the shapes of the curves indicate a heterogeneity of the complexes with respect to the photolytic constant. This is most evident at 355 nm, but it is also found at 365 and 385 nm in experiments where ordinate values below 0.10 are obtained. For the most effective wavelength, 385 nm, the k_p value corresponding to the slope of the initial part of the curve is 1.75×10^{-3} mm² erg⁻¹ (W. Harm, 1970a). Very similar results are obtained *in vitro*, except that the k_p values are 1.5- to 2-fold lower for the same wavelengths, and the apparent heterogeneity is greater (H. Harm and Rupert, 1970b).

Because of the heterogeneity, the initial slope must relate to a weighted average k_p for the different classes of complexes contributing. *In vitro* experiments have shown that this slope has little, if any, dependence on light intensity, temperature (7° to 23°C), and ionic strength (0.078 to 0.15 M), but is about 2-fold higher at pH 7.4 than at pH 6.7 or 6.4 (H. Harm and Rupert, 1970b).

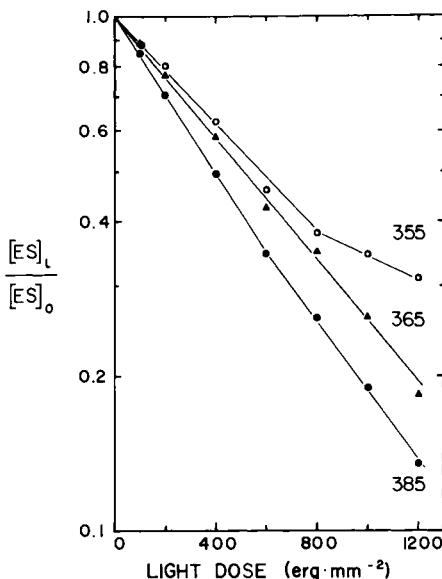


FIG. 16. Measurement of the photolytic constant in *B_{n-1}-160* cells UV-irradiated with 8 erg mm⁻². The graph shows the fraction of nonphotolysed *ES* complexes after illumination at 355 (○), 365 (▲), and 385 (●) nm with light doses shown on the abscissa. (From W. Harm, 1970a.)

5.2 Wavelength Dependence of the Photolytic Constant

The photolytic constant, which is a measure of the efficiency of PER at a particular wavelength (λ), depends on the absorption of light by the complex and the quantum yield with which an absorbed photon causes photolysis. If ϵ is the molar extinction coefficient of the complexes, and ϕ is the quantum yield for the photolytic reaction, it is readily shown (cf. Rupert, 1962b) that

$$\epsilon\phi[\text{liter} \cdot \text{mole}^{-1} \text{cm}^{-1}] = k_p[\text{mm}^2 \text{erg}^{-1}] \cdot \frac{5.2 \times 10^9}{\lambda[\text{nm}]} \quad (8)$$

For the *E. coli* cell we have calculated $\epsilon\phi$ to be 2.4×10^4 liter mole⁻¹ cm⁻¹ for the most effective wavelength (385 nm), while the highest $\epsilon\phi$ values obtained *in vitro* (at 366 nm) are about 1.4×10^4 . The latter figure agrees roughly with an earlier value (Rupert, 1962b) in which a different experimental criterion was employed to determine the photolytic constant of complexes. Although it is not possible with these methods to determine ϵ and ϕ separately, the figures obtained for the product tell us that both must be high. ϵ must be at least as high as the product of the two, since ϕ cannot exceed 1. Thus the ab-

sorbance of the ES complexes around 365 to 385 nm is at least comparable to that of purines and pyrimidines around 265 nm. On the other hand, ϕ is probably greater than 10^{-1} , since any lower ϕ would require an $\epsilon > 10^5$ liter mole $^{-1}$ cm $^{-1}$, which is unlikely for organic structures. It is entirely possible that $\phi \approx 1$.

The dependence of $\epsilon\phi$ on wavelength, shown in Fig. 17, represents an *absolute* action spectrum for the photolytic reaction, in contrast to the usual action spectra, which express only the relative efficiencies of different wavelengths. This action spectrum, as determined with light monochromatic to within ± 2 nm, shows a single broad maximum in the 355–385 nm region. $\epsilon\phi$ is only a factor of 4 lower at 313 nm and a factor of 10 lower at 436 nm than at 366 nm.

5.3 Effects of Experimental Parameters on the Lower Part of the Photolysis Curve

In a semilog plot of $[ES]_L/[ES]_0$ vs. the time of illumination (with monochromatic or polychromatic light) the initial parts of the photolysis curves obtained *in vitro* follow essentially Eq. (7). As the reaction proceeds to values below about 0.5, the curves become concave upward, indicating a decreasing k_p for the remaining complexes. These lower portions of the curves are affected by experimental parameters in ways not yet understood. The complications experienced with the corresponding fraction of the complexes can only be indicated here; for more

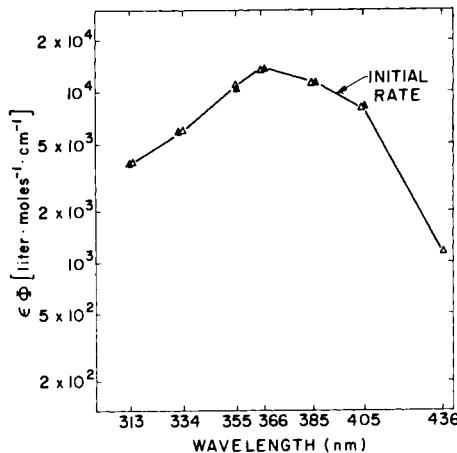


FIG. 17. Absolute action spectrum for PER *in vitro* (cf. text). For simplicity, only the k_p values derived from the initial portions of the photolysis curves were used for calculation of $\epsilon\phi$. The open and closed symbols refer to light intensities differing by about a factor of 10. (Modified from H. Harm and Rupert, 1970b.)

details the reader is referred to the papers by H. Harm (1969) and H. Harm and Rupert (1970b).

An apparently important observation in this connection is shown in Fig. 18. The photolysis curve obtained with increasing time of illumination with continuous white light shows two distinct components. In contrast, when the increasing light dose is supplied by a single flash of increasing strength, the photolytic effect follows essentially a straight line, in accordance with Eq. (7). The dose scales for both kinds of illumination are chosen such that the two curves approximately coincide

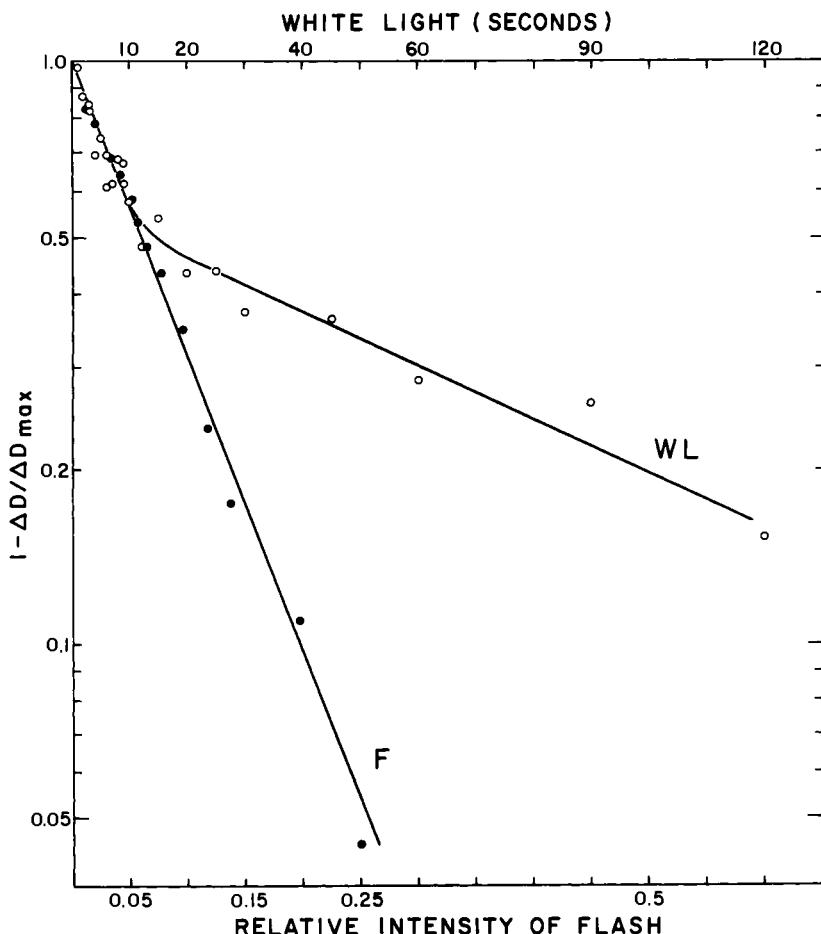


FIG. 18. Fraction of nonphotolysed complexes as a function of the relative light energy delivered at room temperature either as a single flash (●), or as continuous white fluorescent light (○). (Redrawn from H. Harm, 1969.)

for values between 1.0 and 0.5, in order to emphasize the difference in the region below. To understand this difference, we have tentatively considered the possibility that a fraction of the complexes requires absorption of 2 photons within a short time interval. This requirement would be satisfied by intense light flashes but not by continuous illumination at relatively low intensity. Such an interpretation would imply that for low-level continuous illumination the slower decrease below 0.5 involves dark dissociation of many of the complexes requiring two photons, and the formation of new complexes by enzyme molecules which require only 1 photon for photolysis.

Speculative as this explanation is, it is nonetheless interesting that it would account for several other observations which are otherwise hard to understand. For example, the photolysis rate observed with continuous illumination in the lower part of Fig. 18 increases significantly if (1) the temperature is raised, or (2) the intensity of the illumination is lowered, or (3) the illumination is interrupted by dark periods. These findings all can be explained by the assumption that in this part of the curve photolysis requires dark dissociation of the original complexes, and subsequent reformation of complexes which can be photolysed by a single photon.

We have also consistently found that the dark equilibrium level of complex formation is somewhat increased when the PRE preparation is preilluminated with white light. Furthermore, although no photorepair whatever occurs solely by this preillumination, the photolysis rate in the lower portion of the curve is increased.

Since all these effects have been obtained *in vitro*, they might represent artifacts resulting from extraction and purification of PRE. Corresponding investigations *in vivo* have not yet been carried out, but at least the photolysis curves with monochromatic light (Fig. 16) indicate a lower photolytic effect in a minor fraction of the complexes.

5.4 The Photolytic Reaction at Low Temperatures

The effects of low temperatures on the photolytic reaction are most easily surveyed by means of flash illumination, since a sample can be moved from the low-temperature container into the flash apparatus within a second. It is important, however, both for *in vitro* and *in vivo* experiments, that the samples contain 20% glycerol to prevent formation of ice crystals. From Fig. 18, we expect that for a single flash $-\ln ([ES]_L [ES]_0)$ is proportional to $k_p L$. This provides a basis for measuring the relative efficiency of a flash under varying conditions. For the discussion of the following experiments we assume that this simple

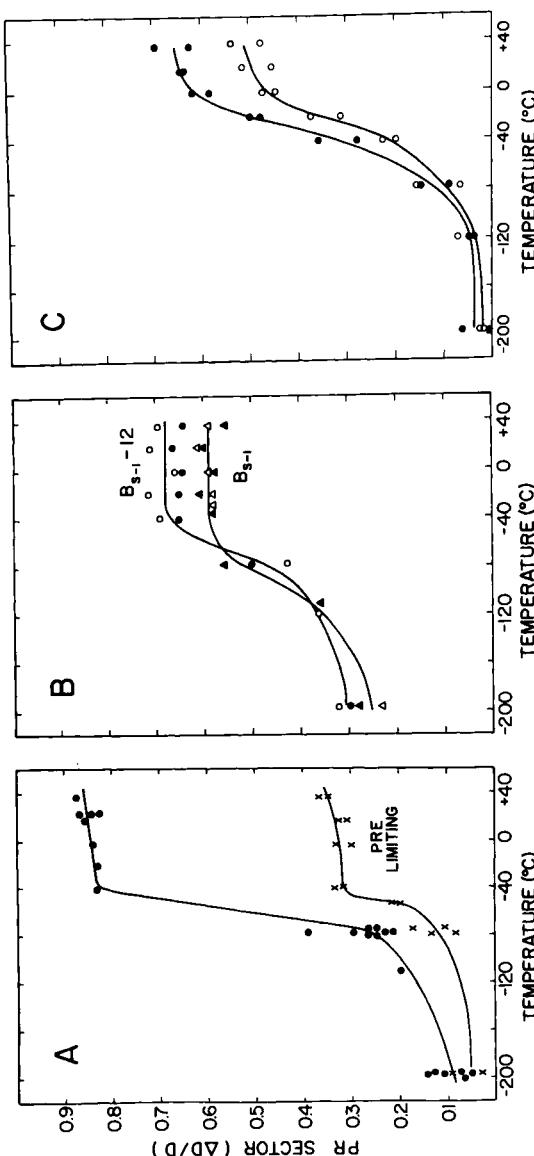


FIG. 19. Temperature dependence of flash PER. *Left panel*, Irradiated *Haemophilus* DNA (2400 erg mm⁻²) with PRE either in excess (●), or limiting (×). *Center panel*, *E. coli* B_{s-1} irradiated with 3.2 erg mm⁻² (▲) or 4.8 erg mm⁻² (△), and B_{s-1}-12 irradiated with 8 erg mm⁻² (○) or 12 erg mm⁻² (●). *Right panel*, Irradiated *Haemophilus* DNA (2400 erg mm⁻²) with PRE in excess, but light energy reduced to 0.135 (●) or 0.065 (○) of the usual flash intensity. (From H. Harm, 1969.)

relationship holds at low temperatures too, although it has not been specifically demonstrated.

If complexes are formed at room temperature, the photolytic effect is essentially the same for a flash given either at room temperature, or after a rapid shift to +37°C or to +5°C. This shows that the photolytic reaction—in contrast to complex formation and dark equilibrium—is temperature-independent within this range. Figure 19 (left and central panels) indicates that essentially the same is found *in vitro* and *in vivo* for temperature shifts down to about -40°C, but that the photolytic effect drops sharply when the flash is given at still lower temperatures (H. Harm, 1969).

However, with a flash of limiting light intensity (0.13 or 0.065 of our usual intensity) a considerable drop is already evident between 0° and -40°C, which continues toward still lower temperatures (Fig. 19, right panel). Control experiments have shown that the complexes are neither destroyed by the low temperature treatment, nor dissociated during the cooling: When such samples are thawed within 20 seconds to 0°C and flashed, they show the PER characteristic of this temperature. Furthermore, if the samples are kept at -78° or -196°C, a number of flashes in sequence increases the total PER effect.

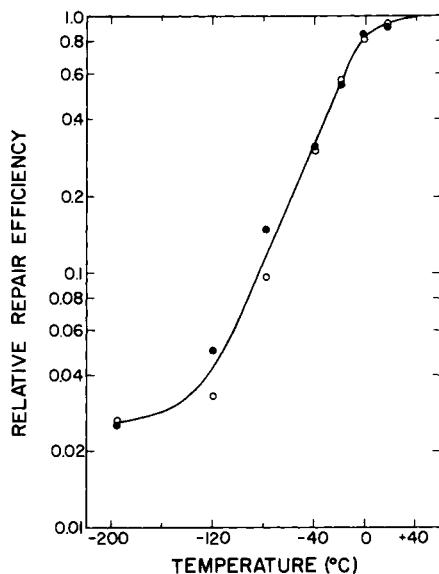


FIG. 20. Relative repair efficiencies of incident photons from light flashes as a function of temperature, where the efficiency at 37°C is defined as unity. Values are calculated from data in the right panel of Fig. 19, averaging the duplicate points for each curve. (From H. Harm, 1969.)

The results obtained with flashes of limiting intensities permit us to calculate the repair efficiencies of incident photons at the various temperatures, relative to the efficiency at 37°C. (For more details, see Appendix in the paper by H. Harm, 1969.) The results of such calculations are shown in Fig. 20. One sees that the essential drop occurs between 0° and -120°C, corresponding to a 20-fold decrease. Evidently the high intensity of a 4-unit flash is sufficient to compensate for the decreasing efficiency between 0° and -40°C, so that at the latter temperature most of the complexes can still be repaired (cf. Fig. 19).

The decrease in the relative repair efficiency indicates a corresponding decrease in k_p (and therefore in the product $\epsilon\phi$). It seems reasonable to interpret this as an expression of conformational changes in the ES complexes at subzero temperatures, affecting either the absorbance or the quantum yield, or both. Such an interpretation seems plausible in view of the fact that the DNA itself undergoes conformational changes at low temperatures, as indicated by the change in the nature of the principal UV photoproducts (Rahn and Hosszu, 1968).

6. Inhibition of Photoenzymatic Repair by Caffeine

The flash photolysis technique offers a way of investigating the interaction of agents other than PRE with the photorepairable lesions in DNA. As an example, the inhibition of photoenzymatic repair by caffeine was investigated (W. Harm, 1970b). This substance is known to inhibit dark repair of lethal UV lesions in phage and bacterial DNA (Sauerbier, 1964; Metzger, 1964); and recent work by Domon *et al.* (1970) showed that caffeine binds to UV-irradiated, but not to unirradiated, *native* DNA. These authors proposed that binding occurs at locally denatured DNA regions resulting from the formation of pyrimidine dimers, since caffeine binds equally well to unirradiated as to irradiated *denatured DNA* (Ts'o and Lu, 1964; Domon *et al.*, 1970). From this work we expected that caffeine would also interfere with PER, and could be useful in the study of this repair. This is indeed the case, but the inhibition is observed only under certain conditions which may be the reason that it has not been noticed earlier.

Figure 21 shows the PER kinetics of B_{s-1} and B/r cells obtained with continuous white light either in the absence or in the presence of 16 mg/ml caffeine. While the PER kinetics are greatly affected by caffeine in B_{s-1} cells, the effect is not observable in B/r cells. PER of UV-irradiated T1 phage infecting either B_{s-1} or B/r cells is greatly inhibited by caffeine, indicating that the difference between B_{s-1} and B/r seen in Fig. 21 does not have a trivial reason, for example, that the B/r cells

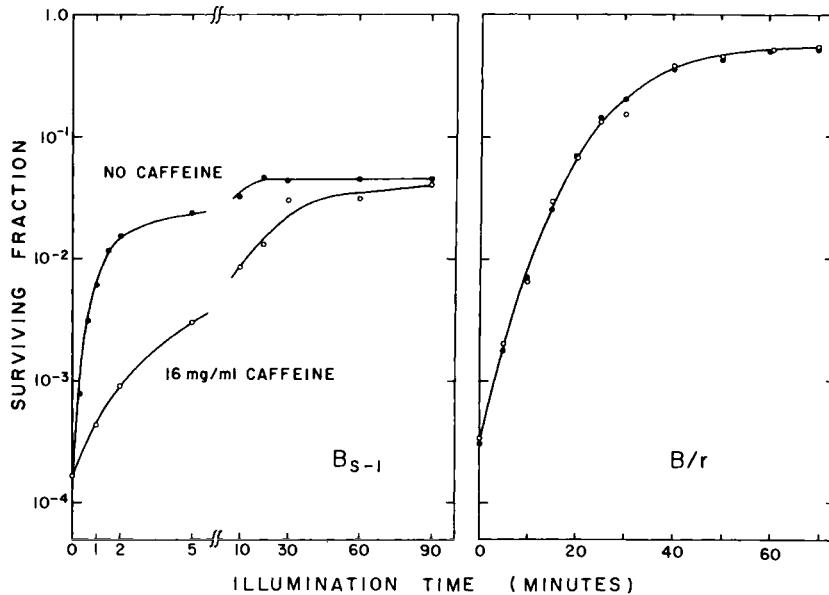


FIG. 21. Photoreactivation of UV-irradiated *E. coli* as a function of illumination with white fluorescent light at 37°C , either in the presence of 16 mg/ml caffeine (\circ) or in its absence (\bullet). Left panel, B_{S-1} cells irradiated with 16 erg mm^{-2} . Right panel, B/r cells irradiated with 1120 erg mm^{-2} . (From W. Harm, 1970b.)

do not take up caffeine from the medium or destroy it after uptake. The explanation will be given at the end of this section.

The photolytic response to a single flash after equilibrium formation of ES complexes decreases with increasing caffeine concentration, as seen in Fig. 22. The following shows that this is the result of a lower equilibrium of complex formation rather than limitation by the flash energy. Similar to the results shown in Fig. 2, we found that the PER effect in the presence of caffeine is the same when either 1, 2, 3, or 4 flash units are discharged simultaneously. Experiments analogous to those for determination of k_1 (Section 4.3) showed that complex formation becomes slower with increasing caffeine concentrations. There is no indication for a threshold at low concentrations, which suggests that the formation of an ES complex can be blocked by a single caffeine molecule.

Thus PRE and caffeine compete for the binding with UV lesions. At the highest inhibitor concentration (16 mg/ml) at least 95% of the lesions not complexed with PRE are "blocked" by caffeine at any given moment. Since the equilibrium of ES complex formation is even under these conditions still considerable, the affinity of PRE to its substrate

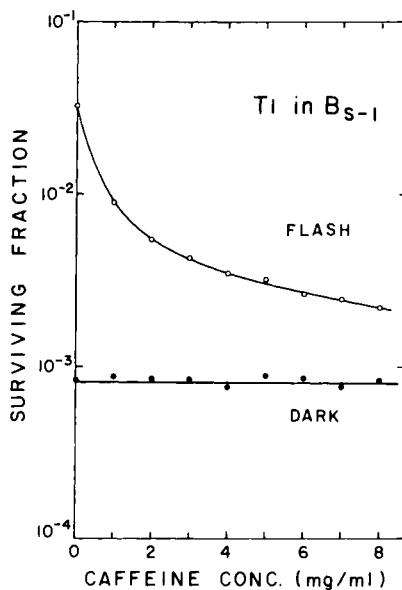


FIG. 22. PER produced by a single flash in phage T1 (UV-irradiated with 280 erg mm⁻² and infecting B₈₋₁ cells) as a function of the caffeine concentration present during complex formation. (From W. Harm, 1970b.)

must be *much* greater than that of caffeine. This is also evident from the fast disappearance of the inhibitory effect after dilution of cells into caffeine-free medium.

It is now understood why caffeine inhibition of PER is not seen in B/r cells illuminated with continuous light of medium intensity. During such illumination there exists a quasisteady state of complexes, which is determined by the rate of complex formation vs. the rate of photolysis. According to Eq. (2), complex formation in a cell with many substrate molecules (like the UV-resistant B/r) is very rapid so that in the steady state almost all of the PRE molecules are tied up in complexed form. Under these conditions slower complex formation in the presence of caffeine would lower only slightly the steady-state concentration of complexes, to which the PER rate is proportional. In contrast, complex formation in UV-sensitive systems (such as B₈₋₁ cells, or T1 phage infecting either B₈₋₁ or B/r) is slow due to the low concentration of substrate molecules, so that in the quasi-steady state only a small fraction of the PRE is in complexed form. Under these conditions a slower complex formation in the presence of caffeine would roughly proportionally reduce the steady-state concentration.

This interpretation has led to two predictions, which have been ex-

perimentally tested (W. Harm, 1970b). Continuous illumination at very high light intensity should decrease the steady-state concentration of ES complexes in B/r so that caffeine inhibition of PER should become observable. Illumination at very low light intensity should increase the steady-state concentration of ES in B_{s-1} cells, thereby decreasing the inhibitory effect of caffeine on the photoreactivation. Both of these predictions have been satisfied by experimental results.

7. Discussion and General Outlook

The results of the flash illumination studies described here are fully consistent with the photoenzymatic repair scheme [Eq. (1)] derived earlier from continuous illumination experiments *in vitro* (Rupert, 1962a,b). They thus confirm its basic correctness both *in vivo* and *in vitro*. The unusual requirement of light energy for converting enzyme-substrate complex into free enzyme and product provides a very direct means for controlling the usually ungovernable second step of an enzymatic reaction. This makes it possible to separate by flash photolysis the complex-formation step from the later reaction of the complex, and to determine the individual rate constants independently under different conditions.

The resulting additional information highlights a feature of the system not realized earlier, and which raises questions about the validity of some conclusions from older experiments. This concerns the transient phase of the reaction. Whenever an enzyme is mixed with its substrate, there is an initial period during which the amount of enzyme-substrate complex increases from zero to a quasi-steady state value, and the rates of change of substrate and product concentration are different from those given by the conventional Michaelis-Menten formulas (see Laidler, 1958; J. R. Bowen *et al.*, 1963). Under the conditions where most enzyme reactions are studied this transient phase is so short that it cannot be detected without taking special pains. However, with the very low concentration of reactants present in the PER systems, this phase may easily dominate the observations. Transient effects are particularly evident when illumination is applied immediately after the reaction mixture is established (or after the cell is irradiated), but they are present under other conditions as well. Hence simple analogies with familiar enzyme kinetics cannot be assumed without explicit consideration of the magnitudes of individual rate constants and reactant concentrations in each experiment.

With due regard for this fact, the PER process, particularly as carried out *in vitro*, is well suited for detailed physicochemical charac-

terization of the formation, dissociation and photolytic reaction of enzyme-substrate complexes. The results described here concerning ionic environment, pH, and temperature dependence of the rate constants k_1 and k_2 , and the effects of low temperature and light intensity on k_3 , can be considered beginnings in this direction. The relatively slow time course of complex formation at the low concentrations usually employed and the precise control of the light-dependent step compensate for the clumsiness of the enzyme assay. On the other hand, the heterogeneous behavior of reactants at each stage of the process and the nonrandom distribution of the substrate in solution impede to some degree such an analysis.

Heterogeneity is evident both *in vivo* and *in vitro* with respect to formation of ES complexes, as well as to their dark dissociation and photolysis. It seems reasonable to associate this with the several varieties of DNA photoproducts known to be present, although it is possible that variations in the PRE molecules themselves (perhaps arising from partial damage during extraction and purification) also contribute to the *in vitro* heterogeneity. At least 3 types of photorepairable lesions are produced in irradiated DNA: TT , CT , and CC dimers, and all these exist with a variety of neighboring bases. R. B. Setlow and Carrier (1966) have shown that during PER the TT dimers disappear appreciably faster than the CT and CC dimers, but it is not known whether this is the result of different rates of complex formation, different rates of photolysis, or both. It is also not known whether the neighboring bases influence the photorepair of a given type of dimer.

The "clustering" of the substrate structures on large macromolecules *in vitro* makes the usual chemical kinetic expressions only approximately applicable, and blurs any fine analyses requiring accurate knowledge of concentrations. From this point of view, it would be desirable to have substrate molecules randomly and independently distributed in solution. However, the enzyme's action on its substrate in DNA, which constitutes the biologically significant process, can be investigated *in vivo* with much less difficulty from these complications.

The results obtained with yeast PRE *in vitro* and with *E. coli* enzyme *in vivo* show many similarities, from which it seems likely that they are representative for PRE in general. In both cases, PRE is only a very small fraction (of the order of 10^{-5}) of the total cell protein. ES complexes formed from either kind of PRE resemble each other with regard to stability in the dark and dissociation in the light. The complexes possess a high absorption coefficient in the spectral range 355–385 nm, and the energy absorbed has at least moderate, and perhaps high, efficiency for producing the repair reaction (monomerization of the

dimers). The forces maintaining the complex are mostly of entropic, rather than enthalpic origin, as has been found for other enzymes interacting with nucleic acids. The intriguing effects of electrolyte concentration on the complex formation remain to be understood, as do the effects of a number of experimental parameters on the light-dependent process for the more slowly repaired complexes. In the latter case, a possible explanation has been proposed (Section 5.3.) which can serve as a basis for further investigation.

For detailed analysis of the steps involved in PER, and for estimation of the number of PRE molecules per cell (or per unit volume *in vitro*), the flash illumination technique has been indispensable, and the insights it affords enable one to understand better the photoreactivation process under continuous illumination. For example, without flash analysis it would have been difficult to explain the different effects of caffeine on the PER kinetics in B/r and B_{s-1} cells. Furthermore, the small number of PRE molecules per cell revealed by the flash studies explains why the total light dose required for maximal PER varies greatly with the intensity at which it is given, since high intensity of illumination is wasteful of light energy. This illumination technique opens the way for studying the regulation of PRE synthesis during cell growth, and for standardization of enzyme units in terms of absolute numbers of active molecules. The simplicity, ready availability, and low cost of the equipment should make it accessible to any laboratory, where it can open considerable areas of both research and instruction. It would appear that this method could become a useful standard routine for photobiological research.

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Chapter 8

PHOTOPERIODISM AND REPRODUCTIVE CYCLES IN BIRDS*†

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1. Introduction

Survival in an annually fluctuating environment, especially at mid and high latitudes, requires that a species have control mechanisms that will cause important functions to occur at appropriate seasons. Most critical is the timing of reproduction. A scheme that causes reproductive

* For the purposes of this chapter we define and restrict the term photoperiodism to the control of annual cycles by mechanisms that, in some way, are driven or entrained by the annual cycle in day length or, at least, some part thereof. This admittedly narrow restriction has been applied not only to limit the chapter to a reasonable length but also to permit the discussion of a set of coherently related phenomena.

† Previously unpublished data on various taxa of *Zonotrichia* come from investigations supported by the National Science Foundation through grants GB-5969X and GB-11905.

activity to occur during the period in which the probability of survival of the young tends to be optimized and in which the energy costs and hazards to the adults tend to be minimized confers an obvious survival advantage. Since birds, like other animals, lack perfect internal clocks and calendars, the timing systems are dependent, in some manner, on external information for their precision. Bearing in mind that physiological preparation for reproduction usually spans several to many weeks, it is clear that environmental information used in the basic timing of the annual reproductive cycle must have reliable predictive value (Farner, 1970a). At mid and high latitudes the single precise annually periodic variable, within the known realm of sensory perception, is day length (Farner, 1964, 1970a; Payne, 1969).* It is therefore not surprising that photoperiodic control of annual cycles, mostly reproductive, has been demonstrated experimentally in at least 50 species in 15 families of birds. The actual number of photoperiodic species is doubtless much greater. Photoperiodic information is clearly of special significance to migrants since other "local information" in the wintering area lacks adequate precision for prediction of the time of optimal breeding conditions in the distant breeding area. Nevertheless, many permanent residents also have well developed photoperiodic controls. It must be emphasized that photoperiodic mechanisms are probably never solely responsible for setting the precise time of reproduction since final adjustments can be made by supplementary and modifying mechanisms (Farner, 1964a, 1967) using various kinds of environmental information. These supplementary and modifying mechanisms often obviously play more prominent roles in resident or nomadic species and populations than in migrants. Although experimental evidence is sparse, extensive field observations suggest very clearly that these supplemental and modifying mechanisms become increasingly important with decreasing latitude, whereas photoperiodic mechanisms become less important or nonexistent, at least as natural functional entities in the timing of the annual cycle (see Immelmann, 1963).

As emphasized above, the central function in the control of annual cycles is the temporal adjustment of the reproductive period. Of further importance is the spacing of mutually exclusive (e.g., reproduction and migration) and ergonomically expensive (e.g., molt and reproduction, molt

*Since there are several recent general reviews of various aspects of photoperiodism in birds (Farner, 1961, 1964a, 1965, 1967, 1970a,b; Farner and Follett, 1966; Loft, 1970; Loft and Murton, 1968; Loft *et al.*, 1970; Wolfson, 1964, 1966), no attempt is made here to consider the historical aspects of the subject nor to present a detailed analysis of the very extensive literature.

and winter thermoregulation) functions. The problems of temporal spacing are especially important among species that breed at high latitudes whether as migrants or residents. Within certain constraints, such as the impossibility of concurrent migration and reproduction, a variety of solutions to the problems of temporal spacing have evolved. For example, in the White-crowned Sparrow, *Zonotrichia leucophrys gambelii*, a rapid, intensive postnuptial molt is inserted between the breeding period and the onset of autumnal migration (Farner, 1964; Morton *et al.*, 1969). An alternate strategy has evolved in the northern populations of the Chaffinch, *Fringilla coelebs*; this involves a prolonged, much less intensive "postnuptial" molt that begins during the nesting period and is completed during southward migration (Blyumental, 1965; Blyumental *et al.*, 1963; Dolnik, 1967; Dolnik and Blyumental, 1967). The daily expenditure of energy in support of molt is thus only a small increment to the greater requirements for feeding of the young and migration.

Among the known photoperiodic species are several, e.g., the European Starling, *Sturnus vulgaris* and the House Sparrow, *Passer domesticus*, that are members of families of low-latitude and equatorial distribution. This, together with a general consideration of avian phylogeny, suggests that avian photoperiodic control mechanisms are of multiple origin and collectively represent a cluster of cases of convergent evolution within, of course, the constraints of the available sensory, neural, and endocrine apparatus. However, because avian photoperiodic mechanisms are still so imperfectly understood, we have, of necessity, chosen here to assume that there is a common basic pattern. Only subsequent research on a variety of cases of apparently diverse evolutionary origins will provide the basis for assessment of the bias that we have created with this assumption.

2. Photoperiodic Control of Reproduction

The photoperiodic control of reproduction can best be viewed in terms of a general avian control scheme (Fig. 1) in which day length has become the primary source of transduced information used by the control centers in the hypothalamus (Fig. 2). Although Fig. 2 is based primarily on *Zonotrichia leucophrys gambelii*, information has been drawn from investigations on about twenty additional species. Much of the neuroendocrinological evidence on which this model is based has been reviewed by Farner *et al.* (1967; see also Wilson, 1967; Stetson, 1969).

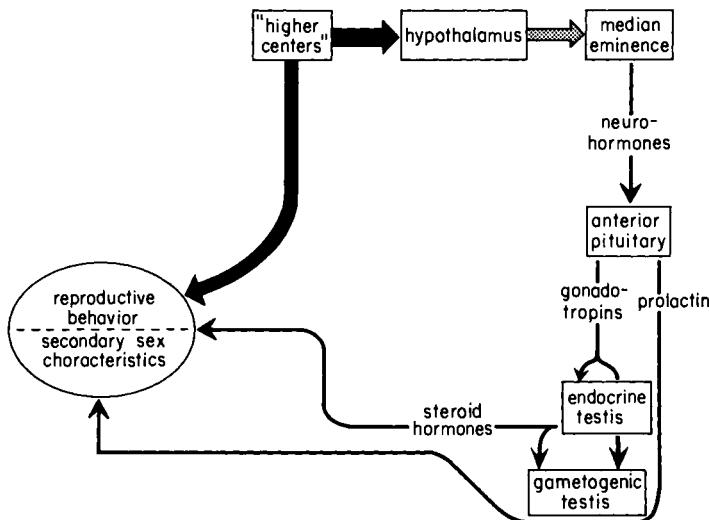


FIG. 1. A generalized scheme of the efferent mechanisms in the control of avian reproductive systems. (Modified from Farner, 1967.)

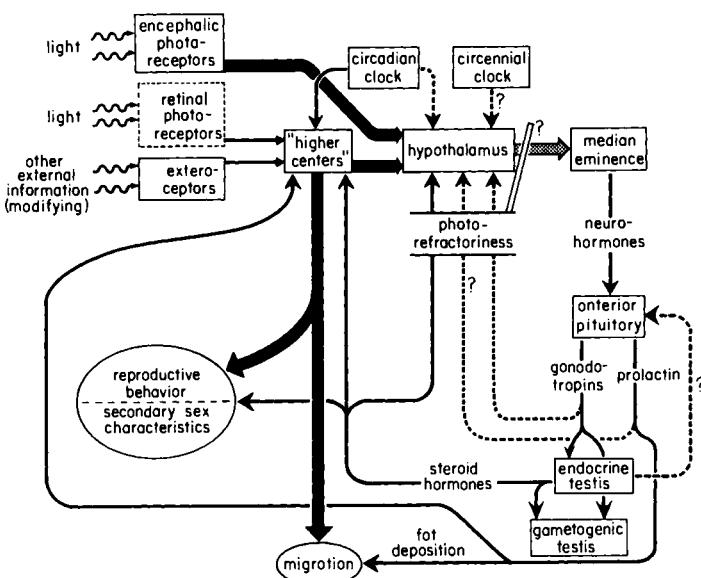


FIG. 2. A schematic representation of neuroendocrine functions and internal information in the control of male reproductive cycles in a photoperiodic species; based primarily on *Zonotrichia leucophrys gambelii*.

2.1 Components of the Avian Photoperiodic Control System

The components are, in a general sense, and within the limits of our present knowledge, shown schematically in Fig. 2. The question of the site of the photoreceptors was first considered more than three decades ago by Benoit (1935a,b, 1938a,b) in his pioneering investigations on the domestic mallard. A protracted series of experiments conducted by Benoit and his colleagues (for summary, see Benoit, 1970) generated data that were interpreted as indicating that both retinal (or ocular) and encephalic (or extraocular) receptors are involved in the photo-stimulation of testicular growth in the mallard. Early experiments (Benoit, 1935a, 1937, 1938a,b) suggested that while the eye could act as a photoreceptor in the photossexual response, testicular growth could be elicited in the absence of eyes. Later experiments, in which the optic tracts were sectioned, demonstrated the existence of deep photoreceptors that transduce the photoperiodic information (Benoit and Kehl, 1939). The site of the nonocular receptors was shown in all probability to be hypothalamic (Benoit, 1938b, 1970); penetration of light to the hypothalamus has been convincingly demonstrated by two methods. (For a resumé of these experiments and for references, see Benoit, 1970.) Although the inferred retinal receptors are not essential to photoperiodic induction of testicular growth in the mallard, Benoit believed that they normally participate in the intact animal and are functionally complementary to the deep receptors. This view was supported by experiments that suggest that the superficial and deep receptors have different spectral sensitivities (Benoit *et al.*, 1952) and perhaps different thresholds of photosensitivity (Benoit *et al.*, 1953c). Perhaps the most suggestive evidence with respect to the existence and complementarity of function of both superficial and deep photoreceptors comes from an experiment in which immature male ducks with severed optic nerves and intact controls were exposed to a range of light intensities. The intact subjects exhibited both a more rapid testicular growth and a lower threshold of response to light intensity than did the operated birds. Benoit interpreted this to mean that the responses of the normal ducks are due to the additive effect of stimulation of two sets of receptors (ocular and non-ocular). We see no simple alternative to this conclusion unless in Benoit's experiments the removal of eyes, or the isolation of the eyes from the orbit with opaque material (Benoit *et al.*, 1953a,b; Benoit and Ott, 1944), substantially reduced the transmission of light to the encephalic receptors as suggested by Underwood and Menaker (1970a).

In *Passer domesticus* the receptor scheme for photoperiodic responses appears at this time, at least, to be somewhat different than in the

mallard. Ivanova (1935) observed no impairment of photoperiodically induced testicular weight in sparrows in which caps had been placed over the eyes. On the other hand, Ringoen and Kirschbaum (1939) found that juvenile males with "caps placed over their heads" did not show appreciable testicular growth when exposed to long photoperiods. Apparently the latter authors did not entertain the possibility that light might penetrate directly to deep receptors and concluded that the ocular region is probably the site of the photoreceptors involved in photoperiodically induced testicular development. The combined observations of Ivanova and of Ringoen and Kirschbaum, and those of Benoit *et al.* (1953a,b) on the penetration of light through the orbit into the hypothalamus in this species, apparently overlooked by recent investigators, suggest a limited role, if any, for the eye in the photosexual response of this species and indicate that extraocular photoreceptors may be essential to the response. Recently Menaker and his collaborators (Menaker and Keatts, 1968; Menaker *et al.*, 1971; Underwood and Menaker, 1970a) have indeed demonstrated that it is highly unlikely that the eyes are involved in the photoperiodic induction of testicular growth in *Passer domesticus*. Their earlier experiments involving bilateral enucleation demonstrated that eyes are unnecessary in the stimulation and maintenance of testicular development. We (unpublished results) have confirmed these results. The most recent investigation of Menaker's group (1971) clearly demonstrate that the photoreception involved in photoperiodically induced testicular growth in this species, at least under low intensities, is only encephalic. In these experiments, two groups of sparrows were exposed to ambient light intensities approximating the threshold value (~ 7 lux) reported by Bartholomew (1949) to elicit measurable testicular growth in response to long photoperiods. India ink was injected under the skin of the head in one group in order to reduce the intensity of light reaching the deeper tissues. Feathers were plucked from the crowns of the second group, thus facilitating light penetration. No testicular growth was observed in the first group in response to LD 16:8 continued for 39 days, whereas significant growth occurred in the second group under the same photoperiodic regime.

Homma and Sakakibara (1971) have concluded that if retinal receptors are involved in the photoperiodic response in the Japanese quail, they require a higher intensity than the encephalic receptors. Oishi *et al.* (1966), employing bilateral enucleation, also found that photoperiodic testicular response of the Japanese quail does not require the visual system.

The role of retinal photoperiodic reception comes under some further

question because of the lack of acceptable evidence for the existence of direct retinohypothalamic pathways (Benoit, 1970; Oksche, 1970), although the existence of direct retinohypothalamic connections is not necessarily crucial to Benoit's arguments for retinal photoreception in ducks.

Clearly the final general assessment of the role of retinal receptors in photoperiodic responses in birds must await further investigations. Of critical importance is a repetition of the experiments of Menaker's group on other species including the mallard. Certainly, retinal receptors do mediate hypothalamic functions in a number of vertebrates (Hollwich, 1964).

The precise sites of the encephalic receptors remain in question. Benoit (1970) has summarized extensive experiments on mallards using quartz rods and optical fibers to transmit light to localized sites of the brain. Using optical fibers and white light at 0.1 lux, the most effective sites were found to be in the vicinity of the hypothalamohypophysial tract, in the paraventricular nucleus, in the suprachiasmatic region, in the tuber and in the median eminence. Illumination of other parts of the hypothalamus was ineffective.

Homma and Sakakibara (1971) have implanted small radioluminescent (surface brightness, $\sim 0.1 \text{ cd m}^{-2}$) into localized regions of the brain and eyes of 4-week-old Japanese quail maintained on short days (LD 8:16). Orange-yellow discs ($610 \text{ m}\mu$) evoked marked testicular development when implanted in the base of the *fissura longitudinalis cerebri* near the hypothalamus, or in the orbit near the optic foramen, the latter confirming the route of penetration proposed by Benoit since intraocular plates gave negative results. Implants in the optic lobes gave positive results, whereas implants in the olfactory lobes were ineffective. When blue discs ($410 \text{ m}\mu$) were employed similar but less consistent results were obtained. These investigations indicate that wherever the encephalic sites are located, they are sensitive to very low light intensities. To the best of our knowledge there is as yet no cytological evidence of specialized photoreceptors in the hypothalamus.

Although the pineal body has been suggested as the photoreceptor organ, the evidence is not convincing (Ralph, 1970). In *Zonotrichia leucophrys gambelii*, for example, pinealectomy has no effect on the photoperiodic testicular response and the development of photorefractoriness (Oksche *et al.*, 1971).

Numerous investigations have established that the photoperiodically active ambient light spectrum covers a range from about $600\text{--}800 \text{ m}\mu$ in intact birds although, with increased intensity, responses can be obtained at wavelengths as low as $480 \text{ m}\mu$ (Benoit, 1970; Benoit and Ott, 1944;

Burger, 1943; Hollwich and Tilgner, 1963; Schildmacher, 1963). This is not surprising in view of the fact that absorption by tissues is an inverse function of wavelength so that much higher intensities would be necessary if the lower wavelengths are to penetrate to the receptor sites in intact birds (see Benoit, 1970; Schildmacher, 1963). While we do not know the true sensitivity of the encephalic receptors, it is clear that the threshold is very low (Homma and Sakakibara, 1971) and that they have a wide spectral sensitivity ($\sim 440\text{--}800\text{ m}\mu$) (Benoit, 1970; Benoit *et al.*, 1950b; Homma and Sakakibara, 1971). Especially since the nature and site of the photoreceptors are unknown, the response to such a broad spectral range gives little basis for speculation about the primary photochemical event.

Because the precise sites of photoreception in the photoperiodic control systems are not known, nothing useful can be said concerning afferent and intermediary pathways. Our knowledge of the final efferent pathways (Fig. 2) has been reviewed in some detail by Farner and Follett (1966), Farner *et al.* (1967), Oksche (1967), and Assenmacher (1958, 1970). In *Zonotrichia leucophrys gambelii* the essential efferent components appear to be some elements of the infundibular nucleus complex, axons that reach the posterior median eminence via the tubero-infundibular tract, the neurohemal junction of the posterior median eminence, and the posterior hypophysial portal vessels that lead to the caudal lobe of the pars distalis (Wilson, 1967; Stetson, 1969; Vitums *et al.*, 1964). The extent to which the anterior median eminence, which also receives some fibers from the tuberoinfundibular tract, is involved is not yet clear. That it has some role seems very probable since its portal drainage, via the anterior portal vessels, is into the cephalic lobe which contains gonadotropin-producing cells (Mikami *et al.*, 1969; Matsuo *et al.*, 1969; Haase and Farner, 1969; Tixier-Vidal, 1963, 1965; Tixier-Vidal and Benoit, 1962; Tixier-Vidal *et al.*, 1962, 1968). The available evidence from *Z. l. gambelii* now indicates that the fuchsinophilic hypothalamic system, i.e., the fuchsinophilic magnocellular nuclei, the hypothalamohypophysial tract, and the fuchsinophilic fibers of the anterior median eminence, are not involved in the photoperiodic control of gonadal growth (Wilson, 1967; Stetson, 1969).

The system, as described above, appears to hold, in principle, also for the Tree Sparrow, *Spizella arborea* (Wilson and Hands, 1968), the Japanese quail (Sharp and Follett, 1969) in which there is now some evidence that the anterior median eminence, but not its fuchsinophilic components, may be involved in release of FSH-like gonadotropin (Stetson, unpublished); and in the nonphotoperiodic domestic fowl (Graber and Nalbandov, 1965; Gruber *et al.*, 1967). It seems also generally consistent with the mechanisms of hypothalamic control of gonadotropic

activity in mammals (e.g., Davidson, 1966; Flerkó, 1963, 1967a,b; Schreiber, 1963; Sawyer, 1964).

Benoit, Assenmacher, and their associates (Assenmacher, 1958, 1970; Benoit and Assenmacher, 1953a,b, 1959; Gogan *et al.*, 1963) have placed surgical lesions in the hypothalamo-hypophysial system of the male domestic mallard and have observed the effects on the photoperiodic testicular response and other endocrine functions. Similar observations have been made on birds with hypophysial autografts. Since the results of these experiments have been summarized recently by Assenmacher (1970), it is sufficient to note here that they have been logically interpreted to place primary importance on the fuchsinophilic system and its components in the anterior median eminence as the final efferent route in the photoperiodic control of the gonadotropic function of the pars distalis. It is difficult at the present state of our knowledge to rationalize the differences that emerge in the experiments with ducks in comparison with those from the other species that have been investigated. It is, of course, possible that differences in methods have resulted in undetected differences in the elements interrupted. Because the anseriform species used by Benoit, Assenmacher, and their colleagues is phylogenetically distant from the passerine and galliform species used by other investigators, one could be tempted to regard the apparent differences in experimental results as a reflection of species differences that have developed with independent evolution of photoperiodic control schemes. However, we agree with Assenmacher (1970) that this explanation should be invoked only after all other possible explanations are rigidly excluded. Certainly the possibility of primitively redundant systems, subsequently altered in the course of evolution, should not be excluded at this time. We have, from time to time, considered the possibility, recently raised by Kordon (Assenmacher, 1970), that the lesions used by Benoit and Assenmacher have invariably involved nonfuchsinophilic elements of the control system in addition to fuchsinophilic elements in which the results of lesions are much more readily detected. Assenmacher (1970) has noted that such a possibility exists and preliminary experiments in this laboratory (Stetson, unpublished) lend evidence thereto. This assumption is consistent with the results of experiments using hypothalamic implants of testosterone (Kordon and Gogan, 1964; Gogan, 1968) but, at least superficially, appears to be inconsistent with the effects of lesions in the median eminence as reported by Gogan *et al.* (1963). The resolution of these apparent differences is confounded by inadequate knowledge of the functions of the two avian gonadotropins and the extent to which their syntheses and secretions are independently controlled functions.

Figure 2 explicitly indicates that the photoperiodic control system

is not the sole mechanism involved in the seasonal control of gonadal function. The role of other sources of information is clearly variable among the photoperiodic species.

2.2 Types of Photoperiodic Control Schemes

As noted above, photoperiodic control schemes may well be of diverse origin although we lack information sufficient even to speculate on the phylogenetic histories of mechanisms. However, it is useful to categorize empirically the "photoperiodic" species in order to assess the results of experiments for their use in model systems. A useful approach at the present state of knowledge is the recognition of three arbitrary, overlapping categories with respect to the role of day length in the control of annual reproductive cycles (Farner, 1970b).

2.2.1 PRIMARY PHOTOPERIODIC SPECIES OR POPULATIONS

These are species in which it has been clearly demonstrated, on the basis of laboratory and confirming field investigations, that day length is the primary component, or at least an important component, of the environmental information used in the control of annual reproductive cycles. Some of these species are obligately photoperiodic; in others, day length appears to be responsible for the timing of events that would otherwise occur without temporal precision. Included in this category are such species as *Junco oreganus*, *Junco hyemalis*, *Zonotrichia leucophrys*, *Zonotrichia albicollis*, *Zonotrichia atricapilla*, *Zonotrichia querula*, *Fringilla coelebs*, *Carpodacus mexicanus*, *Passer domesticus*, *Sturnus vulgaris*, *Agelaius tricolor*, *Agelaius phoeniceus*, *Anas platyrhynchos*, *Columba palumbus*, *Columba oenas*, and many others. This category may include some transequatorial migrants (Engels, 1964; Lofts and Murton, 1968).

2.2.2 SECONDARY PHOTOPERIODIC SPECIES OR POPULATIONS

In these species and populations, day length among the various sources of environment information has a less significant role. Unfortunately there have been very few investigations on populations and species that seem likely to belong in this category. There is considerable circumstantial evidence that the low-latitude natural populations of *Gallus gallus* fall into this category (see Farner, 1970b, for review of evidence). Other possibilities are *Ploceus philippinus* (Thapliyal and Saxena, 1963) and the Costa Rican population of *Zonotrichia capensis* (Epple *et al.*, 1971). Some feral populations of *Columbia livia* (Lofts *et al.*, 1967a) and some introduced populations of *Passer domesticus* apparently fall between categories 2 and 3.

2.2.3 SPECIES OR POPULATIONS WITH PERMISSIVE PHOTOPERIODIC RESPONSES

Species or populations normally subjected to negligible changes in day length, or differences that are too short to measure, clearly cannot use day length as information in establishing the period of reproductive function. Thus far there has been no experimental demonstration that the difference of approximately one hour between the longest and shortest days at a latitude of 10° can serve as the basis for control of an annual reproductive cycle. The changes in gonadal function induced by experimentally altered day lengths, in such species and populations, can only be interpreted as a permissive effect of long days since sufficient changes in day length are not available among the sources of environmental information used in the control of the natural reproductive cycle. Included here are the "long-day effects" on gonadal development in the low-latitude or equatorial populations of *Quelea quelea* (Lofts, 1962) and *Zonotrichia capensis* (Miller, 1965). It is also possible that the photoperiodic effects on reproduction in the domestic fowl and in the Guinea fowl (G. T. Davis and Penquite, 1942; Karapetian, 1955) should be included here. It is interesting to note that the natural equatorial population of *Numida meleagris* in Uganda appears to be reproductively cyclic in areas where the annual change in day length is negligible (Fergin, 1964). Also probably included here should be the "short-day effects" described by Thapliyal and Saxena (1964) for *Munia malacca malacca*. For the species or populations in which such "permissive" effects are demonstrable, it is not argued that day length is unimportant in gonadal function, but rather that it is not significant as a source of environmental information in the control of an annually periodic reproductive cycle. It is interesting to speculate that a day-length "requirement" may be basic to the multiple evolutionary origins of photoperiodic control mechanisms.

2.3 Termination of the Annual Reproductive Period in Photoperiodic Species

The appropriate termination of the breeding period in a seasonal environment is of adaptive significance not only because it avoids the production of young with a lower probability of survival, but also because it permits diversion of energy to other functions such as molt and migration. Thus the termination mechanisms are important in the mutual exclusion of ergonomically intense functions (Farner, 1964).

Although our knowledge is fragmentary, it appears that among the

primary photoperiodic species (Section 2.2.) there may be at least four categories of termination mechanisms.

2.3.1 TERMINATION BY DECREASING DAY LENGTH

It seems possible that this may be the mechanism used by at least some permanent residents with long-breeding seasons involving two or more clutches. The experiments of Lofts *et al.* (1967b) indicate quite conclusively that such is indeed the case in *Columba palumbus*. This appears to be also the case, at least in the females, in *Colinus virginianus* (Kirkpatrick, 1959). Our studies (Farner and Follett, 1966, unpublished) with *Coturnix coturnix* suggest a similar conclusion. Among passerine species the same has been suggested for *Ploceus philippinus* (Thapliyal and Saxena, 1963). The placement of cases in this category, however, requires appropriate caution until a role of negative feedback from gonadal or hypophysial level is excluded experimentally. This is the type E pattern of Lofts and Murton (1968). It seems probable that other environmental information may exert important effects in this category.

2.3.2 TERMINATION BY NEGATIVE FEEDBACK UNTIL LENGTH HAS DECREASED TO NONSTIMULATORY DURATION

This is a hypothetical category since we know definitely of no species in which it has been demonstrated experimentally and rationalized with field observations. It seems that the relatively rapid recovery of the "photoperiodic" testicular response of *Quelea quelea*, as a function independent of day length (Lofts, 1962) suggests that the regression following "photoperiodic" induction of testicular growth could be the result of a simple negative feedback. Although this species appears to be neither a primary nor secondary photoperiodic species, this type of termination of the period of gonadal function may well be of important ecological significance. That negative feedback effects from gonadal and hypophysial levels do occur in birds is clear from the experiments of Kobayashi and Farner (1966) with castrated male *Zonotrichia leucophrys gambelii*. However, this species develops a true photorefractoriness so that the actual natural role of the negative feedback is probably that of limiting the rate of development and ultimate size of the testes.

2.3.3 TERMINATION BY PHOTOREFRACTORINESS

In most of the primary photoperiodic species each period of photoperiodically induced gonadal development and function terminates abruptly with a collapse of gonadal function. Such birds then display no photoperiodic gonadal response regardless of the duration of the daily

photoperiod to which they are subjected, and are designated as photorefractory. Photorefractoriness, apparently first described by Riley (1936) in *Passer domesticus* and by Schildmacher (1938) in *Phoenicurus phoenicurus*, has been studied extensively in *Sturnus vulgaris* (Burger, 1947), in *Passer domesticus* (Threadgold, 1960; L. Vaugien, 1954, 1955), in *Junco hyemalis* and *Zonotrichia albicollis* (Wolfson, 1952, 1958, 1959, 1960, 1966), in *Anas platyrhynchos* (Benoit *et al.*, 1950a; Lofts and Coombs, 1965), in *Carpodacus mexicanus* (Hamner, 1968), and to a lesser extent in other species (Farner, 1959; Laws, 1961; Lofts and Marshall, 1958; Wolfson, 1964, 1966). Of special importance are the investigations of Wolfson which provide an extensive empirical characterization of the photorefractory state and its termination and which demonstrate that there are day lengths, e.g., 12 hours, that may function as "long days" in causing gonadal development in photosensitive birds or as "short days" in the elimination of photorefractoriness. As the critical analysis of Dolnik (1964) indicates, the latter is extremely important in the construction of models for the system involved in the photoperiodic control of gonadal cycles. Of very substantial significance also are the investigations of Hamner (1968) on *Carpodacus mexicanus* which show that the termination of photorefractoriness proceeds equally well under a range of daily photoperiods (6–14 hours), confirm the capability of growth by the testes of photorefractory birds, and demonstrate a period of gradual recovery from photorefractoriness. Hamner also proposes a model that explains, on the basis of a circadian function, the termination of "relative photorefractoriness," i.e., "the increase in photosensitivity," that has been described in other terms for several species (e.g., Benoit *et al.*, 1950a; Dolnik, 1964; Farner and Follett, 1966; L. Vaugien, 1955).

In this category we include types A, B, C, and D of Lofts and Murton (1968) since they seem to represent a continuous spectrum of response rates as functions of day length and of conditions for induction and termination of photorefractoriness.

2.3.4 TERMINATION BY PHOTOREFRACTORINESS— "TRANSEQUATORIAL TYPE"

This is based primarily on an interpretation of the results of Engels (1962, 1964) on a single species, *Dolichonyx orizivorus*. It must be regarded as highly tentative since it seems clear that it cannot be applicable to many transequatorial migrants. This must be viewed as a modification of category 2.2.3 in that it assumes that 14-hour photoperiods fail to restore photosensitivity, whereas 12–13-hour day lengths are effective in so doing. Hence, in this species a few weeks of the photoperiods ex-

perienced in southward migration eliminate photorefractoriness. The rate of response, in terms of gonadal growth, to 14–15 hours photoperiods is substantially lower than that of *Zonotrichia leucophrys gambelii* and other species that winter in the mid-latitudes of the northern hemisphere. This is the type E pattern of Lofts and Murton (1968).

Because, during photorefractoriness, testicular growth can be induced with physiologic dosages of exogenous gonadotropin (Miller, 1949; Hamner, 1968; Lofts and Marshall, 1958), because of the extremely low level of gonadotropin in the pars distalis (King *et al.*, 1966), and because of the failure of the median eminence to react to photoperiodic treatment (Kobayashi and Farner, 1960; Kobayashi *et al.*, 1962), the best hypothesis concerning the site of photorefractoriness is that it is at a hypothalamic or higher level.

Several hypotheses, not all necessarily mutually exclusive, have emerged concerning the etiology of photorefractoriness. A consideration of their relative merits must recognize the probability (Farner, 1964; Farner and Follett, 1966) of multiple evolutionary origins. The hypotheses and models can be categorized as follows:

a. Negative gonadal-hormone feedback on control components. This hypothesis frequently becomes implicit in the consideration of the development of photorefractoriness. Although it is clear that negative feedback functions do exist (Kobayashi and Farner, 1966), and although it is possible to incorporate them into a hypothetical multicomponent model, we find this type of function (contrary to Farner, 1964), even on a nonlinear basis, to be insufficient as the only component in an explanation of the etiology of photorefractoriness. Among the difficulties are (1) first-year birds, which have not had active gonads, are photorefractory in late summer and fall; the elimination of photorefractoriness by short days is empirically identical with that of adults (Farner and Mewaldt, 1955; Laws, 1961); (2) such hypotheses assume identical feedback functions for male and female sex hormones—not impossible, but certainly improbable; (3) a simple negative feedback system under a continuous long-day regime should result in an oscillating system; this is definitely not the case, at least in *Zonotrichia leucophrys gambelii* (Farner and Lewis, unpublished); (4) The courses in change of weight of the adenohypophysis, its gonadotropin activity, and acid-phosphatase activity, and those of changes in activity of the median eminence of photostimulated castrates, although differing slightly from those of photostimulated intact controls, do not differ greatly; they return to levels characteristic of photorefractory birds on schedules very similar to those in photostimulated intact controls (Kobayashi and Farner, 1966; Stetson, Erickson and Farner, unpublished).

b. *Day-length-driven "annual oscillator."* Dolnik (1964) has proposed a model for periodically controlled gonadal function based on a day-length dependent, annually oscillating concentration of a "stimulator," which, contrary to his assumption, cannot be pituitary gonadotropin (King *et al.*, 1966), at least in *Zonotrichia leucophrys gambelii*. With short days (autumn and winter) the day-length-dependent discharge of "stimulator" is at a nonstimulatory level as judged by gonadal response, and is less than the rate of replacement thus resulting in an accumulation of "stimulator." The daily release of "stimulator" is a function of day length and of the accumulated quantity of "stimulator." With stimulatory daily photoperiods the daily discharge of "stimulator" exceeds the rate of replacement and the reserve accumulated during the preceding period of short days is gradually depleted as gonadal growth and function proceeds. During the long days of spring and summer the reserve is gradually depleted with a consequent decrease in daily discharge, when the daily rate of discharge decreases to a threshold level, the gonads regress and the bird is photorefractory. In addition to the fact that the oscillating "stimulator" in this model cannot be pituitary gonadotropin, we find it physiologically improbable that an annual oscillation of a reserve of any substance can be truly basic to photorefractoriness. Nevertheless, the Dolnik model, which rationalizes a wide spectrum of experimental results for *Fringilla coelebs* and other species, has attractive features that should not be discarded at this time.

c. *Shift in phase of the circadian periodicity in photosensitivity.* This is a somewhat simplified version of the hypothesis of Hamner (1968). The hypothesis proposes first the existence of a physiologically unexplained "absolute refractory period" (about 6 weeks in duration in *Carpodacus mexicanus*) during which even continuous light causes no testicular growth. This is followed by a "relative refractory period" during which the birds will not respond to day lengths equal to or shorter than those to which they have been previously exposed. This is explained by a continuing shift in the "photosensitive" phase of the daily (entrained circadian) oscillation in photosensitivity so that it comes to fall in the photoperiod of successively shorter days. This "relative refractory period" corresponds approximately to the steep segment of the curve of increasing photosensitivity of Farner and Follett (1966). The hypothesis does offer a plausible explanation of the heretofore puzzling observations that there is a range of photoperiods that is photo-stimulatory in spring but not in fall after the birds have become photo-sensitive (Hamner, 1968; Wolfson, 1959, 1960, 1964, 1965). The hypothesis has to us the advantage that it must distinguish between an

"absolute refractory period," for which it offers no explanation, and "relative refractoriness," a distinction not required by the Dolnik model. Bearing in mind the Law of Parsimony, we feel intuitively that this distinction may be unnecessary and that there is a continuous, although non-linear change from zero to maximum photosensitivity (see also Farner and Follett, 1966). Although some aspects of the Hamner hypothesis are not entirely clear to us, it does have attractive features. With *Zonotrichia leucophrys gambelii* we (Farner and Lewis, unpublished) have found changes of phase in photosensitivity in fall birds of the nature required by the hypothesis for the period of "relative refractoriness."

d. The "light-lock" hypothesis (Farner and Follett, 1966). This hypothesis assumes that gonadal regression and photorefractoriness are independent events, or at least that photorefractoriness is independent of the gonadal cycle. Such hypotheses were suggested originally by Threadgold (1960) and Laws (1961) on theoretical grounds. The idea that photorefractoriness is independent of the gonadal cycle is made more plausible by the behavior of the pituitary and median eminence of photostimulated castrates (Kobayashi and Farner, 1966; Stetson and Erickson, unpublished). Although Threadgold's suggestions of the involvement of prolactin or a pineal hormone in the independent development of photorefractoriness appear unlikely, at least in *Zonotrichia leucophrys gambelii* (Laws and Farner, 1960; Meier and Dusseau, 1968; Oksche *et al.*, 1971), we know of no evidence that is inconsistent with the basic hypothesis. This hypothesis is not necessarily mutually exclusive with respect to that of Hamner as described above.

e. The fatigue and exhaustion hypothesis. Such hypotheses are either explicit or implicit in many of the older papers (e.g., Burger, 1949; Miller, 1954). Regardless of the level in the control system to which they are applied, they appear highly improbable.

f. Circennial periodicity hypothesis. With the increasing evidence of the occurrence of endogenous circennial cycles in birds and mammals (see Section 2.4), it is also necessary to consider the possibility that photorefractoriness is a component of such a cycle that is entrained at some other phase by a segment of the annual change in photoperiod. Because photorefractoriness can be advanced by several months, or delayed for many, it is difficult at this time, at least, to invoke such an explanation. Also our long-term experiments with *Zonotrichia leucophrys gambelii* give no evidence as yet in support of this hypothesis (Lewis and Farner, unpublished).

It is clear that our knowledge of the nature of photorefractoriness is based very largely on the performance of intact birds subjected to a

variety of photoperiodic treatments. Very little is known about the components of the mechanism involved in its development except that it seems very likely, as noted above, that the site of the block in the response system is most probably at a hypothalamic level.

2.4 "Measurement" of the Duration of the Daily Photoperiod

Of fundamental importance is the mechanism by which the control system senses the duration of a photoperiod. It now seems clear that this is effected by the use of an endogenous circadian function in the "photosensitivity" of the system. Although this type of mechanism, first proposed by Bünning (1936; see also, 1963), had been suggested earlier for several species of plants and insects (see Bünning, 1963, for examples), it remained for Hamner (1963, 1964, 1966) in investigations on *Carpodacus mexicanus* to demonstrate such a mechanism in birds. From the confirming investigations of Wolfson (1965) on *Junco hyemalis*, Menaker (1965) on *Passer domesticus*, and Farner (1964b, 1965) on *Zonotrichia leucophrys gambelii*, it appears that this type of mechanism may be widespread among photoperiodic species although it would be precarious at this time to assert that it is the only type involved in birds. From an evolutionary aspect, it appears to represent the incorporation of a preexisting circadian oscillator, widespread or common among organisms, into the hypothalamic apparatus that controls the gonadotropic activity of the pars distalis. In principle, the type of information that suggests the existence of an endogenous circadian element in the response system is shown in Table I. Here, in experiments with *Z. l. gambelii*, 8-hour photoperiods in a 24-hour cycle were ineffective in stimulating testicular growth whereas 8-hour photoperiods in a 36-hr cycle were highly effective. These results, which are consistent with the much more extensive and sophisticated experiments of Hamner on *C.*

TABLE I
PHOTOPERIODIC TESTICULAR RESPONSES IN MALE *Zonotrichia leucophrys gambelii*
SUBJECTED TO 8-HOUR PHOTOPERIODS IN 24- AND 36-HOUR CYCLES

Age	Number of birds	Light regime hours	k^* (days $^{-1}$)
Adult	11	8L 28D	0.032 ± 0.006
	11	8L 16D	0.00
First-year	10	8L 28D	0.039 ± 0.003
	12	8L 16D	0.00

* Testicular growth-rate constant (Farner and Wilson, 1957) with 95% fiducial limits. For the same light regime, generally, k (first-year) = ~1.2 k (adult).

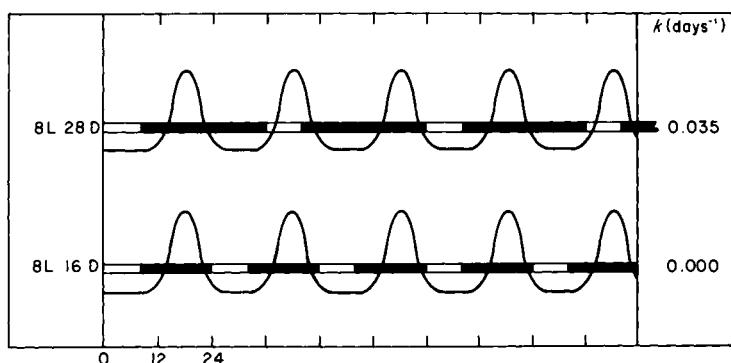


FIG. 3. Evidence of a circadian element in the photoperiodic control of testicular growth in *Zonotrichia leucophrys gambelii* and a hypothetical interpretation thereof (from Farner, 1970a). The white and black sections of the bars represent the light and dark periods, respectively, of the two experimental treatments. K is the testicular growth-rate constant in days^{-1} . The superimposed curves represent a hypothetical circadian cycle (entrained by the 24-hour light cycle) of photosensitivity in the control system.

mexicanus and of Follett and Sharp (1969) on the Japanese quail, can be rationalized if it is assumed that there is an endogenous circadian periodicity in the photosensitivity of the system (Fig. 3) that does not re-entrain immediately with each photoperiod, an assumption that is entirely consistent with the known properties of circadian periodicities. Partial tests of this hypothesis have been accomplished by the use of "scotophase-scan" experiments, similar to those employed by Büning and Joerrens (1962) on *Pierris brassicae*. In birds such experiments have been repeated on *Passer domesticus* (Menaker, 1965), *Zonotrichia leucophrys gambelii* (Farner, 1965), and on the Japanese quail (Follett and Sharp, 1969) (Figs. 4 and 5). In these experiments the scotophase of the 24-hour light-dark cycle is interrupted, at various times for various groups, by a relatively short period of light.

The results, expressed as rates of testicular growth, are best interpreted by a hypothesis that assumes the existence of an entrainable endogenous circadian periodicity in the photosensitivity of the mechanism (Farner, 1965; Menaker, 1965). This recognizes a dual function of the daily photoperiod in the normal photoperiodic response, i.e., that of entrainment of the circadian oscillation in photosensitivity and that of actual stimulation of the system during sensitive phase (Pittendrigh and Minis, 1964). However, very great caution must be applied in the interpretation of "sensitivity curves" obtained in experiments of the Büning-Joerrens type. It is clear that such curves do not describe with completely quantitative accuracy the oscillation in photosensitivity.

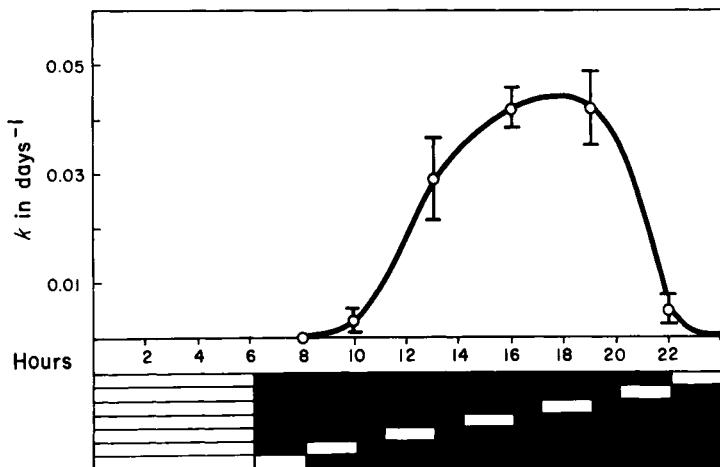


FIG. 4. The effect of "scotophase-scan" schedules on the rate of testicular growth (k) in *Zonotrichia leucophrys gambelii* (from Farner, 1965). Each of seven groups experienced schedules consisting of LD 6:18 plus a 2-hour pulse at the indicated time.

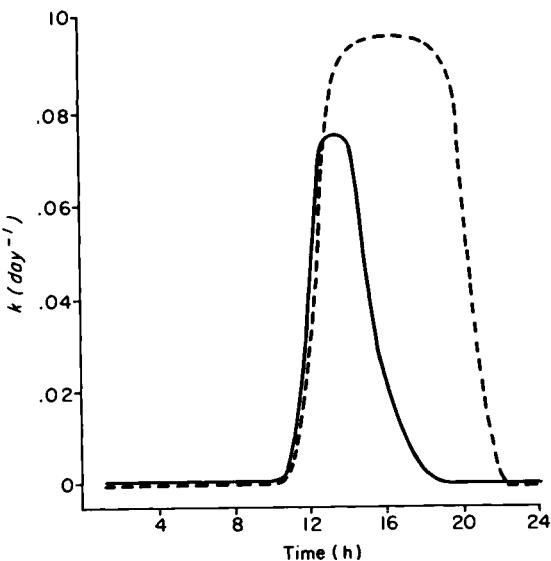


FIG. 5. The effect of "scotophase-scan" schedules on the rate of testicular growth (k) (after Follett and Sharp, 1969). Schedules consisted of LD 6:18 plus a light pulse given at the times indicated on the abscissa. Solid line, 15-minute pulses; dashed line, 2-hour pulses.

since at some point in the course of the scotophase the light interruption assumes the entrainment function of the basic photophase (Menaker, 1965); in other words, the beginning of the light interruption replaces the beginning of the basic photophase as "dawn" to the bird. The very fragmentary evidence available (Farner, 1965; Follett and Sharp, 1969; Hamner, 1964, 1968; Loftus *et al.*, 1970; Menaker, 1965) suggests that the time in the scotophase at which entrainment is taken over by the interrupting light period is a function of the duration of the light interruption; there also appear to be genuine species differences. Thus, making the apparently valid assumption that the oscillators involved in motor activity and photosensitivity are closely coupled, Menaker (1965) could show in experiments with *Passer domesticus* (4-hour basic photophase with 2-hour light-interruptions of the "20-hour" scotophase) occurs at about hour 14 after the beginning of the basic photophase or at about hour 10 of the scotophase.

We (unpublished results) have performed similar experiments with *Zonotrichia leucophrys gambelii* using a 6-hour basic photophase (as in Fig. 4) and 2-hour light interruptions of the 18-hour scotophase with recording of motor activity. Although there are some individual variations, it seems clear that most birds remain entrained to the beginning of the basic photophase with the 2-hour interruption as late as hour 17 after the beginning of the basic 6-hour photophase (11 hours after the beginning of the scotophase). This is clearer when the interrupting 2-hour "light period" consists of one 2-second light flash per minute for 2 hours. This regime perturbs the activity cycle record substantially less than the regime of a 2-hour interruption with continuous light but appears to produce the same gonadal response. With a regime with the 2-hour interruption beginning at hour 19 after the beginning of the basic 6-hour photophase, some individuals entrained to the 2-hour interruption, whereas others remained entrained to the 6-hour photophase. Unfortunately in this species the interpretation of records of motor activity becomes difficult with the development of photoperiodically induced migratory state and the accompanying nocturnal *Zugunruhe*, the analogue of nocturnal migratory flight under natural conditions. Nevertheless it appears that the "sensitivity" curve in Fig. 4 is probably quite accurate between hours 8 and 20 for the conditions used in these experiments. It is moreover possible to rationalize approximately the known empirical relationship between day length and the rate of testicular growth using empirically the function described in Figure 4 and the known "carry-over" function in the response scheme (Farner, 1964, 1965; Farner *et al.*, 1953).

Menaker and Eskin (1967), from a skillful experiment with *Passer*

domesticus, have produced additional evidence in support of the Bünning hypothesis of photoperiodic time measurement by an endogenous circadian period in photosensitivity. Motor activity and photosensitivity were first entrained by use of a daily 14-hour period of very dim, non-stimulatory green light; it was then possible to photostimulate the gonadotropin-releasing apparatus, with consequent gonadal growth, by use of a 75-minute period of bright white light imposed near the termination of the 14-hour period of dim green light; a similar white-light period at the beginning of the green-light period was ineffective.

Recently Loftus *et al.* (1970) have suggested that the natural responses to long daily photoperiods involve a re-entrainment of the photosensitivity oscillator with a consequent shift of the photosensitive part of its function (the "photoperiodically inducible phase" of Pittendrigh, 1966) into the photophase. Although they offer some observations on the Japanese quail that are consistent with this useful hypothesis, there seems, at the moment, no way to subject it to a rigorous test. A comparison of our records of motor activity in *Zonotrichia leucophrys gambelii* subjected to long days and short days, respectively, offers no support for the hypothesis; but this, of course, does not preclude a shift in phase angle between these coupled oscillators. The complexities of phase relationships in the response system are illustrated by the observations of Murton *et al.* (1969) (Fig. 6) that suggest a phase angle difference of several hours in the photostimulated release of LH and FSH in *Chloris chloris* whereas such appears not to occur in *Passer domesticus* (Loftus *et al.*, 1970). This difference in pattern of release of pituitary gonadotropins has been rationalized with ecological and ethological differences between the two species.

As noted above, the daily photoperiod has a double function in photoperiodic responses—(1) It entrains an endogenous, circadian oscillation in photosensitivity; (2) it actually photostimulates the system if it is sufficiently long to extend into the photosensitive phase. Significantly, the investigations of Menaker's group (Menaker and Eskin, 1967; Menaker, 1968; Menaker and Keatts, 1968; Underwood, 1968; Underwood and Menaker, 1970b) on *Passer domesticus* indicate that neither of these functions need involve the eyes.

2.4.1 CIRCENNIAL PERIODICITIES

Although the older literature is replete with fragmentary evidence and suggestions concerning endogenous functions in the annual cycles of birds and mammals, it remained for Aschoff (1955) to offer a clear hypothesis relating the roles of endogenous and exogenous periodicities in annual cycles. There is now reasonable evidence that long-period

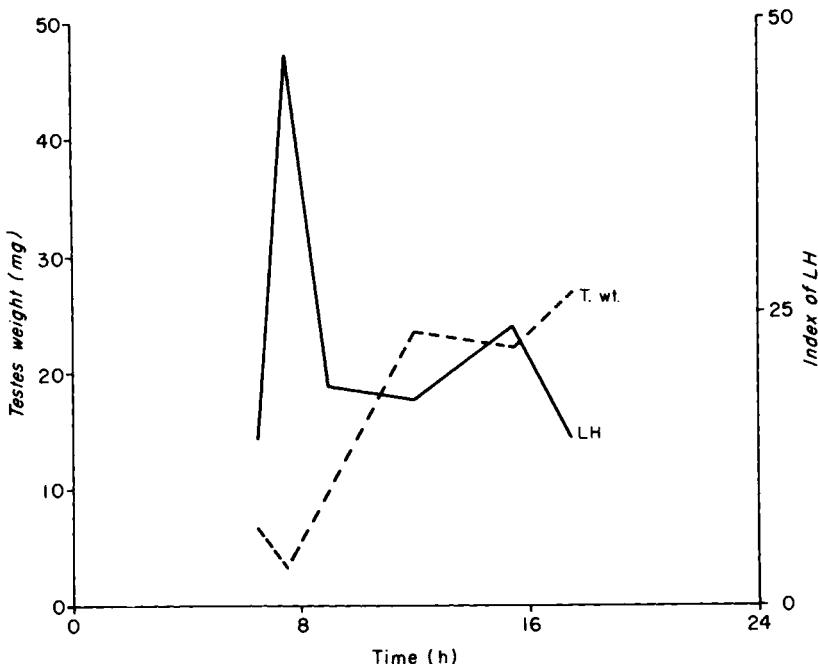


FIG. 6. Effects of asymmetric skeleton photoperiods on testicular growth and plasma LH levels in *Chloris chloris* (Murton *et al.*, 1969). Schedules consisted of LD 6:18 plus a 1-hour light pulse through the course of the scotophase.

endogenous periodicities have evolved in a number of groups of animals (D. E. Davis, 1967; Farner and Follett, 1966; Gwinner 1968, 1969; Heller and Poulson, 1970; King, 1968; Pengelley and Kelly, 1966). The minimum acceptable evidence of the existence of an endogenous circennial periodicity is its persistence through *at least* two accurately timed cycles under constant conditions of light (preferably LL or DD) and ambient temperature (Farner and Follett, 1966; Farner, 1967; Jegla and Poulson, 1970; King, 1968). Unless these conditions are met, it is not possible to exclude the possibility of a pre-programmed cycle that may occur the first year, but that will not recur subsequently in the absence of appropriate external information. Also, if the period length is too close to one year, more than one year is required to exclude the possibility that some annually periodic geophysical cue is controlling the rhythm (Jegla and Poulson, 1970).

Among birds, circennial cycles are reasonably well established experimentally for only a few species. The first suggestive experimental evidence of such in birds was that of Marshall and Serventy (1959) for a transequatorial migrant, *Puffinus tenuirostris*, and that of Benoit *et al.*

(1956, 1959) for the domestic mallard. The data of the latter suggest somewhat irregular endogenous testicular cycles with periods generally less than one year. The data of Merkel (1963) on *Sylvia communis*, also suggest the existence of long-period, endogenous cycles. Berthold (personal communication) reports circennial rhythmicity in molt, body weight cycles, nocturnal activity, and gonadal development in about 75% of the 80 hand-reared sylviids (*Sylvia atricapilla* and *S. borin*) that have been held under constant conditions for two years.

Quelea quelea maintained on LD 12:12 by Loftus (1964) for 29 months underwent testicular cycles with period lengths of about 12½ months. And quite recently Schwab (1971) reported two testicular cycles, the peaks of which were separated by 10 months in *Sturnus vulgaris* maintained on LD 12:12 for a period of 21 months. Schwab found further that this long-period cycle is strongly dependent upon the duration of the photoperiod. Hence, these data (and possibly those of Loftus, 1964) may be open to alternate interpretations. It is possible that this rhythm represents a circennial cycle that is expressed only under photoperiods approximating LD 12:12 it is also possible that the rhythm is somehow generated by this particular photoperiod.

Recently experiments with *Zonotrichia leucophrys gambelii* (King, 1968) have produced evidence of an essentially normal circennial periodicity, but with some shift in phase, in molt and fat deposition under LD 20:4 and in fat deposition, but not in molt, under LD 8:16. On the other hand we (unpublished data) have, as yet, detected no evidence of endogenous gonadal cycles in *Z. l. gambelii* held under constant photoperiodic regimes (LD: 8:16 or 20:4).

The best evidence of a circennial periodicity in birds is that of Gwinner (1968) who has demonstrated that young warblers (*Phylloscopus trochilus* and *P. sibilatrix*, which are transequatorial migrants) maintained on LD 12:12 at constant temperature from September until June underwent prenuptial molt at the same time as controls retained under natural photoperiods either on their breeding or wintering grounds. All three groups developed *Zugunruhe* at the same time and exhibited essentially similar cycles in body weight. Furthermore, birds that hatched late in the year terminated molt and developed both autumnal and vernal *Zugunruhe* later than young hatched earlier in the season. Two *Phylloscopus trochilus* were held on LD 12:12 for 27 months; in both of these, circennial rhythms in molt and *Zugunruhe* persisted through three cycles. The period lengths of these two subjects differed, one being decidedly shorter than one year, thus effectively ruling out the possibility of entrainment by uncontrolled, annually periodic environmental information.

It must be emphasized that circennial cycles are expressions of endogenous time-measurement systems, the mechanisms of which are as

yet totally unknown. Whereas circadian periodicities are primitive and hence apparently characteristic of the entire lineage leading to multicellular organisms, the same is highly unlikely for circennial periodicities since they would not have evolved until there were species in which some fraction of the population survives to an age of one or more years and in which there is a selective advantage in an annual cycle of reproduction and related functions. Furthermore, circadian periodicities are ubiquitous and pervasive among living systems, occurring at cellular and subcellular levels, whereas evidence of circennial cycles at cellular levels does not exist, or at least, have not been demonstrated. It therefore seems highly probable that circennial functions must operate at a higher level of organization, e.g., neuroendocrine. While Gwinner (1968) does not propose a mechanism either for the circennial rhythm or for the actual timing of the natural cycle in *Phylloscopus trochilus* and *P. sibilatrix*, he does suggest that the photoperiodic regime experienced during migration or reproduction at higher latitudes may synchronize the cycle annually. If our present view of the time measurement by photoperiodic mechanisms is correct (Farner, 1965; Farner and Follett, 1966; Hamner, 1963, 1964, 1966, 1968; Wolfson, 1964, 1965) a circadian clock is involved in the annual timing mechanism. It also follows that in certain photoperiodic species, at least, there may be an endogenous circennial periodicity that uses some part of the annual cycle in photoperiod as a *Zeitgeber*.

2.5 The Role of the Pineal Body

Because the epiphysial organs of many lower vertebrates function in photic and photoperiodic responses (Bagnara and Hadley, 1970; Hoffman, 1970), investigators have been intrigued by the possibility of similar roles in mammals and birds. The photobiology of the avian pineal is treated in recent reviews by Quay (1970) and Ralph (1970). Although the avian pineal body has been suggested as a possible site of photoreception (see Section 2.1) and as an endocrine organ that mediates photoperiodically induced gonadal growth and function, most experimental evidence is negative. Although experiments employing pinealectomy have not produced entirely consistent results, we nevertheless know of no instance in which pinealectomy has produced marked changes in gonadotropic functions in adult birds. For young cockerels Shellabarger and Breneman (1950) and Shellabarger (1952) demonstrated a slight progonadotropic effect of the pineal during the first three weeks after hatching and a possible antigonadotropic effect after 40 days. Effects of pinealectomy in both age groups of chicks were reversed by exogenous pineal extracts. In experiments on a markedly

photoperiodic galliform species, the Japanese quail, Homma *et al.* (1967) found no such age effects. In these experiments, pinealectomy of young quail was followed by a more rapid oviducal growth on a stimulatory light regime (LD 14:10); this effect failed to occur in females retained on a nonstimulatory photoperiodic regime. Sayler and Wolfson (1968) found that pinealectomy of immature female quail, but not of males, effects a transient delay in sexual maturation in response to stimulatory photoperiods (LD 16:8 and LL); such effects were not observed under a non-stimulatory regime (LD 8:16). It is interesting that McFarland *et al.* (1968) found that bilateral superior cervical ganglionectomy also delays the onset of oviposition in this species.

In the photoperiodic crowned sparrows (*Zonotrichia*) pinealectomy has no demonstrated effects on gonadal cycles. Gonadal growth rates are unaffected (Oksche *et al.*, 1971), as is testicular regression (Oksche *et al.*, 1971; Donham and Wilson, 1969) and termination of photorefractoriness (Donham and Wilson, 1970; Oksche *et al.*, 1971). However, there appears to be a reduction in levels of pituitary gonadotropins in pinealectomized birds of both sexes (Stetson, Morton, and Erickson, unpublished). Similarly, experiments on *Passer domesticus* in Menaker's laboratory (Menaker *et al.*, 1971) revealed no interference by pinealectomy with photoperiodically induced testicular growth. Electrolytic destruction of the pineal gland of *Carpodacus mexicanus* does not alter either gonadal growth or regression (Hamner and Barfield, 1970).

The reported effects of exogenous pineal principles on photoperiodic and reproductive functions have been reviewed by Ralph (1970) who rightly cautions that the relatively massive doses that have sometimes been employed, make it impossible to rule out nonspecific or even pathological effects.

A number of biochemical functions of the avian pineal gland show diurnal periodicities that appear to be entrained by light (Quay, 1970; Ralph, 1970). What relation these functions may bear to circadian systems or to photoperiodic time measurement in intact birds is unknown. Gaston and Menaker (1968; Gaston, 1969) have found that pinealectomy effectively abolishes the circadian rhythm of locomotor activity normally exhibited by both intact and sham-operated *Passer domesticus* in constant darkness. However, both the apparently arrhythmic locomotor activity pattern of the pinealectomized birds and the free-running circadian pattern of the intact birds are entrained by light-dark cycles of 24-hour duration. The authors suggest that, although other conclusions are possible, the data are consistent with a role of the pineal body as an element in the system involved in the circadian timing of locomotor activity in this species. Unpublished observations by

Gaston and Menaker suggest that the pineal effect on the circadian activity is mediated hormonally, e.g., introduction of pineal homografts into pinealectomized sparrows "immediately" restores the circadian locomotor patterns. The site of photoreception in this phenomenon is unknown, however, encephalic receptors are probably involved since bilaterally enucleated, pinealectomized House Sparrows entrain to light cycles.

Whatever the photoperiodic functions of the pineal gland in birds may be, one may expect some degree of species specificity. Furthermore, considering the great structural diversity of the avian pineal and, indeed, its virtual absence in some birds, it seems possible that many of the reported effects are due more to the trauma and sequelae of surgery, and to other artifacts of treatment, than to the properties of the pineal per se (see Ralph, 1970). At the present time, convincing evidence for a conventional sensory role or an important endocrine role bearing on gonadotropic function is lacking.

2.6 Autumnal Breeding in Relation to Photoperiodic Control

As noted above, most photoperiodic species that breed annually in spring or summer terminate reproduction in a photorefractory state that is not dissipated until late autumn or winter, by which time day length is below stimulatory level and the environment is otherwise unfavorable toward reproduction.

Nevertheless, transient autumnal sexual recrudescence occurs in many resident and at least some migrant species of middle latitudes (see, e.g., Larionov and Zhelnin, 1965; Loftus and Murton, 1968; Marshall and Coombs, 1957; Morley, 1943; Pielowski and Pinowski, 1962). Actual reproduction at this time, however, occurs only under unusual circumstances (Morley, 1943; Orians, 1960; Payne, 1969; Selander and Nicholson, 1962). This phenomenon raises questions concerning the annual termination of reproduction and of photorefractoriness; any general theory of the mechanism of termination of reproduction must accommodate this occurrence of postseasonal reproductive activity.

Certainly photorefractoriness is not an invariable consequence of breeding or of gonadal regression in photoperiodic species (see above). Furthermore, the duration of the photorefractory period varies considerably among different species (Loftus and Murton, 1968; Loftus *et al.*, 1970). As suggested by Farner (1959), sexual recrudescence in the fall may be explained if the refractory period of such species ends relatively early in late summer when photoperiods are still of a stimulatory duration. The continuing shortening of day length, together with a lack of other favorable environmental information, would account for the

transience of the recrudescence. While this suggestion is consonant with the observed facts in some instances (Payne, 1969; Selander and Nicholson, 1962), we must admit that we are largely ignorant of the temporal relations of terminating mechanisms. Investigations of the natural termination of photorefractoriness have not usually been structured to reveal the termination of "absolute" refractoriness (see Hamner, 1968), nor sufficiently sensitive to reveal potential or actual sexual resurgence that might appear before, or even in the absence of, significant gonadal growth (Laws, 1961; Threadgold, 1960).

If autumnal sexual activity has adaptive significance, its nature is by no means clear (Lofts and Murton, 1968) even in the few instances in which such activity actually culminates in reproduction. In most such cases nesting success is probably low (Payne, 1969; Selander and Nicholson, 1962). Because of the apparently recent origin of such patterns of reproduction, and their association with local human activity, we must consider the possibility that autumnal breeding is the result of the failure of the evolved mechanisms of termination of reproduction in the face of other unusually potent local information (Orians, 1960; Payne, 1969; Selander and Nicholson, 1962). Payne (1969) concluded that the low nesting success of *Agelaius tricolor* in the autumn suggests that it may be adaptive only in so far as response to food abundance as a releaser of reproductive activity is adaptive at other times of the year for this somewhat opportunistic breeder. According to Payne, autumnal reproduction in this species before the advent of present agricultural practices, probably occurred less frequently, but with more frequent success in association with grasshopper plagues.

Payne (1969) believes that the majority of the males of *Agelaius tricolor* that breed in fall are those that nested successfully in the early spring. Some males in their second autumn also develop large testes. The fraction of the population that does breed, may simply consist of those that are not photorefractory in late summer. Experimental induction of testicular growth in males was possible only when the photorefractory period was terminated by short-day treatment. However, significant ovarian growth was induced in all experimental females held on natural day lengths in the autumn in response to "simulated rains" and provision of live grasshoppers as food.

Recent experiments (Hamner, 1968; Murton *et al.*, 1970b) suggest that a circadian mechanism may determine the duration of photorefractoriness in *Carpodacus mexicanus* and *Passer domesticus*. Because of this, Hamner (1968) hypothesized that ". . . the timing relationships during the relative refractory period of all birds which experience autumnal gonadal growth may 'reset' too rapidly and bring the shorten-

ing day length and phase of light sensitivity into juxtaposition, causing autumnal recrudescence." This hypothesis is consonant with the generally observed transience of autumnal recrudescence and does not invoke extraphotoperiodic stimuli (i.e., second order control mechanisms). It is too early to evaluate this hypothesis adequately. However, it would seem that this model offers only a partial explanation for the cases in which breeding actually occurs.

Lofts (1970) has postulated that the development of new Leydig cells during the photorefractory phase and subsequent release of androgens near the end of this phase in response to continuing release of LH throughout the refractory period, may account for autumnal sexual resurgence. A possible mechanism, which is not inconsistent with Hamner's model, is provided by recent findings of Lofts and associates (Murton *et al.*, 1969, 1970a) who, as noted above, have demonstrated that FSH and LH may be released at different phases of the circadian cycle. It is thus possible that during the shortening days of autumn, the LH phase of secretion may be engaged to the progressive exclusion of the FSH phase, allowing interstitial cell rehabilitation with limited or no spermatogenesis. The level and perhaps the phases of secretion might, of course, be under some degree of nonphotoperiodic control.

2.7 The Precision of Photoperiodic Controls

Reviewed empirically here are the known quantitative relationships in the photoperiodic control of testicular growth based substantially on our experience with *Zonotrichia leucophrys gambelii*. For a given quality of light at supramaximal intensity, a fixed daily photoperiod (p) of stimulatory duration ($p > \text{ca. } 9 \text{ hr}$ in *Z. l. gambelii*), the growth of the testis from a resting state to about half of maximum weight, i.e., from $\sim 2 \text{ mg}$ to ca. 250 mg in *Z. l. gambelii*) is approximately logarithmic, so that

$$\ln W_t = \ln W_0 + k_p t \quad (1)$$

where W_0 is the resting testicular weight, W_t is the weight after t days of exposure to a stimulatory daily photoperiod of p hours, and k is the logarithmic growth-rate constant (Farner and Wilson, 1957). It has been shown that k is a function of light intensity (I) and wavelength (L), environmental temperature (T), and the time (r) elapsed after the termination of the photorefractory period. The last appears to involve a gradual increase in sensitivity of the response mechanism (Farner and Follett, 1966). If the functions of I and L are maximized for given values of r and T , it is possible to characterize k , as a function of p (Figs. 7, 8, and 9). In *Z. l. gambelii*, for daily photoperiods in the range $p = \sim 9\text{--}18 \text{ hrs}$, the relationship is essentially linear

$$k = m(p - l) \quad (2)$$

where l is the minimum detectable stimulatory photoperiod. In *Z. l. gambelii*, $l = \sim 9$ hours; as noted above, $m = \sim 0.009$. Our data on *Z. l. gambelii* suggest that k may be some positive function of p for $p < 9$ hours but are inadequate for its characterization; obviously the relationship must be nonlinear. There is the possibility that, in the strictest sense, there is no finite threshold value (l) for p . The role of T in the response is slight; no value of T is effective unless $p > \sim 9$ hours (Farner and Wilson, 1957).

If the functions of I and L are maximized (as is the case usually in nature), the appropriate value for r is applied, and the best approximation for T of the natural environment of the population is used for the calculation of an appropriate series of values for k_p , using Eq. (2), the initial part of the natural testicular growth curve can be "predicted" for a population under natural conditions by

$$\ln W_n = \ln W_0 + \sum_{i=1}^n k_i \quad (3)$$

where n is the number of days after the beginning of testicular develop-

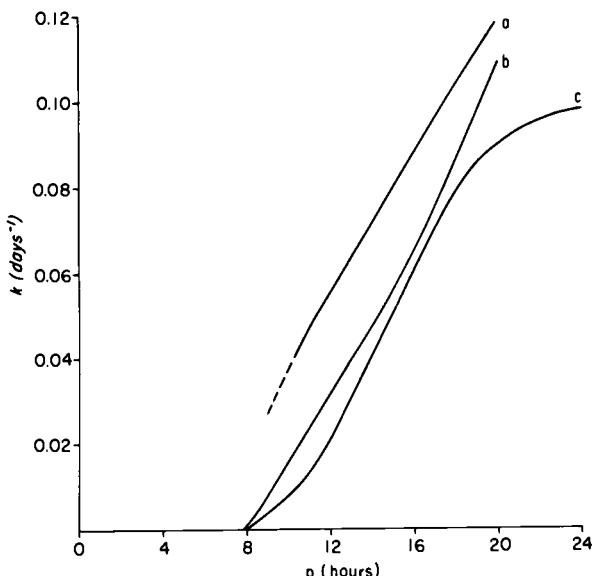


FIG. 7. Rate of testicular growth (k) as a function of the duration of the daily photoperiod (p). (a) *Zonotrichia leucophrys pugetensis* (Lewis, unpublished); (b) *Zonotrichia atricapilla* (Lewis and Farner, unpublished); (c) *Zonotrichia leucophrys gambelii* (Farner and Wilson, 1957).

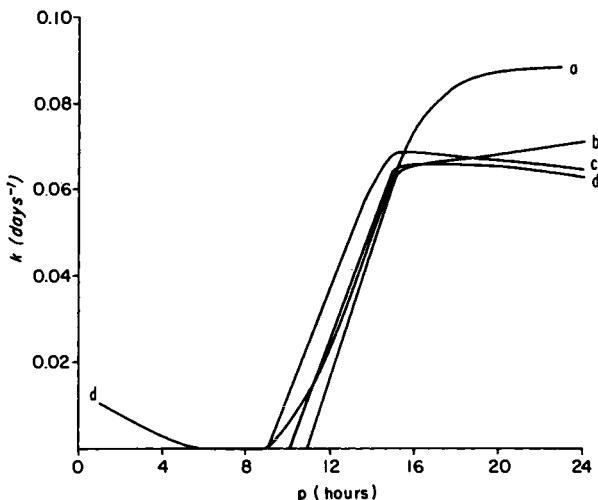


FIG. 8. Rate of testicular growth (k) as a function of the duration of the daily photoperiod (p). (a) *Passer domesticus* (see Middleton, 1965; Threadgold, 1960); (b) *Fringilla montifringilla*, (c) *Chloris chloris*, (d) *Fringilla coelebs* (from Dolnik, 1963).

ment under natural conditions in spring. The "predicted" date for attainment of 100-mg testes in *Z. l. gambelii* is within 10 days of the actual date attained within the population (Farner and Wilson, 1957). We have made similar calculations for *Z. l. pugetensis* and *Zonotrichia atricapilla* (unpublished data). Similar predictions have also been made by Dolnik (1963) for *Fringilla coelebs*, *Fringilla montifringilla*, and *Chloris chloris* and by Payne (1969) for *Agelaius tricolor* and *Agelaius phoeniceus*. These indicate clearly that controlled laboratory experiments

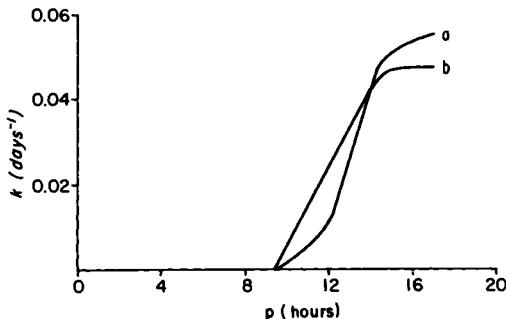


FIG. 9. Rate of testicular growth (k) as a function of the duration of the daily photoperiod (p) based on data presented by Payne (1969). (a) *Agelaius tricolor*; (b) *Agelaius phoeniceus californicus*.

can be meaningful in characterizing the performance of the mechanisms that control the development of the testes under natural conditions.

2.8 Modifying Factors

In considering photoperiodic control mechanisms in the timing of reproduction it is essential to bear in mind that the principal advantage in the use of photoperiodic information is its reliable predictive value. Photoperiodic information induces and sustains the physiological preparation of the reproductive system in such a way that maturational processes, which may require several weeks or even months, culminate at a near optimal time in the annual environmental cycle. To the extent that precision is adaptive, it is obvious that a system so programmed must be relatively insensitive to other environmental information, e.g., ambient temperature, that has less precise predictive value or none. Nevertheless, if the mechanism is not appropriately sensitive to signals that closely reflect the state of the local environment during the culminative stages of the cycle, the population could be subject to competitive disadvantage with populations with more flexible timing systems. Thus it is not surprising that a diversity of modifying information and permissive conditions, some of which are highly species specific, have roles in the final temporal control of the onset of breeding and in the precise chronology of reproduction (Farner, 1964, 1967; Lack, 1950; Loftus and Murton, 1968; Marshall, 1970).

Only rather brief attention is given here to modifying factors since the extensive relevant literature is generally non-experimental and non-quantitative so that precise analyses of the roles of such factors in annual timing schemes are not possible; furthermore, for individual factors it is often not clear whether the effect is exerted principally through the female or through the male (see, for example, Farner, 1964, 1967; Immelmann, 1963; Lehrman, 1961; Marshall, 1970; Nalbandov, 1970; Payne, 1969).

The role of modifying factors may often involve a greater influence on the percentage of a population that breeds than on the actual time of breeding. It is evident, for example, that modifying factors play a limited role in the timing of reproduction in the severely annually periodic environments of high latitudes; arctic breeders are allowed little variability in the timing of the annual cycle. The period during which breeding can take place is restricted to such a degree that the only alternative to an annually precise onset of breeding may be non-breeding. Indeed, nonbreeding among sexually mature arctic birds is not uncommon (Marshall, 1952). Even in the temperate zone, many songbirds begin breeding very early in the spring and according to Lack

(1950), ". . . the nesting disasters which result from abnormally cold spring weather suggest that the safety margin, if any, is small." By contrast, in tropical and arid regions, local environmental factors may be overriding importance in the timing of breeding cycles (Immelmann, 1963; Marshall, 1970).

Several strategies involving differing contributions of modifying factors are employed in the timing of annual reproductive cycles in photoperiodic species. In some, such as *Passer domesticus* (Bartholomew, 1949; Menaker, 1965; Polikarpova, 1940; M. Vaugien and Vaugien, 1961) the photoperiodic response is relatively imprecise and the actual time of reproduction is extensively influenced by other factors. In others, such as *Parus major*, the photoperiodic testicular response is precise under controlled conditions (Suomaleinen, 1937), but is relatively easily modified by other environment factors (Marshall, 1949). *Columba palumbus* is another basically photoperiodic species in which modifiers play an important role in timing the natural testicular and reproductive cycles (Lofts and Murton, 1966; Lofts *et al.*, 1967b; Murton *et al.*, 1963). On the other hand, *Zonotrichia leucophrys gambelii* has a relatively precise photoperiodic testicular response, and modifying factors probably exert important influences only very near the onset of breeding (Farner and Wilson, 1957; King *et al.*, 1966). The rate of testicular growth can be modified slightly as a function of temperature (Farner and Wilson, 1957). Furthermore, the photoperiodic gonadal responses of both male and female *Z. l. gambelii* are not greatly modified by conditions of housing or plane or nutrition (Lewis and Gwinner, unpublished). Populations of *Agelaius tricolor* and *A. phoeniceus* in central California have similar photoperiodic responses, but the former is more of an opportunistic breeder and hence much more influenced by modifying factors than the more regular breeder, *A. phoeniceus* (Payne, 1969).

In general, our present knowledge of the importance of nutritional conditions as information in the timing of reproduction is limited. Payne's data (1969) indicate that *A. tricolor* has a shorter breeding schedule that allows it to use such information as rainfall and local temporary abundance of food in the timing of the culminative stages of reproduction and ovulation. *A. phoeniceus*, which has a more protracted period of territory establishment, courtship, and nest-building seems to derive little critical information from such conditions. Assenmacher *et al.* (1965) found that the testes of Pekin ducks, placed on a strict fast during spring, sustained an involution that was much more severe than that of other parts suggesting the existence of a mechanism that uses poor trophic conditions as information to delay or eliminate the repro-

ductive period. However, partial fasting by photosensitive ducks placed on constant light had no significant effect on testicular development. Realimentation rapidly reversed the effects of fasting. Caloric restriction has little effect on the early stages of testicular development in *Z. l. gambelii* (Farner and Lewis, unpublished) whereas, in *Acanthis cannabina* it appears that the rates of growth and regression are conspicuously affected by reduced caloric intake (L. Vaugien, 1959).

2.9 The Role of Photoperiod in Ovarian Development

Among the photoperiodic species that have been appropriately investigated, it is rather general, and especially in passerine species, that only a partial development of the ovary is induced under artificial conditions by photoperiodic treatment. Females of species in which the male photoperiodic testicular response is strong usually exhibit progressive ovarian and other reproductive changes that are at least approximately equivalent in rate and magnitude to the prenesting changes observed in feral females (for selected data and summaries, see Burger, 1949; Farner, 1959, 1961; Farner and Follett, 1966; Farner *et al.*, 1966; Kern, 1970; Loftus *et al.*, 1970; Payne, 1969; Polikarpova, 1940). Phillips and van Tienhoven (1960) failed to induce demonstrable ovarian growth in *Anas acuta* in captivity by means of photostimulation. Such a complete lack of response in photoperiodic species is rare and was later attributed to "fear" by Phillips (1964), who demonstrated that ventral medial lesions of the archistriatum which reduced "escape behavior," also permit ovarian development. In general, it is vitellogenesis and the culminative stages of follicular development that fail to be induced by photostimulation (Farner *et al.*, 1966; Kern, 1970; Lewis, Gwinner, and Jones, unpublished; Loftus *et al.*, 1970; Payne, 1969). These functions appear to be largely under the control of essential supplementary factors (Farner, 1964), at least some of which are species specific and may involve such environmental information as the presence of an active mate, of nesting material, of a nest site, etc. (see, for example, Hinde, 1965, 1967; Lehrman, 1961, 1964, 1965; Lehrman and Friedman, 1969; Lott *et al.*, 1967; Slater, 1969).

The control of the development and function of the female reproductive system in photoperiodic species is best interpreted in terms of two phases of ovarian development (see, for example, Bissonnette and Zujko, 1936; Cuthbert, 1945; Clavert, 1948; Kern, 1970; Lehrman, 1961; Payne, 1969). There is an initial phase during which there is a *slow growth* of ovules from stage one to stage two of van Durme (see Cuthbert, 1945); this is followed by a phase of *rapid growth* during

which most of the deposition of yolk occurs and during which the largest follicles, i.e., those of the prospective clutch, mature. In a large variety of species, second-stage ovules develop only over a period of several weeks to a few months and it is not until the last 4–11 days preceding ovulation that the final rapid follicular growth begins (*op. cit.*). In *Zonotrichia leucophrys pugetensis*, for example (see Fig. 10), the largest ovule in midwinter is less than 0.4 mm in diameter; on arrival at the breeding grounds the diameter of the largest follicle is about 1.8 mm and just a few days prior to ovulation (~5–6 days in any given individual), the diameter of the largest follicle does not exceed ~2.5 mm.

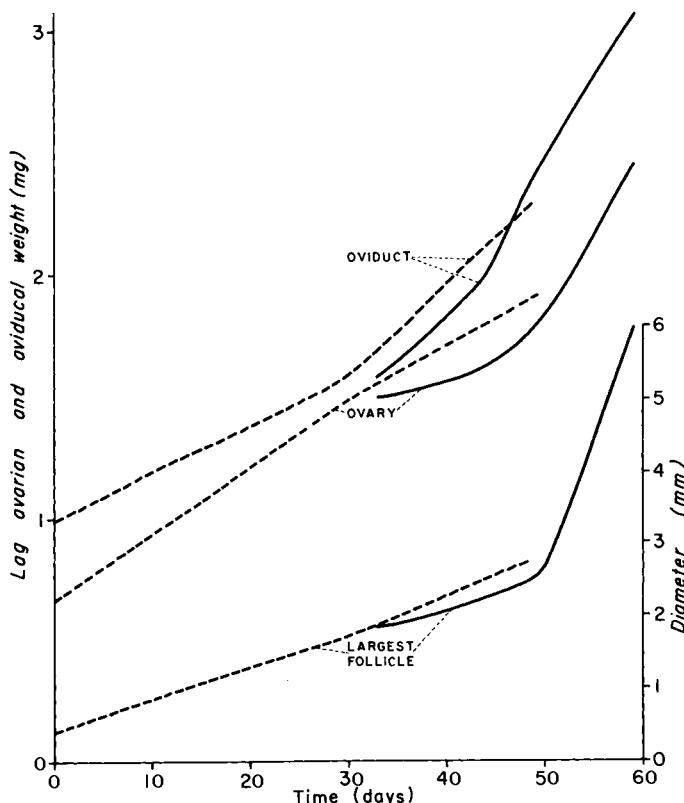


FIG. 10. Ovarian and oviducal growth in adult *Zonotrichia leucophrys pugetensis* in response to photostimulation, LD 18:6 (dashed lines), and in feral adults from the day of arrival (April 21, 1969) on the breeding grounds, Camano Island, Washington, until the peak of the first ovulatory cycle (solid lines) (Lewis, unpublished).

By comparison, photostimulation (LD 18:6) results in ovaries, the largest follicles of which average 2.8 mm in diameter. Furthermore, the ovary so stimulated is an active endocrine organ, as indicated by considerable oviducal growth (Fig. 10) and differentiation, as well as a substantial development of the incubation patch. The experimentally photostimulated female *Z. l. pugetensis* reaches, in terms of these functions, a stage of reproductive development comparable to that of a female several days following arrival on the breeding grounds (Fig. 10). Continuing experiments in our laboratory (Lewis, Farner, Gwinner, and Jones, unpublished) are yielding data that lead to similar conclusions regarding photoperiodic responses of three other taxa of *Zonotrichia*. Payne (1969), from field and laboratory investigations of *A. tricolor* and *A. phoeniceus*, has also reached similar conclusions. He states that ". . . the complete range of follicular development in early spring up to the time of nesting can be accounted for by the photoperiodic response. Apparently the latter stages of ovarian development during nesting are a result of other conditions including presence of a singing male and availability of suitable nest sites."

In summary, the available data suggest that while photoperiodically induced gonadal growth in the female of primary photoperiodic species is not as dramatic as in the male, it fully accounts for all but the terminal few days of maturation. Thus the natural photoperiodic environment is seen to serve the same predictive function in timing the cycle of the female as it does the male. Indeed, it would be strange were this not the case. From a synthesis of the knowledge gained from the many investigations of *Z. leucophrys gambelii* it is apparent that the strategy of this finch (Fig. 11) is basically photoperiodic, the increasing day lengths of spring effecting a progressive development of the testes and a cluster of appropriate responses, e.g., premigratory fattening, migration, etc., so that reproductively competent males arrive on the breeding grounds *in advance* of the time of reproduction. The females respond in similar fashion, but at a slower rate, so that they arrive on the breeding grounds slightly in advance of the optimal reproductive time (but after males have secured territories) and in a physiological state so that the final, rapid culminative changes can occur as soon as breeding conditions are propitious. It is further evident that modifying factors and essential supplementary factors are involved more extensively in the terminal phase of reproductive development in the female. The male is ready to mate at any time after arriving on the breeding grounds. The female, however, through mate selection and because of her sensitivity to the local environment, determines the

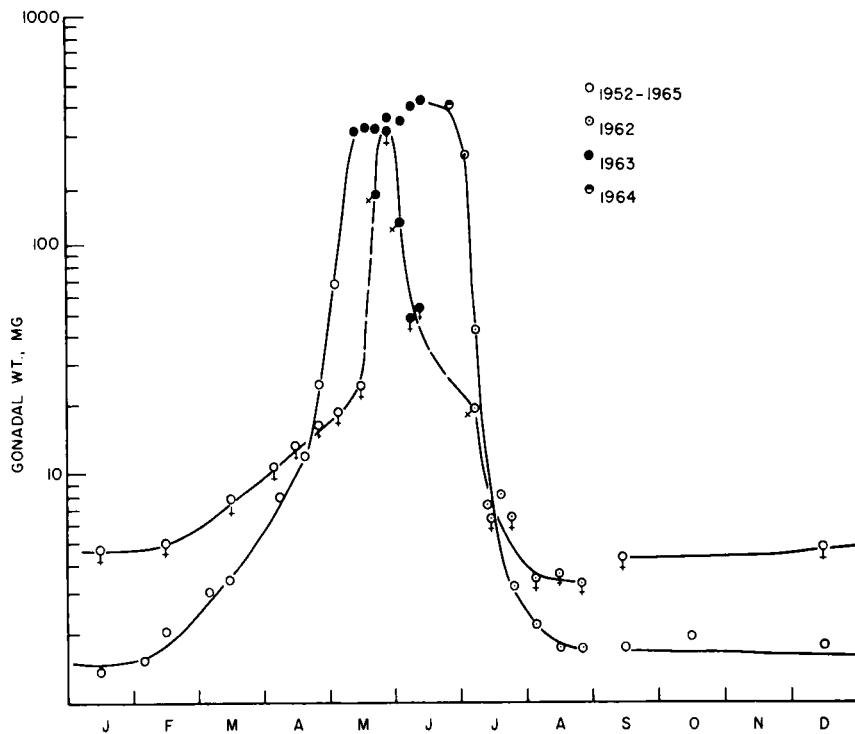


FIG. 11. Annual ovarian and testicular cycles of adult *Zonotrichia leucophrys gambelii*. (From King *et al.*, 1966.)

precise time of reproduction. Because of the greater energy-cost and risks to the female in the production and incubation of eggs the adaptive significance of this scheme is clear.

3. Photoperiodism and Migratory Cycles

The general distribution of migratory behavior among the families, genera, species, and infraspecific groups of birds suggests strongly that this is a phenomenon of multiple origin. Granted this assumption, it then follows that there must be differences in the adaptive significances of the patterns and controls of migration among the species and populations involved. We, therefore, restrict attention to those patterns that involve regular, relatively long-distance flights between well-defined wintering areas and breeding areas. For such migratory patterns it seems clear that adaptive significance lies primarily in the opportunity to exploit an area, inhospitable during the nonbreeding season, that offers improved trophic conditions, including longer days for the feeding

of the young, during the breeding season. The migratory periods are therefore properly regarded as components of the annual reproductive cycle. Because of our own research interests and experience, we draw heavily on the available information for the various taxa of *Zonotrichia* (see, especially, Farner, 1960, 1964a, 1970a; Farner *et al.*, 1968; Helms, 1963; King, 1961a,b, 1963, 1970; King and Farner, 1963, 1965; Mewaldt *et al.*, 1964, 1968; Wolfson, 1954a,b, 1958, 1959, 1964, 1966).

Since many, or most, of the mid- and high-latitude migrants use photoperiodic information in the timing of the vernal development of the reproductive apparatus, it is not surprising that the control of migratory behavior, which must be closely correlated with reproduction, involves photoperiodic controls. Although the early evidence concerning photoperiodic control of migration was obtained from experiments involving the release of photoperiodically treated individuals (Wolfson, 1940, 1942; Rowan, 1929), this approach involves a substantial element of uncertainty and has been replaced largely by experiments in which the birds are retained in captivity. This is possible, especially with nocturnal migrants, because two important physiological parameters characteristic of the migratory state (hyperphagia and fattening; and *Zugunruhe*, the nocturnal restlessness that corresponds to natural nocturnal migration) can be induced and measured quantitatively in caged birds.

As an example of a migrant between mid and high latitudes of the northern hemisphere, the annual cycle of *Zonotrichia leucophrys gambelii* includes nocturnal flights in spring and late summer up to distances of 4000 km. Associated with these migratory periods, and especially conspicuous before and during the vernal migration, is a hyperphagia and a consequent deposition of fat in specific subcutaneous and visceral depots. A similar deposition of fat occurs in captive birds held in outdoor aviaries (King, 1961a,b). Such captive birds also exhibit a nocturnal unrest (*Zugunruhe*) that is usually oriented northward in the spring and southward in late summer (Mewaldt *et al.*, 1964). *Zugunruhe*, the adaptive hyperphagia, and the consequent deposition of fat can be artificially induced in photosensitive birds by subjecting them to long daily photoperiods (King, 1961a, 1970; King and Farner, 1956, 1963; Farner, 1960). Furthermore, these functions fail to develop in birds maintained on chronic short daily photoperiods (Farner, 1964; King, 1961a). Fat deposition does not occur in non-migratory forms and nocturnal activity is also lacking or occurs only to a limited extent (Dolnik, 1966; Eyster, 1954; Farner, 1960; Mewaldt *et al.*, 1968; Gwinne, Farner, and Mewaldt, unpublished; Wolfson, 1942). Patterns of migration and fat deposition similar to those of *Z. l. gambelii* have been

described for a large variety of migrants (for examples, see Nisbet *et al.*, 1963; Odum, 1960a,b, 1965; Odum *et al.*, 1961; Ward, 1963, 1964; for a useful review, see Johnston, 1966). In general, the magnitude of fat deposition is directly related to the duration of the longest migratory flight of the species (Johnston, 1966; Odum, 1960a,b). In species or populations with short migratory flights, fat deposition may be minimal or not detectable as in *Zonotrichia leucophrys pugetensis* (Lewis, unpublished), a population of *Junco hyemalis hyemalis* that winters in North Carolina (Johnston, 1962), and in a population of *Junco oreganus montanus* that winters in eastern Washington (Farner *et al.*, 1961).

In *Zonotrichia leucophrys gambelii* artificial long-day regimes, imposed unseasonally after the termination of photorefractoriness, in addition to causing gonadal growth (see above), induce a vernal-type prenuptial molt (Farner and Mewaldt, 1955) hyperphagia and fat deposition (Farner, 1960; King and Farner, 1956, 1963; King, 1961a,b) and migratory behavior (*Zugunruhe*) (Farner, 1960; King and Farner, 1963). A vernal-type fat deposition has been similarly induced, for example, in *Fringilla coelebs* (Dolnik, 1961), *Zonotrichia albicollis* (Wolfson, 1954), migratory races of *Junco oreganus* (Wolfson, 1942), and *Junco hyemalis* (Wolfson, 1954a) (these investigations did not record motor activity). Both fat deposition and *Zugunruhe* have been shown to be photoperiodically induced in *Fringilla montifringilla* (Lofts and Marshall, 1960). There appears not to be a direct causal relationship between the induction of fat deposition and migration but rather that they are programmed to occur at approximately the same time in many species or populations (King and Farner, 1963; Dolnik, 1970); field studies suggest, however, that they may be rather widely separated in short-distance migrants (Johnston, 1966).

A functional role of the gonads in the control of migration was suggested almost a century and a half ago by Edward Jenner (1824). The suggestion was revived a century later by Rowan (1929) on the basis of observations of the state of interstitial cells in both testes and ovaries of *Junco hyemalis* during the vernal and autumnal migratory periods. Such hypotheses have not gained general acceptance for a variety of reasons (see, for example, Farner, 1955; Weise, 1967) and the results of a variety of castration experiments (e.g., Lofts and Marshall, 1960; Morton and Mewaldt, 1962), have been generally interpreted as suggesting that the gonads do not have an essential role in the development of the migratory state. Morton and Mewaldt, for example, found that both castrated and intact Golden-crowned Sparrows, *Zonotrichia atricapilla*, a strongly migratory species, developed vernal fat deposition and *Zugunruhe* under the naturally increasing day lengths

of spring. The responses, however, were more intense in the controls than in the castrates. Nevertheless, such results suggest that the vernal migratory state does not have a direct causal dependence on gonadal function. Weise (1967), in an investigation on *Z. albicollis*, found that castration before the photostimulatory period had a more depressing effect on fattening and *Zugunruhe* than castration after the beginning of photoperiodic stimulation. He concluded that the role of gonadal hormones is that of a feedback on the hypothalamohypophyseal system. This conclusion rationalizes most of the apparent contradictions in the literature and is consistent with the results of the earlier experiments of Schildmacher and Steubing (1952; also Farner and A. Wilson on *Z. l. gambelii*, unpublished) in which fattening was induced by exogenous androgens.

The rather unsatisfactory state of our knowledge of the processes and control of deposition of migratory fat has been reviewed recently (Farner *et al.*, 1969) and need not be discussed here. Suffice it to say that a series of experiments with *Zonotrichia leucophrys gambelii* and *Z. albicollis* suggest rather strongly that prolactin, interacting with other hormones, has a mediating role both in fat deposition and in the development of migratory behavior (Meier *et al.*, 1969; Meier and Davis, 1967; Meier and Farner, 1964; Meier *et al.*, 1965).

In the paragraphs above we have noted, with respect to the migratory crowned sparrows and certain other nocturnal passerine migrants, that vernal migratory behavior and/or fat deposition can be induced by long daily photoperiods; we have argued that this doubtless represents the normal means of induction of these events in nature. It has been assumed previously (Farner, 1964; Farner and Follett, 1966) that the mid-summer postnuptial molt, fat deposition and migratory behavior are basically physiologic sequelae of the photoperiodically induced vernal events. That this may be somewhat more complex in *Zonotrichia leucophrys gambelii* becomes clear from the observations of King (1968, 1970) that suggest that an endogenous cycle may be involved and that the role of long days in spring may be primarily that of entrainment. This does not preclude the possibility that, etiologically the late-summer events are physiologic consequences of the vernal events. As noted above, however, our observations (Lewis and Farner, unpublished) offer no direct support for the hypothesis of an endogenous cycle insofar as gonadal function is concerned. However, investigations of passerine transequatorial migrants by Gwinner (1967, 1968), Merkel (1961, 1963), and Berthold (unpublished) suggest that such a hypothesis is tenable and clearly, at least for some transequatorial migrants, the most probable explanation.

Although a prenuptial-type molt is clearly a part of the complex of long-day induced events of spring in some of the photoperiodic species (see above), the role of photoperiodic information, if indeed it has one, in the principal molt, is by no means clear. Clearly the strategies for the temporal adjustment of molt to breeding and migration are diverse. The careful review by Payne (1971) indicates that the control mechanisms are equally diverse and that no completely satisfactory generalizations are possible at this time.

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