

Photochemical and Photobiological Reviews

Volume 1

Kendric C. Smith



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Volume 1

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Dedication

This first volume of *Photochemical and Photobiological Reviews* is dedicated to Dr. Arthur C. Giese, Professor Emeritus of the Department of Biological Sciences, Stanford University, in recognition of the inspiration and guidance that he has given to so many photobiologists. His fundamental scientific contributions to photobiology have been highlighted in an article that appeared in *Photochemistry and Photobiology* [13: 1–3 (1971)] to commemorate his retirement in the summer of 1970. Although “officially” retired, he is still actively writing books and publishing research papers. I will try to achieve in this review series the high standards of excellence that so characterize all of Dr. Giese’s work, including his pioneering review series, *Photophysiology*.

*Kendric C. Smith
Editor*

Preface

A stone carving from the 14th century B.C. records that the Egyptian pharaoh Akhenaten (born Amenhotep IV) and his wife, Nefertiti, recognized the importance of sunlight to life. In fact, Akhenaten initiated a monotheistic religion, with Aton, the sun, as God. One of his daughters became the wife of King Tut Ankamon, the spelling of whose name indicates a return to the old religion and an eclipse of interest in photobiology among the pharaohs.

A renewal of interest in photobiology in modern times was climaxed in 1928 by the establishment of an international organization for photobiology under the title Comité International de la Lumière (C.I.L.). Its present title, Comité International de Photobiologie (C.I.P.), was adopted at a meeting in Paris in 1951. The first of a series of international congresses on photobiology was held in 1954 and probably represents the beginning of modern day photobiology.

Medical men were prominent in the activities of the old C.I.L., for the importance of natural sunlight in human health and disease was obvious though not well understood. The bringing together of physicians with physicists, chemists, and biologists from the pure and applied branches of their subjects was the aim of the older C.I.L. and continues to the present day through the C.I.P.

In 1952 a Committee on Photobiology was established in the United States, under the aegis of the National Research Council, to serve as the U.S. section of the C.I.P. Recognizing the need for a national organization for the science of photobiology, the U.S. National Committee for Photobiology sponsored the formation of the American Society for Photobiology. The Society was incorporated 27 July 1972.

Another landmark in the history of photobiology was the establishment in 1962 of the international journal *Photochemistry and Photobiology*, which continues to be the leading journal in this field. Since 1973 it has been the official organ of the American Society for Photobiology.

In 1964 a review series on topics in photobiology was inaugurated by Arthur C. Giese under the title of *Photophysiology*. This highly respected series was discontinued in 1974 after eight volumes when it fell victim to a

corporate reorganization after the acquisition of its publisher by another company.

The science of photobiology is a dynamic multidisciplinary field whose relevance to the needs of man is growing more apparent each day. Publicity about supersonic transports (the SST's), spray cans, and space shuttles and their possible deleterious effects on the stratospheric ozone layer and the possible resultant consequences of enhanced solar ultraviolet radiation on man and his environment have helped to focus attention on both the beneficial and detrimental effects of light. In addition, considerable activity is currently being directed toward harnessing solar energy as one solution to the world energy crisis. Some mechanisms for accomplishing this involve photobiological systems or photochemical models based upon these systems. It would thus seem that modern man has rediscovered the sun and is now actively considering new uses of light rather than thinking of light only as an aid to vision. Photobiology has become a major new scientific field.

In every scientific field it is important that the leaders review the field periodically as a service to the younger scientists in the field and to senior scientists in related fields. Such reviews provide a ready access to the recent literature in the field, and more importantly, they often provide a critical evaluation of the direction that the field is taking and frequently suggest a redirection when appropriate. To accomplish these goals, the present series, *Photochemical and Photobiological Reviews*, was inaugurated.

The American Society for Photobiology has divided the science of photobiology into fourteen subspecialty groups. These subspecialties are organized below in six groups, and another subdivision, New Topics in Photobiology, has been added:

- | | |
|--|--|
| I. Phototechnology
Photochemistry
Spectroscopy | IV. Chronobiology
Photoreception
Vision |
| II. Photosensitization
Ultraviolet and Visible
Radiation Effects | V. Photomorphogenesis
Photomovement
Photosynthesis |
| III. Environmental Photobiology
Medicine | VI. Bioluminescence
New Topics in
Photobiology |

It is our goal that each issue of *Photochemical and Photobiological Reviews* will contain reviews on at least one subject related to each of these six major groups. This goal will not always be met, as exemplified in Volume 1, because unforeseen events can prevent an author from completing a review

by publication time. Nevertheless, we will strive for balanced coverage in each issue.

The goals of the science of photobiology have been divided into four categories*: "(1) The development of ways to protect organisms, including man, from the detrimental effects of light; (2) the development of ways to control the beneficial effects of light upon our environment; (3) the continued development of photochemical tools for use in studies of life processes; and (4) the development of photochemical therapies in medicine."

It is hoped that the articles that appear in *Photochemical and Photobiological Reviews* will not only document and interpret the past but will also help chart the future so that progress in the attainment of the above goals will be swift.

*Kendric C. Smith
Editor*

* K. C. Smith, *BioScience* **4**:45-48 (1974).

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The C₄ Pathway of Photosynthesis: *Ein Kranz-Typ Wirtschaftswunder?*

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1. INTRODUCTION

The continued dependence of man on the productivity of plants for food and structural needs necessitates a serious appraisal of the conditions under

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which plant productivity will be sufficient for future requirements. The need for maximum plant productivity for food has been long appreciated, and reforestation of logged areas is an accepted practice for the production of paper and structural timber. However, plants are now seen to be important, not only as sources of food and shelter, but also as sources of raw energy and starting materials for synthetic processes, and indeed for the maintenance of an adequate O₂:CO₂ balance in the atmosphere. They are also, for the time being, the only significant harvester of solar energy. The climatic conditions under which plants are cultivated vary enormously, and the careful selection of a crop which will produce the maximum yield in a given environment is a criterion of ever increasing significance.

The C₄ plants* are one group with a distinct type of photosynthetic pathway and include such commercially important species as maize, sugar cane, sorghum, as well as a number of crop weeds. The discovery and subsequent biochemical elucidation of the C₄ pathway of photosynthesis has been one of the most significant and popular areas of research in plant biology during the past ten years, but recognition of the pathway as a distinct metabolic entity provided the confluence of a series of unrelated experimental observations, some of which had originated 60 years earlier. Chronologically, the first of these observations concerned the recognition of *Kranz* (wreath) anatomy in the leaves of a number of plants, both monocotyledons and dicotyledons. Later this specialized anatomy was correlated with geographical distribution both with respect to temperature and to water availability. Finally, physiological and biochemical studies led to the realization that a large number of plants, often tropical in origin and possessing Kranz anatomy, fixed CO₂ by a process whose initial product of fixation was a C₄ dicarboxylic acid as contrasted with the C₃ phosphorylated compounds formed by plants using the classical Calvin cycle.

In addition to the features listed above, plants utilizing the C₄ pathway of photosynthesis are characterized by a CO₂ compensation point (that is, the CO₂ concentration at which no net gas exchange occurs) close to zero, their rate of photosynthesis is not affected by the concentration of oxygen, and they have a high light requirement for maximum rates of photosynthesis. Individual leaves of C₄ plants are acknowledged to have a capacity

* Abbreviations: The terms C₃ and C₄ are used to indicate the primary mechanism of carbon fixation by the photosynthetic carbon reduction cycle (Calvin cycle) or by the C₄ dicarboxylic acid pathway. Plants using these pathways are referred to as C₃ and C₄ plants, respectively. AMP, ADP, and ATP abbreviate adenosine mono-, di-, and triphosphates respectively; CAM is Crassulacean acid metabolism; DBMIB, dibromothymoquinone; DCIP, 2,6-dichlorophenolindophenol; FeCN, ferricyanide; MeV, methyl viologen; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglyceric acid; P_i, inorganic phosphate; RuDP, ribulose-1,5-diphosphate.

for photosynthesis about twice as great as leaves of plants employing the C₃ pathway.

It is the purpose of this article to review our current knowledge of plants possessing the C₄ pathway of photosynthesis, to outline their special physiological characteristics and biochemical pathways, and to indicate what biological advantages this group of plants may possess. We have not attempted to assess the present economic importance of C₄ plants in world production, but we hope to provide enough information to indicate whether, biologically, they have the capacity to perform the *Wirtschaftswunder* (economic miracle) which some have claimed.

Some of the early studies on C₄ photosynthesis led to broad generalizations which have not been substantiated and which have left a terminology which is confusing. This is exemplified in descriptions of chloroplast ultrastructure, where bundle-sheath chloroplasts have been described as either granal or agranal. It is very rare in higher plants, however, that a strictly agranal chloroplast is observed under the electron microscope. Similarly, the tendency to group plants as "malate formers" or "aspartate formers" according to the initial products of CO₂ fixation implies an absoluteness which does not exist. We have attempted to avoid such generalizations in the hope that those readers who are not familiar with day-to-day advances in the field will not be misled into accepting them as proven facts.

2. CHARACTERISTICS OF C₄ PLANTS

2.1. TAXONOMY AND EVOLUTION

The C₄ pathway of photosynthesis has not been found in any plant groups lower than the angiosperms, though all plants are likely to have some dark β -carboxylation (i.e., β -carboxylation which does not require light). Among the angiosperms, C₄ photosynthesis has been found in at least 13 families (Downton, 1975). In the monocotyledons, two major subfamilies of the Gramineae and a large proportion of the Cyperaceae are C₄ plants. Among the dicotyledons, many members of the Amaranthaceae and Chenopodiaceae have the C₄ pathway, while a few C₄ species have been found in the Aizoaceae, Boraginaceae, Caryophyllaceae, Compositae, Convolvulaceae, Euphorbiaceae, Nyctaginaceae, Portulacaceae and Zygophyllaceae (Bender, 1971; Downton, 1975; Smith and Epstein, 1971).

The structural and biochemical features of C₄ plants arose polyphyletically, probably selected as advantageous for different reasons in different groups. In dry conditions the C₄ plants have the advantage of better efficiency of water use, especially in environments of high light intensity

(see Section 5.1). This selective advantage is particularly noticeable in the chenopods of arid zones and species which dominate monsoon grasslands, such as those in the Andropogoneae. In vegetation where the carbon dioxide concentration is reduced by photosynthetic activity, C₄ plants can photosynthesize faster in the low CO₂ concentrations and may thus compete effectively with C₃ plants in hot, wet environments, such as those on the periphery of rain forests. The grasses in the Paniceae are thought to have evolved in such conditions (Hartley, 1950, 1958). Apparently, arboreal groups have never been presented with environmental conditions where C₄ characteristics would be advantageous or else these groups have lost the genetic ability to develop the C₄ pathway.

The study of carbon pathways in plants is assisted by the fact that plants discriminate against certain of the naturally occurring isotopes of carbon in the atmosphere during photosynthesis. The reason for this discrimination is obscure, but it can be conveniently estimated by measuring the ratio of ¹³C and ¹²C in the tissue (Abelson and Hoering, 1961). The results are usually expressed as the difference in the ratio of ¹³C to ¹²C between the plant material and some standard source as a per mil of the ratio in the standard:

$$\delta^{13}\text{C}(\text{\%}) = \frac{{}^{13}\text{C}/{}^{12}\text{C sample} - {}^{13}\text{C}/{}^{12}\text{C standard}}{{}^{13}\text{C}/{}^{12}\text{C standard}} \times 1000$$

In C₄ plants, the discrimination results in the relative ¹⁴C content of the plant being slightly lower than that in the CO₂ of the surrounding atmosphere. This depletion is greater in C₃ plants (see Table 1). Discrimination against ¹³C can thus be used to indicate the pathway of photosynthesis occurring in a particular plant and will distinguish, for instance, cane sugar from beet sugar. The values obtained from aquatic plants may be affected by extraneous factors, such as the use of carbon substrates other than CO₂ or isotope discrimination in the environment around the plant.

Fossil plant material may be used to determine the pathway of photosynthesis existing at the time of its growth if care is taken to avoid measuring later carbon seepage. Coal from the Paleozoic era more than 200 million years ago has $\delta^{13}\text{C}$ values that indicate C₃ photosynthesis, thus suggesting that plants which evolved before the angiosperms used the C₃ pathway, as do lower plants which exist today. Similar results were found in coal formed as recently as the end of the Tertiary period, one million years ago (Troughton, 1971). Cave deposits of *Atriplex confertifolia* which are more than 40,000 years old indicated that these species used the C₄ pathway at that time (Troughton *et al.*, 1974).

TABLE 1. Some $\delta^{13}\text{C}$ % Values, Using PeeDee Belemnite Limestone As the Standard

Sample	$\delta^{13}\text{C}$ %	Reference
Atmosphere	-6.7	Craig and Keeling, 1973
C₃ plants		
<i>Bromus kalmii</i>	-30.3	Bender, 1971
<i>Triticum vulgare</i>	-28.5	Park and Epstein, 1961
<i>Lupinus perennis</i>	-28.5	Bender, 1971
<i>Ricinus communis</i>	-30.2	Bender, 1971
<i>Lycopersicum esculentum</i>	-25.2	Park and Epstein, 1961
C₄ plants		
<i>Cynodon dactylon</i>	-15.3	Bender, 1971
<i>Sporobolus poiretti</i>	-13.7	Bender, 1971
<i>Amaranthus retroflexus</i>	-13.3	Bender, 1971

Other studies of C₄ plants have been based on present flora. Balance and coordination among physiological components are essential for optimum expression of complex characters in both natural selection and artificial selection (Hageman *et al.*, 1967). Where interspecific crossing of C₄ and C₃ plants has been successful (*Atriplex rosea* x *Atriplex patula* ssp. *hastata*) (Björkman *et al.*, 1971) efficiency of photosynthesis is less than either parent in all F1 and F2 individuals. There seems little reason to expect species which have only partially "perfected" C₄ photosynthesis to remain among present-day flora, except in very unusual circumstances. One apparent halfway C₄-C₃ species has been described, *Mollugo verticillata* in the Aizoaceae (carpetweed family) (Kennedy and Laetsch, 1974). This family has several C₄ species as well as CAM and C₃ species and many biological variations are evident in *M. verticillata* itself.

Evolutionary specialization of C₄ features can be correlated with deviations from classic Kranz anatomy. In *Triodia* species (spinifex grasses of the arid inland of Australia) the extreme xeromorphy of the leaves is associated with a modification of the arrangement of bundle-sheath cells such that these stretch from one side of the leaf to the other instead of encircling the vascular bundle (Jacobs, 1971).

The phylogenetic origin of C₄ plants in Aizoaceae, Amaranthaceae, Compositae, Euphorbiaceae, Nyctaginaceae, Portulacaceae, and

Zygophyllaceae is unclear, and a detailed reappraisal of these families with respect to C₄ characteristics is necessary.

The Chenopodiaceae family has been studied in relation to C₄ characteristics, notably Kranz anatomy (Carolin *et al.*, 1975). It is an extensive family, with perhaps 100 genera and 1000 species. The boundaries of many of the taxa are ill-defined. "Aqueous" tissue consisting of large thin-walled cells almost totally filled by the vacuole is common in the leaves. In dorsiventral leaves the aqueous tissue occurs in layers under the epidermises outside the chlorenchyma; in concentric leaves it occurs within the ring of chlorenchyma. Carolin *et al.* (1975) suggest that the C₄ species of *Atriplex* (salt bushes) could be derived from the general dicotyledonous dorsiventral C₃ types in the family such as *Chenopodium* (goosefoots) and the C₃ *Atriplex* species, quite separately, from the fleshy C₄ *Kochia* *Bassia* complex (blue bushes, copper burrs). The Kranz cells of the *Atriplex* species have granal chloroplasts, while those in the other group have very few grana in the chloroplasts. This study suggests that taxonomic reappraisal is necessary, particularly in the subfamily Chenopodioideae which includes *Atriplex*, *Kochia*, and *Bassia*. Some reassessment of the migration and evolution of the family is also desirable. For example, it is noted that all the non-Kranz species in the *Atriplex-Obione* genera in the Southern Hemisphere appear to be recent arrivals in the area. This complex of species needs a phytogeographic reappraisal.

It would be desirable to complement such an investigation with studies of carbon metabolism and ecology. For example, some herbarium specimens of *Suaeda fruticosa* (an herb with fleshy leaves widely distributed in salty regions) have Kranz anatomy, others have not. The Kranz cells in a number of species in *Suaeda* surround not one vascular bundle but several bundles set in aqueous tissue, so these cells are not true bundle-sheath cells. The leaf structure of *Sympogma regelii* and at least one *Salsola* sp., both of which lack Kranz anatomy, are evidently reversions from close relatives which possessed Kranz anatomy. All three of these peculiarities would provide interesting material for metabolic study.

With regard to the Chenopodiaceae, it seems likely that integration of the existing knowledge of floral types, embryo types, fruit structures, leaf anatomy, leaf trichome structure, and cytology of the family would provide insights into the evolution of C₄ plants adapted to dry or saline conditions.

C₄ characteristics arose early in the evolution of the Gramineae, so generalizations are possible at subfamily level. Brown and Smith (1972) suggest that C₄ photosynthesis arose in the Mesozoic era before the separation of continents, about 200 million years ago. If this is the case, the C₄ grasses could have had little influence on the coal accumulated at the time.

Whatever the origin of the grasses, the Pooideae (Festucoideae) and Bambusoideae are made up entirely of C₃ plants, while some C₄ plants are found among the Panicoideae and Eragrostoideae (Downton and Tregunna, 1968; Johnson and Brown, 1973; Smith and Brown, 1973). Carolin *et al.* (1973) have carried out an analysis of bundle-sheath anatomy in the Gramineae. The presence of various forms of Kranz anatomy correlates with the mechanism of carbon transport between mesophyll and bundle-sheath cells (Gutierrez *et al.*, 1974a). However, the biochemical groupings of C₄ species do not always coincide with taxonomic groups based on leaf anatomy. Each anatomical group is either made up of species which predominantly make aspartate for transport or of species which make malate for transport. However, the NAD-malic enzyme group and PEP-carboxykinase group are found within the one tribe or even genus (e.g., *Bouteloua*, *Chloris*, and *Sporobolus*) (see Section 3.3).

In the Paniceae, there is difficulty in placing and delimitating the members of the vast genus *Panicum*. It contains C₃ species as well as all biochemical groups of C₄ plants. These difficulties provide better reasons for dismantling the genus than for doubting generalizations about the origin of C₄ photosynthesis in grasses.

Anatomical evidence suggests that C₄ plants are likely to be widespread in the Cyperaceae (Metcalfe, 1971). The bundle-sheath in many sedges is surrounded by a mestome sheath separating it from the mesophyll. As Laetsch (1971) suggested in relation to *Cyperus exulentus*, this structure would pose interesting problems of photosynthetic metabolism, though there is evidence that normal C₄ photosynthesis is operative (Chen *et al.*, 1974). Much of the family Cyperaceae remains to be explored. There are about 3000 species in the family. *Cyperus* itself has about 700 species with diverse chromosome numbers (Rath and Patnaik, 1974). Anatomical and metabolic studies of this family, particularly tropical species, should be rewarding.

2.2. Leaf Structure

The majority of biology texts present leaf anatomy in terms of a transverse section of a dorsiventral leaf. There is palisade mesophyll under the upper epidermis and spongy mesophyll beneath that inside the usually thinner, lower epidermis. The variations from this basic pattern which are presented tend to be extremes of xeromorphy, such as in the needle leaves of *Hakea* (an Australian xeromorphic bush) or the aerenchyma of underwater plants like *Ceratophyllum*. In fact, variation from the dorsiventral pattern is

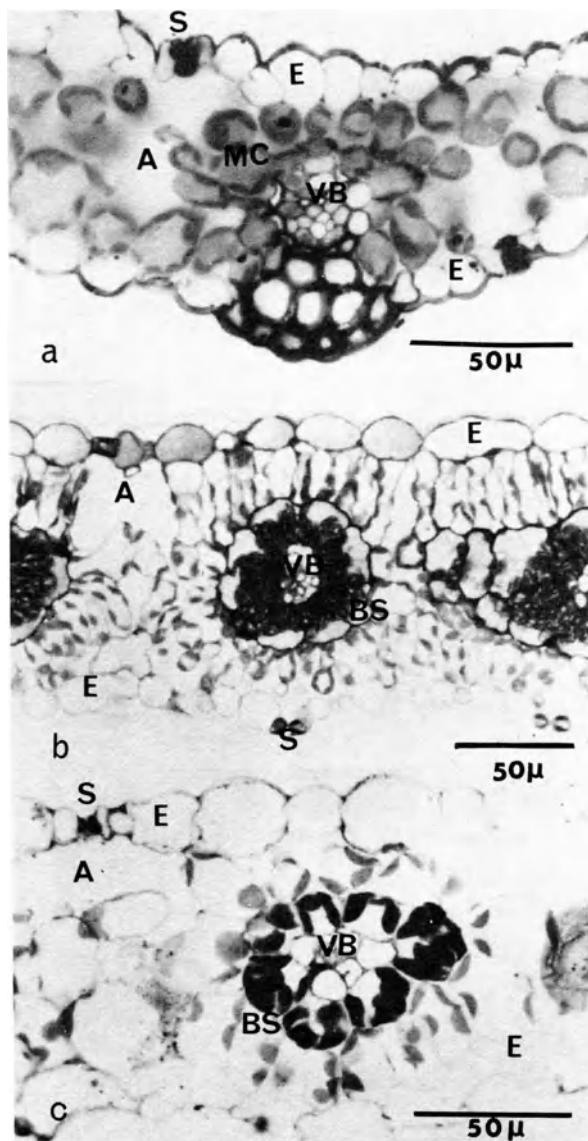


Fig. 1. Light micrographs of transverse sections of small veins of glutaraldehyde-fixed leaves. (a) *Phalaris tuberosa*, subfamily Pooideae, a C₃ grass showing the presence of very few chloroplasts in bundle-sheath cells. (b) *Amaranthus edulis*, family Amaranthaceae, a C₄ amaranth with numerous bundle-sheath chloroplasts located in a centripetal position. (c) *Sorghum bicolor*, subfamily Panicoideae, a C₄ grass with numerous bundle-sheath chloroplasts located centrifugally. Bar represents 50 μ m. Abbreviations: A, airspace; BS, bundle-sheath; E, epidermis; MC, mesophyll chlorenchyma; S, stomate; VB, vascular bundle.

more common than the rule and in many families can be used as a basis for phylogenetic grouping.

Whatever the anatomy of the leaf, the photosynthetic tissues have in common a high proportion of intercellular spaces and frequent small vascular bundles. These small bundles in mesophyll tissue usually are not in contact with intercellular spaces but are completely enclosed in a layer of packed parenchyma. This parenchymatous sheath itself shows variation in different species. Its cells may appear under the light microscope to be empty, very similar to the surrounding mesophyll (Fig. 1a) or with a noticeable concentration of chloroplasts (Figs. 1b,c). In some groups, the bundle-sheath may have more than one layer of cells.

Any of these variations in structure may appear in C₃ or C₄ plants. The distinctive feature of bundle-sheaths in leaves of C₄ plants is the different appearance of organelles in one layer of the sheath. The chloroplasts in this layer are noticeably different in number, size, and/or aggregation from those in the surrounding mesophyll tissue. It is in fresh leaf sections viewed with the light microscope that the description "Kranz" is so appropriate since the chloroplasts of the bundle-sheaths are very prominent.

A comparison of Figs. 1b and 1c shows that the chloroplasts in the bundle-sheaths of C₄ plants may be aggregated towards the center (centripetal position) or towards the periphery of the sheaths (centrifugal). In some species this distinction may not be apparent. Centripetal chloroplasts are common in the bundle-sheath cells of C₄ dicotyledons (Fig. 1b), and centrifugal chloroplasts are common in Panicoid grasses (Fig. 1c). Eragrostoid grasses may have chloroplasts in either position, though it seems that centripetal chloroplasts are found in those Eragrostoids in the NAD-malic enzyme group of C₄ plants, and centrifugal chloroplasts, in the PEP-carboxykinase group (Gutierrez *et al.*, 1974a) (Section 3.3).

Other variations in Kranz anatomy have been mentioned in Section 2.1. Some are associated with increased aqueous tissue in the leaf, others with the I girder of sclerenchyma commonly found in grasses. Chen *et al.* (1974) have studied the biochemistry of *Cyperus rotundus* (nutschell) which, like many sedges, has a distinct colorless sheath separating the green bundle-sheath from the mesophyll tissue. The extra cell layer apparently makes no difference to the enzymology of the cell types.

The presence of different chloroplasts within a leaf implies the existence of different functions. Even palisade mesophyll tissue has been shown to behave differently from spongy mesophyll in transporting photosynthetic products (Mokronosov *et al.*, 1973). Haberlandt (1914), in his classic text *Physiological Plant Anatomy*, suggested the possibility of a hitherto undiscovered division of labor between the chloroplasts in the

bundle-sheath and those in the "girdle cells," the concentric mesophyll cells of some species. This division of labor was not confirmed until late in the 1960s, when these species were shown to have the C₄ pathway of photosynthesis.

2.3. Cell Ultrastructure

A large number, but by no means all, of the C₄ plants are characterized by a dimorphism of their subcellular organelles. While this is most apparent in the chloroplast ultrastructure, variations can also be detected in mitochondria and microbodies. In most higher plants, the chloroplast contains three distinct membrane systems: the outer limiting membrane which is devoid of chlorophyll, an internal membrane system in which chlorophyll is located and which is composed of single lamellae (stroma lamellae), and stacks of lamellae (grana) (Fig. 2a). The first report that mesophyll and bundle-sheath chloroplasts of *Zea mays* (maize, corn) leaves possessed different lamellar structures was that of Hodge *et al.* (1955). These authors demonstrated that the structure of the mesophyll chloroplasts was similar to that of chloroplasts from C₃ plants in that both grana and stroma lamellae were present, whereas those of the bundle-sheath cells were essentially agranal. This initial observation was confirmed by a number of workers, although a few small grana are nearly always detectable in *Zea mays* bundle-sheath chloroplasts (Shumway and Weier, 1967; Rosado-Alberio *et al.*, 1968; Bishop *et al.*, 1971), and structural dimorphism has since been observed in chloroplasts from a wide variety of C₄ plants (Laetsch, 1971; Downton, 1971). Chloroplast dimorphism occurs in both monocotyledons and dicotyledons (Laetsch, 1971, 1974), and Figs. 2 and 3 show the chloroplast ultrastructure in two commonly studied C₄ plants. Both the mesophyll and bundle-sheath chloroplasts of *Amaranthus edulis* (edible or smooth amaranth) contain grana (Fig. 2). *Sorghum bicolor* (great millet, Guinea corn) is representative of the extreme forms of chloroplast structural dimorphism (Fig. 3), and its bundle-sheath chloroplasts are generally characterized as agranal.

It is rare, however, that bundle-sheath chloroplasts which are completely agranal are detected, and it is generally found that a small number of rudimentary grana can be observed even in the chloroplasts of sorghum (Fig. 3b) and *Saccharum officinarum* (sugar cane). The certainty implied by the use of the word "agranal" is to be regretted, and its substitution by a more appropriate word is to be encouraged. The word "paucigranal" (Latin *paucus*, few) will be employed in this article. It is also apparent that imma-

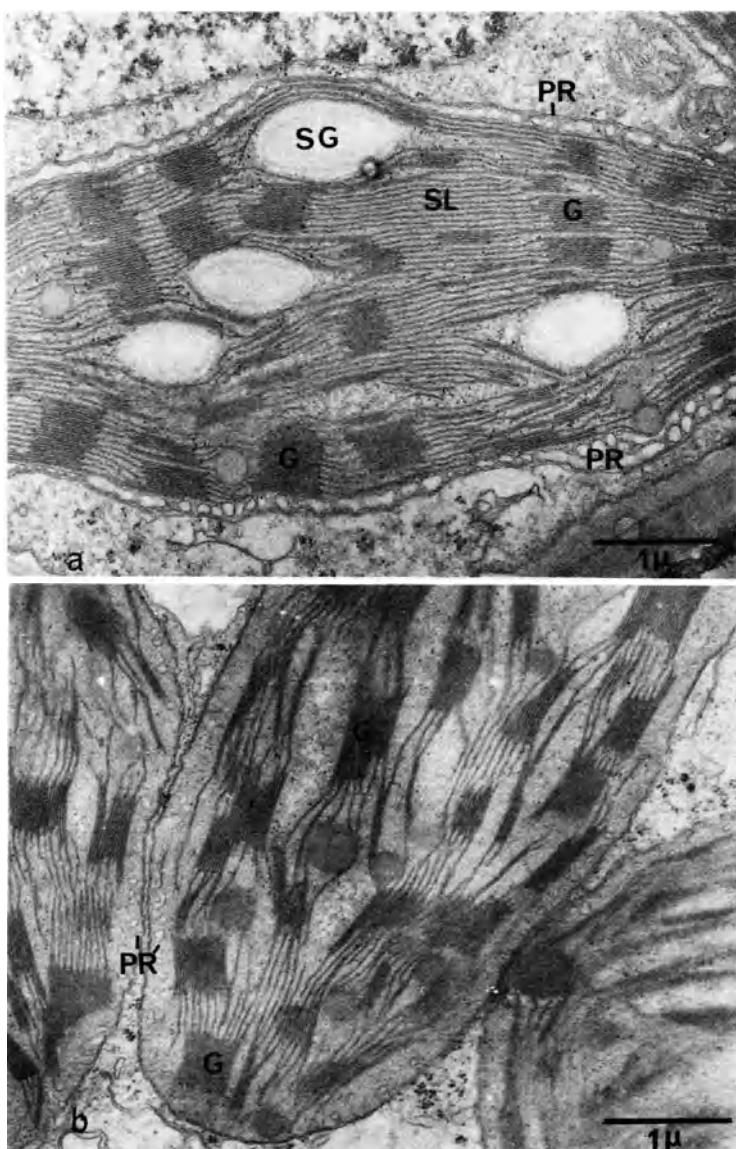


Fig. 2. Ultrastructure of the (a) mesophyll and (b) bundle-sheath chloroplasts of *Amaranthus edulis*. Bar represents 1 μ m. Abbreviations: G, grana; PR, peripheral reticulum; SG, starch granule; SL, stroma lamellae.

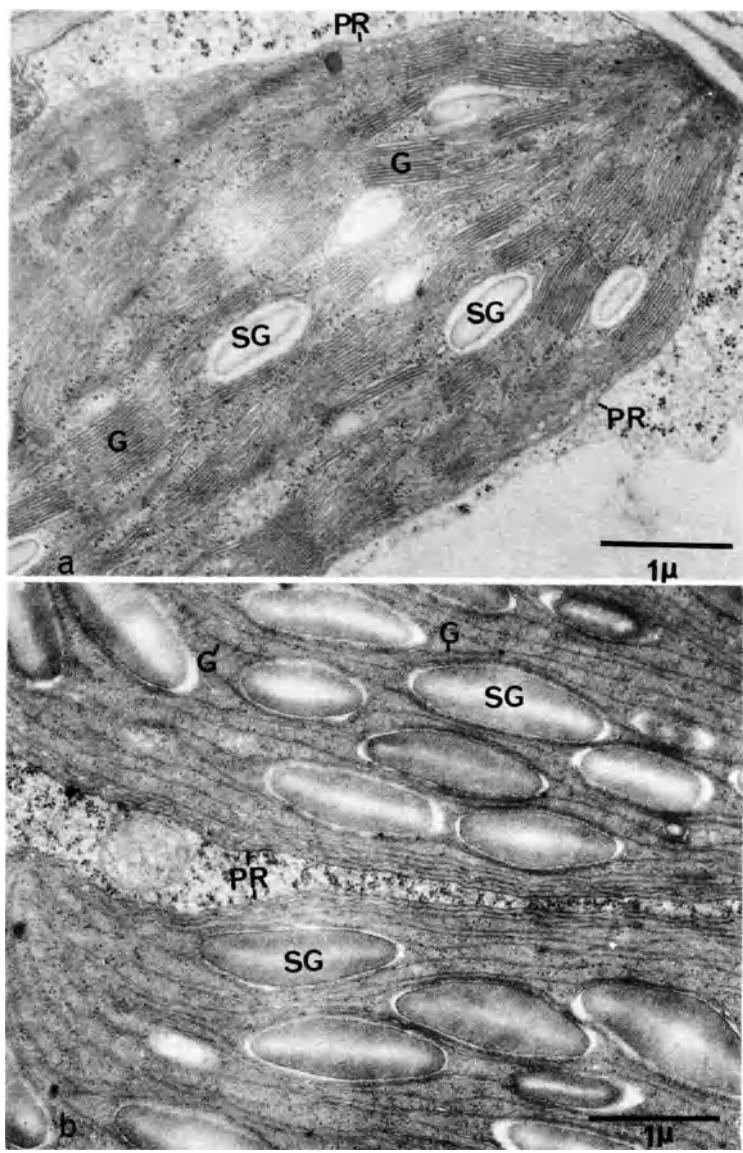


Fig. 3. Ultrastructure of the (a) mesophyll and (b) bundle-sheath chloroplasts of *Sorghum bicolor*. Bar represents 1 μm . Abbreviations as in Fig. 2.

ture bundle-sheath chloroplasts of *Zea mays*, *Saccharum officinarum*, and *Sorghum bicolor* do contain grana, and that the degree of lamellar appressions decreases during maturation (Laetsch and Price, 1969; Downton and Pyliotis, 1971; Andersen *et al.*, 1972). The factors governing this decrease have not been fully elucidated although it has been reported that both light and temperature can influence the degree of appressions. The degree of stacking in bundle-sheath chloroplasts of *Zea mays* and *Amaranthus lividus* (leaden amaranth) is reduced if the light intensity is increased (Brangeon, 1973; Lyttleton *et al.*, 1971). Changes in light intensity and growth temperature can markedly affect the lamellar structure of both mesophyll and bundle-sheath chloroplasts of *Sorghum* and *Paspalum dilatatum* (Dallas grass) (Taylor and Craig, 1971) and chloroplasts of the C₃ plant *Glycine max* (soybean) (Ballantine and Forde).

Chloroplast structural dimorphism does not occur in all C₄ plants, e.g., *Amaranthus edulis* (Fig. 2), but a characteristic feature of both mesophyll and bundle-sheath chloroplasts of C₄ plants is the peripheral reticulum, a network of membranes which is present in both the mesophyll and bundle-sheath chloroplasts (Laetsch, 1971, 1974), (Figs. 2, 3) but whose existence in C₃ plants is still disputed (Laetsch, 1974). The function of this membrane complex is as yet unknown, but its structure is sensitive to changes in light intensity (Lyttleton *et al.*, 1971), and its presence may also be related to the age of the leaf (Laetsch, 1971; Chapman *et al.*, 1975). However, its isolation as a pure membrane preparation, which is probably a requirement for the elucidation of its function, will be a formidable undertaking.

The structural dimorphism of mitochondria is particularly evident in those species which transport newly fixed carbon from mesophyll to bundle-sheath cells primarily as aspartate (Hatch, 1971; Chapman *et al.*, 1975) (Section 3.3). The bundle-sheath mitochondria of *Amaranthus edulis* as shown in Fig. 4 are significantly larger in size and contain more cristae than those in the adjacent mesophyll cells, while there is little difference in size between the mesophyll and bundle-sheath mitochondria of *Zea mays* and *Sorghum bicolor* (Chapman *et al.*, 1975) in which malate is the primary intercellular transporter of carbon. Mitochondria can readily be distinguished from microbodies and peripheral reticulum by their capacity to reduce diaminobenzidine in the dark, a reaction indicative of cytochrome *c* oxidase activity (EC.1.9.3.1) (Fig. 5) (Chapman *et al.*, 1975).

Microbodies occur in both mesophyll and bundle-sheath cells of C₄ plants. They are characterized by the presence of a single membrane and lack internal structure (Fig. 5) and are the intracellular sites of the enzymes of the glycolate pathway (Tolbert, 1971a). It is generally also found that there are more organelles in bundle-sheath cells than in mesophyll cells

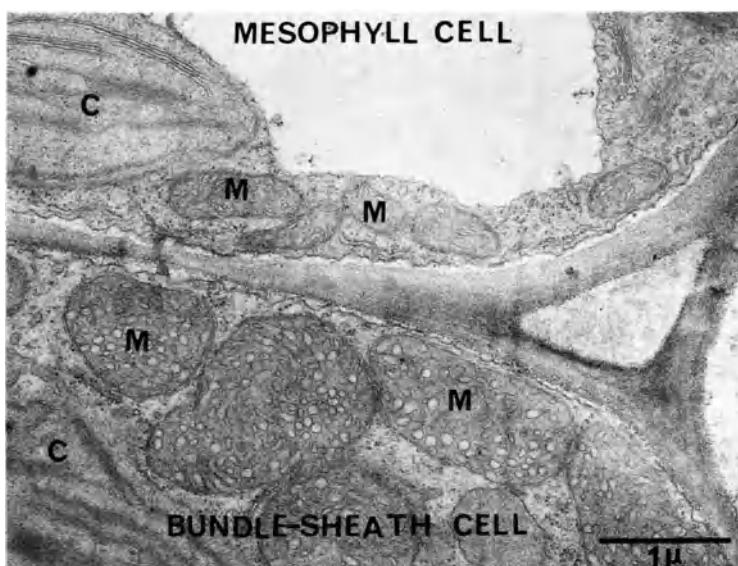


Fig. 4. Size dimorphism of the mitochondria in mesophyll and bundle-sheath cells of *Amaranthus edulis*. Reproduced from Chapman *et al.* (1975) with permission. Bar represents 1 μ m. Abbreviations: C, chloroplast; M, mitochondrion.

(Frederick and Newcomb, 1971; Newcomb and Frederick, 1971; Laetsch, 1971; Hilliard *et al.*, 1971; Liu and Black, 1972), although as Laetsch (1974) has pointed out, the total number of mesophyll cells in a leaf is severalfold greater than that of the bundle-sheath cells and hence, overall, this discrepancy in numbers may not be so significant. A biochemical basis for the structural variations in organelles of C_4 plants is being actively investigated, and evidence will be presented in Sections 3 and 4.

3. BIOCHEMISTRY OF PHOTOSYNTHESIS

3.1. Photosynthetic Electron Transfer

The fixation of carbon dioxide by photosynthetic tissue involves two series of reactions. The first is a light-dependent process, which produces energy stored as ATP and reducing power stored as NADPH, while the second is a series of non-light-requiring reactions which fix carbon into organic compounds and in the process utilize the products of the light-de-

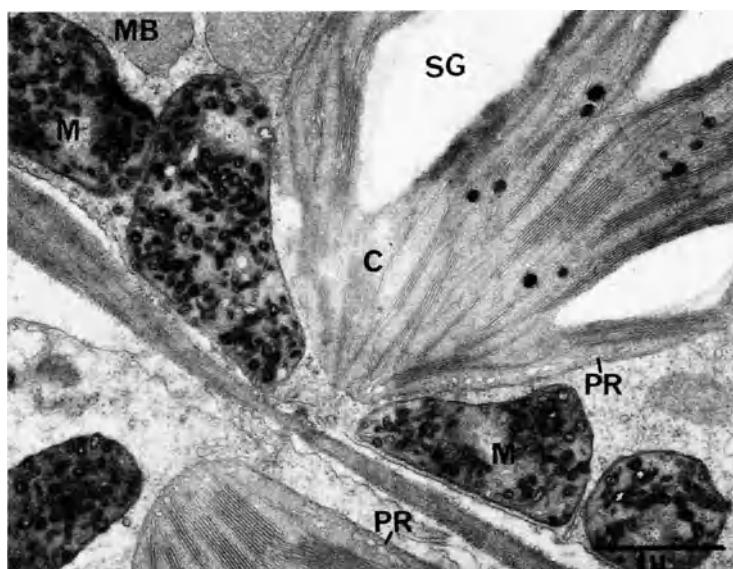


Fig. 5. Cellular organelles in a leaf section of *Amaranthus edulis* treated in the dark with diaminobenzidine at pH 7.5. Only the mitochondria have reduced the dye. Bar represents 1 μ m. Abbreviations: C, chloroplast; M, mitochondrion; MB, microbody; PR, peripheral reticulum; SG, starch granule.

pendent reactions. The light-requiring process, known as photosynthetic electron transfer, is discussed below, while the reactions involved in carbon fixation are described in Section 3.3.

Current concepts of photosynthetic electron transfer in higher plants are almost entirely derived from studies with C₃ plants, but the same basic pathway appears to operate in chloroplasts of C₄ plants. Light-harvesting occurs in the grana and stroma lamellae of the chloroplast, in which chlorophyll is situated. The scheme outlined in Fig. 6 is generally referred to as the Z scheme and is based on the existence of two light-harvesting assemblies which operate in series and are connected by an electron transfer complex (Boardman, 1970, 1971; Bishop, 1971; Avron, 1971). Though there has been little progress in understanding the distribution of the components of the assembly (Trebst, 1974), a large amount of knowledge has accumulated as to their function. The overall complex carries out the transfer of an electron derived from the photochemical splitting of water to ferredoxin, which is further oxidized by ferredoxin-NADP⁺ reductase (EC.1.6.7.1), yielding NADPH. The NADPH serves as a store of reducing power for other chloroplast-located reactions, especially those involved in the fixation

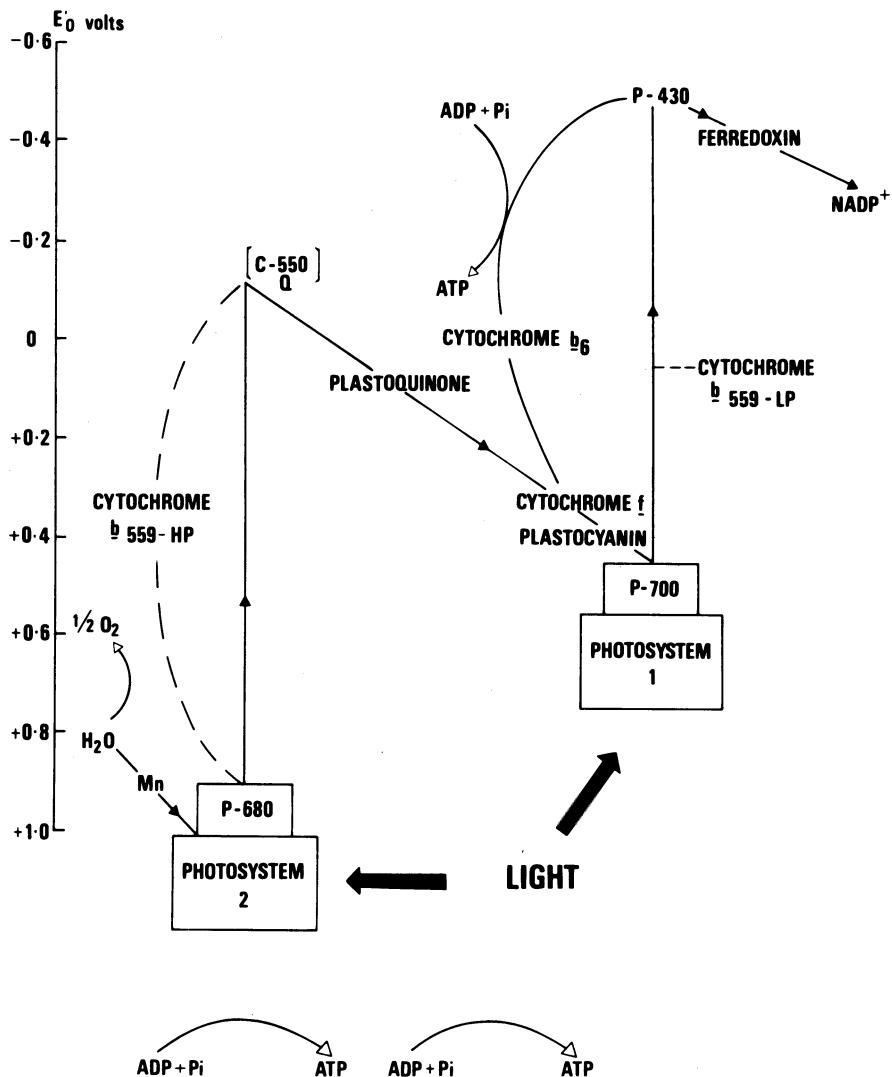


Fig. 6. Outline of the Z scheme of photosynthetic electron transfer.

of carbon dioxide. The two light-harvesting assemblies, designated as photosystems 1 and 2, are thought to consist of a single pigment molecule, designated as the *photochemical reaction center*, around which are clustered a number of additional chlorophyll and carotenoid molecules which serve as light-harvesting antennae and transfer their energy to the photochemical reaction center. In the case of photosystem 1, the photochemical reaction center is a specialized form of chlorophyll *a*, known as P-700, because of its absorption at the wavelength of 700 nm (Ke, 1973). The reaction center of photosystem 2 is thought to be P-680, a form of chlorophyll *a* absorbing at 682 nm, but it is less well-defined than the reaction center of photosystem 1 (Amesz, 1973; Ke *et al.*, 1974). Photosystem 2 absorbs light efficiently only at wavelengths below 700 nm, while photosystem 1 continues to operate efficiently at wavelengths greater than 700 nm. On the basis that there is one P-700 molecule for every 430 chlorophyll molecules in most higher plant chloroplasts, it is suggested that there are about 200 molecules of chlorophyll clustered around each photochemical reaction center of photosystems 1 and 2.

The electron transfer chain connecting the two photosystems contains a number of components. In higher plants, the primary electron donor is water. The absorption of light by the photochemical reaction center of photosystem 2 leads to the reduction of Q, which may be the primary electron acceptor of photosystem 2, and electron flow from water results in the reoxidation of a primary electron donor. It is not yet clear whether Q, which is identified by its ability to quench fluorescence emanating from photosystem 2, is the primary electron acceptor of photosystem 2 or how it is related to C-550, a component identified by Knaff and Arnon, which undergoes a light-induced absorbance change at 550 nm (Knaff and Arnon, 1969; Bendall and Sofrová, 1971). It is clear, however, that both components are close to the reaction center of photosystem 2 (Wessels *et al.*, 1973; Erixon and Butler, 1971). Evidence has been presented that the primary electron acceptor is a bound plastoquinone molecule which is reduced to its semiquinone anion (Witt, 1973; Van Gorkom, 1974) and β -carotene has been implicated in the primary photochemical reaction of photosystem 2 (Okayama and Butler, 1972; Cox and Bendall, 1974).

Electrons from Q are transferred by a number of electron carriers, including plastoquinone, cytochrome *f*, and plastocyanin, a copper-containing protein, to P-700, which is the primary electron donor of photosystem 1 (Fig. 6). Excitation of P-700 leads to the reduction of the primary electron acceptor of photosystem 1, which has been proposed as P-430, a bound form of a ferredoxin-type molecule, identifiable spectrophotometrically (Ke, 1973; Ke and Beinert, 1973; Malkin *et al.*, 1974). The final electron acceptor is the nonheme iron protein ferredoxin, which is subsequently reoxidized

by the enzyme ferredoxin-NADP⁺ reductase, with the formation of NADPH.

The position of a number of the components of the photosynthetic electron transfer chain remains open to question. Plastoquinone is located between the two photosystems (Amesz, 1973), while cytochrome *f* and plastocyanin are located close to photosystem 1, but on the oxidizing side of the photochemical reaction center. The location of these two components with reference to each other is not completely established, although most workers place plastocyanin after cytochrome *f* as, indicated in Fig. 6 (Bishop, 1974). Cytochrome *b*₆ also appears to be associated with photosystem 1 (Boardman, 1971; Plesnicar and Bendall, 1972). Two forms of cytochrome *b*₅₅₉ have been identified, a low-potential form (cytochrome *b*_{559-LP} E_m 70–110 mV) which appears to be located in photosystem 1 (Anderson and Boardman, 1973; Knaff and Malkin, 1973; Plesnicar and Bendall, 1973) and a high-potential form (cytochrome *b*_{559-HP} E_m + 370 mV), which is associated with photosystem 2 (Boardman, 1971; Boardman *et al.*, 1971; Plesnicar and Bendall, 1973). The high-potential form probably lies on a side path or cycle around photosystem 2 and not on the direct path of electron transfer (Boardman, 1971; Boardman *et al.*, 1971; Cox and Bendall, 1972; Hiller *et al.*, 1971; Henningsen and Boardman, 1973; Ben-Hayyim, 1974; Phung Nhu Hung, 1974). Cytochromes *f*, *b*₆, and *b*_{559-LP} can be detected in etiolated leaves (Anderson and Boardman, 1973; Plesnicar and Bendall, 1972), but cytochrome *b*_{559-HP} is only detected after several hours illumination of etiolated leaves (Plesnicar and Bendall, 1972, 1973; Henningsen and Boardman, 1973).

The use of artificial electron donors and acceptors has greatly assisted our knowledge of photosynthetic electron transfer, but some confusion has arisen over the site at which electron donation or acceptance occurs. This is particularly true with the commonly used electron acceptors for photosystem 2, FeCN, and DCIP, which have been shown to accept electrons at more than one site in the electron transfer chain. In chloroplasts possessing an intact electron transfer chain, these two substrates are primarily reduced on the reducing side of photosystem 1. However, if electron flow is inhibited between the two photosystems by compounds such as DBMIB (Böhme *et al.*, 1971), amphotericin B (Bishop, 1973; Nolan and Bishop, 1975), histone (Brand *et al.*, 1972), or potassium cyanide (Ort *et al.*, 1973; Ouitrakul and Izawa, 1973), the site of reduction is shifted to photosystem 2. The use of artificial donors and acceptors has been recently reviewed by Trebst (1972, 1974), Bishop (1974), and Izawa and Good (1972).

Another important approach to a study of photosynthetic electron transfer is by the preparation of subchloroplast particles, which are capable

of partial reactions of electron flow. Two techniques have been widely employed: fractionation of chloroplast membranes with detergents, such as digitonin or Triton X-100 (Boardman, 1970; Park and Sane, 1971; Vernon *et al.*, 1971) and fragmentation by sonication or passage through a French pressure cell (Michel and Michel-Wolwertz, 1970; Sane *et al.*, 1970; Park and Sane, 1971). Differential centrifugation of chloroplasts treated by either of these techniques yields two major fractions with differing photochemical properties. The first, which sediments at about 10,000g, contains both photosystem 1 and photosystem 2 activity, while the second, sedimenting at about 100,000g, contains only photosystem 1 activity. The heavier fractions, prepared by treatment of chloroplasts in the French press, appear to be derived from grana, while the lighter fractions which contain only photosystem 1 activity are apparently stroma lamellae (Sane *et al.*, 1970; Goodchild and Park, 1971). After digitonin treatment of chloroplasts, the smaller fractions probably contain not only stroma lamellae but also some photosystem 1 activity derived from grana, as it has been found that digitonin treatment of grana stacks prepared with the French press will release photosystem 1 activity (Arntzen *et al.*, 1972). Preparations containing only photosystem 2 activity can be prepared by further treatment of the grana fraction (Huzisige *et al.*, 1969; Arntzen *et al.*, 1972; Boardman, 1972; Wessels *et al.*, 1973), and under some conditions, recombination of photosystem 1 and photosystem 2 fractions can lead to reconstitution of photosynthetic electron transfer activity (Arntzen *et al.*, 1972; Huzisige *et al.*, 1969; Ke and Shaw, 1972).

Associated with the reactions of photosynthetic electron transfer is the phosphorylation of ADP to yield ATP. Two types of photophosphorylation have been identified, cyclic and noncyclic. Noncyclic phosphorylation is associated with electron flow from water to NADP⁺, and there is evidence that two phosphorylation sites are involved (Izawa *et al.*, 1973; Ouitrakul and Izawa, 1973; Reeves and Hall, 1973; West and Wiskich, 1973). One site of phosphorylation is located between plastoquinone and cytochrome *f* (Fig. 6), but a second site has been identified which is thought to be close to the reaction center of photosystem 2. Identification of this second site has been greatly aided by the use of inhibitors such as DBMIB and potassium cyanide (Izawa *et al.*, 1973; Ouitrakul and Izawa, 1973; Trebst and Reimer, 1973).

Cyclic phosphorylation is a reaction involving only photosystem 1 and is observed in isolated chloroplasts after the addition of certain cofactors, such as phenazine methosulphate, diaminodurene, and menadione. Cyclic phosphorylation is not inhibited by DCMU, but there appears to be more than one position at which electrons can cycle back into the chain, depending upon the cofactor used (Trebst, 1974). For example, cyclic phosphoryla-

tion in the presence of diaminodurene appears to require plastocyanin, as shown in Fig. 6, but a cycle of electron flow via plastoquinone or P-700 has also been suggested (Trebst, 1974). Cytochrome b_6 also appears to be involved in cyclic phosphorylation (Böhme and Cramer, 1972).

3.2. Electron Transfer in C₄ Plants

The diverse structures of the chloroplasts of C₄ plants is accompanied by variations in photochemical capacity and composition of the chloroplast lamellae (Bishop, 1974). As indicated in Section 2.3, this diversity is most marked in the bundle-sheath chloroplasts whose lamellar structure may contain well-developed grana [e.g., *Amaranthus edulis* (Fig. 2b)] or be paucigranal [e.g., *Sorghum bicolor* (Fig. 3b)]. Techniques have been developed for separating mesophyll and bundle-sheath cells, protoplasts and chloroplasts (Woo *et al.*, 1970; Anderson *et al.*, 1971a; Edwards and Black, 1971a; Kanai and Edwards, 1973a,b), and these have provided much information concerning the photochemical processes involved.

There is excellent evidence that on a chlorophyll basis, paucigranal bundle-sheath chloroplasts have a lower photosystem 2 activity than the mesophyll chloroplasts from the same leaf, and in most cases, a higher photosystem 1 activity (Table 2) (Downton *et al.*, 1970; Woo *et al.*, 1970; Anderson *et al.*, 1971a,c; Arntzen *et al.*, 1971; Bishop *et al.*, 1971; Downton and Pyliotis, 1971; French and Berry, 1971; Mayne *et al.*, 1971; Andersen *et al.*, 1972; Smillie *et al.*, 1972b; Bazzaz and Govindjee, 1973; Ku *et al.*, 1974b). This is exemplified by *Zea mays*, one of the most widely studied C₄ plants containing paucigranal bundle-sheath chloroplasts, in which photosystem 2 activity, measured with a number of substrates, either lipophilic or hydrophilic, is substantially lower in the bundle-sheath chloroplasts than in the mesophyll chloroplasts (Table 2). Bundle-sheath chloroplasts which possess well-developed grana generally have a photosystem 2 activity comparable to that of the mesophyll cells (Table 2). This correlation is not absolute, since bundle-sheath chloroplasts of *Eleusine indica* (crowsfoot grass), which are granal, have less than 25% of the photosynthetic electron transfer activity of mesophyll chloroplasts measured with benzoquinone, DCIP, or ferricyanide (Ku *et al.*, 1974b). These variations in the capacity for photosynthetic electron transfer can also be correlated with other properties of the chloroplast. In many cases the properties of the paucigranal bundle-sheath chloroplasts are similar to those of a photosystem 1 particle derived from a granal chloroplast (Bishop, 1974). Characteristic features of paucigranal bundle-sheath chloroplasts and of photosystem 1 particles include a high chlorophyll $a:b$ ratio and a high P-700:chlorophyll

ratio by comparison with granal chloroplasts (Anderson *et al.*, 1971c; Black and Mayne, 1970; Woo *et al.*, 1971; Holden, 1973). The fluorescence emission properties of the paucigranal bundle-sheath chloroplasts of *Sorghum bicolor* are similar to those of a photosystem 1 particle prepared from spinach chloroplasts in that little fluorescence derived from photosystem 2 can be detected, while the fluorescence properties of the granal mesophyll chloroplast of *Sorghum bicolor* resemble those of whole spinach chloroplasts (Anderson *et al.*, 1971b). The yield of variable fluorescence, with respect to constant fluorescence, by chlorophyll *a* from *Zea mays* bundle-sheath chloroplasts is less than half that of the mesophyll chloroplasts (Bazzaz and Govindjee, 1973). Another characteristic attributed to photosystem 2 is the emission of delayed light, and in *Digitaria sanguinalis* the amount of delayed light emitted by mesophyll cells is more than twice that emitted by bundle-sheath cells (Mayne *et al.*, 1971). The paucigranal bundle-sheath chloroplasts of *Sorghum bicolor* are deficient in cytochrome *b*_{559-HP} (Anderson *et al.*, 1971c) and C-550 (Anderson *et al.*, 1972), both of which are located in photosystem 2 and have a carotenoid composition which is quite different from that of the granal mesophyll chloroplasts (Anderson *et al.*, 1971c). Paucigranal bundle-sheath chloroplast membranes from *Zea mays* also are deficient in certain polypeptides which are present in photosystem 2 enriched fractions of mesophyll chloroplasts, and it has been suggested that these polypeptides may be essential for the assembly of grana (Anderson and Levine, 1974).

Although the above evidence indicates quite strongly that paucigranal bundle-sheath chloroplasts have lower levels of photosystem 2 activity than the corresponding mesophyll chloroplasts, some of the data, particularly those relating to photosynthetic electron transfer (Table 2) must be interpreted with caution. There is good evidence, based on the use of inhibitors such as DBMIB (Böhme *et al.*, 1971), potassium cyanide (Ort *et al.*, 1973; Oitrakul and Izawa, 1973), and amphotericin B (Nolan and Bishop, 1975), that the reduction from water of substrates such as FeCN, DCIP, and benzoquinones involves both photosystems 1 and 2 in chloroplasts containing grana. If, however, the flow of electrons between the two photosystems is blocked, these substrates can accept electrons from photosystem 2 alone. However, in paucigranal bundle-sheath chloroplasts of *Zea mays*, the reduction of ferricyanide appears to involve only photosystem 2, as judged by its insensitivity to amphotericin B (Nolan and Bishop, 1975), its activity curve with respect to pH (Bishop, *et al.*, 1972), and the effect of DBMIB (Bishop and Nolan, 1975). A comparison of the photosynthetic electron transfer activity of mesophyll and bundle-sheath chloroplasts of *Zea mays* is therefore not valid if based on a simple measurement of FeCN reduction. The comparison of FeCN-dependent

TABLE 2. Photochemical Activity of Chloroplasts from C₄ Plants

Chloroplast structure	Photosystem 2		Photosystem 1		Reference	
	Substrate	Activity ^a	Substrate	Activity ^a		
<i>Zea mays</i> Mesophyll Bundle-sheath	Granal Paucigranal	NADP	192 48	NADP	108 384	Bishop <i>et al.</i> , 1972
<i>Zea mays</i> Mesophyll Bundle-sheath	Granal Paucigranal	Benzozquinone	204 3			Ku <i>et al.</i> , 1974b
<i>Zea mays</i> Mesophyll Bundle-sheath	Granal Paucigranal	FeCN	635 142			Nolan and Bishop, 1975
<i>Zea mays</i> Mesophyll Bundle-sheath	Granal Paucigranal	MeV	140 56	MeV	241 778	Bazzaz and Govindjee, 1973

<i>Sorghum bicolor</i>	Granal Paucigranal	DCIP	271 39	NADP	180 426	Arntzen <i>et al.</i> , 1971
Mesophyll Bundle-sheath						
<i>Sorghum bicolor</i>	Granal Paucigranal	NADP	159 0	NADP	38 43	Woo <i>et al.</i> , 1970
Mesophyll Bundle-sheath						
<i>Saccharum officinarum</i>	Granal Paucigranal	Benzoquinone	148 1			Ku <i>et al.</i> , 1974b
Mesophyll Bundle-sheath						
<i>Atriplex spongiosa</i>	Granal Granal	NADP	150 137			Woo <i>et al.</i> , 1970
Mesophyll Bundle-sheath						
<i>Panicum capillare</i>	Granal Granal	Benzoquinone	169 182			Ku <i>et al.</i> , 1974b
Mesophyll Bundle-sheath						

^a The units of activity used vary according to the assay and are not shown in the table. The significance of the data lies in the relative activities of mesophyll and bundle-sheath chloroplasts.

photophosphorylation rates by *Zea mays* mesophyll and bundle-sheath chloroplasts (Anderson *et al.*, 1971a; Polya and Osmond, 1972) may also be in error for similar reasons. Similarly in this laboratory we have found that the photoreduction of NADP⁺ by paucigranal bundle-sheath chloroplasts of *Zea mays* is dependent upon the addition of plastocyanin and ferredoxin-NADP⁺ reductase, while NADP⁺ photoreduction by *Zea mays* mesophyll chloroplasts is only slightly stimulated by these cofactors (Bishop *et al.*, 1972). As mentioned in Section 4.2, one of the characteristics of plants possessing the C₄ pathway is a high light intensity requirement for maximum rates of photosynthesis. Studies with the C₃ plants *Atriplex patula* (spreading orache, fat hen saltbush) (Björkman *et al.*, 1972) and *Sinapis alba* (white mustard) (Wild *et al.*, 1973) have shown that the light intensity during growth can markedly affect the photochemical properties of the plant. The capacity of isolated chloroplasts of *Atriplex patula* to photoreduce DCIP from water varied directly with the light intensity during growth, with respect to both the maximum rate of reduction and the light intensity necessary for saturation of the reaction (Björkman *et al.*, 1972). In contrast, growth of *Zea mays* plants under high- and low-light conditions produced little or no marked changes in photosynthetic capacity or pigment composition of the intact leaf (Wild and Müllenbeck, 1973). However, a difference in the light requirement of isolated *Zea mays* mesophyll and bundle-sheath chloroplasts has been demonstrated. The photoreduction of FeCN, 2,4,6-trichlorophenolindophenol, and NADP⁺ reaches saturation at lower light intensities in mesophyll chloroplasts than in bundle-sheath chloroplasts (Anderson *et al.*, 1971a; Smillie *et al.*, 1972a). Similarly, the mesophyll chloroplasts of a *Zea mays* mutant which were deficient in grana required a higher light for maximum rates of photosynthetic electron transfer than did chloroplasts isolated from a normal plant (Bazzaz *et al.*, 1974). This area is one in which further study would be rewarding, particularly in the case of those plants with granal bundle-sheath chloroplasts.

3.3. Photosynthetic Carbon Metabolism in C₄ Plants

The photosynthetic pathway of carbon fixation, elucidated by Calvin and co-workers, was one in which the initial detectable products of fixation were phosphorylated three-carbon compounds, and the initial fixation step was the carboxylation of RuDP by CO₂ under the influence of the enzyme ribulosediphosphate carboxylase (RuDP carboxylase, EC.4.1.1.39). Reactions leading to the formation of sucrose and starch, which are the major end products of this process, and those involved in the regeneration of RuDP are shown in Fig. 7. One turn of the cycle requires three molecules of ATP and two molecules of NADPH, which are provided by the reactions of

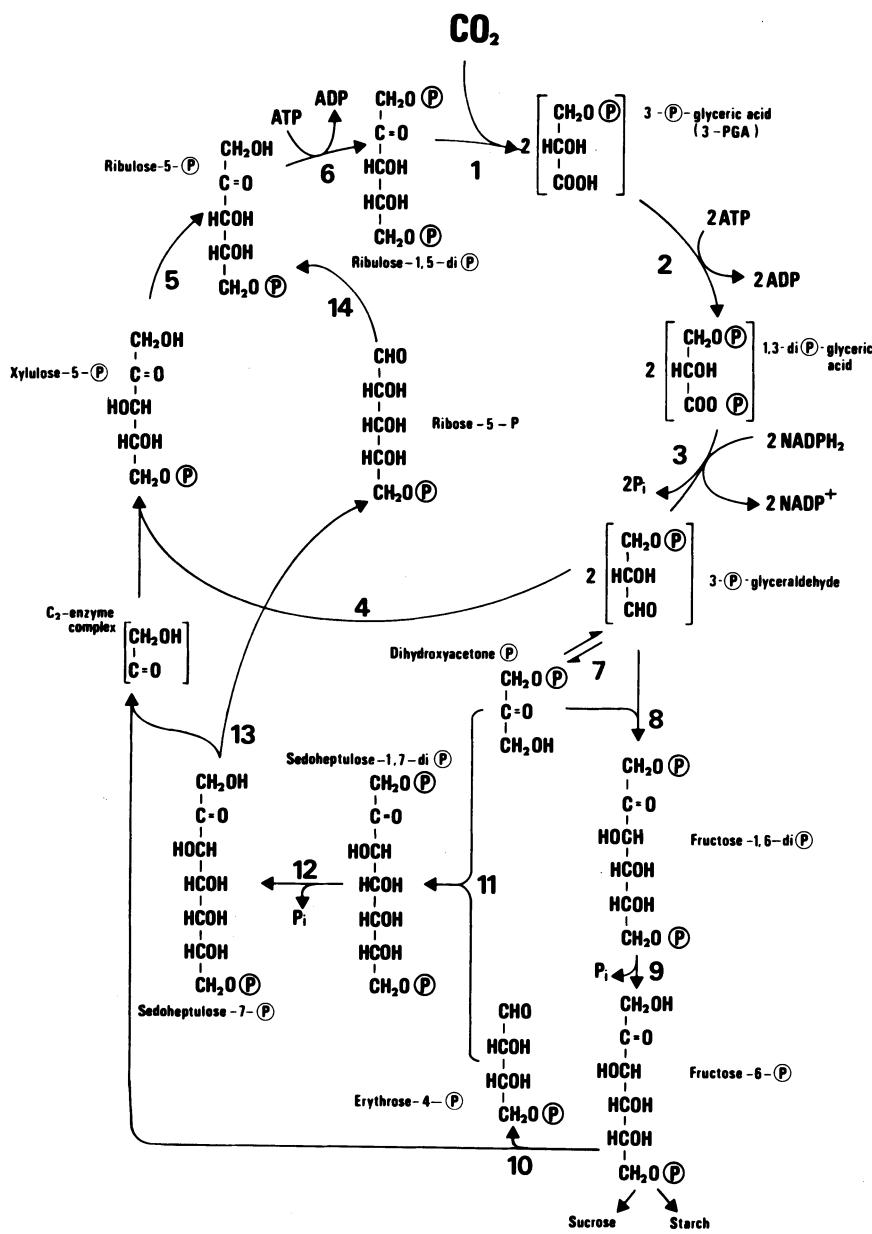


Fig. 7. Photosynthetic carbon fixation by the Calvin cycle. The enzymes catalyzing the individual reactions are: 1, ribulosediphosphate carboxylase (EC.4.1.1.39); 2, 3-phosphoglyceric acid kinase (EC.2.7.2.3); 3, glyceraldehyde phosphate dehydrogenase (NADP⁺) (phosphorylating) (EC.1.2.1.13); 4, a transketolase; 5, ribulosephosphate 3-epimerase (EC.5.1.3.1); 6, ribulose-5-phosphate kinase; 7, triosephosphate isomerase (EC.5.3.1.1); 8, fructoserediphosphate aldolase (EC.4.1.2.13); 9, hexosediphosphatase (EC.3.1.3.11); 10, a transketolase; 11, sedoheptulosediphosphate aldolase; 12, sedoheptulosediphosphatase; 13, a transketolase; 14, ribulosephosphate isomerase (EC.5.3.1.6).

photosynthetic electron transfer. There are numerous reviews of the Calvin cycle (Bassham, 1971; Black, 1973; Heber, 1974; Hatch, 1975), and the individual reactions will not be considered in detail here.

Unlike the reactions of photosynthetic electron transfer which occur in the chloroplast lamellae, photosynthetic CO₂ fixation occurs in the soluble portion (stroma) of the chloroplast. Much interest has been directed towards the enzyme RuDP carboxylase, which can account for as much as half of the soluble protein of leaves (Akazawa, 1970). Although the first readily detectable product of its activity is 3-PGA, there is evidence for a bound six-carbon intermediate (Siegel and Lane, 1973). RuDP carboxylase is a large protein (MW, 0.5×10^6), consisting of a number of subunits, probably eight large and eight small (McFadden and Tabita, 1974). Evidence has been presented that the large subunits are synthesized on chloroplast ribosomes under the direction of chloroplast DNA, while the smaller subunits are synthesized in the cytoplasm under the direction of nuclear DNA (Gooding *et al.*, 1973; Wildman *et al.*, 1973). The enzyme has been considered an inefficient catalyst since its K_m CO₂ *in vitro* is 0.2 to 0.5 mM (Black, 1973), and this belief has been used to account for its high concentration in leaves. Recently however, an *in vivo* form of the enzyme with a K_m CO₂ of about 0.015 mM has been detected in spinach leaves (Bahr and Jensen, 1974). RuDP carboxylase is also capable of synthesizing phosphoglycolate and 3-PGA from RuDP by using O₂ instead of CO₂, and this reaction will be considered in relation to photorespiration (Section 3.5).

The first observations that the initial products of CO₂ fixation in sugar cane and maize were not phosphorylated three-carbon compounds but were four-carbon dicarboxylic acids was made independently by Kortschak and co-workers in Hawaii and by Karpilov in the U.S.S.R. These observations began the biochemical studies which have since linked together the structural and physiological characteristics of those plants now known to possess the C₄ pathway of photosynthesis. In *Saccharum officinarum*, the first stable products in which radioactive CO₂ appeared were malate and aspartate, and only after a lag period was label detected in 3-PGA (Kortschak *et al.*, 1965; Hatch, 1971). Subsequent work in a number of laboratories has confirmed these observations, and a metabolic pathway to explain the results was proposed by Hatch and Slack (1970*a,b*). It is now generally referred to as the C₄ pathway of photosynthesis, and its occurrence has been demonstrated in a wide variety of higher plants (Downton, 1975).

It is important to recognize at the outset that the C₄ pathway is only a modification of the C₃ pathway, probably of recent evolution, and dependent upon the specialized structure of C₄ plants. The effectiveness of the pathway relies on the separation of specific biochemical functions be-

tween the mesophyll and bundle-sheath cells and results in cooperative photosynthesis.

The initial fixation of CO₂ takes place in the mesophyll cells with PEP as the acceptor and produces oxaloacetate, which is rapidly converted to malate or aspartate, with different proportions of these compounds being formed in various plants (Fig. 8). Aspartate is formed from oxaloacetate in the cytoplasm of the mesophyll cell by aspartate aminotransferase (EC.2.6.1.1), with glutamate as donor, while malate is formed by the reduction of oxaloacetate in the mesophyll chloroplast (Hatch and Slack, 1968; Slack *et al.*, 1969; Hatch and Kagawa, 1973). The NADPH for reduction of oxaloacetate can be derived from photosynthetic electron transfer in the mesophyll chloroplast, with oxaloacetate or PEP + bicarbonate acting as the Hill acceptor (Salin *et al.*, 1973, Kagawa and Hatch, 1974a).

The CO₂ thus fixed is transported as aspartate or malate to the bundle-sheath cells, where it is released by decarboxylation. Subsequently it is refixed by RuDP carboxylase in a series of reactions identical to that operating in the C₃ pathway (Fig. 7). Three distinct mechanisms for decarboxylation have been identified occurring in different plants (Fig. 9). The first group of species (NADP-malic enzyme group) contains a high level of an NADP⁺-specific-malic enzyme [malate dehydrogenase (decarboxylating) (NADP⁺), EC.1.1.1.40] in the bundle-sheath chloroplast (Slack *et al.*, 1969; Johnson and Hatch, 1970; Edwards and Black, 1971b; Chen *et al.*, 1973; Huber *et al.*, 1973), and malate is decarboxylated to pyruvate with the regeneration of CO₂ and NADPH. NADP-malic enzyme is also found in photosynthetic cells of other C₄ plants, but in these plants the enzyme has a higher K_m for malate (Nishikido and Wada, 1974). Plants

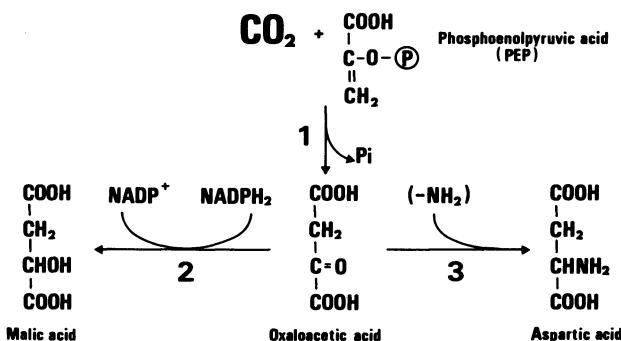


Fig. 8. Primary steps in C₄ photosynthesis. The enzymes catalyzing the individual reactions are: 1, phosphoenolpyruvate carboxylase (EC.4.1.1.31); 2, malate dehydrogenase (NADP⁺) (EC.1.1.1.82); 3, aspartate aminotransferase (EC.2.6.1.1).

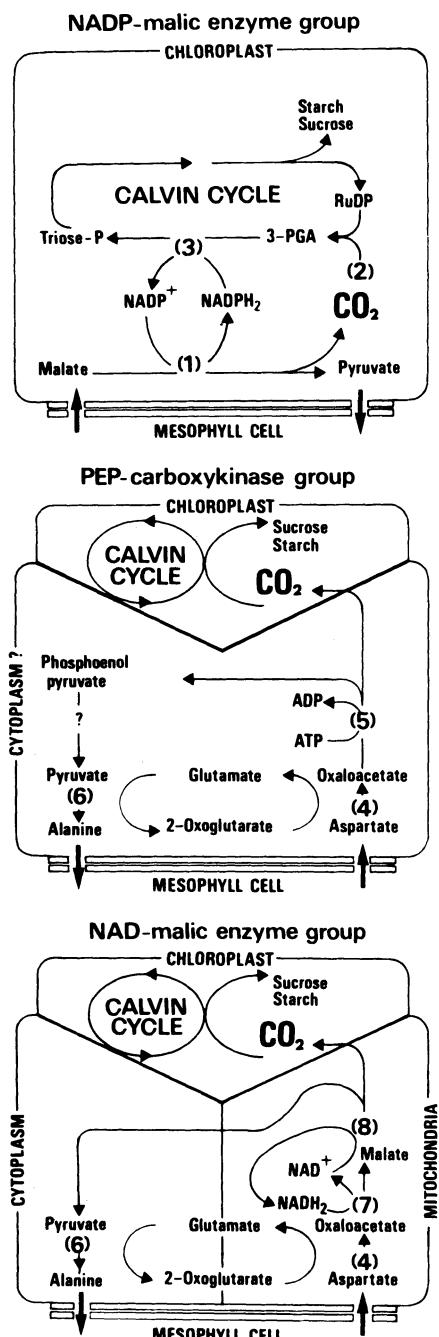


Fig. 9. Decarboxylation and refixation of carbon dioxide in bundle-sheath cells of C₄ plants. The enzymes catalyzing the individual reactions are: 1, malate dehydrogenase (decarboxylating) (NADP⁺) (EC.1.1.1.40); 2, ribulosediphosphate carboxylase (EC.4.1.1.39); 3, glyceraldehyde phosphate dehydrogenase (NADP⁺) (EC.1.2.1.9); 4, aspartate aminotransferase (EC.2.6.1.1); 5, phosphoenolpyruvate carboxykinase (EC.4.1.1.49); 6, alanine aminotransferase (EC.2.6.1.2); 7, malate dehydrogenase (EC.1.1.1.37); 8, malate dehydrogenase (decarboxylating) (EC.1.1.1.39). (Reproduced with permission from Hatch, 1975.)

using this reaction are also characterized by the presence of paucigranal bundle-sheath chloroplasts (Gutierrez *et al.*, 1974a) with a consequent reduction in the capacity of the chloroplast to generate NADPH (Section 3.2). Presumably the need for the reducing capacity to be supplied by photosynthetic electron transfer in bundle-sheath chloroplasts using this decarboxylation process is minimized by its provision from the mesophyll cell.

The second mechanism of decarboxylation occurs in plants whose mesophyll and bundle-sheath cells contain high levels of aspartate aminotransferase (EC.2.6.1.1) and alanine aminotransferase (EC.2.6.1.2) activity (Fig. 9). However, there is a specific isoenzyme for each of the aminotransferases in the two cell types (Hatch, 1973; Hatch and Mau, 1973). In such plants (PEP-carboxykinase group), oxaloacetate is regenerated in the bundle-sheath cell by transamination and then decarboxylated by phosphoenolpyruvate carboxykinase (PEP-carboxykinase, EC.4.1.1.49) (Edwards *et al.*, 1971). The PEP thus formed is probably converted to pyruvate and reaminated to alanine, in a reaction which maintains the amino balance of the cell. Alanine could then be returned to the mesophyll cell and deaminated to pyruvate. The third group of plants (NAD-malic enzyme group) like the second, contains high aminotransferase activity but lacks PEP-carboxykinase. After the reformation of oxaloacetate by deamination of aspartate in the bundle-sheath mitochondria, the oxaloacetate is reduced to malate by the action of an NAD-malate dehydrogenase (EC.1.1.1.37) and the malate decarboxylated by a mitochondrial NAD-malic enzyme [malate dehydrogenase (decarboxylating), EC.1.1.1.39] (Hatch and Kagawa, 1974a,b; Kagawa and Hatch, 1974b; Hatch *et al.*, 1974). It is proposed that the pyruvate thus formed is reaminated to alanine and returned to the mesophyll cell where it can be deaminated to pyruvate. Therefore, such a series of reactions maintains both an amino balance in the bundle-sheath cell and an NAD⁺-NADH balance in the bundle-sheath mitochondria. The plants of the NAD-malic enzyme group are characterized by the presence of large bundle-sheath mitochondria (Fig. 4), e.g., *Amaranthus edulis* and *Atriplex spongiosa* (spongy fruited saltbush), and the biochemical importance of the mitochondria in the decarboxylation reaction thus appear to be reflected in their size.

In addition to their role in the primary fixation of CO₂, it is the function of the mesophyll cells to regenerate sufficient PEP for subsequent carboxylation reactions. As indicated earlier, pyruvate, or alanine which can be converted to pyruvate, is transferred back from the bundle-sheath to the mesophyll cells. The requirement for PEP in mesophyll cells is apparently met by the enzyme pyruvate-P_i-dikinase (EC.2.7.9.1) which catalyzes the phosphorylation of pyruvate in the presence of ATP and inor-

ganic phosphate, to yield PEP, AMP, and inorganic pyrophosphate. Pyruvate- P_i -dikinase has not been detected in C₃ plants (Hatch and Slack, 1968). Two other enzymes adenylate kinase (EC.2.7.4.3) and inorganic pyrophosphatase (EC.3.6.1.1), are also present in substantial amounts in mesophyll cells. These enzymes can convert the AMP and inorganic pyrophosphate, derived from the activity of pyruvate- P_i -dikinase, to ADP and inorganic phosphate which can be used to regenerate ATP by photosynthetic phosphorylation. Experimental evidence for carbon flow through these pathways is described in detail by Hatch (1975). It may be expected that the transport of metabolites from mesophyll to bundle-sheath cells would be a rate-limiting step in the overall process. However, numerous plasmodesmata occur in the walls between mesophyll and bundle-sheath cells (Laetsch, 1971), and Osmond (1971) has calculated that diffusion could maintain metabolite interchange at a sufficient level for the observed rates of photosynthesis.

A criterion of the utmost importance in the series of processes outlined above is the compartmentation of the individual reactions in varying cells and organelles. In particular the locations of the two carboxylating enzymes, PEP-carboxylase (EC.4.1.1.31) and RuDP carboxylase are of particular significance, and their distribution in the leaf has been extensively studied (Edwards and Black, 1971a; Kanai and Edwards, 1973a,b,c; Mamedov and Kovaleva, 1973). In Table 3 the distribution of the carboxylating enzymes from several C₄ grass species is shown. It is apparent that the separation of the two enzymes is essentially complete, and this fact is now generally accepted. Claims that both carboxylating enzymes are present in the mesophyll cells with PEP-carboxylase in the cytoplasm and RuDP carboxylase in the chloroplasts have not been well supported experimentally (Bucke and Long, 1971; Coombs and Baldry, 1972; Coombs *et al.*, 1973a,b,c). Levels of phenols and phenoloxidase activity, also suggested as causes of misleading conclusions (Baldry *et al.*, 1970; Bucke and Long, 1971), have been shown not to affect carboxylase activity significantly (Ku *et al.*, 1974a). Although located almost exclusively in mesophyll cells, PEP-carboxylase does not appear to be a chloroplast enzyme (Hatch and Kagawa, 1973), although RuDP carboxylase is located in the chloroplasts of bundle-sheath cells (Slack *et al.*, 1969), as it is in those of C₃ plants. Pyruvate- P_i -dikinase, adenylate kinase, and pyrophosphatase are located in mesophyll chloroplasts (Hatch and Slack, 1968; Hatch, 1971).

PEP-carboxylase is not unique to the mesophyll cells of C₄ plants but also occurs widely in C₃ plants, although at a low level of activity, and in nonphotosynthetic cells. However, in plants, there appear to be a number of isoenzymes, each of which has a specific function. PEP-carboxylases of the C₃ plant *Atriplex patula* and those of the C₄ plant *Atriplex spongiosa* can be

TABLE 3. Distribution of Carboxylases in C₄ Plants

	Tissue ^a	PEP-carboxylase ^b	RuDP carboxylase ^b	Reference
NADP-malic enzyme group				
<i>Digitaria sanguinalis</i>	WL	475	170	Chen <i>et al.</i> , 1973
	MC	1220	24	
	BS	22	450	
<i>Pennisetum purpureum</i>	WL	1206	332	Ku <i>et al.</i> , 1974a
	MP	2298	3	
	BS	10	654	
<i>Sorghum bicolor</i>	WL	848	57	Kanai and Edwards, 1973b
	MP	1752	1	
	BS	23	272	
<i>Zea mays</i>	WL	660	190	Kanai and Edwards, 1973a
	MP	864	0	
	BS	14	237	
PEP-carboxykinase group				
<i>Chloris gayana</i>	WL	605	244	Gutierrez <i>et al.</i> , 1974b
	MP	856	0	
	BS	29	393	
NAD-malic enzyme group				
<i>Panicum capillare</i>	WL	637	162	Gutierrez <i>et al.</i> , 1974b
	MP	2557	<1	
	BS	12	282	

^a WL, whole leaf; MC, mesophyll cells; MP, mesophyll protoplasts; BS, bundle-sheath cells.

^b Activity measured as ($\mu\text{mol CO}_2$ fixed) $(\text{mg chlorophyll})^{-1} \text{ h}^{-1}$.

readily differentiated on the basis of their physical and kinetic properties (Ting and Osmond, 1973a,b). The properties of the PEP-carboxylase from etiolated *Saccharum officinarum* resemble those of a C₃ plant and contrast markedly with those of the enzyme isolated from green tissue (Goatly and Smith, 1974). In addition, the function of PEP-carboxylase in C₄ plants is clearly that of the primary fixation of CO₂ and the production of photosynthetic intermediates, whereas in C₃ plants the malate derived from PEP-carboxylase activity behaves as an end product.

An additional feature distinguishing the PEP-carboxylase activity of C₄ plants from that of C₃ plants is an increase in its activity on illumination of etiolated leaves. Such increases are characteristic of the many components involved in photosynthetic electron transfer and carbon fixation. Illumination of etiolated *Zea mays* and *Sorghum* leaves gave rise to substantial

increases in PEP-carboxylase activity, while little change occurred in the activity of this enzyme on the illumination of etiolated *Triticum aestivum* (wheat) and *Avena sativa* (oat) leaves (Hatch *et al.*, 1969; Graham *et al.*, 1970). A number of other enzymes involved in C₄ photosynthesis have been shown to increase in activity on illumination of etiolated leaves, including pyruvate-P_i-dikinase and adenylate kinase, which are located in mesophyll chloroplasts (Hatch *et al.*, 1969; Graham *et al.*, 1970), and such nonchloroplast enzymes as the aspartate aminotransferases (Hatch and Mau, 1973) and NAD-malic enzyme (Hatch and Kagawa, 1974b).

The question inevitably arises as to whether all the CO₂ fixed by C₄ plants occurs via PEP-carboxylase or whether RuDP carboxylase can serve as a fixer of atmospheric CO₂. Evidence has been presented that the primary fixation of CO₂ can occur through RuDP carboxylase in *Zea mays* and *Digitaria sanguinalis* (summer grass, crab grass) leaves (Edwards and Black, 1971a; Laber *et al.*, 1974), but that the proportion fixed by this mechanism is small when compared to the PEP-carboxylase pathway. It has also been reported that the proportion of radioactive CO₂ fixed into 3-PGA as compared to that fixed into malate and aspartate, in short-term labeling experiments with *Portulaca oleracea* (common purslane, pigweed), increases as leaves senesce (Kennedy and Laetsch, 1973). However, in this case, the amount of CO₂ fixed by senescent leaves was less than one-tenth of that fixed by mature leaves, and the decrease in total fixation is due to a preferential loss of the PEP-carboxylase mechanism when compared to the RuDP carboxylase mechanism. Certainly there is no increase in the total amount of CO₂ fixed directly by RuDP carboxylase, and the observations may merely reflect the breakdown of cellular integration during leaf senescence. However, a decrease in PEP-carboxylase activity and a concomitant increase in RuDP carboxylase activity, together with a change in the ratio of label fixed into malate and 3-PGA, has been associated with flowering in *Sorghum bicolor* but not in *Pennisetum typhoides* (bulrush millet) (Khanna and Sinha, 1973). It seems likely then that the primary fixation of CO₂ by RuDP carboxylase does occur in C₄ plants, but that it is only a minor pathway when compared to CO₂ fixation by PEP-carboxylase, and that its extent varies between species.

Each of the three groups of decarboxylating mechanisms described above has been identified in several species, and Table 4 shows the relative proportions of the three key activities in a number of plants, including several C₃ species. A detailed comparison of the activities of these enzymes in over 50 species of the Gramineae has been reported by Gutierrez *et al.* (1974a); and this paper also correlates the intracellular location of bundle-sheath chloroplasts with the presence of grana in the bundle-sheath chloro-

TABLE 4. Comparative Aspects of C₄ Acid Decarboxylation^a

	NADP-malic enzyme	PEP- carboxykinase	NAD-malic enzyme
NADP-malic enzyme group			
<i>Digitaria sanguinalis</i>	914	82	89
<i>Pennisetum purpureum</i>	687	64	72
<i>Sorghum bicolor</i>	1129	0	74
PEP-carboxykinase group			
<i>Chloris gayana</i>	14	465	72
<i>Panicum maximum</i>	14	264	142
NAD-malic enzyme group			
<i>Buchloë dactyloides</i>	36	0	389
<i>Eleusine indica</i>	20	0	388
<i>Panicum miliaceum</i>	23	0	393
C₃ plants			
<i>Panicum clandestinum L.</i>	0	0	130
<i>Hordeum vulgare</i>	25	0	128
<i>Triticum aestivum</i>	13	3	40

^a Activity measured as $\mu\text{mol} (\text{mg chlorophyll})^{-1} \text{ h}^{-1}$. Data from Gutierrez *et al.*, 1974a.

plants and the decarboxylating mechanism employed. Thus the members of the NADP-malic enzyme group are characterized by paucigranal bundle-sheath chloroplasts which are located centrifugally in the cell, while members of the PEP-carboxykinase group have granal bundle-sheath chloroplasts located in a centrifugal position and those of the NAD-malic enzyme group have granal chloroplasts located in a centripetal position. This type of biochemical and structural correlation is particularly valuable when it can be correlated with anatomical analyses such as those reported for the Gramineae by Carolin *et al.* (1973).

The relationship between the photosynthetic electron transfer capacity of the bundle-sheath chloroplasts and their capacity for fixation of carbon by the Calvin cycle has been investigated by Osmond (1974). It was found that leaves of *Sorghum bicolor* (which has paucigranal bundle-sheath chloroplasts with low photosystem 2 activity) (Section 3.2) could catalyze the transfer of ¹⁴C from C₄ acids to 3-PGA with equal efficiency when illuminated with 646-nm light (which activates both photosystems 1 and 2) or with 712-nm light (which activates only photosystem 1). In contrast, however, the movement of carbon from C₄ acids to 3-PGA in leaves of *Atri-*

plex spongiosa (which has granal bundle-sheath chloroplasts with good photosystem 2 activity) was dependent upon the activation of both photosystems with 646-nm light, and 712-nm light was relatively ineffective.

The fixation of 1 mol of CO₂ by the C₄ pathway requires 2 mol of NADPH and 5 mol of ATP in those plants of the NADP-malic enzyme and NAD-malic enzyme groups, and 2 mol of NADPH and 6 mol of ATP in the members of the PEP carboxykinase group, while the corresponding process in C₃ plants requires 2 mol of NADPH and 3 mol of ATP. Assuming that there are two sites of phosphorylation in the noncyclic reaction, the photoreduction of 2 mol of NADP⁺ from water would generate, at most, only 4 mol of ATP, which is insufficient for the overall photosynthetic process in C₄ plants. Presumably the deficiency is overcome by cyclic photophosphorylation. Although some authors have questioned the importance of cyclic photophosphorylation *in vivo*, it has been shown that inhibition of cyclic photophosphorylation can affect CO₂ fixation (Klob *et al.*, 1973). It surely cannot be a coincidence that those C₄ plants which have paucigranal bundle-sheath chloroplasts and low photosystem 2 activity in bundle-sheath chloroplasts are also those which use malate for the intercellular transfer of fixed carbon, and thus have a high level of reducing power transferred to the bundle-sheath from the mesophyll chloroplasts. Thus the primary role of such paucigranal chloroplasts could be the generation of ATP by cyclic photophosphorylation without the concomitant generation of NADPH which would be supplied by the noncyclic pathway.

3.4. Crassulacean Acid Metabolism

No comparative discussion of carbon flow during photosynthesis can be complete without mention of the process known as Crassulacean acid metabolism (CAM). This pathway, common to a great number of succulent plants including many *Euphorbia* species and members of the *Bromeliaceae* (pineapple) family, achieves by temporal separation what the C₄ pathway achieves by spatial separation (Fig. 10). During the light period, the stomata of CAM plants remain closed, and there is little assimilation of atmospheric CO₂. However, during the night, CO₂ is assimilated and fixed by PEP-carboxylase, and the oxaloacetate so produced is reduced to malate and stored in the tissue. The carbon atoms of the PEP involved in the initial carboxylation are thought to be derived from starch, the cellular levels of which decrease at night.

In the subsequent light period, malate is decarboxylated and the released CO₂ is refixed by the Calvin cycle (Fig. 10). The mechanism of decarboxylation appears similar to that operating in C₄ plants, in that

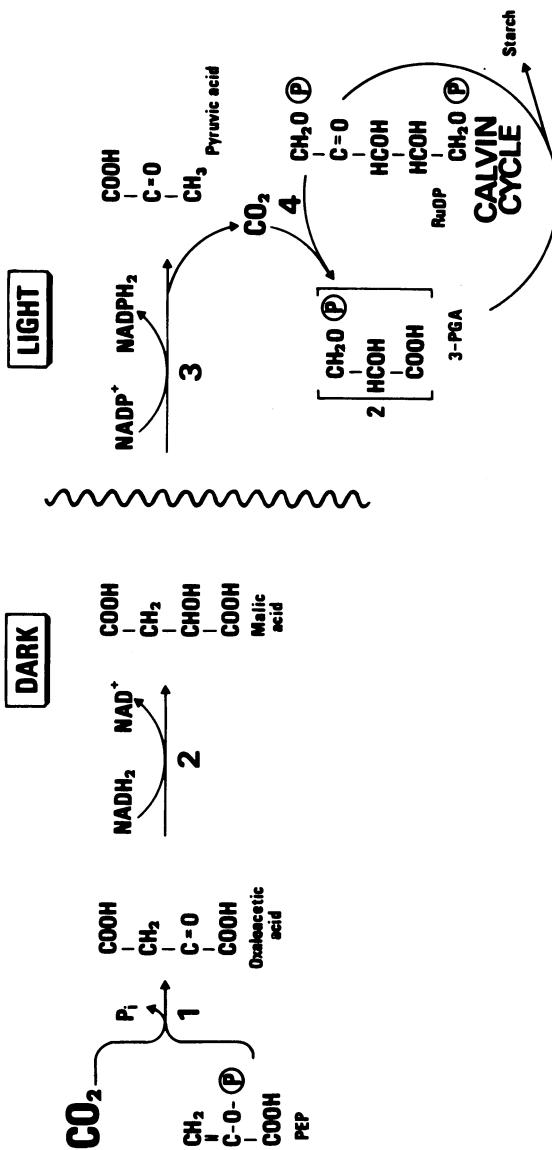


Fig. 10. Carbon fixation during Crassulacean acid metabolism. The enzymes catalyzing the individual reactions are: 1, phosphoenolpyruvate carboxylase (EC.4.1.1.31); 2, malate dehydrogenase (EC.1.1.1.37); 3, malate dehydrogenase (decarboxylating) (NADP⁺) (EC.1.1.1.40); 4, ribulosediphosphate carboxylase (EC.4.1.1.39).

NADP-malic enzyme has been identified in a number of species (Black, 1973). The pyruvate thus formed can be reconverted to PEP by pyruvate- P_i -dikinase, which has been detected in a number of CAM plants (Klüge and Osmond, 1971). In other species an alternative mechanism of decarboxylation may be operative, one similar to that of the second group of C₄ plants, since certain CAM plants lack NADP-malic enzyme but contain PEP-carboxykinase (Dittrich *et al.*, 1973).

CAM plants, when grown under long photoperiods or when night temperatures are high, may photosynthesize during the day by the C₃ pathway (Neales, 1973; Osmond *et al.*, 1973; Allaway *et al.*, 1974). Alternatively, the transfer of the CAM plant *Kalanchoe blossfeldiana* (Tom Thumb) from long photoperiods to short photoperiods results in a switch of carbon fixation from the C₃ pathway to the C₄ pathway (Lerman and Queiroz, 1974).

3.5. Photorespiration

The inhibition of photosynthesis by oxygen was first reported by Warburg in 1920, and has since been demonstrated in a variety of plants. Subsequently Decker (1955, 1959) observed a light-dependent evolution of CO₂ in some species, and it appears that these two observations are related, both being due to the process now referred to as photorespiration (Chollet, 1974). The elucidation of the C₄ pathway of photosynthesis has contributed a stimulus to the study of photorespiration, since C₄ plants do not exhibit the Warburg effect at O₂ concentrations below 21% and do not appear to carry out significant levels of photorespiration.

It has been known for some time that the CO₂ evolved in photorespiration was primarily derived from glycolate (Jackson and Volk, 1970; Tolbert, 1971a; Black, 1973; Zelitch, 1973a), but the major source of this glycolate in C₃ plants has only recently been demonstrated to be the oxygenation of RuDP by RuDP carboxylase, which also has the capacity to act as an oxygenase (Ogren and Bowes, 1971; Bowes *et al.*, 1971; Andrews *et al.*, 1973; Lorimer *et al.*, 1973; Badger and Andrews, 1974). Oxygenase activity has been demonstrated in RuDP carboxylase preparations from a number of sources, including photosynthetic bacteria grown anaerobically (Ryan *et al.*, 1974; McFadden, 1974), and appears to be an inherent feature of the carboxylation enzyme. The oxygenation reaction, which acts competitively with the carboxylation reaction results in the production of 3-PGA and phosphoglycolic acid (Fig. 11). The latter is hydrolyzed by a specific chloroplast-located phosphatase (EC.3.1.3.18) to yield glycolic acid (Fig. 11) (Tolbert, 1971a).

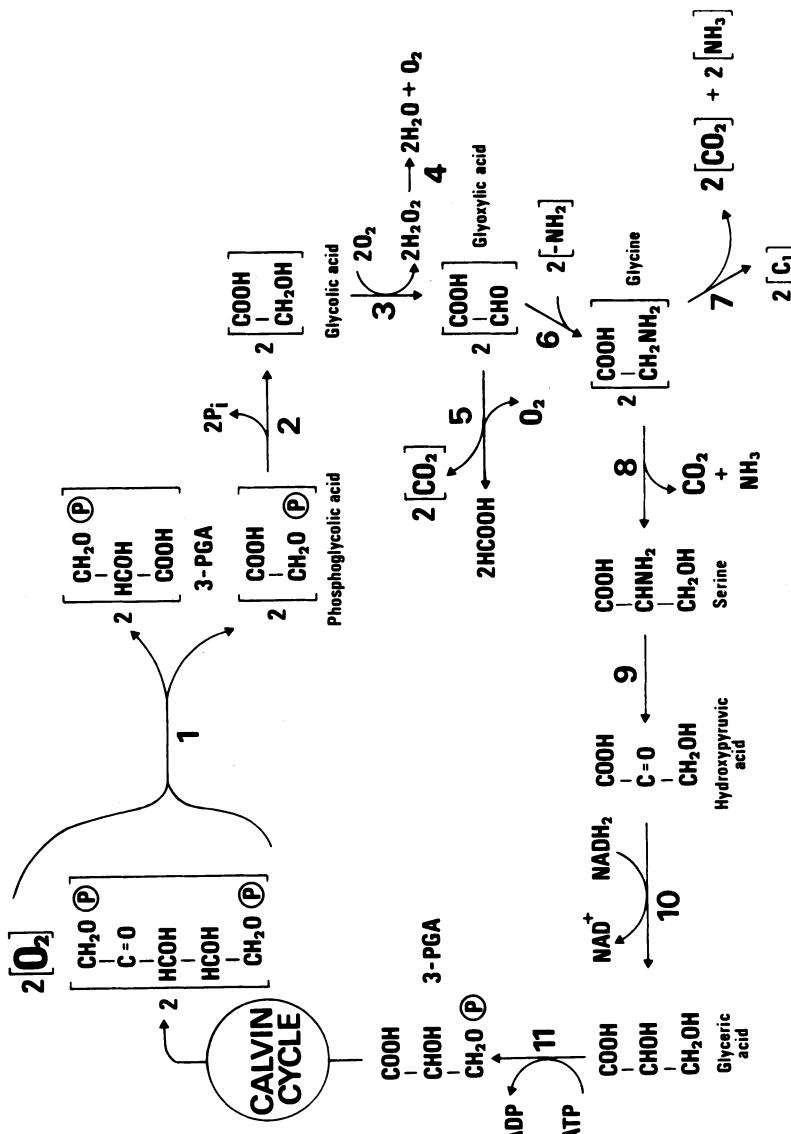


Fig. 11. Reactions of photorespiration. The enzymes catalyzing the individual reactions are: 1, ribulosediphosphate carboxylase acting as an oxygenase; 2, phosphoglycolic acid phosphatase (EC.3.1.3.18); 3, glycolic acid oxidase (EC.1.1.3.1); 4, catalase (EC.1.11.1.6); 5, mechanism unknown; 6, glycine aminotransferase (EC.2.6.1.4); 7, 8, enzymes not well characterized; 9, serine-pyruvate aminotransferase (EC.2.6.1.5); 10, hydroxypyruvate reductase (EC.1.1.1.81); 11, glyceral kinase (EC.2.7.1.31).

Much of the subsequent metabolism of glycolate occurs in the microbodies (peroxisomes) of the leaf cells (Fig. 11). Glycolic acid is first oxidized to glyoxylic acid, with the production of hydrogen peroxide, which is decomposed by catalase (EC.1.11.1.6). Glyoxylate undergoes transamination to produce glycine or, alternatively, can undergo a light-dependent decarboxylation with the production of CO_2 and formic acid (Zelitch, 1972).

There appear to be divergent possibilities for further metabolism of glycine. Two molecules of glycine can be converted to one molecule of serine with the evolution of CO_2 , but this reaction does not appear to occur in microbodies, but rather to be located in mitochondria (Tolbert, 1971*a,b*). The direct decarboxylation of glycine has also been demonstrated (Kisaki *et al.*, 1972). Further metabolism of serine to glyceric acid *via* hydroxypyruvic acid can occur in the microbodies (Tolbert, 1971*a*) (Fig. 11). Finally the phosphorylation of glyceric acid can occur in the chloroplasts, and the 3-PGA so formed may reenter the Calvin cycle.

It is apparent that a substantial proportion of the CO_2 fixed by photosynthesis can be rapidly lost by photorespiration. Any mechanism that would minimize the loss by photorespiration of fixed carbon would be of great advantage to plants, and there is abundant evidence that C_4 plants possess low rates of photorespiration (Jackson and Volk, 1970). Isolated bundle-sheath cells can synthesize glycolic acid (Chollet and Ogren, 1972*b*; Chollet, 1974), and enzymes of the glycolate pathway have been detected in C_4 plants, primarily in the bundle-sheath cells, which contain more microbodies than do the mesophyll cells (Newcomb and Frederick, 1971; Liu and Black, 1972). Oxygen inhibition of CO_2 uptake has been demonstrated in isolated bundle-sheath cells (Chollet and Ogren, 1972*a,b*; Chollet, 1974), and it therefore seems certain that some photorespiration takes place. The difference between C_3 and C_4 plants probably lies in the fact that the bundle-sheath cells are partially protected from atmospheric oxygen by mesophyll cells and also receive CO_2 by decarboxylation at higher concentrations than C_3 plants do from the air. In such a case the competition between O_2 and CO_2 for RuDP is greatly weighted towards CO_2 in bundle-sheath cells, and in addition some of the CO_2 released by photorespiration in bundle-sheath cells could be rapidly refixed in mesophyll cells.

4. COMPARATIVE ASPECTS OF C_3 AND C_4 PHOTOSYNTHESIS

4.1. Temperature

The physiological and biochemical differences between C_3 and C_4 plants which have been outlined in the previous sections lead to a considera-

tion of the relative advantages of each type of photosynthesis. In particular, the temperature requirement and light utilization properties of each type vary markedly and will affect their potential for extended geographic distribution.

There have been numerous demonstrations that the temperature optimum for growth and photosynthesis by C₄ plants lies in the range 30–40°C while that of C₃ plants lies in the range 20–30°C (Black, 1973; Black *et al.*, 1969; Hatch *et al.*, 1971; Ludlow and Wilson, 1971a). These growth optima are also reflected in the specific activity of the primary carboxylating enzymes isolated from plants grown at varying temperatures (Phillips and McWilliam, 1971). Net photosynthesis by C₃ plants generally decreases at temperatures above 30°C (Black *et al.*, 1969; Phillips and McWilliam, 1971), while many C₄ plants appear to be extremely sensitive to temperatures below 10°C, either by day or by night (Taylor and Rowley, 1971). Chlorophyll development in etiolated *Zea mays* leaves is inhibited when the plants are illuminated at 16°C (McWilliam and Naylor, 1967), while leaves of *Triticum aestivum*, a C₃ plant, become chlorotic and die when the plant is grown at a temperature above 34°C (Friend *et al.*, 1962). Exposure of *Sorghum* plants for three days at 10°C leads to an irreversible loss of photosynthetic capacity. Rates of photosynthesis in *Zea mays* and *Paspalum dilatatum* also decrease, although to a lesser extent than in *Sorghum* (Taylor and Rowley, 1971). Damage to *Sorghum bicolor*, *Digitaria smutsii*, and *Paspalum dilatatum* results after one night's exposure to temperatures of 4°C and below. The damage is manifested in the emergence of chlorotic bands on the leaves, due to the production of abnormal plastids in the mesophyll cells, while bundle-sheath cells appear unaffected. Levels of photosynthetic enzymes in the mesophyll cells (such as PEP carboxylase and adenylate kinase) are lower in the chlorotic tissue than in the control tissue, while the level of photosynthetic enzymes located in the bundle-sheath cells (such as RuDP carboxylase and NADP-malic enzyme) were only slightly affected (Slack *et al.*, 1974). Brief exposure of *Sorghum bicolor* to a temperature of 10°C leads to a decrease in the rate at which radioactive CO₂ is released from malate, and after further exposure an increasing proportion of the label accumulates in aspartate (Brooking and Taylor, 1973). Similar alterations in the patterns of isotope exchange have been observed in *Zea mays* exposed to low temperatures (Hofstra and Nelson, 1969a). These changes in carbon flux occur well before any pronounced ultrastructural changes can be observed in leaf organelles (Taylor and Craig, 1971) and while the activities of most enzymes implicated in the C₄ pathway remain relatively unchanged (Taylor *et al.*, 1974).

A number of plants of both C₄ and C₃ types have been shown to undergo discrete changes in physiological and biochemical parameters at about 12°C, and these changes appear to be related to membrane-

associated reactions (Raison, 1973). The changes are detectable as discontinuities in Arrhenius plots and have been observed in PEP-carboxylase activity (Phillips and McWilliam, 1971), photosynthetic electron transfer (Shneyour *et al.*, 1973), and mitochondrial oxidations (Raison, 1973). Such "phase changes" in membrane associated reactions appear to be a property of the membrane lipids, as isolated membrane lipids show discontinuities in Arrhenius plots at the same temperatures as the whole membranes (Raison *et al.*, 1971). Evidence that these measurements on isolated cellular constituents are relevant to the whole plant is shown in Fig. 12. The rate of growth of mung bean (*Vigna radiata* formerly *Phaseolus aureus*), a C₃ plant, shows discontinuities at 28 and 16°C, the same temperatures at which phase changes are detected in the succinoxidase activity of isolated hypocotyl mitochondria (Raison and Chapman, 1976). The temperatures at which such phase changes occur can also be detected by measurement of the mobility of a spin label in the isolated mitochondria, or chloroplasts, although the lipids from the two organelles differ widely, both in their content of individual lipids and in the fatty acid composition of those lipids. The existence of two discontinuities in the Arrhenius plots is interpreted as indicating the onset and completion of a transition of the membrane lipids between a gel phase and a liquid-crystalline phase (Shimshick and McConnell, 1973). The increase in activation energy of enzyme catalyzed reactions below the temperatures at which phase changes occur in membranes presumably causes a marked decrease in the rate of membrane localized processes. Such effects are probably responsible for the accumulation of photosynthetic intermediates in *Sorghum bicolor* (Brooking and Taylor, 1973) and the subsequent destruction of cellular structure and metabolism. The susceptibility of such plants to temperature will obviously place a great restriction on attempts to extend their geographical distribution.

4.2. Light

It is generally accepted that C₄ plants have higher maximum rates of photosynthesis than C₃ plants and that photosynthetic rates in C₄ plants approach their maximum only at light intensities approximating to full sunlight, whereas photosynthesis by C₃ plants reaches saturation at much lower light intensities (Fig. 13) (El-Sharkawy and Hesketh, 1965; Hesketh and Baker, 1967). Nevertheless, this separation is not absolute: Rates of photosynthesis equivalent to those observed in C₄ plants have been observed in some primitive species of *Triticum* (Evans and Dunstone, 1970), *Typha latifolia* (cattail) (McNaughton and Fullem, 1970), and *Helianthus annuus* (sunflower) (Hesketh and Moss, 1963). It has also been found that primitive

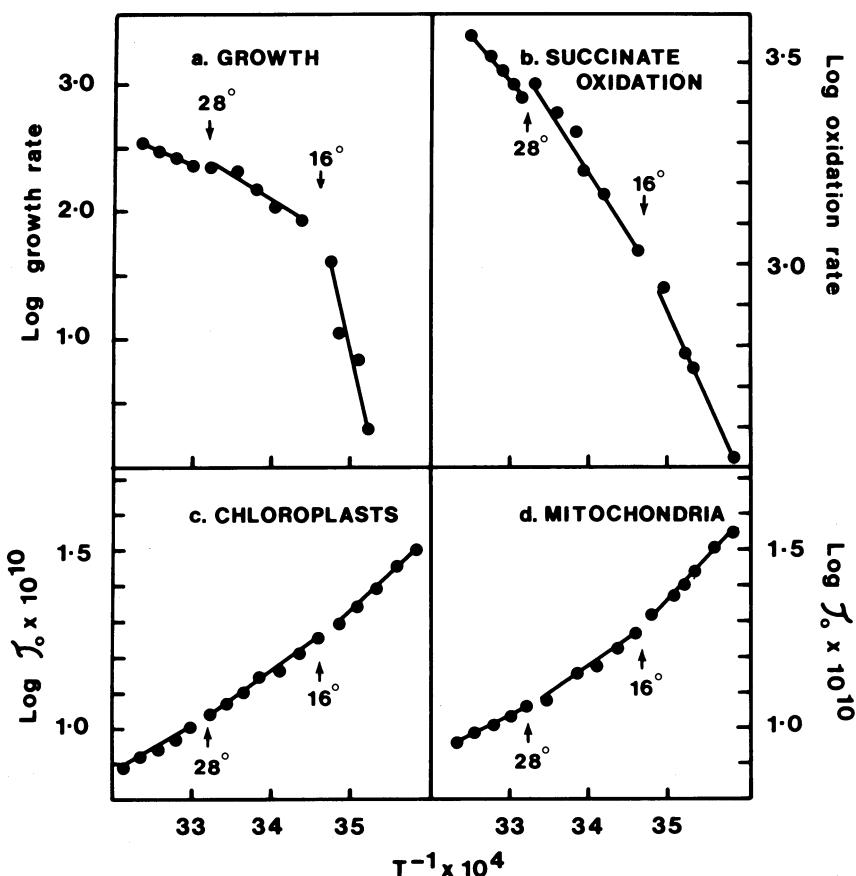


Fig. 12. The effect of temperature on properties of mung bean (*Vigna radiata*). (a) Growth rate, measured as the total length of root and hypocotyl and expressed as millimeter increase per 7 days; (b) succinate oxidation by hypocotyl mitochondria isolated from plants grown at 28°C and expressed as nmol O₂ min⁻¹ ml suspension⁻¹; (c) mobility of the fatty acid spin label, methyl 16-nitroxide stearate in isolated chloroplasts and (d) mitochondria. Mobility was measured as the correlation time, τ_0 (Mehlhorn and Keith, 1972). Discontinuities in the Arrhenius plots can be detected at the same temperatures in all four systems. (Data from Raison and Chapman, 1976.)

species of *Triticum* had higher rates of photorespiration than modern cultivars (Evans and Dunstone, 1970). However, the restriction placed upon individual plants in a community, with respect to the availability of light, means that many of the leaves of a C₄ plant are photosynthesizing at well below maximum capacity.

Several studies have shown that the photosynthetic capacity of C₃

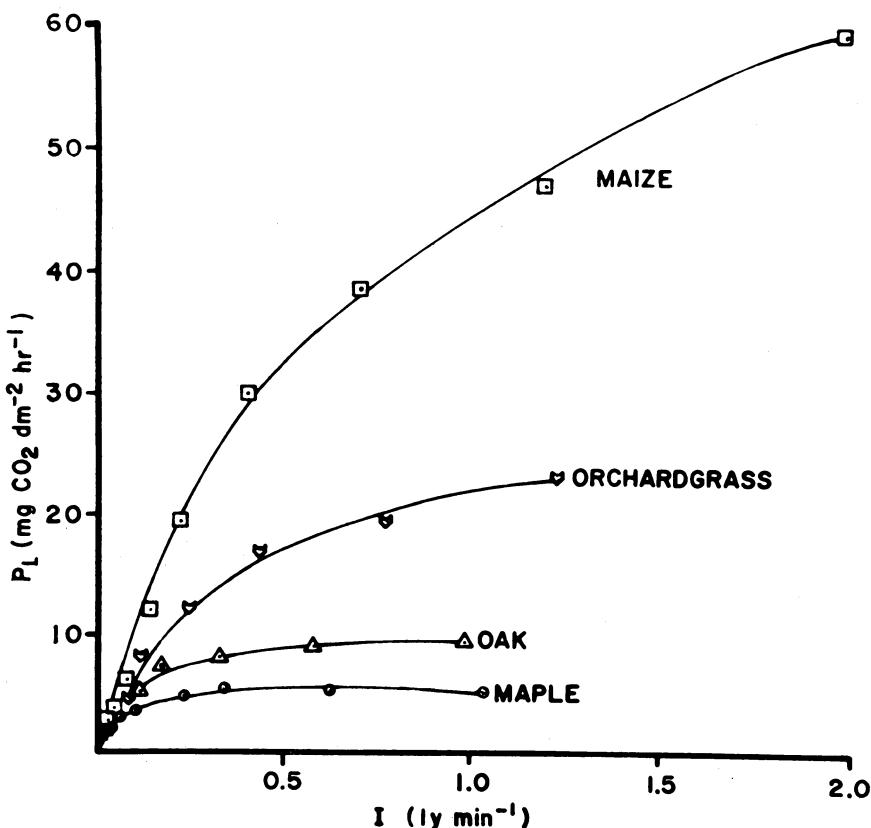


Fig. 13. The effect of light intensity on the rate of photosynthesis by C₃ and C₄ plants. The C₄ species maize (*Zea mays*) is compared with the C₃ species orchardgrass (*Dactylis glomerata*), oak (*Quercus* sp.), and maple (*Acer* sp.). P_l, leaf photosynthetic rate; I, illuminance; ly, langleys. An illuminance value of 1.0 ly min⁻¹ is equivalent to 700 W m⁻². (Reproduced from Hesketh and Baker 1967, with permission.)

plants can vary markedly according to the light conditions under which the plants are grown. The photosynthetic capacity and light intensity for maximum rates of photosynthetic electron transfer of *Atriplex patula* varies directly with the light intensity during growth (Björkman *et al.*, 1972). Plants of *Sinapis alba* (white mustard) grown at low light intensity had a lower photosynthetic rate, a lower compensation point, and a lower respiration rate than those grown at high light. About five days after transfer of plants grown in low light to higher light, maximum photosynthetic activity was obtained (Grahl and Wild, 1972, 1973). Ludlow and Wilson (1972b) observed similar variations in the maximum photosynthetic rate and light satu-

ration curve for a number of C₄ plants grown under different light conditions, but Wild and Müllenbeck (1973) did not detect any effect of light intensity during growth on the photosynthetic rate of *Zea mays*.

Although some differences have been observed in the light intensity curves of isolated photochemical reactions, the significance of these in relation to whole-plant photosynthesis is not clear. The photoreduction of NADP is saturated at much lower light intensities in *Zea mays* mesophyll chloroplasts than in bundle-sheath chloroplasts (Smillie *et al.*, 1972a), and similar results have been found for artificial electron acceptors (Anderson *et al.*, 1971a; Smillie *et al.*, 1972a). Cyclic phosphorylation by isolated chloroplasts (presumably mesophyll) of *Cynodon dactylon* (var. Bermuda grass) requires higher light intensities for maximum rates of ATP formation than does noncyclic phosphorylation (Chen *et al.*, 1969), but the same is true for photophosphorylation by the C₃ plants *Beta vulgaris* (spinach) and *Phytolacca americana* (common pokeweed).

4.3. Photorespiration

The existence in C₃ plants of levels of photorespiration which can result in a substantial loss of fixed carbon, is a severe limitation to productivity and net rates of photosynthesis. The extent to which photorespiration can affect the growth of C₃ plants was elegantly demonstrated by Björkman *et al.* (1967, 1968), who showed that growth of *Phaseolus vulgaris* (bean) and *Mimulus cardinalis* (crimson monkeyflower) under low oxygen resulted in a substantial increase in dry matter production as compared to growth in 21% oxygen. Dry matter production by the C₄ species *Zea mays* was not significantly affected by concentrations of oxygen below atmospheric levels (Björkman *et al.*, 1968), although photosynthetic carbon fixation by *Z. mays* leaves is strongly inhibited at oxygen concentrations approaching 100% (Grishina *et al.*, 1974).

As discussed in Section 3.5, C₄ plants do contain enzymes of the photorespiration cycle, generally located in bundle-sheath cells, and it appears therefore that the major mechanism by which the problem of losses due to photorespiration is overcome, is a maximizing of the CO₂:O₂ ratio in the bundle-sheath cell in the presence of RuDP carboxylase so that competition between the two gases is weighted heavily in favor of CO₂. A second possibility is the refixation of photorespired CO₂ by mesophyll cells. At low O₂ concentrations, the CO₂ fixing capacity of C₄ and C₃ species of *Atriplex* is similar over a wide range of light intensities (Björkman, 1971). A similar condition exists in 21% O₂ at low light intensities, but as the light intensity is increased the net rate of CO₂ fixation by the C₄ species greatly exceeds

that of the C₃ species. Similarly, it has been found that at low light intensities, the rate of photorespiration of *Amaranthus lividus*, a C₄ species, is similar to that of the C₃ plant *Glycine max* (soybean). However, as the light intensity increases, the rate of photorespiration by *A. lividus* decreases, while that of *G. max* remains constant (Laing and Forde, 1971). These observations, when translated to a plant community where most of the leaves will certainly be receiving less than full sunlight for a major part of the day, suggest that in the field the differences in photosynthetic capacity of C₄ and C₃ plants due to photorespiration may be substantially less than those observed in isolated systems in the laboratory.

4.4. Translocation

The characteristics of the C₄ pathway impose two special problems of transport on the leaves of C₄ plants. These are the movement of metabolites between bundle-sheath and mesophyll cells as part of the photosynthetic process and the necessity of coping with a greater rate of photosynthetic production in the movement of carbohydrates to sinks. It has been calculated that simple diffusion through plasmodesmatal connections can account for the flow of metabolites to and from the mesophyll tissue (Osmond, 1971) though direct measurements of the intracellular transfer of intermediates of photosynthesis are not available (Heber, 1974).

It is still uncertain whether the accumulation of assimilates in an illuminated leaf may be responsible for reduction in the net photosynthetic rate of that leaf, as evident from Neales and Incoll's review (1968). It may not be necessary, therefore, to have especially efficient translocation mechanisms for the disposal of assimilates in C₄ leaves. Some C₄ plants have been shown to export photosynthetic products from their leaves more rapidly than do C₃ plants (Hofstra and Nelson, 1969b; Moss and Rasmussen, 1969; Lush and Evans, 1974). Those C₄ plants with centripetal bundle-sheath chloroplasts would be expected to have good translocation to the phloem, simply on the basis of proximity. Further, in a survey of ten grasses it was found that C₄ species had fewer cells between leaf bundles than C₃ species (Crookston and Moss, 1974) though this fact does not necessarily mean a greater cross section area of phloem per leaf (Lush and Evans, 1974).

The presence of starch sheath, caspary strip, and the reaction of cells in the bundle-sheath to various dyes has led to the suggestion that the leaf bundle-sheaths are analogous to endodermis (Esau, 1965). The cells of the bundle-sheath (or sheaths) can differentiate in a number of ways, so the comparison is not always obvious, but such an analogy would imply that

bundle-sheath cells had considerable transport control of both water and metabolites. Certainly, a mass flow of water in plasmodesmata would add extra complications to metabolite flow in the reverse direction.

It has been suggested that the proportions of photosynthetic products stored in starchy bundle-sheaths during the day or the proportions translocated at night are related to the tolerance of night temperatures in different species. The behavior of C₄ plants in this respect has been investigated in only a few species (Lush and Evans, 1974; Hilliard and West, 1970). If a more exhaustive survey were available, it might prove possible to make generalizations connecting the structure of the bundle-sheath, the type of C₄ metabolism, starch accumulation, and temperature tolerance.

5. C₄ PLANTS IN AGRICULTURE

5.1. Water Economy and Salt Relations

Studies leading to the elucidation of the C₄ photosynthetic pathway were first carried out with *Saccharum officinarum* and *Zea mays* and were quickly extended to *Amaranthus* and *Atriplex*. Soon it was apparent that many species of the Chenopodiaceae (a halophilic family) and its weedy offshoot the Amaranthaceae possessed the C₄ pathway. Hot, arid landscapes were found to be dotted with C₄ plants; monsoon grasslands with their long dry seasons were composed entirely of C₄ plants; many drought resisters and evaders were found to be C₄ plants. Clearly, C₄ plants were able to cope with situations of water stress. This assertion does not infer that no C₃ plants possess this ability, nor does it deny that there are other means of coping with water deficit, e.g., CAM, but the situation is worth investigating in view of the importance of water economy in plants. To date, in fact, C₄ plants have been studied in relation to hot arid environments rather than in relation to hot, wet areas, but perhaps this emphasis reflects the present distribution of plant research establishments.

For a time in the development of plant physiology it was thought that plant species had characteristic *transpiration ratios* or *water requirements*, inbuilt ratios of water transpired to dry matter produced. Many painstaking measurements were made. Schröder in 1895 (quoted by Maksimov, 1929) distinguished two groups of cereals with respect to their water requirements: wheat, oats, barley, and rye were classed as less efficient water users than millet, sorghum, and maize. Thus, the reputation of C₄ grasses in this respect is of long standing. Maksimov (1929) extended this list of C₄ plants which made particularly efficient use of water in a study of the physiological basis of drought resistance. A low water requirement was found to

be a better criterion for defining xerophytic species than earlier definitions, which were based on anatomy.

Perhaps the most extensive collection of data on water requirements is that of Shantz and Piemeisel (1927), which has been reassessed and the plants grouped on the basis of their photosynthetic pathway. In the seven-year period over which these experiments were carried out, seasons varied. An Eragrostoid grass had the lowest relative water requirements in hot, dry years, while Panicoid grasses performed better in the humid years, in which evaporation from a free water surface was least. It became clear that C₄ plants used less water than C₃ plants for the same amount of dry matter production (Black, 1971).

Because the C₄ pathway enables the plant to photosynthesize at a higher rate at any given concentration of carbon dioxide in the intercellular spaces, C₄ plants would be expected to be capable to more efficient use of water. But there has been considerable difficulty in showing this difference experimentally on a more analytical level than water requirement. One of the problems is in finding comparable C₃ and C₄ species to study.

At the agronomic level, there is a vast body of empirical knowledge of specific crops, especially under irrigation (Yaron *et al.*, 1973; Hagan *et al.*, 1967; Salter and Goode, 1967), but it is difficult to draw conclusions as to the advantage of C₄ plants on this basis. Similar difficulties arise from experiments where, for instance, maize and sorghum are compared with tobacco and cotton. Even when allied species are compared there are difficulties in interpretation. For instance, Slatyer (1970), in a study of *Atriplex spongiosa* (C₄) and *A. hastata* (C₃), found that *A. hastata* used more photosynthate for production of new leaf than the C₄ species, a difference which could not with certainty be attributed to differing photosynthetic pathways.

A better comparison can be obtained by increasing the number of species studied. Downes (1969) made a survey of seven grasses and found that the C₄ species averaged a higher ratio of assimilation to transpiration than the C₃ species. There was also a tendency for the C₄ plants to keep their high ratio at temperatures up to 30°C. The C₃ plants had their highest value at the lowest temperature used, either 20 or 25°C.

The rate of transpiration from a leaf is directly proportional to the water vapor pressure deficit between the outside air and the intercellular spaces and is inversely proportional to the diffusion resistance along the transpiration pathway from the intercellular spaces to the outside air. For quantitative analysis, this diffusion path is usually divided into discrete segments according to position; that portion from the intercellular spaces through the stomates to the external leaf surface is termed the *stomatal resistance*. The remaining path through the layer of air surrounding the leaf is termed the *boundary layer resistance*. The diffusion of carbon dioxide has

similar resistances plus the intracellular resistance to the site of carboxylation (Milthorpe and Moorby, 1974; Slatyer, 1967). The comparative aspects of interest in the water economy of C₃ and C₄ species include the diffusion paths of carbon dioxide and water, the behavior of stomates, and the temperature of leaves in the sun. In addition, the form of response to water stress and the effect of water stress on final yield need to be considered.

Evidence indicates that stomates of C₄ plants are more sensitive to factors which cause stomatal closure and also have a higher stomatal resistance than those of C₃ plants (Boyer, 1970; Akita and Moss, 1972; Turner and Begg, 1973). Using the simplistic view that carbon dioxide and water have diffusion paths in common through boundary layers and leaf tissue to intercellular spaces, while only carbon dioxide has the extra intracellular resistance to the site of carboxylation, increased stomatal resistance can be viewed as affecting the rate of assimilation less than it affects the rate of transpiration. So, since the stomates of C₄ plants have a tendency partially to close, water-use efficiency would be further increased.

These simplistic explanations become more complicated when one takes into account the different diffusion coefficients of carbon dioxide and water through air, the effect of reduced water loss on leaf temperature and the variability of boundary layers. In one theoretical study (Cowan and Troughton, 1971) it was shown that variability in stomatal resistance could improve the efficiency of water use in plants when the intracellular resistance to carbon dioxide was less than 1 s cm⁻¹. Many C₄ plants have a resistance of this magnitude. Consequently, the fact that the stomates of C₄ plants are more sensitive than those of C₃ plants might account for the more efficient water use of C₄ plants.

Endogenous antitranspirants such as abscisic acid have been found in leaves of C₄ plants; they cause similar effects to those observed in C₃ plants, such as closure of the stomates, and their level increases when the plant is water stressed (Ogunkanmi *et al.*, 1974; Kriedeman *et al.*, 1972; Giles *et al.*, 1974). The level of abscisic acid was found to be higher in a variety of *Zea mays* which was drought resistant than in less resistant varieties (Larque-Saavedra and Wain, 1974). Because of the present difficulties in describing control mechanisms in stomates, it is not surprising that there is insufficient information to say whether there are any distinctive C₄ methods of stomatal control.

When water was withheld from maize for 7 days, the tonoplast ruptured in a quarter of the mesophyll cells. After rewatering, starch could be detected in the bundle-sheath cells next to intact mesophyll within 2 h (Giles *et al.*, 1974). Evidently the Kranz anatomy is well adapted for protection of the photosynthetic tissue near the vascular bundle, both from water deficits and low temperatures (Section 4.1).

There are C₄ species with extreme adaptation to arid conditions, some

of them showing xeromorphic features common to many arid zone species. *Triodia irritans* (gummy spinifex), a perennial hummock grass found in the arid inland of Australia, has rolled, lignified leaves with stomates in deep grooves lined by interlocking papillae (McWilliam and Mison, 1974). *Tidestromia oblongifolia*, a herbaceous perennial in the Amaranthaceae, grows in the deserts of western U.S.A. It has tomentose leaf surfaces and an optimum rate of photosynthesis between 40 and 50°C (Pearcy *et al.*, 1971).

Though it is obvious that water deficits interfere with plant growth, the specific details of the effects at metabolic level are still only understood in general terms whatever the photosynthetic mechanism employed. In these circumstances, it is clearly impossible to describe precisely the "superiority" of C₄ plants with regard to their use of water.

Studies of salt relations of C₄ plants are confounded by the halophilic nature of some C₄ families, e.g., Chenopodiaceae and Zygophyllaceae. However, halophytes are by no means restricted to C₄ groups (Waisel, 1972). Among C₄ plants there are salt requirers, such as *Atriplex vesicaria* (bladder saltbush); salt tolerators, such as *Chloris gayana* (Rhodes grass); and salt sensitive plants, such as *Zea mays* (where "salt" is used in the sense of a higher proportion of sodium ion to potassium ion in the environment than is found in nonsaline environments, as well as higher ionic concentration of both salts). In terms of forage, the salt content can render the plants unpalatable (some *Atriplex* species) but by contrast, the palatability of *Chloris gayana* has been attributed to its salt content (Bogden, 1969). It is impossible to separate which of these isolated observations are attributable to species peculiarities and which are specific properties of C₄ plants.

One characteristic which does appear to be present in C₄ species is an absolute requirement for sodium ions. C₄ species in five families showed leaf lesions and little growth in sodium deficient cultures (Brownell and Crossland, 1972). In leaf discs of *Amaranthus caudatus* (love-lies-bleeding), *Atriplex spongiosa*, and *Zea mays* a mechanism of chloride uptake directly connected with light reactions was found, and this mechanism was apparently lacking in C₃ plants (Luttge *et al.*, 1971). It has been suggested that levels of PEP-carboxylase in *Aeluropus litorales* (a halophytic C₄ grass of Africa and the Middle East) were depleted when sodium chloride was absent (Shomer-Ilan and Waisel, 1973). PEP-carboxylase is known to have a regulatory role in C₄ plants (Hatch *et al.*, 1969; Coombes *et al.*, 1973b), but the evidence concerning the control of its level by sodium chloride nutrition requires better quantitation before a direct connection can be accepted. Investigations into the salt sensitivity of *Zea mays* (Lapina and Bikmukhametova, 1973) need to be extended. The biochemical basis for the necessity of sodium in nutrition of C₄ plants remains an open question.

In view of the increasing use of water of poor quality and the inevitable

salinization of irrigated areas, the salt tolerance of agricultural species is likely to be of increasing importance in the future. Varieties of either C₃ or C₄ plants which can tolerate salinity and economize on water while maintaining productivity will be an undeniable asset. C₄ species would appear to have a better potential for the development of this combination.

5.2. Productivity

The average net carbon dioxide exchange rate of leaves in a basket of C₄ plants exceeds that of C₃ plant leaves in a similar situation. Measured at a CO₂ concentration of about 300 ppm and high illuminance, the values on a leaf area basis are typically 1–3 ng CO₂ mm⁻² s⁻¹ for C₄ and 0.3–2 for nonarboreal C₃ species if unusually productive genera of C₃ plants, such as *Typha*, are excluded. On a chlorophyll basis these rates of carbon dioxide uptake are 3–9 µg mg⁻¹ s⁻¹ for C₄ species and 1–6 µg mg⁻¹ s⁻¹ for C₃ species.

On this basis the C₄ plants have been labeled efficient, highly productive plants of high photosynthetic capacity. Competitiveness of C₄ weeds was attributed to their productivity (Black *et al.*, 1969), and similar credit was given to high-yielding C₄ crops (Stewart, 1970). Gifford (1974) has examined this reputation using data available from the literature, by comparing the capacity of C₃ and C₄ systems at sequential levels of organization from primary carboxylation reactions to economic yield. He considered that there is often little difference between the maximum observed growth rates of C₃ and C₄ plants over short periods and that the difference became even slighter when the comparison was made over longer periods or when yield components of the total production (such as grain) were compared. The biochemical and physiological advantages of C₄ plants grown in their own ideal conditions are attenuated by other factors at higher levels of organization.

The effect of some plants on their carbon dioxide environment is shown in Fig. 14 (Nichiporovich *et al.*, 1973; Uchijima, 1970). As photosynthesis proceeds in a crop each day, the carbon dioxide concentration in the air surrounding the leaves declines, since gas exchange with the atmosphere around the crop is restricted. Much of the leaf surface of *Zea mays* is within the vegetation where gas exchange is limited, so the carbon dioxide level around the leaves can be greatly reduced during the period of highest photosynthetic activity. Under such conditions, the productivity of a crop of *Z. mays* may approximate that of a crop of *Beta vulgaris*, whose leaves are more exposed to the atmosphere. Presumably, however, a C₃ species with the same architecture as *Z. mays* would be unable to maintain productivity

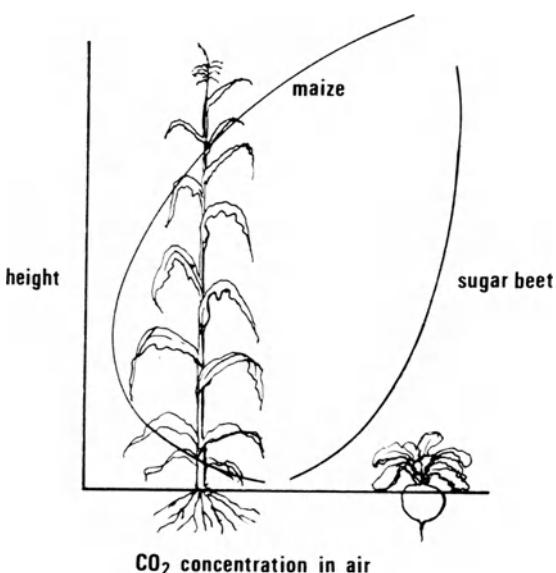


Fig. 14. Carbon dioxide concentration in the air surrounding plants within a crop of *Zea mays* (maize) and a crop of *Beta vulgaris* (sugar beet) at the time of day when carbon dioxide depletion is at its maximum (usually soon after noon). (Data from Nichiporovich *et al.*, 1973; Uchijima, 1970.)

under conditions of low carbon dioxide concentration within the canopy and would thus be at a considerable disadvantage.

Loomis *et al.* (1971) have reviewed the effects of crowding on agricultural ecosystems, and other factors which influence agricultural productivity. They commented that, on an annual basis, productivity can be improved considerably by extending growth duration. Extending duration is a logical objective with forages, sugar crops, and other indeterminant vegetative species. Gifford (1974) noted that in terms of forage yield per annum, C₄ species have a yield 2.5–3.5 times greater than maximum values for C₃ species. This is attributed to the greater length of growing season permitted by the tropical climate, to which the C₄ species are well adapted. In this respect, combinations of C₃ and C₄ pasture species which would take advantage of seasonal variations through the year are a desirable objective. Williams (1974) showed that growth of shortgrass prairies extended through spring and summer as a result of such a combination.

The foregoing comparisons are based on yields of dry matter or carbon material. When forage is used, considerations of its quality, digestibility, and protein content are as important as yield. C₄ grasses when leafy appear to have a lower water content than C₃ grasses, although as maturity approaches this difference becomes negligible (Bailey, 1973).

When digestibility is considered, *Saccharum* is an exception among grasses which grow in warm places, in that it accumulates a high proportion of soluble carbohydrates. C₃ grasses grown in warm climates tend to have

lower dry matter digestibility than when grown in cooler conditions. C₄ grasses have low digestibility whether grown at warm or cool temperatures (Wilson and Ford, 1973; Akin and Burdick, 1973). The difference may be connected with lignin content (Akin and Burdick, 1973) or the different digestibilities of hemicelluloses and celluloses (Ford, 1973). However, it has been suggested that to some extent, lack of knowledge of the agronomy of tropical grasses may account for their reputation as poor-quality forage (Downes *et al.*, 1974). In practice, in areas where C₄ forage grows, production is limited by soil nutrients, particularly nitrogen (Cooper, 1970). The nutrient quality of the forage so grown would inevitably be less than that produced under more favorable conditions.

When conserved as silage, the tropical grasses in general present more problems than temperate grasses. Special care needs to be taken to exclude air, since the fermented product may be based on acetic acid rather than on the lactic acid of conventional silage (Catchpool and Henzell, 1971).

The physical nature of C₄ pasture is such that cows have to graze longer in order to be satisfied (Stobbs, 1973). When given a choice between both types of plants, herbivores, whether insects (Caswell *et al.*, 1973) or sheep and cows (Evans and Tisdale, 1972), tend to consume C₃ species. In one study (Evans and Tisdale, 1972) the selective grazing of sheep and cows led to the replacement of a C₃ species, *Agropyron*, by a C₄ species, *Aristida*.

The quality of temperate pasture has been improved by the use of dicotyledonous plants, and suitable dicotyledonous forage for tropical and temperate regions is being actively sought. Various legumes, all of which are C₃ plants, have been selected to increase the range of their uses, or the productivity of native warm season legumes has been improved. C₄ species have also been considered. For instance, *Amaranthus* species of high digestibility (Alexander *et al.*, 1970) seem suitable for development by the Pirie method (1969) both as forage or for production of protein for human consumption. Leaves of *Amaranthus* have long been used as a vegetable in Africa, but Mugerwa and Bwabye (1974) suggest that the oxalic acid content of *Amaranthus hybridus* ssp. *incurvatus* might restrict its use to ruminants.

Atriplex species have been used as forage for as long as there have been leases on Atriplex rangeland. *Atriplex nummularia* (old man saltbush) had been introduced into California for pastoral purposes by 1898 (Jones, 1969). More recently, attention has been given to the use of *Atriplex* under agronomic conditions (Malcolm, 1969; Goodin and McKell, 1971). There is a wide range of *Atriplex* species to assess for these purposes, and most species probably contain many ecological strains. For instance *A. lentiformis* in western U.S.A. exists in forms adapted to the desert or to the lower temperatures of the coast (Pearcy and Harrison, 1974). But the pa-

latability of *Atriplex* forage is low and its salt content is a problem. The low palatability has in fact protected natural *Atriplex* communities from excessive damage from grazing in some cases. In Australia after rain, *Atriplex* is bypassed and the more palatable short-lived species are eaten (Perry, 1970). However, the chisel-toothed kangaroo rat of the Great Basin in California contrives to eat *Atriplex confertifolia* leaves without taking in the salt. The rat uses its lower incisors to shave off the epidermis bearing the salt bladders, eating what remains (Kenagy, 1973).

There is a need for more agronomic knowledge of the C₄ forage available in tropical and subtropical climates. At present, the greater potential for primary production of these areas cannot be maintained at secondary level because of the poor quality of the forage.

5.3. Plant Breeding

C₄ plants have attracted the attention of plant breeders interested in greater productivity both generally and for the arid zones. Prasad (1973) included C₄ characteristics, deep rootedness, and other features as conducive to consistent performance under water stress in his outline of desirable plant types for breeding programs. Krenzer and Moss (1969) have surveyed the relatives of small grain plants for C₄ characters but without success. Hall (1972) searched unsuccessfully for wild C₄ species of *Beta*, looking for suitable breeding material with the intention of selecting coordinated C₄ progeny once a C₄ cross was introduced into the agricultural species, even if the early progeny yielded less than the parents.

Photosynthetic capacity cannot be assessed visually, and so, as Moss (1970) suggests, it has been neglected in selection programs. Further, as Hanson (1971) showed in *Zea mays* strains of varying productivity, leaf photosynthetic rates are not necessarily correlated with agricultural productivity. At the chloroplast level there was even less connection between photosynthetic rate and productivity (Hanson and Grier, 1973). Similarly Carlson *et al.* (1970) found no positive relation between net leaf photosynthetic rate and pasture production. C₄ isoenzymes, such as those of PEP carboxylase, may be attractive as survey material in progeny, but it has not been shown that their presence is correlated with yield. Methods of mass survey for low compensation point (Goldsworthy and Day, 1970), δ_{13C} ratios (Bender, 1971), or growth in the presence of high O₂ (Heichel, 1973) have been proposed.

It is suggested that the high productivity of C₄ plants may be connected largely with low photorespiration (Marx, 1973; Zelitch, 1973b). Surveys have been made with a view to finding plants with low photorespiration (Ze-

litch and Day, 1973), largely without success. However, inhibitors of photorespiratory reactions have been identified (Zelitch, 1974) and it may be possible with chemical treatment to raise the productivity of C₃ species.

Artificial selection has modified high-yielding characters of some C₄ species. American grain sorghum cultivars had reduced yields under high-temperature regimes as a result of floral and embryo abortion (Downes, 1972). It is apparent that selection for leaf productivity cannot be made in isolation from all other physiological processes, not only of the single plant but of plants competing as a crop. In this respect each individual crop species requires a detailed study such as that of Wallace *et al.* (1972) for *Phaseolus* and Yoshida (1972) for *Oryza sativa* (rice). These studies indicate the many factors which contribute to the economic productivity of a plant, and such knowledge will be a vital factor in future approaches to plant breeding and husbandry.

6. CONCLUSIONS

Although an urgent need for an increase in the yield of plant products is apparent worldwide, there are a number of factors, not related to the net productivity of the plant itself, which must be borne in mind. In many situations, the choice of crop is not determined by the potential yield, but by custom, and assuming present cultures and technology remain dominant, food production is likely to remain based on currently used species. As a source of raw material for synthetic processes, cost per unit rather than overall productivity of the plant will be the determining factor in selection of raw material. Nonetheless, increasing demands for food alone necessitate a careful evaluation of those plants presently cultivated, in order to determine how maximum productivity may be obtained. Even at this stage the relationship between leaf productivity and economic yield are not clearly defined.

We have outlined some of the important properties of plants which utilize the C₄ pathway of photosynthesis and have compared them with those of C₃ plants. Although C₄ plants possess some real advantages in terms of net photosynthesis at the level of the individual leaf, these advantages are largely dissipated at the crop level. Because of their more efficient water use, C₄ plants possess an inherent advantage over C₃ plants in some areas, but to offset advantages in light-harvesting and water use, many commercially important C₄ plants are sensitive to low temperatures, which at present limits their distribution in nontropical areas. Conversely some C₃ plants are susceptible to periods of relatively high temperature.

The elimination of photorespiration from C₃ plants is a mechanism by

which growth rates and productivity could be greatly increased: C₄ plants appear to have achieved this step. Photorespiration seems to be a wasteful process, but we do not yet understand its importance in the production of metabolic intermediates such as glycine and serine or how the inhibition of photorespiration will affect the plant over its whole growth cycle.

It appears that if C₄ plants do prove to have distinct advantages over C₃ plants, then these advantages will lie firstly in their ability to exist in a range of climates which are not conducive to the growth of C₃ plants. Secondly, Gifford (1974) has concluded that the difference in annual productivity of C₃ and C₄ plants (as distinct from that of individual plants under laboratory conditions) is due to the extended growing season which operates in those regions where C₄ plants are grown. If we accept, then, that certain species will grow better in a particular environment, it is clear that the evolutionary differences between C₄ and C₃ plants have already produced a selective distribution, which has resulted in increased natural productivity for both types. Hopefully, our newly gained biochemical and physiological knowledge can be used to increase further that productivity.

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Phycocyanins: Structure and Function

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1. INTRODUCTION

The presence of lamellar structures (thylakoids) is a common feature of the ultrastructure of cells performing oxygen evolving photosynthesis. In eukar-

yotic cells, the photosynthetic lamellae are contained within chloroplasts. In addition to protein-chlorophyll complexes, the thylakoids contain cytochromes, ferredoxin, plastocyanin, carotenoids, various quinones, and a high proportion of galactosylglycerides, phospholipids, and sulfolipids. The bulk of the chlorophyll within the thylakoid serves as a light-harvesting (or antenna) pigment. The trapped light energy is transferred from the antenna pigment to specialized protein-chlorophyll complexes—the “reaction centers”—at which it is converted to chemical energy. The reaction center chlorophylls represent a very small proportion (< 1%) of the chlorophyll within the thylakoid.

In green plants and green algae, the antenna pigments include protein complexes of both chlorophyll *a* and *b*. Some contribution is also made by the carotenoids. Light in the range 500–600 nm is not absorbed effectively by the pigments present in these organisms. Thus, approximately one-third of the visible spectrum is not exploited for photosynthesis. Other classes of algae, however, have accessory pigments that absorb strongly in the range 500–600 nm. The light energy trapped by these pigments is transferred to chlorophyll *a* for utilization in photosynthesis.

Typical accessory pigments include chlorophyll *c*₁ and *c*₂ in eight classes of eukaryotic algae [for a review, see Meeks (1974)], fucoxanthin (Jensen, 1964; Mann and Myers, 1968) in diatoms and dinoflagellates, peridinin (Strain *et al.*, 1971) in the dinoflagellates, and the phycobilin-containing proteins (phycobiliproteins) of the prokaryotic blue-green algae (Cyanophyta), the eukaryotic red algae (Rhodophyta), and of the eukaryotic cryptomonads (Cryptophyceae). Organisms containing these accessory pigments do not contain chlorophyll *b*. At the present time, the phycobiliproteins are the most extensively studied of the extrathylakoidal photosynthetic accessory pigments.

As indicated in Table 1, the prosthetic groups of the phycobiliproteins are open-chain tetrapyrroles. Cyanophytan and rhodophytan phycobiliproteins contain either phycocyanobilin or phycoerythrobilin (or both) as prosthetic groups (Siegelman *et al.*, 1968) (see Fig. 1, for structures). The exceptions are B- and R-phycoerythrins, where a third prosthetic group, phycourobilin, of as yet undetermined structure, has been proposed (O'Carra, 1970; Vaughan, 1964).

The carotenoid accessory pigments, fucoxanthin and peridinin (Fig. 1), though present as protein complexes within the cell, can be removed by extraction with organic solvents. In contrast, the prosthetic groups of the phycobiliproteins are covalently linked to the protein moiety.

All blue-green algae contain allophycocyanin and C-phycocyanin, while all red algae contain allophycocyanin, and R- or C-phycocyanin. Many blue-green algae and some red algae lack phycoerythrin. Of the

TABLE 1. Classification of Major Bilioproteins of Blue-Green and Red Algae^a

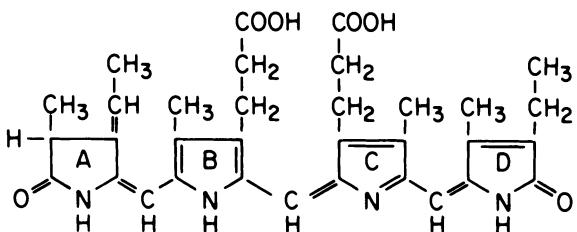
Bilioprotein	Distribution	Major absorption maxima in the visible region ^b (nm)	Fluorescence emission maxima (nm)	References
Allophycocyanin	Blue-green and red algae	650(PCB)	660	Gantt and Lipschultz, 1973
C-Phycocyanin	Blue-green and red algae	620(PCB)	648	Dale and Teale, 1970
C-Phycoerythrin	Blue-green algae	565(PEB)	577	Teale and Dale, 1970
R-Phycocyanin	Red algae	617(PCB) > 555(PEB)	636	Gantt and Lipschultz, 1973
R-Phycoerythrin	Red algae (Blue-green algae?) ^c	567(PEB) > 538(PEB) > 498(PUB)	578	Vaughan, 1964; MacDowall <i>et al.</i> , 1968
B-Phycocyanin	Red algae (Blue-green algae?)	545(PEB) > 563(PEB) > 498(S)(PUB)	575	Gantt and Lipschultz, 1973

^a The data were obtained on dilute aqueous solutions of the native bilioproteins at near-neutral pH.

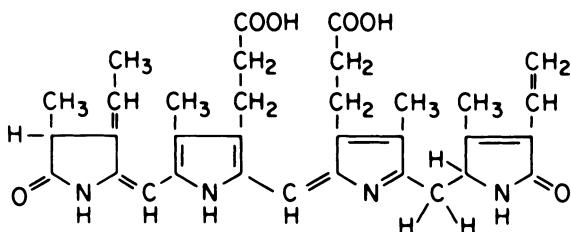
^b The abbreviations, given in parenthesis, are PCB, phycocyanobilin; PEB, phycoerythrobilin; PUB, phycourobilin; S, shoulder.

^c A phycoerythrin characterized by an absorption spectrum with peaks in the visible wavelength region at 500, 547, and 565 nm was isolated from the marine blue-green alga *Trichodesmium thiebautii* (Fujita and Shimura, 1974).

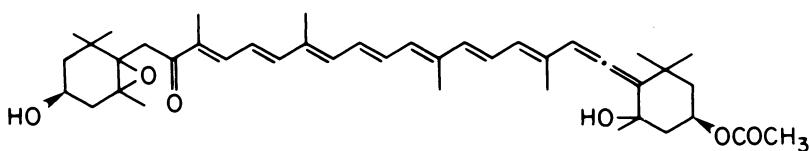
PHYCOCYANOBILIN



PHYCOERYTHROBILIN



FUCOXANTHIN



PERIDININ

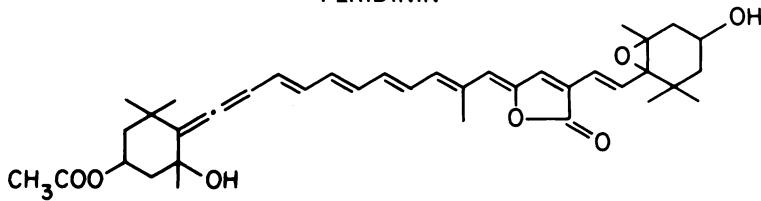


Fig. 1. Chemical structure of phycocyanobilin, phycoerythrobilin, fucoxanthin, and peridinin.

phycobiliproteins, the phycoerythrins show the greatest range of variation in spectroscopic properties (Table 1).

It is appropriate at the outset to consider the basis for the belief that the phycobiliproteins do indeed serve as photosynthetic accessory pigments and to examine briefly the parameters governing the energy transfer

process. The maxima of the absorption spectra of the major phycobiliproteins, phycoerythrin ($\lambda_{\text{max}} \sim 570$ nm) and phycocyanin ($\lambda_{\text{max}} \sim 620$ nm), are well resolved from that of chlorophyll *a* ($\lambda_{\text{max}} \sim 670$ nm). The wide separation of these absorption maxima was utilized by Emerson and Lewis (1942) in a classic demonstration of the high efficiency of utilization of energy absorbed by phycocyanin in photosynthesis in a blue-green alga of *Chroococcus* sp. Emerson and Lewis (1942) compared the quantum yield of photosynthesis (measured as O_2 evolved per incident quantum as a function of wavelength of light) at various wavelengths, with the proportions of light absorbed by the pigment components of the cell at those wavelengths. At 676 nm, where chlorophyll absorbed 94% and phycocyanin 6% of the incident radiation, the quantum yield was found to be 0.085. In the region 560–640 nm, in which phycocyanin absorbed 80% and chlorophyll only 20%, on the average, the quantum efficiency was 0.078.

Determination of the action spectra for oxygen evolution for a number of blue-green and red algae established that light trapped by phycoerythrin, as well as that trapped by phycocyanin, was utilized in photosynthesis with an efficiency approaching 100% (Haxo and Blinks, 1950; Blinks, 1964).

In intact cells of the red alga *Porphyridium cruentum*, it was demonstrated that light absorbed by phycoerythrin sensitized the fluorescence of both phycocyanin and of chlorophyll *a*, suggesting the presence of a stepwise energy transfer path from phycoerythrin to phycocyanin to chlorophyll (French and Young, 1952; Duysens, 1952). Much strong evidence in support of this view has accumulated during the past 20 years and will be alluded to later.

The mechanism of the energy transfer process was first examined by Arnold and Oppenheimer (1950). From a consideration of the absorption spectra of phycocyanin and chlorophyll, Arnold and Oppenheimer (1950) concluded that the only mechanism of importance for the transfer of energy from phycocyanin to chlorophyll was the resonance transfer of energy from one oscillator to another in resonance with it. From the observed efficiency of transfer, the most probable distance between phycocyanin and chlorophyll molecules was estimated to be 40 Å. It should be noted that the existence of allophycocyanin—a biliprotein with an absorption maximum between those of phycocyanin and chlorophyll *a*—was not recognized at the time.

It is now generally accepted that the migration of energy from the biliproteins to chlorophyll *a* does proceed by resonance energy transfer. A theoretical treatment of this form of energy migration has been developed by Förster (1948).

Absorption of a photon by a molecule leads to a promotion of the molecule from the ground state to an electronic excited state. The molecule then undergoes very rapid thermal relaxation to the lowest vibrational

sublevel of a first excited singlet state, without emission of radiation. The expression for the rate of energy transfer from the excited singlet state k_T derived by Förster (1948, 1960, 1965) may be conveniently given in the form (Haugland *et al.*, 1969):

$$k_T = R^{-6} JK^2 n^{-4} k_F (8.71 \times 10^{23}) \text{ s}^{-1}$$

Here R is the distance (in Å) between donor and acceptor chromophores (or, more precisely, the distance between the centers of the donor and acceptor transition moments). J is the spectral overlap integral, a measure of the extent to which the fluorescence spectrum of the donor overlaps the absorption spectrum of the acceptor; the dimensions of J are $\text{cm}^3 \text{ mmol}^{-2}$.* K is an orientation factor. Since the transfer is assumed to result from the coupling of dipole oscillators, the probability of transfer is at a maximum when the donor and acceptor dipoles are parallel. Dipoles at right angles have zero probability of energy transfer. For random orientation between the chromophores, the average value of K^2 is $\frac{1}{3}$. n is the refractive index of the medium between the chromophores. The medium does not affect the transfer process provided that it is transparent at the wavelength at which transfer occurs. k_F is the rate constant for fluorescence emission by the donor in s^{-1} . The transfer rate depends on the strength of the donor dipole, expressed in terms of k_F . The transfer rate is also proportional to the strength of the acceptor dipole, which is a component of the term J .

In Förster's theory, the efficiency of energy transfer, E , defined as the number of quanta transferred to the acceptor divided by the number of quanta absorbed by the donor, is given by the expression

$$E = \frac{(R_0/R)^6}{1 + (R_0/R)^6}$$

where R is the distance between the donor and acceptor chromophores and R_0 is the separation at 50% transfer efficiency.

The transfer efficiency E can be determined experimentally from sensitized fluorescence measurements, and the value of R_0 can be calculated from the donor emission spectrum and the acceptor absorption spectrum. Detailed treatment of the Förster theory may be found in the original publications (Förster, 1948, 1960, 1965), and a brief consideration in an article by Brand and Witholt (1967).

The R^{-6} dependence predicted by Förster's theory has been decisively confirmed in experiments on model systems (Stryer and Haugland, 1967;

* The spectral overlap integral J is given by $J = \int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda / \int F(\lambda)d\lambda$ where $F(\lambda)$ is the fluorescence intensity of the energy donor at wavelength λ , and $\epsilon(\lambda)$ is the molar decadic extinction coefficient (in $\text{cm}^{-1} \text{ M}^{-1}$) of the energy acceptor.

Latt *et al.*, 1965). The linear dependence of k_T on the magnitude of the spectral overlap integral J has also been clearly demonstrated on model systems (Haugland *et al.*, 1969). It is clear, therefore, that the Förster theory does describe satisfactorily the dependence of singlet-singlet energy transfer on spectral overlap and interchromophore distance.

Both the rate and efficiency of energy transfer exhibited by the phycobiliproteins *in vivo* are very high (Table 2). The evolution of these proteins has evidently culminated in molecules which serve as excellent traps of light energy and which also fulfill optimally the spectral overlap, chromophore separation, and orientation requirements of dipole-dipole interaction-dependent energy transfer. One of the major aims of this review is to consider the molecular mechanisms whereby this optimization has been achieved.

2. INTRACELLULAR LOCALIZATION OF BILIPROTEINS IN BLUE-GREEN AND RED ALGAE

Phycobiliproteins are a major constituent of blue-green and red algal cells. In some organisms, e.g., *Anacystis nidulans*, they may represent up to 40% of the cell protein (Myers and Kratz, 1955). The very high concentration of these proteins permits efficient trapping of photons even at low light intensities.

Examination of thin sections of glutaraldehyde-fixed red algal cells revealed the presence of a regular array of granules, *phycobilisomes*, on the outer membrane surface of the chloroplast lamellae (Gantt and Conti, 1966a,b; 1969). This finding is illustrated in Fig. 2, which shows a representative thin section of the unicellular red alga *Porphyridium*

TABLE 2. Excitation Energy Transfer between Pigments in Algal Cells^a

	Transfer time $\tau_t \pm 0.2 \text{ m}\mu\text{s}$	Transfer efficiency (%)
<i>Porphyridium cruentum</i>		
Phycoerythrin → Phycocyanin	0.3	96 ± 3
Phycocyanin → Chlorophyll <i>a</i>	0.5	78 ± 8
<i>Anacystis nidulans</i>		
Phycocyanin → Chlorophyll <i>a</i>	0.3	86 ± 8

^a The algae were in the dark-adapted state during the experiment (Tomita and Rabinowitch, 1962).

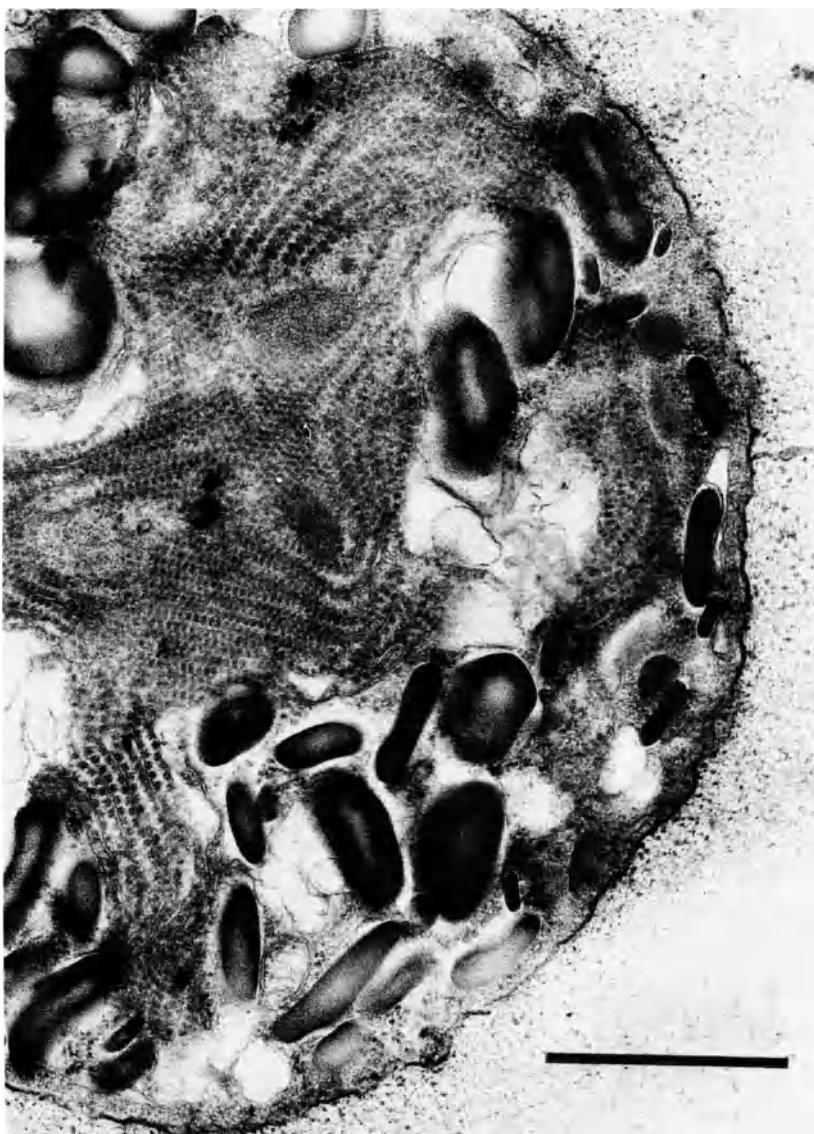


Fig. 2. Electron micrograph of a thin section of a cell of *Porphyridium cruentum*. Note orderly arrangement of granules along the chloroplast lamellae. A regular two-dimensional spacing of the granules (phycobilisomes) is evident in a grazing section over several lamellae. Fixation was in 4% glutaraldehyde in 0.1-M phosphate buffer, pH 6.8, followed by 1% OsO₄. Section was stained with 1% uranyl acetate and basic lead hydroxide after embedding in Epon 812. The bar represents 1 μ m. (Courtesy of E. Gantt.)

cruentum. Similar structures have now been demonstrated in a large number of blue-green algae (see Fig. 3, for example) (Gantt and Conti, 1969; Cohen-Bazire, 1971; Edwards and Gantt, 1971; Wildman and Bowen, 1974) and other red algae (Lichtlé and Giraud, 1970), both in thin sections and by freeze-etching (Lefort-Tran *et al.*, 1973). This striking cytological feature was only found in algae which contained phycobiliproteins.

The inference that the granules do indeed represent the intracellular

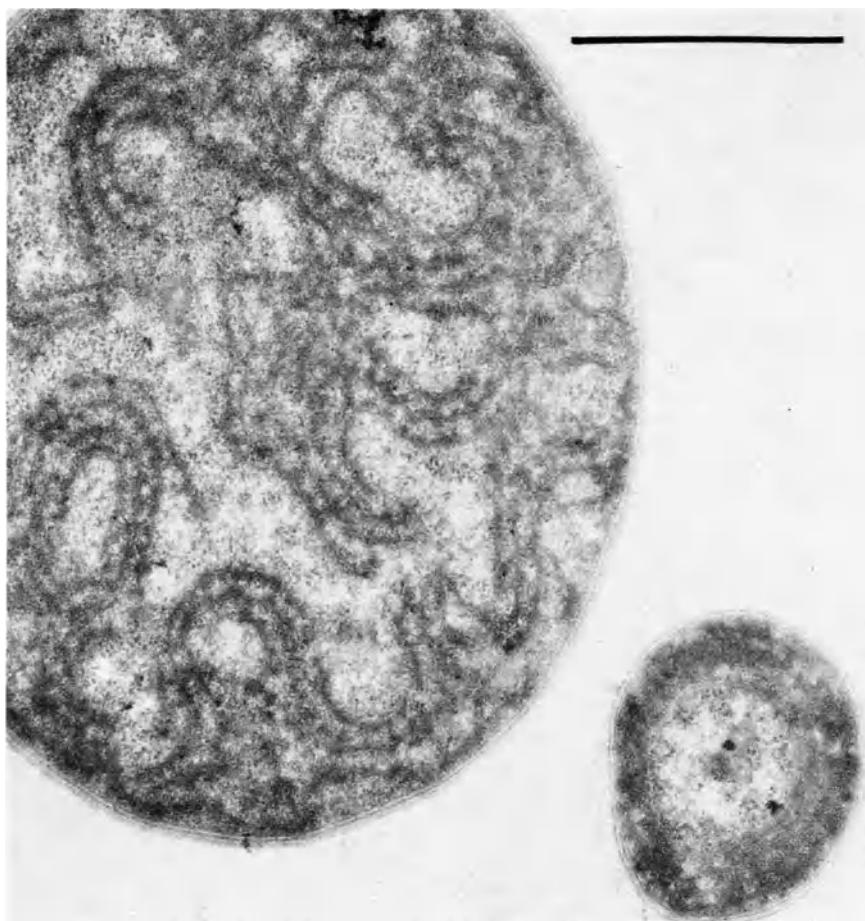


Fig. 3. Electron micrograph of a thin section of *Anabaena* sp., strain 6411, and, at lower right corner, *Synechococcus* sp., strain 6301 (Stanier *et al.*, 1971). Section stained with $\text{KMnO}_4\text{-Pb}$ after embedding in Vestopal. Fixation was in 1% glutaraldehyde followed by 1% OsO_4 . The bar represents 1 μm . (Courtesy of M. and F. E. Eiserling.)

site of phycobiliprotein localization was strongly supported by the demonstration that brief treatment of intact red and blue-green algal cells with glutaraldehyde resulted in the fixation of a high proportion of the phycobiliproteins to the chlorophyll-containing membranes (Cohen-Bazire and Lefort-Tran, 1970). Under these conditions, it was noted that all of the allophycocyanin, but only part of the phycocyanin, was fixed to the thylakoid membrane.

Gantt and her co-workers (Gantt and Lipschultz, 1972; Gray *et al.*, 1973) have developed procedures for the isolation of phycobilisomes. These involve the disruption of algal cells by passage through a French pressure cell in the presence of high salt at pH 7.0, followed by a treatment of the broken cell suspension with the nonionic detergent Triton X-100. The phycobilisomes are then separated by centrifugation on a sucrose step gradient.

Porphyridium cruentum phycobilisomes prepared in this manner appeared as irregularly shaped particles characterized by a long axis of 400–500 Å and a short axis of 300–320 Å (Gantt and Lipschultz, 1972). The phycobilisomes contain each of the phycobiliproteins present in the red algal cell and are relatively stable in the presence of high salt concentrations (0.5 M phosphate, pH 7.0). Slow disruption of phycobilisomes takes place upon decrease in ionic strength of the buffer. Under such conditions, phycoerythrin appears to be the first biliprotein to dissociate from the particle.

Phycobilisomes were also prepared from a blue-green alga of *Nostoc* species by a similar procedure (Gray *et al.*, 1973). All three biliproteins (phycoerythrin, phycocyanin, and allophycocyanin) present in this organism were found in the isolated particles. *Nostoc* phycobilisomes appeared to be globular with a flattened base, about 40 nm in diameter, at the attachment site to the photosynthetic lamellae. The latter conclusion was based on the study of the ultrastructure of vesicle preparations, obtained in the absence of detergent, in which particles believed to be phycobilisomes were attached to the surface of the membranes.

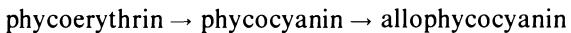
Detailed information on the ultrastructure of phycobilisomes and on the distribution of the individual biliproteins within these particles is lacking. It is possible that the isolation procedure may cause some loss of order in the structure.

Parenthetically, it may be noted that identification of phycobilisomes in thin sections of blue-green algal cells is not always unambiguous. For example, cells of *Anacystis nidulans* contain large numbers of granules (350–700 Å), which upon negative staining appear to be structurally identical with glycogen α -granules. Differentiation between these structures and phycobilisomes is not straightforward (Eiserling and Glazer, 1974).

The distribution of phycobilisomes on the thylakoid surfaces is strikingly regular (see Figs. 2 and 3, for example). The phycobilisome distribution presumably reflects the presence of an ordered array of attachment sites at (or near) the outer membrane surface of the thylakoids. This conjecture is supported by ultrastructural studies on freeze-etched preparations of blue-green and red algal cells (Neushul, 1970, 1971; Lefort-Tran *et al.*, 1973). The fracture surfaces of the thylakoid membranes reveal a regular pattern of granules which appears to be closely correlated with the arrays of phycobilisomes on the outer surface of the thylakoids. Measurements of action spectra show that the phycobiliproteins funnel energy almost exclusively into photosystem 2, and it is tempting to speculate that the granules within the thylakoids, seen in the freeze-etched preparations, reflect the location of photosystem 2 within the photosynthetic lamellae.

As noted above, both the phycobilisomes of *Nostoc* sp. and those of *Porphyridium cruentum* contain several biliproteins. By the measurement of sensitized fluorescence, it is possible to assay these particles for the integrity of the energy transfer process. In both instances, it is found that excitation of phycobilisomes with light of 545 nm, absorbed almost exclusively by phycoerythrin(s), results in emission principally at 675–680 nm, which indicates high efficiency of transfer to allophycocyanin. Upon dissociation of phycobilisomes, emission is observed almost exclusively at 575 nm, the emission maximum of phycoerythrin (Gantt and Lipschultz, 1973; Gray *et al.*, 1973).

These observations taken together with the cross-linking experiments on intact cells (Cohen-Bazire and Lefort-Tran, 1970) and the dissociation studies on phycobilisomes (Gantt and Lipschultz, 1972), support the view that the major path of energy transfer within the phycobilisomes is



and thence to chlorophyll *a*, and that the spatial distribution of the pigments follows the same sequence, i.e., phycoerythrin is the most distant from the membrane surface, whereas allophycocyanin is in close proximity to it.

The conclusion derived from the above studies is that *in vivo* all of the phycobiliproteins are assembled in particles which are attached to the chlorophyll-containing membrane. Moreover, the organization of the proteins within the phycobilisome appears to be consistent with the arrangement indicated by the spectroscopic properties of the individual proteins. This ultrastructural organization at the level of the macromolecular aggregate reflects the requirement to minimize the donor-acceptor separation imposed by the R^{-6} dependence of the rate and efficiency of the energy transfer process.

As might be expected from their location, the presence of phycobilipro-

teins does not appear to be essential to the maintenance of photosystem 1 and 2 activity. Phycobilisomes are a prominent feature of the chloroplast of *Cyanidium caldarium* (Seckbach and Ikan, 1972). A mutant of this organism, lacking both C-phycocyanin and allophycocyanin, is a photoautotroph, and a mutant lacking C-phycocyanin appears to carry out normal photosynthesis (Nicol and Bogorad, 1962; Volk and Bishop, 1968). Arnon *et al.* (1974) have demonstrated that membrane fragments of *Nostoc muscorum* could be almost totally depleted of phycocyanin, with retention of photosystem 1 and photosystem 2 activity.

From two recent studies, it appears, however, that *in vivo* drastic changes in the content or relative amount of the biliproteins have major morphological consequences.

Transfer of fluorescent light-illuminated cultures of the filamentous blue-green alga *Fremyella diplosiphon* to red light causes an abrupt cessation of C-phycoerythrin synthesis. Cells grown in red light are morphologically distinct from those grown in fluorescent light (Bennett and Bogorad, 1973). A change in the accessory photosynthetic pigment composition in response to alterations in the energy distribution in the available light is widespread among blue-green and red algae and is known as complementary chromatic adaptation.

A change in the level of biliproteins may be achieved by growing blue-green algae in the light in the presence of CO₂ and the absence of a nitrogen source. Under these conditions the phycobiliproteins are destroyed, but the viability of the cells is maintained and the chlorophyll content remains high (Allen and Smith, 1969). Lemasson *et al.* (1973) have demonstrated a major difference between the action spectra of such depleted cells and normal cells. In the cells with normal complement of biliproteins, the action spectrum shows little contribution from chlorophyll *a* to the photosynthetic oxygen evolution, whereas in the depleted cells, the action spectrum demonstrates that chlorophyll *a* is functioning as the principal light-harvesting pigment. Lemasson *et al.* (1973) conclude that "the path of energy transfer to photosystem 2 in cyanobacteria can undergo major physiologically induced changes."

3. PHYCOCYANINS

The phycobiliproteins have been the subject of intense study for many years. Much of this work has centered on the distribution and biological function of these proteins, their aggregation properties, and the chemistry of the phycobilins—the tetrapyrrole prosthetic groups. Numerous articles have dealt with these aspects—most recently those of Halldal (1970), Chapman

(1973), and Goodwin (1974). The aim of this review is to examine the properties of a single biliprotein, C-phycocyanin, in detail, with particular attention to those features of the structure of the molecule which relate to its function in the transduction of light energy. A subsidiary aim is to point out those structural features common to biliproteins as a class and those which may provide insight as to the evolutionary origins of this colorful and varied array of macromolecules. To this end, the properties of R-phycocyanin and cryptomonad phycocyanin are also discussed.

3.1. C-Phycocyanin

3.1.1. Structure of the Chromophores

The reviews of Siegelman *et al.* (1968) and of Rüdiger (1970) give very good accounts of the structural studies on the phycobilins. Older work has been summarized by O'hEocha (1962).

A single type of bilitriene pigment is covalently bound to C-phycocyanin. The chromophores may be removed from the protein by a number of procedures including hydrolysis with concentrated hydrochloric acid at room temperature, methanolysis upon refluxing with 90% neutral methanol, and, most recently, cleavage with hydrogen bromide in trifluoroacetic acid at 75°C (Schram and Kroes, 1971). The structure of phycocyanobilin, shown in Fig. 1, is consistent with the results of NMR studies as well as with the products obtained upon chromic acid oxidation of the chromophore (Crespi *et al.*, 1967, 1968; Cole *et al.*, 1967a,b; Rüdiger, 1970). The molecular weight of phycocyanobilin based on this structure is 586. However, the mass spectrum of phycocyanobilin gives a molecule ion at 588 (Crespi *et al.*, 1967; Schram and Kroes, 1971).

On the basis of this fact, and infrared spectroscopy, Schram and Kroes (1971) have proposed a structure for phycocyanobilin which contains two more hydrogens than that shown in Fig. 1. This issue is not resolved, but it is clear that the substituents on the rings are as shown in Fig. 1, and that phycocyanobilin is indeed a bilitriene (Crespi *et al.*, 1967; Cole *et al.*, 1967, 1968) of the IX α configuration (O'Carra and Colleran, 1970).

3.1.2. Nature of the Protein-Chromophore Linkage

Chromopeptides have been isolated after extensive proteolysis of *Phormidium luridum* C-phycocyanin with Nagarse (Crespi and Smith, 1970), and of *Mastigocladius laminosus* C-phycocyanin with pepsin (Byfield and

Zuber, 1972). In both instances, the evidence appears consistent with the presence of a thioether linkage between the protein and the chromophore. Crespi and Smith (1970) proposed that phycocyanobilin is doubly linked to the polypeptide chain, one bond being an ester involving the β -carboxyl group of an aspartyl residue and the hydroxyl group of the enol form of ring A of the bilin (see Fig. 1), and the other bond a thioether derived from a cysteine side-chain to the methine carbon of the ethyldene group at position 2 of ring A of phycocyanobilin.

This proposal is an attractive attempt to provide a rational explanation for the observed stability characteristics of the apoprotein–bilin linkage and the amino acid composition of the chromopeptides. It may be noted that these studies were performed before the presence of three phycocyanobilin moieties per phycocyanin monomer had been recognized (Glazer and Fang, 1973a).

On the basis of chromic acid oxidation studies, under nonhydrolytic conditions, Rüdiger (1970) proposed that phycocyanobilin was linked to the apoprotein through two bonds, one involving ring A, the other either ring B or C (Fig. 1).

Whereas the postulation of a thioether linkage to the chromoprotein is supported by the available data, little can be said about the presence or absence of a second linkage. It should be recognized that an assumption that all three bilin moieties in phycocyanin are covalently linked in an identical manner has no experimental basis, either *pro* or *con*. There is likewise no compelling reason to assert that the bilins are linked to allophycocyanin and C-phycoerythrin in the same manner as to C-phycocyanin.

The presence of either a single thioether bond (Pettigrew, 1972, 1973; Lin *et al.*, 1973), or two thioether linkages from the protein to the heme is unambiguously established in cytochromes *c* from a wide range of organisms (Margoliash and Schejter, 1966). In a chromopeptide isolated from phytochrome, the bilin appears to be linked to the polypeptide through a thioether linkage (Fry and Mumford, 1971). The chromophore of phytochrome is a bilitriene closely related to phycocyanobilin (Siegelman *et al.*, 1968).

3.1.3. Subunit Structure of Phycocyanins

The demonstration that allophycocyanin, C-phycocyanin, and C-phycoerythrin all consist of two dissimilar polypeptide chains, α and β , is comparatively recent (Glazer and Cohen-Bazire, 1971). This generalization has been found to hold true for all cyanophytan and rhodophytan phycobiliproteins which have been subjected to careful study (Vaughan,

1964; Bennett and Bogorad, 1971; O'Carra and Killilea, 1971; Binder *et al.*, 1972; Torjesen and Sletten, 1972; Kobayashi *et al.*, 1972; Gantt and Lipschultz, 1974; Gysi and Zuber, 1974). The sole possible exception known to date is the "big" phycoerythrin of *Porphyridium cruentum*, which appears to have a more complex subunit structure (Gantt and Lipschultz, 1974).

The subunit molecular weights of diverse cyanophytan, rhodophytan, and cryptophytan phycocyanins are listed in Table 3 (see also Fig. 4). The estimates of molecular weight given in Table 3 probably have a precision of $\pm 10\%$. Greater divergence appears to exist between the molecular weights of the α than of the β subunits. Of the cyanophytan proteins examined, the α subunits of *Phormidium luridum* and of *Oscillatoria aghardii* C-phycocyanins have the lowest molecular weights. Both of these organisms belong

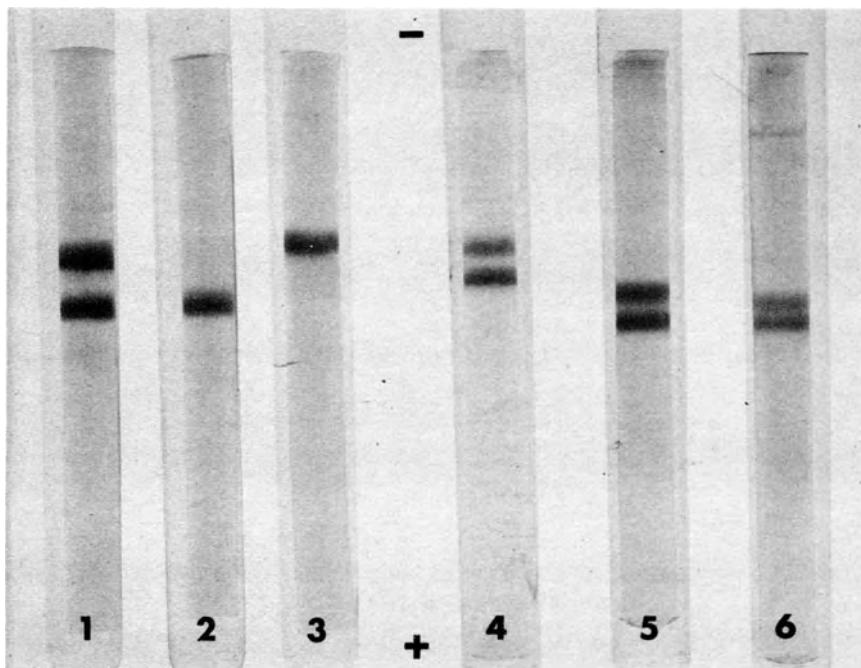


Fig. 4. Polyacrylamide gel electrophoresis, in presence of sodium dodecyl sulfate at pH 7.0, of phycocyanins from unicellular and filamentous blue-green algae and red algae. Gel 1, *Anacystis nidulans* C-phycocyanin, gels 2 and 3, respectively, the α and β subunits of *Anacystis nidulans* C-phycocyanin separated by chromatography in urea solution; gel 4, *Anabaena* sp., strain 6411, C-phycocyanin; gel 5, *Cyanidium caldarium* C-phycocyanin; gel 6, *Porphyridium cruentum* R-phycocyanin. Duration of electrophoresis was different for each of gels 4, 5, and 6, and hence the relative mobility of the bands cannot be compared directly.

TABLE 3. Subunit Molecular Weights of Cyanophytan, Rhodophytan, and Cryptophytan Phycocyanins

Protein	Organism	Subunit α	Molecular weight β	Reference
Blue-green algae				
C-phycocyanin	<i>Synechococcus</i> sp. 6301 (<i>Anacystis nidulans</i>)	17,700	19,000	Glazer and Fang, 1973a
	<i>Aphanocapsa</i> sp. 6701	18,500	20,500	O'Carra and Killilea, 1971
	<i>Phormidium luridum</i>	16,600	20,200	Glazer and Cohen-Bazire, 1971
	<i>Oscillatoria agardhii</i>	11,900	18,500	Kobayashi <i>et al.</i> , 1972
	<i>Anabaena</i> sp. 6411	12,200	14,100	Torjesen and Sleitien, 1972
	<i>Anabaena variabilis</i>	17,100	19,000	Glazer and Fang, 1973b
	<i>Anabaena cylindrica</i>	18,500	20,500	O'Carra and Killilea, 1971
	<i>Nostoc punctiforme</i>	18,500	20,500	O'Carra and Killilea, 1971
	<i>Tolyphothrix tenuis</i>	16,300	17,600	Bennett and Bogorad, 1971
	<i>Fremyella diplosiphon</i>	16,300	17,600	Bennett and Bogorad, 1971
	<i>Calothrix scopulorum</i>	18,500	20,500	O'Carra and Killilea, 1971
	<i>Mastigocladus laminosus</i>	14,000	14,000	Binder <i>et al.</i> , 1972
Red algae				
R-phycocyanin	<i>Cyanidium caldarium</i>	15,500	18,300	Troxler <i>et al.</i> , 1974
	<i>Porphyridium cruentum</i>	16,400	18,400	Gantt and Lipschultz, 1974
Cryptomonads				
Phycocyanin	<i>Hemiselmis virescens</i>	10,000	16,000	MacColl <i>et al.</i> , 1973

to the *Oscillatoriaceae*. This suggests that examination of the sequences of the α subunits may provide useful information on taxonomic relationships among the cyanobacteria.

3.1.4. Separation of the α and β Subunits of C-Phycocyanins

The decomposition of the chromophores of phycocyanin to a variety of colored products, upon denaturation at neutral or mildly alkaline pH, limited the parameters which could be varied in attempts to separate the subunits with intact chromophores. Preparation of subunits which had not suffered chemical modification is a prerequisite to any attempts at renaturation or reconstitution. The most successful procedure employed to date involves chromatography of the subunits at pH 3.0 on the cation-exchange resin Bio-Rex 70 in urea solution in the presence of reducing agent (Glazer and Fang, 1973a,b) (Fig. 4, gels 1-3). Subunits isolated in this manner have been successfully "renatured" and used in reconstitution experiments.

3.1.5. Amino Acid Composition of C-Phycocyanins and of Their α and β Subunits

The amino acid composition of a number of C-phycocyanins is presented in Tables 4 and 5. Examination of these amino acid compositions reveals a number of striking features. The first is the remarkable overall similarity in the amino acid composition of proteins derived from taxonomically unrelated organisms. The second feature is the very high content of aliphatic and acidic residues. Alanine is present in the highest amount and represents roughly one in every six residues; aspartic acid (plus asparagine), about one in ten residues, in each of the proteins examined (Tables 4 and 5). These two residues together with glycine, leucine, glutamic (and glutamine), and serine represent approximately 50% of the amino acid composition.

The amino acid composition of the α subunit also reveals some interesting features. The α subunits of *Synechococcus* sp., *Aphanocapsa* sp., *Anabaena* sp., *Phormidium luridum*, and *Spirulina maxima*, all contain one histidine residue. In contrast, the β subunits of *Mastigocladus laminosus* and of *Oscillatoria aghardii* C-phycocyanins exhibit this feature. This suggests that the assignment of the designation α and β to the subunits of these proteins may have to be reversed when more is known of their amino acid sequence and/or chromophore content (see below).

TABLE 4. Amino Acid Compositions of C-Phycocyanins, and of Their α and β Subunits, of Unicellular and Filamentous Blue-Green Algae^{a,b}

Amino acid	<i>Synechococcus</i> sp. strain 6301			<i>Aphanocapsa</i> sp. strain 6701			<i>Anabaena</i> sp. strain 6411		
	$\alpha\beta$	α	β	$\alpha\beta$	α	β	$\alpha\beta$	α	β
Lysine	13	7	5	12	7	4	11	5	6
Histidine	1	1	0	1	1	0	3	2	0
Arginine	20	7	13	18	7	11	17	7	10
Aspartic acid	42	18	21	34	15	19	34	12	23
Threonine	19	10	9	21	11	11	19	10	9
Serine	24	12	11	27	15	12	21	9	11
Glutamic acid	22	11	11	23	12	11	29	14	15
Proline	11	6	4	10	5	4	11	5	5
Glycine	27	13	13	23	12	11	25	12	13
Alanine	59	25	32	51	22	30	44	21	25
Half-cystine ^c	1.3	n.d. ^d	n.d.	2.5	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	21	8	13	18	6	11	19	5	16
Methionine	3	0.5	3	5	1	4	3	0.2	3
Isoleucine	17	7	10	17	8	10	16	8	9
Leucine	31	16	14	25	13	14	27	12	15
Tyrosine	15	10	5	15	9	6	13	7	5
Phenylalanine	12	6	6	11	6	5	7	3	4
Tryptophan	1	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

^a Data from Glazer and Fang, 1973b.

^b These organisms have been described by Stanier *et al.* (1971).

^c Determined as cysteic acid after performic acid oxidation.

^d n.d. = not determined.

3.2. Physical Properties of C-Phycocyanins

3.2.1. Aggregation Properties

On rupture of algal cells, the phycobiliproteins are released as water-soluble high molecular weight aggregates, the precise size of which depends on the organism under study and on the conditions of isolation, including pH, ionic strength, temperature, protein concentration, and the presence or absence of nonionic detergent (O'hEocha, 1966; Hattori *et al.*, 1965; Craig and Carr, 1968; Neufeld and Riggs, 1969; MacColl *et al.*, 1971; Berns, 1971). At the high molecular weight end of the scale, the aggregates appear to represent intact (or near intact) phycobilisomes (see Section 2). On the basis of electron microscopic studies, the latter could range from about 2 to

TABLE 5. Amino Acid Compositions of C-Phycocyanins, and of Their α and β Subunits, of Filamentous Blue-Green Algae^a

Amino acid	<i>Phormidium luridum</i> ^a			<i>Spirulina maxima</i> ^b			<i>Mastigocladus laminosus</i> ^c			<i>Oscillatoria agardhii</i> ^d		
	$\alpha\beta$	α	β	$\alpha\beta$	α	β	$\alpha\beta$	α	β	$\alpha\beta$	α	β
Lysine	12	5	6	12	9	3	10	5	5	10	3	7
Histidine	2	1	0	1	1	0	2	0	2	2	1	1
Arginine	15	5	11	16	8	8	15	8	7	11	6	5
Aspartic acid	32	11	22	30	18	13	30	15	15	23	11	11
Threonine	15	6	8	17	9	7	16	7	9	13	5	8
Serine	21	9	12	24	14	11	14	8	8	16	6	9
Glutamic acid	17	7	8	27	17	10	25	12	12	19	9	12
Proline	9	3	5	11	6	5	8	3	4	7	3	4
Glycine	24	9	16	24	17	10	18	10	10	22	11	12
Alanine	47	17	30	48	25	22	42	19	20	37	21	17
Half-cystine*	1	1	1	2.25	n.d. ^f	n.d.	3	n.d.	n.d.	4	2	2
Valine	18	6	14	17	8	9	14	9	6	16	9	7
Methionine	10	3	7	9	4	4	3	2	1	5	3	2
Isoleucine	15	5	10	18	11	6	18	9	9	13	6	7
Leucine	21	8	12	26	14	11	23	11	11	20	10	10
Tyrosine	13	5	6	15	11	4	13	5	7	10	3	7
Phenylalanine	8	4	4	8	4	3	6	3	3	7	3	4
Tryptophan	1	1	0	1	1	0	1	n.d.	n.d.	n.d.	0	1

^a Kobayashi *et al.* (1972).^b Analyses on protein prepared from field material, collected at Lake Texoco, Mexico (A. N. Glazer, unpublished results).^c Analyses on protein prepared from field material, collected from a hot spring near Reykjavik, Iceland (Binder *et al.*, 1972).^d Analyses on protein prepared from field material, collected from Lake of Gjersjøen, Norway (Torjesen and Sletten, 1972).

* Determined as cysteic acid after performic acid oxidation.

^f n.d. = not determined.

10 million in molecular weight depending on the organism from which they are obtained (Gantt, 1969; Edwards and Gantt, 1971).

For C-phycocyanin, the hexameric form of the protein $(\alpha\beta)_6$ predominates at protein concentrations higher than 1 mg ml⁻¹ at ionic strengths above 0.01, in the pH range 5–6 (Hattori *et al.*, 1965; Neufeld and Riggs, 1969; Glazer and Cohen-Bazire, 1971; Saito *et al.*, 1974). Under these conditions, the major forms of the protein present appear to be the hexamer and the monomer, and the equilibrium $(\alpha\beta)_6 \rightleftharpoons 6(\alpha\beta)$ lies markedly on the side of the hexamer (Hattori *et al.*, 1965; Saito *et al.*, 1974). For *Plectonema calothricoides* C-phycocyanin in 0.01 M phosphate at pH 5.3, Hattori *et al.* (1965), from spectroscopic measurements, estimated the value of the dissociation constant for this equilibrium at 10^{-7} g-mol liter⁻¹.

At pH 7.0, for most of the C-phycocyanins examined, the major forms of the protein appear to be the trimer and the monomer, and again the equilibrium $(\alpha\beta)_3 \rightleftharpoons 3(\alpha\beta)$ favors the trimer. For *Plectonema calothricoides* C-phycocyanin in 0.01 M phosphate at pH 7.0, the $(\alpha\beta)_3 \rightleftharpoons 3(\alpha\beta)$ equilibrium was characterized by a dissociation constant of 10^{-2} g-mol liter⁻¹ (Hattori *et al.*, 1965). Some representative molecular weight data documenting the above comments is presented in Table 6.

For *Anacystis nidulans*, the major forms of the protein present at pH 7.0 were reported to be the monomer and the dimer (Neufeld and Riggs, 1969). The hexamer is the predominating species at pH 5.5 (Neufeld and Riggs, 1969; Glazer *et al.*, 1973).

The molecular weights for C-phycocyanins at pH 7.0 (Table 6) are somewhat higher than would be predicted from the subunit molecular weights (see Table 3). This suggests that small amounts of a higher aggregate (hexamer?) may be present at pH 7.0. Native phycocyanin is unstable at pH values below 4.0 and higher than 9.0.

Fresh extracts of algal cells contain phycocyanin aggregates characterized by sedimentation coefficients higher than the 11 S value characteristic of the hexamer (Table 6) (see Berns, 1971, for a review; Kessel *et al.*, 1973). Electron microscopic examination of such fresh cell extracts obtained at pH 5.2 from *Anacystis nidulans* showed the presence of both the disc-shaped phycocyanin hexamers, of 120-Å diameter (Berns and Edwards, 1965; Eiserling and Glazer, 1974), and of short rods of varying lengths consisting of stacks of the discs (Eiserling and Glazer, 1974). These presumably represent phycobilisomes in varying stages of disaggregation.

Upon purification of *Anacystis nidulans* phycocyanin, such aggregates dissociate either to the hexamer, or lower aggregates, depending on the pH. Even at high concentrations of pure phycocyanin at pH 5.5, the highest aggregate seen both in the ultracentrifuge (Glazer *et al.*, 1973; Neufeld, 1966), and by electron microscopy (Eiserling and Glazer, 1974), is the hexamer. It

TABLE 6. Molecular Weights and Sedimentation Coefficients of Some Cyanophytan and Rhodophytan C- and R-Phycocyanins

Protein	Organism	Conditions	Molecular weight	Sedimentation coefficient S , 10^{-13} (cm/s) \times (dyn/g)	References
C-Phycocyanin	<i>Synechococcus</i> sp. strain 6301 (<i>Anacystis nidulans</i>)	0.2-M acetate-0.001-M β -mercaptoethanol, pH 5.5	232,000 ^a	...	Glazer <i>et al.</i> , 1973
		0.05-M phosphate-0.001-M β -mercaptoethanol, pH 6.5	127,000 ^a	...	
		0.01-M phosphate, pH 5.3	266,000 ^b	11	Hattori <i>et al.</i> , 1965
Plectonema calothricoides		0.01-M phosphate, pH 7.0	134,000 ^b	6	
		0.05-M phosphate, pH 7.2	138,000 ^b	6.1	Hattori and Fujita, 1959
		0.03-M phosphate, pH 7.0	139,000 ^c	...	Koller and Wehrmeyer, 1974
R-Phycocyanin	<i>Tolyphothrix tenuis</i>	0.1-M phosphate, pH 7.0	103,000 ^a	6	Glazer and Hixson, 1975
	<i>Rhodella violacea</i>	0.02-M phosphate, pH 7.0	105,000 ^b	5.7	Svedberg and Lewis, 1928
	<i>Porphyridium cruentum</i>	0.01-M acetate, pH 4.6	206,000 ^b	11.1	Svedberg and Katsurai, 1929

^a Determined by high-speed sedimentation equilibrium.^b Determined from sedimentation and diffusion coefficients.^c Determined by gel filtration.

is possible, therefore, that the formation of higher aggregation forms of C-phycocyanin is a template-dependent process requiring the presence of another phycobiliprotein (e.g., allophycocyanin) *in an appropriate assembly form.*

3.2.2. Chromophore Content

Detailed analysis of the spectroscopic properties of C-phycocyanin requires knowledge of the chromophore content of the protein, as well as of the distribution of the chromophores between the subunits. In the native biliprotein, the interaction of the chromophore with the protein brings about major changes both in the position and the relative strength of its absorption bands in the visible region of the spectrum. The long-wavelength absorption band of the chromophore (at $\lambda > 600$ nm) both in native C-phycocyanin and allophycocyanin is greatly intensified at the expense of the short-wavelength absorption band (at $\lambda \sim 350$ nm). In the free phycocyanobilin, $A_{612\text{ nm}} : A_{360\text{ nm}}$ is ~ 0.5 (O'hEocha, 1965), while in monomeric C-phycocyanin this ratio is approximately 6 (Glazer *et al.*, 1973).

This alteration in the chromophore spectrum is in the main due to noncovalent interactions with the protein. Upon denaturation by any of a number of agents (urea, ethanol, acid), the absorption maxima of the protein-bound chromophore approximate closely those of the free phycobilin (for a review, see O'hEocha, 1965).

Figures 5 and 6 illustrate this phenomenon. The spectra of native allophycocyanin and C-phycocyanin differ markedly (Fig. 5). Upon denaturation in acid urea, the qualitative differences between the absorption spectra of these proteins are totally abolished (Fig. 6). As may be expected from this result, in acid urea no qualitative difference exists between the absorption spectra of the denatured α and β subunits of C-phycocyanin.

This normalization of the chromophore spectrum upon denaturation permitted the estimation of the chromophore content of the phycobiliproteins. It was determined that C-phycocyanin carries three covalently bound phycocyanobilin chromophores per monomer. Two of these chromophores are attached to the β subunit and one to the α subunit. Allophycocyanin has a single chromophore per subunit (Glazer and Fang, 1973a).

3.2.3. "Renaturation" of the α and β Subunits. Spectroscopic Properties of the Renatured Subunits

After separation by chromatography in urea at pH 3.0, the α and β subunits can be "renatured." This was achieved by gradual removal of urea

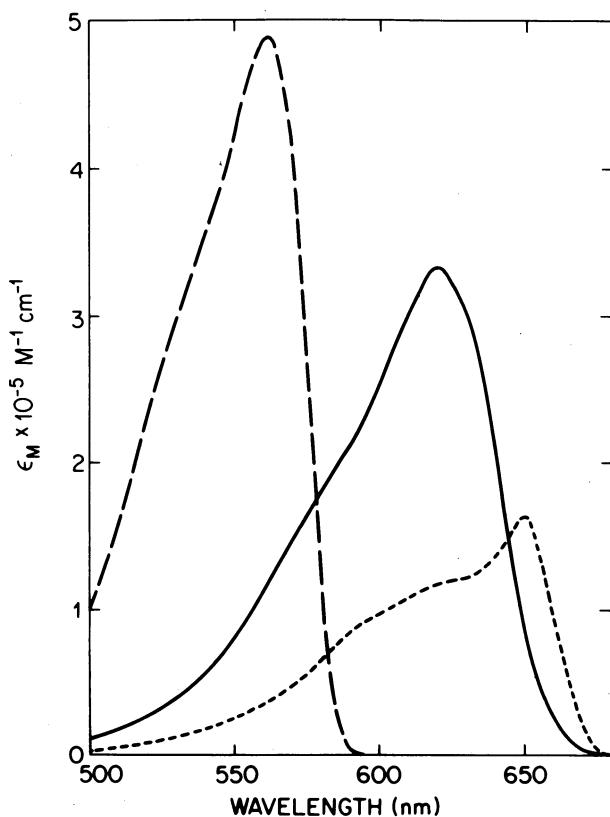


Fig. 5. Absorption spectra in the visible wavelength region of *Aphanocapsa* sp. C-phycerythrin (— — —), *Synechococcus* sp. C-phycocyanin (— — —), *Anabaena* sp. allophycocyanin (-----), all in 0.05-M ammonium acetate at pH 7.0. Molar extinction coefficients (ϵ_M) were calculated per monomer ($\alpha\beta$) molecular weight of each of the proteins.

and appropriate adjustment of pH (Glazer and Fang, 1973b). The renatured subunits give sharp bands upon isoelectric focusing in polyacrylamide gels and symmetrical peaks upon chromatography on diethylaminoethyl-cellulose (Glazer and Fang, 1973b).

Phycocyanin can be reconstituted from the α and β subunits by several procedures (Glazer and Fang, 1973b; Glazer *et al.*, 1973). The reconstituted protein is indistinguishable from the native with respect to a number of properties: chromatographic behavior, isoelectric point, absorption and circular dichroism spectra, acrylamide gel electrophoresis, and electron microscopy of reconstituted hexamers (Glazer and Fang, 1973b; Glazer *et al.*, 1973; Eiserling and Glazer, 1974).

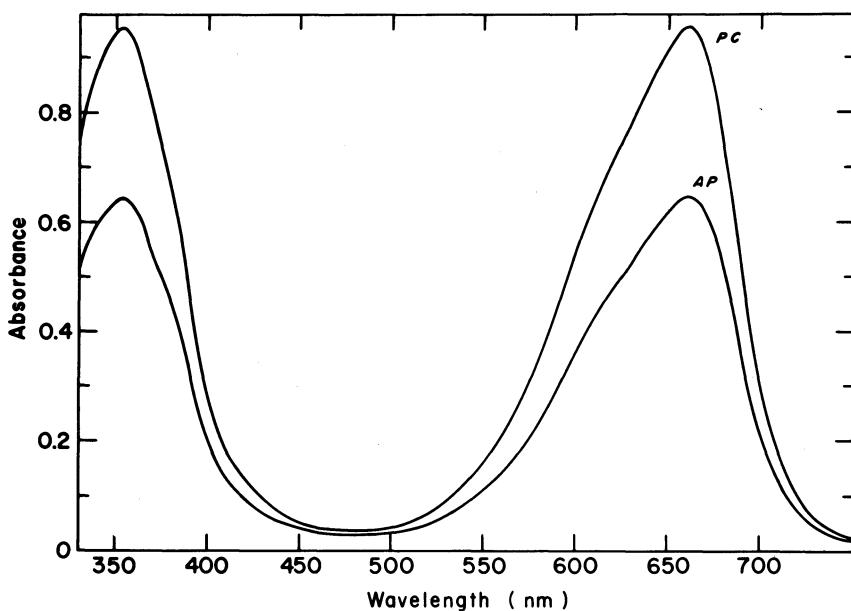


Fig. 6. Visible absorption spectra of *Anacystis nidulans* phycocyanin and allophycocyanin. Both samples were in 8-M urea adjusted to pH 3.0 with HCl. PC, phycocyanin, 0.36 mg ml^{-1} ; AP, allophycocyanin, 0.36 mg ml^{-1} . Light path length: 1.0 cm. (From Glazer and Fang, 1973a.)

The belief that the conformation of the renatured subunits is indeed very similar to that which they possess in the native protein is supported by several lines of evidence. The sum of the absorption spectra of the renatured α and β subunits at pH 7.0 is virtually superimposable upon that of monomeric phycocyanin under the same conditions (see Fig. 7 in Glazer and Fang, 1973b). Moreover, the circular dichroism spectrum, obtained as the algebraic sum of the circular dichroism spectra of the α and β subunits, agrees closely with respect to λ_{\max} values with that of native monomeric C-phycocyanin (Glazer *et al.*, 1973). These findings permit a major conclusion, i.e., that combination of the α and β subunits to form the $\alpha\beta$ monomer of phycocyanin is not accompanied by major changes in the spectra of the chromophores.

The absorption and circular dichroism spectra of the renatured subunits of *Anacystis nidulans* C-phycocyanin have been studied in detail (Glazer *et al.*, 1973). It is apparent that the chromophores are in different environments in the two subunits.

The most striking feature of the absorption spectra is the position of

the long-wavelength absorption maxima, 620 nm for the α and 608 nm for the β subunit. Other noteworthy features are the symmetrical nature of the long-wavelength circular dichroism bands and the good correspondence between the molecular ellipticity maxima, 620 nm for the α and 598 nm for the β subunit and the absorption maxima.

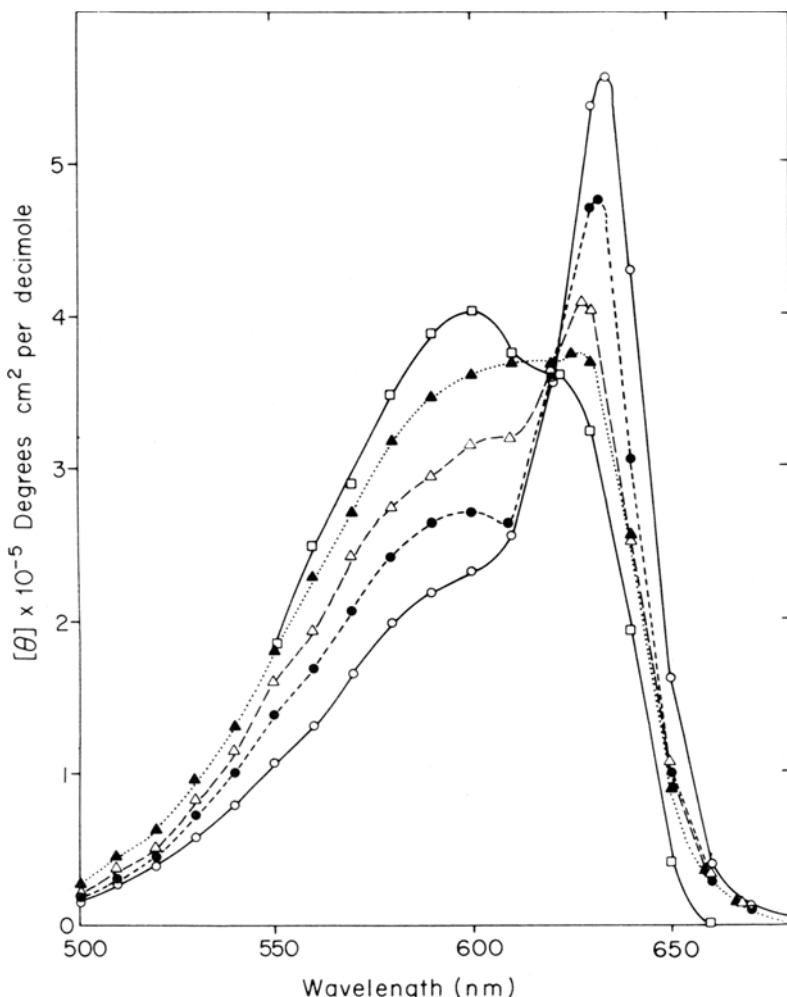


Fig. 7. Concentration dependence of the circular dichroism spectrum of phycocyanin. Spectra were determined at the following protein concentrations: $\circ-\circ$, 2.88×10^{-5} M; $\bullet-\bullet$, 1.44×10^{-6} M; $\Delta-\Delta$, 2.88×10^{-6} M; $\blacktriangle-\blacktriangle$, 1.44×10^{-6} M; $\square-\square$, 7.2×10^{-7} M, in 0.05-M sodium phosphate-0.001-M β -mercaptoethanol at pH 7.0. (From Glazer *et al.*, 1973.)

Fluorescence polarization spectra of phycocyanin reveal the presence of two classes of chromophores, those which absorb maximally in the wavelength region of 600 to 615 nm ("s" type), sensitizing those that absorb maximally at 630–635 nm and emit the energy as fluorescence at higher wavelengths (*f* type) (Teale and Dale, 1970; Dale and Teale, 1970). Dale and Teale (1970) estimated that C-phycocyanin contains twice as many *s* type as "*f*" type chromophores. From the chromophore content of the α and β subunits and their spectroscopic properties, it appears that the β subunit carries two "s"-type chromophores and the α subunit an "f"-type chromophore. In higher aggregates of C-phycocyanin, e.g., hexamer, the fluorescence is emitted by the form absorbing at the longest wavelength.

3.2.4. Spectroscopic Properties

The absorption and fluorescence spectrum, optical rotatory dispersion, and circular dichroism of phycocyanin are sensitive to the state of aggregation of the protein (Bergeron, 1963; Hattori *et al.*, 1965; Scott and Berns, 1965; Neufeld and Riggs, 1969; Dale and Teale, 1970; Glazer *et al.*, 1973). Aggregation results in a shift of the long-wavelength absorption maximum to higher wavelengths, with concomitant increase in the absorption coefficient (Hattori *et al.*, 1965; Glazer *et al.*, 1973) and marked qualitative changes in the optical rotatory dispersion (Boucher *et al.*, 1966) and circular dichroism spectra (Pecci and Fujimori, 1969; Glazer *et al.*, 1973) in this region.

A major consequence of the aggregation of C-phycocyanin is a marked increase in the oscillator strength of the long-wavelength transition and red shift of the absorption maximum. Monomeric phycocyanin at pH 7.0 is characterized by a λ_{\max} of ~ 615 nm and ϵ_M per $\alpha\beta$ of $\sim 2.3 \times 10^5$ M $^{-1}$ cm $^{-1}$, while hexameric phycocyanin at pH 5.5 exhibits a λ_{\max} of 621 nm and ϵ_M of 3.33×10^5 M $^{-1}$ cm $^{-1}$ (Glazer *et al.*, 1973). These spectroscopic changes appear to be a consequence of the interactions of chromophores of one monomer with the chromophores of others within the aggregate. Figure 7 shows the dependence of the circular dichroism spectrum of phycocyanin at pH 7.0 on protein concentration. The presence of an isobestic point at 620 nm shows that only two major spectroscopically distinguishable species contributing to the circular dichroism are present over the concentration range examined. The data presented in Fig. 7 show that aggregation results in the decrease of a pronounced circular dichroism band at 600 nm and the appearance of a new band at ~ 630 nm. This strongly suggests that on aggregation an intermolecular exciton interaction takes place between chromophores on different $\alpha\beta$ monomers.

The effect of aggregation may be seen most strikingly in Fig. 8, which presents the absorption and circular dichroism spectra of hexameric C-phycocyanin. The shape of the absorption spectrum is asymmetric, with a shoulder on the long-wavelength side of the maximum. The circular dichroism band at 600 nm is greatly diminished, and the circular dichroism spectrum is dominated by a strong band centered at 636 nm, markedly displaced from the absorption maximum.

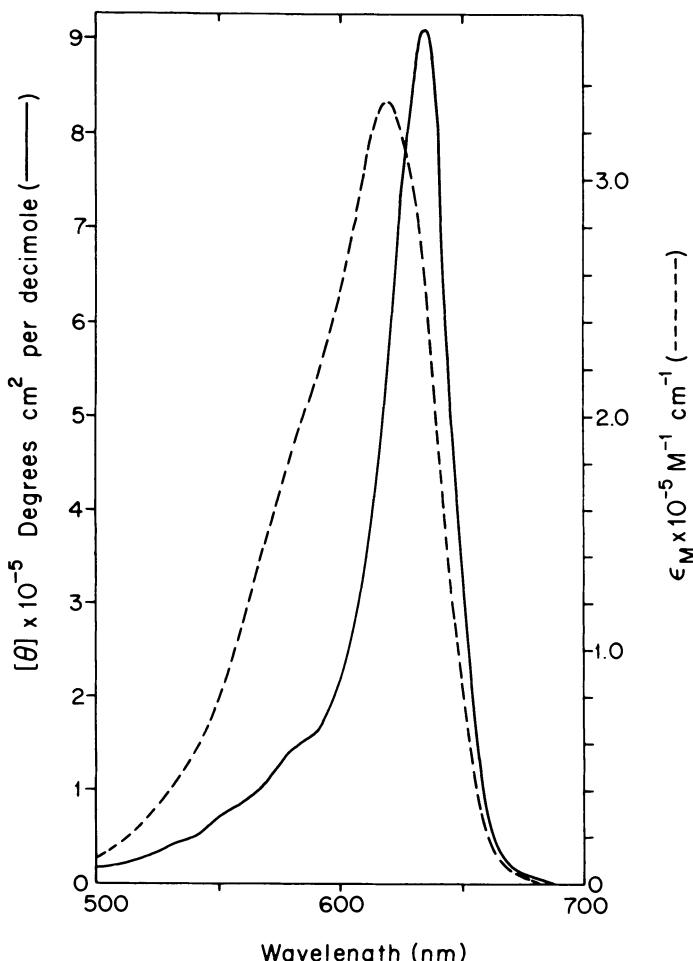


Fig. 8. Long-wavelength visible absorption and circular dichroism spectra of hexameric *Anacyanis nidulans* phycocyanin. The spectra were obtained in 0.2-M sodium acetate–0.001-M β -mercaptoethanol at pH 5.5, and a protein concentration of 2.5×10^{-5} M. (From Glazer *et al.*, 1973.)

The above observations accord well with the fact that hexameric phycocyanin shows very low fluorescence polarization. This demonstrates that energy migration within the hexamer is extremely efficient (Goedheer and Birnie, 1965; Teale and Dale, 1970; Dale and Teale, 1970).

3.3. Immunological Properties of the Phycobiliproteins

Native allophycocyanin, C-phycocyanin, and C-phycoerythrin appear to be immunologically unrelated (Vaughan, 1964; Bogorad, 1965; Bennett and Bogorad, 1971; Glazer *et al.*, 1971*a*). No cross-reactions were detected by the Ouchterlony double-diffusion technique, even when the three antigens were derived from a single algal strain and tested against the three corresponding homologous antisera (Bennett and Bogorad, 1971; Glazer *et al.*, 1971*a*).

However, within a given spectroscopically homologous class of biliproteins, immunological relationships could be readily detected between proteins obtained from both unicellular and filamentous blue-green algae and those derived from the two subclasses of the red algae: the Bangiophyceae and Florideophyceae (Bogorad, 1965; Berns, 1967; Glazer *et al.*, 1971*a*). This is strikingly illustrated in Fig. 9, which compares simultaneously the cross-reactions of C-phycocyanins and allophycocyanins from diverse cyanophytan and rhodophytan sources against a single antiserum in each instance.

It is evident that within a spectroscopically distinct class, the phycobiliproteins display a remarkable conservation of immunological properties.

Cross-reactivity is observed between R- and C-phycocyanins (Bogorad, 1965; Glazer and Hixson, 1975). The cryptomonad biliproteins (see Section 1.3.6) appear totally immunologically unrelated to those of the Cyanophyta and Rhodophyta (Berns, 1967; Glazer *et al.*, 1971*a,b*).

3.4. Hybrid Phycocyanins

The various properties of the C-phycocyanins discussed above suggest a remarkable conservation in the structure of this class of proteins. This phenomenon is notable in two respects. The blue-green algae are among the oldest organisms on earth. Microfossils morphologically indistinguishable from present-day cyanobacteria have been found in formations over 2.6 billion years old (Schopf, 1970; Schopf *et al.*, 1971; Licari and Cloud, 1972). It is a plausible assumption that the phycobiliproteins are equally

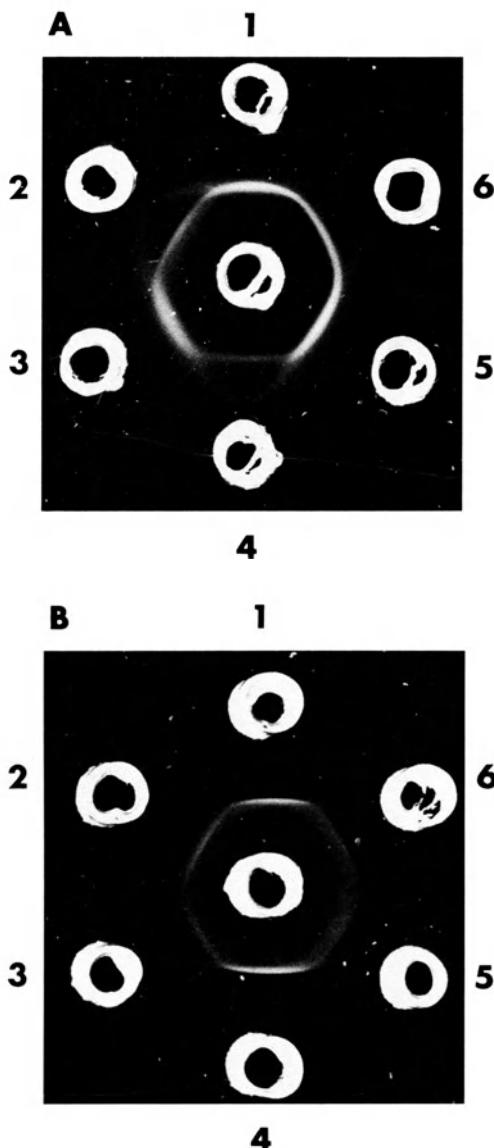


Fig. 9. Demonstration of immunological relatedness of C-phycocyanins and allophycocyanins from unicellular and filamentous blue-green algae, and from red algae by the Ouchterlony double-diffusion technique. In all experiments shown, the antibody wells contained 20 μ l of undiluted anti-serum, and antigen wells 5–10 μ g of protein. (a) Center well contains antiserum to *Synechococcus* sp. (strain 6301) phycocyanin. Wells 1, 3, and 5 contain the homologous C-phycocyanin; well 2, *Cyanidium caldarium* C-phycocyanin; well 4, *Anabaena variabilis* C-phycocyanin; well 6, *Aphanocapsa* sp. (strain 6701) C-phycocyanin. (b) Center well contains antiserum to *Synechococcus* sp. (strain 6301) allophycocyanin. Wells 1 and 4 contain the homologous allophycocyanin; well 2, *Porphyridium cruentum* allophycocyanin; well 3, *Cyanidium caldarium* allophycocyanin; well 5, *Anabaena variabilis* allophycocyanin; well 6, *Aphanocapsa* sp. (strain 6701) allophycocyanin. The agar was buffered with 0.01-M sodium phosphate–0.15-M NaCl, at pH 7.45.

ancient. Indeed, a reasonable correlation can be adduced between the absorption properties of the biliproteins and the energy spectrum of light transmitted through the atmosphere of the primitive Earth (Tilden, 1935). The distribution of homologous biliproteins spans the gap between prokaryotic and eukaryotic cells. This contention does not rely solely on the physical and immunological evidence outlined above, but also on partial amino acid sequence data on C-phycocyanins from prokaryotic and eukaryotic cells (see Section 3.5).

It should be emphasized that the divisions Cyanophyta and Rhabdophyta encompass an enormously varied assemblage of organisms. Even among unicellular cyanobacteria, the *Chroococcales*, the mole percent guanine plus cytosine content of their DNAs covers a span nearly as broad as that of the total range of nonphotosynthetic bacteria (Stanier *et al.*, 1971).

Against this background, it is of interest to explore the reasons for the apparent remarkable evolutionary stability of the structure of phycocyanin. It is clear that the conformation of a phycobiliprotein such as C-phycocyanin must satisfy a number of requirements. The need to confer special environment and conformation on each of the three chromophores represents a major limitation to structural variation. The force of this argument can be readily appreciated upon examination of the relative invariance in the character of residues in contact with the heme in cytochromes *c* from a very wide range of organisms (Margoliash, 1971, Takano *et al.*, 1973). This analogy is particularly appropriate since the apoprotein of cytochrome *c* is similar in size to the subunits of the biliproteins and because of the obvious chemical similarity between the heme and the phycobilin prosthetic groups.

An added requirement in phycocyanin is that of highly specified contacts between the α and β subunits to ensure appropriate separation and orientation of the chromophores. Further restrictions are imposed by the necessity to form $(\alpha\beta)_n$ aggregates of defined structure and the requirement to interact with allophycocyanin, and with phycoerythrin, when it is present.

The hypothesis that the three-dimensional structure of phycocyanin is highly conserved has been tested in solution with respect to one level of organization. The preparation of renatured α and β subunits of phycocyanins from unicellular and filamentous blue-green algae has been discussed above (Section 3.2.3.). The availability of these materials affords the opportunity of performing hybridization experiments. Briefly, hybrid phycocyanins can be prepared in high yield from α and β subunits of C-phycocyanins of unrelated organisms. It was demonstrated that the hybrid phycocyanins consisted of α and β subunits in a ratio of 1:1. Their chromatographic properties absorption spectra and behavior on isoelectric focusing were consistent with the conclusion that these molecules possessed a three-dimen-

sional structure very similar to that of the native proteins (Glazer and Fang, 1973b). This study demonstrates that the conformation and contacts important to the interaction of the α and β subunits have been highly conserved through the long evolutionary history of C-phycocyanin.

3.5. R-Phycocyanin

There are three known types of phycocyanobilin-containing proteins in the red algae: allophycocyanin, R-phycocyanin, and C-phycocyanin (see Table 1). Rhodophytan allophycocyanins and C-phycocyanins are indistinguishable in their major physical and immunological features from their cyanophytan counterparts. A comparison is also available of the N-terminal amino acid sequences of the α and β subunits of C-phycocyanins of the unicellular blue-green alga *Anacystis nidulans* and the eukaryote *Cyanidium caldarium*. Identical residues are present in 22 of 32 positions determined in the two proteins. Substitutions representing single base changes in the codon account for a further five positions (Williams *et al.*, 1974). It should be noted that the assignment of *Cyanidium caldarium* to the Rhodophyta (Seckbach and Ikan, 1972) should still be considered tentative. There is little doubt that the genes coding for the α and β subunits of C-phycocyanin in prokaryotes and eukaryotes arise from the same ancestral gene.

The presence of R-phycocyanin in blue-green algae has thus far not been reported. This molecule occupies a unique position among the biliproteins. It is the only biliprotein which contains both phycocyanobilin and phycoerythrobilin (Chapman *et al.*, 1967) and as such might represent a molecular fossil in the evolution of phycoerythrin from phycocyanin. The absorption spectrum of R-phycocyanin demonstrating the contribution from the two types of bilin chromophores is shown in Fig. 10.

Quantitatively R-phycocyanin is a minor constituent of the biliprotein content of most red algae, e.g., *Porphyridium cruentum* (Gantt and Lipschultz, 1974), but a major constituent in some. For example, R-phycocyanin represents 60% of the biliprotein content of *Porphyra laciniata* (O'Carra, 1965).

R-Phycocyanin is made up of two dissimilar subunits α and β (Fig. 4), and in this respect and in its aggregation behavior it closely resembles C-phycocyanins (see Tables 3 and 6). The amino acid composition of R-phycocyanin and of its subunits is likewise similar to those of C-phycocyanins and their subunits (compare data of Table 7 with those presented in Tables 4 and 5). R-Phycocyanins are immunologically related to C-phycocyanins (Bogorad, 1965; Glazer and Hixson, 1975). In both R- and C-phycocyanin, the α subunit carries a single phycocyanobilin chromophore, and contains

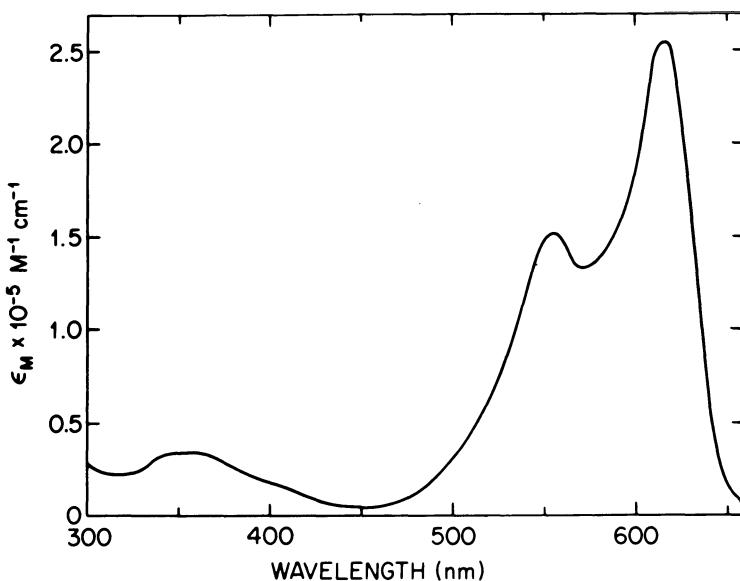


Fig. 10. Absorption spectrum of *Porphyridium cruentum* R-phycocyanin, determined in 0.05-M ammonium acetate buffer at pH 7.0.

histidine and tryptophan. The β subunit of R-phycocyanin carries one phycocyanobilin and one phycoerythrobilin prosthetic group (Glazer and Hixson, 1975). All of these characteristics point to a close evolutionary relationship between R- and C-phycocyanins.

The fluorescence emission maximum of R-phycocyanin is at 636 nm (see Table 1), close to that of C-phycocyanin. It shows efficient energy transfer from the phycoerythrobilin to the phycocyanobilin chromophores. R-Phycocyanin is an excellent subject for future studies on the architecture of a macromolecule which evolved for the effective transfer of energy between a limited number of both chemically distinct and identical chromophores.

3.6. Phycobiliproteins of the Cryptomonads

3.6.1. General Comments

The biliproteins of blue-green algae and of the eukaryotic red algae undoubtedly share a common evolutionary origin. This conclusion rests upon comparison of spectroscopic properties, structure of the chromophores,

subunit structure of the proteins, immunological properties, and isoelectric points, as well as on a limited amount of sequence information (see Section 3.5).

With respect to many of these characteristics, the biliproteins of the cryptomonads lie in a separate class (Glazer *et al.*, 1971b). This is, perhaps, not surprising since the Cryptophyceae are very distant phylogenetically from the Rhodophyceae with respect to a number of other characteristics (Dodge, 1973).

Action spectra for the cryptomonads and the nature of the photosynthetic accessory pigments of these organisms were first described

TABLE 7. Amino Acid Compositions of R-Phycocyanin (*Porphyridium cruentum*), Cryptomonad Phycocyanin (*Chroomonas sp.*), and of Their α and β Subunits

Amino acid	R-phycocyanin ^a			Cryptomonad phycocyanin ^b		
	R-phycocyanin	α subunit	β subunit	phycocyanin	α subunit	β subunit
Lysine	13	7	5	17	8	8
Histidine	1	1	0	1	1	0
Arginine	16	8	8	9	4	7
Aspartic acid	33	17	16	28	11	17
Threonine	20	13	7	10	4	6
Serine	24	14	11	24	5	18
Glutamic acid	29	17	11	17	9	8
Proline	11	7	4	6	3	4
Glycine	24	15	9	19	7	13
Alanine	48	26	20	33	10	20
Half-cystine ^c	3	2	1	11	3	6
Valine	22	9	12	16	5	10
Methionine	9	4	4	4	2	2
Isoleucine	17	10	6	10	4	6
Leucine	28	15	12	19	5	13
Tyrosine	16	12	4	7	2	5
Phenylalanine	7	4	3	5	2	3
Tryptophan	1	1	0	n.d. ^d	n.d.	n.d.
Approx. mol. wt.	36,300			24,000	9000	15,000

^a Data from Glazer and Hixson (1975).

^b Data from MacColl *et al.* (1973).

^c Determined as cysteic acid in hydrolysates of the oxidized protein.

^d n.d. = not determined.

in 1959 (Allen *et al.*, 1959; O'hEocha and Raftery, 1959; Haxo and Fork, 1959). In common with a number of other classes of eukaryotic algae, the cryptomonads contain chlorophyll *c*, and, varying with genus and species, biliproteins whose spectroscopic properties are distinct from those of their cyanophytan and rhodophytan counterparts. A comprehensive tabulation of the properties of cryptomonad phycoerythrins has been presented by Brooks and Gantt (1973). Cryptomonad phycocyanins purified to date all appear to contain at least two types of chromophores, those absorbing at 565 nm and 615–640 nm, respectively (O'hEocha, 1960; MacColl *et al.*, 1973). A pigment related to allophycocyanin has not been reported in the cryptomonads. It is likely that chlorophyll *c* fulfills the role in cryptomonads which allophycocyanin plays in the path of energy transduction in blue-green and red algae.

The most striking difference between the cryptomonad biliproteins and those of blue-green and red algae is in their aggregation behavior. Biliproteins derived from the latter two sources exist in aqueous solution as high molecular weight aggregates (see Section 3.2.1). The cryptomonad biliproteins show little tendency towards self-association at comparable protein concentrations (Allen *et al.*, 1959; Nolan and O'hEocha, 1967; Glazer *et al.*, 1971*b*; MacColl *et al.*, 1973). The cryptomonad biliproteins are immunologically distinct from those of blue-green and red algae (Berns, 1967; Glazer *et al.*, 1971*a*).

3.6.2. Intracellular Location of Cryptomonad Biliproteins

An intrathylakoidal location for the phycobiliproteins of the cryptomonads was suggested on the basis of morphological evidence (Dodge, 1969; Gantt *et al.*, 1971). As may be seen in Fig. 11, finely granular material fills the intrathylakoid lumina.

Treatment of glutaraldehyde-fixed cells with a mixture of proteolytic enzymes (pronase) resulted in correlated removal of the electron-opaque intrathylakoidal material and the release of biliproteins from the fixed cells. The electron-opaque matrix was not removed by acetone-methanol extraction. After this treatment the chloroplast lamellae were no longer evident (Gantt *et al.*, 1971). These observations provide compelling supporting evidence for the intrathylakoidal location of cryptophycean biliproteins.

3.6.3. Cryptomonad Phycocyanins

Three spectroscopically distinct types of phycoerythrin and two of phycocyanin have been observed in different cryptomonads (Allen *et al.*, 1959;

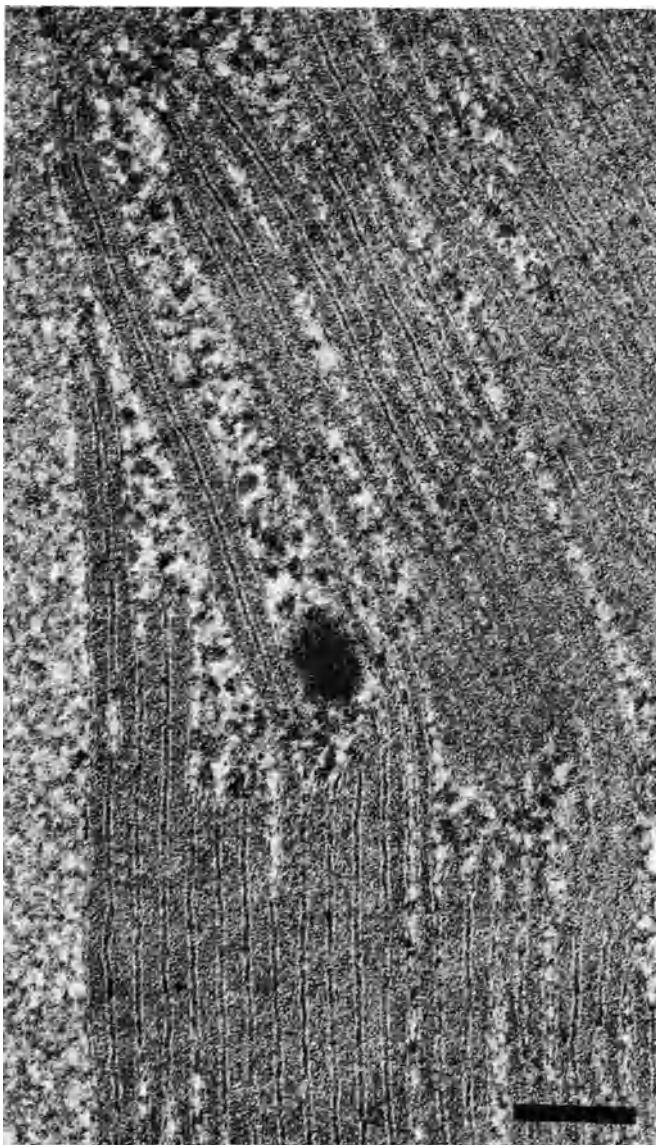


Fig. 11. Electron micrograph showing a portion of the chloroplast of *Cryptomonas* sp. The thylakoids are packed very tightly with a periodicity of 22–24 nm; adjacent thylakoids fuse to form a pseudolamellar system; no interthylakoid lumina are apparent. Finely granular material of relatively high electron sparsity (believed to be biliprotein) completely fills the intrathylakoid lumina. The bar represents 0.1 μm . (From Glazer *et al.*, 1971b.)

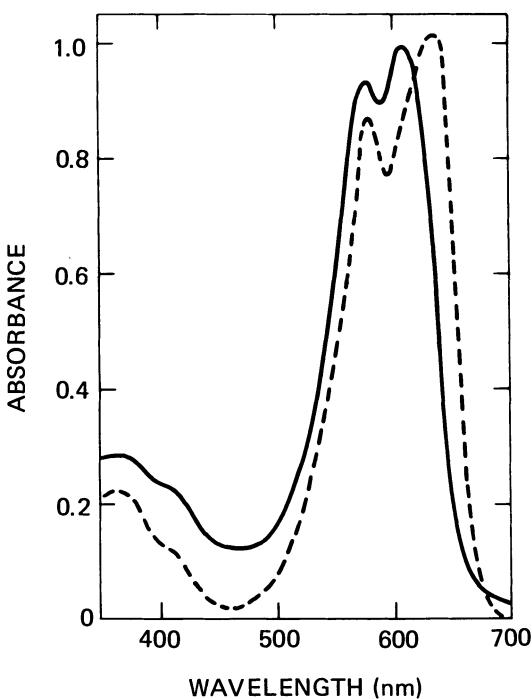


Fig. 12. Absorption spectra in the visible region of phycocyanins derived from two independent isolates of the cryptomonad *Hemiselmis virescens*.

O'hEocha and Raftery, 1959). The spectra of the phycocyanins are shown in Fig. 12. These proteins are characterized by absorption maxima at 583 and 646 nm, and 588 and 615 nm, respectively.

The properties of a *Chroomonas* sp. phycocyanin (583 nm, 646 nm) have been described by MacColl *et al.* (1973). The native protein, of molecular weight of 50,000, dissociated in the presence of sodium dodecyl sulfate to subunits of molecular weights of 10,000 and 16,000. On the basis of these data, as well as of the amino acid analyses shown in Table 7, a structure $\alpha_2\beta_2$ was proposed for the native protein.

Cryptomonad phycoerythrins are also composed of α and β subunits of molecular weights similar to those given above for *Chroomonas* sp. phycocyanin (Glazer *et al.*, 1971b; Brooks and Gantt, 1973). As previously noted for C- and R-phycocyanins, the fluorescence of cryptomonad phycocyanin is emitted from the longest-wavelength-absorbing chromophore (MacColl *et al.*, 1973).

The intracellular location of cryptomonad biliproteins, their limited self-association, and lack of immunological relatedness to rhodophytan and cyanophytan biliproteins all argue for a distinct evolutionary origin for these chromoproteins. However, the fact that cryptomonad biliproteins share the same general subunit structure and type of prosthetic group with those of the Cyanophyta and Rhodophyta suggests that this may not be the case. The cryptomonad biliproteins may well be derived from an ancestral molecule which diverged very early in the evolution of the biliproteins.

3.7. Evolution of the Biliproteins

A hypothetical scheme can be formulated for the evolutionary relationships among certain of the biliproteins. This scheme (see Fig. 13) is based on the information on the sequence of the biliproteins in the energy transfer path to chlorophyll as well as the physical and chemical evidence presented briefly in this review.

The postulation that allophycocyanin was the evolutionary precursor of C-phycocyanin, which in turn preceded C-phcoerythrin, is based on several lines of evidence. First, allophycocyanin and phycocyanin (but not phycoerythrin) are universally present in blue-green and red algae. Both the physical location of the biliproteins and the energy transfer order follow the sequence phycoerythrin → phycocyanin → allophycocyanin. It would appear plausible that in the evolution of the biliproteins the appearance of the acceptor would in each instance precede that of the donor molecule. In terms of the number of chromophores and chromophore-chromophore interaction, allophycocyanin is simpler than either C-phycocyanin or C-phycoerythrin (Glazer and Fang, 1973a; Glazer and Hixson, 1975).

The postulation of a gene duplication event early in the history of biliprotein evolution relies on the amino acid sequence homology between the α and β subunits both of prokaryotic and eukaryotic C-phycocyanins (Williams *et al.*, 1974; Troxler *et al.*, 1975). This phase of the evolution of the biliproteins is formulated in a manner strictly analogous to that established for other multichain proteins, e.g., hemoglobin. Separation of the subunits of allophycocyanin on a preparative scale has been reported recently (Gysi and Zuber, 1974), and sequence information will soon be available on this class of proteins.

Since the difference in the molecular weights of the β subunits of allophycocyanins and C-phycocyanins derived from the same organism is relatively small, it appears reasonable to suggest that the evolution of C-phycocyanin resulted from mutational events leading to the generation of an additional chromophore attachment site on the β subunit of allophycocyanin.

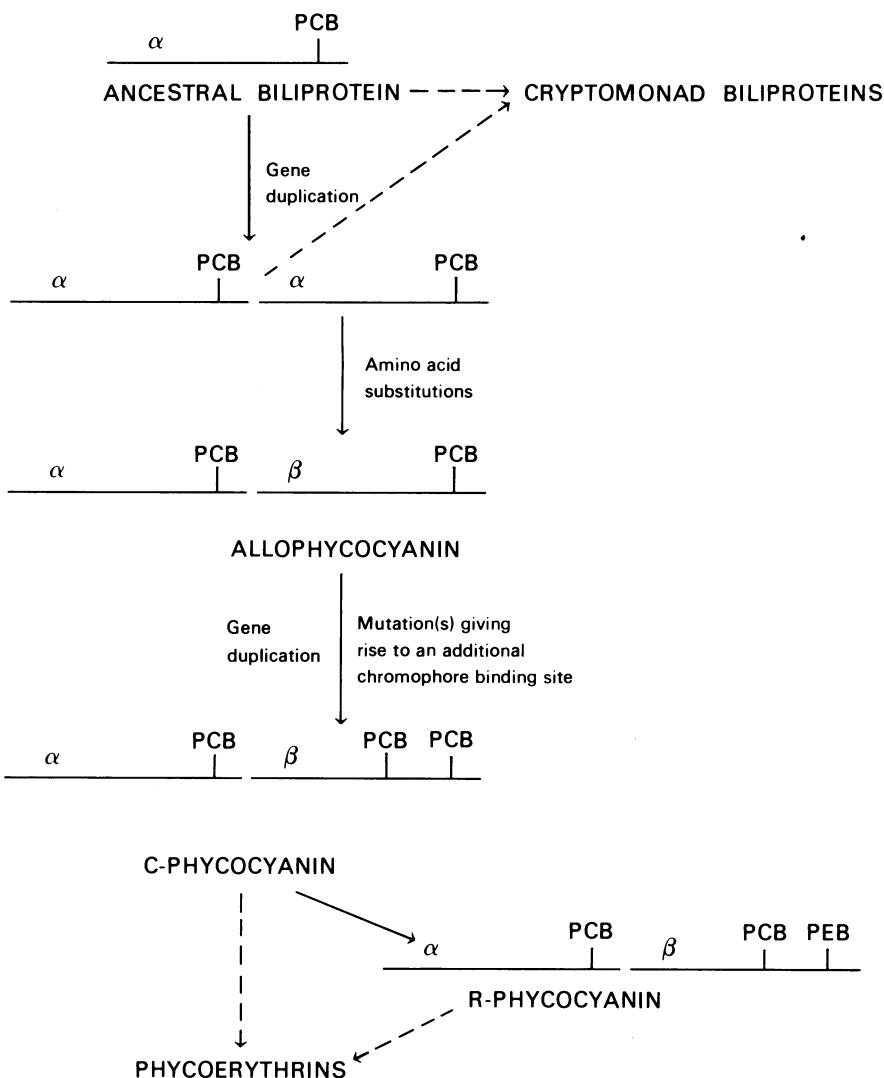


Fig. 13. Hypothetical scheme for evolutionary relationships among several biliproteins: PCB and PEB denote, respectively, phycocyanobilin and phycoerythrobilin.

The connection between C- and R-phycocyanins is clear both from immunological and physico-chemical evidence (see Section 3.5).

The dotted lines between either C-phycocyanin or R-phycocyanin and phycoerythrins indicate that at present no decision can be made as to which of the two classes of phycocyanins served as an evolutionary precursor to

the phycoerythrins. It is possible that phycoerythrins may have arisen by both of the paths indicated.

The biliproteins of the cryptomonads exhibit the same type of subunit structure and serve the same biological function as do those of the blue-green and red algae. However, there are ample grounds for the belief that they are but distantly related to the cyanophytan and rhodophytan biliproteins (see Section 3.7). This is the basis for the suggested assignment of the possible points of divergence of these proteins (see dotted lines) from the ancestral biliprotein very early in the evolution of these macromolecules.

The simplified outline presented should not be regarded as a definitive scheme, but rather as a unifying proposal which can be subjected to test by comparison of amino acid sequences of appropriate biliproteins.

A modest beginning along these lines hints at possible homology between the sequences of C-phycocyanins and *Porphyridium cruentum* B-phycoerythrin (Harris and Berns, 1974, cited by Troxler *et al.*, 1975).

Rapid evaluation of possible interrelationships might result from a comparison of immunological relationships among fully denatured subunits of the various biliproteins.

3.8. Concluding Remarks

In comparison with many other chromoproteins (cytochromes *c*, ferredoxins, hemoglobins, etc.), the phycobiliproteins are still a little studied group of proteins. However, even with the limited information available, some correlation of structure with biological function can be achieved for C-phycocyanin.

It is easy to account for the aggregation behavior of the biliproteins in functional terms. The aggregation into phycobilisomes and the attachment of these structures to the photosynthetic lamellae is mandatory in view of the R^{-6} dependence of the rate and efficiency of the energy transfer process.

The hexamer $(\alpha\beta)_6$ of C-phycocyanin is definitely an intermediate in phycobilisome assembly. The formation of this aggregate from the $\alpha\beta$ monomer has obvious functional correlates. The hexamer absorbs maximally at 621 nm with an ϵ_M of 330,000 M⁻¹ cm⁻¹, while the monomer absorbs at ~615 nm with an ϵ_M of ~23,000 M⁻¹ cm⁻¹. The hexamer, therefore, absorbs energy more efficiently and its fluorescence emission has greater spectral overlap with the absorption spectrum of the acceptor than does that of the monomer. These changes in spectroscopic properties represent contributions to the optimization of the parameter *J* in the Förster equation.

The spectra of the chromophores in the separate α and β subunits are very different from those of free phycocyanobilin. The protein-bilin interac-

tion leads to enhancement of the long-wavelength (~ 600 nm) band at the expense of the short-wavelength band (~ 360 nm), i.e., the bilin linked to the native subunits absorbs light less strongly in the region of the chlorophyll Soret absorption band and more strongly in the region where chlorophyll is nearly transparent, i.e., the bilin spectrum is rendered more nearly complementary to that of chlorophyll *a*.

The attachment of chromophores to two dissimilar polypeptide chains provides different environments for the individual chromophores with associated differences in absorption spectra. The absorption maximum of the β subunit chromophores is lower than that of the α subunit chromophore. Consequently, the energy absorbed by the β subunit chromophores is funneled through the α subunit to the next acceptor. The probable advantage of this arrangement is that the sensitizing chromophores on the β subunits can harvest light with greater efficiency at lower wavelengths with no loss in the overall efficiency of energy transfer from the $\alpha\beta$ monomer.

The optimization of the interchromophore orientation parameter K has not been discussed because of the dearth of relevant data. However, it should be noted that the phycobiliproteins exhibit powerful aggregation-dependent circular dichroism bands in the visible region of the spectrum. It is likely that when crystallographic data on these proteins become available, it will be found that the mutual orientation of the chromophores is also optimal for energy transfer.

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Transmission of Solar Radiation into Natural Waters*

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1. INTRODUCTION

Physical and biological measurements in aquatic environments are becoming more accurate and precise as instruments and experimental techniques improve. These improved measurements go hand in hand with an increasingly quantitative description of the relationship between solar radiation, the optical properties of natural waters, and aquatic photoprocesses (e.g., Bannister, 1974; Kiefer and Austin, 1974; Morel and Smith, 1974; Patten, 1968). As man becomes increasingly aware of his own, often adverse, impact upon the aquatic environment, the techniques of optical oceanography

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assume greater importance as a means for the rapid and accurate assessment of this environment (e.g., Gibbs, 1974; Jerlov and Steemann Nielsen, 1974; Smith *et al.*, 1973; Clark *et al.*, 1969). It is timely, therefore, to formulate a consistent set of fundamental radiometric quantities, units, and nomenclature that will have consistent applicability to limnology and oceanography, as well as to photochemistry and photobiology.

The primary emphasis of this chapter will be (1) to interrelate the fundamental quantities, units, and nomenclature which are in current use in the two scientific fields of optical oceanography and photobiology; (2) to present the additional quantities used in optical oceanography for describing the transmission of radiant energy into natural waters; (3) to evaluate critically the existing data for the spectral attenuation coefficient within the wavelength region from 200 to 800 nm, for both pure fresh water and "pure" sea water; (4) to present data for the diffuse attenuation coefficient for irradiance (200–800 nm) for several natural water types.

2. FUNDAMENTAL QUANTITIES, UNITS, AND NOMENCLATURE

In order to determine quantitatively the energy available underwater for aquatic photoprocesses, it is necessary to adopt concepts for the measurement of radiant energy that are biologically meaningful and radiometrically well-defined.

* Dosimetric concepts in photobiology have recently been discussed in detail by Rupert (1974). As pointed out by Jagger (1974), Rupert's article was developed through the activity of the U.S. National Committee for Photobiology (NAS-NRC). It was presented in an earlier version at the Sixth International Congress of Photobiology held in August, 1972, in Bochum, Germany, and, while not yet an official recommendation, was published (Rupert, 1974) to widen the circle of critical participants interested in dosimetric terms and nomenclature that relate to photobiology with the hope that a final recommendation would be ready by the time of the International Congress of Photobiology in Rome in 1976. With these points in mind this review adheres to the recommendations of Rupert (1974) with respect to photobiological quantities, units, and nomenclature.

Radiometric quantities, as used in oceanography, have been standardized by the International Association of Physical Oceanography (IAPO). A Committee on Radiant Energy in the Sea set up by IAPO in 1960 published a recommended standard terminology on underwater optics (IAPO, 1964; UNESCO, 1965). Jerlov (1968), who chaired this IAPO Committee, has republished and discussed the recommended terminology. Preisendorfer (1960), a coopted member of this committee, contributed a remarkably complete and precise review of radiometric concepts as used in

hydrologic optics. When discussing radiometric quantities, as used in optical oceanography, the standard terminology as recommended by IAPO (1964) will be used.

The units used, in both photobiology and oceanography, are expressed in the International System of Units (Système International or SI). This system has been published by the International Bureau of Weights and Measures (BIPM, 1970) and by the National Bureau of Standards (NBS, 1972). As noted by Rupert, the SI is currently the only internationally agreed upon system of units for expressing scientific data.

Table 1 summarizes the radiometric and dosimetric quantities characterizing the flow of radiant energy and gives abbreviated defining equations which are in use in both oceanography and photobiology. Only incoherent unpolarized radiation of a specific wavelength is considered, since it is kept in mind that the interaction of radiant energy with matter will depend upon the wavelength distribution of the radiant energy. Jerlov (1968) and Rupert (1974) may be consulted for more complete details. Ivanoff (1974 and references therein) discusses polarization measurements in the sea.

The quantity of energy transferred by radiation, represented by the symbol Q , is measured in the SI derived unit joule (J). (This is the electromagnetic energy which may be derived from Maxwell's equations, and as such, provides the link between radiometry and electromagnetic theory.) Radiant flux dQ/dt is defined as the time rate of flow of radiant energy and is measured in the SI derived unit watt (W).

In oceanography the distribution of natural radiance, $L(z, \theta, \phi)$, around a point at a fixed depth z underwater is called a radiance distribution. In this notation $L(z, \theta, \phi)$ is the energy per unit time per unit area per unit solid angle incident upon a point from the direction (θ, ϕ) . Here θ is the zenith angle, and the azimuthal angle ϕ is the angle between the plane of the sun and the flow of energy. Detailed data for radiance distributions in natural waters have been published by Tyler (1960) and Smith (1974).

The irradiance $E(z)$ at a depth z is the radiant flux incident on an element of surface divided by the area of that element. $E_d(z)$ is the downwelling irradiance, i.e., it is the flux incident per unit area measured on a horizontally oriented cosine collector facing upward (Smith, 1969). Similarly, $E_u(z)$ is the upwelling irradiance. These quantities are defined in terms of the radiance distributions by the following equations:

$$E_d(z) = \int_0^{2\pi} \int_0^{\pi/2} L(z, \theta, \phi) \cos \theta d\omega \quad (1)$$

$$E_u(z) = \int_0^{2\pi} \int_{\pi/2}^{\pi} L(z, \theta, \phi) |\cos \theta| d\omega \quad (2)$$

TABLE 1. Summary of Radiometric and Dosimetric Quantities Characterizing Flow of Radiant Energy

Radiometric quantity (units)	Oceanographic symbol and defining equation ^a	Dosimetric quantity (units)	Suggested symbol ^b
Wavelength [nm]	λ	Wavelength [nm]	λ
Quantity of radiant energy [J]	Q	Quantity of radiant energy [J]	Q
		Energy fluence [$J\ m^{-2}$]	$F = \int_{4\pi} L\ d\omega \int dt$
		Photon fluence [(# photons) m^{-2}]	$P = \frac{1}{h\nu} \int_{4\pi} L\ d\omega \int dt$
Radiant flux [$W = J\ s^{-1}$]	$\left(\frac{dQ}{dt} \right)$		
Radiance [$W\ m^{-2}\ sr^{-1}$]	$L = \frac{d^2 (dQ/dt)}{dA \cos \theta\ d\omega}$	Radiance [$W\ m^{-2}\ sr$]	L
Irradiance [$W\ m^{-2}$]	$E = \frac{d}{dA} \left(\frac{dQ}{dt} \right)$		
Scalar irradiance [$W\ m^{-2}$]	$E_0 = \int_{4\pi} L\ d\omega$	Energy fluence rate [$W\ m^{-2}$]	$\frac{dF}{dt} = \int_{4\pi} L\ d\omega$
Photon scalar irradiance [(# photons) $m^{-2}\ s^{-1}$]	$\frac{E_0}{h\nu} = \frac{1}{h\nu} \int_{4\pi} L\ d\omega$	Photon fluence rate [(# photons) $m^{-2}\ s^{-1}$]	$\frac{dP}{dt} = \frac{1}{h\nu} \int_{4\pi} L\ d\omega$

^a Terminology recognized by the Committee on Radiant Energy in the Sea (of the International Association of Physical Oceanography, IAPAO) as given by Jerlov (1968).

^b Rupert (1974).

where $d\omega = \sin \theta d\theta d\phi$. Downwelling irradiance is one of the most commonly measured quantities in optical oceanography. This is because it has been easier to measure E_d than to measure other radiometric quantities. In oceanography, useful values of E_d are dependent upon the often stated (and frequently correct) assumption that the ocean is horizontally stratified and, therefore, that radiant energy varies primarily with depth.

Scalar irradiance (energy fluence rate) is the integral of a radiance distribution at a point at depth z over all directions about the point

$$E_0(z) = \iint_{4\pi} L(z, \theta, \phi) d\omega \quad (3a)$$

This is equivalent to the energy fluence rate, e.g.,

$$\frac{dF(z)}{dt} = \iint_{4\pi} L(z, \theta, \phi) d\omega \quad (3b)$$

The scalar irradiance ($\text{W}\cdot\text{m}^{-2}$), when divided by the velocity of light in the medium, yields the total amount of radiant energy per unit volume of space at the given point, i.e., the radiant energy density ($\text{J}\cdot\text{m}^{-3}$).

Several factors favor scalar irradiance as the parameter that best expresses the radiant energy available for aquatic photoprocesses. It provides a measure of radiant energy incident upon a small sample volume when all directions of incidence are equally weighed. It is a scalar quantity, not defined with respect to some particular geometrical orientation, thus it allows all laboratory and field measurements to be directly compared on an absolute basis. In addition, it is a well-defined and standardized physical quantity, amenable to theoretical description and mathematical manipulation, and possessing standardized units. A number of authors have described scalar irradiance meters for laboratory and oceanographic work (Curie, 1961; Maddux, 1966; Sasaki *et al.*, 1966; Rich and Wetzel, 1969; Smith and Wilson, 1972; Booth, 1974).

3. OPTICAL PROPERTIES OF WATER

The quantities listed in Table 1 can be used to describe the energy available to or incident upon a sample volume which, in a natural aquatic environment, consists of a volume of water (fresh or salt) together with its attendant dissolved and suspended material. In order to evaluate photo-processes which are concerned with the absorption of radiant energy within these small natural hydrosol volumes, it is necessary to make use of the standard definitions and operational concepts of radiometry.

As defined by Preisendorfer (1960) and discussed by Tyler and Prei-

sendorfer (1962), ocean optical properties may be divided into two classes, called *inherent* and *apparent*. An optical property is *inherent* if its operational value at a given point in a medium is invariant with changes of the radiance distribution at that point. An *apparent* optical property is one for which this is not the case. Examples of inherent optical properties are the coefficients of beam attenuation, absorption, and scattering and the volume scattering function (for more complete discussion see Tyler and Preisendorfer, 1962; Jerlov, 1968). The diffuse attenuation coefficient for irradiance, defined below in Section 3.2 is an example of an apparent optical property.

3.1. Inherent Optical Properties of Pure Water

Transmittance is defined as the ratio of transmitted radiant flux to incident radiant flux. This definition can be applied to any light field, diffuse, specular, or a mixture of both, provided the measurements of incident and transmitted flux are for the same geometrical distributions of radiant energy.

Beam transmittance T is a limiting case of the general concept and refers to the transmittance of a (collimated) beam, the diameter of which is small compared to its length. Over a fixed path length r

$$T = e^{-cr} \quad (4)$$

where c is the total attenuation coefficient, defined as the sum of two generally independent terms

$$c = a + b \quad (5)$$

The total scattering coefficient b refers to that part of beam attenuation due to scattering, and the total absorption coefficient a refers to that part of the beam attenuation due to conversion of radiant flux into other forms of energy. The total scattering coefficient in Eq. (5) may be obtained independently by integrating the measured volume scattering function $\beta(\theta)$ over all angles about a fixed sample volume

$$b = \iint_{4\pi} \beta(\theta) d\omega \quad (6)$$

It is frequently useful to distinguish between the forward-scattering coefficient b_f , which relates only to scattering in the forward direction (0° to 90°), and the backward-scattering coefficient b_b , which relates only to scattering in the backward direction (90° to 180°). These coefficients are defined in

the following equations:

$$b_f = 2\pi \int_0^{\pi/2} \beta(\theta) \sin \theta d\theta \quad (7)$$

$$b_b = 2\pi \int_{\pi/2}^{\pi} \beta(\theta) \sin \theta d\theta \quad (8)$$

$$b = b_f + b_b \quad (9)$$

Equation (5) has been expressed in more detail (see, for example, Jerlov, 1968, Table XIII)

$$c = a_w + a_d + a_p + b_w + b_d + b_p \quad (10a)$$

$$c = c_w + a_d + a_p + b_d + b_p \quad (10b)$$

where the subscripts signify: w , pure water; d , dissolved and colloidal material; p , particulate matter. This separation of c into components permits the introduction of laboratory determinations of a_w and b_w for pure water, thus simplifying Eq. (10) and providing greater insight into the significance of the dissolved and particulate matter contained in a given sample of ocean water.

Laboratory methods have also been employed for the study of suspended particles (e.g., Van de Hulst, 1957; Kerker, 1969) and colloidal solutions (e.g., Kruyt, 1952; Mysels, 1959). In these laboratory studies the need for accurate values of the optical properties of pure water is achieved by use of dual beam comparisons, where a blank cell of pure water is used as a standard alongside a cell containing the material under investigation. This dual beam technique does not lend itself easily to *in situ* oceanographic investigations and, thus, there is a need for accurate pure water values of the absorption and scattering coefficients for use as comparison standards.

3.2. Apparent Optical Properties of Natural Waters

For many important problems of interest in limnology and oceanography, the (beam) attenuation coefficient does not provide the information of greatest interest. Of prime importance to marine biology is the total amount and spectral distribution of solar radiant energy penetrating to various depths in natural waters. For the purpose of *in situ* radiant energy studies in aquatic environments several apparent optical water properties are used. These apparent optical properties, while they vary with the geometrical composition of the radiant energy field, describe the penetra-

tion of solar radiation into natural waters and can be used to characterize these waters.

Preisendorfer (1961) gives precise mathematical definitions of the apparent optical properties and justifies their usefulness on the bases that their gross behavior with depth exhibits striking regularities which are reproducible in medium after medium, that it is possible to formulate theoretical interrelationships between apparent and inherent optical properties, and that the use of apparent optical properties makes many practical oceanographic problems amenable to solution (Gordon *et al.*, 1975).

To describe the penetration of solar radiation into natural waters, we use the (diffuse) attenuation coefficients for irradiance, defined as

$$K(z) = \frac{-1}{E(z)} \frac{dE(z)}{dz} \quad (11)$$

or, alternatively,

$$\frac{E(z_2)}{E(z_1)} = \exp [-K(z_2 - z_1)] \quad (12)$$

where K has units of reciprocal length, z_2 and z_1 are the depths (increasing positively with increasing depth) at which $E(z_2)$ and $E(z_1)$ are measured. A K -type function may be defined for downwelling, upwelling, or scalar irradiances by adding subscripts d , u , or o , respectively to the appropriate parameters in the above equations. In addition, a K -type radiance function may be similarly defined as the logarithmic depth derivative of the radiance, $L(z,\theta,\phi)$, for a fixed direction (θ,ϕ) . Physically, the K functions are the quantities that specify the individual depth dependence of irradiance (or radiance) functions. Historically, the K functions were derived from the experimental fact that, in general, radiant energy decreases exponentially with depth. In principle, the K functions may be subdivided into several principal components [as was done for c in Eq. (10)] representing the separate contributions to diffuse attenuation (J. E. Tyler and R. C. Smith, in preparation.)

The depth dependence of the K functions, for monochromatic radiant energy, is influenced by the depth dependence of the radiance distribution $L(z,\theta,\phi)$. Near the surface the radiance distribution underwater is a complex combination of collimated direct sunlight plus diffuse skylight. With increasing optical depth this distinction becomes lost, with the radiance distribution becoming increasingly diffuse. Also, with increasing depth the position of maximum radiance in the underwater light field moves away from the direct refracted angle of the sun toward the zenith, and the radiance distribution approaches a more stable geometrical shape which de-

pends upon the inherent optical properties of the water (Smith, 1974 and references therein).

As a consequence of these changes in the underwater radiance distribution, the diffuse attenuation coefficients for irradiance vary with depth, even in water which is uniform with depth. This variation is relatively large near the surface but becomes small below a few optical depths, where the K functions approach a constant value. The dependence of K on the light field, as well as on the composition of the water, means that K is not an unambiguous constant in the near-surface layers of the ocean and cannot properly be used to classify surface waters. However at greater depths, the light field becomes more stable, and the value of K becomes more nearly a property of the water alone. With these restrictions in mind, K can be used in classifying ocean waters.

Following the work of Schuster (1905), a number of authors (Preisendorfer, 1958a,b and references therein) have utilized and modified the classical two-flow analysis of radiant energy penetration into natural waters. From Schuster's classical theoretical analysis, as modified by Preisendorfer, the following equations relating the inherent and apparent optical properties are heuristically useful:

$$c \simeq K + b_f \quad (13)$$

$$K \simeq Da + b_b \quad (14)$$

From these equations it follows that

$$c \simeq (K + b_f) \geq K \simeq (Da + b_b) \geq a \quad (15)$$

In the derivation of these equations, an optically homogeneous plane-parallel medium in steady state has been assumed (i.e., that L , c , β depend spatially only on depth z). In the original presentation by Schuster, the diffuse component of the radiance distribution was assumed to be uniform at all depths, which is contrary to experimental results in natural environments. Preisendorfer (1958a,b) eliminated this assumption by introducing a distribution function D which, in general, varies with depth as the radiance distribution varies. In the classical limit for a uniformly diffuse radiance distribution we have $D = 2$, while for a single collimated beam incident normally upon the medium we find $D = 1$. To obtain the simple heuristic Eqs. (13), (14), and (15), the backward-scattering coefficient was assumed to be small compared to the absorption coefficient. This assumption allows second-order backward-scattering terms to be neglected.

Equation (13) shows that the total beam attenuation coefficient c is larger than the diffuse attenuation coefficient for irradiance K . This occurs because scattering removes flux from a collimated beam. In contrast, an ir-

radiance measurement in a natural environment includes forward-scattered radiant energy as well as radiant energy which has not been absorbed. Equation (14) shows that K is greater than the absorption coefficient a due to the addition of the (relatively small) backward-scattering coefficient. Of greater importance, causing K to be larger than a , is the effect of the distribution function. This function accounts for the experimental fact that the absorption of uniformly diffuse radiation ($D = 2$) passing through a thin layer of a medium is twice the absorption of collimated radiation ($D = 1$) passing normally through the same thin layer.

It is of interest to consider Eqs. (13), (14), and (15) for two limiting situations. First, if the absorption in the medium tends to zero ($a \approx 0$), the total attenuation coefficient may be approximated by the total scattering coefficient ($c \approx b$); whereas, K tends to be approximated by the relatively small backward-scattering coefficient ($K \approx b_b \sim 0$). Thus, without absorption, the diffuse attenuation of irradiance in a natural medium is relatively small (compared to the beam attenuation.) As a second limiting example, consider a medium in which the total scattering becomes relatively small ($b \approx 0$). In this situation the total attenuation coefficient may be approximated by the absorption coefficient ($c \approx a$), while K may be approximated by Da ($K \approx Da$), which, for collimated radiant energy ($D = 1$), becomes equal to the absorption coefficient ($c \approx K \approx a$). The hope that this last approximation is true, without critical evaluation of the assumptions involved to obtain it, was an early source of confusion in the published literature concerned with the "absorption" or "attenuation" properties of water.

4. DATA

With respect to the penetration of the sun's radiant energy into ocean and lake waters, we are primarily concerned with *in situ* measurements of the diffuse attenuation coefficient for irradiance $K(\lambda)$. Values of $K(\lambda)$ have already been obtained for a wide variety of natural waters and environmental conditions (see Section 4.2 below). Now, with the possibility of increased levels of solar ultraviolet radiation reaching the earth's surface due to anthropogenic changes in the earth's atmosphere (Green *et al.*, 1974; Environmental Studies Board, 1973), it has become important to be able to predict reliably the penetration of ultraviolet radiation into natural waters. However, since radiation below approximately 300 nm is strongly absorbed by transmission through the atmosphere (Moon, 1940), the $K(\lambda)$ functions for this spectral region must be obtained by indirect methods.

The K functions can be theoretically related to the inherent optical properties [e.g., Eqs. (13), (14), and (15)] and hence, in principle, can be

derived from them. In practice the required experimental information necessary to relate accurately inherent and apparent optical properties has seldom been completely determined. Nevertheless, since direct K data are not available, laboratory measurements of the beam attenuation coefficient $c(\lambda)$ for pure fresh water and artificial sea water provide at least some information which may be used to estimate the maximum depths of penetration of ultraviolet radiant energy into oceans and lakes.

In the following we will first critically review the available data for the spectral attenuation coefficient of pure (fresh and sea) water. We will then present data for the spectral diffuse attenuation coefficient for irradiance for a variety of natural water types. The consistency of these data will then be discussed with respect to the penetration of radiant energy into natural waters.

4.1. Total Attenuation Coefficient $c(\lambda)$

In Fig. 1 data for the total attenuation coefficient of pure water, as a function of wavelength between 200 and 800 nm, are presented. (For a review of data published prior to 1931, see Dorsey, 1940; James and Birge, 1938; Sawyer, 1931. For more recent reviews see Morel, 1973; Hale and Querry, 1973; Jerlov, 1968; Irvine and Pollack, 1968). An inspection of these data shows that the reported values of the various investigators disagree by more than their stated (or implied) accuracies and precision. In order to obtain a basis for selecting the more reliable data from these widely scattered values, it is useful to consider the potential sources of error in $c(\lambda)$ measurements. This will allow us to establish criteria with which to judge the relative limits of error of the data shown in Fig. 1.

4.1.1. Discussion of Error

The spread in the data shown in Fig. 1 is due to two primary factors. First, the optical properties of water are precisely defined by current theory, and experimental difficulties are commonly encountered when attempting to devise experimental methods or instruments to make measurements that conform to the theoretical concepts. Second, it is notoriously difficult to prepare "optically pure" water, and (within the spectral region under discussion) the absorption coefficient is small and the scattering coefficient very small in comparison to the potential contribution to absorption and scattering from impurities.

In addition to these fundamental sources of error, many of the early

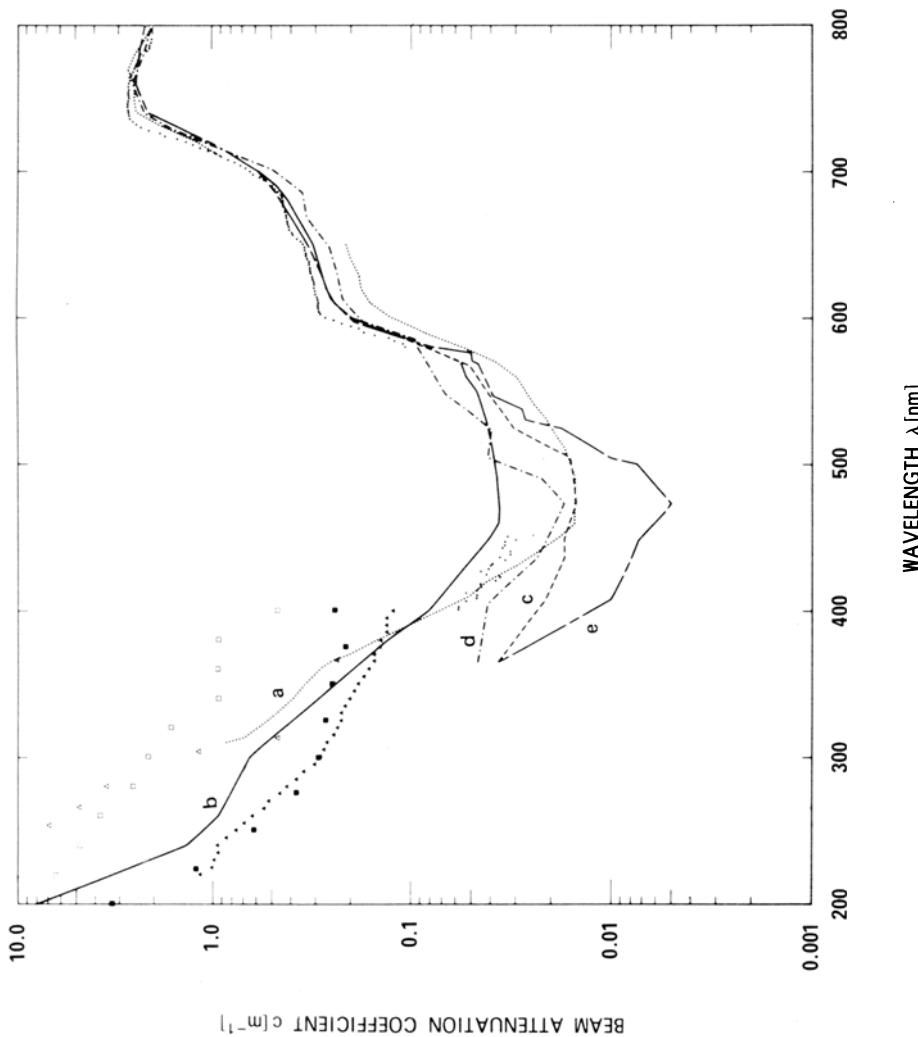


Fig. 1. Total attenuation coefficient c (m^{-1}) of pure water as a function of wavelength, λ (nm). (Δ Δ Δ), Hulbert (1928) (254–366 nm); Curve (a), Sawyer (1931) (310–650 nm), Curcio and Petty (1951) (710–800 nm); (\square \square \square), Hodgman (1933) (220–400 nm); Curve (b), Dawson and Hulbert (1934) (200–400 nm), Hulbert (1945) (400–700 nm); Curve (c), James and Birge (1938) (365–800 nm); Curve (d), James and Birge (1938) (365–800 nm); Curve (e), Clark and James (1939) (365–800 nm); (\blacktriangle \blacktriangle \blacktriangle), Le-noble and Saint-Guily (1955) (220–400 nm); (\blacksquare \blacksquare \blacksquare), Armstrong and Boalch (1961a,b) (200–400 nm); (.....), Sullivan (1963) (400–450 nm, 580–790 nm).

investigators failed to distinguish between the absorption coefficient and the beam attenuation coefficient. A reappraisal of these early data must therefore be made in light of current knowledge before we attempt to use it in modern theory or to compare it with more recent data.

In the measurement of beam transmittance, any radiation that does not conform to the theoretical definition [Eq. (4)] becomes spurious flux and must be eliminated. Since beam transmittance is a measure of the total attenuation ($a + b$), the experimental measurement must (in theory) exclude all scattered radiant energy. No real instrument can conform to this ideal requirement while maintaining a finite receiver aperture. Thus the error due to scattering in the medium is implicit in the experimental measurement of beam transmittance. In addition, experimental instruments generally require windows, sample cells, etc., in the beam, and these components frequently are an even greater source of scattering than the water itself. Spurious flux of all kinds must be carefully understood and evaluated if serious inaccuracies are to be avoided in the determination of c .

Tyler *et al.* (1974) have reviewed the theoretical basis for the measurement of beam transmittance of water. Following Preisendorfer (1958a,b, 1960), they use the phenomenological theory of radiative transfer to develop a theoretical relationship between the error due to the collection of spurious forward-scattered radiant energy and the dimensions of the transmissometer beam. Errors in the measurement of beam transmittance have also been reviewed by Morel (1974) and Jerlov (1968). Latimer (1972) carried out an extensive numerical survey of the influence of the geometry on a transmittance optical system. A related study of the effects of photometer geometry on direct measurements of scattered radiant energy was reported by Hodkinson and Greenfield (1965). These authors examined the effects of the parts of the photometric optical system both before and after the sample vessel and the effect of particle size, refractive index, and absorption coefficient upon the experimentally determined results.

There are special problems associated with preparing optically pure water for measurement. Hickman *et al.* (1973) have reviewed the problems of preparing pure water and have noted that today it is almost universally realized that absolute purity is unattainable. However, in order to obtain optical data on distilled water, special care must be taken to ensure a high degree of optical purity (Cohen and Eisenberg, 1965; Kratochvil *et al.*, 1965; Litan, 1968). In particular, to approach optical purity, the water must be free of suspended particulate material and of fluorescent impurities.

The presence of suspended particles invariably leads to a marked dissymmetry (about 90°) in the angular dependence of the volume scattering function $\beta(\theta)$, (Kerker, 1969; Van de Hulst, 1957). While a symmetric scattering function does not necessarily guarantee optical purity (Morel, 1966), an asymmetric scattering function does imply suspended scattering

particles within the liquid. The presence of even very small quantities of suspended particles (larger than the wavelength of the irradiating energy) causes the narrow-angle forward-scattering to increase by orders of magnitude.

Cohen and Eisenberg (1965) have suggested that a major source of error in the experimental determination of the optical properties of water appears to be due to the presence of small amounts of fluorescent materials, which are extremely difficult to completely eliminate from polar liquids such as water. They emphasize the problem caused by these materials by observing that the fluorescence from a solution containing 10^{-6} g of fluorescein per ml of water is about 200 times larger than that from the water itself.

Other factors contributing to confusion and discrepancies within the published data on optical properties of water can be traced to nonstandard units and nomenclature. Some workers, for example, have defined transmittance [Eq. (4)] with respect to the base 10 rather than to the base e . Others have failed to carefully distinguish between the total attenuation coefficient and the absorption coefficient. In the case of some distilled water results, the distinction between c and a has been neglected on the basis that the total scattering coefficient within the visible spectrum is roughly an order of magnitude less than the absorption coefficient. The assumption that scattering is negligible compared to absorption has led several workers apparently to ignore the experimental problems associated with spurious scattered flux.

Because of limited information concerning experimental details, it is usually difficult, when examining the published data, to assess quantitatively the relative accuracy of the various data. In contrast, the relative precision of the various data can be estimated.

R. W. Austin (private communication, this laboratory) has discussed the experimental precision of attenuation coefficient measurements and has shown [using Eq. (4)] that

$$\Delta c = \frac{-1}{r} \frac{\Delta T}{T} \quad (16)$$

or

$$\frac{\Delta c}{c} = - \frac{e^{cr}}{r} \Delta T \quad (17)$$

Here ΔT is the photometric error in the measurement of beam transmittance, r is the path length over which the beam transmittance is measured, and Δc is the error in the beam attenuation coefficient caused by ΔT . Equation (17) gives the fractional error in the determination of c due to ΔT . This

fractional error $\Delta c/c$ is a minimum (for a fixed ΔT) when the path length equals the reciprocal of the attenuation coefficient ($r = 1/c$). The fractional error increases when the instrument path length is not "optimized" for the range of c values being measured. This is shown in Fig. 2, where Eq. (17), for two values of ΔT and several fixed path lengths, have been plotted.

As an example, consider a 1-m path length instrument capable of 1% photometric precision. The plotted curve ($r = 1 \text{ m}$ and $\Delta T = 0.01$) shows that the fractional error in determining c for such an instrument is a minimum (2.7%) when $c = 1.0 \text{ m}^{-1}$. However, if $c = 0.1 \text{ m}^{-1}$, the fractional error is 11%, and if $c = 0.01 \text{ m}^{-1}$, this error rises to over 100%. Alternatively, we can say that if c (as determined over a 1-m path length with $\Delta T =$

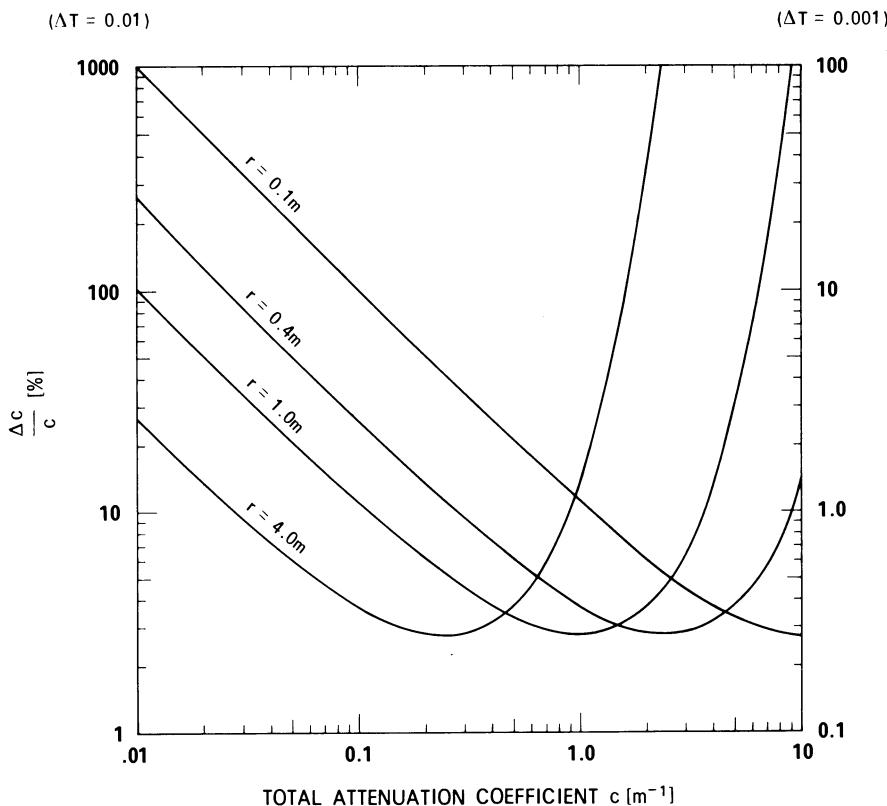


Fig. 2. Plot of Eq. (17) for two values of ΔT and four fixed path lengths of 0.1, 0.4, 1.0, and 4.0 m. In this figure the left-hand ordinate gives the fractional error in c for $\Delta T = 0.01$, the minimum photometric error that could be expected using techniques of photographic photometry. The right-hand ordinate is for $\Delta T = 0.001$, a photometric error that could be achieved using modern experimental equipment.

0.01) has a value between 0.4 and 5.0 m⁻¹, the fractional error will be less than 27% (ten times the optimized minimum). Conversely, if *c* is outside this range of values, the fractional error will be greater than 27%.

Thus, for each instrument with a fixed path length there corresponds a range of *c* values which may be most precisely determined. Measurements of *c* values outside this optimum range will have relatively large random errors. In evaluating the published data, we have arbitrarily chosen this optimum region to be that range of *c* values for which $\Delta c/c$ is less than ten times the minimum value of *c* (i.e., less than 27% fractional error). We consider data with poorer precision to be significantly less reliable.

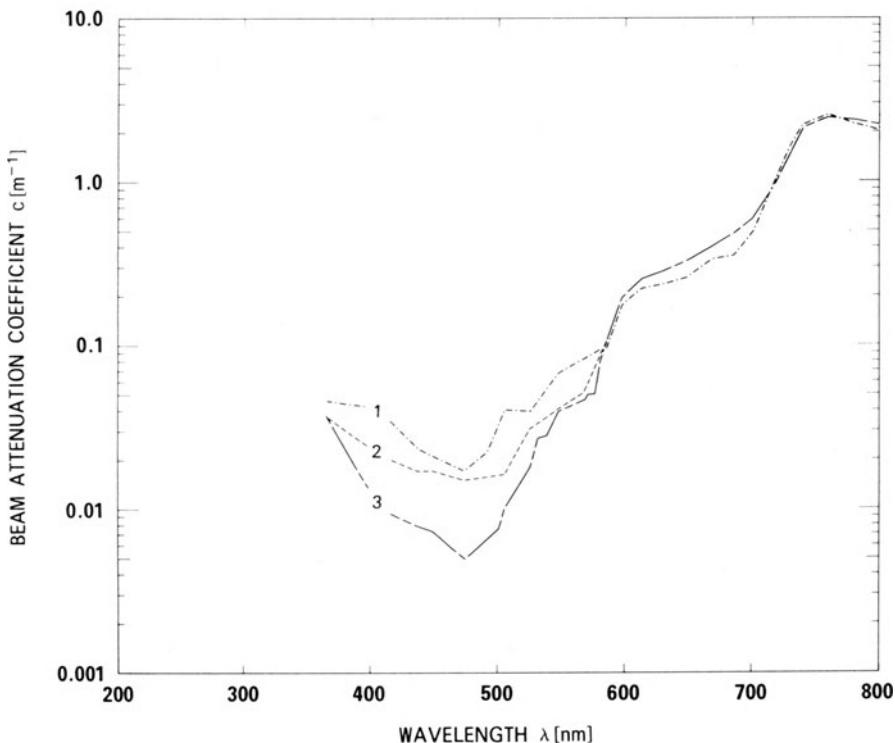


Fig. 3. Total attenuation coefficient *c*(m⁻¹), of pure water as a function of wavelength, λ (nm). Curve 1 from data of Clark and James (1934), Table IV, obtained with an 0.97-m pathlength blackened ceresin-lined tube. Curve 2 from data of James and Birge (1938), Table X, obtained with a 2-m blackened paraffin-lined tube. Curve 3 from data of James and Birge (1938), Table I [republished by Clark and James (1939), Table IV], obtained with an 0.97-m silver-lined tube. Curves 2 and 3 overlap for wavelengths greater than 600 nm. Notation for these curves is the same as used in Figs. 1 and 4.

4.1.2. Pure Fresh Water $c(\lambda)$

We now have an objective basis with which to select the more precise and accurate data from among those shown in Fig. 1. Consider, as a specific example, the data of James and Birge (1938) and Clark and James (1939) shown in Fig. 3. All of the data shown in this figure were obtained with the same experimental apparatus: a prism monochromator to disperse the radiant energy, attendant optics for forming a parallel beam, a tube containing the water under test, and a thermopile and galvanometer to measure the transmitted radiant energy. This apparatus could accommodate water sample tubes of variable length and internal lining.

The data shown in Fig. 3 may be qualitatively considered for relative accuracy. Curves 1 and 3 are plots of data that were obtained with the same cell length ($r = 0.97$ m) and experimental procedure save for one important difference. The tube used to obtain the data for curve 3 was lined with silver, whereas, the data for curve 1 was obtained with a sample tube, the inside of which was blackened by treatment with ammoniacal copper carbonate and then lined with ceresin wax. It seems clear, with the advantage of hindsight, that the silver-lined tube, as compared to the blackened tube, enhanced the systematic error due to the increased collection of forward-scattered flux. Transmittances measured with the silver-lined tube were thus systematically higher (due to this scattered radiant energy), and the corresponding attenuation coefficients lower, than similar measurements obtained with the blackened tube. Thus, we consider the data shown by curve 3 to be less accurate than the data shown by curve 1.

The relative precision of the data shown in Fig. 3 may be quantitatively evaluated by making use of Eq. (17) as shown in Fig. 2. The photometric error in the measurement of the beam transmittance, ΔT , was about 0.01 (Clark and James, 1939; footnote, p. 45). This corresponds closely to the example discussed in Section 4.1.1. Thus, by comparing the data shown in Fig. 3 with the curve of $r = 1$ m in Fig. 2, it is seen that data points having values that lie below $c = 0.04 \text{ m}^{-1}$ lie outside the optimum range for precise measurement. The data points of curve 3 which are lower than $c = 0.01 \text{ m}^{-1}$ may be expected to have fractional errors in excess of 100%. From the range of $c(\lambda)$ values obtained by James *et al.* we can expect that the data obtained with the 2-m sample tube (curve 2, Fig. 3) will be more precise than those obtained with the 1-m path length tube.

In selecting what we judge to be the more reliable data (shown in Fig. 4) we have included only those $c(\lambda)$ data of James *et al.*, which exhibit fractional random errors of less than 27%. We consider the "black tube" data to be more accurate than the "silver tube" data and the 2-m path length data to be more precise than the 1-m path length data. Thus, we consider

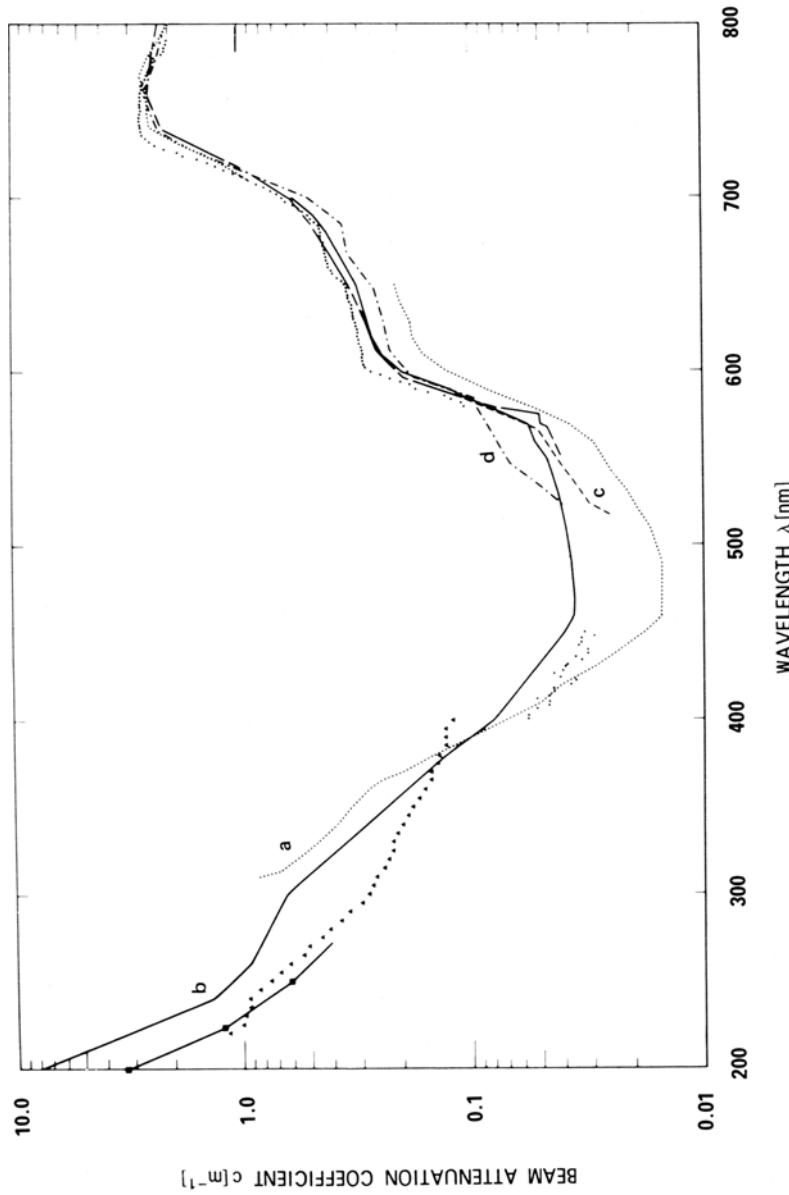


Fig. 4. Selected data for the total attenuation coefficient $c(\text{m}^{-1})$ of pure water as a function of wavelength, $\lambda(\text{nm})$. Only data judged to have relatively higher precision and accuracy are included in this figure (see text for details). These selected data are also given in Table 2. Curve notation follows that of Figs. 1 and 3; Curve (a), Sawyer (1931); Curve (b), Curcio and Petty (1951); Curve (c), Dawson and Hulbert (1945); Curve (d), James and Birge (1938); Curve (d), Clark and James (1939); ($\blacktriangle \blacksquare$), Lenoble and Saint-Guilly (1955); ($\blacksquare \blacktriangle$), Armstrong and Boalch (1961a,b); ($\cdots \cdots$), Sullivan (1963).

curve 2 to be James' most reliable data. While these are not his most frequently quoted data (James and Birge, 1938, Table I), they are the data he and Birge used (1938, Table X) as a standard of comparison for all their other lake water studies.

In a similar manner we have evaluated the path length precision and estimated relative accuracies of all the data shown in Fig. 1. This provides a selection of data we judge to have greatest reliability. These data are plotted in Fig. 4, where the range of discrepancy in the data has been reduced, but hardly eliminated, by this selection.

The data of Hulbert (1928) and Hodgman (1933) have not been included in Fig. 4 because they used path lengths too short (0.0015 and 0.021 m by Hulbert and 0.02 m by Hodgman) to obtain precise results. This judgment is consistent with Hulbert's later (Dawson and Hulbert, 1934) assessment of this early work.

Sawyer (1931) gives a historical review of data obtained prior to 1931 and includes a discussion of the errors involved in beam transmittance measurements. However, he apparently did not consider errors due to narrow-angle forward-scattering and made little attempt to reduce or eliminate stray or scattered flux. It is likely that his values for the attenuation coefficient are systematically low, especially for the smaller $c(\lambda)$ values. The precision of Sawyer's measurements was limited to that obtainable by photographic photometry ($\Delta T \geq 0.01$). However, he utilized various sample tubes up to 5 m in length in order to minimize his random errors. Thus, his data are relatively precise throughout the full spectral region (305–625 nm) of his results. We include Sawyer's data in Fig. 4, but note that his values are likely to be systematically low.

The data of Dawson and Hulbert (1934) and Hulbert (1945) would appear to be among the more reliable and more complete for $c(\lambda)$ between 200 and 700 nm. Dawson and Hulbert's 1934 data were obtained with a quartz spectrograph with use made of photographic photometry. These data, primarily in the ultraviolet region of the spectrum, made use of 0.35- and 2.72-m path length sample cells. Dawson and Hulbert (1934) noted that for "wavelengths from about 400 to 520 nm, water is so transparent that the absorption cannot be determined with 2.72-m tube." Later Hulbert (1945) obtained precise data within this spectral region (400–520 nm) by utilizing a 3.64-m sample cell. This work was especially important because, for the first time, an attempt was made to determine the total scattering coefficient simultaneously with the attenuation coefficient, thereby permitting the absorption coefficient to be evaluated. Hulbert's data are the only data by a single investigator to span the full range of both the ultraviolet and visible regions of the spectrum. His data provide the shape of the $c(\lambda)$ curve,

without discontinuities, for the full spectral region considered in this review. All of Hulbert's (1934 and 1945) data are included in Fig. 4.

Curcio and Petty (1951) determined, with relatively good precision and good accuracy, the attenuation coefficient for liquid water in the near infrared. Their data, from 700 to 800 nm, have been included in Fig. 4.

Lenoble and Saint-Guilly (1955) measured the attenuation coefficient of distilled water, using the techniques of photographic photometry, between 200 and 400 nm. They used sample tubes 1- and 4-m long, which, as can be seen from Fig. 2, are optimally chosen for $c(\lambda)$ values between 0.1 m^{-1} and 1.0 m^{-1} . The data of Lenoble and Saint-Guilly appear to be among the more reliable for the ultraviolet region of the spectrum and have been included in Fig. 4. One aspect of these ultraviolet data and those of Armstrong and Boalch discussed below, is that these data do not seem to confirm the slopes of the curves drawn through data for the visible region. While the data of Lenoble and Saint-Guilly match those of Hulbert at 380 nm, they become progressively lower than Hulbert's at shorter wavelengths. The shape of their $c(\lambda)$ curves is thus dissimilar.

Armstrong and Boalch (1961a,b) measured $c(\lambda)$ for fresh and artificial sea water between 200 and 400 nm utilizing a spectrophotometer and a 0.1-m path length. Because of this short path length we consider the data to be relatively precise only for values of c greater than 0.4 m^{-1} . We have included in Fig. 4 only these values and only after applying a correction of several percent to their original data which is necessary to account for loss of light by reflection at the end windows of their sample cell (this correction is mentioned but not made by Armstrong and Boalch.) This data of these workers indicate that there is a relatively large difference between the attenuation coefficients of pure fresh and pure artificial sea water in the ultraviolet region of the spectrum. This is in agreement with the earlier observations of Hulbert (1928) and Lenoble (1956a,b).

Sullivan (1963) determined $c(\lambda)$ for pure fresh water and artificial sea water for the visible spectrum with a spectrometer and photomultiplier detector. The photometric error in the measurement of beam transmittance for his data is probably less than that of earlier workers. Sullivan used path lengths of 0.63 and 1.32 m. He did not present data in the spectral region where c is less than about 0.03 m^{-1} . We judge the relative precision of his data to be better than other published $c(\lambda)$ data. Sullivan took special care to work with a small-diameter parallel beam and to "minimize the effects of stray light." He used water that had been triply distilled over quartz and took extra care during the measurement to maintain the optical purity of his water. For these reasons we judge the accuracy of Sullivan's data to be relatively high.

The selected data plotted in Fig. 4 have also been listed, for the sake of

accuracy and completeness, in Table 2. Also included in Table 2 is the average value of the beam attenuation coefficient, $\bar{c}(\lambda)$, the standard deviation of this mean value $\delta c(\lambda)$, the fractional standard deviation ($\delta c/\bar{c}$), and the number of selected data values, n , at each wavelength. These mean values of the beam attenuation coefficient, along with dashed curves one standard deviation on each side of \bar{c} , are plotted in Fig. 5. It must be noted that the shape (in particular discontinuities) of the $c(\lambda)$ curve is in part determined by the overlapping of the spectral data of the various investigators. Further, while we have selected the more reliable data, we know of no quantitative way to weight the data of the various investigators. Thus, we have not been able to obtain a properly weighted $\bar{c}(\lambda)$ value.

The statistical average of the selected data (Table 2 and Fig. 5) is given

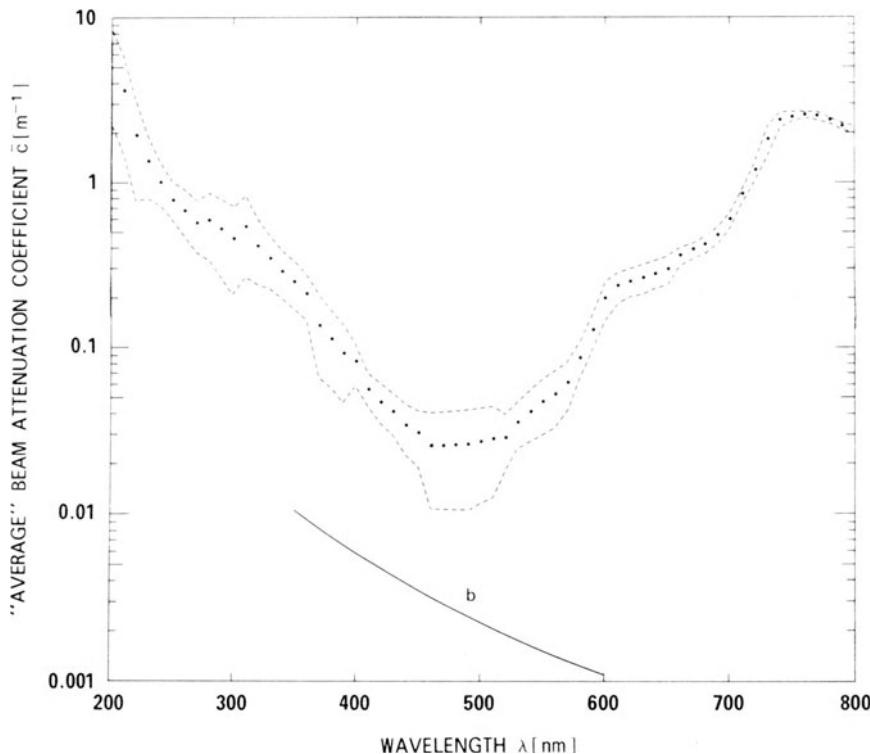


Fig. 5. Average beam attenuation coefficient for pure water as a function of wavelength for the selected data plotted in Fig. 4 and listed in Table 2. The dots indicate average values of $c(\lambda)$, the dashed curves are one standard deviation on either side of $c(\lambda)$. Discontinuities in the curves are due to overlapping of the spectral data of the various investigators. The solid curve $b(\lambda)$ is a plot of Morel's (1966) data for the total scattering coefficient of pure water.

TABLE 2. Beam Attenuation Coefficient $c(\lambda) (\text{m}^{-1})$, Pure Water

λ (nm)	Sa ^a 1931	DH ^b 1934	JB ^{b,c} 1938	CJ 1939	Hu 1945	CP 1951	LS 1955	AB ^{b,d} 1961a,b	Su 1963	n	\bar{c}	δc	$\frac{100\delta c}{\bar{c}}$
200	8						3.2			2	5.6	3.4	61
210		5.2					2.1			2	3.7	2.2	60
220		3.3					1.14	1.4		3	1.95	1.2	60
230		2.0					0.98	1.0		3	1.36	0.56	41
240		1.35					0.93	0.78		3	1.02	0.30	29
250		1.0					0.75	0.61		3	0.79	0.20	25
260		0.92					0.62	0.50		3	0.68	0.22	32
270		0.79					0.51	0.41		3	0.57	0.20	35
280		0.77					0.41			2	0.59	0.26	43
290		0.71					0.34			2	0.53	0.26	50
300		0.64					0.28			2	0.46	0.26	55
310	0.84	0.53					0.26			3	0.54	0.29	53
320	0.58	0.43					0.23			3	0.41	0.18	43
330	0.46	0.35					0.22			3	0.34	0.12	35
340	0.38	0.28					0.20			3	0.29	0.09	32
350	0.33	0.23					0.18			3	0.25	0.08	32
360	0.28	0.19					0.16			3	0.21	0.06	30
370	0.20	0.16	0.034				0.15			4	0.136	0.071	52
380	0.148	0.13	0.030				0.14			4	0.112	0.055	49
390	0.099	0.11	0.026				0.13			4	0.091	0.045	49
400	0.072	0.08					0.12	0.058	5	0.082	0.023	28	
410	0.050		0.070				0.047		3	0.056	0.013	23	
420	0.041		0.061				0.038		3	0.047	0.013	27	
430	0.030		0.053				0.040		3	0.041	0.012	28	
440	0.023		0.046				0.032		3	0.034	0.012	34	
450	0.018		0.040				0.033		3	0.030	0.011	37	
460	0.015		0.036						2	0.026	0.015	58	
470	0.015		0.036						2	0.026	0.015	58	
480	0.015		0.037						2	0.026	0.015	59	
490	0.015		0.037						2	0.026	0.016	60	
500	0.016		0.038						2	0.027	0.016	58	

510	0.017	0.039	2	0.028	0.016	56
520	0.019	0.027	0.040	0.029	0.011	37
530	0.021	0.033	0.045	0.042	0.011	31
540	0.024	0.038	0.057	0.044	0.014	34
550	0.027	0.043	0.069	0.047	0.017	37
560	0.030	0.047	0.076	0.053	0.019	37
570	0.038	0.056	0.085	0.066	0.020	32
580	0.055	0.087	0.093	0.084	0.020	23
590	0.085	0.135	0.125	0.120	0.127	25
600	0.125	0.21	0.182	0.197	0.196	27
610	0.160	0.24	0.22	0.24	0.23	21
620	0.178	0.27	0.23	0.27	0.25	20
630	0.181	0.29	0.24	0.28	0.26	21
640	0.20	0.31	0.25	0.29	0.28	20
650	0.21	0.33	0.27	0.31	0.29	20
660		0.37	0.30	0.34	0.35	13
670		0.41	0.34	0.38	0.39	10
680		0.46	0.35	0.41	0.45	12
690		0.52	0.40	0.47	0.51	12
700		0.60	0.50	0.58	0.65	11
710		0.80	0.74	0.9	0.90	10
720		1.04	1.06	1.2	1.38	13
730		1.46	1.63	1.8	2.31	20
740		2.16	2.25	2.4	2.70	10
750		2.30	2.40	2.5	2.68	6.6
760		2.45	2.56	2.5	2.69	4
770		2.40	2.40	2.7	2.52	4
780		2.32	2.25	2.5	2.35	4
790		2.26	2.13	2.2	2.05	4
800		2.20	2.02	2.0	3	3
					2.07	5.3

^a Sa, Sawyer (1931); DH, Dawson and Hulbert (1934); JB, James and Birge (1938); CJ, Clark and James (1939); Hu, Hulbert (1945); LS, Curcio and Petty (1951); AB, Armstrong and Boalch (1961a, 1961b); Su, Sullivan (1963); *n*, number of *c* values averaged; \bar{c} , arithmetic average; δc , standard deviation; $100\delta c/c$, fractional standard deviation (%).

^b Determined, for even values of wavelength, by a linear interpolation between original data points.

^c Two-meter, black paraffin-lined tube (JB, Table X).

^d As corrected by Smith and Tyler.

primarily to indicate the range of uncertainty in the $c(\lambda)$ data as a function of wavelength. The fractional standard deviation is 5–10% for wavelengths between 800 and 700 nm, 10–20% for wavelengths between 700 and 600 nm, 20–40% for wavelengths between 600 and 500 nm, and rises to values as high as 60% for wavelengths between 500 and 200 nm. Also included in Fig. 5 is a plot of Morel's (1966) data for the total scattering coefficient of pure water, $b(\lambda)$. As noted in Section 4.1.1, the scattering coefficient for pure water is roughly a factor of 10 less than the attenuation coefficient for wavelengths less than 500 nm. For wavelengths greater than 500 nm, b is several orders of magnitude less than c . Thus, while b is small compared to c , it is a prime candidate as a source of increased systematic experimental error for wavelengths below 500 nm.

Even after selecting to retain only the more precise and accurate data (Fig. 4), there is not a good agreement among the results of various investigators. In view of the theoretical interest in the optical properties of pure water (Morel, 1974; Litan, 1968 and references therein) and the potential application of these pure water properties to a variety of important processes in limnology and oceanography (Hutchinson, 1957; Jerlov, 1953, 1968; Jerlov and Steeman Nielsen, 1974 and references therein), it is surprising that our knowledge of them is so uncertain. Our discussion of $c(\lambda)$ data has been undertaken, in part, because there are few $K(\lambda)$ data in the ultraviolet region of the spectrum and none below 310 nm. We must therefore try to utilize $c(\lambda)$ data to determine theoretically $K(\lambda)$ for the ultraviolet spectral region. Thus, an uncertainty in c is theoretically related to our uncertainty in estimating the penetration of radiant energy into natural waters. For example, a factor of 3 uncertainty for the value of c (at 300 nm) is the difference between a fixed amount of radiation at this wavelength penetrating to a depth of 10 m (estimated using the high value of c) vs. 30 m (using the low value), or to a depth of 20 vs. 60 m, 30 vs. 90 m, etc. These differences have potential ecological significance.

4.1.3. Pure (Artificial) Sea Water $c(\lambda)$

A number of workers have included in their study of pure fresh water an investigation of pure sea water. Several techniques have been used to obtain "pure" sea water. Hulbert (1928), Armstrong and Boalch (1961a,b), and Sullivan (1963) combined the appropriate chemicals with carefully distilled water so as to simulate the average salt composition of sea water. Lenoble determined the beam transmittance of distilled water (Lenoble and Saint-Guilly, 1955) and of various concentrated solutions of the independent

sea salts (Lenoble, 1956*a,b*). The transmittance of pure sea water, of average salinity, can then be calculated from these data. Clark and James (1939) filtered an actual sample of sea water from an oceanographic region (Sargasso Sea) known to be relatively free of particulate matter.

Data of $c(\lambda)$ for "pure" sea water are plotted in Fig. 6 and listed in Table 3. The data given in Fig. 6 and Table 3 have been selected by use of the same criteria as used in Section 4.1.2 for selecting $c(\lambda)$ data for pure fresh water. There are no data between 400 and 500 nm, the spectral region of maximum transmittance, which conforms to our selection criteria.

The data of Sullivan (1963) for pure sea water are relatively precise and accurate. As noted by Sullivan, the attenuation of distilled and artificial sea water differs little in the spectral region above 500 nm. In contrast, as shown by Hulbert (1928), Armstrong and Boalch (1961*a,b*), and Lenoble and Saint-Guilly (Lenoble and Saint-Guilly, 1955; Lenoble, 1956*a,b*), the salts in sea water increasingly absorb radiation with decreasing wavelength. The magnitude of this difference between fresh and sea water for $c(\lambda)$ in the ultraviolet region of the spectrum may be seen by comparing corresponding

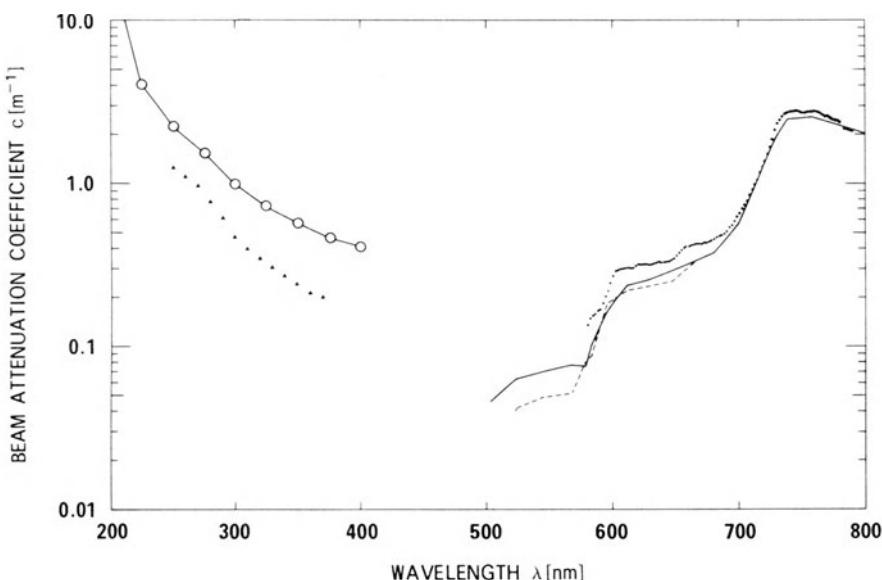


Fig. 6. Beam attenuation coefficient of "pure" sea water as a function of wavelength: (—), Clark and James (1939), Sample 5; (-----), Clark and James (1939), Sample 7; (▲ ▲ ▲), Lenoble and Saint-Guilly (1955), Lenoble (1956*a,b*); (○ ○ ○), Armstrong and Boalch (1961*a,b*); (.....), Sullivan (1963).

TABLE 3. Beam Attenuation Coefficient $c(\lambda)$ (m^{-1}),
 "Pure" Sea Water

λ (nm)	CJ ^{a,b,c} 1939	L ^d 1956	AB ^{b,e} 1961	Su 1963
200			21.8	
210			10.7	
220			5.5	
230			3.6	
240			2.8	
250		1.26	2.22	
260		1.10	1.90	
270		0.97	1.64	
280		0.77	1.39	
290		0.60	1.17	
300		0.47	0.99	
310		0.40	0.86	
320		0.35	0.75	
330		0.30	0.68	
340		0.27	0.62	
350		0.24	0.57	
360		0.21	0.52	
370		0.20	0.48	
380			0.45	
390			0.43	
400			0.41	
560	0.062			
570	0.066			
580	0.079		0.133	
590	0.123		0.167	
600	0.182		0.27	
610	0.25		0.30	
620	0.232		0.32	
630	0.242		0.31	
640	0.256		0.33	
650	0.272		0.36	
660	0.306		0.41	
670	0.342		0.42	
680	0.372		0.45	
690	0.440		0.50	
700	0.553		0.65	
710	0.83		0.91	
720	1.22		1.37	
730	1.88		2.31	
740	2.45		2.73	
750	2.50		2.70	

TABLE 3. Continued.

λ (nm)	CJ ^{a,b,c} 1939	L ^d 1956	AB ^{b,e} 1961	Su 1963
760	2.51			2.73
770	2.40			2.54
780	2.54			2.38
790	2.13			2.10
800	1.96			

^a Legend: CJ, Clark and James (1939); L, Lenoble (1956) and Lenoble and Saint-Guilly (1955); AB, Armstrong and Boalch (1961a, 1961b); Su, Sullivan (1963).

^b Determined, for even values of wavelength, by a linear interpolation between original data points.

^c Filtered Sargasso Sea water, average of samples 5 and 7.

^d Calculated using data from Lenoble and Saint-Guilly (1955) and Lenoble (1956).

^e As corrected by Smith and Tyler.

data in Tables 2 and 3 (or Figs. 4 and 6). While there are insufficient data to compute an average $c(\lambda)$ curve for pure sea water, we can conclude that there is less than a few percent difference between fresh and sea water above 400 nm. Below 400 nm the values of $c(\lambda)$ for sea water will be increasingly larger than $c(\lambda)$ for fresh water, with sea water values becoming 3–10 times larger than those for fresh water at values between 200 and 300 nm.

4.2. Diffuse Attenuation Coefficient for Irradiance, $K(\lambda)$

As discussed in Section 3.2, the diffuse attenuation coefficient for irradiance can be used to describe the penetration of solar radiation into natural waters. Values of $K(\lambda)$ have been obtained for a wide variety of natural waters and environmental conditions (Morel and Caloumenos, 1974, 1973; Morel, 1973; Smith, 1973; Smith *et al.*, 1973; Burr and Duncan, 1972; Tyler and Smith, 1970, 1967; Kampa, 1970; Halldal, 1969; Smith and Tyler, 1967; Duntley, 1963; Lenoble, 1956c; Ivanoff, 1956; Jerlov, 1950, 1951; Jerlov and Koczy, 1951). Earlier *in situ* spectroradiometric measurements have been referenced and reviewed by Tyler (1965) and Jerlov (1968). The early spectral measurements of the sun's radiant energy underwater employed broad-band optical filters for wavelength selection and are not accurate in comparison to more recent measurements (Tyler, 1959).

4.2.1. Discussion of Error

The errors involved in determining the spectral diffuse attenuation coefficient for irradiance of natural waters have been discussed in detail by Tyler and Smith (1970, 1967) and Tyler (1959). Briefly, the principal errors are caused by light "leakage" due to insufficient wavelength isolation, spectral bandwidth shifts due to the strong selective absorption of water, and inability to collect radiant energy in accordance with the theoretical concept of irradiance. Since $K(\lambda)$ is an apparent optical property dependent upon changes in the environmental radiance distribution, care must also be exercised when intercomparing data which has been obtained by various investigators or obtained under different environmental conditions.

Environmental variability, when not itself a subject of investigation, may require that measurements of underwater spectral irradiance be time averaged or otherwise corrected to account for this variability. Environmental variability may be caused by changes in sun angle, variable cloud cover, high sea states, and inhomogeneities (in space and/or time) of the water mass under investigation.

In order for an underwater spectral irradiance instrument to be accurate, it must make measurements which conform to Eq. (1). This requires that a properly designed underwater cosine collector be constructed (Smith, 1969) and that a spectral calibration be performed by means of a standard of spectral irradiance (Stair *et al.*, 1963). The numerical value of $K(\lambda)$ may be determined from relative values of spectral irradiance since only ratios of $E_d(z)$ are required [Eq. (12)]. However, it has been shown (Smith, 1969) that inexact collector geometries can cause systematic errors in the determination of $K(\lambda)$ as a function of depth. In contrast to laboratory measurements of $c(\lambda)$, it is relatively easy to obtain large (and thus optimum) path lengths ($z_2 - z_1$) in the natural environment.

The most critical requirement for accurate underwater spectral irradiance measurements is due to the selective absorption of water itself (Tyler, 1959). In the Sargasso Sea, for example, the ratio $E_d(450)/E_d(650)$ is more than a million to one at a depth of 40 m. Thus efforts to measure E_d at wavelengths removed from the wavelength of maximum transmittance are beset with formidable problems. To be accurate, underwater spectroradiometers must be designed to have extremely low levels of stray light from spectral regions outside the indicated wavelength bandwidth. This problem has been discussed in detail by Tyler and Smith (1970).

Figure 7 shows representative plots of $E_d(\lambda_{\max})$ as a function of depth for four different ocean waters. The wavelength of maximum irradiance transmittance (minimum K) is denoted λ_{\max} . The slope of these curves (on a semilogarithmic plot) gives $K(\lambda_{\max})$ [Eqs. (11 and 12)]. As noted in Section

3.2, the diffuse attenuation coefficient for irradiance (i.e., the slope of the curve in Fig. 7) varies as the radiance distribution changes with depth. Below several optical depths ($K \cdot z$) in homogeneous water, the K value approaches a constant (asymptotic) value (Preisendorfer, 1959). This occurs within a few meters of the surface for high K values and after several tens of meters for low K values.

For the data presented in Section 4.2.2, we have endeavored to include only "asymptotic" $K(\lambda)$ values. The data are thus comparable and can be used to typify the waters under study. However, it must be noted that $K(\lambda)$ values nearer the surface are, in general, smaller than asymptotic values (the radiance distribution is less diffuse), and an asymptotic value of $K(\lambda)$

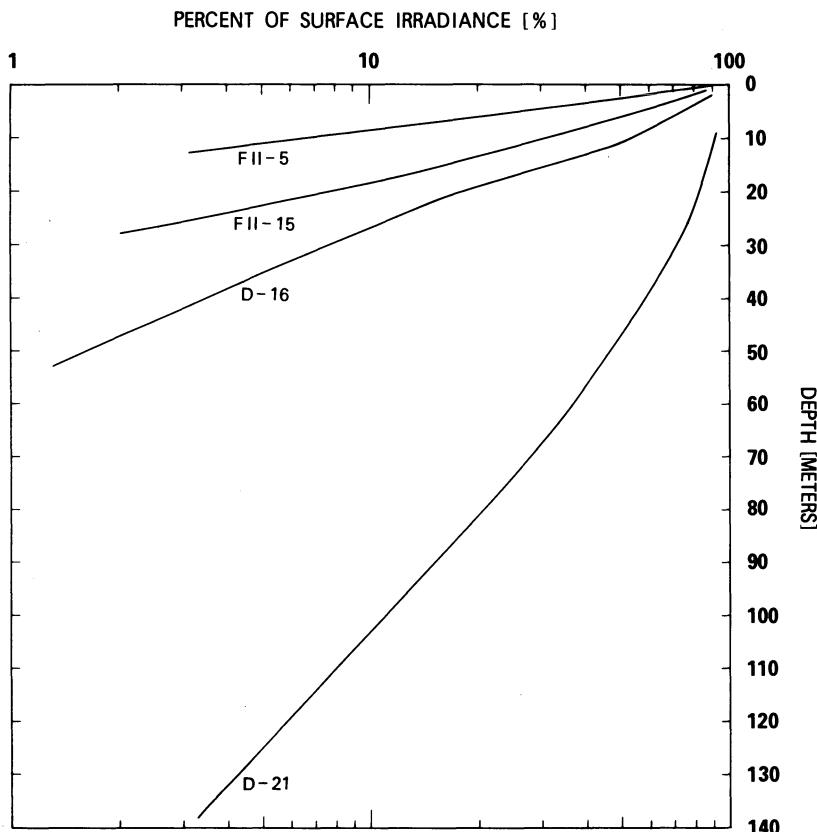


Fig. 7. Downwelling irradiance at the wavelength of maximum transmittance, $E_d (\lambda_{\max})$, as a function of depth z . Data are from Discoverer expedition, May, 1970, (Tyler, 1973) and Fresnel II Cruise, March, 1971, and correspond to the similarly labeled data in Fig. 8.

will thus underestimate the real amount of radiant energy penetrating into natural waters.

In order to minimize errors, the calibration of any radiant energy measuring device should be considered a maintenance procedure. This is especially true of instrumentation which must withstand the rigors of the ocean environment. Instrumentation components are unreliable and cannot be trusted to remain stable over long periods of time. Consequently, every period of *in situ* data acquisition should be preceded and followed by a formal calibration which should be treated as part of the data. The accuracy of published data may, in part, be assessed with respect to this calibration procedure.

4.2.2. Representative Natural Waters $K(\lambda)$

Diffuse attenuation coefficients for irradiance, for several representative natural waters, are plotted in Fig. 8. Of particular interest are the data obtained in the Sargasso Sea. This relatively barren ocean gyre, in the southwestern area of the North Atlantic, is regarded by oceanographers as having among the clearest of ocean waters. Thus $K(\lambda)$ data from this region are expected to show the lowest values (hence maximum values of irradiance transmittance) to be obtained in ocean waters. The Sargasso Sea data provide a basis with which other more productive ocean areas may be compared, and data from this area may be regarded as the natural analogue of "pure" sea water.

Spectral irradiance measurements have been made in the area of the Sargasso Sea by several investigators (Jerlov, 1950, 1951, 1968; Ivanoff *et al.*, 1961; Neuymin *et al.*, 1961; Kullenberg *et al.*, 1970; Tyler and Smith, 1966, 1967, 1970; Lundgren *et al.*, 1971; Morel, 1973; Smith, 1973). Two instruments, utilizing dispersing systems which permit spectral irradiance measurements of relatively high accuracy, are the Scripps spectroradiometer (Tyler and Smith, 1966, 1970) and the French spectroirradiance meter (Bauer and Ivanoff, 1970). The Scripps spectroradiometer utilizes a double Ebert grating monochromator to minimize stray light within the instrument and has been calibrated to measure absolute values of spectral irradiance from 360 to 740 nm. Absolute accuracy, a function of the vicissitudes of a particular cruise and time between calibrations, is usually less than $\pm 10\%$ (350–400 nm) and $\pm 6\%$ (400–740 nm). The relative precision is plus or minus a few percent under favorable environmental conditions. The French spectroirradiance meter utilizes a Bausch and Lomb grating monochromator as the dispersing element and measures relative spectral irradiance. The relative spectral values are placed on an absolute basis by

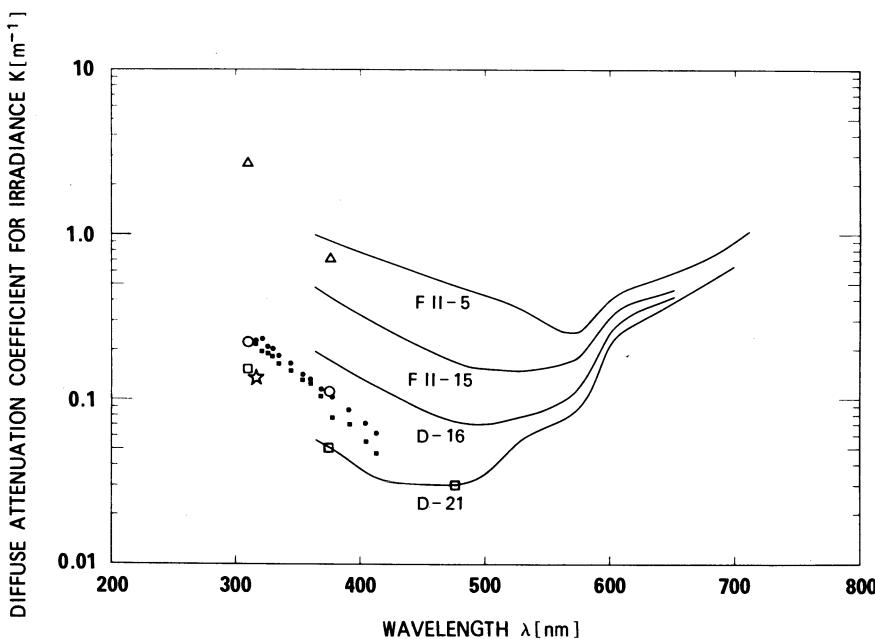


Fig. 8. Diffuse attenuation coefficient for irradiance, for selected water types, as a function of wavelength: *D*-21, Discoverer expedition 1970 (Tyler, Smith, Morel, 1973); Open ocean, low productivity [0.025 mg/m³ Chlorophyll *a* (Chl *a*), Baird, 1973] waters of the Sargasso Sea (25°43' N, 65°41' W); *D*-16, Discoverer expedition, 1970, low productivity (0.45 mg/m³ Chl *a*) coastal waters (6°27' N, 79°57' W); *FII*-15, Fresnel II Cruise (R. C. Smith and J. E. Tyler, unpublished); Moderately productive (2.2 mg/m³ Chl *a*, Berman and Kiefer, private communication) coastal waters (20°41' N, 105°26' W); *FII*-5, Fresnel II Cruise. Highly productive coastal [10 mg/m³ Chl *a*] waters of Bahia Concepcion (Gulf of California (26°43' N, 111°53' W)]; □, Jerlov (1950, 1951) Eastern Mediterranean Sea; ☆, Calkins (1975), clean ocean waters near Puerto Rico; ○, Jerlov, open ocean [as attributed to Jerlov by Steemann Nielsen (1964)]; △, Jerlov, coastal waters; ■, ●, Lenoble (1954, 1955, 1956c) relatively clear waters of the Mediterranean Sea. The chlorophyll *a* values given are the average concentration in the water column above the depth at which *K*(λ) was determined.

employing a calibrated narrow-band irradiance collector in conjunction with the special instrument.

On the Discoverer Expedition, May, 1970, the French (Bauer and Ivanoff, 1970) and American (Tyler and Smith, 1966) instruments were used to obtain spectral irradiance data in the Sargasso Sea at the same stations (Morel, 1973; Smith, 1973). The curve labeled *D*-21 on Fig. 8 is a plot of the *K*(λ) values calculated from the data obtained with these two instruments. Earlier data, obtained at 310, 365, and 475 nm by Jerlov in the very clear waters of the Eastern Mediterranean Sea (1950) (indicated by the three

open squares) are also included. Jerlov isolated a few narrow spectral bandwidths by carefully combining liquid and glass filters along with a selenium rectifier cell. These data are among the few which have been determined with relative good accuracy using filters to select bandwidth.

The Sargasso Sea and other very clear ocean water data comprise a consistent and relatively accurate ($\pm 20\%$) set of $K(\lambda)$ values, from 310 to 700 nm, for an important specific ocean water type. These data represent the lowest (or at least near the lowest) asymptotic $K(\lambda)$ values to be expected in the world's oceans. These data are also the easiest to specify uniquely since they represent a natural limit. The $K(\lambda)$ values of the Sargasso Sea can be used, with due regard to depth variations shown in Fig. 7, to estimate the maximum penetration of radiant energy to depths in ocean waters.

Because of the recent interest on the possible enhancement of solar UV-B (280–320 nm) at the earth's surface (Environmental Studies Board, 1973; Green *et al.*, 1974), Calkins (1975) has measured the penetration of UV-B into various natural waters. A Robertson meter (Robertson, 1972; CIAP, 1975), which in water has a dominant wavelength of about 315 nm and a bandwidth (FWHM) of about 14 nm, was used for this work. An evaluation of a Robertson meter by the present authors indicates that this instrument is capable of accurate irradiance measurements (at 315 nm) to values of 0.1% of noon surface irradiance. The clearest ocean water measured by Calkins, off the coast of Puerto Rico, gave a $K(315 \text{ nm}) = 0.14 \text{ m}^{-1}$. This clear ocean water value (the "star" in Fig. 8) is consistent with the value of $K(310 \text{ nm}) = 0.15 \text{ m}^{-1}$ obtained by Jerlov (1950) in very clear sea water. Values of $K(315 \text{ nm})$ obtained by Calkins, for other natural waters, ranged from this low of 0.14 m^{-1} to over 20 m^{-1} in Delaware Bay.

Smith and Tyler (1967) and Smith *et al.* (1973) have argued that the waters of Crater Lake, Oregon, are a natural analogue of pure fresh water. When $K(\lambda)$ values of the Sargasso Sea and Crater Lake are compared for the same optical depths, it is found that they are the same within the accuracy of measurement ($\sim 20\%$). Data below 360 nm, where a difference between fresh and sea water is expected, have not been obtained for Crater Lake. In the early work of Smith and Tyler (1967) on Crater Lake the published K values are for near-surface nonasymptotic depths and are therefore not directly comparable to the data presented here.

It is only the clearest ocean waters that can be uniquely specified. The $K(\lambda)$ values for other natural water types will be greater than those given for the Sargasso Sea and will vary with the physical and biological conditions of the water. We have selected three other representative water types based upon the average amount of chlorophyll *a* in the water column above the depth at which $K(\lambda)$ was determined. Morel and Smith (1974) have shown that a good correlation exists between chlorophyll *a* and the wavelength for

TABLE 4. Diffuse Attenuation Coefficient for
Irradiance $K(\lambda)$ (m^{-1}), Clear Ocean Water

λ (nm)	J ^a 1950	C 1975	ST 1975
310	0.15		
315		0.14	
350			0.059
360			0.056
370			0.053
375	0.051		
380			0.049
390			0.043
400			0.037
410			0.033 ₅
420			0.031 ₈
430			0.030 ₉
440			0.030 ₅
450			0.030 ₃
460			0.030 ₀
465	0.030 ₅		
470			0.029 ₈
480			0.029 ₈
490			0.031 ₀
500			0.034 ₅
510			0.040 ₀
520			0.049 ₅
530			0.058
540			0.063
550			0.066
560			0.072
570			0.080
580			0.094
590			0.135
600			0.22
610			0.26
620			0.28
630			0.31
640			0.34
650			0.37
660			0.43
670			0.46
680			0.51
690			0.57
700			0.63

^a J, Jerlov (1950); C, Calkins (1975), clear ocean water near Puerto Rico; ST, Smith and Tyler, calculated for this review from data of Smith (1973) and Morel (1973).

which the spectral irradiance has a maximum value, $\lambda_{H_{\max}}$. The curves in Fig. 8 are representative of coastal waters having low, medium, and high productivity (D-16, FII-15, FII-5, respectively).

In situ measurements in the ultraviolet region of the spectrum are very limited. We have included in Fig. 8 the data of Jerlov (1950) and Lenoble (1954, 1955, 1956c). An ultraviolet spectrograph, enclosed in an underwater housing with a quartz window and the technique of photographic photometry were used by Lenoble to obtain values of underwater radiance. The solid squares and circles in Fig. 8 give Lenoble's values for radiance K , rather than irradiance K , obtained in the Mediterranean Sea. Radiance and irradiance K values are strictly comparable only when both are obtained below depths where the K functions become asymptotic. While the data of Lenoble have not been uniquely matched to similar data in the visible region of the spectrum, her data serve to indicate the trend of $K(\lambda)$ in the ultraviolet region of the spectrum.

The combined data in Fig. 8 may be used [again with due regard for the behavior of $K(\lambda)$ as shown in Fig. 7] to calculate the transmission of solar radiation into natural waters. The choice of $K(\lambda)$ values may be made on the basis of chlorophyll *a* concentration or on the basis of $\lambda_{H_{\max}}$. $K(\lambda)$ data for clear ocean waters have been listed in Table 4.

5. SUMMARY

When the authors began this review it was hoped that the $c(\lambda)$ data for pure fresh and salt water could be used, together with theory and in conjunction with Crater Lake and Sargasso Sea $K(\lambda)$ data, to calculate a consistent set of $K(\lambda)$ values for the clearest natural waters, including an extrapolation into the ultraviolet region of the spectrum where experimental data do not now exist. The penetration of ultraviolet radiation into natural waters, due to possible increased levels of solar ultraviolet radiation reaching the earth's surface as a result of anthropogenic changes in the earth's atmosphere, could then have been reliably predicted. After attempting a number of theoretical techniques using available $c(\lambda)$, $b(\lambda)$, and $K(\lambda)$ data, it has been concluded that such an extrapolation of $K(\lambda)$ data cannot be done from the present data with credibility. As shown in Section 4.1, values for $c(\lambda)$ are known only within a factor of 3–10 in the ultraviolet spectral region. A careful comparison shows that in the blue region of the spectrum the $c(\lambda)$ and $K(\lambda)$ curves have different shapes and are diverging with decreasing wavelength. Even after accounting for the wavelength dependence of the total scattering coefficient, which influences $c(\lambda)$ more than $K(\lambda)$, it is not possible to make a satisfactory fit, much less an extrapolation.

tion, of the data. It would appear that direct experimental determination of the distribution functions (or the complete radiance distributions) will be necessary before an accurate extrapolation of the $K(\lambda)$ data can be made.

The data presented in Section 4 may be used to calculate the transmission of radiant energy into the four selected natural water types (Figs. 7, 8) which range in chlorophyll-*a* concentrations from 0.029 to 10 mg m⁻³. The data for the Sargasso Sea (Fig. 8 and Table 4) represent a clean natural water with which to compare other waters.

The techniques of optical oceanography can provide an accurate description of the flow of radiant energy into natural waters. At the present time both the spectral and geometrical distributions of underwater radiant energy have been determined for various waters. This information makes it possible to determine the radiant energy available for underwater photoprocesses and also to determine the radiant energy actually utilized in aquatic photosynthesis. Detailed information on the underwater photic environment makes it possible to relate the structure and function of underwater photoreceptors (e.g., chloroplasts or the eyes of fish) to spatial and spectral characteristics of the radiant energy distribution. Collaborative efforts of photobiology and optical oceanography may soon provide a much clearer understanding of aquatic photoprocesses.

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Light and Diurnal Vertical Migration: Photobehavior and Photophysiology of Plankton

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1. INTRODUCTION

Many phytoplankton and zooplankton species display a daily pattern of vertical movement, generally termed *diurnal vertical migration* (DVM). The photoresponses of these organisms become functionally significant during such migrations, since light is agreed to be the dominant environmental factor controlling DVM. Light underwater, however, is a complex environmental stimulus, various aspects of which can participate in initiating, controlling, and/or orienting DVM.

This review will concern the DVM patterns of plankton and the implications of light in these movements. Related topics that will be considered are the photobehavior of plankton as modified by internal and external factors; the photophysiology of the behavioral responses, with emphasis on intensity, wavelength, and polarization sensitivity; and the interrelationships of these different areas. An exhaustive literature survey is not attempted due to the size and diversity of this field, but rather recent work and areas of extensive research within each planktonic group are stressed.

1.1. Underwater Light Conditions

The physical characteristics of light underwater have been extensively reviewed (e.g., Jerlov, 1968, 1970; Jerlov and Steeman Nielsen, 1974; Hutchinson, 1957) and will not be discussed here in detail. Four general aspects of light underwater, however, are pertinent when considering behavioral photoresponses of plankton in relation to vertical movement.

(a) *Intensity*. Of importance are the ambient light flux or irradiance, its rate of change in time and space, and the angular distribution of light or radiance. Intensity at any moment in time decreases with depth due to scattering and absorption, and the intensity at any depth will vary over the day. The overall intensity to which an organism is exposed will affect its degree of light adaptation and thereby its sensitivity to light. In addition, ir-

radiance and its rate of change can regulate the type of behavioral response displayed, while angular distribution influences the orientation direction.

(b) *Spectral composition of the light.* This will vary with water type and depth and is important as it relates to the spectral sensitivity of the organism. For pure water and that found in the clearest lakes and oceanic waters, the wavelengths of maximum transmission are around 470–475 nm. As the water contains more particles and yellowing substances, the wavelength transmission maximum shifts to longer wavelengths. Thus, in clearest coastal water, the maximum is at about 500 nm, while in average coastal water and in inshore water the maxima range from 525 to 550 and 575 to 600, respectively (Jerlov, 1968). A similar situation occurs in lakes with increased dissolved substances and suspended matter (Hutchinson, 1957). With depth the spectral distribution of available light is increasingly composed of those wavelengths that are maximally transmitted.

(c) *Polarization.* Even though elliptical polarization exists underwater (Ivanoff and Waterman, 1958a), only linear polarization will be considered, since it is the type of polarization to which organisms are known to respond (Waterman, 1973). Although much of the light underwater is linearly polarized, the percentage depends on depth, turbidity, wavelength, nearness to the bottom, cloud cover, the sun's position, and direction of sight. In addition, a distinct polarization pattern exists underwater which depends mainly on the sun's position, the cloud cover, and the depth (Waterman, 1954, 1955; Waterman and Westell, 1956; Ivanoff and Waterman, 1958b; reviewed by Ivanoff, 1974). While polarized light underwater may be used as an orientation cue for vertical movements (Umminger, 1968a), it is theoretically most useful for positioning the sun during horizontal movements involving a sun compass orientation (Waterman, 1974b).

(d) *Day-night cycle.* This can be important for establishing the timing of biological rhythms and for seasonal cycles.

1.2. Behavioral Photoresponses

Motile organisms show a variety of rather stereotyped behavioral responses to light. The descriptive terminology for phytoplankton can vary with investigator, while for zooplankton it usually conforms to that proposed by Fraenkel and Gunn (1961). Terms used in this review are defined here. *Photokinesis* is a nondirectional response to a light stimulus. It is dependent upon intensity and occurs as a change in swimming velocity (*orthophotokinesis*) and/or a change in the rate of change in direction (*klinophotokinesis*). A positive orthophotokinesis is an acceleration of swimming velocity upon an intensity increase, while a negative response is the reverse. Positive and negative klinophotokinesis occur similarly.

TABLE 1. Phytoplankton Diurnal Vertical Migration Patterns

Species	Source
Dinoflagellates	
Type I. Dawn migration	
<i>Cachonina niei</i> (L) ^a	Eppley <i>et al.</i> , 1968
<i>Ceratium furca</i>	Hasle, 1954; Eppley <i>et al.</i> , 1968
<i>Ceratium fusus</i>	Hasle, 1954
<i>Gonyaulax polyedra</i> (L)	Hasle, 1950; Eppley <i>et al.</i> , 1968; Walsh <i>et al.</i> , 1974
<i>Gymnodinium nelsoni</i>	Goff, 1974
<i>Gymnodinium</i> sp.	Pomeroy <i>et al.</i> , 1956
<i>Peridinium</i> sp.	Pomeroy <i>et al.</i> , 1956
<i>Peridinium triquetrum</i>	Hasle, 1954
<i>Prorocentrum micans</i>	Hasle, 1950, 1954; Eppley <i>et al.</i> , 1965
Dinoflagellates: unidentified	Yentach <i>et al.</i> , 1964
Type II. Dusk migration	
<i>Ceratium fusus</i>	Hasle, 1950; Taylor <i>et al.</i> , 1966
<i>Ceratium tripos</i>	Hasle, 1950
<i>Exuviaella baltica</i> ^b	Wheeler, 1966; Marshall and Wheeler, 1965
<i>Peridinium brevi</i> ^b	Taylor <i>et al.</i> , 1966
Type III. Dawn and dusk migration	
<i>Ceratium hircus</i> ^b	Taylor <i>et al.</i> , 1966
<i>Dinophysis caudata</i> ^b	Taylor <i>et al.</i> , 1966
<i>Peridinium divergens</i> ^b	Taylor <i>et al.</i> , 1966
<i>Pyrodinium bahmense</i>	Taylor <i>et al.</i> , 1966; Soli, 1966
Fresh water flagellates	
Type I. Dawn migration	
<i>Endorina elegans</i>	Rüttner, 1943
<i>Gonyostomum semen</i>	Cowles and Brambel, 1936
<i>Volvox aureus</i>	Utermöhl, 1925

^a (L) Laboratory study; all others are field studies.

^b Tentative evaluation of pattern since data are only available for part of the day.

Phototaxis (or *phototropotaxis* in phytoplankton studies) is a directional movement in relation to the direction of a light stimulus. A positive response is movement toward the light source, while movement away is negative. The comparable directional response to a pattern of linearly polarized light is *polarotaxis* (Waterman, 1966). Another type of response has created some controversy over its description (Feinleib and Curry, 1970a) and is observed when microalgae experience an abrupt change in light intensity. In this situation organisms can show either a brief cessation of movement or a reversal in the direction of movement with subsequent movement in a new direction. Since this response is unoriented with respect to the stimulus light, it is not a true taxis, although it is frequently called a *photophobotaxis*. A more appropriate term is a *photophobic response*, as suggested by Diehn (1973). A positive photophobic response is a cessation of or reversal in the direction of movement with a sudden decrease in light intensity; if this occurs upon an intensity increase, the response is negative.

2. PHYTOPLANKTON

In reviewing the behavior and physiology of photoresponses by phytoplankton, discussion will be primarily limited to free-swimming photosensitive unicellular microalgae which are potentially capable of vertical movements due to their motility, i.e., dinoflagellates, euglenoids, and volvocales. Other phytoplankters are reported to respond behaviorally to light, e.g., coccolithophorids (Mjaaland, 1956) and diatoms (Nultsch, 1956; Nultsch and Wenderoth, 1973). These organisms, however, are not extremely motile, and the contribution of their light responses to movements in nature is unknown. Aspects of responses to light by phytoplankters have been reviewed repeatedly (Bendix, 1960; Clayton, 1964; Feinleib and Curry, 1971a; Halldal, 1962, 1964; Haupt, 1959, 1965, 1966; Nultsch, 1970); methods for studying behavioral responses are covered by Hand and Davenport (1970).

2.1. Diurnal Vertical Migration Patterns

Diurnal vertical movements by phytoplankton are well documented among many marine dinoflagellates and a few freshwater forms. There are three apparent patterns of migration (Table 1). *Dawn migration* involves a rise during the day with either a descent or a dispersion at night. The ascent begins either before or shortly after dawn with a rise to minimum depth. The descent begins in the evening usually before sunset and either continues

into the night down to a maximum depth or the cells become uniformly distributed in the water column through the night. In *dusk migration*, the reverse pattern is observed: there is a descent to maximum depth during the day and a rise to minimum depth beginning near sunset which can continue into at least part of the night. *Dawn-dusk migration* involves a rise and descent during both day and night. A rise occurs in the morning followed by a descent in the afternoon. A second rise begins at the end of the day, usually before sunset, which can continue into the night with a descent before morning. This pattern is only well studied in *Pyrodonium bahmense* (Soli, 1966), but is suggested in other species.

Both the ascents and descents involve active swimming movements. Admittedly, sinking can contribute to a descent, but at least for *Gonyaulax polyhedra* the contribution appears small. Estimates of the vertical migration distance traveled by *Gonyaulax* during the descent beginning in late afternoon are around 10 m (Hasle, 1950; Eppley *et al.*, 1968; Walsh *et al.*, 1974), yet a measured sinking rate for growing cells is only about 2.8 m/day (Eppley *et al.*, 1967). Measured swimming speeds range around 1 m/h (Hand *et al.*, 1965; Hasle, 1950) and are more compatible with the distance traveled. Thus the descent must involve active movement.

2.2. Photoreceptors

In neither dinoflagellates (Dodge, 1971) nor volvocales (e.g., Walne and Arnott, 1967) which are reported to be photoresponsive, is there a distinct organelle suggestive as being the light receptor. Nevertheless, several dinoflagellates have structures which certainly resemble photoreceptors. *Glenodinium foliaceum* has a lamellar body which lies just below the base of the longitudinal and transverse flagella (Dodge and Crawford, 1969), and dinoflagellates of the family Warnowiaceae (Greuet, 1964; Mornin and Francis, 1967) possess a complex eyespot consisting of a retinoid and lens capable of focusing light onto the inner part of the pigment cup (Francis, 1967). Unfortunately, photoresponses by these dinoflagellates have not been demonstrated. From a fine structural study of the volvocales *Chlamydomonas*, Walne and Arnott (1967) suggest that the photoreceptor may be the clear region of cytoplasm lateral to the stigma. However, supportive experimental evidence is needed.

In *Euglena*, Engelmann (1882) originally suggested that the photoreceptor was located at the anterior end of the organism. The organelles located there, one or both of which could be the photoreceptor, are (1) the paraflagellar body which is the swelling at the base of the locomotory flagellum and (2) the stigma (also termed eyespot) which is on a red-orange organelle situated lateral to the locomotory flagellum base. The compelling

evidence favoring the paraflagellar body as the photoreceptor has been reviewed by Feinleib and Curry (1971a) and centers on Gössel's (1957) work with mutants. Chlorophyll-free mutants having a paraflagellar body, either with or without a stigma, were phototactic, whereas *Astasia*, which lacks both a paraflagellar body and stigma, is unresponsive. *Astasia*, however, lacks other organelles, one of which may be the unrecognized photoreceptor (Feinleib and Curry, 1971a). Demonstration that the paraflagellar body contains a pigment having absorption characteristics resembling those predicted from action spectra would perhaps clarify the situation, but for technical reasons this has not been accomplished.

Alternately, the stigma is suggested as the photoreceptor (Batra and Tollar, 1964; Strother and Wolken, 1961; Wolken, 1967; Wolken and Shin, 1958). Evidence against this suggestion is that euglenoids lacking a stigma are phototactic (Gössel, 1957; Diehn and Tollar, 1966b). Furthermore, for *Euglena gracilis* the action spectra for photoresponses do not correspond to the absorption spectra of the carotenoid pigments in the stigma (Kivic and Vesk, 1972).

Considering stigmata in other phytoflagellates, their structure and cellular placement are not consistent between species (reviewed by Dodge, 1969). In dinoflagellates fewer than 5% have a stigma (Dodge and Crawford, 1969), and many species which lack a stigma are phototactic (Halldal, 1958; Hand *et al.*, 1967; Forward, 1974). Furthermore, stigmaless *Chlamydomonas* (Hartshorne, 1953) and *Chilomonas* (Luntz, 1931a), are also photoresponsive. Thus most evidence indicates that the stigma is not the photoreceptor in phytoflagellates.

2.3. Mechanism for Orientation to a Directional Light Source

In a converging light beam, phytoflagellates actually swim toward the light source not the most intense point (Buder, 1917; Halldal, 1959; Sachs and Mayer, 1961). This indicates definite orientation and directional swimming. Theories on the mechanism for orientation to a light source (reviewed by Feinleib and Curry, 1971a; Hand and Davenport, 1970) are limited by uncertainty about the photoreceptor location in many forms.

Detailed investigations of individual cells indicate that the behavioral sequence in some dinoflagellate species (Hand *et al.*, 1967; Hand, 1970; Forward, 1974a) and in the volvocales *Chlamydomonas reinhardtii* (Feinleib and Curry, 1971b) consists of an initial cessation of movement (negative photophobic response) upon stimulation with higher light intensities, followed by directional movement toward the light. The importance of the initial photophobic response to subsequent directional movement is unresolved. Nevertheless, using the dinoflagellate *Gyrodinium dorsum*, Hand

(1970) found that during movements subsequent to the initial stopping a cell aligns the anterior end directly toward the light source. In addition, using evidence from orientation to mono- and bidirectional stimuli, Hand (1970) argues that two receptors are present in a cell which perceives the light at two points in space and one point in time.

In the colonial flagellate *Volvox*, upon directional stimulation flagellar activity ceases in the anterior cells on the stimulated side. Unstimulated cells continue to beat, which causes orientation and movement toward the light source (Hand and Haupt, 1971; Huth, 1970). Thus, orientation by *Volvox* is due to an asymmetrical distribution of flagellar beating.

Orientation has been most extensively studied with *Euglena*. Because a receptor site is hypothesized, a mechanism for orientation is clearly suggested and supported by experimental observations. The most widely accepted explanation for phototactic orientation is the intermittent shading hypothesis originally advanced by Mast (1911) (reviewed by Feinleib and Curry, 1971a; Diehn, 1973). Swimming cells rotate about their longitudinal axis, which results in the stigma rotating around the paraflagellar body. Thus, if stimulated from the side, the stigma will intermittently shade the paraflagellar body. Shading causes a shock or photophobic response which realigns the cell's longitudinal body axis. This photophobic response continues to occur with each rotation until the relative position of the stigma, paraflagellar body, and light source are such that shading will no longer occur. In this case, the cell is aligned, either toward or away from the light. Orientation, therefore, consists of a series of nonoriented photophobic responses.

Thus although the stigma in *Euglena* is not the photoreceptor, it is important for orientation. The universality of this orientation mechanism is questionable, since some phototactic phytoflagellates lack stigmas. However, other organelles within the cell may function as shading bodies, e.g., chloroplasts (Halldal, 1958). This may also be true for *Euglena*, since Kivic and Vesk (1972) in reanalyzing older microspectrophotometric and action spectra data for *Euglena* argue that the chloroplasts must also be used for shading.

2.4. Photoresponses of Phytoplankton

Some vertical movements during the day are due to behavioral responses to daily changes in underwater light conditions (Hasle, 1950, 1954; Cowles and Brambel, 1936; Soli, 1966; Wheeler, 1964). Support for this statement is based on several observations. First, when studied in the laboratory those species showing DVM also display phototactic responses to light. Second, the timing of the distributional changes corresponds to

light intensity changes over a day. Third, alterations in daily light due to climatic conditions can alter migration, e.g., clouds lessen the intensity of migration (Hasle, 1954; Eppley *et al.*, 1968); on an exceptionally clear sunny day movement away from the surface at noon is observed (e.g., Goff, 1974). Fourth, the light transmission characteristics of the water can influence the migratory pattern. The migration pattern for *Ceratium fusus* (Hasle, 1950, 1954) illustrates this. At Ormya, where the depth at which 1% of the surface intensity occurred at 8–10 m, *C. fusus* showed a dusk migration pattern (Hasle, 1950), while at Frognerkilen the 1% depth was at 3 m, and the dawn migration pattern was observed (Hasle, 1954). Fifth, the light from the moon can alter the migration pattern at night (Cowles and Brambel, 1936).

Before considering the physiology of the photoresponses, internal and external modifying factors will be discussed. Specifically, these include circadian rhythms, temperature, inorganic ions, nutrients, and gases.

2.4.1. Internal Modifying Factors (Circadian Rhythms)

In many phytoflagellates, photoresponsiveness is not constant over a 24-h day, but shows circadian rhythmicity. The generalized sequence is to exhibit maximum responsiveness during the day with a minimum at night. Rhythms have been clearly demonstrated for three types of phytoplankton: dinoflagellates, euglenoids, and volvocales. Two species of dinoflagellates upon transfer from a 12h:12h light-dark (LD) cycle to constant lighting conditions show a clear circadian rhythm in responsiveness (Forward and Davenport, 1970; Forward, 1974a). In both cases the timing of the rhythms was similar, with the maximum photoresponse occurring at about the time for the beginning of the light phase. However, the physiology of the rhythm seemed quite different between species. In *Gymnodinium splendens* an identical rhythm was seen for the initial photophobic response and the subsequent positive phototactic response to 453-nm light (Forward, 1974a). In contrast, when stimulated with 470-nm light *Gyrodinium dorsum* had no rhythmicity in the initial photophobic response; cells just showed an increase in sensitivity over time upon transfer from 12:12 LD to DD where DD is constant dark (Forward, 1975). A clear rhythm was observed, however, if prior to testing for sensitivity to blue light the cells were briefly irradiated with red light (Forward and Davenport, 1970). Thus for *Gymnodinium* the rhythm involves the actual photoreceptor, while for *Gyrodinium* the rhythm is probably associated with an accessory phytochrome pigment (Forward, 1975).

Cultures of *Chlamydomonas* growing under either 12:12 LD or upon transfer from a 12:12 LD cycle to continuous light show a maximum response during the time of the early light phase, with a slight decline at the end of this phase and depressed responsiveness at night (Bruce, 1970, 1973).

A similar rhythm in photoresponsiveness by *Euglena* was originally observed by Pohl (1948) and extensively investigated by Bruce and Pittendrigh (1956, 1958). The observational method employed by Bruce and Pittendrigh could not clearly distinguish between a rhythm in motility and in light responsiveness. Subsequent work by Brinkmann (1966) demonstrated a rhythm in both and showed that much of the amplitude in Bruce and Pittendrigh's results was due to the motility rhythm.

There is an obvious relationship between vertical migration and circadian rhythms in photoresponses; the organisms are maximally sensitive during the day when the greatest amount of light is available as an orientation cue. Cells usually show maximum responsiveness during the first half of the day, which correlates with either upward (Table 1; dawn migration, dawn-dusk migration) or downward movements (Table 1; dusk migration). In all cases studied photoresponsiveness declines by the end of the day phase. In nature vertical movements in the late afternoon, are observed, which might indicate that at this time light is not an important orientation cue. An observation that also suggests that light is not totally necessary as a stimulus during DVM comes from the work of Eppley *et al.* (1968) on *Cachonia niei*. If cells are entrained on a 12:12 LD cycle and transferred to a small tank under constant dark conditions, they displayed an apparently normal up and down migration pattern for at least 24 h. This important result certainly suggests rhythms in behaviors other than photoresponsiveness are involved in DVM. Experiments of this type with other species are needed.

2.4.2. External Modifying Factors

2.4.2.1. Temperature

During DVM by the dinoflagellate *Gymnodinium nelsoni*, no general pattern of environmental temperature change correlated with the migratory pattern (Goff, 1974). In the laboratory relatively little work has been done on the effect of temperature on photoresponses. Early studies concerned temperature alterations in the upper intensity range which caused changes in the sign of phototaxis from positive to negative (*inversion intensity*). In some volvocales, Mast (1918, 1919) showed that the direction of temperature change corresponded to the direction of change in the inversion intensity. Mainx (1929) found that the inversion intensity always decreased regardless of the direction of the temperature change. More recently, investigators have studied changes in photoresponses of phytoplankton to a fixed intensity light stimulus at different temperatures. In all cases (Mayer and Poljakoff, 1959; Diehn and Tollin, 1966a; Diehn, 1970) responsiveness does vary, and an optimal temperature range can be described. Due to the

methods in measuring photoresponses, however, a clear separation of the effect of temperature on motility from that on photoresponsiveness is not possible. Nevertheless, temperature does alter the apparent response and can be important in nature. Unfortunately, the environmental implications of these studies is unclear. An unstudied but potentially interesting area of investigation is photoresponses in temperature gradients.

2.4.2.2. Inorganic Ions and Nutrients

The balance of inorganic ions can alter phototaxis. Halldal (1959) in his extensive study with *Platymonas* found that the sign of phototaxis changed depending upon ionic ratios. In all ionic concentration combinations of K^+ and Mg^{++} with no Ca^{++} present the cells were positively responsive, while Ca^{++} combined with low concentrations of K^+ caused cells to be negative, and in combination with high K^+ concentrations they were positive. *Platymonas* was immobile in medium, lacking both Mg^{++} and Ca^{++} . Similarly, the phototactic sign of *Chlamydomonas* could be altered by bicarbonate (Marbach and Mayer, 1970). If the pH was held constant, positively phototactic cells become negative and *vice versa* upon the addition of bicarbonate.

Stavis and Hirschberg (1973) found the inorganic ions required for phototaxis by *Chlamydomonas reinhardtii* were Ca^{++} and either K^+ , Cs^+ , Rb^+ , or NH_4^+ which were different from those necessary for motility, i.e., Ca^{++} or Mg^{++} . While the importance of these ionic requirements is interesting from a physiological viewpoint, their environmental implication remains to be determined. Nitrogen, however, may be very important for DVM and perhaps deserves further study. NH_4^+ is one of the ions necessary for phototaxis by *Chlamydomonas* (Stavis and Hirschberg, 1973). Correspondingly, when nitrogen limited, the dinoflagellate *Gonyaulax polyhedra* shows no DVM, but within one day after its addition this species resumes migration. *Cachonina niei* continues to migrate when nitrogen limited, but the pattern is different since the cells fail to aggregate during the day in the upper half-meter of the water column as seen for the controls (Eppley *et al.*, 1968).

2.4.2.3. Gases

The effect of gases on photoresponses is relatively unstudied. Halldal (1959) found changes in O_2 or CO_2 tension did not alter photoresponses by *Platymonas*. Motility by *Chlamydomonas* is stopped by a stream of CO_2 (Sachs and Mayer, 1961). However, in this study the pH was not measured. Halldal (1959) with *Platymonas* and Tsubo (1960) with *Chlamydomonas* found motility to depend dramatically on the pH. Whether gases are environmentally important in phototaxis remains to be determined.

2.5. Physiology of Photoresponses

2.5.1. Light Intensity

2.5.1.1. Photokinesis

The older literature contains reports of no change in the speed of movement, or orthophotokinesis, by phytoflagellates with light intensity change (Mainx, 1929; Mast and Graves, 1922) as well as reports of definite change with intensity (Mainx and Wolf, 1939; Holmes, 1903; Oltman, 1917; Mast, 1926; Luntz, 1931a,b, 1932). More recently, using *Euglena*, Wolken and Shin (1958) found that orthophotokinesis increased independent of phototaxis when ambient white light was changed from 2 to 40 fc. Since it took 15 min to see the effect, a direct relationship between speed and intensity perception by the photoreceptor is questionable. Feinleib and Curry (1971b) measured swimming speeds by *Chlamydomonas* during phototaxis and found that for 2-day-old cultures, speed during a positive response did not vary, but during a negative response it increased with intensity. Thus swimming speed may change with stimulus intensity, and this variation could lead to accumulation in an area where swimming is slow. Considering DVM, any link between orthophotokinesis and vertical movements and/or accumulation at particular depths is unknown.

2.5.1.2. Phototaxis

The minimum light intensity necessary for a positive phototactic response varies with species. In light-adapted *Chlamydomonas reinhardtii* using a broad-band pass blue-green filter, a clear response by individual cells occurs at about $7 \mu\text{W cm}^{-2}$, while when measuring a population response, $0.1 \mu\text{W cm}^{-2}$ is sufficient (Feinleib and Curry, 1971b). The lowest intensity at which a measurable response to 460-nm light is observed by *Euglena gracilis* is $0.05 \mu\text{W cm}^{-2}$ for 0.25 s (Diehn and Tollin, 1966b). Whether the cells were light or dark adapted is not clear. The light-adapted dinoflagellate *Gymnodinium splendens* is clearly less sensitive since the minimum intensity for a response to 453-nm light is $30.0 \mu\text{W cm}^{-2}$ (Forward, 1974a). Curiously, both *Euglena* and *Chlamydomonas* possess stigmata which are lacking in *Gymnodinium*. Whether this anatomical difference contributes to the difference in sensitivity is unknown, but nevertheless, Halldal (1958) indicated that forms without a stigma showed less precise phototactic orientation.

An obvious concern when considering vertical migration is the relationship between intensity sensitivity and underwater light intensities. Forward (1974a) predicted the underwater light intensity in the early morning at 450

nm in Jerlov type 5 coastal water to be sufficient for phototaxis by *Gymnodinium splendens* at depths above 15 m. This depth is close to night-time depths reported for natural populations (Eppley *et al.*, 1968; Hasle, 1950, 1954). Since this dinoflagellate is much less sensitive than either *Euglena* or *Chlamydomonas*, sufficient light would be available for migration by these organisms under similar conditions. However, DVM by planktonic *Euglena* and *Chlamydomonas* remains to be demonstrated, and true light intensity measurements during migration in the field are needed.

The phototactic responses of some phytoflagellates to different light intensities adhere to the *Weber Law* (Halldal, 1958; Mainx and Wolf, 1939; Diehn and Tollin, 1966b). In addition, the *Weber-Fechner Law* is supported as responsiveness is linearly related to the logarithm of the stimulus intensity over some intensity range (*Chlamydomonas reinhardii*, Feinleib and Curry, 1971b; *Euglena gracilis*, Diehn and Tollin, 1966b; *Gymnodinium splendens*, Forward, 1974a). In their detailed study of this relationship, Feinleib and Curry (1971b) found that for *Chlamydomonas* the increase in the apparent positive phototaxis as the intensity increased was due to an increase in the number of cells responding and the directness of their swimming paths, not a change in swimming speeds.

Frequently, the direction of phototaxis varies with intensity, the common pattern being a positive response at low intensity, which reverses to negative at high intensities. The light level at which this change occurs is termed the inversion or transition intensity. For 2-day-old light-adapted *Chlamydomonas* the inversion intensity is about $10^3 \mu\text{W cm}^{-2}$ blue-green light (Feinleib and Curry, 1971b), while in light-adapted *Euglena* the intensity is about $2 \times 10^4 \mu\text{W cm}^{-2}$ white light (Diehn, 1969b). In both of these species the reversal occurs very rapidly, requiring only a few seconds of stimulation. A reversal in dinoflagellates is unreported. Although this implies that the response is absent among dinoflagellates, it is likely that this is due to the lack of studies using high-intensity stimulation and the lack of investigations of species which avoid the surface during the day according to their DVM patterns.

At the inversion intensity investigators find no net movement of a phytoplankton population with respect to light (Buder, 1917; Feinleib and Curry, 1971b). This may result due to random orientation by the cells as suggested by Buder (1917). Alternatively, through studying individual cells, Feinleib and Curry (1971b) found the lack of movement by *Chlamydomonas* was due to equal numbers of cells showing positive and negative phototaxis.

The laboratory studies of phototactic response versus intensity relate directly to vertical migration. A rise during the day (dawn migration) could be a positive phototaxis. During the ascent the overhead intensity continually increases and similarly responsiveness would increase. This, of

course, assumes the intensity is not too high. If this were the case and the intensity was above the inversion level, a negative phototaxis could result in a descent as is observed for some species at the beginning of the day (dusk migration) and for others on a clear day at noon (Goff, 1974; Hasle, 1954; Cowles and Brambel, 1936). Some phytoplankters aggregate at a particular midday depth, which could be the depth of the inversion intensity since laboratory populations show no net movement at this intensity.

Vertical movements which investigators find difficult to attribute to behavioral responses to light alone are ascents early in the night (e.g., Hasle, 1950; Soli, 1966), ascents beginning before sunrise, and descents at the end of the day beginning before sunset (Eppley *et al.*, 1968; Goff, 1974; Hasle, 1950, 1954). The involvement of light responses cannot be totally ruled out of these night movements until studies are made of behavioral intensity sensitivity and accurate measurements or calculations are done of underwater light intensities at the nighttime migratory depths. For zooplankton, calculations of this type indicate that sufficient light is available for phototaxis near the surface at night (e.g., Crisp and Ritz, 1973). The late afternoon descent is most difficult to attribute to a photoresponse. It could result from a negative phototaxis to a low light intensity; however, such a response is unknown for phytoflagellates. Alternately, it may represent a response to gravity. Geotaxis is reported in some flagellates (reviewed by Halldal, 1962), but its importance in vertical movements is unknown. It is also possible that the cells may have a circadian rhythm in cellular density (Seliger *et al.*, 1962) or in intracellular organelle positions as reported for *Pyrocystis* (Swift and Taylor, 1967). Either of these could alter the center-of-buoyancy–center-of-gravity relationship and orient the cells upward or downward during random swimming (Eppley *et al.*, 1968).

2.5.2. Wavelength

2.5.2.1. Spectral Sensitivity

The results from recent action and response spectral studies are shown in Table 2. For a listing of older determinations, refer to Jahn and Bovee (1967). Generally, spectral sensitivity by phytoplankton agrees with Engelmann's (1882) observation that *Euglena* is most responsive to blue-green light. Primary maxima usually occur between 450 and 500 nm, with responses to wavelengths beyond about 550 nm usually poor or nonexistent.

The pronounced sensitivity to blue-green light (450–500 nm) corresponds to wavelengths that penetrate best in clear and semiclear water. This correspondence has limited applicability, however, since many species live in coastal waters and lakes where light of maximum transmission is

shifted to longer wavelengths. Clearly the spectral sensitivities of *Prorocentrum micans* and *Cryptomonas* sp. (Table 2) are adapted to such habitats, but these species are exceptions. Nevertheless, considering the intensity sensitivity of phytoplankters and the daytime underwater light intensity levels at blue-green wavelengths in coastal water at the depths attained during DVM, sufficient light is probably available for photoresponses (e.g., Forward, 1974a).

2.5.2.2. Photoreceptor Pigments

The pigments used in photoreception are present in such small amounts that neither extraction nor microspectrophotometry are feasible for identification. Therefore, action or response spectra are used to indicate the pigment absorption characteristics. At best the generalized type of pigment can be determined while its true identity is usually speculative. Several serious problems with action and response spectra determinations are the following: (1) distorted spectra can result due to shading pigments; (2) in some studies there is inadequate control over stimulus wavelength and intensity; (3) inadequate intensity measurements prohibit reliable results.

The action and response spectra suggest that different phytoplankters use different photoreceptive pigments with three main types indicated. In dinoflagellates and in *Platymonas subcordiformis* a carotenoid photoreceptor is suggested by the visible maxima plus the lack of a maximum in the near UV (Forward, 1973, 1974a; Halldal, 1958, 1961). A maximum also occurs in the UV region at around 280 nm, so the pigment is probably a carotenoprotein. In volvocales other than *P. subcordiformis* the action spectra determinations do not permit a clear statement about the identity of the photoreceptor pigment.

Although *Euglena* has been most extensively studied, early determinations are in poor agreement (Diehn and Tollin, 1966a; Büning and Schneiderhöhn, 1956; Wolken and Shin, 1958). The more recent studies by Diehn (1969a,c) are the most convincing and thus will be discussed. He proposes that the action spectra for a positive phototaxis and a positive photophobic response result from the composite absorption by screening and photoreceptive pigments, while that for the negative photophobic response reflects the true reception by the photoreceptor. The visible maxima plus the broad UV peak centering on about 365 nm for a negative photophobic response lead to identification of the pigment as a flavin. This is further supported by the observation that potassium iodide, a quencher of flavin excitation states, inhibits phototaxis at concentrations down to 10^{-2} M (Diehn and Kent, 1970).

Recently, a third type of pigment has been hypothesized for photoreception in *Cryptomonas* sp. In contrast to most other phytoplankton the action spectrum maximum occurs at 560 nm. Since the cells contain

TABLE 2. Spectral Sensitivity of Phytoplankton

Species	Response studied	Maximum (nm)	Source
Class: Dinophyceae			
<i>Gonyaulax catenella</i>	+ Phototaxis	475	Halldal, 1958
<i>Gyrodinium dorsum</i>	+ Phototaxis	470	Hand <i>et al.</i> , 1967
	Initial photophobic or stop response after red or white light irradiation	470, 280	Forward, 1973
	Initial photophobic response after far-red irradiation	490, 320	Forward, 1973
<i>Gymnodinium splendens</i>	+ Phototaxis	450, 280	Forward, 1974a
	Initial photophobic response	450, 280	Forward, 1974a
<i>Peridinium trochoideum</i>	+ Phototaxis	475	Halldal, 1958
<i>Prorocentrum micans</i>	+ Phototaxis	570	Halldal, 1958
Class: Chlorophyceae			
<i>Chlamydomonas reinhardtii</i>	± Phototaxis	503 (minor 443)	Nultsch <i>et al.</i> , 1971
<i>Chlamydomonas snowiae</i>	+ Phototaxis	465–480 (minor 500)	Mayer and Pojarkoff, 1959
<i>Dunaliella cf. euchlora</i>	+ Phototaxis	493 (shoulder 435)	Halldal, 1958

<i>Dunaliella salina</i>	- Phototaxis	493 (shoulder 435)	Halldal, 1958
<i>Dunaliella viridis</i>	+ Phototaxis	493 (shoulder 435)	Halldal, 1958
<i>Platymonas subcordiformis</i>	± Phototaxis	495, 275 (minor peaks 400, 335)	Halldal, 1961
<i>Stephanoptera gracilis</i>	- Phototaxis	493 (shoulder 435)	Halldal, 1958
<i>Volvax minor</i>	- Phototaxis	492	Lunz, 1931a
Class: Cryptophyceae			
<i>Cryptomonas</i> sp.	+ Phototaxis	560 (shoulder 490)	Watanabe and Furuya, 1974
Class: Euglenophyceae			
<i>Euglena gracilis</i>	+ Phototaxis	480, 375 495, 425 (shoulder 475)	Diehn, 1969a Bünning and Schneiderhöhn, 1956
	- Phototaxis	450-520 420, 490 415 (shoulders 450, 475, 510)	Diehn and Tollin, 1966a Wolken and Shin, 1958 Bünning and Schneiderhöhn, 1956
	+ Photophobic response	480, 375	Diehn, 1969a
	- Photophobic response	480, 450, 412, 365	Diehn, 1969a, c

phycoerythrin which has an absorption maximum in this region, Watanabe and Furuya (1974) speculate that this is the photoreceptor pigment.

2.5.2.3. Accessory Pigments

Shading pigments have only been thoroughly investigated in *Euglena*. As discussed previously, positive phototactic orientation probably occurs through periodic shading of the paraflagellar body by the stigma. In *Euglena gracilis* the absorption characteristics of the stigma pigments have been examined by microspectrophotometry (Strother and Wolken, 1960; Gössel, 1957; Wolken, 1967), however distortion due to chlorophyll absorption is a problem. A suspension of stigma granules averts this problem and shows broad absorption from 430 to 530 nm. A pigment extraction in cyclohexane from these granules has maximum absorption at 450 nm, with smaller peaks at 420 and 480 nm (Batra and Tollin, 1964; Tollin, 1973). The pigments present were identified as carotenoids with major contribution by β -carotene, cryptoxanthin, and lutein.

The action spectrum for positive phototaxis in *Euglena* has maxima at 480 and 375 nm. Diehn (1969a) suggests that both peaks result from composite absorption by the photoreceptor pigment and shading pigments. Carotenoids in the stigma which are oriented parallel to the long axis of the organism account for the maximum at 480 nm. Stigma pigments, however, show minimal absorption below 400 nm (Batra and Tollin, 1964) and thus cannot account for the peak at 375 nm. The pigment responsible for this maximum is possibly a flavin oriented perpendicular to the cell's long axis, but its location in the cell is unknown (Diehn, 1969a).

The photoresponses by the dinoflagellate *Gyrodinium dorsum* are unusual because prior exposure to red and/or far-red light alter the intensity sensitivity and action spectrum (Table 2) for the initial photophobic response (Forward, 1970, 1973). The effect of red and far-red light suggests involvement of a phytochrome pigment which is supported by the red/far-red reversibility and the action spectra (Forward and Davenport, 1968; Forward, 1973). A pigment extraction, however, is needed for absolute verification. A phytochrome interaction with phototaxis is unreported in other phytoplankton, but it does participate in chloroplast phototaxis in desmids (reviewed by Haupt and Schönbohm, 1970), and Lipps (1973) present tentative evidence for its involvement in cell division rate in four phytoplankton species. The action spectra maxima for the red and far-red absorbing forms of the phytochrome in *Gyrodinium* are somewhat unusual (Forward and Davenport, 1968), since they occur at shorter wavelengths (620, 700 nm) than those for terrestrial plants (665, 730 nm) (Butler *et al.*, 1964). An intriguing theory is that this shift represents an adaptation to the light trans-

mission characteristics of sea water which allows greater penetration of wavelengths shorter than 730 nm (Forward, 1975).

2.5.2.4. Multiple Photoreceptor Pigments

The possibility exists that different photoreceptive pigments may be utilized for different photoresponses. In dinoflagellates the action spectra for the initial photophobic response and subsequent phototaxis are identical (Hand *et al.*, 1967; Forward, 1974a), which indicates participation of the same pigment. Similarly, action spectra for positive and negative phototaxis by *Platymonas subcordiformis* are the same (Halldal, 1961). In this case, however, the sign of phototaxis in response to a blue-green light stimulus can be reversed by relatively short-term (3 min) exposure to light of other wavelengths. A negatively phototactic culture in a particular inorganic ionic solution can be changed to positive by exposure to 400–540-nm and 660–700-nm light, while a positively phototactic culture can be changed to negative after exposure at 560–630 nm (Halldal, 1960). Clearly different pigments are involved in this reversal, but there is no evidence that the basic positive or negative response itself involves different photoreceptor pigments. In another volvocale *Chlamydomonas reinhardtii*, two photoreceptive pigments are suggested by the observation that positive phototaxis occurs in response to 436 and 546 nm, while the response is negative to 577 nm (Feinleib and Curry, 1971a). This latter response cannot be reversed by lowering the intensity, but it is not consistently observed, and a definitive study is needed. The action spectra in *Euglena* vary depending upon the photoresponse studied (e.g., Büning and Tazawa, 1957; Diehn, 1969a,c). However, this probably results from the participation of shading pigments in different responses rather than from multiple photoreceptor pigments. Therefore, except for the tentative report by Feinleib and Curry (1971a), evidence does not exist for multiple photoreceptive pigments used for different photoresponses.

2.5.2.5. Ultraviolet Light Perception

The effects of UV light on phytoplankton is a recent concern (Broderick and Hard, 1974). It has been suggested that underwater UV light may influence movements during the day, particularly avoidance of the surface at midday (e.g., Hasle, 1954; Cowles and Brambel, 1936). Observational evidence to the contrary comes from the laboratory study of diurnal vertical migration by *Cochonina niei* (Eppley *et al.*, 1968). This species avoids the surface layer at midday under artificial lights that produce neither excessive intensity nor UV light.

Considering the physiology of photoresponses as indicated from the action and response spectra studies (Table 2, Halldal, 1967), some phytoflagellates can respond to UV wavelengths, and at sufficiently high intensities phototactic responses can reverse from positive to negative. This reversal would of course depend on the spectral sensitivity of the organism and the spectral distribution and light intensity underwater. However, there is no evidence for an independent UV photoreceptor pigment involved in phototaxis. The only case of a distinct UV absorbing pigment is a shading pigment in *Euglena* (Diehn, 1969a,c), but this pigment participates in positive phototaxis and the positive photophobic response.

2.5.3. Polarized Light Sensitivity

Except in *Euglena*, responses to polarized light by phytoplankton are unstudied. Using *Euglena*, Wolken and Shin (1958) showed greater swimming velocity in linearly polarized light than in light not passed through the polarizing filter. Altered photoresponses to polarized light led Bound and Tollin (1967) to conclude that the pigments in the stigma were aligned parallel to the long axis of the organism and that the photoreceptor itself was probably randomly oriented. In an extension of this work, Diehn (1969a,c) demonstrated that there are two pigment systems used for shading the photoreceptor which are oriented at right angles to one another. Judging from the apparent success with polarized light in pigment identification in *Euglena*, work with other phytoplankters might benefit from use of this stimulus. In addition, since much of the light underwater is polarized, this is potentially an additional unconsidered factor in light-oriented vertical movements.

3. ZOOPLANKTON

The discussion of the photobiology of zooplankton will center on forms known to show DVM, i.e., adult crustaceans and chaetognaths, which are planktonic throughout their entire life histories (*holoplankton*), and larvae of benthic living adults (*meroplankton*), of fish, and of holoplankton. Adult fish will not be considered, since their photobiology has been extensively reviewed elsewhere (Blaxter, 1970; Lythgoe, 1966, 1972; Woodhead, 1966). There will also be no discussion of zooplankton photoreceptors or pigments involved in photoreception since separate reviews would be required for each topic.

3.1. Diurnal Vertical Migration Patterns

DVM by adult zooplankton has been reviewed by Banse (1964), Ringelberg (1964), Segal (1970), and Vinogradov (1968), while DVM by specific animal groups has also been considered, i.e., copepods (Marshall and Orr, 1955, 1972), crustaceans (Bainbridge, 1961; Cushing, 1951), euphausiids (Mauchline and Fisher, 1969), and freshwater zooplankton, particularly *Daphnia* (Hutchinson, 1967). Among the larvae of holoplankton, migrations have been noted for copepods (Marshall and Orr, 1955, 1972), euphausiids (Mauchline and Fisher, 1969), and fish larvae (Woodhead, 1966). Meroplankton display weak migrations (Banse, 1964), however the distance traveled may be small compared to the accuracy of vertical sampling.

A great deal of variation in the migration pattern is observed between different species, between individuals of the same species at different locations, and even for the same organisms on different days (Bainbridge, 1961). Any classification system for migration patterns would, therefore, seem contrived. Nevertheless, Hutchinson (1967) defines three migration types which will be noted: (1) *nocturnal migration*, characterized by a single daily ascent, with minimum depth reached between sunset and sunrise; (2) *twilight migration*, in which a rise to minimum depths occurs at both sunset and sunrise; and (3) *reverse migration*, when the animals rise to a minimum depth during the day and descend to a maximum depth at night. The migration types which are most commonly observed are nocturnal migration and twilight migration, which is considered by Cushing (1951) to be the basic pattern. The descent phase of zooplankton migration probably has a passive sinking component (e.g., Rudjakov, 1970). The vertical ascent, however, results primarily from active swimming, and the distances traveled are compatible with swimming velocities (e.g., Mileikovsky, 1973; Hardy and Bainbridge, 1954).

3.2. Photoresponses of Zooplankton

In attempting to establish how light influences zooplankton DVM, the first consideration will be how responsiveness to light is modified by factors other than light. The importance of internal factors (age, sex, circadian rhythms, and feeding) as well as external factors (pressure, salinity, and temperature) will be discussed.

3.2.1. Internal Modifying Factors

3.2.1.1. Age and Sex

The vertical depth distribution of a species in the water column can vary with age. This has been reviewed in general (Vinogradov, 1970; Banse, 1964), for cladocera and copepods (Hutchinson, 1967; Cushing, 1951), for crustaceans (Bainbridge, 1961), for euphausiids (Mauchline and Fisher, 1967), and for fish larvae (Woodhead, 1966). Most commonly, animals living in deep water have juveniles which live nearer the surface. On the other hand, if older individuals live near the surface, the young live deeper. An example is the copepod *Calanus finmarchicus* in which gravid females remain higher in the water column and show a more extensive DVM than immature females (Marshall and Orr, 1960). A difference in light sensitivity is suggested to explain the occurrence of individuals of different ages at different depths (Cushing, 1951; Banse, 1964).

Changes in light-oriented behavior with age are seen in adults, e.g., young female *Daphnia* are positively phototactic, but become negative subsequent to the release of the first brood of young (Clarke, 1932). The most consistently observed changes in phototaxis, however, are those occurring during larval development of meroplankton (reviewed by Thorson, 1964) and of fish (reviewed by Woodhead, 1966). Several distinct developmental patterns are seen. For meroplankton the most common pattern is a change from positive phototaxis in early stages to negative in later stages, as Thorson (1964) lists 47 species from 33 genera that display this pattern. In addition, he lists 7 species as having larvae which are positive early in development but become indifferent to light at later stages. In contrast, no change in phototactic sign during development occurs in 15 species from 11 genera which are positive throughout their development (Thorson, 1964). A reverse change is noted in elvers of the fish *Anguilla vulgaris* which change from negative to positive before entering the rivers from the sea (Bückmann *et al.*, 1953, cited in Woodhead, 1966).

Among the larvae of benthic invertebrates, an early positive phototaxis is useful for moving the larvae up into the water column as plankton. This is clearly demonstrated by Dingle's (1969) work with stomatopod larvae. Upon hatching, the larvae pass through three propelagic stages while residing within the cavity occupied by the female. During these stages they show a negative phototaxis and will grasp any surface with which they come in contact (thigmokinesis). Upon molting into stage IV larvae, they become free swimming and positively phototactic and thereupon enter the plankton.

The maintenance of a positive response throughout all larval stages is characteristic of intertidal barnacles (Thorson, 1964) and most intertidal

brachyuran crabs (Hyman, 1920; Herrnkind, 1968; Sulkin, 1971; Forward and Costlow, 1974). Those intertidal crabs which are exceptions have larvae which reverse to negative at later stages (Welsch, 1932; Christensen and McDermott, 1958). Presumably this positive response contributes to positioning the larvae up in the water column, thereby increasing the possibility of their being swept into the intertidal zone. However, after contacting the intertidal substrate, the photoresponses of some barnacles can reverse to negative at the time for attachment, e.g., *Balanus balanoides* (Crisp and Ritz, 1973).

The change to negative phototaxis late in development is characteristic of larvae of subtidal benthic adults. The adaptive significance of this change is obvious, since a negative phototaxis would move the larvae lower in the water column and increases the likelihood of contacting subtidal substrate at the time for metamorphosis.

As for differences between sexes, studies of DVM in nature show that males and females display different patterns (review of fresh water studies, Hutchinson, 1967; *Calanus finmarchicus*, Marshall and Orr, 1955). This could result from different sensitivity to light as for example ovigerous female *Daphnia* are more negatively phototactic than are males (Rose, 1925). However, more studies are needed to support this suggestion.

3.2.1.2. Circadian Rhythms

The daily changes in light underwater could alone provide the stimulus for daily vertical movements of zooplankton. On the other hand, endogenous circadian rhythms in activity and in responsiveness to light, pressure, and/or gravity could also contribute to vertical movements over a 24-h period. In this case light is still important, since the alternating underwater light-dark (LD) cycle probably acts as the *zeitgeber* for entraining the rhythm.

Field observations of *Calanus* (Hardy and Paton, 1947) and of siphonophores (Moore and Corwin, 1956) led to the postulation that endogenous rhythms were involved in DVM. Much earlier, Easterly (1917b), studying three species of copepods in the laboratory, found a rhythm in swimming activity, which resulted in the greatest numbers of animals being at the surface between 1800 and 2000 h, with a descent thereafter. Since these investigations, laboratory studies have involved either observation of movements in vertical columns under LD cycles, constant light (LL), and/or constant dark (DD), or observations of photoresponses throughout the day.

In vertical columns Harris (1963) demonstrated rhythmic movement by *Daphnia magna* and *Calanus finmarchicus* under DD. In this case, any

change in vertical position could be due to a rhythmic response to pressure or gravity and/or an activity rhythm. *Daphnia* displayed a clear circadian rhythm in vertical movement since a rise in the column occurred during the time of the original day phase with a descent during the time for night. Although *Calanus* showed the opposite migratory pattern, an endogenous circadian rhythm was only suggested since only one cycle of movement was reported. Similarly, Grindly (1972) stated that the copepod *Pseudodraptomus* had a weak rhythm in vertical movement under DD; however no data were presented. Studies on *Acartia tonsa* (Schallek, 1942), on *Neomysis americana* (Herman, 1962), and on *Cyclops vernalis* (Umminger, 1968a) found no rhythms in vertical movement under DD.

Under constant low-level overhead light, vertical movement could result from the same rhythms as under DD, but a rhythm in responsiveness to light could contribute. For *Daphnia* Harris (1963) again found a clear rhythm in vertical movement. Photoresponsiveness was probably important, in this case, since both the timing of maximum and minimum depths and the period length were different from those under DD. Enright and Hamner (1967) looked at changes in abundance at the surface of a 2.5-m deep tank in animals subjected to a 12:12 LD cycle and then transferred to LL. Clear endogenous rhythms leading to consistent increase in abundance at the surface during the time for the night phase and decrease during the time for the day phase were seen for an amphipod *Nototropis* sp., an isopod *Exosphaeroma* sp., a cumacean *Cyclaspis* sp., and for peltidiad copepods. Positive phototaxis was a factor in the cumacean and copepod distributions, since increased abundance at the surface was noted at all times under LL as compared to distribution under an alternating LD cycle. Similarly, Grindley (1972) reported a rhythm in vertical movement by a copepod under a natural LD cycle which is weakly maintained under LL. Enright and Hamner (1967) also found 18 species which had exogenous rhythms, as they showed rhythmic migration patterns only under a LD cycle and not under LL. In addition, six species showed no migration even under the LD cycle.

Other studies indicate that circadian rhythms in photoresponses are evident in some animals. Rimet (1960) presented limited data suggesting that *Daphnia* has a rhythm in phototaxis. More recently, Ringelberg and Servaas (1971) clearly established its existence. Under a 12:12 LD cycle female *D. magna* showed maximum phototactic response during the light phase and a minimum at night. Under LL the rhythm in phototaxis continued with a period of about 28 h, which was similar to that found by Harris (1963).

When stimulated with a constant intensity white light stimulus, both fed and starved barnacle nauplii showed strong positive phototaxis in the morning and afternoon, with a negative response around noon (Singarajah

et al., 1967). It is difficult to assess whether a true endogenous rhythm is responsible for these responses, since the entrainment photoperiod is not stated and only the cycle during the day phase is shown. Nevertheless, either a reversal in the sign of phototaxis or a change in sensitivity to light is implied by the result. In contrast, Umminger (1968a) found no reversal in phototactic sign by the copepod *Cyclops vernalis* over the light phase of a 12:12 LD cycle; the animals remained negatively phototactic.

An intriguing rhythm in polarotaxis by the copepod *Cyclops vernalis* is suggested by the work of Umminger (1968a). Copepods were maintained on a 12:12 LD cycle, and responses to linearly polarized light were tested during the light phase. Swimming, which is perpendicular to the direction of polarization (e-vector direction) early in this phase, changes to parallel in the middle, and reverts back to perpendicular at the end. Although Umminger (1968a) suggested that an endogenous rhythm underlies this response change, the animals were not under LL or DD conditions and the responses were only followed through the time of the light phase. In testing six other copepod species, he failed to find a similar change in polarotaxis (Umminger, 1968b).

Thus many zooplankters exhibit endogenous circadian rhythms in activity and in photoresponsiveness. The fact that they are not observed in other species may not imply their absence, but only that those special experimental conditions necessary for display of these rhythms were absent (e.g., Umminger, 1968a). The rise during the night phase and descent during the day phase seen in some experiments with animals in vertical columns (Harris, 1968; Enright and Hamner, 1967), are similar to nocturnal migrations in nature. It is unclear, however, as to the rhythms in which behaviors underlie these movements. Rhythms in photoresponses do exist and can contribute when light is available. For example, the change in responses by barnacle larvae (Singarajah *et al.*, 1967) and by the copepod *Cyclops vernalis* (Umminger, 1968a) over a light phase could lead to DVM throughout the day. However, rhythmic movements observed under DD imply the involvement of other factors, such as activity rhythms and/or rhythms in responses to pressure and gravity. To assess fully their contribution to migrations, studies of the possible rhythms in each of these behaviors are needed.

3.2.1.3. Feeding

Photoresponses can be altered by feeding. *Daphnia* are more photo-negative after feeding (Clarke, 1932), and if starved show only the blue color dance to all wavelengths of light (Section 3.4.2.2) (Baylor and Smith, 1957). This is consistent with work on nauplii of the barnacles *Balanus*

balanoides and *Elminius modestus* (Singarajah *et al.*, 1967). Positive phototaxis is most pronounced in starved nauplii which show increased negative phototaxis subsequent to feeding.

The presence of food can also alter photoresponses. Lucas (1936) found that both the copepod *Eurytemora hirundooides* and the mysid *Neomysis* avoid light in the presence of abundant diatoms. In contrast, Bainbridge (1953) observed *Calanus finmarchicus* and *Hemimysis lamornae* to swim upward in a vertical tube illuminated obliquely from above in the presence of certain phytoplankton species. He does not indicate whether the animals were fed or starved prior to experimentation. If phytoplankton are added to a suspension of starved *Daphnia*, upon stimulation with light only the red color dance is observed, regardless of wavelength (Baylor and Smith, 1957). While the presence of the actual phytoplankton cells may contribute to the photoresponse alterations, the metabolites alone are also effective. Hence, in the presence of medium from the dinoflagellate *Prorocentrum micans*, starved barnacle nauplii display increased positive phototaxis and fed nauplii, stronger negative responses (Singarajah *et al.*, 1967).

Considering DVM, the consistent laboratory observation of increased positive phototaxis by starved animals could lead to an ascent into surface waters (assuming sufficient light is available) where more food is perhaps available. Once fed, a descent could result from a negative phototaxis, which is frequently observed in sated zooplankton. Although this model is overly simplified, it is somewhat supported by field studies of the chaetognath *Sagitta elegans* (Pearre, 1973), which indicate that this species migrates to the surface area to feed and descends once food is captured. Pearre (1973) postulates that the migratory ascent and descent involve responses to light and gravity, the direction and dominant response being regulated by the level of satiation and ambient light.

3.2.2. External Modifying Factors

3.2.2.1. Pressure

The direction of phototaxis can be influenced by changes in hydrostatic pressure. Qasim and Knight-Jones (1957) reported that the copepod *Temora longicornis* and barnacle nauplii move toward a light with an increase in pressure and away with a decrease. This general result was confirmed by Rice (1964) in his extensive work with many species. He assigned animals to different response types depending on their reactions to white light upon experiencing a pressure change of 500–1000 mbar. Six species showed "type 2" response, in which they moved toward a horizontal light in response to a pressure increase, but either moved away or decreased activity upon a pressure decrease. However, in vertical light (above or below) or

darkness, movements were oriented entirely with respect to gravity. Of the 31 species showing a "type 3" response, all moved toward the light (positioned either vertically above or below or horizontally) upon a pressure increase, while in response to a pressure decrease 25 species stopped swimming and sank, three actively moved away, and three did both.

More recently, Lincoln (1970, 1971) found a pressure increase to cause a small initial upward movement by *Daphnia magna* under overhead illumination, while the reverse occurred with a pressure decrease. He felt the movements resulted from a kinesis and not a directional response. After the initial response, the normal DVM pattern to an increase and decrease in overhead light intensity continued unaltered. Similar movements were observed by Ennis (1973) for lobster larvae upon pressure changes in a vertical tube under overhead light.

Thus the original observations by Qasim and Knight-Jones (1957) are consistently reported. The importance of these responses is probably as a mechanism for maintaining a particular depth in the water column.

3.2.2.2. Salinity

Observations on DVM in areas of salinity gradients and stratification are reviewed by Banse (1964) and for euphausiids by Mauchline and Fisher (1969). The general conclusion is that salinity gradients do not pose insurmountable physical or physiological barriers to migration, but for some animals under some circumstances they may modify the migratory pattern, probably through changes in behavior. These changes can take the form of altered phototaxis. Thorson (1964) lists larvae of 11 species that are normally positively phototactic, but when subjected to a salinity decline become negative. The amount of salinity change necessary to induce a phototactic reversal varies with species, and salinity acclimation exists, since a return of positive phototaxis after time in low salinity can occur. Responses of this type could contribute to an avoidance of low-salinity surface water.

3.2.2.3. Temperature

In laboratory studies temperature change can induce a rapid reversal in the sign of phototaxis. Among meroplankton, Thorson (1964) lists 14 species that are positively phototactic normally, but become negative upon a temperature rise. Alternately, larvae of *Balanus perforatus* (Ewald, 1912) and *Squilla* sp. (Frankel, 1931), which are normally negative become positive when exposed to a temperature decline. Also *Mytilus edulis* larvae become indifferent to light when exposed to elevated temperatures (Bayne, 1964), and a similar response occurs in *Bugula* larvae when subjected to lower temperatures (Lynch, 1947).

The same general response trends are observed in adults. For *Daphnia* Smith and Baylor (1953) obtained results similar to those by Rose (1925) and Clarke (1932) in which they found animals that were positively phototactic at 15°C became negative at 30°C and showed a stronger positive response at 0–5°C. In addition, Clarke (1932) found that older individuals that were negatively phototactic normally reversed to positive phototaxis upon lowering the temperature. Among adult copepods Easterly (1919) obtained the same trends for *Calanus finmarchicus*, but the reverse responses occurred for *Acartia clausi* and *A. tonsa* (Easterly, 1917a). Thus the most general effect of temperature is that an elevation will reverse phototaxis from positive to negative, and a lowering will reverse negative to positive.

Considering temperature in nature and effects on DVM, Cushing (1951) reviewed the older literature and cited cases where animals did and did not migrate through thermoclines. Subsequently, Lewis (1954) studying euphausiids presented data which indicate DVM itself is not related to temperature, but the lower limits of migration are related to the depth of the low-temperature isotherm. This agrees with Banse's (1964) assessment that "temperature may modify the patterns particularly near the upper and lower range of migration."

The obvious relationship between laboratory and field observations is that elevated temperature most often encountered high in the water column will reverse positive phototaxis to negative and thereby could restrict further ascent. Lower temperatures experienced in deep water can reverse negative phototaxis to positive, which could restrict further descent.

3.3. Physiology of Photoresponses

Three aspects of light underwater which can be important during DVM are intensity, wavelength, and polarization. Thus the physiology of photoresponses will be discussed in relation to these aspects of light.

3.3.1. Light Intensity

The role of light intensity in behavioral responses is very important, since the stimulation intensity can determine the response itself while the past exposure intensity (level of light or dark adaptation) can determine the level of photosensitivity. Usually, there is a positive phototactic response to low or moderate intensities; this reverses to negative as the intensity becomes very great (reviewed for crustaceans by Pardi and Papi, 1961; for meroplankton by Thorson, 1964). The possible evidence that this occurs in nature is the absence of many zooplankton at the surface during the day, when the surface intensity is high, and the descent by animals near the sur-

face on clear moonlit nights (Ritchie, 1954; Ritz, 1972b; Moore, 1950). Similarly, in laboratory studies of DVM under natural sunlight, animals avoid the surface and descend during the times of high sunlight intensity (e.g., Blaxter, 1973).

Representative intensities at which the reversal from positive to negative phototaxis occurs are 5.4×10^3 -lux sunlight for dark-adapted stage II *Balanus balanoides* larvae (Crisp and Ritz, 1973) and $3.3 \times 10^3 \mu\text{W cm}^{-2}$ 499-nm light for dark-adapted stage I larvae of the crab *Rhithropanopeus harrisii* (Forward, 1974b). However, in stages II, III, and IV larvae of *Rhithropanopeus*, a reversal is not seen at this intensity, but only a decline in positive phototaxis at stimulus levels above $10 \mu\text{W cm}^{-2}$ at 499 nm. Ritz (1972a) observed a similar decline in positive phototaxis by lobster larvae to a random response at the highest experimental white light stimulus intensity of $4.6 \times 10^3 \mu\text{W cm}^{-2}$. For both *Rhithropanopeus* and lobster larvae, a negative response may occur at higher stimulus intensities (Forward and Costlow, 1974; Ritz, 1972a). Nevertheless, the reversal from positive to negative phototaxis does not occur abruptly at a particular intensity level, but is interspersed with a period of reduced directional orientation to light.

Among larvae of the crabs *Uca* (Herrnkind, 1968) and *Rhithropanopeus* (Forward, 1974b; Forward and Costlow, 1974) and of three fish species (Blaxter, 1968, 1969) the opposite reversal is observed, in which animals are positively phototactic to moderate intensities and respond negatively at low intensities. This may be a common phenomenon among crustacean larvae since eight other species show this pattern (Forward, unpublished). For the crab *Rhithropanopeus* the intensity at which reversal occurs is about $6\text{m} \times 10^{-1} \mu\text{W cm}^{-2}$ at 499 nm at all larval stages (Forward, 1974b; Forward and Costlow, 1974), while for the herring larvae this varies with age, i.e., just hatched, 0.75-lux white light; 1 week, 2.3×10^{-1} lux; and 2–3 weeks, 5.4×10^{-1} lux (average values from Blaxter, 1968). Curiously, this response is only observed in light-adapted crustacean larvae (Forward, 1974b; Forward and Costlow, 1974; Herrnkind, 1968) and in dark-adapted fish larvae (Blaxter, 1968, 1969), which thereby leads to different speculation regarding its functional significance. In *Rhithropanopeus* Forward (1974b) suggests that the negative phototaxis to low light acts as a shadow reflex, possibly for predator avoidance. Based solely on phototaxis, the hypothesized DVM of this organism consists of a rise at sunrise, light adaptation during the day, and a descent at sunset due to negative phototaxis to low overhead light intensities. This then is a reverse migration pattern. For herring and plaice larvae, the migratory descent observed by Blaxter (1973) at dawn (nocturnal migration pattern) could result from a negative phototaxis to low early morning intensities after dark adaptation during the night.

Some lower light intensity threshold values for phototaxis by zooplankton living mainly in coastal waters are shown in Table 3. Comparable

TABLE 3. Intensity Thresholds for Zooplankton Phototaxis

Species	Threshold	Source
INVERTEBRATES		
Phylum: Arthropoda		
<i>Class: Crustacea (larvae)</i>		
<i>Balanus balanoides</i>		
Stage I (+) ^a	$1.99 \times 10^{-6} \mu\text{W cm}^{-2}$ @ 522 nm	Barnes and Klepal, 1972
Stage I (-)	6.5×10^{-4} lux WL ^b	Crisp and Ritz, 1973
Stage II (+)	4.2×10^{-5} lux WL	Crisp and Ritz, 1973
Cyprid (+)	2.5×10^{-6} lux WL	Crisp and Ritz, 1973
<i>Elminius modestus</i>		
Stage I (+)	$1.44 \times 10^{-6} \mu\text{W cm}^{-2}$ @ 522 nm	Barnes and Klepal, 1972
<i>Balanus crenatus</i>		
Stage I (-)	1.2×10^{-4} lux WL	Crisp and Ritz, 1973
Stage II (-)	6.3×10^{-5} lux WL	Crisp and Ritz, 1973
<i>Panulirus longipes cygnus</i>		
Stage I (+)	$4.4 \times 10^{-6} \mu\text{W cm}^{-2}$ WL	Ritz, 1972a
<i>Rhithropanopeus harrisi</i>		
Stage I (L) ^c (-)	$5 \times 10^{-3} \mu\text{W cm}^{-2}$ @ 499 nm	Forward, 1974
Stages II-IV (L) (-)	$5 \times 10^{-3} \mu\text{W cm}^{-2}$ @ 499 nm	Forward and Costlow, 1974

Class: Crustacea (adults)
Praeaus neglectus (+) $2.9 \times 10^{-6} \mu\text{W cm}^{-2}$ @ 420–540 nm Nicol, 1959

VERTEBRATES

Teleost larvae

<i>Clupea harengus</i>	Newly hatched (-)	2.1×10^{-6} lux WL	Blaxter, 1968
	1 week (-)	2.1×10^{-6} lux WL	Blaxter, 1968
	2–3 weeks (-)	8.7×10^{-6} lux WL	Blaxter, 1968
<i>Pleuronectes platessa</i>	Newly hatched (-)	10^{-4} lux WL	Blaxter, 1969
	1 week (-)	4×10^{-6} lux WL	Blaxter, 1969
	2–3 weeks (-)	2×10^{-6} lux WL	Blaxter, 1969
<i>Solea solea</i>	2 weeks (-)	10^{-6} lux WL	Blaxter, 1968
	3 weeks (-)	4×10^{-6} lux WL	Blaxter, 1968
	6 weeks (-)	3×10^{-6} lux WL	Blaxter, 1968

^a Sign of phototaxis, + positive, - negative.

^b White light.

^c Light-adapted; all other species were dark-adapted.

values for deep ocean forms would be interesting, but the problems of altered behavior in the laboratory make their determinations difficult, if not impossible. Although the units differ with investigator, general thresholds are about 10^{-5} to 10^{-6} lux and 10^{-5} to $10^{-6} \mu\text{W cm}^{-2}$. An important question is at what depth is sufficient light available for orientation, especially at night. Crisp and Ritz (1973) calculated the depth at which 520 nm attenuates to 10^{-5} lux under different environmental conditions in various water types. Even under conditions of no moon at night with heavy clouds in Jerlov type VII coastal water, light was available for orientation down to 2 m, while in type IV oceanic water there was sufficient light to 11.4 m. Naturally, any further improvement in conditions lowers the depth. DVM by animals below those depths where sufficient light is available for orientation remains to be explained (Waterman, 1974a), but for animals moving within these depths, phototactic orientation could occur over an entire 24-h day.

Light adaptation clearly raises the threshold for phototaxis which causes those intensities that evoke a maximum response to shift to higher values (Clarke, 1932; Crisp and Ritz, 1973; Forward and Costlow, 1974). During DVM light adaptation is speculated to influence the migration depth as some animals tend to rise slightly over the day (Clarke and Bachus, 1956; Harris and Wolfe, 1955; Siebeck, 1960).

Considering photoresponsiveness to different intensities, it is not surprising that over some intensity range the Weber-Fechner Law applies, and response is related to the logarithm of the stimulus intensity. This has been frequently demonstrated and is reviewed for Crustacea by Pardi and Papi (1961). The percent change in light intensity (Weber fraction) necessary for initiating a behavioral response is of interest in nature for an animal moving vertically in a light gradient. For *Daphnia* the Weber fractions vary with intensity; Heberdey and Kupka (1942) report 2.8% at 400 lux, 9% at 1000 lux, and 12% at 1500 lux. Most recently, Ringelberg *et al.* (1967) also found the Weber fraction to vary with intensity for white light below $40 \text{ erg cm}^{-2} \text{ s}^{-1}$, but for intensities above this level it was relatively constant at about 3%.

Blaxter (1972), using larvae of the sole, measured the percent difference in the light intensities of two horizontal lights necessary to evoke a phototactic response. The values varied from 60 to 200% over the intensity range 10^{-1} to 10^{-5} lux. His percentages were calculated to allow values greater than 100%. To apply this information to DVM, Blaxter (1972) further calculated that the vertical distances larvae would have to move in Jerlov type III and VII coastal water to experience these changes in intensity would be 1–6 m. More calculations of this type would be interesting since the depth of some animals during the day corresponds to the depth of a particular light

intensity level or isolume, and the distribution range around this isolume may relate to the Weber fraction at that intensity level.

In nature evidence for the relationship of intensity to DVM comes mainly from studies of the daily changes in the *deep* (or sonic) *scattering layer* (DSL) depths as measured by echo sounders. The animals responsible for these layers have been determined in some studies, e.g., euphausiids (Boden *et al.*, 1961; Boden and Kampa, 1965) and not in others (e.g., Blaxter and Currie, 1967). Clearly, light intensity affects the depths at which these layers occur, since clouds (Boden and Kampa, 1967; Blaxter and Currie, 1967; Dietz, 1962) and artificial lights (Blaxter and Currie, 1967) can alter their depths.

Furthermore, considerable evidence exists that deep scattering layers are associated with particular isolomes. In the San Diego trough *Euphausia pacifica* follows an isolume at 475–480 of $1 \times 10^{-4} \mu\text{W cm}^{-2}$ (Boden *et al.*, 1961). In the Saanich Inlet, British Columbia, the same organisms are prevented from descending below 100 m by a low oxygen layer at this depth. An apparent consequence is that the isolume followed is at 494–502 nm of $1.2 \times 10^{-1} \mu\text{W cm}^{-2}$ (Boden and Kampa, 1965). Perhaps the best demonstration of a DSL descending with an isolume at dawn and ascending at sunset is presented by Boden and Kampa (1967) for a migratory layer measured southeast of Fuertaventura in the Canary Islands. The isolume followed was $5 \times 10^{-4} \mu\text{W cm}^{-2}$ at 474 nm, and animals were never more than ± 12 m from this isolume. At midday this range represented intensities $3.5\text{--}7.5 \times 10^{-4} \mu\text{W cm}^{-2}$, which from bottom to top is a 114% change.

Recently, with more refined equipment and a greater number of observations Kampa (1970a) reported that four layers in the eastern North Atlantic (Kampa, 1970b) and two in the Gulf of California (Kampa, 1970c) had midday depths associated with an isolume of about $3\text{--}5 \times 10^{-5} \mu\text{W cm}^{-2} \text{ nm}^{-1}$ at 480 nm. At most, the differences in the isolume intensity in these areas was only sixfold. The studied regions were separated by great distances, yet the animals were at the depth of a relatively constant isolume, not an absolute depth. The biological significance of this particular isolume is unknown.

3.3.1.1. Role of Intensity in DVM

The historical development of the theories for the manner in which light acts during DVM is reviewed by Ringelberg (1964). Clearly, light intensity underwater is considered most important during DVM. The most pervasive theory is that animals are following a particular optimum light intensity level or isolume (Russell, 1927). The study by Boden and Kampa (1967) showing that a deep scattering layer follows a specific isolume during

its descent at dawn and ascent at sunset clearly supports this theory. In addition, the midday depths of deep scattering layers are associated with specific isolomes which are very similar in different oceanic regions (Kampa, 1970a,b,c). In disagreement with this theory are observations that during an ascent and descent deep scattering layers can move at different rates from that of the isolome (Clarke and Bachus, 1956, 1964). They can also follow lower intensity isolomes during the ascent than during the descent which implies differential light adaptation prior to the movements (Bary, 1967; Boden and Kampa, 1965). In addition the enigma of DVM by bathypelagic animals living at depths where the perception of sun and sky irradiance is questionable remains to be explained (Waterman, 1974a).

Furthermore, the underwater light cues involved in initiating vertical movements are not resolved. On the one hand, if animals are following a particular isolome, it is theorized that an ascent (due to positive phototaxis and/or negative geotaxis) begins once the light level decreases to a particular intensity below the ideal isolome and a descent (due to negative phototaxis and/or positive geotaxis) begins once the light level reaches a particular upper level with undirected movements occurring at intensities between these (Russell, 1927; Rose, 1925; Ewald, 1910; Waterman, 1974a). Alternately, the cue for initiating vertical movement is attributed to the rate and duration of the change in light intensity from the ambient intensity (adaptation intensity), which itself can change over a day. This hypothesis was originally considered by Clarke (1930) and was investigated in depth for *Daphnia* (Ringelberg, 1964, 1966; Daan and Ringelberg, 1969).

Upstreaming as performed by *Daphnia* in the afternoon is related to the rate and duration of light intensity decrease (Ringelberg, 1964), while downstreaming as occurs in the morning is similarly related to intensity increases (Daan and Ringelberg, 1969). However, the initial ascent observed by some animals during the early morning as the intensity increases remains to be explained (Pearre, 1973). Bary (1967) found migration by a DSL in the Saanich Inlet to deviate from an isolome and could interpret the pattern in terms of responses to the rate of relative change in light intensity as proposed by Ringelberg (1964). However, in Bary's (1967) study light intensity measurements were not regularly taken, but were mainly calculated from other work. As pointed out by Kampa (1970a), this is a precarious method.

3.3.2. Wavelength

3.3.2.1. Spectral Sensitivity

Spectral sensitivity maxima for zooplankton, shown in Table 4, have been determined either from behavioral photoresponses, from electrophysio-

TABLE 4. Spectral Sensitivity of Zooplankton

Species	Method	Maximum (nm)	Source
INVERTEBRATES			
Phylum: Arthropoda			
Class: Crustacea (Larvae)			
Subclass: Cirripedia			
<i>Balanus amphitrite</i> (C) ^a	+ phototaxis	530-545	Visscher and Luce, 1928
<i>B. improvisus</i> (C)	+ phototaxis	530-545	Visscher and Luce, 1928
<i>B. balanoides</i> (C)	+ phototaxis	520-530	Barnes and Klepal, 1972
Stage I			
<i>Elminius modestus</i> (C)	+ phototaxis	450-480S ^b	Barnes and Klepal, 1972
Stage I			
<i>Palaeonetes vulgaris</i> (C)	+ phototaxis	520-530	Barnes and Klepal, 1972
<i>Palaemonetes longipes cygnus</i> (C and UO)	+ phototaxis	470-510	White, 1924
Stage I			
<i>Rhithropanopeus harrisi</i> (C)	+ phototaxis	470, 555, 615	Ritz, 1972a
Stages I-IV			
Stage II	polarotaxis	400, 500	Forward and Costlow, 1974
		500	Via and Forward, 1975
Class: Crustacea (Adults)			
Order: Mysidacea			
<i>Mysis relicta</i> (FW)	light-induced + geotaxis	395, 515	Beeton, 1959
<i>Neomysis americana</i> (C)	color choice	515	Herman, 1962

Continued

TABLE 4. Continued.

Species	Method	Maximum (nm)	Source
Order: Euphausiacea <i>Euphausia pacifica</i> (DO)	pigment extract	462	Kampa, 1955
	ERG	460, 490, 515	Boden <i>et al.</i> , 1961
	ERG	465, 495, 515	Boden and Kampa, 1965
<i>Meganyctiphantes norvegica</i> (DO)	pigment extract	460–465	Fisher and Goldie, 1959
	ERG	460, 490, 515	Boden <i>et al.</i> , 1961
<i>Nematoscelis difficilis</i> (DO)	ERG lower lobe	490	Boden <i>et al.</i> , 1961
<i>Nematoscelis megalops</i> (DO)	ERG upper lobe	460–470, 530	Boden <i>et al.</i> , 1961
<i>Stylocheiron maximum</i> (DO)	pigment extract	465	Fisher and Goldie, 1961
<i>Thysanopessa raschii</i> (DO)	pigment extract	470	Fisher and Goldie, 1961
<i>Thysanopoda acutifrons</i> (DO)	pigment extract	460–465	Fisher and Goldie, 1961
Order: Decapoda	pigment extract	480	Fisher and Goldie, 1961
<i>Pleuroncodes planipes</i> (C)	pigment extract	503	Fernandez, 1973
	ERG	523	Fernandez, 1973
<i>Acanthephyra haackeli</i> (DO)	pigment extract	480	Fisher and Goldie, 1961
<i>Sergestes articus</i> (DO)	pigment extract	475	Fisher and Goldie, 1961
<i>S. robustus</i> (DO)	pigment extract	470	Fisher and Goldie, 1961
Order: Diplopoda			
<i>Daphnia pulex</i> (FW)	eye movement	400–420	Robert <i>et al.</i> , 1958
	dermal sensitivity	520	
	visual sensitivity	500–550	Viaud, 1951
	– phototaxis	450 ^c	Viaud, 1951
	+ phototaxis		Young, 1974
<i>Daphnia magna</i> (FW)	eye movement	550–600	
	stimulate side of head	400–500	
	stimulate top of head		

Phylum: Mollusca

Class: Cephalopoda
Loligo pealei (C)
 Larvae

+ phototaxis

470-510

White, 1924

Phylum: Rotifera

Asplanchna priodonta (FW)
Polyarthra remata (FW)
Filinia longiseta (FW)

+ phototaxis
 + phototaxis
 + phototaxis

440, 520-580
 440, 520-540
 360, 440-480

Menzel and Roth, 1972
 Menzel and Roth, 1972
 Menzel and Roth, 1972

440, 520-580
 440, 520-540
 360, 440-480

White, 1924

VERTEBRATES

Teleost Larvae

Clupea harengus (C)
 at hatching
 1 week
 2-3 weeks
Pleuronectes platessa (C)
 at hatching
 1-½ weeks
 2-4 weeks
Solea solea (C)
 3 weeks

- phototaxis
 - phototaxis
 - phototaxis

450, 521, 605
 485
 450, 521

Blaxter, 1968
 Blaxter, 1968
 Blaxter, 1968

Blaxter, 1969
 Blaxter, 1969
 Blaxter, 1969

480-600

Blaxter, 1969

^a Habitat: C—coastal; UO—upper ocean; FW—freshwater; DO—deep ocean.

^b S—shoulder.

^c Point of maximum sensitivity, but no shorter wavelengths were tested.

logical measurement, or from visual pigment extractions. The various methods sometimes yield different results, e.g., absorption maxima for extracted pigments are usually at shorter wavelength than those derived from electrophysiological spectral sensitivity determinations (Fernandez, 1973). Nevertheless, generalized conclusions emerge from this table concerning the relationship between maximum sensitivity and the transmission of light from the sky and sun in an animal's environment (Goldsmith, 1972).

Animals living in coastal water or in fresh water where longer wavelengths are transmitted better than in clearest oceanic (maximum at 470 nm) or pure water (maximum at 475 nm) (Jerlov, 1970) tend to have their main spectral maxima in the region 500–600 nm. This is especially true for larvae, but also holds for adult crustaceans and rotifers. Among the deep ocean crustaceans, the main spectral sensitivity maxima usually occur in the blue-green between 460 and 495 nm. This corresponds to the transmission characteristics of oceanic water and also for many euphausiids the wavelengths of greatest intensity light at the oceanic depths inhabited (Boden, 1961; Kampa, 1961).

A comparison of euphausiids of the species *Euphausia pacifica* found in the San Diego Trough and in the Saanich Inlet provides an interesting example of spectral sensitivity as related to the light transmitted to the depths inhabited. In the San Diego Trough the peak in the spectrum of transmitted sun and sky light throughout the diurnal cycle is 475–490 nm (Boden *et al.*, 1961). In the Saanich Inlet, animals are limited to depths above 100 m due to an oxygen-deficient zone below this depth. Above 100 m the peak in transmitted wavelengths is 494–502 nm (Boden and Kampa, 1965). Animals from both areas have spectral sensitivity curves of the same shape, but for San Diego animals the main maxima are at 465 and 495 nm, while in the Saanich Inlet they are at longer wavelengths, 495 and 515 nm. Boden and Kampa (1965) suggest that the shift results from different concentrations of the screening pigment astaxanthin.

Several exceptions (Table 4) to the generalized pattern deserve comment. Phyllosoma larvae of the lobster *Panulirus longipes cygnus* have equivalent maxima at 470 and 555 nm. Ritz (1972) speculates that this may be an adaptation to the light transmission characteristics of the multiple areas inhabited by the larvae, since they hatch in coastal waters and are transported by currents into oceanic circulation. The rotifers *Asplanchna* and *Polyarthra* have maximum sensitivity at 520–580 nm, while *Filinia* is most responsive at 440–480 nm. Menzel and Roth (1972) speculate that the difference in sensitivity results from the habitats in which the animals occur. *Asplanchna* and *Polyarthra* live near the surface, where longer wavelengths are available, while *Filinia* lives much deeper, where longer wavelengths would be filtered out. This of course assumes the fresh water inhabited is very clear.

3.3.2.2. Differential Responsiveness

For some zooplankters different wavelengths can initiate different behavioral responses. Color dances, originally reported by Smith and Baylor (1953), demonstrate this phenomenon. Under red light (greater than 600 nm) presented from above, they found four cladoceran species to display a vertical hop and sink behavior with low swimming velocity and horizontal movement (red dance). When exposed to blue light (less than 500 nm) the behavior changed as the linear velocity became 3–5 times greater, and the organisms swam perpendicular (horizontal) to the line of the light direction (blue dance). In later work Baylor and Smith (1967) reported this response in five other crustacean species and Dingle (1962) in nine species.

Such studies imply that these animals have independent red and blue sensitivity. Recently, Young (1974) confirmed this for *Daphnia magna*. Using the eye movement following response as a behavioral indicator of light sensitivity, he found that if stimulated through the side of the head, the action spectrum maximum was in the region 550–600 nm. However, if stimulated through the top of the head, peak sensitivity was at 400–500 nm.

Smith and Baylor (1955) suggest that the functional significance of color dances is food finding. Under blue light animals would swim horizontally, thereby increasing the chance of moving into an area occupied by phytoplankton. Since phytoplankters filter out blue wavelengths, leaving yellow and reds, in their presence a hop and sink behavior would be initiated. This would increase the random motion, and thereby the chance for contact with food. Also, the organism's relative position is maintained since horizontal movement is reduced. This speculation is somewhat confirmed by the observation that when a Lucite dish containing phytoplankton is placed over a tray containing the animals and this assembly illuminated from above with white light, the organisms accumulate under the phytoplankton (Smith and Baylor, 1953; Baylor and Smith, 1957; Dingle, 1962). However, Dingle (1962) found no correlation between species displaying color dances and food habits.

Another possible wavelength-specific behavior was noted by Herman (1962) observation that the opossum shrimp, *Neomysis americana*, is specifically attracted to 515-nm light even when presented with light of other wavelengths at higher intensities. Although this suggests wavelength selection, the spectral sensitivity of the animal may be such that intensities of the other wavelengths were not high enough to be photoreceptively equivalent to the light at 515 nm.

As for the specific effects of and responses to UV light, exposure to UV light from the sun can kill zooplankton, e.g., copepods (Huntsman, 1924; Klugh, 1929, 1930) and fish larvae (reviewed by Blaxter, 1970). Physiologically, UV light injures oyster larvae (Aboul-Ela, 1958), while a

short exposure to sunlight causes a decline in heart rate by *Calanus* (Harvey, 1929). However, some degree of adjustment to the injurious effects of sunlight is possible, since *Calanus* from broods that live at the surface are less affected by sunlight than those which live deeper (Marshall and Orr, 1955). Also, some fish larvae are transparent so that light absorption is reduced, while other species appear to have their central nervous systems protected from adverse sunlight effects by pigment spots (Breeder, 1962). As for avoidance of UV, negative phototaxis to UV light is reported for barnacle larvae (Loeb, 1906, 1908) and for *Daphnia* (Baylor and Smith, 1957; Moore, 1912; Schultz, 1928). This perhaps also accounts for the descent observed by Aboul-Ela (1958) by oyster larvae under overhead UV irradiation in a 1-m tank.

3.3.3. Polarized Light Sensitivity

Behavioral responsiveness to linear or plane polarized light is well-known among many animals showing DMV. Although polarotaxis is extensively documented for adult crustaceans (summarized by Waterman, 1973), very little work has been done with their planktonic larvae. Via and Forward (1975) studied the ontogeny of responses by larvae of the crab *Rhithropanopeus harrisii* and found polarotaxis only in stage II and III zoea. Among Mollusca, Jander *et al.* (1963) demonstrated polarotaxis by larvae of the cephalopod *Sepioteuthis lessoniana*. Polarization sensitivity by fish larvae is unstudied but perhaps exists, since some adult fish respond behaviorally to this stimulus (Forward *et al.*, 1972; Forward and Waterman, 1973; Kleerekoper *et al.*, 1973; Waterman and Forward, 1970, 1972b).

In most cases polarotaxis was tested under a vertical beam of linearly polarized light, and behavioral responses consisted of body axis alignment or directional swimming. Responsiveness was at stereotyped angles of parallel, perpendicular, and/or $\pm 45^\circ$ to the e-vector. As elegantly demonstrated by Jander and Waterman (1960), this response is a true orientation to polarized light and not to the light intensity patterns created by polarized light. Most experiments were done under conditions of 100% polarization, which exceeds the maximum reported in nature of about 60% (Ivanoff and Waterman, 1958b; Ivanoff, 1974). An important consideration is the minimum percent polarization that animals can perceive. Few studies exist, but at least for *Daphnia* orientation is possible when light is 20% polarized (Waterman, 1974b). This tentatively indicates that the percent polarization in nature is sufficient for perception.

As for spectral sensitivity of polarotaxis by planktonic animals, the only study is that by Via and Forward (1975) on larvae of the crab *Rhithropanopeus*. The response spectrum appears identical to that for phototaxis (Forward and Costlow, 1974), with the main maximum at 500 nm.

Polarization perception is potentially most important in localization of the sun for horizontal movements involving sun compass orientation (Waterman, 1974a,b). Yet polarization underwater may also serve as a cue for vertical movements. Only Umminger (1968a) in experiments with the freshwater copepod *Cyclops vernalis* has seriously considered the involvement of polarotaxis in DVM. In his experiments the polarization pattern was presented horizontally with the e-vector also horizontal. This pattern crudely simulated that found underwater at low latitudes around noon and in the direction of the sun at all times of the day (Waterman, 1954). Early morning conditions were simulated by having the overhead intensity greater than the horizontal intensity. As the horizontal intensity was increased, he found that the copepods swam vertically downward perpendicular to the e-vector. At midday the conditions of equal overhead and horizontal intensity provoked swimming parallel to the e-vector, or in a horizontal plane with little vertical movement. To simulate the late afternoon, the horizontal intensity was decreased and swimming was perpendicular to the e-vector, with the predominant direction upward. Thus responses to polarized light oriented horizontally vary dependent upon the horizontal versus overhead intensity ratio plus the direction of the intensity change. These responses lead to a descent early in the day, no vertical movement at midday, and an ascent at the end of the day which are similar to the nocturnal migration pattern of some copepods. The major problem with this study is that the polarization pattern is much more complex underwater. However, that aspect of the pattern used as a cue for vertical movements may only be the constant horizontal e-vector in the direction of the sun. Unfortunately, other species of copepods, when tested, do not display these variable responses (Umminger, 1968b).

4. CONCLUSION

A final comparison of phytoplankton and zooplankton might be instructive and perhaps contribute to an understanding of how light functions as a stimulus for vertical movements. Admittedly, extrapolations between laboratory studies on one species and field studies on another is a questionable procedure, but it does offer some interesting correlations.

Physiological studies indicate that zooplankton are the most sensitive to light, as their intensity thresholds for phototaxis are several orders of magnitude below those for phytoplankton. This is certainly not unexpected, since zooplankton have more sophisticated photoreceptors. Also by migrating over greater distances, they occur at depths where the light intensity is greatly reduced. Phytoplankton occur at relatively shallow depths, and

thereby experience higher light intensities which agrees with their higher light thresholds.

As for wavelength sensitivity, primary maxima for phytoplankton usually occur at 450–500 nm. Among deep sea zooplankton, maxima are similar (460–495 nm), while those for coastal and freshwater forms are higher (500–600). For zooplankton, spectral sensitivity corresponds to the wavelength region of maximum transmission for the particular water mass inhabited. Spectral sensitivity of phytoplankton, however, corresponds to the wavelength transmission in oceanic water, yet many species inhabit coastal or freshwater areas. This discrepancy may be a consequence of living near the water surface, where alterations in the spectral composition of sun and sky light due to absorption and scattering are less than at greater depths.

The general patterns of DVM displayed by zooplankton and phytoplankton are similar, i.e., reverse migration by zooplankton corresponds to the dawn migration by phytoplankton; nocturnal migration, to dusk migration; and twilight migration, to dawn–dusk migration. Among zooplankton species the DVM patterns are variable, but twilight migration may be the basic pattern, while for phytoplankton the dawn migration is most commonly observed.

From field studies of DVM by zooplankton and simultaneous measurements of environmental parameters, changes in ambient underwater light conditions clearly correspond to changes in depth. Light may act as an *initiating*, a *controlling*, and/or an *orienting* cue in the vertical movements (Bainbridge, 1961). Speculation as to aspects of light used for these three purposes is now possible based on information from laboratory studies.

Although the light intensity level may be important in initiating DVM of zooplankton, laboratory experiments have clearly established only that the rate and direction of the change in intensity can initiate vertical movements. As a controlling factor, ambient light intensity is of primary importance, since its level can determine the type of photoresponse displayed and the level of photosensitivity. Perhaps most important as a controlling factor is the contribution made by circadian rhythms. Experiments indicate that endogenous circadian rhythms in vertical movements do exist, but further studies are needed to differentiate the contributions by rhythms in activity and in responsiveness to light, gravity, and/or pressure. In addition, various external and internal factors can modify photoresponses and thereby act as controlling influences. Those conditions that normally induce positive phototaxis are consistently encountered low in the water column, i.e., moderate- and low-intensity light, a decrease in temperature, an increase in hydrostatic pressure, and reduced food. Alternately, those conditions that frequently reverse the sign of phototaxis from positive to negative are found near the surface, i.e., high-intensity light, UV light, a temperature increase, a decrease in hydrostatic pressure, low salinity, and more food.

For orienting vertical movements, the radiance distribution pattern is certainly a contributing factor. In addition, the polarization pattern can provide a directional cue.

Unfortunately, studies of migrations by phytoplankton combined with accurate underwater light intensity measurements have not been performed in the field. Laboratory investigations to date have centered primarily on the mechanisms of orientation to light and on the physiology of photo-responses, with relatively little concern for their ecological implications. Consequently, it is difficult to determine the initiating, controlling, and orienting factors during DVM, although they may be similar to those for zooplankton. Cues initiating DVM, are unstudied. Ambient light intensity and circadian rhythms are probably the most important controlling factors. Light intensity influences the speed of movement as well as the sign of phototaxis, while the timing of maximum photoresponsiveness in the circadian rhythm of photobehavior consistently occurs during the day phase. Various external photoresponse modifying factors such as temperature and nutrients may also be controlling factors. Since phytoplankton are able to orient to a directional light source, the radiance distribution pattern can provide an orientation cue for vertical movements. The usefulness of the polarization pattern, however, remains to be demonstrated.

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Sunlight and Melanin Pigmentation*

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1. INTRODUCTION

When viewed from the perspective of photobiology, melanin pigmentation of human skin can be described in two categories: the first, *constitutive* or *intrinsic skin color*, and the second, *facultative* or *inducible skin color* (Quevedo *et al.*, 1974). Constitutive skin color designates the genetically determined levels of cutaneous melanin pigmentation in accordance with the

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genetic programs of the cells in the absence of direct or indirect influences (e.g., solar radiation, hormones, or other environmental factors). Facultative skin color characterizes the increase in melanin pigmentation above the constitutive level and arises from the complex interplay of solar radiation and hormones upon the genetically endowed melanogenesis of the individual. The facultative skin color change brought about by solar radiation is commonly referred to as "suntan."

2. CONSTITUTIVE MELANIN PIGMENTATION

2.1. Biology of Melanin Pigmentation

The melanin pigmentary system of the human skin is based on two cell types: dendritic melanocytes and nondendritic keratinocytes. The melanocytes synthesize specialized organelles called melanosomes within which the pigment melanin is contained. Melanosomes are transferred to the keratinocytes and transported by these cells to the epidermal surface or stratum corneum. The production and the transfer of these chromoprotein-carrying organelles, the melanosomes, is a complex process involving the structural and functional organization of both melanocytes and keratinocytes. This functional unit, consisting of one melanocyte and approximately 36 keratinocytes, is known as the *epidermal-melanin unit* (Fitzpatrick and Breathnach, 1963; Hadley and Quevedo, 1966; Frenk and Schellhorn, 1969) (Fig. 1). Information gathered within the past five years indicates that the functional activity of the multicellular epidermal-melanin unit, rather than the melanocyte alone, is the focal point for the determination of skin color. This unique symbiotic relationship results in a uniform and wide distribution of pigment granules throughout the entire epidermis, although the melanocyte population of the epidermis is no more than 10–25% of the population of the keratinizing basal cells (Szabo, 1967a). The structural basis of normal melanin pigmentation of mammalian skin depends on the following factors: (1) the formation of pigment granules, the melanosomes; (2) the melanization of melanosomes, involving the synthesis of the enzyme tyrosinase and the enzymatic oxidation of tyrosine into melanin; (3) the movement of the melanosomes from the protoplasmic mass, the perikaryon, into the dendrites of the melanocytes; (4) the transfer of these melanosomes into the keratinizing epidermal cells, the keratinocytes; (5) the incorporation of melanosomes by these cells either as single discrete particles or as melanosome complexes; (6) the degradation of the melanosomes within keratinocytes; and (7) the rate of exfoliation of keratinocytes. Although each of these factors plays an important role in

understanding the nature of hyperpigmentation arising from the complex interplay of light and the tanning ability of the individual, only factors such as the formation, melanization, and transfer of melanosomes will be discussed (Fig. 1).

2.1.1. Formation of Pigment Granules

The melanocyte synthesizes melanosomes within which the pigment melanin is contained. Four stages of melanosomal development are currently recognized (Fitzpatrick *et al.*, 1967, 1971*a,b*; Toda and Fitzpatrick, 1971; Quevedo *et al.*, 1974; Jimbow and Kukita, 1971; Jimbow, *et al.*, 1971; Jimbow and Fitzpatrick, 1974; and reflect the degree of its melanization.

Stage I describes a spherical, membrane-delineated vesicle that

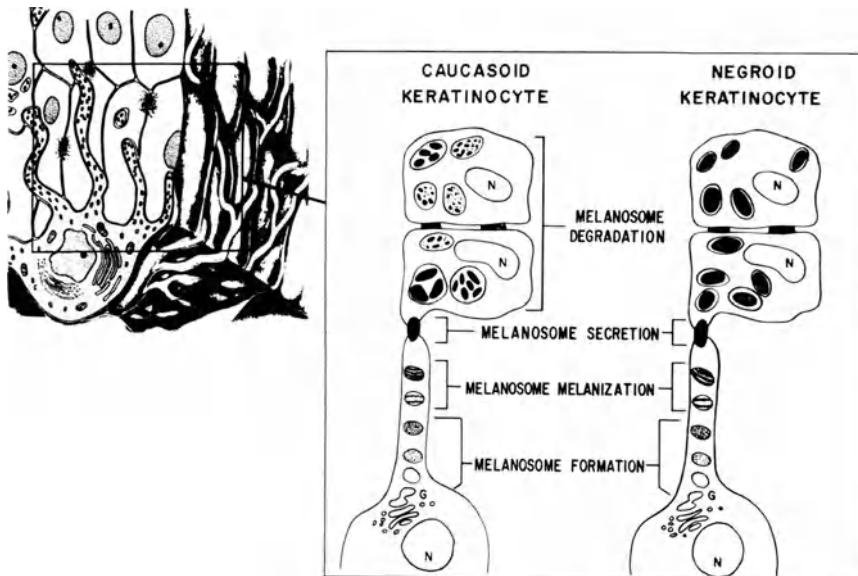


Fig. 1. Epidermal melanin unit illustrating the structural and functional basis of normal melanin pigmentation of skin. Only four processes involved in melanogenesis are illustrated: formation of melanosomes, melanization of melanosomes, secretion and transfer of melanosomes, and degradation of melanosomes. The left half of the figure shows a melanocyte with its branched dendrites in contact with several keratinocytes. The right half of the figure shows an epidermal melanocyte-keratinocyte unit of a Caucasoid and a Negroid subject. Note the difference between melanosomes in the Negroid and Caucasoid keratinocytes. In the Negroid keratinocyte, the melanosomes are discrete; in the Caucasoid keratinocytes, groups of two or more melanosomes are aggregated within membrane-limited, lysosome-like organelles, and melanosomes often show evidence of degradation.

contains tyrosinase and (or) filaments that have a distinct 10-nm (100-Å) periodicity. The formation of melanosomes in this initial stage of development is regulated by the Golgi apparatus, the Golgi-associated endoplasmic reticulum, and rough endoplasmic reticulum. A stage I melanosome is believed to contain tyrosinase, structural proteins, membranes, and possibly certain auxiliary enzymes. It is speculated that polypeptides of the enzyme tyrosinase are synthesized on ribosomes and that the phospholipid-containing proteins are fashioned into membranes by the Golgi complex.

Stage II describes an oval organelle in which numerous membranous structures become evident, with or without cross-linking, and show a distinct 10-nm periodicity.

Stage III reflects an oval organelle in which melanin synthesis has begun through the enzymatic oxidation of tyrosine. Melanin accumulation occurs on the inner membrane, and the 10-nm periodicity evident earlier in stage II becomes partially obscured.

Stage IV reflects the end stage of development of melanosomes in which the deposition of melanin has resulted in the obliteration of the internal structure of melanosomes in stages II and III. The organelle is electron opaque and shows electron-lucent bodies at its periphery (Jimbow and Fitzpatrick, 1974a).

2.1.2. Melanization of Melanosomes

Melanin synthesis takes place within the melanosomes (Seiji *et al.*, 1963a,b; Fitzpatrick *et al.*, 1967; Moyer, 1966). The copper-containing enzyme tyrosinase, present in these organelles, catalyzes the oxidation of both monohydric (tyrosine) and dihydric phenols (3,4-dihydroxyphenylalanine) to orthoquinones. Molecular oxygen acts directly as the hydrogen acceptor in the reactions catalyzed by tyrosinase. In contrast to melanins of plant origin, which are generally described as *catechol melanin* in type, the mammalian melanins are *indole* in type and are composed basically of indole-5,6-quinone units. According to the classic Raper-Mason scheme of enzymic melanin formation from tyrosine and 3,4-dihydroxyphenylalanine (dopa) critically reviewed by Mason (1967), melanin is thought to be formed from tyrosine → dopa → dopa-quinone → dopa-chrome → 5,6-dihydroxyindole → indole-5,6-quinone → melanin through the polymerization of indole-5,6-quinone units (a homopolymer of indole-5,6-quinone linked through a single bond type). However, several chemical studies of natural melanins (Nicolaus, 1968; Swan, 1963, 1964; Robson and Swan, 1966; Hempel, 1966) using ³H-labeled and ¹⁴C-labeled precursors have revealed that mammalian melanins are not composed entirely of indole-5,6-

quinone units, but are complex polymers (a heteropolymer or a random polymer) consisting of several different monomers that may be coupled by various bond types of dopa-quinone, dopa-chrome, 5,6-dihydroxyindole, and 5,6-dihydroxyindole-2-carboxylic acid at various oxidation levels held together by a variety of bond types. Pyrrole units, carrying carboxyl groups, in addition to phenolic or quinonoid groups or both, are also present, and some of them represent carboxylated terminal units. The redox state of melanin polymer is equally important. The polyquinonoid melanin can have oxidized (quinonoid), as well as reduced (phenolic) forms of indole-5,6-quinone. These remarks relate only to the synthesis of the black-brown melanin (eumelanin) and much less is known about the yellow and red pigments in mammals (phaeomelanins) which differ from eumelanin. Eumelanin is insoluble in almost all solvents and is resistant to chemical treatments, whereas pheomelanin is soluble in dilute alkali (Duchon *et al.*, 1968; Fitzpatrick *et al.*, 1971*a,b*). It is believed that dopa-quinone formed from the oxidation of tyrosine by tyrosinase interacts with the sulfur-containing amino acid, cysteine, to form cysteinyl-dopa from which phaeomelanin is derived by pathways yet incompletely understood (Fitzpatrick *et al.*, 1971*b*, Quevedo *et al.*, 1974).

2.1.3. Transfer and Dispersion of Melanosomes

We have discussed the fact that the epidermal melanocytes are in symbiotic relationship with the epidermal keratinocytes. The importance of this symbiotic relationship can be well recognized when one examines the distribution of melanosomes after their transfer into keratinocytes in various races (Mottaz and Zelickson, 1967; Klaus, 1969). Electron microscopic observations of human epidermis obtained from people of various racial backgrounds have revealed that melanosomes occur within keratinocytes as discrete granules (nonaggregated form) or as aggregates of two or more discrete granules within membrane-limited bodies (Hori *et al.*, 1968; Szabo *et al.*, 1969; Toda *et al.*, 1972; Wolff and Konrad, 1971; Olson *et al.*, 1973). In Caucasoids and Mongoloids, the melanosomes are almost always found in groups (Fig. 1) and resemble the membrane-limited vacuoles that have been identified as phagolysosomes (Mishima, 1967*a,b*; Hori *et al.*, 1968). In the melanosome complexes of fair-skinned Caucasoids, the melanosomes are loosely packed and there is some granular substance located between them. In Mongoloids and Orientals, the groups of melanosomes are usually very tightly packed, with little or no substance between the melanosomes. After UV irradiation, the keratinocytes of Mongoloids and Caucasoids usually still contain melanosome complexes

(Szabo *et al.*, 1969). However, the number of groups increases, and there is a tendency toward increased numbers of melanosomes inside the melanosome complexes. The keratinocytes of Negroids (African and American) and Australoids (Szabo *et al.*, 1969; Mitchell, 1968; Toda *et al.*, 1972) contain mostly single melanosomes, and only occasionally does one find groups and doublets of melanosomes inside keratinocytes (Fig. 1). This aggregation or nonaggregation of melanosomes in keratinocytes appears to be a size-dependent phenomenon (Toda *et al.*, 1972; Wolff and Konrad, 1971) inasmuch as ellipsoidal melanosomes smaller than 0.6×0.3 nm in size are usually arranged in groups of two or more and show evidence of degradation. Unlike those of Caucasoids and Mongoloids, the melanosomes of Negroids and Australian aborigines are larger ($0.7-0.8 \times 0.3-0.4$ nm), and they usually do not form such aggregated complexes within the keratinocytes but are found as single, discrete bodies (Toda *et al.*, 1972; Olson *et al.*, 1973; Wolff and Konrad, 1971; Wolff *et al.*, 1974). Thus, variations in the size of melanosomes and the distribution pattern of melanosomes in the keratinocytes significantly influence the color of the skin. When melanosomes are aggregated and are few in number and small in size, they will contribute less to the scattering and absorption of impinging light than when they are singly dispersed, greater in number, and large in size (Table 1).

2.2. Normal Skin Color and Human Racial Color Differences

The most obvious difference between the various human races is the variation in the color of the skin. The factors that determine the skin color of normal skin include: (a) a reflection coefficient of skin surface; (b) absorption coefficient of epidermal-cell and dermal-cell constituents; (c) scattering coefficients of various cell layers; (d) thickness of the individual cell layers (stratum corneum, epidermis, and dermis); (e) the concentration of UV light and visible light absorbing components such as proteins (keratin, elastin, collagen, lipoprotein), melanin, nucleic acid, urocanic acid, carotenoids, hemoglobin (reduced and oxidized), and lipids; (f) the number and spatial arrangement of melanosomes and melanocytes; (g) the number and spatial arrangement of blood vessels and the relative quantity of blood cells (reduced and oxidized hemoglobin) flowing through the vessels. Pigmentation of the skin, as viewed clinically, is principally related to the variation in the content of melanin in the epidermis. If melanin pigment were absent from the skin, as in Vitiligo, the color of skin in all races would appear to be milk-white.

It appears therefore, that the color of skin is determined by the func-

TABLE 1. Relationship between Constitutive Skin Color and Size and Distribution Pattern of Melanosomes in Habitually Exposed Skin

Skin color	Size of melanosomes	Melanization of melanosomes	Tyrosinase activity in melanocytes	Distribution of melanosomes in epidermal keratinocytes	Approximate number of melanosomes per basal keratinocyte*
Heavily pigmented skin of African and American Negroes and Australian Aborigines	0.7-0.8 μm X 0.3-0.4 μm	Fully melanized, predominantly in stage IV	Marked	Single, non-aggregated	400 \pm 35
Moderately pigmented skin of Mongoloids (American Indians, Orientals)	0.5-0.7 μm X 0.2-0.4 μm	Moderately melanized stages III and IV	Moderate	Mixed, non-aggregated as well as aggregated	250 \pm 50
Moderately pigmented skin of Caucasoids (East Indians, Italians, Egyptians)	0.5-0.7 μm X 0.2-0.4 μm	Moderately melanized stages III and IV	Moderate	Predominantly aggregated	200 \pm 5
Lightly pigmented skin of Caucasoids (fair-skinned Americans, British, French, Germans, etc.)	0.4-0.6 μm X 0.2-0.4 μm	Partially melanized stages II and III	Weak	Predominantly aggregated	100 \pm 50

* Based on random calculations of 50 keratinocytes of basal layer.

tional state of pigment-producing cells, the melanocytes. One of the most obvious questions to ask is how the function of melanocytes is related to phenotypical coloration, a coloration determined genetically (constitutive) or produced by environmental factors (facultative). On the macroscopical level one can qualitatively assign color grades (e.g., black, brown, olive, moderately fair, very fair, freckled, etc.) based upon the quantity of melanin in the epidermis. At the level of light microscopy, when skin biopsies are examined, one can also distinguish the variations in skin color by the presence of more or less melanin in the skin (Gates and Zimmerman, 1953). The color of human skin derives from the visual impact of the total melanin content of the epidermis and is influenced by the reflection, absorption, and scattering of the impinging radiation on the surface of the skin. But the racial origin or the background of an individual cannot be ascertained by the mere visual color of the skin based on its melanin content. A representative of the Mediterranean race or an Asiatic Caucasoid may be labeled as "white" during winter but as "colored" by the end of a sunny summer. It is also true that neither a numerical count of melanocytes nor any other histologic characteristic as seen by light microscopy in the paraffin-embedded skin sections could reveal the racial origin of the skin specimens. The epidermal melanocyte system of various human races has been investigated by Staricco and Pinkus (1957), Szabo (1954, 1959, 1967*a,b*), Mitchell (1963), and Toda *et al.*, (1973). These investigators compared the melanocyte density of single representatives of various "colored" groups with the average melanocyte frequency of white Caucasians and observed that the colored races do not have more melanocytes than white Caucasians. Careful studies of human skin, particularly of the unexposed regions of the body, have revealed that racial differences in skin color are not due to differences in the number and distribution of melanocytes but are due to characteristic differences in the rate at which melanosomes are produced by melanocytes and transferred and distributed in keratinocytes (Fitzpatrick *et al.*, 1965, 1971*a,b*; Toda *et al.*, 1973; Pathak, 1967; Pathak *et al.*, 1971; Quevedo *et al.*, 1974).

Racial color differences can be recognized at the ultrastructural level and involve: (a) differences in the localization or distribution pattern of melanosomes in the keratinizing malpighian cells, either in the aggregated or in the nonaggregated form, or as a combination of aggregated and nonaggregated forms; (b) variation in the number of melanosomes in the epidermal melanocytes and keratinocytes; (c) differences in the size of melanosomes; (d) differences in the degree of melanization of melanosomes; and (e) differences in the degradation of melanosomes due to variations in the hydrolytic activity of these organelles. Variations in the hydrolytic activity of the melanosomes can influence the degradation of melanosomes. The

lighter skin color of Caucasoids may result from the degradation of melanosomes, a phenomenon that has been observed to occur within keratinocytes (Hori *et al.*, 1968; Szabo *et al.*; Toda *et al.*, 1974).

3. FACULTATIVE MELANIN PIGMENTATION: ACTION OF LIGHT

Solar radiation profoundly influences skin color. Increased melanin pigmentation which occurs after exposure of human skin to sunlight or to UV light from artificial sources is familiarly known as "tanning." Tanning of the skin involves two distinct photobiologic processes: (1) immediate tanning (IT), sometimes referred to as immediate pigment darkening (IPD) reaction, and (2) delayed tanning (DT) (Fig. 2). The biophysical, biochemical, and ultrastructural bases of these two processes will be reviewed briefly with special emphasis on the effects of single and multiple exposures to UV radiation on (a) changes in the number of melanocytes (Figs. 3, 4); (b) synthesis of melanosomes, i.e., the number of melanosomes and their size; (c) melanization of melanosomes; and (d) the transfer of melanosomes to keratinocytes; concomitant changes in keratinocytes concerning (e) the number melanosomes transferred; and (f) the distribution pattern of melanosomes within the keratinocytes.

3.1. Immediate Tanning Reaction

IT can best be seen in pigmented individuals or in the previously tanned areas of fair-skinned individuals. IT can be induced both by long-wave UV (315–400 nm) and visible light (400–700 nm). UV-A (315–400-nm) radiation is more effective in the induction of IT than is visible radiation (Pathak, 1967; Pathak *et al.*, 1962*a,b*; Pathak and Stratton, 1969; Jimbow and Fitzpatrick, 1975; Jimbow *et al.*, 1974*a,b*, 1975*a,b*). UV-B radiation (290–315 nm), the sunburn-producing spectrum, does not stimulate IT as effectively as UV-A radiation. This selective induction of IT by UV-A is related to the depth of penetration and absorption of this radiation at the dermo-epidermal junction. The skin begins to be hyperpigmented with 5–10 min of midday summer sun exposure and can be maximally pigmented with 1 h of irradiation. When the skin is withdrawn from exposure to light, the hyperpigmentation fades rapidly within 30–60 min, and thereafter the color usually fades gradually, so that after 3–4 h the irradiated areas are barely hyperpigmented. Sometimes, however, after prolonged sun exposure, 90–120 min, skin may remain hyperpigmented for as long as 36–48 hr, after which time newly synthesized melanin (new melanogenesis or DT) begins to hy-

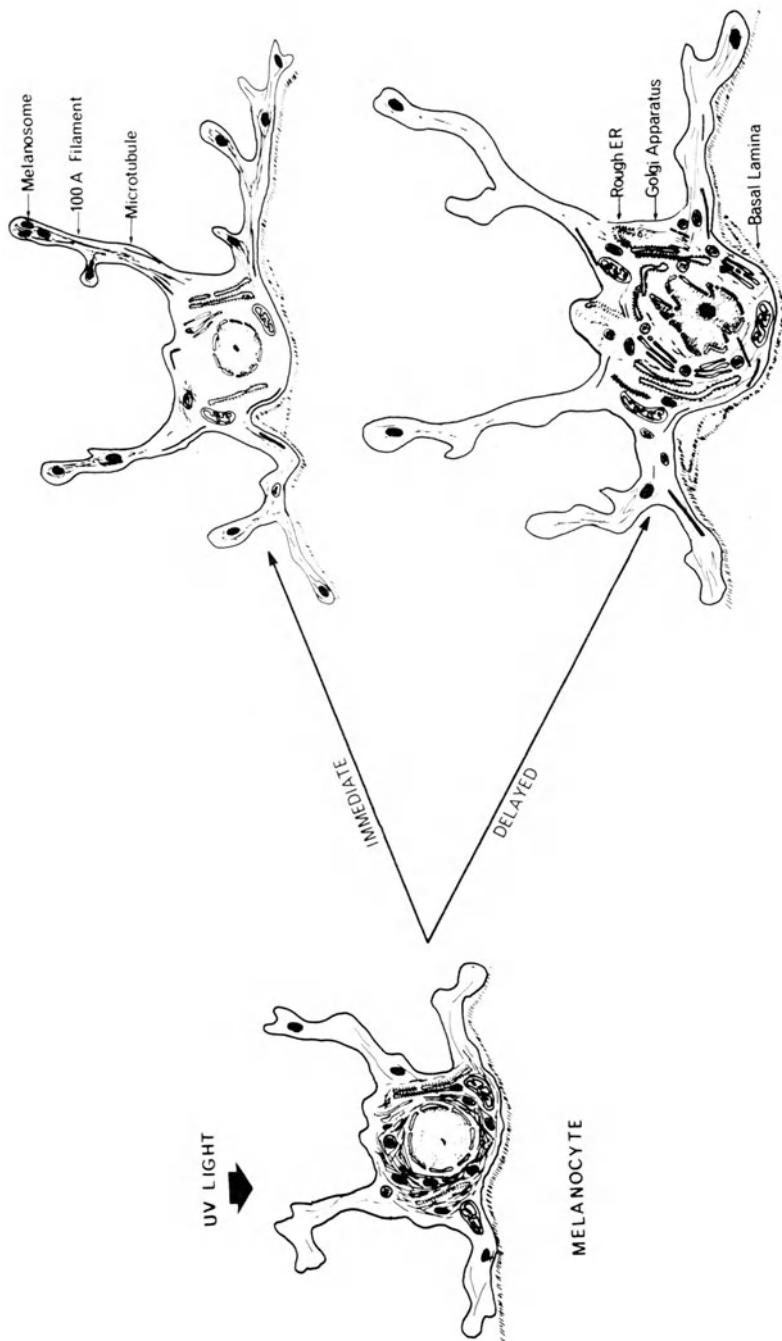


Fig. 2. Two photobiologic processes in melanin pigmentation stimulated by sunlight are illustrated: immediate tanning (IT) and delayed tanning (DT) reaction. The left side of the figure shows a melanocyte from the unexposed skin. This melanocyte shows a round or oval nucleus. Few melanized melanosomes, and dense aggregates of fine filamentous structures around the nucleus. Top right shows a melanocyte after induction of IT. Melanocytic thin 10-nm filaments are hardly seen in the perinuclear area; they can be seen in the dendritic processes. The melanosomes are closely intermingled with 10-nm filaments. Lower right shows a hypertrophic melanocyte with well-developed dendrites after DT.

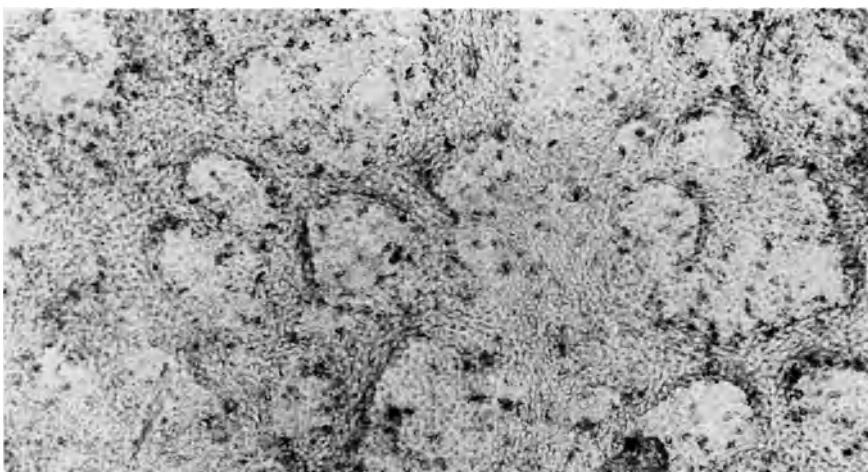


Fig. 3. A low-power view of dopa-incubated epidermal preparation from the unexposed Caucasoid skin. Biopsy was initially incubated in 2N NaBr, epidermis was then split from the dermis and reincubated in dopa solution (Starrico and Pinkus, 1957). The perikaryon of the melanocyte is small and the dopa reaction is weak.

perpigment the skin. The residual hyperpigmentation of IT is due to the redistribution of the existing melanosomes within the keratinocytes. Inasmuch as the IT reaction is a rapid phenomenon and can be induced in a matter of a few minutes, it appears that IT results from changes in melanosomes already existing in the melanocytes and keratinocytes of skin and is brought about by a combination of several of the events described below.

3.1.1. Photooxidation of Preformed Melanin

An immediate photooxidation of already existing melanin polymer occurs through the generation of semiquinonelike free radicals in melanin (Pathak, 1967; Pathak and Stratton, 1968). As stated earlier, the indole-5,6-quinone units in the melanin polymer can exist in different stages of oxidation. The comparatively reduced state of melanin is evidenced by a brown or lightly tanned color of the skin, and the comparatively oxidized form can be recognized as a dark brown or black color. One of the most important properties of melanins is their stable, free radical character which is ascribed to the semiquinonoid form of 5,6-dihydroxyindole that is stabilized by resonance throughout the highly conjugated polymer. The free radical content depends upon the degree of melanization and oxidation (Mason *et al.*, 1960; Blois *et al.*, 1964; Pathak, 1967; Stratton and Pathak, 1968; Pathak and Stratton, 1969). Prior to irradiation the lightly tanned skin

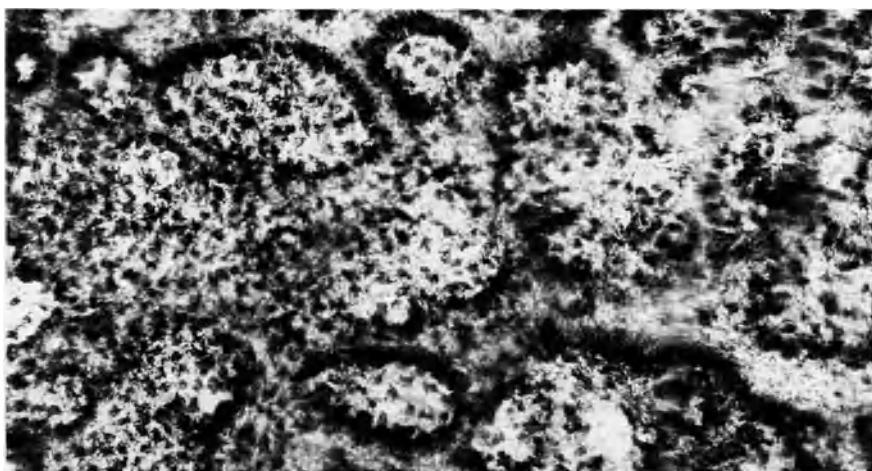


Fig. 4. A low-power view of dopa-incubated biopsy preparation at day 5 after exposure to UV radiation (150 mJ/cm^2 , 290–320 nm). The intensity of the dopa reaction is markedly increased, the perikaryon of the melanocyte is hypertrophic, and there is marked arborization of the dendritic process.

exhibits a weak electron paramagnetic resonance signal characteristic of melanin free radicals. Immediately after irradiation with UV and visible light, the color of the skin changes to dark brown or black and one can detect a significant increase in the semiquinonelike free radicals in the melanin polymer, suggesting that an immediate oxidation reaction is also occurring in the polymer. Furthermore, the unexposed skin of fair individuals contains melanosomes which are partially melanized and can be recognized in stages II and III of their development. After induction of IT, however, one can often detect melanosomes in stages III and IV (highly melanized form). The unexposed skin of darkly pigmented individuals (e.g., Mongoloids, Negroids, and pigmented Caucasoids) already contains melanosomes in stages III and IV. Their melanosomes are mostly in stage IV when the IT reaction is induced.

3.1.2. Changes in the Distribution Pattern of Melanosomes in the Epidermis

In the melanocytes of unexposed skin, the melanosomes are usually aggregated around the nucleus and are rarely seen in the dendritic processes (Figs. 2, 5, 6). After the induction of IT, however, the melanosomes become prominent in the dendritic processes (Figs. 2, 7, 8; Jimbow *et al.*, 1973, 1974a). There is also a definite change in the number and dispersion pattern

of melanosomes in keratinocytes after the IT reaction. A random count of melanosomes in keratinocytes with nuclei located in the basal layer of four subjects showed a statistically significant increase in the number of melanosomes per keratinocyte after the IT reaction. These findings suggest that during the IT reaction there was a rapid transfer and redistribution of pigmented melanosomes from the melanocytes to the keratinocytes. It is equally possible that this redistribution and variations in the melanosomal number discussed above reflect changes in the sol-gel property of the cytoplasm and also an increment in the negative charge of the melanosomes due to the absorption of radiant energy.

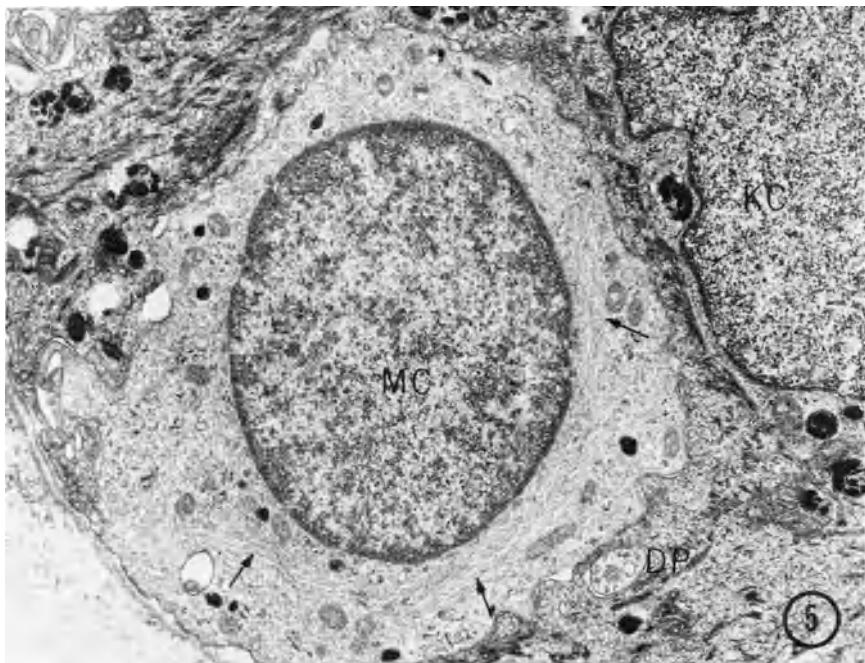


Fig. 5. An electron micrograph of unexposed skin of buttock from a Mongoloid subject. The melanosomes are mostly in melanizing stages (stages II and III). Dense aggregates of 10-nm filamentous structures (see arrows) around the oval nucleus can be seen. On the right side of the figure is a keratinocyte (KC); DP = dendritic processes, MC = melanocyte. ($\times 10,200$)

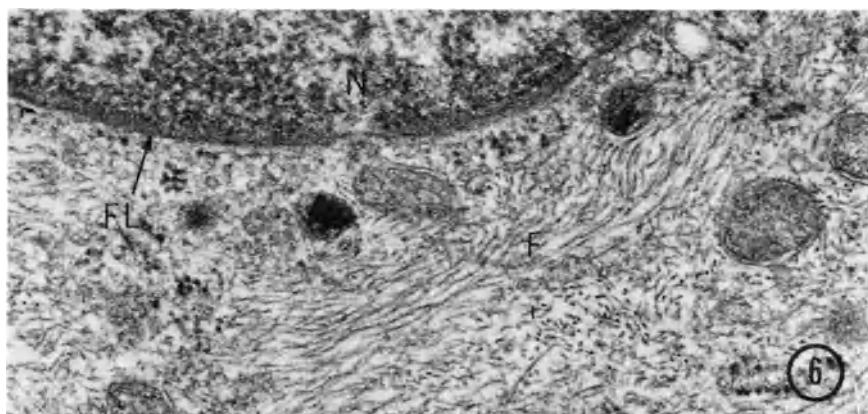


Fig. 6. A higher-power view of a portion of the melanocyte shown above. Around the nucleus, an aggregate of 10-nm filaments (F) and melanosomes in stages I-III can be seen. FL = fibrous lamina of the nucleus (N).

3.1.3. Changes in the Distribution Pattern of 10-nm Melanocytic Filaments

Recent observations of Jimbow and Fitzpatrick (1975a) and Jimbow *et al.* (1973, 1975a) have indicated that human melanocytes contain 10-nm-diameter filaments. These melanocytic filaments, and probably microtubules also, play a prominent role in the IT reaction and provide a motive force for rapid movement and transfer of melanosomes from melanocytes into the keratinocytes (Figs. 2-8). This hypothesis is based on the following observations. Prior to the induction of IT, the melanocytes from the habitually nonexposed regions of the body in the skin of Caucasoids, Mongoloids, and Negroids exhibit: (a) few melanosomes, (b) numerous 10-nm-diameter filaments, and (c) a few microtubules (25-27 nm in diameter) characteristically aggregated around the nuclei. *Per contra*, significant changes in the distribution pattern of these organelles in IT involved (a) prominence of dendritic processes laden with 10-nm-diameter filaments; (b) translocation of melanosomes from the perikaryon to dendritic processes which forms a concourse of melanosomes in the bundles and a meshwork of microfilaments; (c) a few microtubules in the extended processes of dendrites; and (d) a concomitant increase in the number of melanosomes in the keratinocytes (Jimbow and Fitzpatrick, 1975; Jimbow *et al.*, 1973, 1975a).

Thus, IT reflects changes in the existing melanosomes and does not involve the new synthesis of melanosomes. The most noticeable changes include (a) photooxidation of melanin, (b) marked change in the distribution pattern of melanocytic filaments and microtubules as well as melanosomes characterized by shifting and dispersion of these filaments and tubules from the perinuclear area to the dendritic processes of melanocytes, and (c) a

recognizable decrease in the number of melanosomes in the perikaryon accompanied by an increase in the number of melanosomes in the keratinocytes.

3.2. Delayed Tanning Reaction and Hyperpigmentation of the Skin

Delayed tanning is a process which involves the production, transfer, distribution, and, to a limited extent, degradation of melanosomes (Jimbrow *et al.*, 1974*a,b*, 1975*b*). The degree of melanin pigmentation that occurs following exposure of human skin to solar radiation varies to a certain

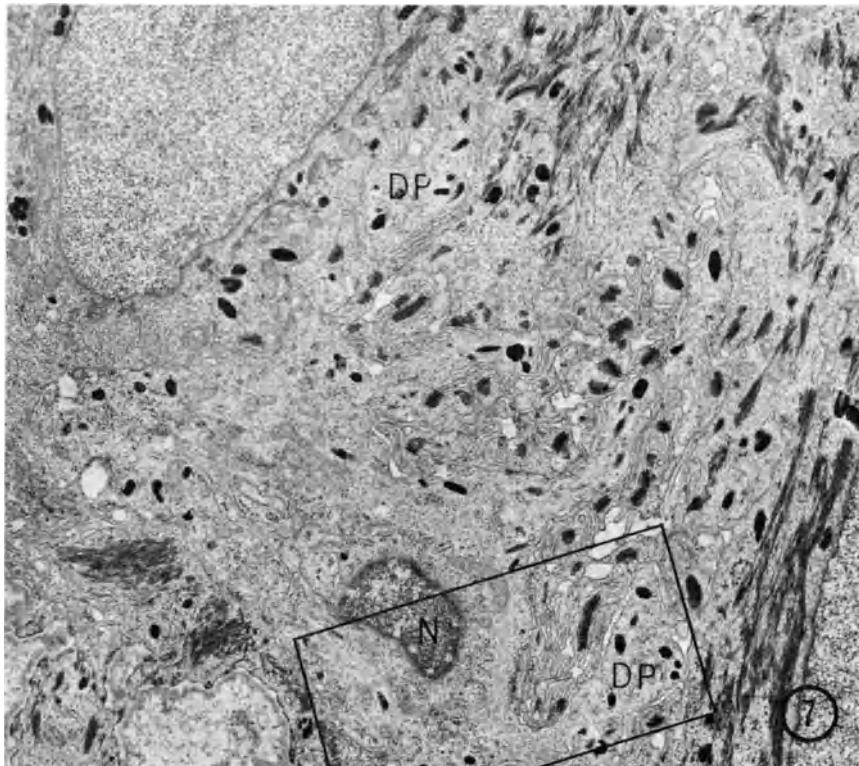


Fig. 7. A melanocyte from Mongoloid buttock skin after the IT reaction. The specimen was taken from the same subject shown in Figs. 5 and 6. The dendritic processes (DP) of the melanocytes are well developed and extend into the keratinocytes. The melanosomes become less aggregated in the perinuclear area (N = nucleus) and are now prominently seen in the dendritic process. The melanocyte contains more melanized melanosomes than those shown in Fig. 5 before exposure.

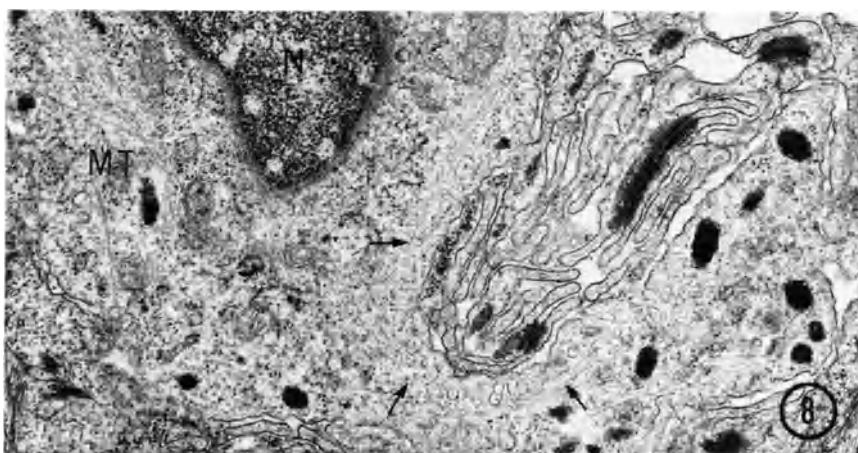


Fig. 8. High-power view of the portion of the melanocyte shown in a rectangular box in Fig. 7. The nucleus (N) is indented and shows nuclear pores. The 10-nm filaments appear to be stemming from perinuclear area to the tip of dendrites (see arrows). Microtubules (MT) are located in the periphery of the cytoplasm.

extent with the total dose of solar radiation received, but more importantly it is regulated by the genetically controlled functional capacity of the epidermal melanin unit of the individual. Genes control the structure of the melanosomes, the level of tyrosinase activity, the polymerization process of the indolequinone and other intermediates, and the development of the dendrites that transfer the melanosomes to the keratinocytes. Solar radiation or UV radiation from artificial sources influences the genetically controlled normal melanin pigmentation (facultative color) of the skin in one or more of the following ways: (a) an increase in the number of functional melanocytes (dopa-positive) as a result of proliferation of melanocytes, and also possibly the activation of the dormant or resting melanocytes (Figs. 3, 4); (b) hypertrophy of the melanocytes and increased arborization (branching) of the dendrites of melanocytes; (c) augmentation of melanosome synthesis manifested by an increase in the number of melanosomes both in the melanocytes and in malpighian cells (keratinocytes) (Figs. 8, 9). The number of fully melanized melanosomes (stage IV) is increased both in the melanocytes and the associated pool of keratinocytes. Even the number of early and intermediate stage (partially melanized, stage I and II) melanosomes is increased; (d) an increase in tyrosinase activity due principally to the synthesis of new tyrosinase in the proliferating melanocytes; (e) an increase in the transfer of melanosomes from melanocytes to keratinocytes as the result of increased turnover of keratinocytes; (f) an increase in the size of melanosomes and also an increase in the size of the melanosome complex. This is, however, greatly dependent on the racial complexion and

genetic background of the individual. These observations are illustrated in Figs. 9 and 10 and summarized in Table 2.

Pigmentary responses in individuals who are exposed to sun can be grouped into the following five categories:

- I. (Easy burn and no tan): People who sunburn very easily and do not show visually recognizable evidence of tanning (e.g., very fair skin, red hair, blue eyes, freckled skin, people with Celtic background—Irish and Scottish).

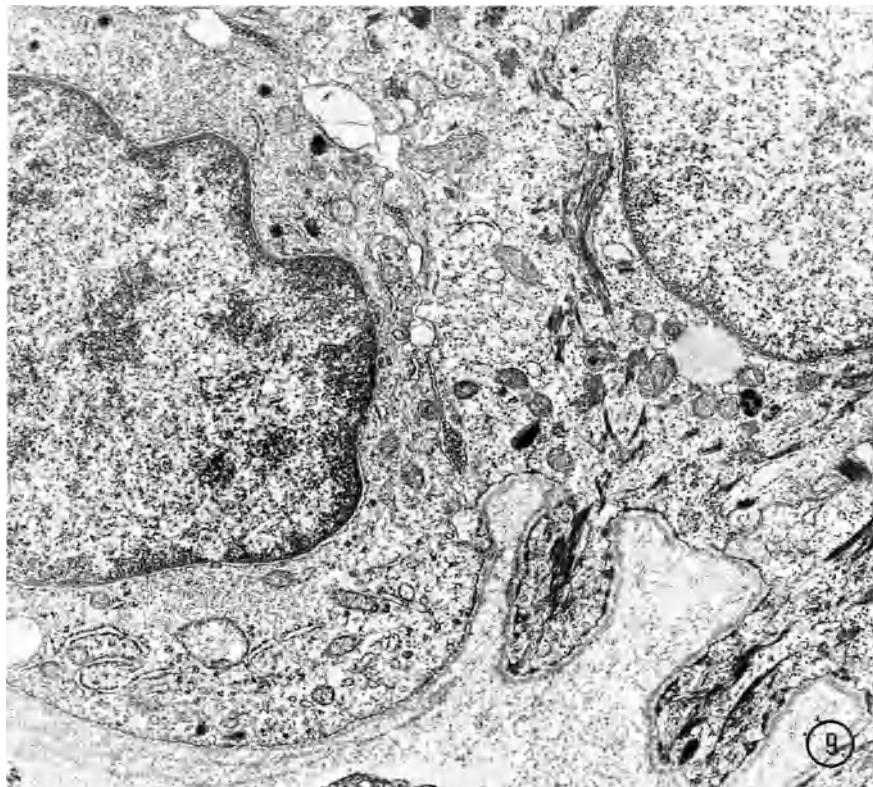


Fig. 9. An electron micrograph of a melanocyte and a keratinocyte from an unexposed Caucasoid back skin prior to UV irradiation. On the left side of the figure one can see a melanocyte with few melanosomes in stages II and III of their development. Notice the perinuclear distribution of 10-nm filaments. The keratinocyte (right side) shows few melanosomes which are in aggregated or complex forms.



Fig. 10. An electron micrograph of a Caucasoid back skin during the delayed tanning (DT) reaction induced by UV-A radiation (315–400 nm). The specimen was taken from the same subject shown in Fig. 9. There are three melanocytes laden with numerous, highly melanized (stage IV) melanosomes. Note the increased number of transferred melanosomes in the keratinocytes. The melanosomes are either singly dispersed or are aggregated and forming complexes. Both UV-B (290–315 nm) and UV-A plus 8-MOP can induce similar changes (see Table 2).

- II. (Easy burn and slight tan): People who sunburn easily and tan slightly (e.g., moderately fair skin, people with blond hair, blue-green, or hazel eyes).
- III. (Burn and then tan): People who burn moderately in the beginning, acquire a tan readily, and then do not generally burn on subsequent sun exposures (e.g., "brunette skin," olive skin, medium color skin).

TABLE 2. Changes in Epidermal Melanin Unit in Delayed Tanning Reaction after Repeated Exposures to UV-B, UV-A, or UV-A plus Oral 8-Methoxysoralen (8-MOP)

Nature of observations	Nonexposed skin	Exposed skin after UV-B, UV-A, or UV-A plus oral 8-MOP
<i>Macroscopic</i>		
Degree of visual pigmentation	+	Increased with UV-B ++, UV-A +++, UV-A plus 8-MOP +++; UV-A within 48 h
Onset of visual pigmentation	Continuous at steady state	UV-B within 72 h UV-A plus 8-MOP within 96-120 h
Degree of erythema reaction	None	Minimal with UV-A, moderate with UV-B, most with UV-A plus 8-MOP, but minimal with UV-A plus trimethylpsoralen.
<i>Light microscopic</i>		
Melanin granules	Barely visible in fair Caucasoids but easily visible in Negroids and Mongoloids	Increased in all races; most with UV-A plus 8-MOP
Number of melanocytes	About 700-950/mm ² in unexposed skin and 1000-1300/mm ² in habitually exposed skin; no racial differences in population density	Increased 2-3 times within 7-10 days; most remarkable increase in UV-A plus 8-MOP
Perikaryon of melanocytes	Small	Markedly enlarged
Dendrites of melanocytes	Poorly developed	Prominent and marked arborization
Dopa reaction	Weak	Strong; UV-A plus 8-MOP > UV-A; UV-A ≥ UV-B
Tyrosine reaction	Barely detectable or absent	Increased and easily detectable in all races
<i>Electron microscopic</i>		
<i>In melanocytes:</i>		
Number of melanosomes	Few	Markedly increased

Continued

TABLE 2. Continued.

Nature of observations	Nonexposed skin	Exposed skin after UV-B, UV-A, or UV-A plus oral 8-MOP
Melanization of melanosomes	Predominantly unmelanized in fair-skin (stages I-III); melanizing forms (stage III-IV) in dark skin	Melanized melanosomes (stages III and IV) markedly increased. In UV-A and UV-A plus 8-MOP most of the melanosomes are fully melanized; in UV-B melanosomes are in various stages of melanization
Size of melanosomes	Small (400-500 nm in long axis) Caucasoids; large in Negroids (500-700 nm in long axis)	Some increased; some are 700-800 nm even in Caucasoids
Distribution of melanosomes	Perinuclear and rarely in dendrites	Variable (diffused in perikaryon and dendrites); more prominent in dendrites particularly with UV-A or UV-A plus 8-MOP
10-nm (100 Å) filaments	Dense, perinuclear aggregation	Diffusely scattered in perikaryon and dendrites
Golgi apparatus	Poorly developed	Well developed, marked increase in size and number
Rough endoplasmic reticulum	Poorly developed	Well developed
Microtubules	Perinuclear	Perinuclear and in dendrites
<i>In keratinocytes:</i>		
Distribution pattern of melanosomes	Aggregated (melanosome complexes) in Caucasoids, nonaggregated in Negroids and Australians, and mixed (aggregated and single) in Mongoloids.	Slightly altered (more single and aggregated melanosomes) in Caucasoids and Mongoloids; in Negroids nonaggregated form
Ratio of nonaggregated versus aggregated form of melanosomes	Predominantly aggregated in Caucasoids; non-aggregated in Negroids	Increased ratio of nonaggregated form, particularly with UV-A plus 8-MOP
Number of melanosomes per melanosome complex	5-6 melanosomes in Caucasoids	Fewer, 3-4 melanosomes in Caucasoids; most prominent decrease UV-A plus 8-MOP

TABLE 2. Continued.

Nature of observations	Nonexposed skin	Exposed skin after UV-B, UV-A, or UV-A plus oral 8-MOP
Autophagic vacuoles in melanocytes and keratinocytes	Absent or very rare	Usually seen in UV-B and UV-A plus 8-MOP treated skin
Lipid droplets	Absent or very rare	Usually seen in UV-B and UV-A plus 8-MOP treated skin

- IV. (No burn and good tan): People who do not burn readily but tan substantially; their eyes and hair are most likely dark (e.g., pigmented Caucasoids, Orientals, and others).
- V. (Never burn and markedly tan): Markedly pigmented people (African and American Negroes, Australian Aborigines) who generally never burn but get profusely dark skinned after sun exposure.

3.3. Action Spectrum for Melanogenesis

Wavelengths shorter than 320 nm, which cause sunburn (erythema), are considered to stimulate melanogenesis or delayed tanning most effectively (Blum, 1955, 1959). The action spectrum for sunburn induced by exposure to sunlight has a maximum at 300–307 nm; that for sunburn induced by UV radiation from artificial sources has a maximum at 250–254 nm (8-h response). The erythema effectiveness gradually decreases at 270–280 nm (a distinct trough is seen at 280 nm due to absorption by proteins of the stratum corneum) and shows a distinct rise at 290–293 nm. The erythema effectiveness subsequently decreases rapidly at 313–320 nm. Wavelengths longer than 320 nm are weakly erythemogenic (Pathak and Epstein, 1972; Ying *et al.*, 1974). It requires nearly 800–1000 times more energy to produce a minimally perceptible erythema ($20\text{--}30 \text{ J cm}^{-2}$) at 320–400 nm than is required to produce a similar degree of erythema reaction at 290–300 nm (about $20\text{--}30 \text{ mJ cm}^{-2}$). If the erythema reaction were directly related to melanogenesis, the findings discussed above would suggest that the maximum efficiency for melanogenesis should be at 250–254 nm, followed

by slightly less response at 290–315 nm, and long-wave UV and visible radiation would be least effective in the stimulation of melanogenesis. It is, in fact, quite the opposite. Germicidal radiation is significantly less melanogenic than UV-B (290–315 nm) or UV-A (315–400 nm) radiation. The pigmentation produced by 254 nm is less intense and of shorter duration than that produced by UV-B or UV-A radiation (Parrish *et al.*, 1972). Several hundred multiples of the minimal erythema dose (MED) exposures of UV-C radiation ($\lambda < 280$ nm) will not produce any blistering reaction nor any intense pigmentation response, while as little as 3–6 times the MED exposure to 290–315-nm radiation may cause an intense erythema and pigmentation reaction. For example, an exposure to 254-nm radiation equivalent to $30 \times$ MED will produce a maximum of grade 2+ pink erythema response and a minimal tan (grade +, light brown tan). On the other hand, an exposure to 297-nm radiation equivalent to $2-5 \times$ MED will produce a grade 2+ pink erythema response and a moderate tan (grade ++, medium brown tan). An exposure dose of $10 \times$ MED at 297 nm can produce a grade +++, deep brown tan. In recent studies (Ying *et al.*, 1974; Jimbow *et al.*, 1974*a,b*, 1975*b*; Willis *et al.*, 1972), it was observed that UV-A (315–400 nm) was less erythemogenic and induced less intracellular degenerative change than UV-B. UV-A was found to be more effective in the induction of new melanogenesis than was UV-B. For a long time it has been generally believed, and more or less firmly stated in the dermatologic literature, that melanogenesis (DT) is optimally initiated by UV light of the so-called erythema spectrum (i.e., by UV-B). As early as 1962, Pathak *et al.*, showed that irradiation of human skin with long-wave UV radiation (UV-A), and to a limited extent visible light, would not only stimulate IT but also new melanogenesis. Subsequent studies by Langner and Kligman (1972) and Willis *et al.*, (1972) reemphasized the profound stimulation of melanin pigmentation by UV-A. In fact, UV-A appears to be more effective in the induction of new melanogenesis than UV-B radiation (Jimbow *et al.*, 1974*a,b*, 1975*b*). Thus, the generally held concept of the initiation of melanogenesis only by UV-B should be modified; long-wave UV light (UV-A) must be included in the melanogenic spectrum. It must be stressed, however, that if one were to estimate the quantum efficiency for melanogenesis by UV-B and UV-A, it would be apparent that UV-B is more efficient in the induction of melanogenesis than is UV-A. It requires approximately a minimum of $50-100 \text{ mJ cm}^{-2}$ to stimulate melanogenesis by UV-B, whereas a minimum of $10-12 \text{ J cm}^{-2}$ of UV-A is required to stimulate melanogenesis. It would appear, therefore, that the experimentally observed marked stimulation of melanogenesis by UV-A, both by Jimbow *et al.*, (1974*a,b*, 1975*b*) and Langner and Kligman (1972), is due to the fact that the less energetic photons associated with UV-A radiation (about 70–

80 kCal mol⁻¹) cause less cellular degeneration than does UV-B (about 95–100 kCal mol⁻¹). Differences in the depth of transmission and absorption of UV-A and UV-B radiation within the epidermis are also important factors in the differential activation of the epidermal melanocytes. In fair-skinned individuals, most of the impinging UV-B radiation (about 75–80%) is absorbed by the nonviable multicellular layer of the stratum corneum. About 10–15% is absorbed by the viable cells of the malpighian layer (keratinocytes), and about 7–10% will transmit through the dermo-epidermal junction and be absorbed in the papillary dermis (Pathak and Epstein, 1971; Pathak and Fitzpatrick, 1974). It is the absorbed radiation at the dermo-epidermal junction where the melanocytes are localized that stimulates or activates melanogenesis. UV-A radiation, on the contrary, can penetrate deeper through the dermo-epidermal junction. Nearly 20–35% of the impinging radiation will penetrate through the epidermis and reach the hairbulb region. It appears that the better stimulation of melanogenesis by UV-A than by UV-B is due to the selective activation and proliferation of melanocytes both at dermo-epidermal junction and in the hairbulb. Many of the ultrastructural changes reflecting formation, melanization, transfer, and degradation of melanosomes that take place in skin following repeated treatments with either UV-B, UV-A, or UV-A plus 8-MOP (8-methoxysoralen) are listed in Table 2. For details concerning the stimulation of pigmentation by UV-A plus 8-methoxysoralen, a potent photosensitizing agent of skin, the reader is referred to the articles by Pathak *et al.* (1974) and Pathak and Fitzpatrick (1974).

Visible radiation (400–700 nm) and infrared radiation ($\lambda > 750$ nm) are extremely weak in the induction or stimulation of melanogenesis. A single exposure in the range of 25–30 J cm⁻² of visible light will not stimulate melanogenesis. Repeated exposures of skin to either visible radiation or to infrared radiation may stimulate some melanogenesis but most of the stimulation is secondary to the effects of heat resulting from prolonged absorption of the radiant energy.

3.4. Effect of Single and Multiple Exposures of UV Radiation on Human Melanocytes

Photobiologic processes that lead to hyperpigmentation of skin following a single exposure to UV-B or UV-A are different from those that result from multiple exposures to UV-B or UV-A radiation. The increased melanin pigmentation after a single exposure to either UV-B or UV-A radiation primarily reflects changes in the functional activity of the melanocytes, whereas the hyperpigmentation induced by multiple exposures

to either UV-B or UV-A reflects changes not only in the functional activity of the melanocytes but also the numerical changes in the epidermal melanin units (i.e., in the melanocytes and the associated pool of keratinocytes). A single exposure to either UV-B or UV-A causes none or minimal change in the number of functional melanocytes, but reveals an increment in the synthesis, melanization, and a transfer of melanosomes (Pathak *et al.*, 1965; Jimbow *et al.*, 1974*a,b*, 1975*b*). Multiple exposures to either UV-A or UV-B cause a marked increase in (a) the number of melanocytes; (b) the number of melanosomes synthesized; (c) the degree of melanization of the melanosomes as a result of an increase in tyrosinase activity; (d) the number of melanosomes transferred to the keratinocytes; (e) a distinct alteration in the size of some of the melanosomes (some of the newly synthesized melanosomes are larger in size than those in the unexposed skin); and (f) alteration in the distribution pattern of melanosomes in keratinocytes as a result of a change in the size of some of the melanosomes.

4. PHOTOPROTECTIVE ROLE OF MELANIN

In pigmented peoples, there exists a unique light absorbing and filtering system that minimizes the impact of photons on the vulnerable viable cells of the epidermis. The "white" population possesses a much less protective neutral density filter known as melanin, while the albino skin has virtually no neutral density filter, and more UV light penetrates such skin. In people with absence of melanin or in people who sunburn easily and tan poorly, skin will develop, early in life, abnormal changes caused by sunlight: wrinkling, keratoses, telangiectasia, and skin cancer (Fitzpatrick *et al.*, 1974). The onset of the cancerous or other abnormal changes is directly related to the degree of sun exposure and the latitude at which the person resides (i.e., sun intensity times the duration of exposure), and is inversely related to the amount of melanin in the skin. Among races with dark skin, in which melanin pigment effectively filters UV radiation, there is very little skin cancer. This photoprotective role of melanin is attributable to its presence in the particulate and nonparticulate form in the epidermis. In the stratum corneum most of the melanin is usually in the nonparticulate, amorphous form, although in certain individuals who are heavily pigmented, one does find a few melanosomes scattered randomly in the nonviable, horny cells. Most of the particulate forms of melanosomes are believed to be degraded due to the presence of hydrolytic activity associated with the melanosome complexes or in the outer membrane of the discrete organelles. Melanin present in the melanosomes is nondegradable (Hori *et al.*, 1968). The rest of the viable cells of the epidermis contain melanin-laden melanosomes in the particulate form.

The photoprotective role of melanin is accomplished by the following physical and chemical properties of the biochrome (Pathak and Fitzpatrick, 1974):

- a. Melanin absorbs UV and visible radiation and can act as a neutral density filter. Melanins isolated from human hair, melanoma, and other biologic materials show high absorption without any characteristic peaks or absorption bands in the UV, visible, and near-infrared region (200–2400 nm). This absorption increases in the shorter wavelengths in the UV spectrum and appears to be due to highly conjugated system in the polymer.
- b. Melanin-laden melanosomes attenuate the impinging radiation by scattering; this scattering involves any process that deflects electromagnetic radiation from a straight-line path and results in the attenuation of radiation. This increases the total absorbing path through which the UV radiation must pass. For particles with the dimensions of the order of wavelength of light in the UV spectrum ($0.3\text{ }\mu\text{m}$), the impinging light may be scattered according to the Rayleigh relation (scattering is inversely proportional to the fourth power of the incident light). Maximum scattering occurs when the wavelength of light approaches the diameter of the particle. For particles larger in size than the wavelength of the incident light (e.g., melanosomes which are $0.3\text{--}1.0\text{ }\mu\text{m}$ in size), the scattering relationship is quite complex, and more incident light will be scattered in the forward direction than in the backward direction.
- c. Melanin absorbs the radiant energy in the UV and visible spectra and dissipates the absorbed energy as heat. In this regard it is of interest to point out the hypothesis of McGinnes and Proctor (1973) that melanin in the cell may serve as a device by which it may convert the energy of the excited states into heat by a phenomenon known as photon-photon conversion. This hypothesis implies that melanin polymer can act as an amorphous semiconductor in which coupling of phonons (i.e., vibrational modes of the melanin polymer) to its excited electronic states plays a role in the dissipation of energy absorbed from the impinging radiation.
- d. Melanin can also utilize the absorbed energy and undergo immediate oxidation through the generation of semiquinonoid free radicals (Pathak and Fitzpatrick, 1974).
- e. Melanin, as a stable free radical, with its ability for oxidation and reduction, can act as a biologic electron exchange polymer and minimize the impact of the impinging photons on the other vulnerable cell constituents (e.g., DNA) (Pathak and Fitzpatrick, 1974). The free radicals in melanin are quite stable and the unpaired electrons seem to be limited to localized regions of the polymer or are stabilized by a large number of resonance structures. Because of the unpaired electrons in melanin it may in effect serve as a one-dimensional semiconductor, where any bound protons

serve as electron traps. A free flow of charge in the form of electrons is then possible through the melanin (Longuet-Higgins, 1960; Pathak and Stratton, 1968; McGinnes *et al.*, 1974). It is known that UV irradiation increases the spin concentration in biological tissues such as human skin. Trapping of free radicals which could disrupt the metabolism of living cells is thus feasible in presence of stable free radicals in melanin polymer.

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Visible Light Therapy of Neonatal Hyperbilirubinemia

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1. INTRODUCTION

In 1877 General Augustus J. Pleasonton proposed, in a remarkable address to the Philadelphia Society for Promoting Agriculture, that blue light would cure certain diseases and increase the yield of crops and the fecundity of domestic beasts. His proposals were buttressed with the results of his own research—an endeavor permitted the educated amateur in those days. He published his work in a book, tastefully bound and printed in blue (Fig. 1). Within a year a preposterous craze for blue light spread across the country which lasted for over a decade. Interest in blue light (sunlight filtered through blue glass) gradually waned, and for the next 70 or more years the medicinal virtues of light (except heliotherapy for the treatment of tuberculosis) were accepted only by cultists and quacks. Perhaps because of this unfortunate, almost ludicrous history, American physicians rejected any

THE
INFLUENCE
OF THE
BLUE RAY OF THE SUNLIGHT
AND OF THE
BLUE COLOUR OF THE SKY,
IN DEVELOPING ANIMAL AND VEGETABLE LIFE;
IN ARRESTING DISEASE, AND IN RESTORING HEALTH IN ACUTE AND
CHRONIC DISORDERS TO HUMAN AND DOMESTIC ANIMALS,
AS ILLUSTRATED BY THE EXPERIMENTS OF
GEN. A. J. PLEASONTON, AND OTHERS,
Between the years 1861 and 1876.

Addressed to the Philadelphia Society for Promoting Agriculture.

"Error may be tolerated, when reason is left free to combat it."—*Thomas Jefferson.*
"If this theory be true, it upsets all other theories."—*Richmond Whig.*

PHILADELPHIA:
CLAXTON, REMSEN & HAFFELINGER, PUBLISHERS.
1877.

Fig. 1. "Phototherapy" 100 years ago. The first American treatise on the uses of blue light.

thought of light as a mode of medical treatment and ignored its use in the management of jaundice in the newborn infant, a condition which, if unchecked, can be crippling to a severe degree.

2. THE NATURE AND FREQUENCY OF NEONATAL HYPERBILIRUBINEMIA

Hyperbilirubinemia in neonates, also known as jaundice of the newborn, is a common condition usually uncomplicated and self-limited. Some estimate that over 50% of all infants develop at least mild jaundice during the first week of life. In the majority of newborns the condition is "physiologic," or "developmental," and caused, it is thought, by an inability of the infant to conjugate and excrete bilirubin as rapidly as it is produced. This leads to the accumulation of the yellow pigment, bilirubin, in the skin, fat, and organs of the body, and to elevated concentrations in the blood plasma. A more significant degree of hyperbilirubinemia is caused by hemolytic disease of the newborn due to maternal-fetal blood group incompatibility, and by sepsis, or blood-borne infection. Less common causes of hyperbilirubinemia are also known, such as congenital deficiency of the red cell enzyme glucose-6-phosphate dehydrogenase (G-6-PD), congenital deficiency of the conjugating liver enzyme uridine diphospho (UDP) glucuronyl transferase (Crigler-Najjar), congenital spherocytic anemia, and even an association with breast feeding.

In itself, jaundice is only a symptom and not a disease. Its treatment, often independently of its cause, is necessary because at plasma concentrations of 10 mg dl^{-1} or above in low birth weight infants (Gartner *et al.*, 1970; Harris *et al.*, 1958; Stern *et al.*, 1970) and of 15 mg dl^{-1} or more in full-term infants (Boggs *et al.*, 1967) there is danger of bilirubin encephalopathy and irreversible brain damage from toxic injury to brain cells by bilirubin molecules, resulting in brain cell death, noted especially in the basal ganglia. This damage may be of varying degrees of severity, from almost undetectable neurologic injury manifest by learning impairment (as from visual-motor perceptive dysfunction) to the condition known as "kernicterus," from the German description of yellow nuclei, the most marked staining of the brain by bilirubin. If not fatal in the newborn period, kernicterus leads to the development of cerebral palsy, deafness, and other serious neurologic deficits.

The toxic effects of unbound bilirubin in its lipid-soluble and unconjugated form upon the cells of the brain is permanent and may be so devastating to the central nervous system that efforts to reduce the levels of

circulating bilirubin and the amount in tissues must be vigorous, immediate, and effective. No reliable estimates of the number of infants who develop hyperbilirubinemia of a degree of severity to require treatment is available, but it is thought that between 7 and 10% of all newborns exhibiting jaundice are in this category. If this conservative appraisal is even nearly correct, then at least 150,000 neonates a year receive or should receive treatment for hyperbilirubinemia.

3. ORIGIN, TRANSPORT, AND FATE OF BILIRUBIN

Bilirubin is a tetrapyrrole, derived principally from the degradation of the heme moiety of hemoglobin. It appears first as a lipid-soluble nonpolar substance, whose immediate precursor is the greenish pigment biliverdin. The yellow pigment bilirubin is transported in the blood by binding to albumin on two specific sites, a primary locus and a secondary site of much weaker binding affinity. Upon reaching the liver, bilirubin is taken up by a ligand (protein X, Y) in the liver cells and there conjugated to a glucuronide by the enzymatic action of UDP-glucuronyl transferase, produced in the smooth endoplasmic reticulum of the cells (Arias, 1972). Once conjugated, the pigment is excreted into the bile canaliculi within the liver, thence in the bile to the gall bladder and then to the intestine. From there it is excreted in the feces after further breakdown. Some portion, however, is deconjugated by β -glucuronidase, which is produced in the intestinal mucosa, and in the unconjugated form reabsorbed. This, in brief, is the entero-hepatic circulation of bilirubin. A small amount of bilirubin is excreted in the urine, and other conjugates of much less importance exist. An excellent review of the subject of hemoglobin degradation has been published (Miescher and Jaffe, 1972).

Bilirubin is also bound to some degree to red blood cells and to β -lipoprotein in plasma. It will circulate in a free, unbound state in plasma if it is present in such concentration as to exceed available binding sites or if it meets competition for these sites by other substances with a greater affinity for albumin, such as salicylate, sulfisoxazole, benzoate, novobiocin, some vitamin K analogues, and stabilizers found in certain parenteral preparations of antibiotics. The ability of these substances to displace bilirubin from the weak secondary binding sites on albumin, and perhaps from erythrocytes, thus promoting the circulation of clinically dangerous high concentrations of unbound bilirubin, has led to the careful examination of most medicaments administered to neonates and to the interdiction of the use of such substances with this capacity in the newborn period.

When the rate of production of bilirubin exceeds the rate of uptake by

the liver, the rate of conjugation of the pigment, the rate of excretion, or all of these, bilirubin accumulates in plasma, and, because it is circulated throughout the body, reaches almost all tissues. Normal plasma concentrations, even in the newborn, rarely exceed 1.5 or 2.0 mg dl⁻¹, and above that it is released to fatty tissues and skin, especially collagen. When plasma concentrations rise above 5.0–6.0 mg dl⁻¹ the quantity of pigment then transferred to skin gives it a yellowish cast we know as jaundice. The higher the concentration in plasma the more bilirubin is diverted to these tissue sites and the deeper the jaundice appears. In newborns the yellow skin hue appears first on the face, and spreads to trunk and extremities as plasma levels increase. The visible expression of jaundice is usually the first indication that hyperbilirubinemia ensues. Once detected, the only proper way of monitoring and managing the condition is by appropriate measurement of plasma levels, since tissue concentrations in skin are only very roughly correlative with actual circulating levels at any one time. Bleaching of the pigment by phototherapy complicates the picture.

4. HYPERBILIRUBINEMIA IN THE NEWBORN

The premature infant is characteristically hypoalbuminemic, and therefore has fewer binding sites than are found in the full-term infant. Moreover, the small preterm infant frequently develops some type of respiratory distress syndrome, the most severe form being hyaline membrane disease. In this condition an impermeable fibrinous membrane forms which lines the alveoli of the lungs, and this membrane severely limits the absorption of oxygen by the lung and the release of carbon dioxide upon exhalation. At birth the prematurely born infant is acidotic, the pH of his blood frequently being below the normal hydrogen ion concentration of 7.35, and this state of acidosis is aggravated as body temperature falls after delivery. It is even further accentuated if ventilatory perfusion is compromised by hyaline membrane disease, pneumothorax, aspiration pneumonia, or any other condition of the neonate in which pulmonary function is diminished. Acidosis in turn weakens the binding of bilirubin to albumin, consequently increasing the possibility that the pigment, in a free and unconjugated state, will circulate at high concentration and thus reach the brain.

Hyperbilirubinemia is a frequent symptom appearing in the infants of diabetic mothers and in small-for-dates (i.e., small for gestational age) infants. In some newborns with a congenital deficiency of G-6-PD, an enzyme necessary for erythrocyte respiration, hemolysis of the red cells may be prompt and severe soon after birth, which results in the rapid elevation of

plasma bilirubin levels (Milbauer *et al.*, 1973). It will be found in infants with moderately large cephalhematomas (the commonly encountered accumulations of blood under the scalp from minor vessel rupture during delivery, or from other aggregations of blood leaked from vessels either spontaneously or from birth trauma). In many infants an unusually high volume of red blood cells in the vascular system (plethora) will result from an excessive transfusion of placental blood into the infant before the umbilical cord is clamped upon delivery. This red cell hypervolemia accelerates the normal rate of destruction of erythrocytes and will usually lead to hyperbilirubinemia.

5. METHODS OF MANAGEMENT OF HYPERBILIRUBINEMIA— ALTERNATIVES TO PHOTOTHERAPY

The simple correction of jaundice in the newborn is not sufficient therapy in itself. Although most instances of hyperbilirubinemia are presumed due to inadequate capacity of the maturing enzyme systems to conjugate bilirubin taken up in the liver and mere symptomatic treatment is deemed enough, a serious underlying disease must be ruled out lest other necessary therapies be omitted.

Prevention of bilirubin encephalopathy can be accomplished only by a reduction of the circulating free pigment. Traditionally, that is to say, since the late 1940s, this has been effected by exchange transfusion, which is the replacement of the infant's blood with that of an adult donor drawn within at most 72 h of the exchange. As a rule of thumb, the transfusion requires the exchange of twice the total circulating blood volume of the infant recipient. The infant with severe hemolytic disease may need three, five, or even more exchange transfusions to ensure a safely maintained reduction of plasma bilirubin concentration.

Various methods have been tried to avoid multiple exchange transfusions, which do incur some degree of risk even in practiced hands.

Infusion of human serum albumin (Odell *et al.*, 1962) has been employed with temporary success. The albumin provides fresh binding sites for free bilirubin in the plasma and also tends to alter the blood-tissue gradient by pulling bilirubin back into the circulation. If followed by exchange transfusion within 45–60 min, there may result an increased efficiency of the withdrawal and discard of the offending bilirubin. The efficacy of this measure is, however, transient.

An effort to interrupt the entero-hepatic circulation of bilirubin by its adsorption on agar was successful in the hands of Poland and Odell (1971). By tightly binding bilirubin excreted in the bile while still in the gut, and

thus preventing its deconjugation and reabsorption, a gradual reduction of plasma bilirubin concentration with increased excretion was achieved. The technique has not been useful in other hands, however (Maurer *et al.*, 1973; Arrowsmith *et al.*, 1975; Romagnoli *et al.*, 1975).

The use of orotic acid and aspartic acid, precursors of UDP-glucuronic acid, itself a substrate in bilirubin conjugation, were initially thought to be effective in reducing neonatal jaundice (Kintzel *et al.*, 1971; Matsuda and Shirahata, 1966), but subsequently failed to maintain their promising results (Schwarze *et al.*, 1971, Arrowsmith *et al.*, 1975).

The oral administration of a classic adsorbant, activated charcoal (Ulstrom and Eisenklam, 1964) to reduce neonatal bilirubin levels was not conspicuously successful.

Uridine diphosphoglucose (UDPG) was found useful in the treatment of neonatal jaundice (Caredu and Marini, 1968; Marini, 1969) but is costly and must be administered intramuscularly. This mode of treatment never found favor.

Phenobarbital is known to induce microsomal enzymes such as UDP-glucuronyl transferase, among others, to increase the conjugation of bilirubin and augment its excretion in the newborn (Catz and Yaffe, 1962). When given to the mother for two or more weeks before delivery of her infant and to the infant itself for several days after birth, the incidence of neonatal jaundice is significantly reduced (Trolle, 1968). When phenobarbital is given to infants alone without pretreatment of the pregnant mother it has a less marked influence upon neonatal jaundice (Stern *et al.*, 1970; Yeung and Field, 1969). Its effect in reducing serum bilirubin concentration is slow, taking several days, if, indeed, any effect is noted at all (Cunningham *et al.*, 1969). It seems to have less efficacy in low-birthweight infants (Vest *et al.*, 1970) and when administered after bilirubin levels are already elevated (Trolle, 1968). It is not widely used today, in part because phototherapy is effective and does not require the administration of a sedative drug whose dosage must be carefully monitored due to the inefficient metabolism of barbiturates by the neonate.

6. PHOTOTHERAPY OF NEONATAL JAUNDICE

The only method of treating neonatal hyperbilirubinemia other than exchange transfusion, that not only has had success but notable utility is phototherapy. In 1958, Cremer and his colleagues published the first scientific study of the use of visible light for the reduction of hyperbilirubinemia in the newborn. This new modality was seized upon by many physicians in Europe and South America as an alternative to exchange transfusion, which

at that time had a mortality of about 5%. Perhaps because of the disrepute of light therapy from its association with cultists and those of tenuous scientific pretensions, Cremer's phototherapy technique was ignored in the United States, despite a steady accumulation of data on the effect of light on hyperbilirubinemia and its clinical efficacy (Berezin, 1960; Broughton *et al.*, 1965; Corso *et al.*, 1964; Ferriera *et al.*, 1960; Caredu and Marini, 1968; Mellone, 1960; Obes-Poleri and Hill, 1964; Peluffo *et al.* 1962).

A paper by Lucey *et al.* (1967) describing the effectiveness of phototherapy in the prevention of hyperbilirubinemia in prematurely born infants (who are singularly vulnerable to this condition) was almost solely responsible for the rapid acceptance of the method on the North American continent following its presentation and eventual publication (Lucey *et al.*, 1968).

It is difficult to realize the sudden impact that the report of Lucey and his colleagues had on the pediatric community and in short order upon medical practitioners at large. Phototherapy units were quickly manufactured by a number of entrepreneurs (some quite literally in basement workshops), by individual hospitals, and then by medical supply companies to meet the demand that arose.

Unfortunately, many infants were irradiated by phototherapy units of haphazard construction and variety. Some physicians were under the misconception at first that phototherapy was accomplished by UV not visible light. Most had little idea of the type or amount of light to be used, or for how long. A phototherapy unit of hemicylindrical shape, containing 10 lamps is shown in Fig. 2.

To this day the optimum irradiation of infants for the correction of hyperbilirubinemia has not been established. As is the case with so many aspects of therapy, this is still largely empirical. Nonetheless, phototherapy has come into nationwide use while academic centers, aware of the potential importance of this new-old treatment, have been examining its rationale, studying its effects on bilirubin itself, and at last exploring the fundamental influences of visible light on the human organism. Pediatricians, in short, have discovered that the science of photobiology, previously the concern of botanists, chemists, agronomists and others, is now within the compass of medical scrutiny.

Bilirubin is photochemically altered if exposed *in vitro* or *in vivo* to light at wavelengths which include its absorption peaks (Blondheim *et al.*, 1962), notably about 445–450 nm in serum. Bilirubin may be found in almost all tissues of the body, especially those of lipid constitution, if hyperbilirubinemia is severe and of more than transient duration.

In practice, phototherapy is the whole-body irradiation of an infant by banks of 8–10 fluorescent lamps, some portion of whose emission is in the

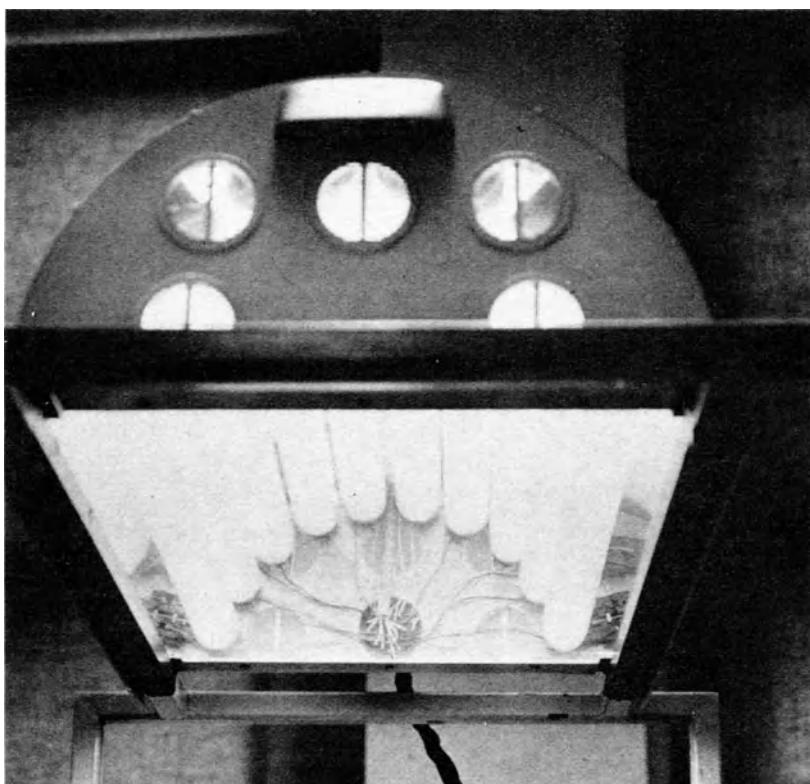


Fig. 2. One type of phototherapy unit, of hemicylindrical design, with venting for the dissipation of heat from the ten fluorescent lamps it contains: a Plexiglas shield 0.5-in. thick covers the open side to block the small but significant amount of near-UV emission of the lamps.

blue range of the visible spectrum i.e., between 400 and 490 nm. It is important to point out that some UV light is emitted by fluorescent lamps and must be blocked, for the prolonged exposure of the infant's skin to UV radiation may be injurious (Pathak and Stratton, 1969). Elimination of UV irradiation of the skin by phototherapy units in common use is easily accomplished by placing a Plexiglas shield between the light source and the infant. The shield also serves to protect the infant should a lamp shatter. Such shielding has operational disadvantages, however. The internal heat generated by closely apposed lamps in phototherapy units is trapped and excessive heat is then radiated to the infant. Proper venting of the phototherapy unit or incorporation of a fan in the unit to force dissipation of the heat is of great value in overcoming thermal buildup. The principal

disadvantages of heat are rapid degradation of the phosphor coating in some lamps requiring their replacement after only 200–300 h of use (Sisson *et al.*, 1970b), particularly noted in the type designated "blue" (F20T12/B), and a marked increase in body temperature and consequent increase of insensible water loss through the skin in perspiration and lungs in respiration by the radiated infant (Wu and Hodgman, 1974).

The increase of insensible water loss, amounting to as much as 2.0 ml kg⁻¹ h⁻¹, imposes an extraordinary burden on the infant's homeostatic mechanisms. The ability of the newborn immature infant to compensate for increased water loss is greatly limited, and measures to correct this loss challenge the physician in attendance, but the infant most of all.

The jaundiced newborn is usually placed beneath a phototherapy unit at a distance of 45–50 cm and is kept unclothed, undiapered, with only the eyes shielded with an appropriate opaque eyemask. Masking of the eyes is necessary for it has been shown in animal studies (Dantzker and Gerstein, 1969; Noell *et al.*, 1966; Sisson *et al.*, 1970a) that the exposure of the retina to fluorescent light, whether "daylight" (F20T12/D), "blue" (F20T12/B), or special monochromatic blue (F20T12/BB) will cause destruction of the photoreceptors of the retina after as little as 12 h. In more recent studies in newborn piglets exposed to "daylight" and "special blue" lamps in an oxygen-rich atmosphere (40% and 100%) for 24 h, severe retinal vascular and receptor changes were observed (Sisson, 1973b). The presumption is that the eyes of infants under phototherapy should be protected from the damaging effect of the light lest they be blinded. The possibility exists, moreover, that phototherapy will exert an additive injury to the unshielded eyes if given at the time an infant is receiving supplemental oxygen for respiratory distress. The use of an opaque eye mask is suggested since up to 20% of phototherapy light will penetrate the ordinary soft white cotton eye pad and about 3–5% of the incident light will penetrate an infant's eyelid (S. C. Glauser and T. R. C. Sisson, unpublished data).

Most phototherapy regimens are conducted by constant irradiation, except perhaps for interruption during feedings. Some investigators advocate alternating periods of phototherapeutic irradiation with periods of exposure only to normal nursery lighting. One study of intermittent therapy (Maurer *et al.*, 1973) used 12-h periods of phototherapy and 12 h off the treatment, and demonstrated these intervals to be less clinically effective than constant phototherapeutic exposure. The provocative suggestion has been made by Indyk (1975) that pulsed light irradiation may be entirely effective in bilirubin destruction and prevent undesirable photochemical side reactions that might occur under constant irradiation.

Exposure of jaundiced infants to sufficiently energetic light emissions in the 400–490-nm range, of at least 0.9 μW cm⁻², will cause a visually de-

tectable fading of the yellowish skin color due to jaundice within 2–3 h. A decline of plasma bilirubin concentration will be encountered within 1–3 h. However, in those instances of excessively rapid bilirubin production, as in moderately severe hemolytic disease, reduction of plasma concentrations may not be noted for 4–6 or more hours. The effect of phototherapy using a broad-spectrum blue light source (F20T12/B) in a controlled study of 67 infants is illustrated in Fig. 3.

When phototherapy has failed to result in a plasma bilirubin decrement

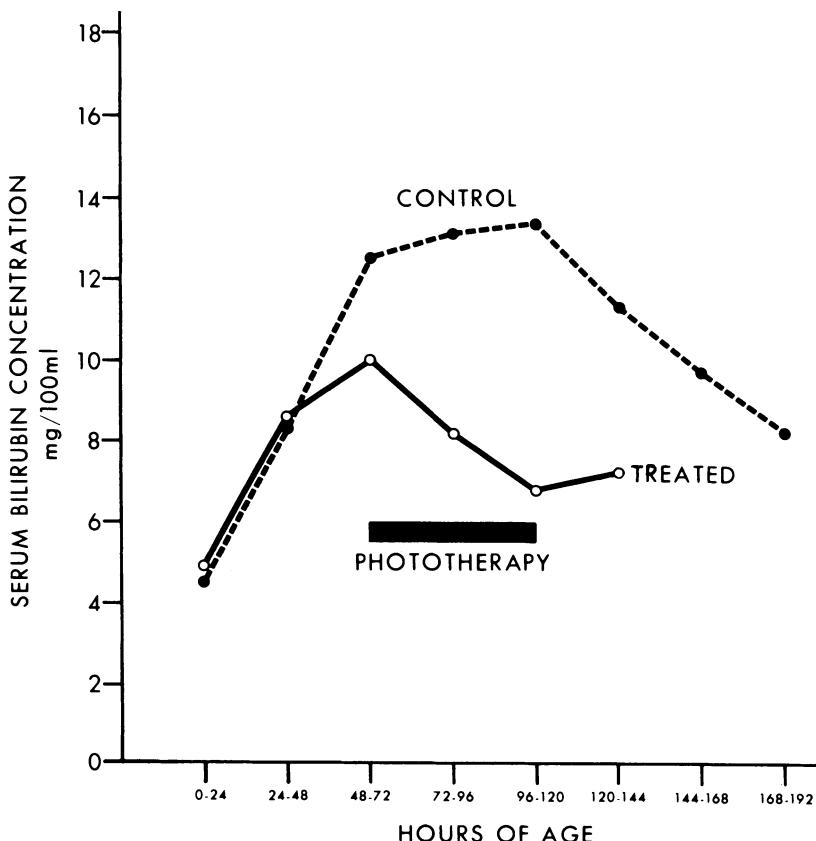


Fig. 3. The effect of phototherapy of neonatal hyperbilirubinemia upon the mean serum bilirubin concentrations of 32 infants compared with that of 33 hyperbilirubinemic infants who received no treatment. Phototherapy was initiated between 48–72 h of age when serum concentrations reached 10 mg/dl and was followed by a prompt and dramatic reduction of serum bilirubin. In contrast, untreated control infants had a mean concentration that continued to rise until 96–120 h before a decline was observed, and the reduced levels of the treated group were not reached in controls until 168–192 h. (From Sisson, 1973a.)

(Patel *et al.*, 1970), the failure may be due to a rate of bilirubin production which exceeds both the infant's capacity to conjugate and excrete it and the ability of the light to degrade it. Therefore, light sources with only a small energy output in the range which includes the absorption maximum of bilirubin have appreciably less efficiency in producing a rapid breakdown of bilirubin *in vivo* than light sources of greater photic energies in the critical spectral range. This has been observed clinically by many practitioners when phototherapy lamps have become overaged with consequent loss of output.

A relationship has been shown to exist between the radiant intensity used for phototherapy and the rate of decline of plasma bilirubin concentration (Mims *et al.*, 1973; Sisson *et al.*, 1972) (Table 1). It is obvious from the rapid bleaching of skin jaundice under light that the photobreakdown of bilirubin does occur in the skin itself. However, plasma bilirubin must also be degraded since the reduction of circulating plasma bilirubin exceeds the calculable amount of pigment in skin and continues to decline after visible jaundice has faded. It is an acknowledged fact that light penetrates tissues quite deeply, although scatter is pronounced. The transmission of both white and blue light through the abdominal wall of the living rat and pig has been shown to be about 20% (range from 16–23%) of incident light (Sisson and Wickler, 1973). Viggiani *et al.*, (1970) have reported a significant transmission of light through the skin, skull and brain tissue of the rat. One may conclude that the loci of action of light upon bilirubin are not confined to skin alone, but probably include at least the peripheral vascular bed and possibly more deeply placed tissues.

The use of phototherapy to prevent rather than to treat hyperbilirubi-

TABLE 1. Decrement of Mean Serum Bilirubin Concentration under Three Fluorescent Phototherapy Light Sources, in 72 Newborn Infants^a

Lamp type	Flux $\mu\text{W cm}^{-2}$ (420–490 nm)	No. infants	Mean bilirubin decrement mg/dl per 24 h				Total decrement
			1st day	2nd day	3rd day		
Daylight F20T12/D	0.32	24	0.86	0.23	1.50	2.59	
Blue F20T12/B	0.91	24	1.50	1.20	1.41	4.11	
Special blue F20T12/BB	2.90	24	2.56	2.32	1.80	6.68	

^a Sisson *et al.* (1972).

nemia has had some adherents, but since it is now generally agreed that the consequences of indiscriminate exposure of newborns to light of high intensity are largely unknown, prophylactic phototherapy for the present, at least, should be discouraged.

7. LIGHT SOURCES FOR PHOTOTHERAPY

The light sources in use in this country for phototherapy are fluorescent lamps, usually "daylight," "blue," and Vitalite, all broad-spectrum lamps with a significant but varying energy output in the blue range. The last named lamps also emit substantially in the near-UV region. A narrow-spectrum lamp, emitting only between 420 and 470 nm, is also in use. Their relative spectral distributions are shown in Fig. 4.

It has been demonstrated that light in the blue portion of the visible spectrum is the most efficient for the photodestruction of bilirubin in the rat (Sisson *et al.*, 1970b) and that there is a dose-response relationship between the light energy of irradiation and bilirubin catabolism (Mims *et al.*, 1973; Sisson *et al.*, 1972). Since there are actual and potential side effects of the intense light exposure in phototherapy that may be undesirable, it is reasonable to seriously consider the suggestion (Behrman, 1974) that a light source be employed which will offer the most efficient photodegradation of bilirubin in the shortest period of time, yet not include extraneous wavelengths which might fall within the action spectra of other substances in the body.

Whether or not intermittent light treatment should replace constantly administered phototherapy has not been decided. It depends, in part, with what seriousness the potential hazards of photosensitization, mutagenesis, and carcinogenesis are taken, how severe the individual case of hyperbilirubinemia may be, or how one may philosophically view phototherapy.

8. PHOTOCHEMISTRY OF BILIRUBIN

The mechanisms of the photodegradation of bilirubin *in vivo* are not yet completely understood, although there is good evidence that it is photooxidative in nature (Ostrow, 1971; Ostrow *et al.*, 1974). An illustration of its photodecomposition by spectrophotometric analysis, demonstrating loss of optical density with increasing length of exposure to light, is shown in Fig. 5.

In vitro studies of the photodegradation of this pigment indicate that a series of photoproducts are produced as a result of the photooxidative

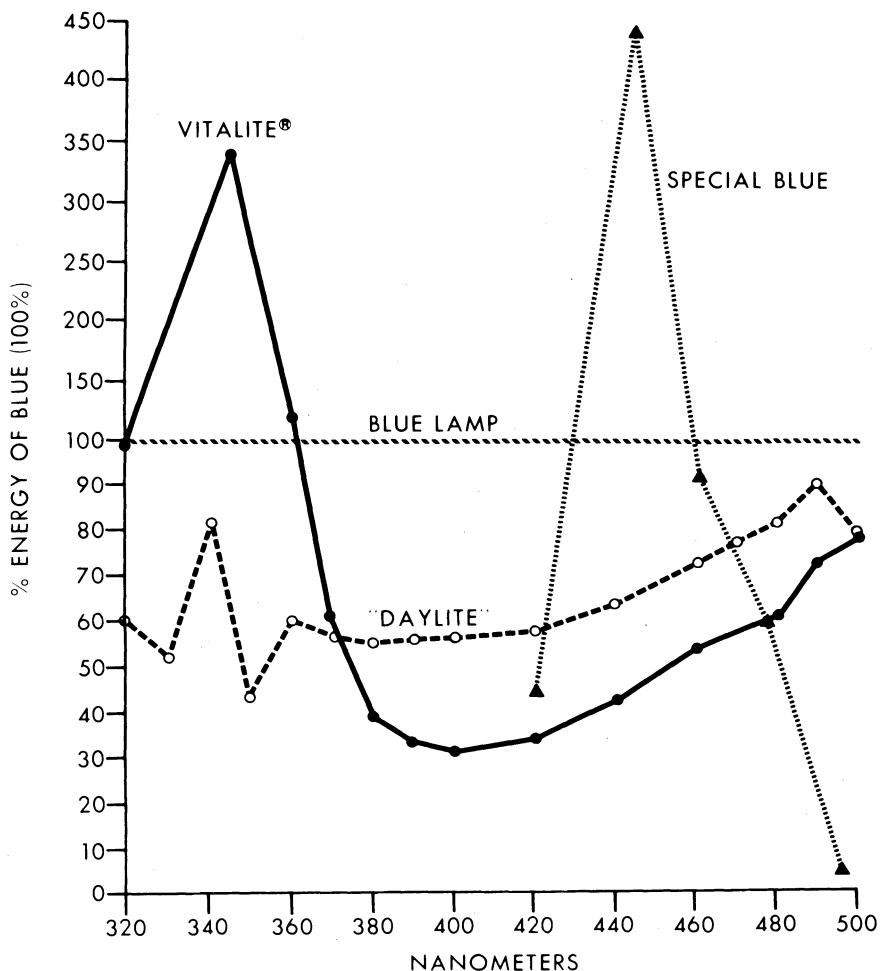


Fig. 4. A comparison of the emissions of three fluorescent lamps, "daylite" F20T12/D, "Vitalite," and a special "blue" F20T12/BB whose emission is 420-470 nm, with a standard "blue" F20T12/B whose energy output at each nanometer is taken as 100%. There is a significant difference in the emission of these lamps in the blue range of the spectrum. (Modified from Sisson *et al.*, 1970b.)

processes (Ostrow and Branham, 1970). It has also been demonstrated (McDonagh, 1974) that bilirubin is a weak photosensitizer when excited by light. The photooxidation of bilirubin is accelerated by the presence of photosensitizers such as riboflavin, (Kostenbauder and Sanvordeker, 1972), methylene blue, rose bengal, or hematoporphyrin (McDonagh, 1974). Bilirubin, then, may sensitize its own companion molecules by photodegradation

in vitro, and may do so *in vivo*, although this has not been demonstrated directly.

Biliverdin is produced by photodestruction of bilirubin (Lightner, 1974), but it is not believed that this reaction is greatly significant (Cowger, 1974), for other products are formed at a faster rate and in larger amounts, and, in any event, biliverdin is not an intermediate in the process.

Davies and Keohane (1970) demonstrated that the first photoproduct of bilirubin photobreakdown *in vitro* (in human serum bilirubin) is a com-

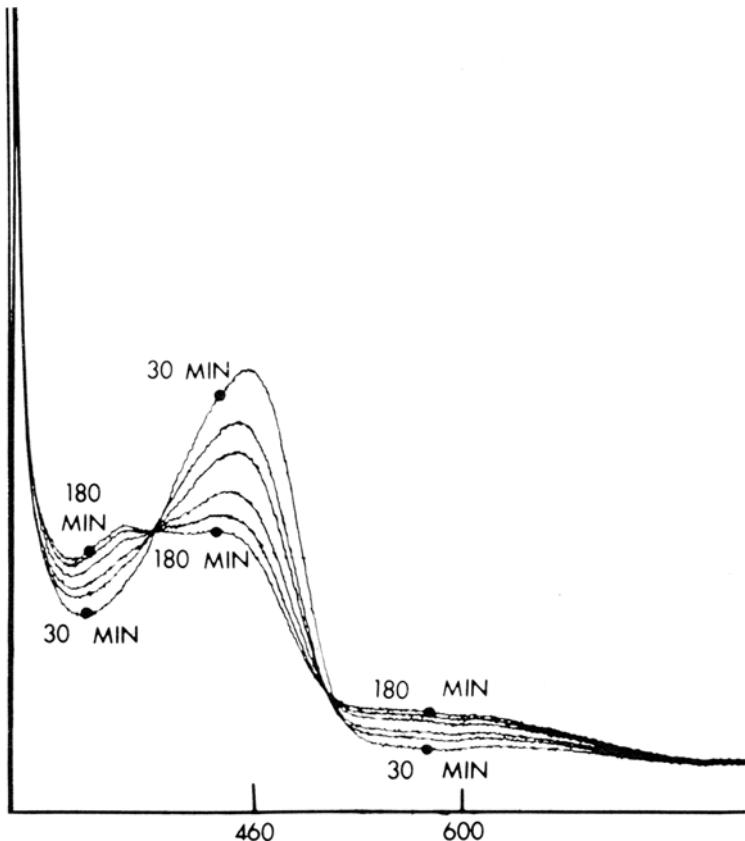


Fig. 5. The principal absorption peak of bilirubin (15 mg/dl) in a buffered solution (Sorenson's buffer, pH 7.4) is at 460 nm. Reduction in optical density (ordinate) at increasing lengths of exposure to light from broad-spectrum blue fluorescent lamps (F20T12/B) from 30 to 180 min, demonstrates the photodecomposition of bilirubin to its largely colorless photoproducts. Exposure time of bilirubin samples is noted on the differential spectrophotometric tracing illustrated. (T. R. C. Sisson, unpublished results.)

pound absorbing at 490 nm. It has but a fleeting existence since it is, itself, highly photoreactive. Ostrow and Branham (1970) have shown that when bilirubin is irradiated in an alkaline medium a variety of products of differing chromatographic properties appear. They quite properly raised the sound proposition that the photoproducts found *in vitro* may not be the same as those found after the photoreaction of bilirubin *in vivo*.

It is now clear that phototherapy of the jaundiced rat (Ostrow *et al.*, 1974) and jaundiced infant (Lund and Jacobsen, 1974) causes an increased excretion of unconjugated bilirubin in the bile of both species.

Besides the significantly augmented excretion of unconjugated bilirubin, several other pigments are excreted (Ostrow *et al.*, 1974): dipyrroles; photorubin, photopurpurin, and other as yet incompletely characterized products. These photocompounds are not themselves conjugated, but are rapidly excreted.

It is not known, moreover, whether the same products of phototherapy develop in the jaundiced newborn human as in the jaundiced Gunn rat. Although this congenitally jaundiced animal is the best model available for study, it does after all differ from the human. Unlike the newborn infant, it has no ability to conjugate bilirubin, but does have a normal hepatic uptake and biliary excretion of the pigment, which is to some degree defective in the neonate.

9. TOXICITY OF BILIRUBIN PHOTOPRODUCTS

Toxicity of the photoproducts of bilirubin has not been demonstrated. Johnson and Schutta (1970) demonstrated the cellular toxicity of bilirubin in neurone tissue culture, but not when such cultures, in a bilirubin-laden milieu, were irradiated, which indicated that the photodestruction of bilirubin spared the cells. Had the tissue shown injury, then one could presume photoproduct toxicity and/or a direct reaction of the cells to light, but this did not occur.

Since other products of the photodegradation of bilirubin are rapidly excreted and are not in high concentration in plasma or tissues, their toxicity is doubtful. Further studies of these photobreakdown products may prove them to have more subtle toxic effects on cells than these investigations would reveal, though it will be technically difficult to determine. Until evidence appears by either clinical or laboratory investigation only speculative concern can be expressed.

Whole-body irradiation of infants by visible light results not only in the gradual disappearance of jaundice in the skin, but also in the reduction of plasma bilirubin concentration to the end that levels toxic to the brain are prevented. It has not been conclusively proven that neurological damage is

absolutely forestalled by phototherapy, but a long-term follow-up study of irradiated infants reported by Lucey *et al.* (1973) indicated that there was no demonstrable neurologic deficit at age four. A study of the effect of light irradiation on congenitally icteric Gunn rats from birth (Sisson *et al.*, 1974) showed normal convulsive threshold, absence of signs of kernicterus, and normal brain size and color compared with unirradiated icteric littermates. One must conclude, therefore, that phototherapy can protect the infant from the encephalopathy of hyperbilirubinemia.

10. COMPLICATIONS OF PHOTOTHERAPY

Bilirubin is only one of many compounds in blood and body tissues that can be affected by visible light irradiation. Not only may individual compounds be vulnerable to photochemical activation, but metabolic processes themselves may be altered by the effects of light on essential enzymes, metabolites, and other constituents.

It has been shown, for instance, that the tryptophan-kynurenone pathway is disturbed in jaundiced infants treated with light (Rubaltelli *et al.*, 1974) because of the photodestruction of the metabolites of tryptophan. Levels of glutathione, essential for the respiration of red blood cells, measured in blood are reduced by phototherapy (Blackburn *et al.*, 1972).

The normal concentration of riboflavin, vitamin B₂, in whole blood is reduced *in vivo* by phototherapy of newborn infants, and in some neonates a deficiency of G-6-PD in red cells has been demonstrated (Sisson *et al.*, 1976). This has been observed both *in vitro* and *in vivo* under both broad-spectrum white and narrow-spectrum blue radiation. The light-induced enzyme deficiency shown in these studies has resulted in a clinical exacerbation of red cell hemolysis during phototherapy and a consequent failure of treatment.

It is entirely possible that other important enzymes are affected, particularly the iron-containing cytochrome oxidases, which are known to be photoactive and to absorb light near the absorption peak of bilirubin (Horie and Morrison, 1963).

Plasma amino acids themselves appear to be unaffected by phototherapy (Sisson *et al.*, 1976), but verification of this by other investigators would be most useful.

There are known clinical side effects of phototherapy, usually minor. Most of these have been reported following the use of broad-spectrum fluorescent light (Table 2). Disturbing effects have been noted in other, non-human, biological systems.

In vitro studies have demonstrated that albumin is partially denatured by light irradiation; thereby its capacity to bind bilirubin is decreased (Odell

TABLE 2. Side Effects of Phototherapy

Determined *in vitro*

1. albumin denaturation
2. diminished riboflavin levels
3. G-6-PD activity loss
4. glutathione reductase activity loss
5. mutagenesis in cell cultures

Determined *in vivo* (animals)

1. retinal damage
2. increased liver glycogen in rats
3. retarded gonadal growth (not function) in rats

Determined *in vivo* (human infants)

1. excess body heat from thermal output of lamps
2. temporary growth retardation
3. increased insensible water loss
4. transient hemolysis (uncommon)
5. loose, discolored stools
6. transient skin rash
7. reduction of whole blood riboflavin
8. alteration of tryptophan—kynurenine metabolism
9. alteration of biologic rhythms
10. physical hazards from inappropriate phototherapy—unit construction
11. increase in gut transit time
12. increase in respiration
13. increase in peripheral blood flow
14. decrease in circulating platelets

et al., 1970). However, recent studies (Cashore *et al.*, 1975) indicate that the bilirubin binding capacity of serum in newborns is unaffected by phototherapy. It has been shown (Peak *et al.*, 1973) that visible light has the ability to inactivate purified DNA and to kill bacteria in cultures (Webb and Lorenz, 1970). It is believed that when the bilirubin-DNA complex in mammalian cells is irradiated by broad-spectrum light containing near-UV radiation that the bilirubin is activated, thus causing a cleavage of bonds within the DNA (Speck, 1974).

Blue light, narrow-spectrum irradiation of *Escherichia coli* organisms results in the production of cell mutation (Webb and Malina, 1970). In a study of the effect of light of a spectral distribution between only 400–500 nm on cultures of *Streptococcus pyogenes* (Storer *et al.*, 1974) it was found that the light, comparable in intensity to that used in neonatal phototherapy, caused defects in bacterial cell walls and a decrease in the multiplication of the organism in culture.

Red blood cells maintained in a medium containing bilirubin and irradiated with broad-spectrum light lose potassium and undergo hemolysis (Odell *et al.*, 1972). This suggests that erythrocyte damage in an *in vitro* system is mediated by bilirubin photosensitized oxidation of some essential constituent of the cell membrane. Other *in vitro* studies of red cells from newborn infants have shown (Blackburn *et al.*, 1972) that visible light irradiation causes the cells to swell and to lose glutathione (essential for their respiratory metabolism). That these same dire effects are imposed on the cells of the infant under phototherapy is highly speculative since no direct evidence yet exists.

The platelet cells, or thrombocytes, in the blood have recently been shown to be affected by visible light irradiation. Maurer *et al.* (1975a) have demonstrated in rabbits that exposure to both daylight-type fluorescent light and to broad-spectrum blue light causes a shortened life span of these cells, so essential for blood clotting, and also increases the rate of platelet production in direct proportion to the irradiance at 460 nm. In jaundiced newborn infants treated with daylight fluorescent lamps, there was an absolute decrease in the number of circulating platelets which rebounded to normal levels after cessation of phototherapy (Maurer *et al.*, 1975b). This is a significant finding, for many preterm infants have a low platelet count to begin with, and if this is reduced further by phototherapy, the chance of a coagulation defect in such infants is enhanced.

As noted before, the heat generated by the lamps of many phototherapy units causes an increase of insensible water loss and an increase of body temperature. It is not difficult to correct the design of phototherapy units to dissipate the heat and avoid these complications.

It was believed at one time that phototherapy caused retardation of growth in both Gunn rats and infants. Studies have been conducted since that have disproven this idea (Hodgman, 1975; Kendall *et al.*, 1970; Sisson *et al.*, 1974).

Many newborns undergoing phototherapy develop a blotchy, red skin rash, transient and self-limited. The rash disappears with the fading of jaundice in the skin. The cause of the rash is not known, but it may be due to the photosensitizing action of the bilirubin attached to collagen and lipid in skin tissues. It has been established that cutaneous bilirubin is rapidly broken down to water-soluble products by light (Kapoor *et al.*, 1973; Rubaltelli and Carli, 1971) and it is generally agreed that, at least in the initial stages of phototherapy when skin is laden with the pigment, the major portion of the photodestruction of bilirubin occurs in those tissues (Kapoor *et al.*, 1973; Vogl, 1974). It is during this event that the rash most often appears.

It may be useful to comment again that, although the photochemical

effects of light exposure do take place in skin, nonetheless light penetrates even more deeply. It does not seem logical to consider that the reduction of plasma bilirubin concentrations can be the result only of the breakdown of tissue-bound bilirubin in the skin, for photocatabolism of other substances found in circulating red cells is simultaneously accomplished. This would indicate that the vascular bed of the skin is also reached by the light of phototherapy, and that circulating plasma and red cell constituents including bilirubin are acted upon.

The development of loose, green stools during phototherapy is commonly observed. The cause has not been definitely determined, but it is clear from the work of Rubaltelli and Largajolli (1973) that there is an increase in gut transit time during light exposure of jaundiced infants.

In an interesting study of newborns treated with light because of jaundice, Oh *et al.* (1975) discovered that peripheral blood flow increased, as did the infants' respiratory rates. These investigators concluded that the increased flow of blood was evidence of peripheral vasodilatation—a mechanism to increase body heat loss—and this, with elevated respiratory rates, would account for an increase of insensible water loss *not* related to overheating of the infants caused by poorly ventilated phototherapy units.

Observation of animals and humans over many years and in many centers has led to our present knowledge of the capability of changes in lighting regimens to shift or otherwise alter circadian and ultradian rhythms. It is reasonable to assume that phototherapy, especially when constantly administered, will do so. It has been shown, for instance, that plasma human growth hormone rhythm in newborns so treated differs from that of infants kept in a cycled light-dark environment (Sisson *et al.*, 1975). Whether this is due to vision blockage by eye masks or to the light exposure is not clear.

11. THE CLINICAL APPLICATION OF PHOTOTHERAPY

During the early days of phototherapy in this country the question was raised as to the possible interference with irradiation by skin pigment (melanin) in those infants of Negro ancestry. It has since been determined by several investigators (Ballowitz and Avery, 1970; Porto *et al.*, 1969; Valdes *et al.*, 1971) that the presence of melanin, even in marked degree, does not attenuate the effect of visible light on hyperbilirubinemia.

The clinical effectiveness of phototherapy differs in respect to the type of jaundice treated. The common variety, developmental (physiologic) jaundice, is particularly responsive to light. In this condition bilirubin accumulates in blood and tissue because the mechanisms for its conjugation and

excretion are inadequate, or thought to be so. The prematurely born infant, even more than the full-term, has not developed a full capacity to manage an adequate hepatic uptake of bilirubin, and/or to produce enough UDP-glucuronyl transferase for bilirubin conjugation in the liver. Thus, excretion is limited until uptake and conjugation reach sufficient competency, which does not occur for several days after birth. Although the mean plasma concentration of bilirubin, which peaks between the third and fifth days of life, does not exceed 12 mg dl^{-1} , many infants have maximum levels above 15 mg dl^{-1} . It has been reported that in full-term infants having concentrations of 15 mg dl^{-1} or more, nearly 50% exhibited at least minimal neurologic deficits by four years (Johnson and Boggs, 1974). It is well-known that premature infants may develop bilirubin encephalopathy at concentrations of about 10 mg dl^{-1} (Harris *et al.*, 1958; Stern and Denton, 1965; Gartner *et al.*, 1970). If instituted at levels below these, phototherapy does prevent further accumulation of bilirubin, reducing plasma concentrations within 2–5 days of treatment (Elliott *et al.*, 1974; Sisson, 1973a; Tabb *et al.*, 1972).

Many infants have maternal-fetal blood group incompatibility which results in the transplacental passage of maternal antibodies to the infant and in consequent hemolysis of its red cells; this leads to an extraordinarily rapid production of bilirubin. Most frequently it is the result of major blood group incompatibility, A-B-O, with the transfer of maternal antibodies to the infant's type A or B cells. The resulting hemolysis is usually mild or only moderate in degree. A significant number of these infants suffer severe hemolytic disease, however, and phototherapy is highly successful in management of the resultant jaundice (Kaplan *et al.*, 1971; Sisson *et al.*, 1971). Table 3 lists the effect of light on bilirubin concentrations of infants with this incompatibility.

TABLE 3. Mean Daily Decrement of Total Plasma Bilirubin Concentration in Full-Term and Premature Infants with A-B-O Incompatibility under Phototherapy
(10 Lamps F20T12/B = $1.1 \mu\text{W cm}^{-2}$)^a

Number of subjects	Mean birth weight, g	Mean conc. start of Rx, mg dl^{-1}	Mean plasma bilirubin decrement/24 h, mg dl^{-1}		
			0–24 h	24–48 h	48–72 h
32	1956	9.0	1.98	1.80	1.60
35	3112	9.8	1.90	1.31	1.76

^a Sisson *et al.* (1971).

Sensitization of an Rh-negative mother to the red blood cells of an Rh-positive fetus causes an even more severe hemolytic process in the fetus-infant. The hyperbilirubinemia in this type is partially dealt with by phototherapy and most often requires repeated exchange transfusions. These may be reduced in number if phototherapy is used as adjunctive treatment, not the sole mode of therapy (Moller and Ebbesen, 1975; Shennan, 1974). In hemolytic disease of the newborn, other factors such as anemia and the need for the removal of maternal antibodies dictate the use of exchange transfusion, and, except in the mild form, preclude phototherapy alone.

Sepsis in the newborn is a devastating condition, and, in its course, causes severe hemolytic episodes. Indeed, jaundice may be the first definite symptom. Phototherapy is used successfully, usually, to correct the accompanying hyperbilirubinemia and is of great value for those infants too small and too ill to tolerate the stress of exchange transfusion.

Plethora is frequently observed in the neonate, particularly following a placental transfusion during the birth process. A mild to moderate degree of hyperbilirubinemia results from the rapid destruction of excess erythrocytes in the infant's circulation, and phototherapy is singularly useful in correcting the elevated bilirubin levels.

Uncommon causes of jaundice in infants, such as congenital spherocytic and nonspherocytic anemia, characterized by fragile red cells spherical rather than normally biconcave in shape, biliary atresias, the congenital deficiency of G-6-PD in red blood cells (common in the Mediterranean region, much less so here), and congenital deficiency of UDP-glucuronyl transferase respond to phototherapy to some extent, and in some for only as long as the treatment is continued (Callahan *et al.*, 1970; Gorodischer *et al.*, 1970; Karon *et al.*, 1970).

12. CONCLUSION

At this point, when optimal exposure both in energy and time is unknown, and when the full effects of phototherapy have yet to be discovered, it would seem wisest to use the treatment as one would a new drug.

Under this concept, phototherapy is like a medicament which has obvious efficacy, but of which little is known regarding its metabolism, site and mode of action, potential hazards, or, most important of all, dosage. In effect, the use of phototherapy is just short of empirical.

Inasmuch as the rate of bilirubin photodestruction is related to the amount of radiant energy of the light source, the lamps selected should have spectral emission in the region of bilirubin absorption. The amount of

photic energy irradiating the jaundiced infant should be known, and the clinical response monitored. Only in this way can experience with the treatment yield necessary information in respect to the most effective "dose," and eventually the optimum intensity and method of irradiance.

In our studies we have observed that boosting radiant energy of blue lamps fivefold produces neither increased breakdown of bilirubin *in vivo* nor accelerated rate of decline (as measured by plasma concentrations) compared with these effects at a customary irradiance of 2.1 mW cm^{-2} at 420–490 nm (Sisson *et al.*, 1972).

A further consideration is that light emission which will degrade bilirubin will also affect other body constituents, perhaps to a degree that presents an unwanted result. One must decide if the intensity and length of exposure to phototherapy should be regulated in a manner to limit the possibly undesirable effects on other substances. This, too, is analogous to the decisions made as to how, when, and how much of a drug is to be administered.

In the end, the use of phototherapy should be governed by weighing the benefits known to accrue against the risks of known or potential side effects. If the benefits do outbalance the risks, and as far as we know now they do, then the actual technique of phototherapy must be managed so as to reduce hazards to a minimum.

Phototherapy is unquestionably effective in correcting hyperbilirubinemia of the newborn. It is, in a sense, noninvasive in contrast to exchange transfusion and carries less risk of mortality if any at all. Morbidity associated with phototherapy seems to be low, and this makes it an even more attractive alternative to transfusion.

Phototherapy is now in universal use, and in other parts of the world has been an entrenched and successful therapeutic modality for over 15 years. It will not be readily abandoned by the host of practitioners who have found it so effective and apparently safe on mere suspicion of hazard. This makes it even more urgent that the photobiology of visible light in the mammal receive concerted study now. The implications of *in vitro* studies which only indicate that phototherapy may be injurious but are far from proving it in the newborn infant, require that suitable studies be undertaken in an expanded program of human photobiological research.

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Physiological Responses of *Escherichia coli* to Far-Ultraviolet Radiation*

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Although this entire volume is dedicated to Arthur Giese, I wish to make a special dedication of this chapter to him. To do so has special meaning for me because as his graduate student in 1947 I was introduced to UV photobiology.

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Theories concerning the nature of the action of light upon living forms are as old as recorded history. Since they are attempts to correlate observations with the aggregate of known facts, they are but reflections of the philosophy of their times (W. T. Bovie, 1918, *J. Med. Res.* 34:271).

1. INTRODUCTION

The primary target for far-ultraviolet (UV) radiation* on cells has repeatedly been shown to be DNA, and the relationship between DNA damage and lethal and mutagenic effects has been intensively studied and reviewed. This chapter surveys the ways in which the lethal and other responses of *Escherichia coli* to UV irradiation can be modified by pre- and postirradiation conditions. The principal emphasis will be on papers that have appeared since the review of the subject by Rupert and Harm (1966).

This review is almost completely restricted to the effects of UV radiation on a single bacterium, *Escherichia coli*, and on bacteriophage that infect this host. While these restrictions simplify the reviewer's task, they do not permit a comparative approach either between the effects of UV and ionizing radiations or among the responses of various prokaryotes and eukaryotes to radiation. To limit the scope further, only a few *in vitro* DNA

* The far-UV region of the electromagnetic spectrum lies between 190 and 300 nm. Most of the experiments described in this review have been carried out with a low-pressure mercury arc (emitting mainly at 254 nm) as a far-UV source.

repair studies have been included, and coverage of the biological effects of UV radiation on repair-deficient mutants is limited to a few strains on which most of the work has been done. Neither UV mutagenesis nor photoreactivation has been given separate coverage. Among other topics not included is the sensitization of cells to UV radiation by substitution of bromouracil for thymine.

The title of this review reflects the writer's long-standing conviction that if we are to understand how radiation affects cells, the causes of physiological responses must be understood. Evidence has accumulated that a number of responses of *E. coli* to UV radiation are induced in the sense that operons are derepressed and specific proteins are synthesized (see Section 12). Thus, induction of operons could be the basis for many physiological responses to UV radiation. Death may be viewed as the ultimate physiological response of the irradiated cell, and, although it is one of the biological indicators most frequently used to study the effects of UV radiation, the specific causes of death resulting from radiation damage are not known. Our ignorance of the causes of cell death represents a gap in our present understanding of molecular and cellular radiation biology. One of the objectives of this review is to put this problem in new perspective.

2. PHOTOCHEMICAL DAMAGE TO DNA: BIOLOGICAL SIGNIFICANCE

UV irradiation of *E. coli* cells causes many photochemical changes in DNA; these have been reviewed by Smith (1964) and by Setlow and Setlow (1972).

Pyrimidine dimers are formed in greatest quantity, are easiest to measure, and are apparently of the greatest biological importance. The cyclobutane ring characteristic of these dimers is formed at normal temperatures between adjacent pyrimidine dimers in DNA. Since their discovery, these photoproducts have played a central role in the design and interpretation of experiments in far-UV photobiology.

Pyrimidines also participate in the formation of other photoproducts, notably heteroadducts such as the thymine-cytosine azetidine derivative, 6-4'-(pyrimidine-2'-one)-thymine, known as PO-T (Wang and Varghese, 1967). The biological significance of PO-T in *E. coli* is not clear since no increase in survival resulted upon photochemical removal of PO-T (Ikenaga *et al.*, 1970) by 313-nm irradiation (Patrick, 1970). DNA-protein cross-links also form in UV-irradiated *E. coli* (Smith, 1962), and there is evidence that these cross-links can lead to lethality (Smith *et al.*, 1966).

3. MEASUREMENT OF CELL SURVIVAL

Studies on the response of *E. coli* to photochemical damage of its DNA have from the beginning relied upon plating methods and survival curves. These methods involve diluting cell suspensions irradiated with various UV fluences and spreading known volumes on growth medium solidified with agar. Survival curves, obtained by plotting the log of the surviving fraction of colony-forming units versus fluence have various shapes depending on the strain, conditions of irradiation, pre- and postirradiation treatments, the composition of plating medium, and the conditions of incubation of the plates. Survival curves usually have either constant or variable negative slopes. Among the curves with variable slopes two in particular deserve comment. In one type, the fraction of survivors decreases exponentially at low fluences, and above some fluence decreases at a lower rate. The discontinuity in slope is often attributed to a mixed population of cells with different radiation sensitivities. The other type is characterized by a shoulder with nearly zero slope at relatively low fluences of UV radiation and a relatively steep (more negative) slope at higher fluences.

A survival curve poses two important questions with which this review is concerned. Why did the nonsurviving cells die and why did the surviving cells continue to proliferate? At the outset it should be emphasized that the interpretation of the shapes and slopes of survival curves is very difficult since the matter of either survival or death is so complex.

Survival curves have been useful for comparing radiation sensitivities of various repair-deficient strains since differences in survival may be greater at one fluence than another. The sensitivities are usually expressed as the $1/e$ fluence (dose). A survival curve of a simple exponential type has the equation $N/N_0 = e^{-\sigma F}$, where N_0 and N are the numbers of viable cells in the unirradiated culture and in the irradiated culture at a given fluence, respectively. F is the fluence, and σ is the slope of the survival curve. When the product of F and $\sigma = 1$, the fraction of surviving cells is $1/e$ or 0.37. Thus the $1/e$ fluence (dose) is one which reduces survival to 37%. Note that the slope σ can be found by taking the reciprocal of the $1/e$ fluence. The $1/e$ fluence is called a lethal hit, although modifying effects of repair and secondary effects of UV radiation on cells make the precise use of the term meaningless.

For survival curves with shoulders, the fluence reducing survival to 37% has no more importance than any other percent, but the exponential part of the curve can be treated the same way as a simple exponential curve to find a $1/e$ fluence. Alper *et al.* (1962), introducing the term D_0 , defined as the dose (fluence) that reduces the surviving fraction in the exponential part of the curve from a given value to 37% of that value.

A number of interpretations of the shapes of survival curves are covered in this review. These interpretations and proposed causes of cell death are summarized in Section 13.

4. DNA REPAIR PROCESSES AND GENETICS OF RADIATION SENSITIVITY

4.1. Excision Repair

A number of excellent review articles on DNA repair have been published. Therefore, the subject will be treated briefly to provide some background concerning the mechanisms of the known dark-repair processes of UV-damaged DNA and how these mechanisms relate to radiation sensitivity. There are two well-known dark-repair systems in *E. coli*, the excision repair system and the recombination or postreplication repair system. Excision repair, the better understood of the two, was discovered by Setlow and Carrier (1964) in the B strains and by Boyce and Howard-Flanders (1964a) in the K-12 strains. According to present models, the first step in this process is the recognition of a pyrimidine dimer or other lesion in a single strand of DNA. Recognition is followed by endonucleic incision on the 5' side of the dimer and then excision (through cleavage of one or more phosphodiester bonds by an exonuclease) of a short oligonucleotide which contains the dimer. The gap created by removal of the dimer may be enlarged by the same or a different exonuclease before a DNA polymerase, beginning at the free 5' end of the open strand and using the complementary strand as a template, lays down nucleotides in the same sequence as before irradiation. There is evidence that DNA polymerase I is capable of both the excision and polymerizing steps (Kelly *et al.*, 1969). The polymerizing step, called "repair replication" (Pettijohn and Hanawalt, 1964), is followed by the action of a DNA ligase which forms a phosphodiester bond between the 5' end of the short, newly synthesized segment and the 3' end of the contiguous strand of DNA. In *E. coli* the DNA ligase uses NAD as a cofactor (Olivera and Lehman, 1967; Zimmerman *et al.*, 1967). The enzymology of excision repair has been reviewed by Grossman (1974).

Among the first radiation-sensitive mutations in *E. coli* for which genetic mapping was carried out were those designated *uvrA*, *uvrB*, and *uvrC*. Although the genes are widely dispersed on the map of the *E. coli* chromosome (Howard-Flanders *et al.*, 1962; Rörsch *et al.*, 1963; van de Putte *et al.*, 1965; Howard-Flanders *et al.*, 1966), the three mutant strains are phenotypically similar in that they all fail to excise dimers from their

DNA (Howard-Flanders *et al.*, 1966). The $1/e$ fluence for *uvrA* is about 1.5% that for the wild-type strain (Howard-Flanders and Boyce, 1966); the *uvrB* and *uvrC* strains are less sensitive than *uvrA* (Howard-Flanders *et al.*, 1966). The failure of a given strain to excise pyrimidine dimers should result in the absence of repair replication as has been demonstrated for *uvrA* cells by Cooper and Hanawalt (1971) and by Billen and Carreira (1971).

Although all the *uvrA*-, *B*, and -*C* mutants are phenotypically similar, evidence has been obtained which indicates that the *uvrC* mutant has unique characteristics. Recently, Braun and Grossman (1974) isolated an endonuclease from *E. coli* that is specific for UV-irradiated DNA and is not detectable in *uvrA* and *uvrB* strains; it is however present in the *uvrC* strain. Braun and Grossman (1974) also found another endonuclease that acts preferentially on UV-irradiated DNA in all three *uvr* mutants. After UV irradiation, the *uvrC* mutant develops breaks in its DNA which are not repaired within an hour after irradiation (Kato, 1972); Kato suggested that there might be an intermediate step between incision and excision controlled by the *uvrC* gene product. A similar finding was made by Seeberg and Johansen (1973) on *uvrC* cells superinfected with UV-irradiated λ phage. Seeberg and Rupp (1975) found that when a *uvrC* mutant with a ligase deficiency was irradiated, even more single-strand breaks developed than in ligase-proficient *uvrC* cells. Also the same number of breaks were formed in UV-irradiated *uvrC polA12* cells (having a temperature sensitive DNA polymerase I) at the restrictive temperature, as in the *uvrC* cells. It was concluded that the *uvrC* gene product interacts with the ligase system and that in *uvrC* mutants many breaks are abortively sealed at dimer sites without excision of the dimer.

The absence of DNA polymerase I activity in the *polA1* mutant has a small effect on its excision ability but, presumably because the enzyme retains its 5'-3' exonucleolytic activity (Lehman and Chien, 1973), DNA is excessively degraded (Boyle *et al.*, 1970) during uncontrolled enlargement of excision gaps. In addition to the 5'-3' exonuclease (exonuclease VI) associated with DNA polymerase I, two other 5'-3' exonucleases may play parts in the excision repair and DNA degradation processes: exonuclease VII (Chase and Richardson, 1974) and the 5'-3' activity associated with DNA polymerase III (Livingston and Richardson, 1975). Exonuclease VII can also degrade single-stranded DNA in the 3'-5' direction and can, in contrast to exonuclease VI, degrade long stretches of DNA. Cooper and Hanawalt (1972a,b) have demonstrated both short patch repair, requiring DNA polymerase I, and long patch repair requiring the *recA* and *recB* gene products. Exonuclease VII could be responsible for creation of large gaps for the long patch repair. A temperature-sensitive mutant *polAex1* has been isolated that possesses normal DNA polymerase I DNA-synthesizing

activity and full 5'-3' nuclease (exonuclease VI) activity at 30°C but greatly reduced exonuclease VI activity at the lethal temperature of 43°C (Konrad and Lehman, 1974). The mutant is sensitive to UV radiation at 30°C.

It might be expected that repair replication would be reduced in a *polA1* mutant, but instead more is seen than in the *polA⁺* parent (Cooper and Hanawalt, 1972a). On the basis of excision and repair replication experiments with three different DNA polymerase I mutants of *E. coli* B, each deficient in one or both of the enzyme's two known functions, Glickman (1974) suggests that the excessive repair replication seen by Cooper and Hanawalt is carried out by another DNA polymerase. A key point in Glickman's analysis is that one of the DNA polymerase mutants, *resA1* of *E. coli* B (Kato and Kondo, 1967; Kato, 1972) can neither excise dimers (Kato, 1972; Glickman, 1974) nor carry out repair replication (Glickman, 1974). DNA polymerase III is able to fill excision gaps in *polA1* cells (Youngs and Smith, 1973a), and cells deficient in both polymerases show reduced ability to carry out this process (Sedgwick and Bridges, 1974). In addition, studies with a polymerase mutant deficient in both DNA polymerase I and III suggest that DNA polymerase II plays a limited role in the excision repair process (Tait *et al.*, 1974). The results of the experiments described above support the conclusion that both *uvr* and *pol* gene products play roles in excision repair, and the finding of equal UV sensitivity in *uvr* and *uvr polA1* strains (Monk *et al.*, 1971) further supports this conclusion for DNA polymerase I.

The mutants *uvrA*, *uvrB*, and *uvrC* are quite sensitive to UV radiation but only slightly sensitive to ionizing radiation. The *uvrE* mutants, originally isolated by Mattern (1971) (see van Sluis *et al.*, 1974 for review and nomenclature), also fall into that category but they are strains with high mutagenic rates (Mattern, 1971; Smirnov *et al.*, 1972). The *uvrE* mutants, including *uvr502* (Smirnov *et al.*, 1972), do not degrade their DNA excessively after UV irradiation and are affected primarily in the excision repair system (Smirnov *et al.*, 1973; van Sluis *et al.*, 1974). They do not excise pyrimidine dimers as rapidly nor to as great an extent as wild-type strains and may have the excision repair process impaired in a closely succeeding step (van Sluis *et al.*, 1974).

The model for excision repair summarized in the first paragraph of this section is probably essentially correct, but as more is learned the enzymology process appears increasingly complex. For example, the inter-relations between the *uvr* mutants and other mutants involved in DNA repair have been studied in various repair-deficient mutants by Youngs and Smith (1973b) and Youngs *et al.* (1974a). Based on viability studies and the extent of repair of single-strand breaks that presumably arise during incision, at least three branches of excision repair are proposed.

4.2. Recombination Repair

The other presently known dark-repair system is called *recombination* or *postreplication* repair was discovered by Rupp and Howard-Flanders (1968). DNA of *uvr* (excision-deficient) strains is apparently synthesized using the undamaged template between pyrimidine dimers; thus for a time during the postirradiation period there are gaps in the DNA opposite the dimers in the parental strand. The gaps in the daughter strands are thought to be filled by sections of DNA removed from the noncomplementary parental strand which has the same polarity (Rupp *et al.*, 1971). The *uvr* cells are fully capable of genetic recombination, and the enzymes involved in this repair process are probably common to those used in some steps in recombination. Evidently in irradiated cells exchanges take place that involve other than dimer-free regions of parental DNA, because endonuclease-sensitive sites peculiar to UV-irradiated DNA are found after a time in the newly synthesized daughter strands (Ganesan, 1974). Ganesan suggests that perhaps the residual dimers are diluted out in succeeding postirradiation generations.

Other evidence for the persistence of dimers after UV irradiation in *uvr* (*Hcr*⁻) cells was reported by Bridges and Munson (1968). They found that up to 3.75 generation times after UV irradiation, mutations could be prevented by photoreactivation treatment; such treatment is effective only on pyrimidine dimers (Setlow, 1967).

Smith (1971) has reviewed the role of the *rec* genes in DNA repair in *E. coli*. There are at least three genes involved in postreplication repair, *recA*, *recB*, and *recC*. *RecA* cells (Fig. 1) are more sensitive to UV irradiation ($1/e$ fluence of 4.5 erg mm^{-2}) than either *recB* or *recC* ($1/e$ fluence of 25 erg mm^{-2}) (Howard-Flanders, 1968b; Willetts and Mount, 1969). Excision of pyrimidine dimers occurs in all *rec* mutants, but only 75% of the dimers excised by the *recA*⁺ parent are excised by the *recA*⁻ mutant (Shlaes *et al.*, 1972). Smith and Meun (1970) found that short strands of DNA were synthesized in all *rec*⁺ and *rec*⁻ cells and that this synthesis occurred even in double mutants carrying a *uvr* gene. However, completion of repair, as indicated by joining of the short strands, took place in *recB* and *recC* but not in *recA* mutants after a fluence of 63 erg mm^{-2} . Genetic recombination is absent in *recA* strains (Clark, 1967; Clark and Margulies, 1965; Howard-Flanders and Theriot, 1966), but some remains in *recB* and *recC* strains (Low, 1968; Willetts and Mount, 1969). *RecA* strains rapidly degrade their DNA after UV irradiation (Clark *et al.*, 1966; Howard-Flanders and Theriot, 1966). The effect of the *recA* and other genes on this process is discussed in Section 5.1.

Devoret (1974) has pointed out that recombination repair may be

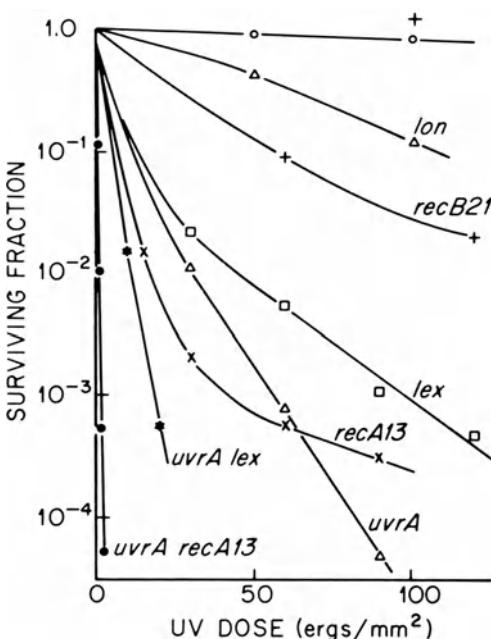


Fig. 1. The fractions of bacteria able to form colonies when plated on complete medium plotted against UV fluence for various mutants of *E. coli* K-12. These are all strains derived from the multiple auxotrophic strain AB1157. The strain numbers are as follows: AB1157 (+); AB1899 (*lon*); AB2470 (*recB21*); AB2463 (*recA13*); AB2494 (*lex*); AB1886 (*uvrA*); AB2474 (*uvrA lex*); and AB2480 (*uvrA6 recA13*). (Modified from Howard-Flanders, 1968b.)

divided into two processes. One is postreplication repair, in which the cell uses a recombination mechanism on its own DNA duplex. The other recombination repair process involves two DNA duplexes and seems to be operative in multiplicity reactivation (Luria, 1947), marker rescue or cross-reactivation (Doerman, 1961), and prophage reactivation (Jacob and Wollman, 1953), all of which take place in unirradiated hosts. Multiplicity reactivation and marker rescue of radiation-damaged phage take place in a host that does not contain a prophage, while prophage reactivation occurs in a host that contains a prophage.

Postreplication repair in *E. coli* K-12 has been divided into multiple pathways. Three separate pathways have been shown to be controlled by the *uvrD*, *exrA* and *recB* genes (Youngs, *et al.*, 1974b).

4.3. Repair Involving *lex* and *recA* Genes

Another mutant, *lex* or *exrA*, is sensitive to UV irradiation and may be involved in postreplication repair. This gene, along with *recA*, is very important in the responses of *E. coli* cells to UV irradiation, and it seems worthwhile to summarize the work which shows that *lex* and *exrA* are probably the same mutants or mutants in the same cistron. Howard-

Flanders and Boyce (1966) gave the name *lex* to an *E. coli* K-12 mutant that was also sensitive to both UV radiation and X rays. The strain B_{s-1} was shown to carry an *exr* mutation for radiation sensitivity in addition to the *Hcr* character (Mattern *et al.*, 1966; Greenberg, 1967). The *exr* gene in B_{s-1} was similar to *lex* in its behavior (Donch and Greenberg, 1968a). The *exr* gene which was linked to *malB* was designated *exrA* by Donch *et al.* (1970) to distinguish it from the other *exr* mutations (in the same paper the genotype of B_{s-1} was given as *uvrB exrA*). The *exrA* mutant was thought to be the same as *lex* (Donch *et al.*, 1970). Donch and Greenberg (1974a,b) found a number of other similarities which make it highly probable that *lex* and *exrA* are mutations in the same cistron. In this review the mutations in B strains will be referred to as *exrA* (*lex*) when the cited reference has used *exrA*. In all other cases *lex* will be used.

The *recA* and *lex* mutants each have high radiation sensitivity and the ability to carry on host-cell reactivation of irradiated phage (Howard-Flanders and Boyce, 1966). In addition, these mutants do not show a number of special responses including mutagenesis, induction of prophage, UV reactivation of irradiated phage, filament formation in *lon* cells, and maintenance of stability of DNA (DNA is rapidly and extensively degraded in *recA* and *lex* cells). These responses and others by *recA⁺* and *lex⁺* cells, the so-called *rec-lex* effects, will be documented and discussed in other sections. One significant difference between the two mutants is that *lex* mutants show near-normal recombination but *recA* mutants do not (Howard-Flanders and Boyce, 1966).

The extensive degradation of DNA (Clark *et al.*, 1966; Howard-Flanders and Boyce, 1966) and lack of mutagenesis (Witkin, 1967b, 1969b; Defais *et al.*, 1971; Miura and Tomizawa, 1968) in both *recA* and *lex* cells led Witkin (1969b) to hypothesize that excision repair is practically error-free and that the *recA* and *lex* gene products are involved in an error-prone repair system which probably entails a gap-filling function in newly replicated strands opposite dimers. Mount *et al.* (1972) constructed *lex⁺/lex* merodiploids and found that the *lex* phenotype was retained; they concluded that *lex* is dominant and suggested that since it is dominant, the phenotype results from a *lex* gene product. Mount and Kosel (1973) suggest that this finding, as well as the observation that *lex* mutants are also sensitive to ionizing radiation (Howard-Flanders and Boyce, 1966) and methylmethanesulfonate (Mount *et al.*, 1972), weakens the hypothesis of Witkin that mistakes are made during gap filling in the DNA strand opposite dimers in *lex⁺* cells. They feel that *lex* mutants are defective in some repair process that involves more than filling gaps opposite dimers in UV-irradiated DNA. Moody *et al.* (1973) concluded from the similar responses to UV by *lex⁺* *recA* and *lex recA* strains that the *lex* and *recA* genes are involved in the

same repair pathway. Donch and Greenberg (1974b) came to the same conclusion working with *exrA⁺ recA* and *exrA recA* strains.

With the knowledge of a number of *rec-lex* responses to UV radiation, Witkin's idea of *recA* and *lex* genes being involved in an error-prone repair system has been expanded. All of these responses are now viewed as being induced by UV in the sense of derepressing an operon. Only in the case of the λ lysogen is derepression actually known to occur. The total pattern of these responses has led Witkin and George (1973) and Radman (1974) to propose the existence of an inducible, error-prone repair system dependent upon the *recA⁺* and *lex⁺* genes. Radman (1974) has termed this system SOS repair.

Still another dark-repair system, proposed by Bridges (1972), involves repair and removal of a "stalled" DNA replication point. The evidence supporting this proposal will be introduced in the section on DNA synthesis.

5. DEGRADATION OF DNA AND RNA

5.1. DNA Degradation

The nature of excision and repair replication in excision-type repair makes it clear that some DNA is degraded in repair-competent cells. At biological fluences of UV radiation, the number of nucleotides expected to be removed from the DNA of these cells would be fairly small and that from the DNA of *uvr* cells much smaller. Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964a) reported a 10% loss of acid-insoluble material from the DNA of UV-irradiated *E. coli* B/r and K-12, respectively. Later Boyce and Howard-Flanders (1964b) found three times that amount lost in wild-type *E. coli* K-12, but only a small amount from *uvr* cells. Swenson and Schenley (1974a) found a negligible loss from UV-irradiated *E. coli* WP2 *Hcr⁺* and WP2 *Hcr⁻* mutant cells (B/r tryptophan-requiring derivatives) grown on minimal glycerol medium. Evidently the recombinational repair system does not normally involve extensive DNA degradation, but *recA* cells degrade their DNA extensively after UV irradiation (Clark *et al.*, 1966; Howard-Flanders and Theriot, 1966); degradation is less than that for wild-type cells in irradiated *recB* and *recC* cells (Howard-Flanders and Theriot, 1966; Emmerson, 1968). There is no relationship between DNA degradation and survival in UV-irradiated wild-type and *recB* cells (Emmerson, 1968). Likewise, the extensive degradation by *recA* cells does not appear to be the cause of death because a multiple mutant such as *recA recB recC* has about the same radiation sensitivity as *recA*, but degradation of DNA is greatly reduced (Willets and Clark, 1969).

the *recB* and *recC* genes seem to be jointly responsible for the production of an ATP-dependent deoxyribonuclease, exonuclease V (Oishi, 1969; Buttin and Wright, 1968; Barbour and Clark, 1970; Goldmark and Linn, 1970). The *recA* gene may be needed for the production of an inhibitor of that enzyme (Marsden *et al.*, 1974; see discussion later in this section).

Another mutant showing rapid and extensive degradation after UV irradiation is *lex* (Howard-Flanders and Boyce, 1966). The *lex* and *exrA* genes are thought to be in the same cistron (see Section 4.3). When *recB* and *recC* mutations are present in *exrA* mutants, DNA degradation is greatly reduced (Donch and Greenberg, 1974b). Thus *exrA* and *recA* may be jointly responsible for the regulation of the *recBC* nuclease.

Suzuki *et al.* (1966) were the first to report extensive DNA degradation in an *E. coli* strain; strain *B_{s-1}* shows rapid degradation after UV irradiation, probably because of the *exrA* mutation. The behavior of strain *B_{s-1}* is anomalous since it is also excision-deficient because of a *uvrB* mutation; no incisions would be expected to be made in the absence of the UV-specific endonuclease (Braun and Grossman, 1974). Kato and Kondo (1970) found the expected result, little degradation, in *recA uvr* strains. The small amount of degradation that did take place in *recA uvr* cells seemed to be at the replication fork since the DNA pulse labeled with radioactive thymidine prior to UV irradiation became acid soluble. Horii and Suzuki (1970) suggested that the extensive degradation in *recA* cells was a secondary effect. Earlier experiments of Horii and Suzuki (1968) on amino-acid-starved *recA* cells suggested such an explanation since UV-irradiated wild-type and *recA* cells in stationary phase showed no significant differences in the breakdown of DNA compared to that which occurred spontaneously in unirradiated cells. Starvation for essential amino acids, another case where there was an absence of replication forks, caused the same effect. Yonei and Nozu (1972) worked with still another mutant of the *recA* type, NG30 (Kato and Kondo, 1969), and found quite different results. The DNA degradation which occurred in resting-phase NG30 cells, and in a double mutant NG30 *uvrC* strain, was greatly reduced by caffeine. They concluded that DNA degradation occurs not only at the replication forks but at sites at which dimers are excised.

Caffeine also reduced the initial rate but not the final amount of breakdown in UV-irradiated strain B and had no effect on degradation in *E. coli* *B_{s-2}* (Grigg, 1970). Strain *B_{s-2}* is *lon lex* (Donch *et al.*, 1968). Shimada and Tagaki (1967) found that 1% caffeine inhibited DNA breakdown by 90% in a mutant that could not carry out host-cell reactivation on λ phage and that underwent excessive postirradiation degradation of DNA. When this strain was infected with UV-irradiated λ , DNA velocity sedimentation studies suggested that caffeine prevented the incision of phage DNA by the

repair endonuclease. The studies of Roulland-Dussoix (1967) on repair-competent *E. coli* infected with UV-irradiated λ phage show that caffeine interferes with the degradation of the phage DNA; this interference is attributed to the fact that both exo- and endonucleases are inhibited by caffeine (Roulland-Dussoix, 1967).

Other *E. coli* repair-deficient mutants also degrade DNA excessively after UV irradiation, for example, *uvrD* (Ogawa *et al.*, 1968), *polA1* (Boyle *et al.*, 1970), and *resA1* (also deficient for DNA polymerase I) (Kato and Kondo, 1970). The introduction of the *recB* gene into a *polA1* strain does not affect the amount of DNA degradation after UV irradiation (Strike and Emerson, 1974); this leads to the conclusion that exonuclease V is not involved in DNA degradation in the *polA1* strain. The amber codon-terminated fragment of DNA polymerase I of *polA1* retains the 5' to 3' exonuclease activity (Lehman and Chien, 1973), and this activity may be responsible for DNA degradation after UV irradiation (Strike and Emerson, 1974). Alternatively, exonuclease VII (Chase and Richardson, 1974) or the 5'-3' exonuclease associated with DNA polymerase III (Livingston and Richardson, 1975) may be involved in postirradiation breakdown of DNA in *polA1* strains. Green *et al.* (1971) measured DNA breakdown and survival in a *resA1* strain under growing and stationary conditions and found that both were more sensitive under the latter condition, but there was not a good correlation between amount of breakdown and lethality. The mutant *resA1* has lost polymerizing activity, but the presence or absence of the 5'-3' exonuclease activity associated with polymerase I has not been established. Kato (1972) found that the introduction of a *recB* gene into a *resA1* strain completely stopped UV-induced DNA degradation but increased UV sensitivity. Kato (1972) states that *resA1 recB* has a low level of DNA polymerase I activity.

The *recBC* genes are responsible for synthesis of exonuclease V, and this enzyme seems to be responsible for normal DNA degradation in X-irradiated *E. coli* (Emmerson, 1968; Youngs and Bernstein, 1973). Pollard and Randall (1973) presented evidence that a radiation-induced inhibitor keeps DNA degradation in check and that UV radiation, as well as ionizing radiation, acts as an inducer. When rifampicin was added to γ -irradiated *E. coli* 15 T⁻L⁻ or to UV-irradiated *E. coli* B prior to irradiation with γ rays, the amount of DNA degradation was increased. If rifampicin was added to the *E. coli* B culture 35 min after UV irradiation, it had little effect. Rifampicin had little influence on the extensive DNA degradation in *E. coli* B_{s-1} [*uvrB exrA (lex)*]. The induced synthesis of the proposed *recBC* nuclease (exonuclease V) inhibitor has been shown to be dependent upon the *recA* and *lex* genes (Marsden *et al.*, 1974). Figure 2 shows representative results of experiments in which various repair-competent and repair-defi-

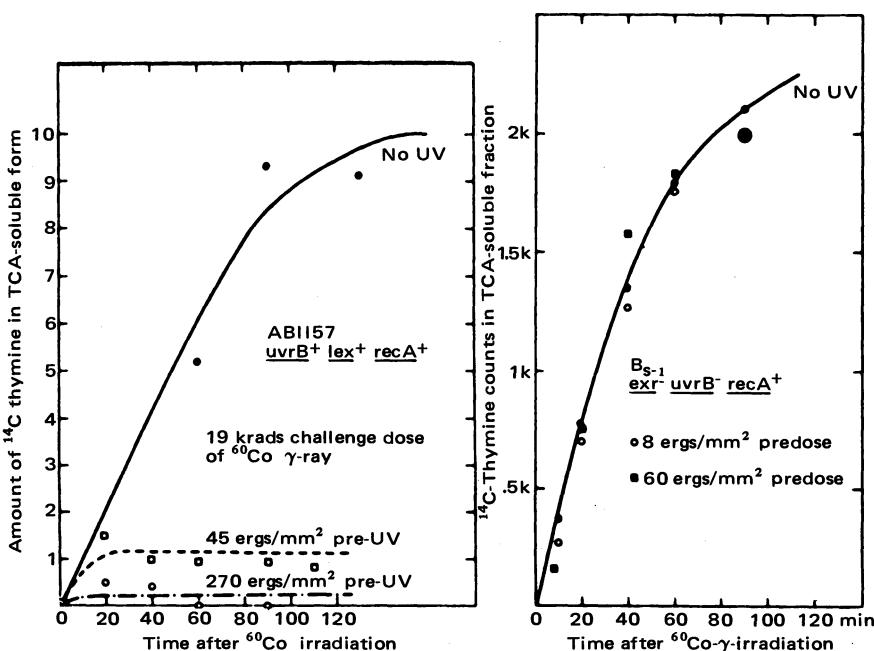


Fig. 2. The effect of pre-UV fluence and 60 min of incubation on the effect of ^{60}Co γ irradiation on the DNA of cells: (a) Wild-type *E. coli* K-12; (b) *E. coli* B_{s-1} . The cells have been labeled in the DNA with $[^{14}\text{C}]$ thymine, and the amount of label rendered acid soluble is plotted as time elapsed after the γ irradiation. (From Marsden *et al.*, 1974.)

cient mutants were UV irradiated, incubated for 60 min, and then γ irradiated. Wild-type *E. coli* K-12 cells (AB1157) were protected by UV irradiation against DNA degradation, but B_{s-1} was not; only mutants containing *recA* or *lex* mutations were not protected by pretreatment with UV radiation against the extensive DNA degradation caused by γ irradiation. Because the induced inhibitor is probably a protein, post-UV-irradiation starvation of cells for an amino acid prevents the protective action of UV irradiation against the degradation of DNA normally caused by the subsequent γ irradiation (Pollard *et al.*, 1974). This result is also consistent with an earlier one of Frampton and Billen (1966) in which postirradiation starvation of γ -irradiated cells for essential amino acids caused more degradation of DNA than in unstarved cells. When γ -irradiated cells are protected against DNA degradation by previous induction with UV radiation, increased repair of γ -radiation-produced single-strand breaks in DNA is observed (Tolun *et al.*, 1974).

5.2. RNA Degradation

UV radiation also causes the degradation of RNA in *E. coli* B and B/r to acid-soluble materials (Pečevsky-Kućan and Kućan, 1970). The degradation begins about 70 min after UV irradiation and increases exponentially with fluence and then saturates at 1500 erg mm^{-2} . At 500 erg mm^{-2} about 20% of the RNA is degraded by 150 min after UV irradiation. Similar results have been obtained for *E. coli* B/r by W. D. Fisher, R. L. Schenley, and P. A. Swenson (unpublished data). Doudney (1969) found that starvation for an essential amino acid after UV irradiation causes a large fraction of the ribosomes to be degraded, but he found no degradation in the irradiated cells if there was no starvation.

6. BIOCHEMICAL AND PHYSIOLOGICAL CONSEQUENCES

6.1. DNA Synthesis

One of the first consequences of the formation of UV photoproducts in *E. coli* DNA is on DNA synthesis. Kelner (1953) first showed that DNA synthesis is much more sensitive to UV radiation than are either RNA or protein synthesis. Kelner's results as well as those of others (Kanazir and Errera, 1954; Hanawalt and Setlow, 1960; Setlow *et al.*, 1963) showed that DNA synthesis is almost completely inhibited for a time after irradiation and then increases in rate. It was thought for several years (Setlow and Carrier, 1964; Swenson and Setlow, 1966) that upon irradiation the *E. coli* chromosome continued normal semiconservative replication up to a dimer that acted as a block, that excision repair was random, and that resumption of synthesis marked completion of excision repair. Later it was shown by Rupp and Howard-Flanders (1968) with *uvr* cells that excision-deficient strains were able to synthesize DNA throughout the chromosome; dimers acted as blocks to synthesis, but synthesis could be initiated beyond the dimer, continue to the next dimer, and then repeat the process. This synthesis between dimers, plus that of repair replication, probably accounts for the small amount of synthesis detected by radioactive thymine incorporation during the time synthesis is inhibited by relatively high fluences of UV radiation. During the first 15 min after receiving a relatively low fluence (35% survival), 93% of DNA synthesis is semiconservative and 7% is repair replication (Billen *et al.*, 1967).

Smith and O'Leary (1968) have placed emphasis on methodology and manner of plotting the data in radioactive thymine-incorporation studies.

They strongly recommend that cells be labeled with the radioactive precursor both prior and subsequent to irradiation. Likewise, they prescribe that postirradiation incorporation data be plotted on semilog paper. Setlow and Setlow (1970) have indicated that the choice of methodology (prelabeling or no prelabeling) and the type of plot should depend on which points the investigator wishes to emphasize. Doudney (1971a) pointed out that radioactive thymine incorporation studies done with thymine auxotrophic strains give kinetics which reflect more accurately the true kinetics for DNA synthesis than do studies with prototrophic strains. The latter strains have internal pools of nonradioactive precursors of DNA, and these precursors may compete with the radioactive thymidine entering the cells from the external medium.

Setlow *et al.* (1963) reported that low fluences of UV radiation to *E. coli* B_{s-1} allowed DNA synthesis to proceed for a time, dependent upon fluence, and then stopped it permanently. This early DNA synthesis could not have been easily measured if the DNA were even moderately labeled with radioactive thymidine prior to UV irradiation. Smith's (1969) observation on *E. coli* B_{s-1} is that at all UV fluences between 2.5 and 10 erg mm⁻² DNA synthesis continues for as long as 300 min. Rupp and Howard-Flanders (1968) examined postirradiation DNA synthesis in a *uvrA* strain and found that, although DNA synthesis was delayed at fluences between 22 and 45 erg mm⁻², the process did not stop. Rudé and Doudney (1973), working with an *Hcr* derivative of *E. coli* B/r, reported that at fluences between 20 and 30 erg mm⁻² DNA synthesis continued for about 60 min after UV irradiation and then ceased. The glucose minimal media used in both experiments were quite similar, and there seems to be no obvious reason for the differences. The results of Rupp and Howard-Flanders (1968) are in accord with their finding of synthesis of new DNA strands with gaps. The results of Rudé and Doudney (1973) are in accord with the findings of Swenson and Schenley (1974a) that UV radiation causes cessation of respiration of *Hcr*⁻ cells about 60 min after UV irradiation.

Doudney (1965) and Smith (1969) have reported that the duration of inhibition of DNA synthesis in *E. coli* is dependent upon the fluence of UV radiation; the maximum duration of the inhibition is 45 min, approximately one division time. At doses lower than this "critical dose" (Doudney, 1965) of about 400 erg mm⁻², the rate of DNA synthesis upon resumption is about equal to that of the unirradiated control. At higher doses these rates are progressively lower. In agreement with the work of Suzuki *et al.* (1966), the amount of DNA degradation that occurs, even after doses exceeding the critical dose, is not significant. Doudney (1959) and others (Harold and Ziporin, 1958; Drakulić and Errera, 1959) have shown that protein synthesis is necessary for resumption of DNA synthesis in UV-irradiated *E. coli*.

Chloramphenicol added within 15 min after a UV radiation fluence that delays DNA synthesis for 45 min completely prevents the resumption of synthesis. Addition of the antibiotic at later times permits resumption, but at rates less than in its absence. If chloramphenicol is added after resumption of synthesis, little effect on DNA synthesis is observed (Doudney, 1965). Similar results were obtained by Drakulić and Errera (1959). Acriflavine also prevents reinitiation of DNA synthesis (Doudney *et al.*, 1964) and, when added at later times after UV irradiation (Doudney, 1966b), it loses its effectiveness as does chloramphenicol (see above). It is interesting that excision takes place in the presence of chloramphenicol (Swenson and Setlow, 1966), but is interfered with by acriflavine (Setlow, 1964).

Doudney (1973a) has examined in more detail the effects of chloramphenicol on DNA replication in irradiated cells (160 erg mm^{-2}) and has found that low concentrations (2–10 $\mu\text{g/ml}$) allow about 80% of the synthesis required for full DNA replication. This result, and that of Lark and Lark (1964) with amino acid-starved cells, suggests that a low concentration allows the completion of a round of replication upon resumption of DNA synthesis but prevents initiation of the next round.

The antibiotic rifampicin prevents initiation of RNA synthesis in *E. coli* and when added to cultures within 13 min after UV irradiation, it prevents DNA synthesis from resuming (Doudney, 1973b). This observation is consistent with the previous conclusion of Doudney (1959, 1965) that a protein must be induced to reinitiate DNA synthesis after UV irradiation. Addition of rifampicin between 15 and 40 min after irradiation limits DNA synthesis to a 50% increase, whereas if added at 60 or 70 min the increase is limited to 100% (Doudney, 1973). These limitations lend support to Doudney's (1968) idea that, in UV-irradiated cultures, DNA replication is in synchrony upon resumption of DNA synthesis. Doudney (1968) has shown that cell division begins immediately after a doubling of the DNA; this last point is difficult to reconcile with the observation by Doudney and Young (1962) that some of the cells do not replicate their DNA at all.

Doudney (1968) suggests that stoppage of DNA synthesis is caused by irreversible damage to the replication site, and that recovery of DNA synthesis occurs because protein synthesis takes place and causes reinitiation of DNA synthesis at the chromosome origin. Billen (1969) has shown that after UV irradiation the original replication point is inactivated and is not restored in the dark to its original activity; instead new sites of initiation of semiconservative DNA synthesis appear preferentially at the fixed origin of DNA replication. This premature reinitiation at new replication sites could result in abnormal gene dosage or membrane-DNA rearrangements, either of which might be lethal (Billen and Carreira, 1971). Inhibition of protein

synthesis by withdrawal of an essential amino acid prevents the appearance of new replication sites (Hewitt and Billen, 1965). Hewitt and Gaskins (1971) have studied DNA replication in a *uvr* strain of *E. coli* and, in contrast to repair-competent cells, found that the synthesis after irradiation is semiconservative and involves no new replication sites.

Photoreactivation (PR) treatment with visible or near-UV light causes early resumption of DNA synthesis (Kelner, 1953; Setlow *et al.*, 1963; Doudney, 1965) as well as increased viability. Doudney (1974) gave PR treatment to UV-irradiated *E. coli* cultures at various times after UV irradiation. With UV fluences near the critical level, PR treatment given up to 5 min before normal resumption causes earlier resumption; if given 5 min after normal resumption no effect was seen. At higher UV fluences where the rate after resumption was lower, the times of effectiveness and noneffectiveness of the PR treatments were the same; when effective, the PR treatment brought about an increased rate of DNA synthesis but not an earlier resumption. The loss of photoreversibility indicates that within a 10-min period pyrimidine dimers cause irreversible damage to sites in DNA concerned with reinitiation of DNA synthesis. The data do not enable us to say whether the supposed damaged replication sites repaired by PR treatment are those at the replication fork or at the chromosome origin.

Doudney (1966a) found that at low fluences of UV radiation, photo-reactivation treatment caused DNA synthesis to be nearly like that of unirradiated cells. At critical fluences, however, where dark recovery of DNA synthesis was apparently complete, little PR effect was observed; at fluences greater than the critical one, where resumption of synthesis was at a low rate, PR treatment caused earlier resumption at a faster rate. Thus, there is photoreversibility of dark-reparable damage, nonphotoreversibility of dark-reparable damage, and photoreversibility of damage that is not repaired in the dark. Doudney also tested the effect of chloramphenicol on cells that received a critical fluence of UV radiation, followed by PR treatment and chloramphenicol. In spite of the PR treatment, the chloramphenicol almost completely stopped the resumption of DNA synthesis. Thus, the reinitiation of DNA synthesis appears to involve lesions other than pyrimidine dimers or involves dimers that are not accessible to the PR enzyme.

Bridges (1972) proposed that reinitiation of DNA synthesis in UV-irradiated cells, after stalling at the replication point, involves a separate dark-repair system. His argument rests on many of the points reviewed in this and the previous section. Bridges suggests that synthesis stops because of single-strand gaps that arise during repair and that these gaps may be remote from the replication site. DNA synthesis in *recA* cells, for example, is drastically inhibited by a single ^{32}P decay (Cairns and Davern, 1966) or by

very low doses of ionizing radiation (Gray *et al.*, 1972). Bridges (1972) showed that both acriflavine and chloramphenicol interfere with recovery, i.e., survival, of repair-competent cells to the same extent (the shoulder of the survival curve is lost). Chloramphenicol does not interfere with excision, and acriflavine is thought to have effects other than interference with excision. These agents have in common the ability to prevent resumption of DNA synthesis after UV irradiation; Bridges proposed that this is the means by which recovery is prevented. He proposed that these agents interfere with a process concerned with repair of the DNA replication point, and that this process is dependent upon the functions of *recA⁺* and *uvr⁺* gene products.

One would expect, in a culture of irradiated cells containing a heterogeneous population of cells destined to survive and die, that some cells might continue to synthesize DNA longer than others. Doudney and Young (1962) found that in an *E. coli* culture irradiated to 20% survival, most cells synthesized a round of DNA as indicated by distribution of a density label, but some cells did not carry out any replication. Some years later Billen and Bruns (1970a) reported that in some UV-irradiated cells DNA which had been replicated one or more times failed to continue to be copied. This dampening of DNA replication cycles over several generations is probably reflected at the molecular level as lethal sectoring (Haefner and Striebeck, 1967) in which, after one or two divisions, only one of the daughter cells will continue to divide. Billen and Bruns (1970a) suggest that both of these phenomena may indicate failure of the passage of genetic information to daughter cells.

Radman *et al.* (1970) found evidence (see section on reactivation of bacteriophage) that at low fluences of UV radiation to λ phage, recombination repair processes were more important than excision repair but, at fluences high enough to cause inhibition of DNA synthesis, excision repair became more prominent. This finding suggested that inhibition of DNA synthesis after UV irradiation may play a role in the timing of repair processes so as to enhance excision repair and increase survival. Radman and Errera (1970) investigated the possibility that excision repair and survival could be increased in an *E. coli* strain (GY935) thermosensitive for DNA replication at a nonpermissive temperature (41°C). The UV-irradiated cells grown at 32°C were irradiated and held in liquid growth medium for various times at 41°C, but plating and subsequent incubation were carried out at 32°C. Under these conditions higher survival was obtained than when all operations were at 32°C. When acriflavine was present during the 41°C regime for the mutant cells, the survival increase did not occur. A temperature increase after UV irradiation for wild-type and *uvrB* strains of *E. coli* did not cause a survival increase.

6.2. RNA and Protein Synthesis

The processes of RNA and protein synthesis are much less sensitive to UV irradiation than that of DNA synthesis (Kelner, 1953). Hanawalt and Setlow (1960) did fluence-effect curves on RNA and protein synthesis and found a shoulder on the curve relating the log of the rate of RNA synthesis to the fluence, but the rate of protein synthesis fell off with increasing fluence in a simple exponential fashion; the kinetics of RNA and protein synthesis were linear during the time DNA synthesis was blocked. Swenson and Setlow (1966) also found linear kinetics for RNA and protein synthesis in UV-irradiated *E. coli* and reported that, when DNA synthesis resumed, the rate of RNA and protein synthesis also increased. The simplest explanation for these observations is that UV-induced lesions (mainly pyrimidine dimers) in DNA are widely spaced, and that between lesions RNA synthesis can take place. During the time of inhibition of DNA synthesis the amount of template for RNA synthesis remains constant, but when DNA synthesis resumes, RNA and protein synthesis increase proportionately.

The effects of UV irradiation have been examined on the synthesis of three general classes of RNA molecules, messenger RNA, transfer RNA, and ribosomal RNA. Wainfan *et al.* (1963) found that the synthesis of particulate (ribosomal) RNA was more sensitive than transfer RNA to UV radiation. This finding was also made by Sibatani and Mizuno (1963), who found too that the synthesis of 16S RNA was more sensitive than that of 23S RNA. The latter finding was also made by Kroes *et al.* (1963) in experiments in which ^{32}P pulses were used. When the ^{32}P pulse was followed by a cold chase, a 4S component appeared, and its synthesis was more sensitive to UV radiation than either 23S or 16S RNA. Brdar *et al.* (1966) followed the kinetics of synthesis of 50S ribosomes and 23S RNA in UV-irradiated *E. coli*. Synthesis was partially inhibited during the time that DNA synthesis was blocked; thereafter, synthesis of these RNA species was normal. Matsuzaki and Nozu (1969) studied the formation of ribosomal subparticles in UV-irradiated $\text{B}_{\text{s}-1}$ cultures. They found that the synthesis of 16S RNA particles was resistant to UV-irradiation and that some smaller particles were formed which were not components of 30S ribosomal subunits.

Just as pyrimidine dimers may act as terminating lesions for DNA synthesis, they also act as transcription-terminating lesions for RNA. Michalke and Bremer (1969) did sedimentation experiments and showed that pieces of RNA synthesized by irradiated cells are smaller than those synthesized by unirradiated cells. Their model involves every pyrimidine dimer being a transcription terminator and thereby causing the RNA polymerase to drop off the DNA template when the dimer is reached. A

consequence of the synthesis of shortened messenger RNA molecules is that abnormally short polypeptide chains are synthesized (Brunschede and Bremer, 1969); this finding and the kinetics of protein synthesis show that shortened messenger RNA molecules are released normally from ribosomes upon completion of translation.

Sauerbier and co-workers have used the early termination of RNA chains for mapping the sequence of genes in transcription units of phage T4 (Hercules and Sauerbier, 1973, 1974), of T7 (Bräutigam and Sauerbier, 1973, 1974) and of *E. coli* (Hackett and Sauerbier, 1974). The method is based on the knowledge that genes in a transcription unit are transcribed sequentially, with the formation of a single-messenger RNA chain. Since a lesion in the DNA terminates transcription of the gene in which it lies and prevents transcription of genes lying beyond it, synthesis of the protein coded by the gene most distal from the initiation site in the transcription unit is most strongly inhibited. Likewise, the gene most proximal to the initiation site is most weakly inhibited, so that the slopes of the inactivation curves (% of normal synthesis vs. fluence) indicate the relative distance from the promoter site in the transcription unit. Thus, the method can be used to verify the position of genes mapped by other methods. Hackett and Sauerbier (1974) followed the synthesis of these ribosomal RNA components and have, by obtaining the inactivation constants, determined the gene order to be 16S, 23S, and 5S. This order agrees with that obtained by other mapping methods and shows that some of the results of earlier work on the inhibition of ribosomal RNA synthesis (see above) have a rational explanation. The principle of this method of determining the sequence of genes in transcription units explains the results of Starlinger and Kölisch (1964), who measured the ability of UV-irradiated *E. coli* cells to synthesize the three enzymes coded for by the *gal* operon and found that the sensitivities to UV radiation were in the same order as the order of the genes in the operon.

Several studies have been carried out on the effects of UV radiation on the induced synthesis of β -galactosidase. Inactivation of any of several genes in the *lac* operon by UV radiation could prevent synthesis upon induction, but the main cause is catabolite repression (Bowne and Rogers, 1962; Pardee and Prestidge, 1963, 1967), designated UV catabolite repression by Swenson (1972). Catabolite repression of the *lac* operon in unirradiated cells is most pronounced when the cells are grown on glucose, although all carbon sources cause repression (Magasanik, 1961). Glycerol is a weak catabolite repressor and is usually used for *in vivo* studies where the maximum synthesis upon induction is desired. Addition of cyclic 3',5'-adenosine monophosphate (cAMP) to induced cells under strong catabolite repression (glucose present) causes rapid synthesis of β -galactosidase (Perlman and

Pastan, 1968; Ullmann and Monod, 1968). Further details on the mechanism of catabolite repression are given in Section 12.

For studies on the effects of UV radiation on β -galactosidase synthesis, cells are usually grown on minimal medium with glycerol as a carbon source and supplemented with a low concentration of casamino acids. To prevent catabolite repression after UV irradiation, the cells are incubated in a medium lacking glycerol; under these conditions the effects of UV radiation are assumed to be mainly on the structural gene, although Pardee and Prestidge (1967) have not ruled out the possibility of cytoplasmic damage being involved. Pardee and Prestidge (1967) attempted to follow the repair of DNA lesions involved in the inhibition of the β -galactosidase-synthesizing system of *E. coli*. They induced cells and irradiated them 6.5 min later. There was partial inhibition of synthesis, and within 10 min recovery was complete. The UV fluence was 480 erg mm^{-2} , and unless there was preferential repair of genes in the *lac* operon, repair of DNA could not account for this rapid recovery. An alternative explanation is that the β -galactosidase messenger RNA already synthesized at 6.5 min is inactivated by UV radiation (Swenson and Setlow, 1964), and the recovery period represents time for the damaged messenger RNA to be destroyed and new messenger RNA to be formed.

Swenson (1972) irradiated *E. coli* B/r cells under conditions that assured severe UV-induced catabolite repression (glycerol and casamino acids both present) at 520 erg mm^{-2} ; synthesis of β -galactosidase was strongly inhibited for about 100 min and then increased. Addition of cAMP immediately after UV irradiation partially reversed the inhibition, thus supporting the contention that UV radiation inhibits the induced synthesis of β -galactosidase by catabolite repression. UV irradiation caused respiration and growth to cease after about 60 min (Hamkalo and Swenson, 1969), and under these nutritional conditions growth resumed at about 160 min. Thus, the differential synthesis (enzyme/mass) was low for about the first 100 min after UV irradiation and then increased dramatically. This increase was an indication of the release of the *lac* operon from catabolite repression. Photoreactivation and cAMP treatment are effective in causing an increase in the synthesis of β -galactosidase when given before 100 min. At this same time, PR treatment is no longer effective on growth. Thus, between 90 and 100 min, the *lac* operon is released from catabolite repression, and it may be the time at which repair of DNA is complete.

Pardee and Prestidge (1967) found that in the absence of catabolite repression (glycerol absent) a fluence of 1000 erg mm^{-2} inhibits induced β -galactosidase synthesis by 90%. Swenson's (1972) data (74% inhibition after 520 erg mm^{-2}) are in good agreement, if it is assumed that the rate of synthesis decreases exponentially with fluence; if so, the $1/e$ fluence in each

case should be about 400 erg mm⁻². Pardee and Prestidge (1967) estimated that a fluence of 300 erg mm⁻² should put, on the average, one dimer into the structural gene for β -galactosidase; therefore it seems reasonable that termination of transcription in this gene by pyrimidine dimers accounts for inhibition under these experimental conditions. Pardee and Prestidge (1967) and Swenson (1972) also found that, under conditions of catabolite repression (glycerol and casamino acids present), sensitivity of induced β -galactosidase synthesis to UV radiation is increased at least fourfold. Thus dimers in DNA, at sites other than the structural gene, may cause repression of the *lac* operon. Since UV-induced catabolite repression disappears as the result of photoreactivation treatment, pyrimidine dimers must be involved. UV catabolite repression of the *lac* operon is an example of how UV-radiation damage to DNA causes physiological responses through repression of operons. Another example is that of the arabinose operon (Bhattacharya, 1974) as indicated by the partial reversal of the inhibition of synthesis of *L*-arabinose isomerase by cAMP. Rifampicin completely blocked the reversing action of cAMP; this indicates that UV radiation inhibits induced enzyme synthesis at the transcription level.

Although most of the available evidence indicates that dimers act solely as blocks to terminate transcription, there is at least one study that presents results consistent with UV-induced photoproducts being transcribed. Yonei *et al.* (1969) determined the base composition of rapidly labeled RNA in UV-irradiated *E. coli* B/r (3500 erg mm⁻²) and B_{s-1} (100 erg mm⁻²). In both cases the purine-pyrimidine ratio became larger and the guanine-cytosine content smaller. In the B/r cells the ratio returned to normal after 2 h of incubation in the dark, but did not for *E. coli* B_{s-1}.

6.3. Respiration and Growth

Kelner (1953) examined the respiration of UV-irradiated *E. coli* B/r cells in his classic studies on photoreactivation of various processes inhibited by these radiations. Unlike DNA synthesis, which was severely inhibited by UV radiation, respiration in a manner similar to RNA and protein synthesis was only partially inhibited. Respiration was increased as a result of photoreactivation, and while the effect was not great, Kelner concluded that DNA in some way controlled the respiration of the cell.

In the 15 years or so that followed, the effect of UV radiation on respiration of *E. coli* was largely ignored by UV photobiologists, probably because under the conditions in which experiments were done, with rich media, and often with stationary cells, the effects produced were ill-defined.

Hamkalo and Swenson (1969) found that log phase *E. coli* B/r cells

grown on glycerol and 0.05% casamino acids respond to UV irradiation in a unique way with respect to respiration. During the first 60 min, respiration goes on at a near-normal rate and then ceases completely for a time, dependent upon fluence. Swenson and Schenley (1970a) later showed that when the B/r cells were grown on minimal medium with glycerol as a carbon source, a UV fluence of 520 erg/mm^{-2} (1.0% survival) caused respiration to be almost completely inhibited for several hours after being turned off 60 min after UV irradiation (Fig. 3); at lower fluences respiration resumes at times dependent upon the fluence. Respiration and growth are closely coupled processes in all cases except where protein and/or RNA synthesis are inhibited (Swenson and Schenley, 1970a,b). Hamkalo and Swenson (1969) observed that respiration ceased at about the time that DNA synthesis resumed, and hypothesized that the slowing down of metabolism takes place so as to correct for the unbalanced growth that occurred during inhibition of DNA synthesis. If resumption of DNA synthesis were the result of completion of DNA repair and also triggered the cessation of respiration, inhibition of repair by caffeine should result in continuous respiration. Instead respiration shuts off at 60 min (Swenson

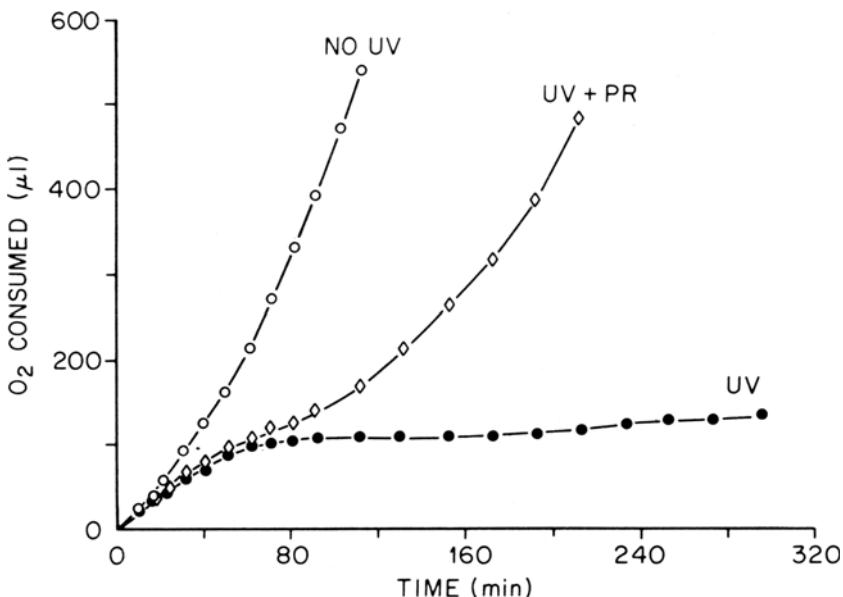


Fig. 3. Effects of UV radiation and of UV irradiation followed by photoreactivation (PR) treatment on the respiration of *E. coli* B/r. The cells were grown on minimal medium with glycerol as a carbon source. The UV fluence was 520 erg mm^{-2} ; the photoreactivating fluence at 405 nm was $4.8 \times 10^6 \text{ erg mm}^{-2}$. (Modified from Swenson and Schenley, 1970a.)

and Schenley, 1970a). From the work of Hamkalo and Swenson (1969) there were several reasons to believe that cessation of respiration was a consequence of UV radiation damage to DNA: (i) The period of cessation is greatly reduced by photoreactivation treatment (see also Fig. 1). (ii) Respiration does not cease in *E. coli* B_{s-1}, a strain in which DNA degeneration is extensive after UV irradiation (Suzuki *et al.*, 1966). (iii) After very high UV fluences (e.g., 7000 erg mm⁻² to *E. coli* B/r), respiration does not cease. (iv) Stationary-phase B/r cells do not cease respiration. If DNA were involved in a respiratory control system, the effect would be produced through RNA and protein. Swenson and Schenley (1970a) found that chloramphenicol, which prevents protein synthesis, keeps respiration from ceasing in irradiated cells (Fig. 4a). Rifampicin, which inhibits initiation of RNA synthesis (Hartman *et al.*, 1967; Maitra and Barash, 1969), has the same effect (Swenson and Schenley, unpublished) as does infection of irradiated cells with an amber mutant of T4 bacteriophage (Swenson and Schenley, 1970a). T4-infected cells are known to stop synthesis of bacterial RNA and proteins within 2 min after infection (Rouviere *et al.*, 1968). The treatment of UV irradiated cells with 5-fluorouracil (FUra) (Swenson *et al.*, 1974) also cause respiration to be maintained. FUra presumably acts by causing a miscoding error in a messenger RNA (Champe and Benzer, 1962; Horowitz *et al.*, 1960; Edlin, 1965; Rosen, 1965); thermal treatment may act by inactivation of a protein required for cessation of respiration. None of these treatments that maintain respiration are effective in causing resumption of respiration once it is turned off. Swenson and Schenley (1972) have proposed a model in which there is a gene under repressor control that codes for a protein involved in the control of respiration in unirradiated cells. Normally repressor action prevents formation of the protein. In an irradiated cell the control gene is derepressed and excess protein is formed, which causes respiration to cease. The 60-min period after UV irradiation is required for derepression, formation of the protein, and whatever intermediate events lead to cessation of respiration. Any interference with synthesis of RNA and protein or with the synthesis of authentic RNA should prevent the intermediate events from taking place, and respiration should be maintained.

Swenson and Schenley (1974a) tested the effect of UV radiation on the respiration of several repair-deficient mutants of *E. coli* strain WP2, a tryptophan-requiring derivative of *E. coli* B/r (Fig. 5). The *Hcr*⁻ strain which cannot carry out excision repair responds to UV irradiation in the same way as B/r and the WP2 strain, but much lower fluences are required (Fig. 5b). Respiration does not cease in *recA* or *lex* (*exrA*) cells after they have been given UV fluences between 30 and 500 erg mm⁻² (Figs. 5c and 5d,

respectively), and this lack of cessation was attributed to destruction of the respiratory control system through DNA degradation. However, another explanation is suggested by the fact that a number of UV-induced phenomena interpreted as being caused by derepression of operons do not occur in *recA* or *lex* cells (see Witkin and George, 1973, for summary). This point, as well as the significance to cell survival of cessation of respiration in *B/r*, *B*, and *uvr* cells, and maintenance of respiration by FURA and thermal treatments, will be considered later.

The immediate biochemical cause of cessation of respiration in UV-irradiated cells is not known, but associated with the cessation is the disappearance from these cells of pyridine nucleotides, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) (Swenson and Schenley, 1970b). Suspicion that pyridine nucleotides were involved was aroused as a result of experiments on cell-free respiration by fragments of the sonicated unirradiated and irradiated cells. Such fragments were tested for their ability to oxidize glycerol and a variety of metabolic intermediates. Unirradiated cell fragments showed good oxygen consumption with glycerol and all intermediates, but fragments of irradiated cells that had stopped their respiration before sonication could respire only when α -glycerophosphate or succinate was present. These substrates are the only ones whose dehydrogenases do not require pyridine nucleotides to transfer electrons to the electron transport system and eventually to molecular oxygen. Swenson and Schenley (1970b) further showed that at about 30 min after UV irradiation, pyridine nucleotides began to disappear from irradiated cells (Fig. 6); in the case of cells grown on glycerol where respiration and growth virtually ceased, they disappeared completely. In other cases, such as for cells grown on glycerol plus casamino acids and on glucose, the cessation of respiration and growth appear transitory (Figs. 6a, 6b) and, after an initial drop in the pyridine nucleotide level, these cofactors are replenished (Fig. 6c). The pyridine nucleotides are lost, unaltered, to the suspending medium (Schenley and Swenson, unpublished data). The loss is selective in that no other nucleotide or its di- or triphosphate is lost as completely as pyridine nucleotides (R. L. Schenley, J. X. Khym, and P. A. Swenson, unpublished data). In *E. coli* B_{s-1} , a strain in which respiration does not cease, pyridine nucleotide levels remain high (Swenson and Schenley, 1970b).

Fluences of UV radiation that cause almost complete cessation of respiration in *E. coli* *B/r* cells grown on glycerol cause only transient inhibitory effects when glucose is the carbon source; these transient effects are similar to those seen for glycerol-grown cells given lower fluences. Pyridine nucleotide losses by the irradiated glucose-grown cultures are also transitory (Swenson and Schenley, 1970b), and it has been demonstrated by the

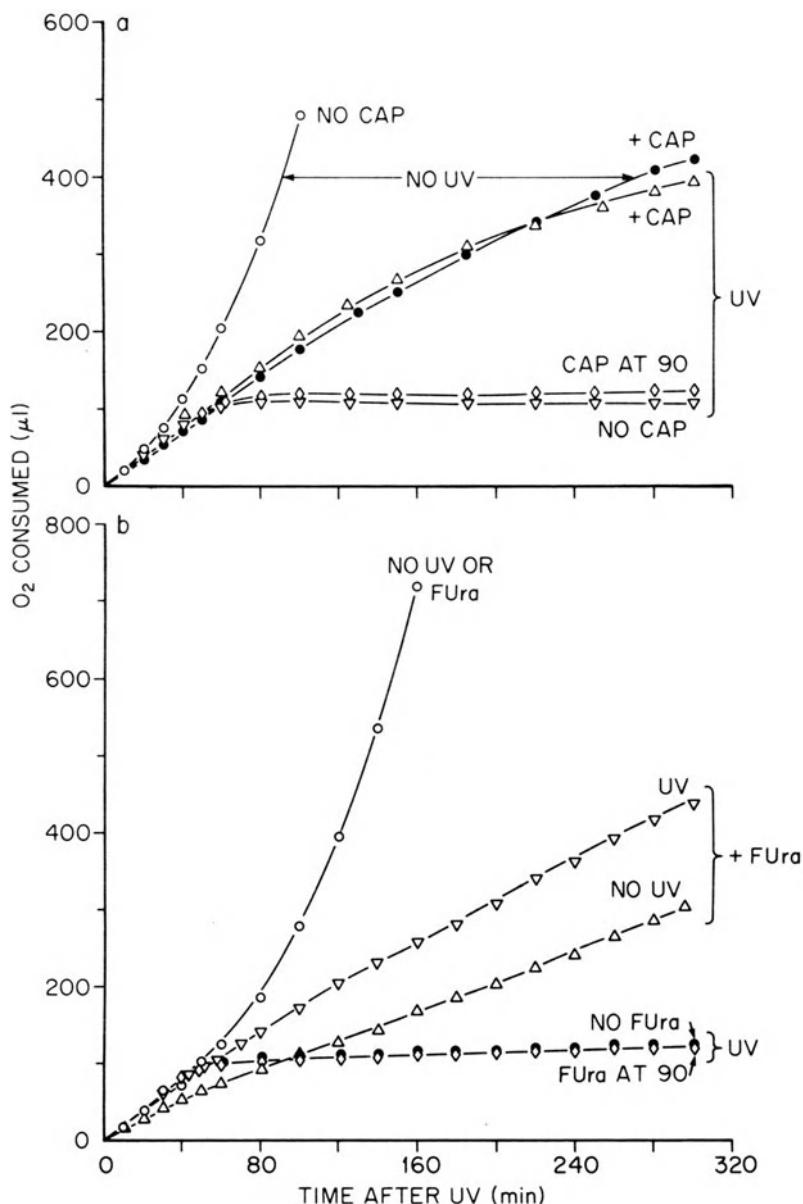


Fig. 4. The effect of (a) chloramphenicol (CAP) at $100 \mu\text{g ml}^{-1}$ and (b) 5-fluorouracil (FUra) at $50 \mu\text{g ml}^{-1}$ on respiration of UV-irradiated cells grown on minimal medium with glucose as a carbon source. CAP or FUra was added either immediately after UV irradiation or 90 min later. The UV fluence was 520 erg mm^{-2} . (From Swenson and Schenley, 1970a.)

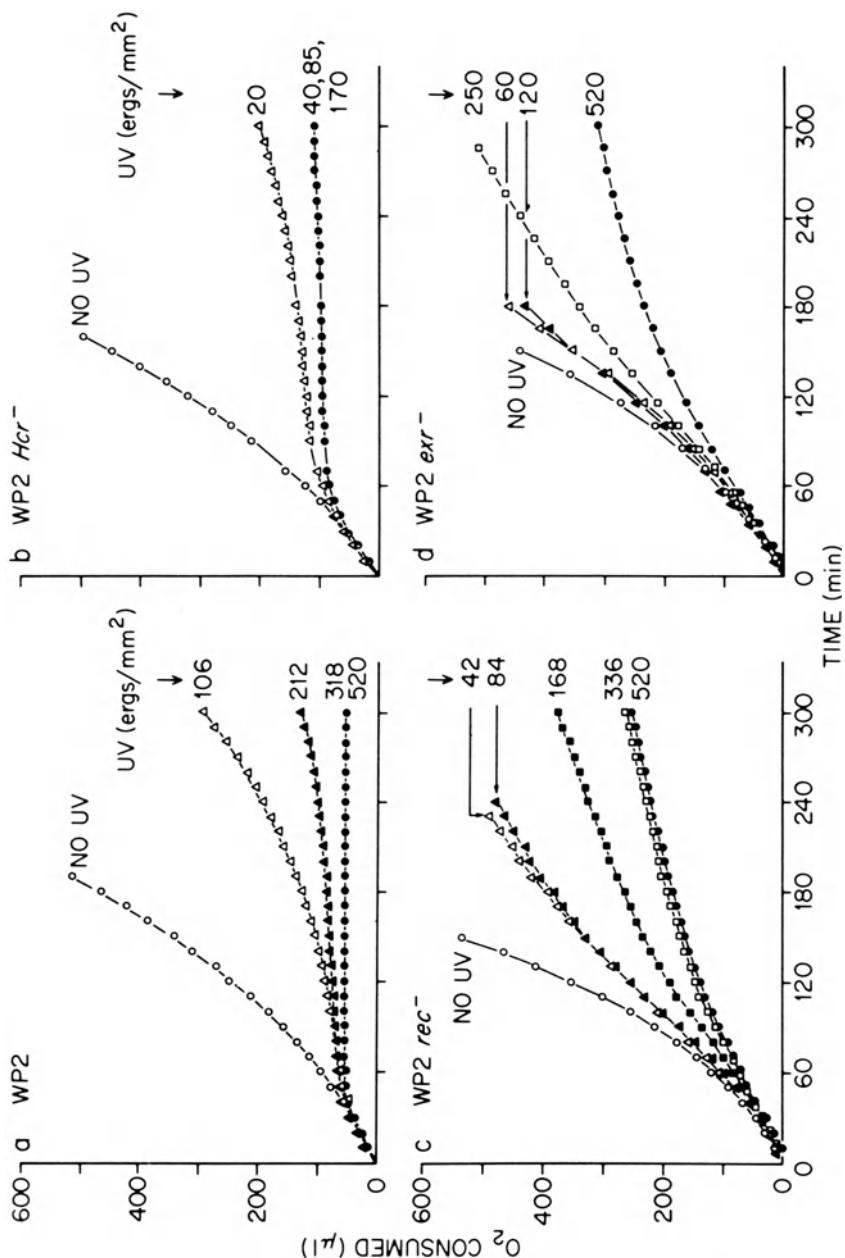


Fig. 5. Effects of UV radiation on respiration of *E. coli* WP2 and three repair-deficient mutants. All cells were grown on minimal medium containing glycerol as a carbon source. (Modified from Swenson and Schenley, 1974a.)

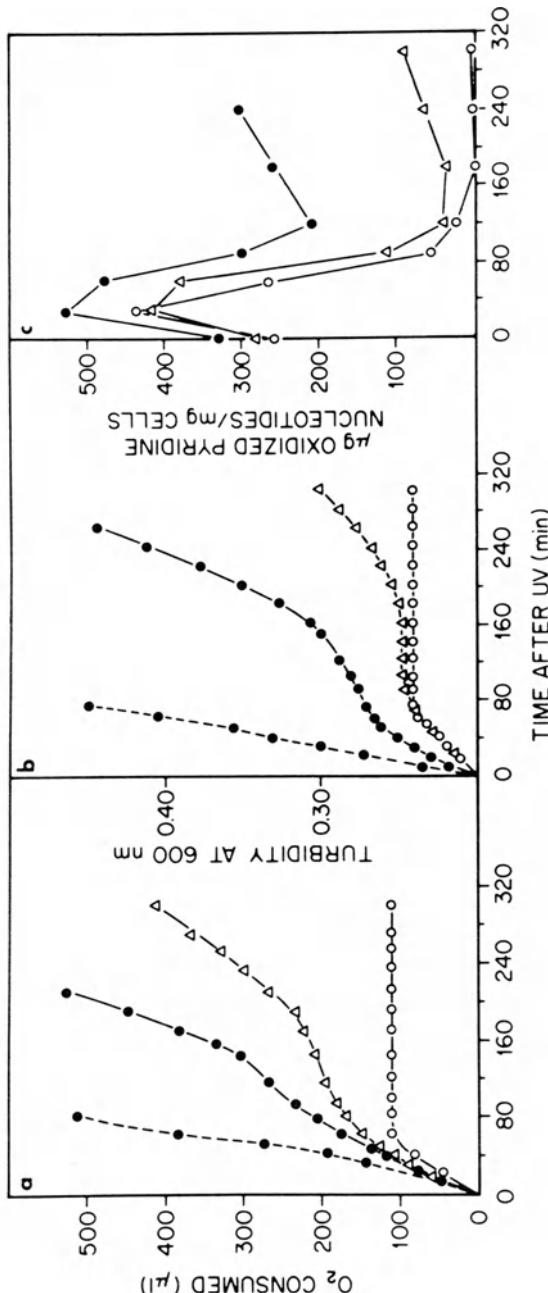


Fig. 6. Respiration, growth, and oxidized pyridine nucleotide levels in UV irradiated *B/r* cells that had been grown on three different media: (a) respiration; (b) growth; (c) oxidized pyridine nucleotides on the basis of cell mass. Symbols: (●—●) no UV; (○—○) UV M63gly; (Δ—Δ) UV M63gly + 0.05% casamino acids; (●—○) UV M63 glu. The UV fluence was 500 erg mm^{-2} . M63gly and M63glu denote a minimal medium containing glycerol and glucose, respectively, as carbon sources (from Swenson and Schenley, 1970b).

use of the detergent Triton X-100 that some irradiated cells in the glucose-grown cultures have stopped respiration irreversibly (see Swenson and Schenley, 1974b; and Section 6.4). Swenson (unpublished data) has recently found evidence that the respiration control system affected by UV radiation is subject to catabolite repression, and this may explain the different effects of UV radiation on glycerol-grown and glucose-grown cells (see Section 12). Respiration kinetics of *E. coli* B/r cells grown on nutrient broth do not show evidence for transient cessation of respiration but studies with the detergent Triton X-100 indicate that some cells do stop irreversibly (see Section 6.4). In rich media, growth by unaffected cells is so rapid that it masks cessation of respiration by a few cells. At high fluences of UV radiation the initial respiration and growth rates are less than those of unirradiated cells but soon the rates are equal.

Under their experimental conditions, other investigators have not found the shutting off of respiration after UV irradiation. Kössler (1971), e.g., grew *E. coli* B/r and B_{s-1} in rich medium and after irradiation with very large fluences of UV radiation (8.2×10^5 erg mm⁻²) suspended the cells in phosphate buffer plus glucose for measurement of respiration. These high fluences of UV radiation would be expected to destroy the genome and with it the respiratory control system of *E. coli* B/r; even at lower fluences the proposed control system could not function under conditions where RNA and protein cannot be synthesized. Kössler's findings were that strains B/r and B_{s-1} each had its respiration rate decreased by 50% as a result of the UV irradiation. When these cells were irradiated in the presence of 5-mM adenosine, a much higher survival resulted. This protection is doubtless caused by the absorption of far-UV radiation by this nucleoside.

In one of the few studies on the effects of ionizing radiations on respiration in *E. coli*, Billen *et al.* (1952) found that X rays (60 kR) caused some inhibition of respiration of strain B/r but did not cause this process to cease. The cultures were grown to stationary phase in nutrient broth, washed, irradiated, and suspended in phosphate buffer with glucose or other carbon sources for respiration studies. Swenson *et al.* (1971) found that when log-phase *E. coli* B/r cells were grown on minimal medium with glycerol as a substrate, 30 kR of γ radiation caused respiration to stop about 40 min after irradiation. This dose gives the same survival, about 1.0%, as the fluence of UV radiation (520 erg mm⁻²) which shuts off respiration. A similar response has been obtained with methylmethane sulfonate and mitomycin C at concentrations and exposure times that give 1.0% survival (P. A. Swenson and J. M. Boyle, unpublished data).

Growth of bacterial cells in liquid suspensions is usually measured by turbidity; increases in turbidity indicate increases in cell size, cell numbers, or both. Growth, so defined, is fairly closely coupled to respiration in unirradiated and UV-irradiated repair-competent *E. coli* cells (Hamkalo and

Swenson, 1969) and in *Hcr⁻* derivatives (Swenson and Schenley, 1974a). When respiration stops or resumes, corresponding growth changes take place. Coupling of these processes ceases after a time, depending upon fluence, in *recA* and *lex* (*exrA*) cells; respiration does not cease, but growth does (Swenson and Schenley, 1974a). Perhaps with the degradation of DNA in these strains, control over synthesis and growth processes is completely lost.

Under certain conditions growth is delayed in UV-irradiated cells in that for a time after irradiation the rate of growth is less than the control. Takebe and Jagger (1969) have studied this delay in resumption of the normal growth rate in *E. coli* B and found that the action spectrum resembles that obtained for killing, which indicates that the chromophore is either RNA or DNA. Growth delay by near-UV radiation is much more pronounced than by far-UV radiation and occurs at fluences that are lethal to only a small percentage of cells (Jagger, 1972).

Growth and cell division are not necessarily the same processes; the term "division" was used by Takebe and Jagger (1969) to mean cytokinesis. Thus, an increase in cell numbers or in viable cells indicates cell division. In a study on the effects of UV radiation on synchronously growing *E. coli* B/r cells, Helmstetter and Pierucci (1968) found that although DNA synthesis stopped for essentially all cells, some cell division continued for another 20 min after UV irradiation. This division was attributed to cells that had completed their rounds of DNA replication prior to UV irradiation. An initial increase in viability by irradiated cells incubated in liquid medium for a time before plating has been observed (Barner and Cohen, 1956; Okagaki, 1960; Smith, 1969; Swenson and Schenley, 1970b), and the increase is followed by a plateau or by a small drop in viability. This loss in viability is discussed in detail in Section 10.8. At later times after UV irradiation, dependent upon fluence, cell division resumes, as indicated by an exponential increase in viable cells (Smith, 1969; Swenson and Schenley, 1970b; Boyle and Swenson, 1971).

Witkin and George (1973) have measured delays in cell division after UV irradiation of both wild-type and *polA1* bacteria by a method involving respreading on agar plates. At a given fluence, the delay is longest for the *polA1* cells and is attributed to the reduced ability of these cells to close gaps opened by excision enzymes. As previously mentioned, the excision rates are approximately the same in the two strains (Boyle *et al.*, 1971).

6.4. Cell Envelope Changes

Ionic and nonionic detergents have little effect on certain respiring bacteria, but when poisoned with potassium cyanide (KCN), an inhibitor of the

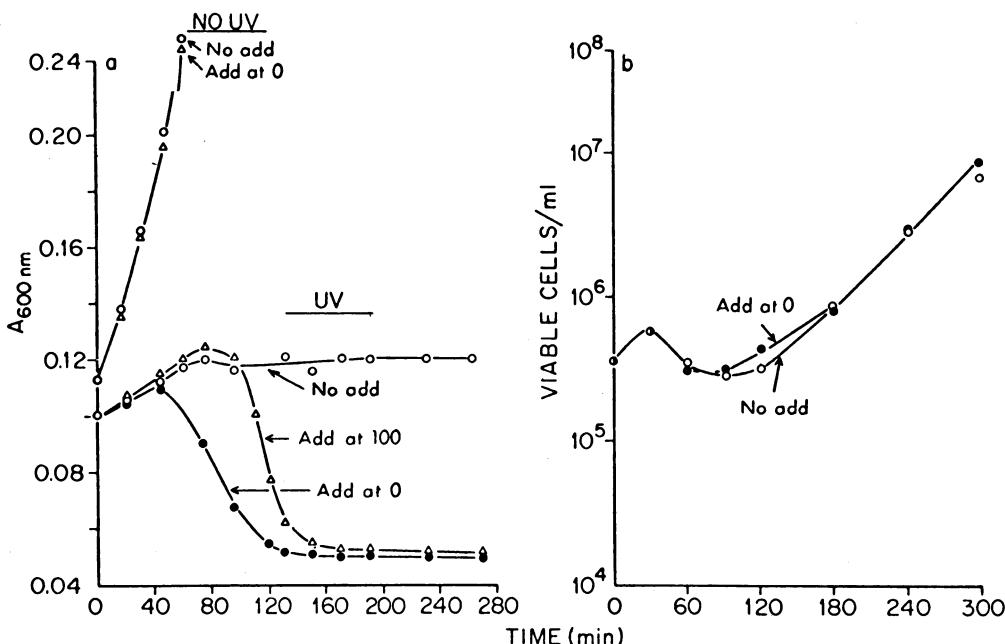


Fig. 7. Effects of Triton X-100 on the turbidity and viability time course of UV-irradiated (520 erg mm^{-2}) *E. coli* B/r cultures: (a) Turbidity; the times after UV irradiation at which the detergent was added are indicated. (b) Viability time course curves. Irradiated cultures with and without detergent were incubated in liquid minimal growth medium containing glycerol and were plated on the same growth medium plus agar at various times after UV irradiation. The final concentration of Triton X-100 was 0.7%. (Modified from Swenson and Schenley, 1974b.)

cytochrome system, the plasma (cell) membranes of *Halobacterium cutirubrum* (Lanyi, 1972) and of *E. coli* (Bolle and Kellenberger, 1958; Woldringh and van Iterson, 1972) are rapidly made soluble as indicated by turbidity losses. *E. coli* B/r cells under anaerobic conditions also are sensitive to treatment with Triton X-100 (Swenson and Schenley, 1974b). The loss of turbidity of detergent-treated *E. coli* cells under conditions where respiration was inhibited suggested that the loss would also occur when cells had their respiration shut off after UV irradiation. Figure 7a shows that this expectation was correct and that when Triton X-100 was added immediately after irradiation (520 ergs mm^{-2}), there was a delay of about 40 min before the loss of turbidity began (Swenson and Schenley, 1974b). At low UV fluences that permit resumption of respiration and growth, the detergent causes an initial loss in turbidity followed by an increase. The loss is interpreted as due to the destruction of plasma membranes of cells that have

stopped respiring; the rise is interpreted as growth of cells whose respiration does not cease. The detergent has no effect on the viability of either respiring unirradiated or irradiated cell cultures (Fig. 7b); thus, Swenson and Schenley (1974b) attribute the loss in turbidity to the dissolution of plasma membranes of cells that have irreversibly stopped respiration and are dead. The loss of turbidity of UV-irradiated cell suspensions in the presence of Triton X-100 has been observed in several growth media and is interpreted as an early indication of radiation cell death.

The reasons for the vulnerability of the plasma membrane to detergent action in nonrespiring cells is not known, but may be caused by respiratory enzymes being oriented differently in the membrane when respiration is stopped. The detergent-induced turbidity losses after UV irradiation may not be some unique consequence of UV-induced damage to DNA. KCN and anaerobiosis also make B/r cells sensitive to detergent (Swenson and Schenley, 1974b). It does not appear that UV radiation causes direct damage to any part of the cell envelope because respiration does not stop in UV-irradiated *recA* and *exrA* (*lex*) cells, and there is no loss in turbidity caused by Triton X-100 (P. A. Swenson and R. L. Schenley, unpublished data).

7. REACTIVATION OF BACTERIOPHAGE IN *ESCHERICHIA COLI* CELLS

7.1. Host-Cell Reactivation

Bacteriophage irradiated outside the host cells, including T1, T3, T7, and λ , must have their DNA repaired by a bacterial repair system if they are to survive; the process leading to this survival is called host-cell reactivation (HCR). The effect was discovered by Garen and Zinder (1955); the work on this subject through 1966 has been reviewed and summarized by Rupert and Harm (1966). Only *E. coli* that are *uvr*⁺ can carry on HCR; the strains that cannot or that do so to only a very small extent are sometimes designated *Hcr*⁻. Ellison *et al.* (1960) showed that *Hcr*⁺ can be experimentally distinguished from *Hcr*⁻ strains by plating complexes of irradiated host-cell reactivable phage and unirradiated bacteria and observing the slopes of the phage survival curves. Criteria for HCR are best given by quoting Rupert and Harm (1966): "(1) a relative resistance to UV of a nucleic acid infecting a cell, which is lost by 5-BU substitution for thymine in this DNA, by certain inhibitory substances or by sufficiently high radiation doses applied to the cell or by cellular mutation and which is (2) accompanied by a relative UV resistance of the cell itself, which is lost in the

presence of the same inhibitors or by the same cellular mutations acting under (1)." Examples of inhibitors of HCR are acriflavine (Feiner and Hill, 1963) and caffeine (Metzger, 1964; Sauerbier, 1964) which, as has been discussed in Section 1, are thought to interfere with excision repair of DNA.

The inability of *Hcr*⁻ cells to excise dimers from their own UV-irradiated DNA led to the correct inference that excision repair of irradiated phage DNA was carried out by bacterial host enzymes. This point was established by Boyle and Setlow (1970); however, the rate of dimer excision from bacterial DNA is 30 times faster than from phage DNA. In *Hcr*⁻ cells these authors observed the disappearance of 10–20% of the phage dimers; this disappearance of dimers may be attributed to their removal through recombination repair by host enzymes and by the *red* recombination system of λ phage (Signer and Weil, 1968). Competition experiments showed that host-cell enzymes preferentially excise dimers from host-cell DNA.

Liquid-holding recovery (LHR) processes are those in which UV-irradiated cells, held for a time in buffer or in other liquids, show increases in survival (Roberts and Aldous, 1949). The process involves, as will be elaborated on in Section 10.1, the same type of repair (excision repair) as is involved in HCR. UV-irradiated T3 complexed with *Hcr*⁺ cells and held in buffer or in broth plus chloramphenicol show good LHR effects. No LHR of T3 is obtained with *Hcr*⁻ cells (Martignoni and Harm, 1972). It will be recalled that chloramphenicol does not interfere with excision (Swenson and Setlow, 1966).

When UV-irradiated T1 bacteriophage infect *E. coli* B/r (*Hcr*⁺), a two-component survival curve is obtained; at low fluences of UV radiation inactivation is roughly exponential, but at higher fluences a break in the curve appears as the phage continue to be inactivated exponentially at a lower rate (Harm, 1967). Caffeine and acriflavine in the plating media caused the slope of the first component of the inactivation curve to decrease, but the slope of the second component did not change. Similar kinetics were generated when the phage were absorbed to irradiated host cells (Harm, 1974a). Upon increasing the concentration of acriflavine or caffeine or increasing the fluence to the host bacteria, the phage survival decreases, but the final slopes of the second component of the phage inactivation curves remain constant. Harm (1974a) found that maximum photoreactivation of absorbed complexes resulted in increased phage survival for all UV fluences to phage and changed the inactivation curve to one with a slight shoulder and a greater slope than the final slope on the nonphotoreactivated complexes. Maximum liquid-holding treatment of the complexes in buffer-plus-glucose for 210 min gave the same result (Harm, 1974b). When *B_{s-1}* cells were used as hosts for UV-irradiated T1, the phage inactivation curves did not show the second, less sensitive component at high fluences. Caffeine treatments (Harm, 1967,

1974a) and photoreactivation treatments (Harm, 1974a) caused simple dose-modifying effects.

These results and others led Harm (1974b) to propose that there are in B/r cells two modes of excision repair that lead to host-cell reactivation of T1 phage. The first is the most efficient and depends on the occurrence of a critical intracellular event necessary for all lesions to be repaired by this mode. If this event does not occur in a phage, the second, less efficient mode, becomes operative. He suggests that the critical event could involve an interaction between phage DNA and a structural element of the cell.

Even strain B_{s-1} cells are able to support the growth of irradiated phage T1, and it is of interest to know whether any of this phage survival is due to repair of DNA by these Hcr⁻ hosts. Harm (1973b) heavily UV irradiated B_{s-1} cells and found that the shoulder of the survival curve largely disappeared and the slope of the exponential region of the curve decreased. From this result he concluded that unirradiated Hcr⁻ cells confer a minor recovery effect on irradiated T1 phage. This minor recovery is not affected by caffeine or acriflavine and therefore is not thought to be due to normal HCR (excision repair) processes. He suggested that heavily preirradiated *E. coli* B_{s-1} cells are absolutely repairless and that, if this is so, one or two dimers properly placed should have a lethal effect. A single dimer in the region concerned with prereplication processes of the functional strand, or one dimer in each strand, should be lethal; if either strand is dimer-free, the phage should survive. The theoretical inactivation curve developed according to this model agrees very well with the experimental results.

One of the consequences of UV irradiating phage is that upon infection of an unirradiated *E. coli* cell there is a delay in the time required for the normal phage burst to appear (Luria, 1944; Garen and Zinder, 1955). Harm (1965a) studied this delay using UV-irradiated phages T1 and λ (both subject to HCR) and T4 (not subject to HCR) which infected either strain B (Hcr⁺) or B_{s-1} (Hcr⁻) host cells. In each case where several phage strains were irradiated to give the same survival on an Hcr⁺ and Hcr⁻ host, the delay of phage growth and growth kinetics on each host were the same. Since a greater growth delay was obtained on Hcr⁻ hosts than on Hcr⁺ hosts when phage were given identical UV fluences, Harm concluded that HCR repairs presumptive nonlethal lesions in DNA. Possible nonlethal effects of UV radiation may be the slowing down of transcription and DNA replication.

The influence of the polA1 mutation on host-cell reactivation of λ phage was studied by Klein and Niebch (1971). As for the bacteria themselves, the sensitivity of the UV-irradiated phage, when infecting this host mutant, was intermediate between those obtained when infecting the wild type (which permitted the highest survival) and a uvr strain. Also, as in

the bacteria themselves (Monk *et al.*, 1971), the sensitivity of the phage was the same when infecting the double mutant host (*polA1 uvr*) as when infecting a *uvr* host. These results support the idea that the *uvr⁺* gene and *polA1⁺* gene are both involved in the same excision repair pathway.

The way in which the repair work was shared by the excision and recombination repair systems on UV-irradiated λ DNA was studied by Radman *et al.* (1970). λ phages rely entirely upon the *E. coli* host for excision repair, but the λ *red* gene product provides information for the phage's own recombination repair system (Signer and Weil, 1968). The *red⁺* and *red⁻* phages were irradiated and used to infect *E. coli* strains that had all combinations of *rec⁺* or *rec⁻* and *uvr⁺* and *uvr⁻* genotypes. From the shapes and slopes of the eight survival curves, Radman *et al.* (1970) concluded that at low UV fluences most of the repair of UV-irradiated DNA is done by the recombination system and at high UV fluences most is done by the excision system. The increased sensitivity of *red⁻* compared to *red⁺* phage in any bacterial genotype indicated that the *red* system functioned in the repair of its irradiated DNA.

Radman *et al.* (1970) also studied the effect of inhibition of phage DNA synthesis on the repair of the phage DNA. Synthesis was inhibited in a temperature-sensitive phage mutant [λ cIO₂₈(ts)] that synthesizes DNA normally but stops doing so at 42°C. Phage survival increased in wild-type and *rec uvr⁺* cells as a result of holding the phage host complexes at 42°C for 30–100 min, but the *uvr* cells, whether *rec⁺* or *rec⁻*, were not affected. It appears that excision enzymes work best in the absence of DNA replication.

Radman *et al.* (1970) have developed a model in which the extent of repair by the two repair systems is controlled by the degree to which DNA synthesis is inhibited by UV radiation. Up to a certain "critical" fluence little inhibition takes place and, because semiconservative replication is going on, recombination repair is dominant. Above the critical fluence DNA synthesis is inhibited, allowing excision repair to take place; their idea is that for maximum efficiency of repair the timing of resumption of DNA replication is important. Fornili *et al.* (1971) have developed equations that generate survival curves that fit the experimental points obtained when UV-irradiated λ (*red⁺* and *red⁻*) infect wild-type *rec⁺* and *uvr⁺* and *rec uvr* cells of *E. coli*. Radman and Errera (1970) have also shown that inhibition of DNA synthesis in UV-irradiated *E. coli* favors excision repair.

7.2. *v*-Gene Reactivation

Certain bacteriophage, notably T4, carry the gene *v⁺* (formerly the *u⁺* gene; see Rupert and Harm, 1966, for review), which codes for an endonuclease specific for pyrimidine dimers (Friedberg and King, 1971). Bac-

teriophage T2 differs from T4 in not possessing the v^+ (u^+) gene (Luria, 1949; Streisinger, 1956), and, for reasons that are not understood, neither strain can be host-cell reactivated. See Rupert and Harm (1966) for a discussion of v -gene reactivation in the T-even phage. In a biological *tour de force* Harm (1968a) has put the v^+ gene system to work repairing the DNA of UV-irradiated B_{s-1} cells, thus increasing the survival of this Hcr^- strain. This feat was accomplished by infecting the cells with T4 phage irradiated with a UV fluence high enough to prevent phage development and host lysis but not high enough to inhibit the v^+ gene product, which functioned to produce an excision enzyme for the deficient B_{s-1} cells. In another series of experiments Harm (1973a) caused T4-mediated v^+ gene reactivation of phage T1 in an Hcr^- cell. The Hcr^- cells were infected with UV-irradiated T1 phage and then superinfected with heavily irradiated T4 phage. Harm (1973a) also found that caffeine and acriflavin, at concentrations that inhibit HCR completely, affect v -gene reactivation little or not at all. Witte (1972) has also shown that repair functions controlled by phage T4D are insensitive to caffeine. Several other T4 loci which appear to control repair are the x and y genes (Rupert and Harm, 1966; Boyle and Symonds, 1969).

7.3. Capacity of UV-Irradiated Cells to Support Phage Growth

Low fluences of UV radiation to many host bacteria have little effect on the capacity (Benzer and Jacob, 1953) of these cells to support growth of unirradiated phage. For example, when *E. coli* cells were irradiated with fluences nearly 30 times that required for one lethal hit, 50% of them showed some support of T-even phage growth (Anderson, 1948). Boyle and Swenson (1971) showed that under conditions where 520 erg mm^{-2} reduced survival of *E. coli* B/r to about 1%, the capacity to support growth of T4 phage did not begin to show reduction until about 5000 erg mm^{-2} . In the usual capacity study, cells are infected immediately after UV radiation. Boyle and Swenson irradiated *E. coli* B/r, grown with glycerol as a carbon source, and infected them with T4 phage at various times to see what effect the turning off of respiration had on capacity. The capacity remained near 100% for about 30 min after irradiation, then fell off exponentially until 120 min after irradiation, and then increased exponentially. Thirty minutes after UV irradiation is about the time at which cells become sensitive to detergent, which indicates, as Swenson and Schenley (1974b) have shown, that respiration has stopped irreversibly in the cells so affected. At 120 min the survivors in the irradiated population begin to divide, and the ratio of viable phage to viable bacteria is nearly constant, which indicates that only surviving (respiring) bacteria support phage growth.

When UV-irradiated T4 phage infects wild-type and *polA1* cells, the

phage survival is slightly reduced on *polA1* cells, as shown by George and Rosenberg (1972). However, these authors found that the capacity of UV-irradiated *polA1* cells fell off very rapidly to 0.0001% over a fluence range that hardly affected the *polA⁺* cells. Similar results were found by Monk *et al.* (1971) using bacteriophage λ .

7.4. UV Reactivation

Weigle (1953) discovered that when λ phage was irradiated with a constant UV fluence and then used to infect *E. coli* K-12S cells irradiated with varying fluences of UV radiation, the phage survival was greater at low UV fluences than with no UV at all. Other phages, including T3, but not T2 and T5, show UV reactivation (now sometimes called Weigle reactivation). *Hcr⁺* cells show the greatest amount of UV reactivation (UVR) and, because Harm (1963) found it to be present but greatly reduced for T3 and λ in some *Hcr⁻* cells (12S *Hcr⁻* and CSyn⁺) and absent for T3 in B_{s-1} (where no HCR was observed), he suggested that HCR and UVR were related. Harm (1963) proposed that UVR involved enhanced HCR activity. Kneser *et al.* (1965) argued this point because a combination of treatment with caffeine and substitution of 5-bromouracil (BrUra) for thymine in phage DNA fails to stop UVR of λ in four different *Hcr⁻* strains; they proposed that UVR involves a mode of repair different from what they termed "ordinary HCR." Harm (1965b) subsequently pointed out that Kneser *et al.* (1965) did not stop all HCR with these treatments to *Hcr⁺* cells and that even some *Hcr⁻* cells have some residual HCR which is not inhibited by caffeine or BrUra substitution. Kneser (1968) seemed partially to reconcile these two points of view when he worked with phage λ and strains of *E. coli* K-12 deficient in various combinations of *rec* and *Hcr⁻* functions. A surprising result, the significance of which was not appreciated at the time, was that only strains that were *recA* did not show UVR (Fig. 8). Because of the similarity of UV survival curves for strains B and *rec* of strain K-12, and also for B_{s-1} and *rec Hcr⁻* of strain K-12, he supposed that strain B and the *rec* mutant of K-12 lacked a common reactivation mechanism (K reactivation). The K-reactivation mechanism was thought to be the same as for UVR and residual HCR of λ ; UVR of T3 was thought to be handled by the "ordinary" HCR mechanism. Radman and Devoret (1971) demonstrated that *uvrA*, *uvrB*, and *uvrC* cells were all capable of carrying out UVR of λ bacteriophage. It is now well established that maximum UVR takes place in excision-deficient strains at a fluence that is about one-tenth that of excision-proficient strains (Radman and Devoret, 1971). Boyle and Setlow

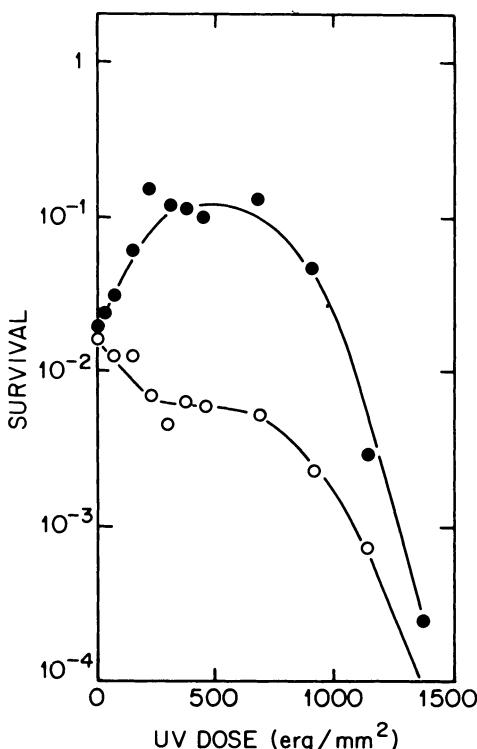


Fig. 8. Survival of UV-irradiated phage λ vir given a constant fluence of UV radiation (920 erg mm^{-2}) on UV-irradiated *E. coli* K-12 strains given increasing UV fluences. Symbols: (●) W3102 *rec*⁺; (○) 152 *rec*. (From Kneser, 1968.)

(1970) presented evidence that argued against UVR being the result of enhanced HCR, presumably involving enhanced dimer excision. They found, with an *Hcr*⁺ host infected with irradiated λ phage, that the rate of excision of dimers in λ DNA decreased as the number of host dimers increased (presumably due to a competition effect) over the range of UV fluences to the host that brought about UVR.

When phage λ infects *E. coli* cells, the linear phage DNA is converted to a covalent circular molecule. Shimada *et al.* (1968) isolated the closed circular molecules from infected cells, irradiated the DNA, and performed transfection assays on unirradiated and irradiated *uvr*⁺ bacteria. More closed circles were formed in the irradiated bacteria. No DNA damage was detected in these molecules by spheroplast transfection assay. An increase in the number of closed circular molecules was prevented by chloramphenicol, which indicated that UVR requires protein synthesis.

That UVR of irradiated phage does not take place in *recA* cells was

also shown by Ogawa *et al.* (1968) and Miura and Tomizawa (1968). The *lex* gene also is necessary for UVR (Defais *et al.*, 1971). As previously mentioned, both *recA* and *lex* strains extensively degrade their DNA after UV irradiation (Howard-Flanders and Boyce, 1966). The presence of the *recB* or *recC* genes in combination with *recA* prevents the degradation of both phage and bacterial DNA, but has no effect on phage survival (Kerr and Hart, 1972).

UVR through genetic recombination has been proposed several times (Garen and Zinder, 1955; Kellenberger and Weigle, 1958), and Hart and Ellison (1970) found support for this notion in that there is a good correlation between the percentage of host-phage homology and the extent of UVR. Blanco and Devoret (1973) designed an experiment to test the hypothesis that UVR of λ is due to a recombination between the DNA of irradiated phage and that of the host. They compared the extent of UVR in normal host cells and those containing an integrated but noninducible prophage. There was a slight increase in UVR in the prophage-containing cells, but less recombination between the prophage and infecting phage was seen in irradiated than in unirradiated cells. Thus, recombination does not appear to be responsible for UVR.

Although, shortly after the discovery of the *polA1* mutant, there were reports that it carried out little (Paterson *et al.*, 1971) or no (Ogawa, 1970) UV reactivation of phage λ , Caillet-Fauquet and Defais (1972) later demonstrated that phage λ is definitely UV-reactivated in this strain and suggested that the previous workers used UV fluences on phage and bacteria which were too high to elicit a good response. As previously mentioned, the only mutant strains known to be unable to carry out UV reactivation are *recA* and *lex*, and these strains cannot be mutagenized by UV radiation. The finding by Caillet-Fauquet and Defais (1972) that the *polA1* mutant can UV-reactivate phage λ is in line with the finding of Witkin (1971) that mutagenesis is normal in *polA1*.

The UVR discussed up to this point involves direct damage to the host DNA prior to phage infection or phage DNA transfection. Indirect UVR of UV-irradiated phage λ is brought about in an unirradiated $F^- lac\ E. coli$ bacterium into which is transferred Hfr or $F^+ lac^+$ DNA from an UV-irradiated donor bacterium (George *et al.*, 1974). The indirect UVR, shown in Fig. 9, appears in every way to be qualitatively the same as the direct type. The *lac⁺* gene in the donor DNA was for the purpose of determining the efficiency of conjugation.

In his original work on UVR, Weigle (1953) showed that phage mutagenesis accompanied the increase in phage survival. The *recA⁺* (Miura and Tomizawa, 1968) and *lex⁺* (Defais *et al.*, 1971) genotypes increase UV

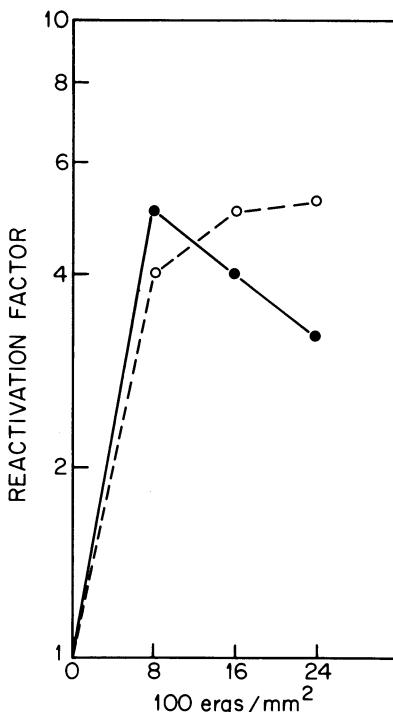


Fig. 9. Indirect UV-reactivation was determined on *E. coli* GY688 mated with UV-irradiated Hfr GY1151 (○) and with *F-lac⁺* GY854 (●). The titer of phage λ used was 3×10^6 ml⁻¹; its survival was 1×10^{-2} in this experiment. The percentages of recipients having acquired *Lac⁺* were 59% (cross GY854 \times GY688); 17% (cross GY1151 \times GY688). The reactivation factor is plotted on the ordinate, against the UV fluence given to the donor on the abscissa. (From George *et al.*, 1974.)

mutagenesis of phage. These genes are also necessary for UV-induced mutation in *E. coli* (Witkin, 1967b; Miura and Tomizawa, 1968; and Witkin, 1969a,b). Indirect UV induction of prophage λ mutations has been achieved by conjugation of F^- (λ) recipient cells with UV-irradiated F' donor cells (Miura, 1974).

7.5. Prophage Induction by UV Radiation

Thus far we have discussed UV radiation effects on irradiated virulent phage. Phage λ is a temperate phage and may either retain its integrity free of the DNA of the host or, when repressed, become integrated into the bacterial genome and become a lysogen (Hershey, 1971). Low fluences of UV radiation cause induction of the lysogen or prophage (Lwoff *et al.*, 1950). This process involves inactivation of the repressor and excision of phage DNA from that of the host. The λ then behaves like a virulent phage, multiplies, and lyses the host cell (Borek and Ryan, 1973). The induction process

does not take place in cells that are *recA* (Fuerst and Siminovitch, 1965; Brooks and Clark, 1967; Hertman and Luria, 1967) or *exrA* (*lex*) (Donch *et al.*, 1970, 1971).

The radiation-sensitive strain B_{s-1} isolated by Hill (1958) is unable to carry out HCR on UV-irradiated phage T1 (Hill and Feiner, 1964) presumably due to its inability to excise pyrimidine dimers (Setlow and Carrier, 1964). The gene responsible for these deficiencies is *uvrB* (Donch *et al.*, 1970). A λ prophage in B_{s-1} (λ) cannot be induced by UV radiation (Donch *et al.*, 1970); this is due to the *exrA* (*lex*) mutation (Greenberg, 1967). The *exrA* (*lex*) mutation, along with the *uvrB* gene, gives *E. coli* B_{s-1} additional sensitivity to UV radiation.

As with indirect UVR, indirect induction of λ has been achieved in an F^- host by conjugation with a UV-irradiated F^+ donor (Borek and Ryan, 1960). The indirect induction requires transfer of an entire UV-damaged replicon (Devoret and George, 1967; Rosner *et al.*, 1968; George and Devoret, 1971).

With this background we now turn to the effect that a prophage in an unirradiated host has on the repair of an irradiated infecting phage.

7.6. Prophage Reactivation

The abilities of unirradiated and UV-irradiated virulent mutants of phage (λv) to form plaques when infecting *E. coli* host cells with and without an integrated λ prophage were studied by Jacob and Wollman (1953). They found that when both host strains were infected with unirradiated λv , the same number of plaques was observed but, when infected with irradiated λv , more plaques appeared with the lysogenic strain than the nonlysogenic strain. Prophage reactivation, as it is called, occurs only when the prophage in the host is noninducible, and the effect is small compared with that of host-cell reactivation. Prophage reactivation occurs equally well in *uvr⁺* and *uvr* cells (Hart and Ellison, 1970; George and Devoret, 1971) and in *lex⁺* and *lex* cells (Blanco and Devoret, 1973). According to Hart and Ellison (1970), prophage reactivation does not occur in *recA* cells; but Blanco and Devoret (1973) reported that *recA* cells showed about 25% of the reactivation of *recA⁺* cells. Blanco and Devoret (1973) further characterized prophage reactivation when they found that no mutations were produced despite a high rate of recombination between the irradiated infecting phage and the prophage in the unirradiated cell. In addition, they found that the presence of the *red* mutation (absence of an exonuclease involved in recombination) in the infecting phage prevented prophage reacti-

vation in *recA* lysogenic bacteria. Blanco and Devoret (1973) concluded, as had Hart and Ellison (1970), that prophage reactivation involves recombination between the infecting phage and the prophage, and pointed out that other repair processes such as multiplicity reactivation (Luria, 1947) and cross-reactivation (marker rescue) (Doermann, 1961) also involve recombination. The important difference lies in the fact that for prophage reactivation only the infecting phage is irradiated.

8. FILAMENT FORMATION

The emphasis in this review is on death and on recovery of repair-competent cells from UV-radiation damage. Much of the older work was done with strain B and its derivatives but, with the advent of knowledge concerning the genetics of radiation sensitivity and repair, the use of K-12 strains became popular. The main reason is that K-12 is a sexual line and offers ease of genetic manipulation in constructing special multiple mutants. The disadvantage of the nonsexual B line has been offset to some extent by the use of universal transducing phage for the transference of genes into strain B and its derivatives. Many postirradiation treatments used for enhancing recovery of *E. coli* from UV irradiation were discovered with strain B and are much more effective in this strain than in its B/r (radiation resistant) derivative. Before considering these conditions, the differences in radiation sensitivity between strains B and B/r will be discussed in terms of the formation of filaments after irradiation.

Escherichia coli B and its derivative, strain B/r, are repair-competent strains, but the latter is more resistant to UV radiation (Witkin, 1946). Strain B forms long nonseptate filaments upon incubation in growth medium after low fluences of UV radiation (Witkin, 1947; Errera, 1954) or other treatments that cause inhibition of DNA synthesis. Filament formation is genetically controlled, and the phenotypes of *E. coli* B and B/r cells are sometimes designated as *Fil*⁺ and *Fil*⁻, respectively. Howard-Flanders *et al.* (1964b) isolated a radiation-sensitive K-12 strain, *lon*, that formed filaments after exposure to UV or ionizing radiation. The mutants formed watery mucous colonies when plated on rich solid media. The *lon* gene may be identical with *capR*, a gene that regulates mucopolysaccharide synthesis (Markovitz, 1964; Markovitz and Rosenbaum, 1965; and Markovitz and Baker, 1967). Like *E. coli* B, the survival curve of a K-12 *lon* strain is exponential at low fluences of UV radiation and shows a discontinuous slope at about 10% survival (Howard-Flanders *et al.*, 1964b). A *lon uvr* strain is much more sensitive to UV radiation than one with either single mutation,

and the fact that the *lon* mutant can carry on host-cell reactivation shows that the two mutants are radiation-sensitive for different reasons (Howard-Flanders *et al.*, 1964a).

Phage can transduce genes from strains B and B/r into wild-type K-12 strains, and the radiation-sensitive colonies selected for are *lon* and mucoid (Donch and Greenberg, 1968b; Donch *et al.*, 1969). The nonfilamentous character of B/r cells is due to a mutation (*sul*) which suppresses the *lon* gene (Donch *et al.*, 1969). The *lon* mutant is deficient in the ability to carry out cross-wall formation during septation after UV irradiation (Howard-Flanders *et al.*, 1964b) and after X irradiation (Adler and Hardigree, 1964). Filaments may be formed equivalent to 100 normal cells containing apparently normal nuclear bodies (Adler and Hardigree, 1965). DNA, RNA, and protein synthesis continue during growth and filament formation and are present in the same quantities and ratios as in normal cells (Deering, 1958). Significantly, introduction of the *lon*⁺ allele into an irradiated *lon* cell causes cell division (Walker and Pardee, 1967). Very low fluences of UV radiation that have only slight effects on survival will cause filament formation in nearly every cell of an *E. coli* B culture (Deering and Setlow, 1957). Thus, many filaments eventually divide; others grow to a critical length and then lyse (Kantor and Deering, 1966). Walker and Pardee (1968) envisioned an overproduction, in *lon* cells, of a cell envelope precursor that is diverted to mucopolysaccharide production in unirradiated cells plated on minimal media. In irradiated cells this precursor is thought to inhibit the functioning of a process concerned with a link between DNA replication and septum formation.

Witkin (1967a) pointed out the similarities between prophage induction and filament formation in *lon* cells. Both are mass effects caused by low fluences of UV radiation and by other agents that inhibit DNA synthesis. In the case of UV radiation, fluences that produce 10–20 pyrimidine dimers per bacterium will trigger off these events, suggesting that damage anywhere in DNA is effective. Pyrimidine dimers seem to be the effective lesions because both effects are prevented by photoreactivation. Protein synthesis is required for each to occur because chloroamphenicol after UV irradiation prevents them from taking place. In the case of λ induction it is certain that early proteins must be synthesized for excision of the λ prophage. Specific proteins involved in filament formation are not known, but Witkin (1967a) hypothesized that a protein which inhibits cell division was formed upon derepression of an operon. Witkin's hypothesis of filament formation states that as long as the operon is derepressed and growth continues, filaments will form. When the operon is once more repressed, presumably upon completion of repair of the DNA, the inhibitor protein

ceases to be formed, and division of the filaments occurs. Division may occur if the threshold concentration of the inhibitor falls below a certain level in a growing filament. If a filament reaches the critical length before repair or dilution occurs, recovery is not complete and death results.

Other similarities between UV-induced filament formation and prophage induction have been found. Filament formation in *lon* cells does not take place in cells that are *recA* (Green *et al.*, 1969b) (see Fig. 10) or *lex* (Donch *et al.*, 1968), nor does induction of integrated prophage λ take place in cells that are *recA* or *lex* (see Section 7.5). Despite the fact that *lon* cells do not form filaments after UV irradiation, they are mucoid on rich, solid medium when they carry *recA* (Green *et al.*, 1969b) or *lex* (Donch *et al.*, 1968) mutations.

Survival through division of filaments in UV- or X-irradiated *lon* cultures has been increased by adding cell extract from *E. coli* B/r (Fisher *et*

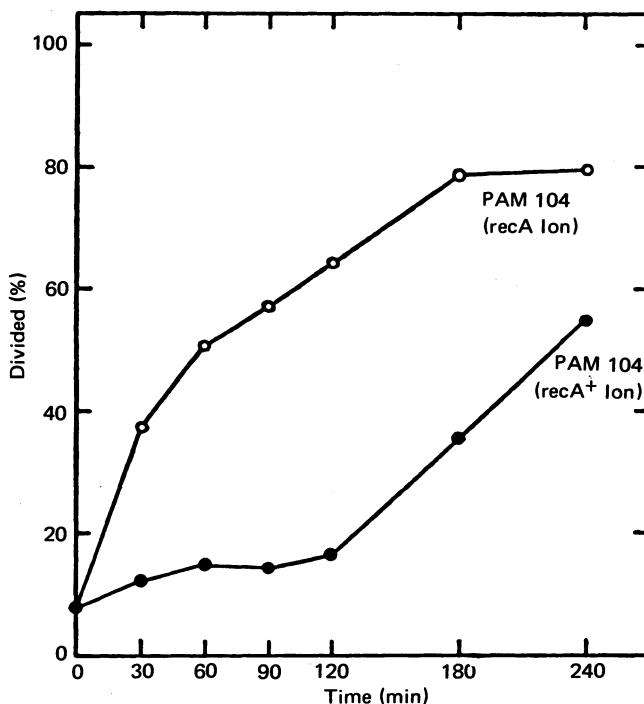


Fig. 10. Effects of UV irradiation (10 erg mm^{-2}) on cell division in *recA*⁺*lon* and *recA**lon* cultures. (From Green *et al.*, 1969b.)

al., 1969). These results give credence to the notion that *lon* cells are deficient in a cell-division substance. Division of filaments, followed by colony formation, can also be brought about in a number of ways to be discussed in Section 10. Most of the pre- and postirradiation treatments are also effective in increasing survival of *E. coli* B/r.

9. INFLUENCE OF GROWTH PHASE ON SURVIVAL AFTER UV IRRADIATION

Escherichia coli B, like other bacteria, is more sensitive to UV irradiation during the growth phase than during the stationary phase (Durham and Wyss, 1956; see Zelle and Hollaender, 1955, for earlier observations). The possibility that the amount of initial damage to DNA of *E. coli* 15 T⁻A⁻U⁻ cells (which require thymine, arginine, and uracil, as well as proline and methionine) varied in the different growth stages was investigated by Ginsberg and Jagger (1965). They gave photoreactivating treatment to log-phase and early stationary-phase cultures and found identical photoreactivable sectors, identical rates of photoreactivation after given UV fluences, and identical linear relationships for total light required for maximum photoreactivation as a function of UV fluence. Thus the amount of UV-induced DNA damage is independent of growth phase.

The sensitivity to UV radiation of *E. coli* B/r and B_{s-1} cells at various times during growth, from log phase to stationary phase, was measured by Morton and Haynes (1969). The survival of B/r cells grown on nutrient broth, irradiated at a constant fluence, and plated on nutrient agar, fluctuated widely over a 12-h incubation period. They were least sensitive during the early log phase, which showed, in fact, a slight loss in sensitivity compared to log phase. During late log phase the sensitivity increased nearly 100-fold. As the cells entered stationary phase they became less sensitive again. Survival-curve data indicated that changes in sensitivity were caused primarily by variations in the shoulders of the survival curves rather than by changes in the final slopes. The extra sensitivity during late log phase can be reversed by transfer of the cells to fresh medium. Compared to strain B/r, the sensitivity of strain B_{s-1} cells to UV radiation is relatively constant as a function of growth phase. Morton and Haynes (1969) suggest that the variations in sensitivity of B/r cells during various growth phases is caused by differences in intracellular concentrations or activities of repair enzymes.

This type of approach was enlarged upon by Tyrrell *et al.* (1972), who used four strains of *E. coli* K-12: wild-type, *uvrA*, *recA*, and the double

mutant, *uvrA recA*. The strains were grown in nutrient medium and, after irradiation, plated on nutrient agar. Fluences were selected that reduced survival of 24-h-old cells to 0.1%. The response pattern for viability vs. time over a 12-h period was quite similar to that obtained by Morton and Haynes (1969) for strain B/r, except that during early log phase a larger increase in survival was observed; the dramatic increase in sensitivity during late log phase was seen, as was the decrease in sensitivity as the cells entered stationary phase. The *uvr* and *uvr rec* strains showed similar responses; after a small initial decrease in sensitivity during early log phase, the sensitivities increased to their original levels during late log phase and remained constant. The *rec* strain displayed an immediate increase in sensitivity that extended throughout the log phase and then increased steadily. The changes in sensitivity seemed to be associated more with changes in the shoulder rather than in the slope of the curves.

The relative constancy of sensitivity of the *uvr* cells during early log phase suggests (Tyrrell *et al.*, 1972) that the level of activity of *rec* system enzymes is fairly stable. The increase in sensitivity of the *rec* cells during this period suggests that activity of excision-system enzymes decreases; the decrease in sensitivity during stationary phase suggests the opposite is the case. These notions are supported by the fact that both the *uvr* cells and the *uvr rec* cells have the same pattern of UV response to equivalent fluences over the entire growth period.

In studying the UV radiation sensitivity at different stages of growth, one is presumably working with rather uniform cultures at late stationary phase, but while the cultures are growing there is a distribution of cells of different ages in the culture. With the increased study of DNA replication and cell division during the past decade, and with the knowledge that the physiological state of the cell determines its radiation sensitivity, it is natural to inquire about the UV sensitivity of the cell at various stages during the cell cycle. Two papers have dealt in detail with this topic and they give different answers to the question. Helmstetter and Uretz (1963) used synchronously dividing populations of *E. coli* B and found a sharp drop in sensitivity in the middle and at the end of the division cycle. Kubitschek *et al.* (1973) separated *E. coli* B/r and B_{s-1} cells by size (and thus cell age) on sucrose gradients and found no differences in sensitivity throughout the cell cycles for either strain. Helmstetter and Uretz (1963) used rapidly growing cultures while Kubitschek *et al.* (1973) grew their cells slowly with a limited amount of glucose to ensure that cells were largely uninucleate and that DNA was replicated over only part of the cell cycle. It would seem that these latter conditions would accentuate any differences occurring during cell growth. Kubitschek *et al.* (1973) feel that the model for cell killing

which best fits their results is that UV-induced lesions in newly replicated DNA are not lethal. The results of neither of these papers seem to be in accord with the results of Morton and Haynes (1969) and Tyrrell *et al.* (1972) with growing, unseparated, and unsynchronized cultures.

10. POST-UV-IRRADIATION TREATMENTS

10.1. Liquid Holding

The work on recovery processes prior to 1965 has been reviewed by Rupert and Harm (1966). In general, I shall not deal in detail with papers covered in that review except for key ones which laid the foundation for, or which seem especially relevant to, recent studies.

Although earlier workers noticed similar effects, the work of Roberts and Aldous (1949) showed clearly the large recovery effects that could be brought about by holding UV-irradiated *E. coli* B cells in a variety of liquid media before plating. Phosphate buffer is often employed to minimize the growth and division of the cells. In the cell line of *E. coli* B, repair competent strains such as B (Roberts and Aldous, 1949; Jagger *et al.*, 1964) and B/r (Harm, 1968c) show liquid holding recovery (LHR), but strain B_{s-1} responds only slightly (Castellani *et al.*, 1964; Harm, 1968c). Repair competent *E. coli* K-12 strains, their *uvr*, *recB*, and *recC* derivatives do not exhibit LHR, but five *recA* strains and *rec-56* do show the effect (Ganesan and Smith, 1968a). These results and others (Ganesan and Smith, 1969) indicate that, in *E. coli* K-12 strains, the *uvr⁺* gene is necessary but not sufficient for LHR, but that the *recA* mutation allows LHR to be observed.

It was pointed out by Ganesan and Smith (1968a) that some repair (probably excision repair) is necessary for demonstration of LHR, and that repair is inhibited by yeast extract (in the plating agar). If plated on minimal medium, many of the cells that would be inhibited on yeast extract recover and only a small liquid-holding effect is seen. Ganesan and Smith (1968a,b) added a low concentration of yeast extract (0.075%) to *recA* cells in liquid-holding buffer and almost completely inhibited, without delay, any further LHR (Fig. 11). A similar observation had been made on *E. coli* B cells by Alper and Gillies (1960a), who incubated irradiated cells in buffer or broth for 45 min before plating and found that survival was highest for cells incubated in buffer. The inhibiting action of yeast extract on LHR is prevented completely in *recA* cells when chloramphenicol is also present in the buffer (Ganesan and Smith, 1968b). DNA is extensively degraded during the liquid-holding period when yeast extract is present. Degradation is less when chloramphenicol is also present, but it is still greater than when

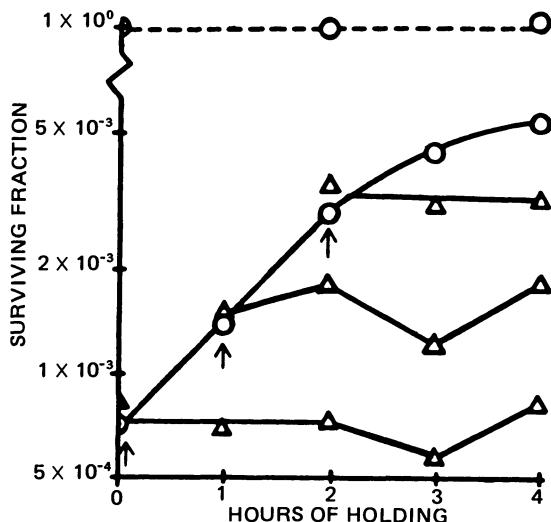


Fig. 11. Inhibition of LHR in *E. coli* B by yeast extract. Cells were held in buffer (○). Yeast extract (Δ), 0.075% (final concentration), was added at the times indicated by the arrows. Solid lines indicate unirradiated cells; dotted lines, the unirradiated controls. (From Ganesan and Smith, 1968a.)

the irradiated cells are in buffer alone. Thus DNA degradation and its prevention does not entirely account for the actions of yeast extract and chloramphenicol on LHR in *recA* cells.

The requirement of the *uvr⁺* gene in *E. coli* K-12 for both HCR and LHR strongly suggests that both reactivations are brought about by the excision repair system. This idea is substantiated by the fact that both HCR (see Section 7.1) and LHR (Harm, 1966) are inhibited by caffeine and acriflavine, inhibitors of the excision repair system. Harm (1966) has proposed that LHR is most likely to occur in irradiated cells where HCR (excision) repair on a given plating medium does not reach its full extent.

Although there is a great deal of circumstantial evidence implicating excision repair in LHR, it is appropriate to note that in only one case has excision been measured under conditions approximating those under which liquid-holding experiments are usually carried out (Setlow and Carrier, 1964). It is perplexing that under starvation conditions excision is completely inhibited in *E. coli* B/r for at least 1 h after irradiation (Setlow and Carrier, 1968).

The course of LHR, obtained by plating samples at various holding times, is roughly exponential until complete LHR is reached (Harm, 1966). As in any other procedure in which plating is used to measure repair or

recovery by the ability to form a colony, one must wait 24 or more hours for the colonies to be large enough to count. While the end result is clear, the course of events is not: repair and recovery are complex processes and one does not know how much happened in the liquid medium and how much happened on the plate. Harm (1966) determined in the following way that most if not all of the LHR in UV-irradiated *E. coli* B cells takes place in the liquid medium. Liquid holding of irradiated cells was carried out in buffer, and the cells were plated on solid medium with or without caffeine. Immediately after UV irradiation, the viable count was 10-fold higher on the plates not containing caffeine, but 4 h later almost the same count of viable cells was obtained on both types of plates. Thus, all the LHR took place in the liquid medium. Further evidence was obtained in the same type of experiment by including caffeine in the liquid medium and plating on solid medium without caffeine. Caffeine held the number of potentially viable irradiated cells fairly constant during liquid incubation. Apparently repair and recovery processes were held in check by caffeine, but any cell capable of surviving in the absence of caffeine was capable of completing its repair and recovery events after being transferred to a plate without caffeine. When irradiated cells were held in liquid medium containing caffeine and then transferred to solid medium also containing caffeine, some of the cells capable of surviving on plates without caffeine were unable to complete repair and recovery processes and died. Similar results were obtained with acriflavine (Harm, 1966).

The conclusion by Harm (1966) that LHR events take place in the liquid medium is reinforced by unpublished experiments of Jagger (cited by Harm, 1966) that the division delay of UV-irradiated cells after plating is the same for cells plated immediately as for cells given LHR treatment for several hours; likewise, there was no difference in the growth patterns of the two groups of cells after being placed in nutrient liquid medium. All of these results argue against the hypothesis (Jagger *et al.*, 1964) that LHR caused growth delay on the plate, thereby allowing extra time for repair of DNA.

It is well-known that lethal effects of UV radiation can be reversed by photoreactivation (PR) treatment and that this reversal with visible light (e.g., 405 nm) is due to the monomerization of pyrimidine dimers in DNA (J. K. Setlow, 1967); there is a large overlap between the population of irradiated cells affected by PR treatment and those affected by LHR treatment in *E. coli* B. Likewise, in *E. coli* B there is complete overlap between photo-protection (PP) and LHR (see Section 11). In these studies on overlapping effects the PR treatment was given immediately after UV irradiation. Harm (1968c) used PR treatment after various times of LHR treatment, assuming that any dimers still unexcised during LHR treatment would be susceptible to

splitting and would lead to survival (Fig. 12). After 600 erg mm⁻², *E. coli* B cells required some 60 h for maximum LHR to occur (Harm, 1968c); PR treatment plus LHR treatment gave relatively constant total viability at all times during LHR treatment. For UV-irradiated B/r cells (1000 erg mm⁻²), LHR increased linearly for about 96 h, but the total viability for LHR and PR treatments dropped rapidly until, at about 45 h, PR treatment no longer had an effect (Harm, 1968c). Thus, PR repairs lesions that would otherwise be dark repaired through LHR, given sufficient time. The loss of PR ability and total viability between 0 and 40 h could indicate that dark repair of a given lesion is only partially complete; incision or excision may have occurred, thus making the lesion irreparable by PR treatment. One other assumption must be made: completion of dark repair of the lesion can take place only in the buffer medium. As Harm (1968c) puts it, excision repair (during LHR) does not always end up in (complete) dark repair. Dose

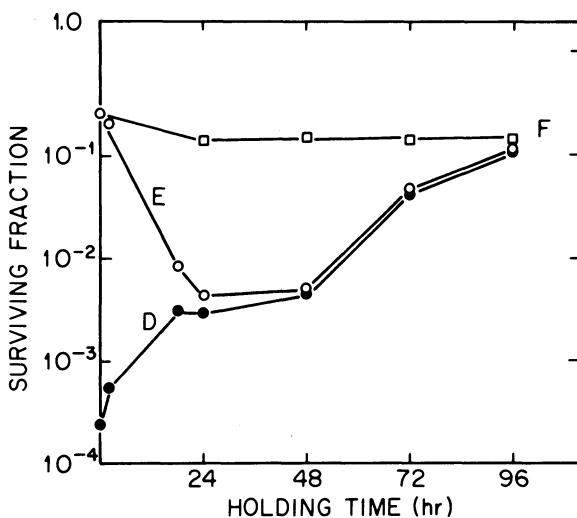


Fig. 12. Survival of *E. coli* B/r, UV-irradiated with 1000 erg mm⁻², as a function of the period of holding after irradiation. The letters refer to the particular conditions outlined in the scheme below.

- (D) UV —→ LH(*t*) —→ plating
- (E) UV —→ LH(*t*) —→ PER —→ plating
- (F) UV —→ PER —→ LH(*t*) —→ plating

In this scheme, UV stands for irradiation with a fluence of 1000 erg mm⁻²; PER stands for photoenzymatic repair during a 50-min illumination; and LH(*t*) stands for liquid-holding for varying times *t*, represented by the abscissa. Several control curves (A, B, and C) have been eliminated from the original figure. (Modified from Harm, 1968c.)

(fluence) fractionation (sector) irradiation studies described below show that complete repair can take place in buffer. In agreement with Ganesan and Smith (1968a, 1969), Harm (1968c) showed that *E. coli* K-12 *uvr* cells showed no LHR and *rec* cells did. *E. coli* B_{s-1} showed some LHR and could still be photoreactivated after 120 h of LHR. The strain showing the least LHR was *recA uvrA*.

LHR studies show that even repair-competent cells under certain laboratory conditions do not efficiently carry out their repair and recovery processes. Harm (1968b) has advanced the idea that mutual interference of repair enzymes with each other at lesions close to each other might prevent repair. He has shown (Harm, 1968c) that, for large total UV fluences, very high survivals of *E. coli* B/r were obtained (Fig. 13) by lowering the fluence rate from $8 \text{ erg mm}^{-2} \text{ s}^{-1}$ to $0.022 \text{ erg mm}^{-2} \text{ s}^{-1}$. The cells seemed to repair the lesions almost as fast as they were put into the DNA. In another experiment, Harm (1968b) used sector irradiation, fractions of a total fluence given sequentially but with each fraction of fluence separated by periods of LHR. Such a procedure gave much larger survival for any cumulative fluence than for the same fluence followed by a single liquid-holding treatment to give the maximum recovery effect. Both of these experiments show

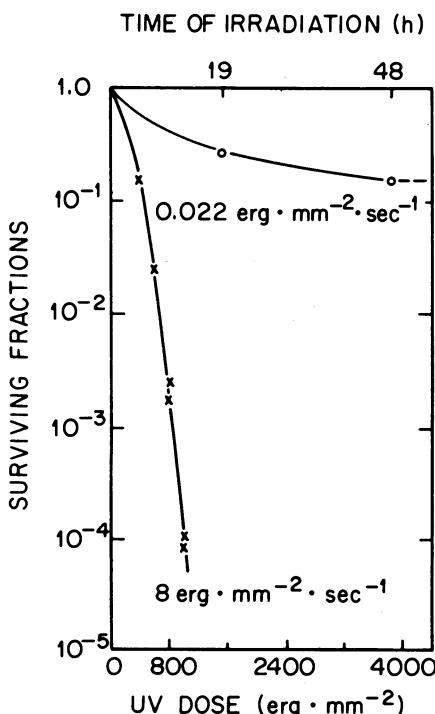


Fig. 13. Survival of UV-irradiated *E. coli* B/r as a function of fluence at two fluence rates. (Modified from Harm, 1968c.)

that the numbers of lesions in DNA at any time, either because of the rate of their generation or because of intermittent creation and removal, have a profound effect on the extent of repair. Harm (1968b) assumed that when many lesions were put into DNA at the same time as when a large uninterrupted fluence was given at a high fluence rate, the probability of two dimers in opposite strands being near to each other would be quite high. Upon excision and enlargement of the gaps, a double-strand break in the DNA would result. Harm developed equations for the probability of two overlapping gaps to bring about such a potentially lethal event. Such calculations seem to explain the shoulder in the B/r survival curve. The analysis suggests that cells are more likely to die because of unsuccessful repair than because of either inaccurate repair or lesions that cannot be repaired. Harm suggests that the latter group of lesions may account for the nonsurvivors in low-dose-rate and sector-irradiation experiments.

Liquid-holding and photoreactivating treatments have also been used in combination by Moss and Davies (1974) to provide data to explain the responses to radiation by wild-type and *recA* strains of *E. coli* K-12. It will be recalled that in *E. coli* K-12 the LHR process depends on the *uvr* gene product and is seen only in *recA* cells (Ganesan and Smith, 1968a). Moss and Davies (1974) showed that UV-irradiated, wild-type cells may even lose viability during liquid-holding treatment if the UV fluence is too high; if photoreactivating treatment is given after varying lengths of liquid-holding treatment, the photoreactivability is eventually completely lost. These authors use Harm's model (Harm, 1968b) for irradiation death, in which overlapping excised regions are lethal events. Thus, they propose that in the wild-type cells the excision system normally repairs a certain number of dimers and the postreplication repair system completes the repair of DNA. During LHR, the excision system repairs a greater number of dimers than normal and the chance of lethal overlapping excised regions is increased. Photoreactivating treatment given immediately after UV irradiation decreases the number of dimers without creating strand breaks, but if LHR precedes PR treatment, the latter becomes progressively more ineffective. Although in *recA* strains only excision repair is operative after PR treatment, one is dealing with fewer lesions that must be repaired, and therefore the probability of overlapping excised regions is lowered. *recA*, with a two-component survival curve, shows a less steep slope in the higher fluence range than in the lower range. Moss and Davies (1974) find that the higher UV fluences cause the growth rate to be lower immediately after irradiation and suggest that this growth delay allows more time than normal for completion of excision repair. They developed an equation for the probability of the occurrence of overlapping excised regions in UV-irradiated *recA* cells that are given minimal liquid-holding treatment. The experimental points for survival after various UV fluences fit closely to the theoretical curve.

Bonura and Smith (1975) presented experimental evidence that double-strand breaks arise enzymatically during excision repair in UV-irradiated wild-type (*uvr⁺*) *E. coli* K-12 cells, and suggested that these may be lethal events. The breaks begin to appear about 15 min after radiation and are maximal 65 min later. The number of DNA molecules having double-strand breaks increased with fluence between 20 and 120 erg/mm². *E. coli* K-12 *uvr⁺* cells with mutant genes for DNA repair (*recA*, *recB*, *polA1*, and *exrA*) showed a greater number of these breaks after uv irradiation than wild-type cells, but no double-strand breaks appeared in the DNA of *uvrA* cells after fluences between 50 and 160 erg/mm².

In this section we have seen several interpretations of survival curves based on inability of repair systems to cope with an excess number of defects in DNA. Haynes (1966) proposed that the shoulder on survival curves was caused by the operation of repair systems, and that as fluences became high enough, repair was less efficient. Achey and Billen (1969) followed repair replication in *E. coli* after various fluences of UV radiation and found that there was a linear relationship between the fluence and the amount of synthesis up to 200 erg mm⁻²; above that fluence the amount increased slowly with fluence. A similar finding was made for the number of single-strand breaks (or alkali labile bonds) remaining in DNA after time was allowed for repair to take place. Achey and Billen (1969) suggest that the shoulder in the UV survival curve for *E. coli* B/r may be interpreted as due to efficient functional dark-repair systems; the linear part of the survival curve is interpreted as a region in which the capacity of the cells to carry out some step (perhaps repair replication), after the initial incision, is exceeded.

Another evidence for the falling off of repair efficiency with an increasing number of photoproducts is that above a certain fluence, as the number of thymine dimers increases in the DNA of UV-irradiated *E. coli* the fraction of dimers excised becomes smaller (Setlow, 1964). Boyle and Setlow (1970) confirmed this point and showed that at relatively low fluences, corresponding to those in the shoulder region of the inactivation curve of *E. coli* B/r, the rate of dimer excision was proportional to the number of dimers formed (Fig. 14). When the rate of excision became relatively constant, inactivation of cells occurred at an increasing rate.

10.2. Plating Media

Another way of enhancing recovery of colony forming ability after UV irradiation of certain *E. coli* strains grown on a complex medium is to plate the cells on a minimal rather than a complex medium. The effect, first

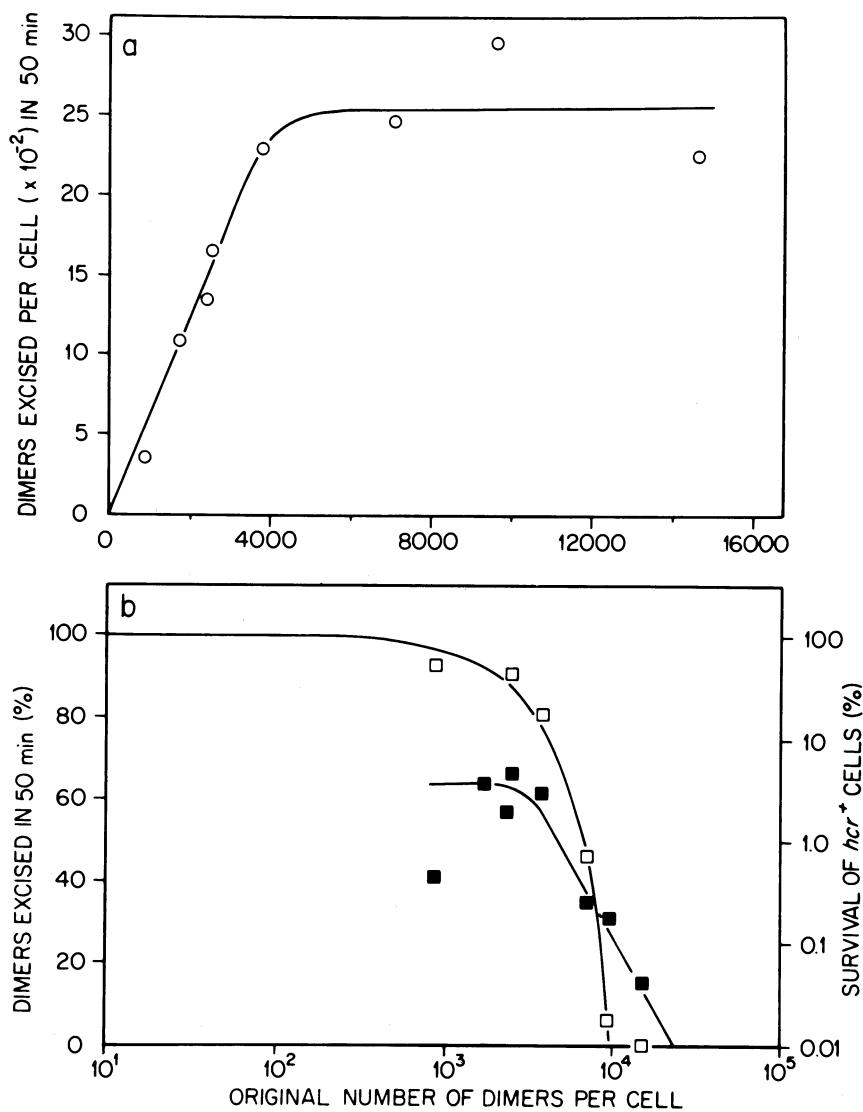


Fig. 14. Inhibition of excision by large UV fluences. (a) Number of dimers excised per cell from bacterial DNA during 50-min incubation of *Hcr*⁺ cells is plotted as a function of the number of dimers originally present in each cell. (b) The fraction of dimers excised per *Hcr*⁺ cell in 50 min versus number of dimers originally present in each cell. (■), excision of dimers from bacterial DNA; (□), survival curve of *Hcr*⁺ cells. (Modified from Boyle and Setlow, 1970.)

described for *E. coli* B by Roberts and Aldous (1949), since has been found in several repair deficient mutants of *E. coli* K-12. The term "minimal medium recovery" (MMR) was used by Ganesan and Smith (1970) to describe the effect in *E. coli* K-12 *uvr* strains but is used in this review for all plating medium effects where survival after UV radiation is higher on minimal than on complex media. Roberts and Aldous (1949) grew *E. coli* B on nutrient broth before UV irradiation, and then plated on minimal and nutrient agar plates. Although stationary and log phase cells gave inactivation curves of different shapes, the cells plated on minimal medium always showed the greatest recovery. Similar results for *E. coli* B were reported by Alper and Gillies (1960a) and by Hill and Simson (1961).

Alper and Gillies (1958) reported that when UV-irradiated *E. coli* B cells were plated on Difco nutrient agar and Oxoid blood agar, survival was lower on the Oxoid blood agar (the richer of the two media). Subsequently, Alper and Gillies (1960a) found that, for several media including a minimal one, survival of *E. coli* B after UV irradiation was highest on the media where growth was the slowest. This idea has had great influence on radiation biology over the last 15 years. The conditions for LHR and MMR seem to operate on UV-irradiated *E. coli* B cells in the same way. This notion is supported by the observations by Alper and Gillies (1960a) and by Ganesan and Smith (1968a,b) that rich nutrients such as yeast extract interfere with LHR.

Another indication of the relationship between LHR and MMR in *E. coli* B is seen in an experiment of Alper and Gillies (1960b). They incubated UV-irradiated cells in liquid nutrient broth and synthetic medium for various times up to 5 h, and then plated them on agar plates containing either medium. During the 5 h incubation period in liquid nutrient broth, the "recovery" effect produced by plating on minimal plates was completely lost. Likewise, during incubation in liquid minimal medium, the extra loss in viability produced by plating on nutrient broth plates disappeared altogether.

For other derivatives of strain B, slow growth after UV irradiation does not always favor high survival. For example, Roberts and Aldous (1949) found that survival of UV-irradiated *E. coli* B/r initially grown on nutrient broth was identical on both plating media, but Alper and Gillies (1958, 1960a), doing the same kind of experiment, found it was higher on rich than on minimal medium. Boyle and Swenson (1971) and Swenson and Schenley (1970b) have found that survival of UV-irradiated B/r cells is invariably higher on richer growth and plating media. On the other hand, B/r cells show a large LHR effect (Harm, 1968c). As has already been mentioned, B_{s-1} shows only slight LHR, and for MMR (Hill and Simson, 1961) the effect at most is only slight. It seems clear that in *E. coli* B, MMR is in-

fluenced by the unsuppressed *lon* gene as in *E. coli* B and that suboptimal growth conditions do not always enhance survival of *E. coli* B/r.

No MMR effect is seen for wild-type (repair-competent) *E. coli* K-12 strains, but Ganesan and Smith (1968a, 1969, 1970) have found such responses by *uvr* and certain *rec* mutants. The effect for *uvr* cells (Ganesan and Smith, 1970) is shown in Fig. 15. Although *uvr* cells do not carry on LHR (Ganesan and Smith, 1968a, 1969), some kind of repair or recovery process goes on in liquid minimal medium that eliminates the MMR plating effect. Ganesan and Smith (1971) irradiated *uvr* K-12 cells, allowed them to recover in liquid minimal medium, and plated at intervals on rich and minimal agar medium. Plating cells that received 63 erg mm⁻² on rich medium gave lower survivals, presumably because of inhibition of a recovery step, until 5 h had elapsed. During the incubation in liquid minimal media for these 5 h, the number of viable cells measured on minimal plating medium remained relatively constant; after that time the number increased exponentially. This division delay was shortened by PR treatment. DNA synthesis continued during the 5-h period, and the newly synthesized DNA was discontinuous until about 4 h had elapsed. Thus, repair and recovery of ability to divide required about the same amount of time.

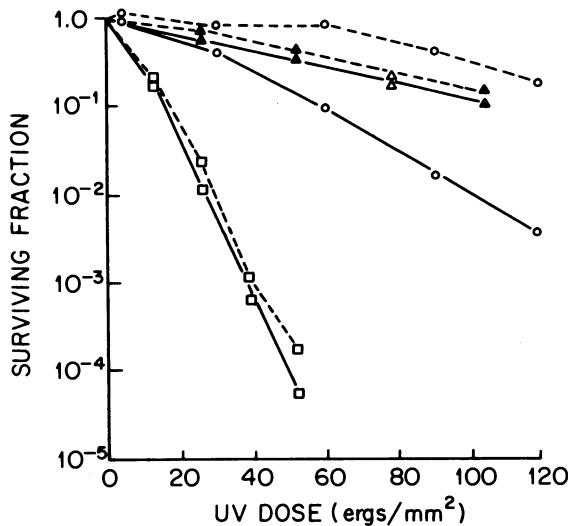


Fig. 15. Survival of *E. coli* K-12 SR87 (*recB21 uvrB5*) compared to its parents, AB2499 (*rec+* *uvrB5*) and JC5743 (*recB21 uvr+*). Cultures growing exponentially in minimal medium were irradiated and plated on yeast extract nutrient broth agar (solid lines) or minimal medium agar (broken lines). Symbols: (○) AB2499; (△) JC5743; (□) SR87. (From Ganesan and Smith, 1970.)

It will be recalled from Section 10.1 that LHR in *E. coli* K-12 occurs only in *recA* strains. Ganesan and Smith (1968a) studied LHR and the effects of plating on rich (containing yeast extract) and minimal medium on *rec* strains of *E. coli* K-12; five *recA* strains showed both LHR and greater recovery on MMR. One strain, *recB21*, showed little LHR but did show significant MMR. Strain *recC22* showed neither effect. Ganesan and Smith (1968a) have suggested that yeast extract, which inhibits LHR and MMR in *recA* strains, does so by inhibiting some repair process, presumably excision repair.

In summary, several *E. coli* strains, when grown on complex media, exhibit biological responses to UV radiation involving higher survival when plated on minimal than on complex media. These responses, collectively termed MMR, have been seen in *E. coli* strain B and in *uvr* and certain *rec* strains of *E. coli* K-12. Although the responses of all strains are outwardly similar, the underlying biochemical reasons are probably quite different.

Van der Schueren *et al.* (1974) warned that impurities in certain agars used for plating irradiated cells may inhibit repairs and give spurious results. Wild-type and *polA1* cells were most sensitive to these impurities; *recA*, *recB*, *exrA*, and *uvrB* cells were not affected. When incubated for 60 min in liquid minimal medium supplemented with only required nutrients, the wild-type and *polA1* cells became insensitive to the impurities in this agar. This fact, plus the lack of initial sensitivity of *uvrB*, *recA*, *recB*, and *exrA* cells led van der Schueren *et al.* (1974) to suggest that the impurities were inhibitors of a branch of the excision repair system that involves the *rec* and *exrA* genes.

In this and the previous two sections, we have seen that the sensitivity of repair-competent *E. coli* B/r cells depends on the phase of growth, that survival can be increased by liquid-holding treatment (Harm, 1968c), and that survival of irradiated B/r cells grown on rich medium is lower when plated on minimal than on rich medium (Alper and Gillies, 1960a). In the Alper and Gillies experiments the lower survival on minimal medium was caused by the loss of a shoulder in the survival curve and not by a change in the slope of the curve.

Rude and Alper (1972) extended these observations by using *E. coli* B/r cells grown on glucose minimal medium and, after irradiation, plated on media of various degrees of richness. Glucose-grown cells always yielded a survival curve with a shoulder when plated on glucose minimal plates; when these irradiated cells were plated on lactose minimal or arabinose minimal plates, exponential curves resulted. However, when plated on minimal plates (no carbon source) with casamino acids added, a shouldered curve like that on glucose minimal plates resulted. It is interesting that when cells are grown on nutrient broth and plated on the same type of solid

medium and on glucose minimal plates, the survival curves are shouldered and exponential, respectively (as was shown also by Alper and Gillies, 1960a). In these experiments of Rudé and Alper (1972), holding the washed, irradiated cells on water-agar plates for several hours before final plating on the less rich medium yielded a shouldered curve similar to that obtained by plating immediately on the richer medium. One interpretation is that more efficient repair is favored for the cells plated immediately on the richer medium. Such an interpretation is difficult to reconcile with the situation for *E. coli* B, where increased survival on minimal medium is usually attributed to enhanced repair under slow growing conditions. Rudé and Alper (1972) developed a different model based on the fact that in a nutrient medium the synthesis of many enzymes is repressed and, upon a shift down to a minimal medium, multiple derepression occurs (Schaechter, 1973). They do not speculate on the types of enzymes that are involved in the survival decrease but present a thought-provoking experiment which involved a wild-type strain, with an inducible *lac* operon, and a mutant constitutive for the production of β -galactosidase and other enzymes in that operon. Both strains were grown on glucose and plated on either glucose minimal or lactose minimal agar. All curves were shouldered and nearly identical except for the wild-type strain plated on lactose minimal agar. Thus, operons that may be repressed and derepressed appear to play a role in survival after UV irradiation. This point will be explored further in Section 12.

10.3. Chloramphenicol

Alper and Gillies (1958) showed that UV-irradiated *E. coli* B cultures survived best under suboptimal growing conditions. They obtained strong support for this hypothesis when they demonstrated that survival increased if irradiated cells were plated for a short time on chloramphenicol agar before transfer to nutrient agar (Gillies and Alper, 1959). They also found that if UV-irradiated *E. coli* B cells were incubated on nutrient agar for a short time before transfer to chloramphenicol agar and subsequently to nutrient agar, the survival increase was even greater. The greater efficacy of chloramphenicol treatment on increasing survival of *E. coli* B was also shown by Suzuki and Iwama (1960) and Drakulić *et al.* (1966). Brown and Gillies (1972) used the delayed chloramphenicol treatment on UV-irradiated, log-phase *E. coli* B cells and followed the number of single cells, filament dividing cells (clones), and ghosts (lysed filaments). Compared to the untreated cells, the numbers of filaments and ghosts were reduced and the number of colony formers (from filaments and nonfilamentous cells)

was increased. Brown and Gillies (1972) proposed that lysis of filaments in irradiated *E. coli* B occurs because cell-wall synthesis cannot keep pace with general cell growth. Chloramphenicol, they suggest, allows cell-wall thickening while preventing cell growth; the result is that filaments do not lyse when the chloramphenicol is removed. The walls of *E. coli* filaments are different from those of normal cells (Weinbaum, 1966), and chloramphenicol does not inhibit cell wall formation in certain bacteria other than *E. coli* [see Brown and Gillies (1972) for references]. Another suggested way in which the inhibition of growth by chloramphenicol brings about rescue in *E. coli* B cells is by permitting more time for repair of lesions that inhibit cell division. Morozov and Myasnik (1970) incubated *E. coli* B_{s-1} in liquid medium plus chloramphenicol before plating, and found that for exposure times up to 4 h viability increased as the exposure time to the antibiotic increased. They attributed the increase to the *Fil*⁺ or *lon* gene. Marshall *et al.* (1973) tested the effects of temporary treatment with chloramphenicol on the survival after UV irradiation of various repair-deficient strains of *E. coli* K-12. The cells were irradiated in stationary phase and no preliminary incubation on nutrient agar was carried out before the chloramphenicol treatment of 0–6 h. Wild-type and *recB* cells were not rescued, nor were those of the double mutants *uvrA recA* and *uvrA exrA*. On the other hand, *lon*, *recA*, *exrA*, and the double mutant *recA exrA* cells were all rescued after UV irradiation. The *lon* cells of *E. coli* K-12 are genetically similar to *E. coli* B cells which have an unsuppressed *lon* gene, and the temporary chloramphenicol treatment may be acting in the same manner on both strains. The involvement of the excision repair system in the rescue of *E. coli* B is suggested by the effectiveness of caffeine in preventing the rescue. The rescue of *recA*, *exrA*, and *rec exr* cells suggests that chloramphenicol rescue, like LHR and MMR, is dependent upon the *uvr* gene, but any such conclusion is weakened by the fact that caffeine does not prevent the rescue.

Although chloramphenicol treatment after UV irradiation rescues *E. coli* B cells, similar treatment to *E. coli* B/r cells after UV irradiation has been reported to kill additional cells (Alper and Gillies, 1960a). This effect on strain B/r as well as on strain 15 T⁻ was studied in detail by Okagaki (1960) and was found to require an energy source, although the effect was independent of the presence or absence of a nitrogen source. Bridges (1972) reported that post-UV chloramphenicol treatment of *E. coli* B/r WP2 (a tryptophan-requiring strain of *E. coli* B/r) removes the shoulder from the survival curve. This result is consistent with the observation that *E. coli* B/r, when plated on two different media after UV irradiation, shows the higher survival on the one providing the faster growth (see Section 10.2. on plating medium effects).

Ganesan and Smith (1972) tested for the effects of post-UV incubation

with chloramphenicol on various repair-competent and deficient strains of *E. coli* K-12. Survival of UV-irradiated wild-type, *uvrA*, *uvrB*, and *uvrC* cells was decreased by the antibiotic, but *recA* and *recB* cells were not affected. Chloramphenicol had little effect on postreplication repair of DNA in UV-irradiated *uvrB* cells, as indicated by the ability of the cells to repair discontinuities in newly synthesized DNA, a function controlled by the *recA⁺* gene (Smith and Meun, 1970). The inference drawn by Ganesan and Smith was that chloramphenicol interfered with a *recB* function that required protein synthesis.

Youngs *et al.* (1974) have shown other postirradiation effects of chloramphenicol on DNA repair in UV-irradiated *E. coli* K-12 cells grown on minimal medium. This antibiotic interferes with the closing of incision breaks of wild-type and *polA1* cells, but has little effect on *recA*, *recB*, or *exrA* cells. Chloramphenicol accelerated degradation of DNA in wild-type and *polA1* cells under these growth conditions but produced little additional breakdown in *recA* or *recB* cells.

The effects of pre-UV treatments of cells with chloramphenicol will be covered in Section 11.2.

10.4. Starvation for Essential Nutrients

The last two sections were devoted to the effects on survival of post-UV treatments that limit the synthetic abilities of the cell: liquid-holding treatment, in which irradiated cells are usually held in a buffer, and treatment with chloramphenicol. Gillies (1961) deprived auxotrophic *E. coli* B mutants of a required nutrient (e.g., tryptophan) after UV irradiation and found an increase in viability. Post-UV incubation of stationary-phase cells for 30 min on complete synthetic medium before withdrawal of tryptophan negated most of the effectiveness of the starvation treatment. There may be something in the complete synthetic medium which prevents repair and/or recovery, but the effect probably is not the same as the rich medium effect on LHR observed by Ganesan and Smith (1968a). In a similar series of experiments Kos *et al.* (1965) showed increased viability upon withholding tryptophan from a tryptophan-requiring strain of *E. coli* B, and a larger increase during the absence of uracil from a strain of *E. coli* B that requires uracil. However, in a K-12 methionineless strain, viability dropped more than 10-fold in 90 min when this amino acid was omitted from the medium. The results with *E. coli* B were consistent with those of almost all experiments reported: When growth and synthesis were inhibited after UV irradiation, an increase in viability took place.

For *E. coli* B/r a mixed set of responses occurred when, after irradia-

tion, required amino acids were withheld from several amino acid auxotrophs (Forage and Gillies, 1969). Histidine-requiring mutants showed decreased survival, a proline mutant showed an increase, and methionine- and tryptophan-requiring mutants showed no change. No correlation was observed between the amount of protein synthesis and survival or killing, but the amount of RNA synthesized immediately after UV irradiation seemed to make a difference. RNA synthesis was highest with histidine (where killing was the greatest) and lowest with proline, tryptophan, and methionine, where survival was increased or unaffected.

The effects of pre-UV starvation for essential nutrients will be covered in Section 11.3.

10.5. Acriflavine

Treatment of *E. coli* cells with acridine dyes during or after UV irradiation produces different effects. In general, if the dye is added after irradiation, full recovery is prevented, and if it is present during irradiation, a protective effect is seen. Both effects may be related to the fact that the dyes bind to nucleic acids by intercalating between stacked bases (Lerman, 1964). In the first effect, the dye might be expected to interfere with the repair of photoproducts formed in DNA by UV radiation and in the second to influence the rate of photoproduct formation.

Witkin (1961) studied the effects of acriflavine on the induction and fixation of mutations. Mutations of tryptophan auxotrophs to prototrophy were about 50 times as frequent when the cells were plated on media containing acriflavine. Likewise liquid incubation of irradiated cells in the presence of the dye for 90 min prevented mutation frequency decline. If the dye was removed before 90 min, the mutation frequency declined to its full extent. The importance of this paper is the DNA-repair hypothesis proposed by Witkin: UV-induced damage to DNA produces mutations if not repaired; mutation frequency decline represents repair without replication, and acriflavine, by binding to DNA, prevents this repair.

Witkin (1963) then showed that acriflavine in the plating medium reduced survival of UV-irradiated cells, and she used PR treatment to test whether the lesions responsible for lethality and mutation to prototrophy were the same. Four times as much photoreactivating light is required to prevent mutations when acriflavine is present than when absent, but the dye had no effect on the PR of UV-induced killing. Thus, the lesions are different for the two events. Feiner and Hill (1963) irradiated T1 bacteriophage with various fluences of UV radiation and infected *Hcr⁺* and *Hcr⁻* host bacteria, plating the complexes on normal and acriflavine-

containing agar. Acriflavine increased the rate of inactivation for *Hcr⁺* cells, but the rates of inactivation for *Hcr⁻* cells were not affected. Alper (1963) reported that acriflavine had the effect of reducing survival of *Hcr⁻* as well as *Hcr⁺* *E. coli* cells. On the other hand, Harm (1967), in a comparative study of the effects of acriflavine and caffeine on repair processes, reported that both the *Hcr⁺* and *Hcr⁻* cells showed decreased abilities to support the growth of irradiated λ phage when plated on acriflavine. In the same paper, Harm confirmed the earlier finding of Alper (1963) that acriflavine in the plating medium reduced the survival of *Hcr⁻* as well as *Hcr⁺* cells.

Additional studies with acriflavine which indicate that it interferes with HCR repair (excision repair) are found in Section 10.5. It is significant, however, that there is only one piece of biochemical evidence that acridine dyes do have an effect on repair of DNA. Setlow (1964) found that the rate of dimer excision was reduced if the UV-irradiated cells were incubated in the presence of acriflavine.

Before taking up the influence of the biological effects of UV irradiation of cells in the presence of acridine dyes, it is pertinent to examine briefly the photochemical aspects of the problem. Beukers (1965) and later Setlow and Carrier (1967) showed that one of these dyes, proflavine, when present during irradiation of DNA, prevented formation of cyclobutane-type pyrimidine dimers. Setlow and Carrier concluded, on the basis of studies on monomerization of dimers by short-wavelength UV radiation (239 nm), that proflavine prevents dimer formation by producing structural deformities in DNA and that energy transfer is not involved. Sutherland and Sutherland (1969) presented evidence that dimer formation is prevented by the quenching action of proflavine and that energy is transferred from the singlet state of the DNA to the dye. Whatever the mechanism by which acridine dyes in the irradiation medium prevent dimer formation, it seems clear that this inhibition is involved in its protective action in *E. coli* cells.

Webb and Petrussek (1966) UV irradiated *E. coli* B/r in the presence of acridine orange; the most important finding with regard to the subsequent discussion is that the bacteria were protected from the lethal effects of the irradiation. It should be noted that under anoxic conditions even greater protection was obtained.

Alper and Hodgkins (1969) carried out experiments on a variety of resistant and sensitive mutants of *E. coli* B (B/r, B_{s-8}, and B_{s-1}) and found that if acriflavine was added 30 min prior to irradiation, the cells were protected in an exact fluence-modifying way, but if the dye was added after irradiation, they were not. Exact fluence modification means that if one takes survival data after UV irradiation of an *E. coli* culture with and without acriflavine present, a fluence scale can be found for each set of

points such that both sets will fall on the same survival curve. The point is illustrated in two figures from Alper and Hodgkins' paper. Figure 16a shows fluence-response curves for *E. coli* B-H for which the ratios of fluence scales in the absence and presence of acriflavine is 9:1. Figure 16b shows that exact fluence modification by acriflavine occurs for a wide variety of repair-proficient and deficient strains of *E. coli*. There are at least two strains of *E. coli* B, B_{s-2} and B_{s-11}, that do not show fluence-modification protection after UV irradiation; these strains have been found to contain inducible colicin factors (Alper *et al.*, 1972a). As brought out in Section 11.4, some colicin factors decrease and others increase or leave unchanged the resistance of *E. coli* cells to UV radiation. Alper *et al.* (1972a) suggested that degradation of DNA after induction of the colicins could account for the sensitivity of B_{s-2} and B_{s-11} to UV radiation.

The presence of λ prophage in *E. coli* K-12-6 (λ) also prevents exact fluence modification under the conditions described above (Alper *et al.*, 1972b), and it is inferred by these workers that the lack of protection is due to killing of the bacteria by induced prophage. Since *E. coli* B shows exact fluence modification upon being UV irradiated in the presence of acriflavine, Alper *et al.* (1972a) suggested that induction of a defective

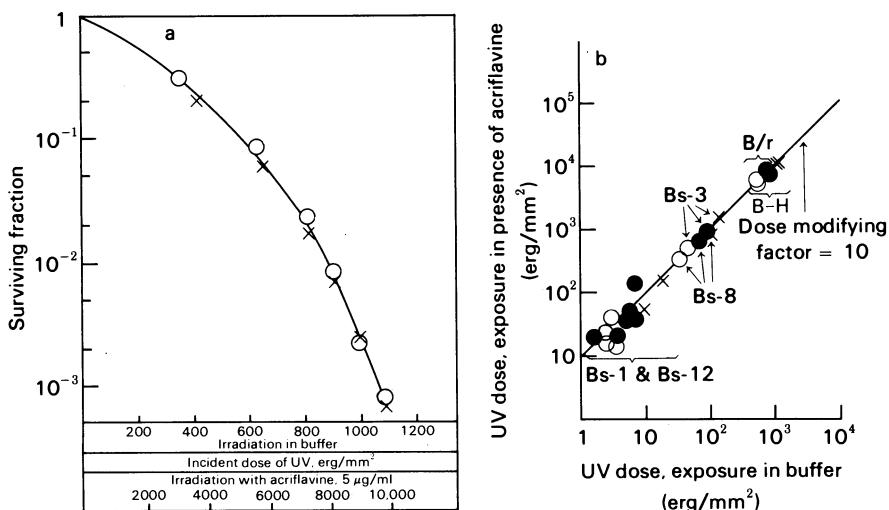


Fig. 16. Survival of *E. coli* strains irradiated in the presence and absence of acriflavine. (a) Survival curves for *E. coli* B-H. Symbols: (○), irradiated in buffer; (×), irradiated in presence of acriflavine. (b) Fluences of UV radiation which give equal survival in the presence and absence of acriflavine. Symbols for various survival levels: (Δ) 10%; (\circ) 1.0%; (\times) 0.1%. The concentration of acriflavine was $5 \mu\text{g ml}^{-1}$. (From Alper and Hodgkins, 1969.)

prophage, as suggested by Rupert and Harm (1966), does not account for the UV sensitivity of this strain. For the same reason, they ruled out Witkin's proposal (Witkin, 1967a) for induction of a lethal (division-inhibiting) protein to account for filament formation and enhanced killing of *E. coli* B. Since filaments will normally form after very low fluences of UV radiation, they would probably form even if the effective fluences were reduced by acriflavine. If the acriflavine reduces the effective fluence, there seems to be no reason why the induction of a division-inhibiting protein should be affected differently from any other protein. Of course the same point could be argued for the induction of colicins or of λ unless one infers, as do Alper *et al.* (1972b), that these inductions involve lesions other than pyrimidine dimers. It is interesting that the departures from exact fluence modification involve DNA that is not normally part of the *E. coli* genome.

It has already been pointed out that acriflavine inhibits the excision of diners in irradiated cells and that is the reason usually given to explain the lower survival of UV-irradiated cells plated on acriflavine-containing medium. Alper *et al.* (1972b) reported that these survival curves do not show exact fluence-modifying effects, and it seems clear that something other than lowering of repair rates is involved. Furthermore, acriflavine appears to bind tightly to DNA, and it is expected that if interference with repair were involved in the post-UV treatment it should also be involved in the pre-UV treatment and should prevent exact dose-reducing effects. Pre-UV treatment with acriflavine must be of at least 30-min duration to cause maximum protection effects, presumably to allow time for entry into the cell and binding to DNA. Yet, incubating irradiated cells in buffer with acriflavine prior to plating gives no protective or inhibitory effect (Forage and Alper, 1973). It appears that acriflavine is acting in several different ways and not exclusively on repair of DNA.

10.6. Caffeine

Caffeine in the plating medium of UV-irradiated cells produces two effects which strongly suggest that repair of DNA is inhibited by this purine analog; survival is lowered and an increase in mutation is observed (Witkin, 1959). In addition, caffeine decreases the survival of certain UV-irradiated phage by inhibiting host-cell reactivation (HCR) (Sauerbier, 1964). As emphasized previously, HCR seems to involve the excision repair system, and the fact that caffeine inhibits the excision of pyrimidine dimers from DNA supports this idea (R. B. Setlow, 1967; Setlow and Carrier, 1968; Sideropoulos and Shankel, 1968). Caffeine binds to irradiated native and denatured DNA but not to unirradiated native DNA (Domon *et al.*, 1970). It

also interferes with the binding of the photoreactivating enzyme to irradiated DNA (Harm, 1970). Local denatured regions in the vicinity of pyrimidine dimers may be the sites for caffeine binding. The effects of caffeine on the degradation of DNA have already been discussed in Section 5.

The section (10.1) on LHR described experiments with caffeine and acriflavine in which Harm (1966) showed that most of the LHR took place in liquid rather than during subsequent incubation on the plate. In these experiments with *E. coli* B, caffeine in the liquid-holding medium (buffer) prevents about 50% of the increase in viability gained during a 90-min period by cells in the absence of caffeine. Acriflavine was much more effective. Witkin (1959) had shown earlier that when UV-irradiated *E. coli* were treated with caffeine, the division delay normally obtained in nutrient broth was increased in proportion to the concentration of caffeine. After UV irradiation of *E. coli* B/r Swenson and Schenley (1972) used caffeine in minimal liquid growth medium containing glycerol and found essentially no increase or decrease in the number of viable cells over a 5-h incubation period before plating in the absence of caffeine. In the absence of caffeine, an exponential increase in viability began at 120 min (see Fig. 18, Section 10.7). They inferred that caffeine inhibited repair during the entire liquid incubation period, but that when cells that would normally have completed their repair and recovery events were plated in the absence of caffeine, they would still form colonies. The viability time-course curves for *Hcr*⁻ cells incubated in liquid growth medium after UV irradiation are like those of B/r cells for fluences giving comparable survival (Swenson and Schenley, 1974a), but caffeine has no effect on these kinetics (P. A. Swenson, unpublished data). This result is consistent with the finding that at low caffeine concentrations there is no lethal synergism between UV radiation and caffeine in *Hcr*⁻ cells (Clarke, 1967; Witkin and Farquharson, 1969). At higher concentrations of caffeine, lethal synergism was seen, perhaps due to effects on recombination repair (Witkin and Farquharson, 1969; Grigg, 1972). The ineffectiveness of caffeine in preventing the increased viability of UV-irradiated B/r cells during 5-fluorouracil reactivation is discussed in Section 10.7.

It is pertinent to mention that growth, cell division, respiration, and viability of unirradiated cells are affected only slightly by caffeine at the concentrations used in the experiments described here.

The comparative effects of caffeine and acriflavine on the inhibition of recovery of UV-irradiated *E. coli* cells were further studied by Harm (1967). He used several *Hcr*⁺ strains, B/r, B/r(λ), B, etc., and found that the more sensitive ones, B/r(λ) and B, show larger inhibitory effects than B/r. This extra inhibition was attributed to the absence of sufficient early repair necessary to prevent some lethal event, for example, lysis in B/r(λ).

Survival of *Hcr*⁻ cells is also decreased by the presence of both acriflavine (see Section 10.5) and caffeine, and Harm (1967) suggested two possible causes. The first is inhibition of residual repair activity persisting in the presence of caffeine, and the second is a synergistic effect between UV radiation and caffeine, and between UV radiation and acriflavine. Strains that are *Hcr*⁻ show some residual host-cell reactivation of phage T1 and this reactivation is inhibited by these chemical agents (Harm, 1967).

10.7. 5-Fluorouracil

5-Fluorouracil (FUra) is toxic to *E. coli* when the cells are incubated with this analog of uracil for a long time. For example, when included in the plating medium at a concentration of 0.50 µg/ml, fewer than 1 cell in 10⁵ survives (P. A. Swenson, unpublished data). However, the cells can tolerate FUra for several generation times during which growth is slowed down and the division time increases. FUra is incorporated into RNA and not DNA and is known to cause coding errors in messenger RNA (see Section 6.3). Ben-Ishai *et al.* (1962) first showed that incubation of *E. coli* strain W with FUra prior to UV irradiation increased cell survival compared to untreated controls. Ben-Ishai *et al.* (1965) found that in unirradiated *E. coli* cells FUra causes accumulation of RNA that is rapidly labeled with ¹⁴C-5-FUra and is inferred to be messenger RNA. The accumulation of this messenger RNA seems to be associated with the radiation resistance that developed. When the labeled messenger RNA was chased with cold uracil, the labeled FUra moved into ribosomal and transfer RNA. The authors stated that this "chase" procedure resulted in a decrease in radiation sensitivity.

Ben-Ishai and Zeevi (1967) studied the protective effects of incubation with FUra prior to UV irradiation in various strains of *E. coli* and found that only those that form filaments after irradiation are protected. Thus *Fil*⁺ B cells (*lon*), but not *Fil*⁻ B/r cells (where the *lon* gene is suppressed) show the effect. The increase in viability is associated with the fragmentation of filaments after postirradiation incubation of the irradiated *lon* cells. It was suggested that the inhibiting effects of the FUra on macromolecular synthesis were causing a growth delay and that this delay leads to higher survival.

Brozmanová *et al.* (1968) tested the effects of post-UV incubation of *E. coli* 15T⁻ cells with FUra. They treated irradiated samples for various times after UV irradiation and found about a five-fold increase in the number of viable cells over the untreated ones 1 h after UV irradiation. Thus, the *lon* gene is not essential for FUra reactivation brought about by post-UV incubation with this agent.

Boyle *et al.* (1971) studied the relationship between reactivation and the maintenance of respiration in UV-irradiated *E. coli* B/r cells grown on glycerol. Figure 17a shows the time-course curves for viability of irradiated cells without and with FUra (0.5 µg/ml) treatment. When the analog is not present, an exponential increase in viability due to cell division begins about 120 min after UV irradiation. The FUra-treated B/r cells undergo a rapid early increase (50-fold) in viability, followed by a plateau and another late increase. At higher concentrations the increase in viability is greater, but prolonged treatment in liquid medium before plating is lethal to the cells (J. M. Boyle, R. L. Schenley, and P. A. Swenson, unpublished data). Figure 17b shows that if the plateau levels for viability of irradiated cells treated

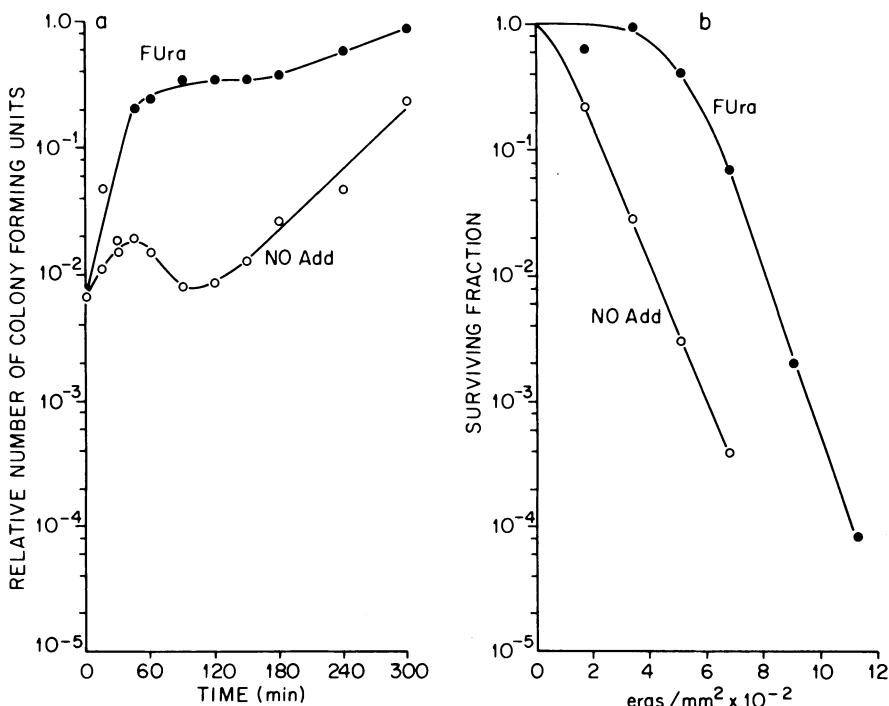


Fig. 17. Effects of 5-fluorouracil (FUra) on the viability of UV-irradiated *E. coli* B/r cells. (a) Viability time-course curves in which the number of colonies developing on the plate after sampling of cultures during a 5-h period after irradiation are compared to the original number obtained before incubation of nonirradiated cells. The UV fluence was 520 erg mm⁻²; the concentration of the FUra was 0.5 µg/ml. (b) Survival curves: "No add" means that irradiated cells were plated immediately after UV irradiation. "FUra" means that irradiated cells were incubated in liquid medium containing FUra for 60 min (the plateau level) before plating. The concentration of FUra was 50 µg ml⁻¹. (Modified from Boyle *et al.*, 1971.)

with this high concentration of FUra are plotted vs. fluence, a time-course curve with a very large shoulder is obtained in which the exponential decrease does not begin until a fluence of about 370 erg mm⁻² is reached (Boyle *et al.*, 1971). All other FUra studies by these authors and summarized in this review have been carried out with concentrations of 0.5 µg/ml.

In Section 6.3 attention was called to the fact that UV-irradiated cells lose their pyridine nucleotides; the loss of nicotinamide adenine dinucleotide (NAD) places a cell in double jeopardy because of its roles in electron transport and as a cofactor for polynucleotide ligase, an enzyme believed responsible for carrying out the last step in excision repair (see Section 4.1). Boyle *et al.* (1971) showed that of the single-strand breaks arising as a result of excision in UV-irradiated cells, many are not closed, perhaps because of the lack of NAD in cells that have stopped respiring. These investigators further showed that treatment of UV-irradiated cells with FUra causes pyridine nucleotide to be retained and that some of the single-strand gaps which appear in the irradiated population become closed.

Swenson and Schenley (1972) used caffeine to inhibit the excision repair system to see what effect such inhibition would have on the time course for viability of UV-irradiated cells in the presence and absence of FUra. Figure 18 shows that cell division is completely inhibited by caffeine for up to 5 h, but when cultures are treated with FUra and caffeine, a considerable early increase in viability still takes place. The second (late) increase in cell division does not occur in the presence of caffeine. The interpretation of these data is as follows: At time zero after UV irradiation about 1% of the cells are able to respire, as well as to complete all repair and recovery processes and to form a colony on solid medium. In the absence of FUra, caffeine in liquid medium inhibits repair and, although these cells do not divide in liquid, when plated in the absence of caffeine they complete their repair and recovery processes and go on to form colonies. A further interpretation is that a fraction of the irradiated cells die of causes associated with respiration failure.

FUra, by maintaining respiration and normal levels of pyridine nucleotides (Boyle *et al.*, 1971), enables cells that otherwise would have died to complete their repair and recovery processes and to form colonies. These cells behave toward caffeine in the same way as the regular survivors in the absence of FUra. The early increase in viability shown by FUra-treated cells (which is not affected by caffeine) is attributed to the maintenance of respiration and pyridine nucleotide levels. The late increase in viability is attributed to a delayed cell division, perhaps due to the presence of the FUra.

Swenson *et al.* (1972) confirmed the work of Ben-Ishai and Zeevi (1967) regarding the protecting action of FUra treatment before UV ir-

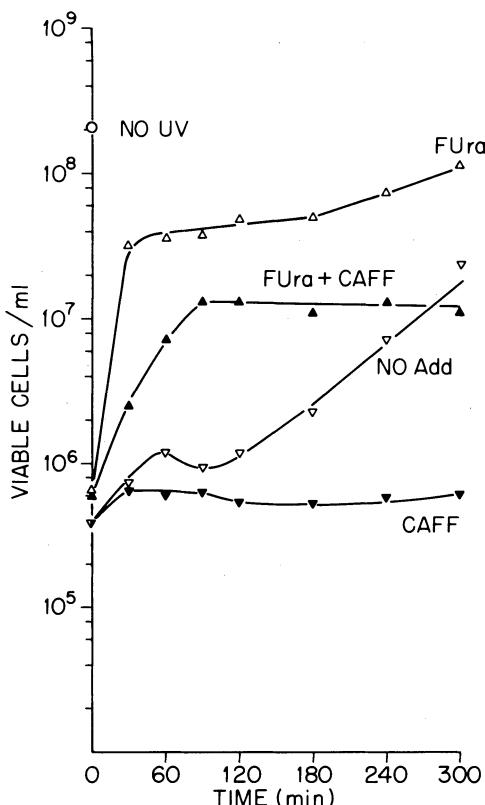


Fig. 18. Effects of 5-fluorouracil (FUra) and caffeine (CAFF) in the liquid culture medium on the viability of *E. coli* B/r cells after UV irradiation (520 erg mm⁻²). The concentration of FUra was 0.5 µg ml⁻¹ and that of CAFF 1 mg ml⁻¹. (From Swenson and Schenley, 1972.)

radiation on *Fil*⁺ (*E. coli* B) but not on *Fil*⁻ (*E. coli* B/r) cells. These experiments involved incubation of the cells with FUra for a period before irradiation and then plating the cells *directly after* UV irradiation. The FUra preirradiation treatment of B and B/r cells caused respiration to be maintained, and led to viability time-course curves for both strains (Swenson *et al.*, 1972) similar to those obtained by post-UV treatment with FUra (Boyle *et al.*, 1971). Swenson and Schenley (1970a) had previously hypothesized that FUra given after UV irradiation caused maintenance of respiration because of incorporation of this uracil analog into the messenger RNA of a protein involved in the cessation of respiration. If this model were correct for the pre-UV treatment case, the FUra might be accumulated in the nucleotide pool of the cells or incorporated into labile messenger RNA. After UV irradiation the FUra in the pool would be available for making the fraudulent messenger RNA that could not function in cessation of respiration. This hypothesis was supported by the results

of the following experiment. After the irradiation of cells previously treated with FUra, a large excess of uracil was added to the cell suspension to swamp out the FUra; the observation was that respiration did not cease and that the viability kinetics were approximately those of untreated, irradiated cells. Thus, it is clear that FUra has two effects involving increased viability of UV-irradiated *E. coli*. The first is produced by pretreatment with FUra and occurs in *Fil*⁺ but not *Fil*⁻ cells. This is the effect described by Ben-Ishai and Zeevi (1967) and is obtained by plating irradiated cells immediately after UV irradiation. The second is independent of the *Fil*⁺ phenotype and is obtained by either pretreatment or posttreatment of UV-irradiated cells. This second effect involves obtaining improved viability kinetics after UV irradiation and also seems to involve maintenance of respiration.

10.8. Pantoyl Lactone

Pantoyl lactone (PL) is an intermediate in pantothenic acid synthesis and has an effect of reversing the inhibition of cell division in certain bacteria. Grula and Grula (1962) inhibited cell division by D-amino acids, penicillin, and UV radiation in a species of *Erwinia*, and used PL to cause cell division to occur. Van de Putte *et al.* (1963) were able to inhibit filament formation in *E. coli* B by PL treatment whether the filament formation was caused by UV radiation, crystal violet, or ³²P decay. Van de Putte *et al.* (1963) with UV-irradiated, and Adler and Hardigree (1964) with X-irradiated *E. coli* K-12 cells obtained increased survival when PL was included in the plating medium. Green *et al.* (1969a) compared the action of PL on survival of *lon*⁺ and *lon* strains in the presence and absence of the *uvr* mutation; some increase in survival was effected by PL in all strains, but the largest effects were obtained with the *lon* mutants. Thus, PL may affect division in both *lon*⁺ and *lon* cells.

Harm (1966) UV-irradiated *E. coli* B cells and placed them under LHR conditions at 37°C for various lengths of time up to 3.5 h, and then plated them in the presence and absence of PL. Although both treatments seemed to be effective on a common group of cells in the irradiated culture (so-called overlapping effects), PL appeared to be more effective than LHR. Evidently LHR did not occur fast enough to overcome the inability of the cells to divide. PL in the plating medium enabled even the cells plated immediately to divide if they could complete accurate repair. Thus, LHR was not necessary for these cells. The overlapping of viability increases due to PL and those due to thermal treatment (Harm, 1966) is discussed in Section 10.9.

When UV-irradiated *E. coli* cells are incubated in liquid medium and plated at various times after irradiation there is usually an increase in viability during the first hour. This increase is followed by a decrease (called secondary death), but the viability does not fall below the original level (see Fig. 17a, Section 10.7). Finally, the cell viability begins to increase exponentially. Such an increase and subsequent decrease were reported by Barner and Cohen (1956). They interpreted the secondary death as due to the same causes as thymineless death; in each case DNA synthesis was severely inhibited while RNA and protein synthesis continued. Barner and Cohen suggested that with both UV-irradiated and thymine-deprived cells, death was due to unbalanced growth. In the case of UV radiation this notion was supported by the fact that in the absence of the carbon source (glucose), or in the presence of 5-methyl-tryptophan, the initial increase took place but the decrease did not (Okagaki *et al.*, 1960). These authors pointed out that unbalanced growth ought to occur in the entire population but that secondary death involves only a small fraction of the population. Also, DNA synthesis, which might be expected to prevent death through unbalanced growth, occurs at about the time secondary death begins. These and other considerations led Okagaki *et al.* to conclude that unbalanced growth did not lead to secondary death.

Other workers have also called attention to the similarities between thymineless death and UV-induced death in *E. coli*. For example, Cummings and Taylor (1966) showed that *Fil*⁺ (i.e., *lon*) mutants are more sensitive to both UV irradiation and thymine starvation than are *lon*⁺ strains. Bazill (1967) protected *E. coli* B_s *thy*⁻ cells against thymine starvation by incubating them with PL during the starvation period. This result is consistent with a relationship between DNA synthesis and cell-wall synthesis. Bazill (1967) argues that this relationship is disturbed in *lon* cells and causes a weakening of the cell wall. As support for his hypothesis he cites, among other work, that of Gallant and Suskind (1961), who starved a *lon*⁺ *thy*⁻ *E. coli* strain for thymine before exposing them to UV radiation; the UV inactivation curve was a simple exponential type similar to that of a *lon* strain. The interpretation is that the cell-wall synthesis of the *lon*⁺ cells was disturbed by thymine starvation, so that they assumed a *lon* phenotype.

Donachie and Hobbs (1967) have found evidence suggesting that thymineless death is completely reversible, and that death occurs after the cells have been plated on agar. Evidently the cells are fragile, because they do not die if they are not plated. Similar results were reported by Bresler *et al.* (1973), but contradictory evidence was presented by Nakayama and Couch (1973), who found the reversibility effect only under special plating conditions.

The so-called secondary death (loss in viability after an initial increase)

occurs in both B and B/r strains, and can be eliminated in both by PL (Swenson *et al.*, 1972, 1974). Since this treatment also promotes cell division in both strains, the suggestion has been made by Swenson *et al.* (1974) that the cells involved in secondary death may be those that have completed DNA replication and are about to divide at the time of irradiation (Helmstetter and Pierucci, 1968). These cells may be particularly sensitive to handling during the dilution and plating procedure. It was further suggested that PL may reduce the fragility of these cells and promote survival. This interpretation is consistent with the findings of Bazill (1967) on the rescue of thymine-starved cells by PL and with the interpretations of Donachie and Hobbs (1967) and Bresler *et al.* (1973) on the fragility of thymine-starved cells.

10.9. Thermal Treatment

In survival studies done with UV-irradiated *E. coli*, the plates are usually incubated at 37°C and, in the case of strain B/r, it makes little difference if the plates are incubated at an elevated temperature such as 42°C. However, for strain B it does make a difference in that survival is considerably higher at the elevated temperature (Anderson, 1949, 1951). Rupert and Harm (1966) summarized the work on thermal reactivation and concluded that the thermal treatment affects only those UV lesions which cause strain B to be more sensitive than B/r.

Harm (1966) found that when *E. coli* B cells were plated immediately after UV irradiation and given thermal treatment (plate incubated at 44°C), or incubated at 37°C on plates containing PL, the PL treatment produced the largest increase in survival. Both of the treatments were more effective than 3.5 h of LHR treatment followed by plating at 37°C. Small increases in viability were seen when LHR treatment preceded the two plating treatments, but the levels reached after 3.5 h of LHR treatment were about the same. Combined thermal and PL treatments upon plating immediately after UV irradiation gave the highest survival (about 100-fold greater than the control), and LHR prior to plating gave no additional increase.

In the work just discussed, as well as that reviewed by Rupert and Harm (1966), thermal treatment was given while the irradiated cells were on the plate, and, as is clear from other "recovery" studies, the irradiated cells often behave differently in liquid than on solid medium. This point is made clear by Swenson *et al.* (1974), who have shown that incubation of UV-irradiated cells in liquid medium at 42°C causes an increase in viability which they term "liquid thermal reactivation." The thermal reactivation studies were an outgrowth of work which showed that respiration in UV-irradiated

B/r and B cells is maintained when the cells are incubated in liquid medium at 42°C contrasted to the cessation which occurs at 37°C. This result is similar to that in which FUra treatment causes respiration to be maintained. In terms of the model for how UV radiation interferes with respiratory control, it has been suggested that the respiratory control protein may be thermolabile. There is no evidence to support or contradict that hypothesis.

Viability time-course studies with UV-irradiated B/r cells incubated in liquid growth medium at 42°C yield a pattern similar to that obtained with FUra-treated cells. There is a rapid initial rise in viability, a plateau, and a second rise somewhat later. The early increase in viability brought about by liquid thermal reactivation is not as extensive as FUra reactivation. The rate of early increase in viability in both FUra and liquid thermal reactivation is greater than can be accounted for by cell division. In each case the initial rise, but not the second, occurs in the presence of caffeine. Also, the second rise occurs earlier if the reactivating agents (FUra and elevated temperature) are withdrawn after 60 min. One interpretation of the thermal experiments on *E. coli* B/r is the same as that proposed for the FUra experiments. Maintenance of respiration enables some cells, which would otherwise have died, to complete their repair and survive. The plateau in the viability time-course curve is interpreted as a division delay caused by the thermal treatment. There are additional similarities between FUra and thermal reactivation which support the above interpretation regarding the relationships among respiration, repair, and viability. For example, both FUra and thermal treatments maintain high pyridine nucleotide levels in irradiated cells, and with each treatment the closure of some gaps in the single strands of DNA (presumably brought about by the excision process) is completed.

The effects of liquid thermal treatment on the viability time-course patterns of *E. coli* B (Swenson *et al.*, 1974) have been found to differ considerably from those of B/r, and this difference may be due to the fact that the growing cells form filaments. After an initial early increase in viability, as in B/r, there follows a decrease in viability (rather than a plateau as measured with B/r), and then a rapid exponential increase in the number of viable cells; the decrease may represent the thermal sensitivity of a group of filament-forming cells. PL prevents the decrease, and a plateau precedes the exponential increase. The difference between the zero time survival and that at the plateau is a measure of the degree of liquid thermal reactivation. This reactivation is approximately equal to that of plate thermal reactivation and the observation probably means that both treatments affect the same processes in the same group of cells. FUra treatment of UV-irradiated strain B cells yields a viability time-course curve that is similar to that for

strain B/r (Swenson *et al.*, 1972), and it is interesting that when thermal treatment is given concurrently with FUra treatment the response is the same as with FUra alone (Swenson *et al.*, 1974).

The shape of the viability time-course curves of strain B at 42°C (Swenson *et al.*, 1974) is in all probability due to the fact that this strain forms filaments upon UV irradiation. When given a low UV fluence, large numbers of filaments are observed, but when given a high UV fluence (in minimal medium with glycerol as a carbon source), respiration shuts off and allows practically no filaments to form (Swenson *et al.*, 1972). Treatments that favor maintenance of respiration also favor filament formation in *E. coli* B. It seems paradoxical at first that FUra and thermal treatment, which favor filament formation, also produce large viability increases, but it must be remembered that filament formation does not necessarily lead to death.

10.10. Other Post-UV-Irradiation Treatments

The post-UV-irradiation treatments discussed in Sections 10.1 to 10.9 are, for the most part, those in which considerable work has been done. A number of chemical compounds have effects that may be no less important but which have received little attention. Among those that reduce survival when added to UV-irradiated repair-competent cells are pyronine Y, coumarin, 6,9-dimethyl 2-methylthiopurine (Grigg, 1972), 8-methoxy-psoralen (Bridges, 1971), chloroquine (Yielding *et al.*, 1970), ethidium bromide (Shankel and Molholt, 1973), reductone (Alcantara Gomes *et al.*, 1970), 5-bromouracil (Aoki *et al.*, 1966), and 2,4-dinitrophenol (van der Schueren and Smith, 1974). In all cases the interpretation was that interference with repair processes is involved. Although decreased survival was produced by 2,4-dinitrophenol on UV-irradiated *E. coli* K-12 cells, van der Schueren and Smith (1974) did not observe it in a *uvr* strain, and they concluded that one of the excision repair pathways is blocked by this agent.

11. PRE-UV-IRRADIATION TREATMENTS

11.1. Photoprotection

Near-UV radiation (300–400 nm) given to *E. coli* cultures before far-UV radiation protects the cells against the lethal effects of the latter (Weatherwax, 1956; Jagger, 1960). As in the case of several other phenomena involving increased survival after far-UV irradiation, this effect

was discovered in *E. coli* B, and the work described herein has been performed on this strain unless specifically stated otherwise. Near-UV radiation causes a number of interesting physiological effects on *E. coli* which deserve attention in their own right. One effect of near-UV radiation is the transient inhibition of growth (Jagger *et al.*, 1964). Hollaender (1943) found such inhibition with mixed long-wavelength (near-) UV and short-wavelength visible light, and it was doubtlessly the near-UV component that caused the effect. In certain cases, inhibition of growth may be due to the inhibition of respiration; consistent with this interpretation is the observation that synthesis of DNA, RNA, and protein in *E. coli* B/r are all transiently inhibited immediately after irradiation with near UV (Swenson *et al.*, 1975). RNA synthesis is the most sensitive (Swenson *et al.*, 1975; T. V. Ramabhadran, personal communication). Under certain conditions, however, near-UV radiation inhibits growth without affecting respiration (J. Jagger, personal communication). All these transient responses, as well as the inhibition of cell division (Phillips *et al.*, 1967), are caused by fluences of near-UV radiation that produce only small lethal effects on repair-competent *E. coli* (Jagger, 1967); at larger fluences, which produce pyrimidine dimers, lethal effects are more prominent (Tyrrell, 1973).

Photoprotection in *E. coli* B occurs equally well if a given near-UV fluence is delivered to *E. coli* at 3 or 37°C (Jagger, 1960); in addition, the effect produced is independent of fluence rate over a 16-fold range (Jagger, 1960). These facts suggest that some cellular component is destroyed photochemically. A component of the electron transport chain would seem to be a likely candidate as a photolabile component (chromophore) (see Jagger, 1972, for summary), but there seems to be no correlation between the extent of photochemical destruction of suspected chromophores that absorb selectively in this spectral region and the biological effects observed Werbin *et al.*, 1974). Recent work by Ramabhadran *et al.* (1975) indicates that 4-thiouridine in transfer RNA is the chromophore and target for growth delay caused in *E. coli* by near-UV radiation. Ramabhadran and Jagger (1975) have evidence that partial inactivation of certain species of tRNA affects the regulation of RNA synthesis in a way similar to that of amino acid starvation. Thus, growth delay may result from a shutoff of RNA synthesis.

The action spectrum for photoprotection has a peak at 338 nm (Jagger *et al.*, 1964) as do the action spectra for both growth (Jagger *et al.*, 1964) and division delay (Phillips *et al.*, 1967), and these facts lend strong support for the idea that a cause and effect relationship exists between photoprotection and growth and division inhibition. Additional support is in the finding by Lakchaura and Jagger (1972) that 2,4-dinitrophenol causes a growth delay in unirradiated *E. coli* B cells and increased survival when in the plat-

ing medium of far-UV-irradiated cells. This concentration of 2,4-dinitrophenol is much lower than that used by van der Schueren and Smith (1974) to decrease survival of UV-irradiated *E. coli* K-12 cells.

If near-UV radiation photoprotects cells by causing growth and division delays, the near-UV treatment should be effective whether given before or after far-UV radiation injury. Near-UV radiation given after far-UV radiation does reduce the lethal effects of the latter (Jagger and Stafford, 1965), as does visible light (see Rupert, 1964 and J. K. Setlow, 1967 for reviews). The action spectrum for PR, as the effect caused by the post-UV treatment with near-UV radiation is called, shows a region of high photon efficiency between 320 and 420 nm, with minor peaks at about 330, 350, and 375 nm (Jagger and Latarjet, 1956). Two types of PR are represented in this action spectrum (Jagger and Stafford, 1965). Type I, brought about by the longer, primarily visible, wavelengths, is photoenzymic in nature and involves the monomerization of cyclobutane-type pyrimidine dimers in DNA (J. K. Setlow, 1967). Because of the enzymatic nature of the events following the absorption of light, type I photoreactivation is affected by temperature and fluence rate (see Rupert, 1964, for review). Type II PR does not involve monomerization of pyrimidine dimers (Jagger *et al.*, 1969) and has all the characteristics of the photoprotection system (Jagger and Stafford, 1965). These systems were resolved by using discriminating wavelengths—405 nm for type I and 334 for type II (indirect) applied to a normal strain which lacks the photoreactivating enzyme. The latter strain can be photoreactivated by 334-nm but not by 405-nm radiation (Jagger and Stafford, 1965).

A proposed way in which photoprotection and type II PR reduce the lethal effects is through the transient growth inhibition (growth delay), thus allowing the UV-irradiated cells a longer time for repair than would normally be the case (Jagger *et al.*, 1964). Far-UV radiation itself causes a growth delay, and sequential near-UV and far-UV treatment cause a longer delay than either by itself (Jagger *et al.*, 1964). As has been discussed previously, LHR also involves growth inhibition, and the evidence is quite strong that increased repair of pyrimidine dimers takes place. Just as LHR and type I PR cause the same total of viability increases when given singly or in combination (complete overlap of effects) (Castellani *et al.*, 1964), there is almost complete overlap between photoprotection and LHR (Jagger *et al.*, 1964). These considerations have led to the conclusion that photoprotection involves an enhancement of excision-type repair.

Photoprotecting treatment causes early division in far-UV-induced filaments of *E. coli* B (Kantor and Deering, 1967). It must not be concluded, however, that photoprotection is associated primarily with prevention of filament formation, because strains such as *E. coli* B/r, in which the *lon* gene is suppressed, and *E. coli* K-12 *recA* strains, which are *lon*⁺, can also

be photoprotected. Strain B/r shows photoprotection only when the strain is growing in log phase (Jagger and Stafford, 1965; Witkin, 1963). Photoprotection is not seen in strain B_{s-1} (Jagger *et al.*, 1964).

Swenson *et al.*, (1975) have studied photoprotection in *E. coli* B/r cells under the same conditions they used to study the effects of far-UV radiation alone on respiration, growth, and viability. Respiration and growth, closely coupled processes, continue for 1 h after UV irradiation and then cease for a time, dependent upon fluence. At a fluence of 520 erg mm⁻² at 254 nm, respiration of the culture almost completely ceases for several hours. Illumination with near-UV radiation prior to far-UV radiation reduces the initial rate of respiration to that of cultures receiving near-UV radiation alone, but does not allow respiration to be turned off as completely as with far-UV radiation alone. The kinetics of respiration, growth, DNA, RNA, and protein synthesis of the photoprotected cells are all like those of a culture that received a much lower fluence of far-UV radiation than 520 erg mm⁻². Many single-strand breaks, presumably arising as the result of excision of pyrimidine dimers, persist in the DNA of UV-irradiated cells. These breaks are effectively closed in the cells receiving photoprotection treatment. Swenson *et al.* (1975) measured the excision of dimers and found that the rate of excision and the extent of excision were slightly lower in the photoprotected cells.

In a photoprotection experiment, the viability is normally measured immediately after UV irradiation and, if the treatment is effective, the viability of the cells is greater after the photoprotection treatment. In a viability time-course experiment, the irradiated cells are allowed to incubate in liquid growth medium for various times before plating. If division delay plays a role in photoprotection, the kinetics would be perturbed in some way. Swenson *et al.* (1975) showed that the time-course curves, though vertically displaced from each other, are almost exactly alike, with the exponential increase due to cell division beginning at about 120 min in each case.

The unprotected and photoprotected cells have the same number of photoproducts in their DNA. The respiration, growth, and synthesis data show that the cells behave as though they received a lower fluence of UV radiation, yet the viability data show that repair and recovery events are completed at about the same time regardless of photoprotection treatment. Swenson *et al.* (1975) offered the following interpretation based on the hypothesis that UV radiation interferes with a respiration control system and causes respiration to be shut off: the near-UV radiation affects this control system in some way; thus it prevents cessation of respiration and the associated lethal events.

Another line of evidence suggests that growth delay is *not* the primary

factor in photoprotection. Lakchaura (1972) studied photoprotection in various repair-deficient mutants of *E. coli* K-12; rich nutritional conditions were used. Repair-competent (*uvr⁺ rec⁺*) and excision-deficient (*uvrA*) cells showed little or no response to photoprotection treatment prior to far-UV radiation, but *recA* cells showed a large photoprotection effect (Fig. 19). All three strains showed strong inhibition of growth by near-UV radiation alone. Lakchaura (1972) suggested that in *E. coli* K-12 strains growth inhibition is a necessary but not sufficient condition for photoprotection; in addition, the strain must be capable of excision-resynthesis repair and be deficient in some other type of repair, e.g., recombination repair.

We have seen that there are at least three conditions under which photoprotection takes place and each involves a mutant strain or cultural condition that gives low survival after UV irradiation. In the first case, *E. coli* B (*lon*) cells have cell division inhibited after UV irradiation. The second case, for *E. coli* K-12 *recA* cells, involves the absence of an important repair system. In the third case, B/r cells are grown on minimal glycerol medium, and UV radiation causes lethal events associated with

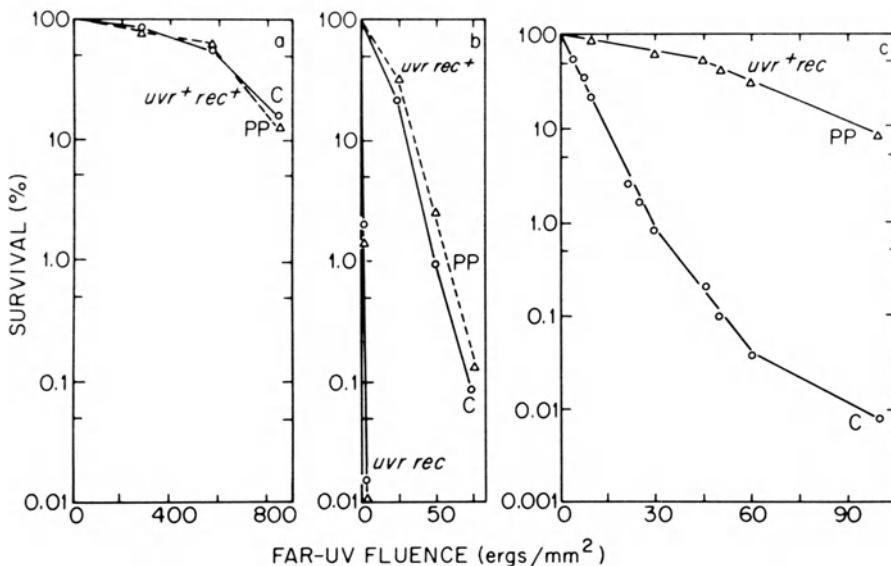


Fig. 19. Effects of photoprotection (PP) treatment (near-UV-pre-illumination) on the survival of far-UV-irradiated repair-competent and repair-deficient strains of *E. coli* K-12. Curves labeled C received no PP treatment. The PP treatments of near-UV radiation for the various strains were: *uvr⁺ rec⁺*, *uvr rec⁺*, and *uvr rec*, 3×10^6 erg mm⁻²; *uvr⁺ rec*, 3.81×10^6 erg mm⁻². The PP curves in panel (c) have been corrected for the small amount of killing by high fluences of near-UV radiation. (Modified from Lakchaura, 1972.)

respiratory failure. Growth and division delays, to allow extra time for repair, have been proposed as the common mechanism for photoprotection in *E. coli* B (Jagger *et al.*, 1964) and *E. coli* K-12 *recA* (Lakchaura, 1972) (cases 1 and 2). The evidence of Swenson *et al.* (1975) that photoprotecting treatment prevents death due to causes associated with respiratory failure (case 3) suggests that there may not be a common mechanism for photoprotection. It has been pointed out earlier in this section that the identical action spectra for growth delay, division delay, and photoprotection in *E. coli* B give strong support for the growth-delay hypothesis. However, even if the action spectra for photoprotection in cases 2 and 3 above turn out to be the same as for case 1, there is another reason for exercising caution in assigning a role to growth and division delays in the photoprotection processes in all three cases. A 334-nm maximum for action spectra is not restricted to growth delay, division delay, and photoprotection; the action spectrum for the inhibition of the induced formation of tryptophanase also has a peak at 334 nm (Swenson and Setlow, 1970), and there is little reason to relate this inhibition to photoprotection or to growth delay. The induced inhibition of the induced synthesis of tryptophanase is far more sensitive than growth. Perhaps the action spectra for these various processes are alike because the same or similar chromophores participate in as many different independent biological processes.

11.2. Chloramphenicol

Although B/r cells and wild-type K-12 cells are killed by post-UV-irradiation treatment with chloramphenicol, pre-UV-irradiation treatment with this antibiotic gives protection to *E. coli* B/r cells and to certain repair-deficient derivatives (Rude and Doudney, 1973) grown on arabinose. This protective effect is similar to that produced by pre-UV-irradiation starvation for an essential amino acid (see Section 11.3). The chloramphenicol and amino-acid-starvation pretreatments are also effective in protecting *Hcr⁻* and *rec* cells. The maximum protection effects to both *Hcr⁺* and *Hcr⁻* cells are produced by 3 h of pretreatment with either chloramphenicol (5 µg/ml) or starvation for an essential amino acid. Protection is also seen for repair-deficient *Hcr⁻ recA* cultures, with maximum protection when the cells are incubated with 5 µg/ml of the antibiotic. The protective effect on the repair-competent (*Hcr⁺ rec⁺*) strain increased the shoulder on the survival curve, but on the repair-deficient strain both the shoulder and final slope were affected. Pretreatment with chloramphenicol and prestarvation for essential amino acids cause about the same maximum increases in viability for UV-irradiated *Hcr⁺* cells, but chloramphenicol causes a greater

increase than amino acid starvation for UV-irradiated *Hcr⁻* cells. There was no difference in the amount of DNA synthesized by the *Hcr⁻* strain during the two pretreatments.

Rudé and Doudney (1974) investigated the UV resistance in streptolydigin-sensitive strains of *Hcr⁺* and *Hcr⁻* cells in an effort to learn whether the greater increase in survival of *Hcr⁻* cells during pretreatment with chloramphenicol compared to amino acid prestarvation could be due to some aspect of RNA metabolism. (Streptolydigin inhibits RNA synthesis in strains sensitive to this antibiotic). Streptolydigin and chloramphenicol pretreatment gave the same large viability increases to *Hcr⁺* cells, but the streptolydigin effect was much smaller than the chloramphenicol effect for the *Hcr⁻* cells. Thus there seems to be a special RNA-dependent UV-survival enhancement mechanism in *Hcr⁺* (*uvr⁺*) cells.

11.3. Starvation for Essential Nutrients

When *E. coli* auxotrophs are starved for essential nutrients such as thymine, uracil, or amino acids, the cells exhibited unbalanced growth. In the absence of other complications, one might expect that any of these starvation conditions would slow down growth after UV irradiation and lead to higher survival because more time would be available for repair to take place. When thymine-requiring strains of *E. coli* are incubated in liquid medium in the absence of thymine, many of the cells die (Cohen, 1971). Gallant and Suskind (1961) found that the survivors were more sensitive to UV radiation than were log-phase cells prior to starvation. Log-phase cells yielded a multihit curve, whereas the thymine-starved cells exhibited a single-hit exponential curve, with the final slopes the same. Mukherjee *et al.* (1971) also found that the final slopes of the two curves were the same, but Anderson and Barbour (1973) found both a loss of the shoulder and a change to a more negative slope. In all of the above experiments the greatest extent of inactivation was to between 0.5 and 5%. In the fluence range giving 0.5 and 5% survival for two thymine-starvation periods, Smith *et al.* (1966) also found more negative slopes of exponential curves than for log-phase cells; at higher fluences, however, these survival curves became less negative, which indicated the presence of a more resistant fraction of cells. At even higher fluences, as inactivation approached 0.05%, the slopes once again became more negative, with values close to that of log-phase cells. To determine if different repair systems were involved in the changes in the survival curves after thymine starvation, Anderson and Barbour (1973) also worked with *uvr* and *rec* strains. The change in sensitivity was less in *uvrB* and *recA*, *recB*, and *recC* cells than in wild-type cells, and this

change was due mainly to a disappearance of the shoulders of the survival curves. Anderson and Barbour (1973) suggested that after thymine starvation the coordination of *rec* and *uvr* repair is lost.

Ginsburg and Jagger (1965) compared the UV sensitivity of *E. coli* 15 TAU in three types of cultures: log phase, early stationary phase, and log-phase cultures starved for arginine and uracil prior to irradiation. The stationary and starved cells showed identical sensitivity to UV radiation, but this sensitivity was less than that of log-phase cultures. They concluded that starvation caused the log-phase cells to go into stationary phase. PR experiments indicated that the initial lesions in all three cultures were the same, but that the stationary-phase and starved cells were better able to repair the lethal damage. Considered by itself, this work suggests that the starvation and growing to stationary phase slows down growth and that these cultures have more time to repair than do log-phase cultures. Hanawalt (1966) took a different view in his work on *E. coli* strain TAU. He interpreted the large shoulder which develops on the UV survival curve of cells starved for arginine and uracil as due to all cells having completed their round of DNA replication (Hanawalt *et al.*, 1961) before irradiation. He suggested that some of the UV-irradiated log-phase cells reinitiated DNA synthesis before completion of repair replication and that the resulting strand breaks (gaps) in the DNA were lethal events.

Billen and Bruns (1970b) also looked at the starvation-induced resistance enhancement (SIRE) problem from the standpoint of the DNA replication cycle. They found a large SIRE effect (brought about through amino acid starvation) in *E. coli* B/r *Hcr*⁺ cells, but only a small effect in the *Hcr*⁻ strain. They concluded from this result, as did Hanawalt (1966) from work with *E. coli* B_{s-1} (which is *uvrB exrA*), that the major system responsible for SIRE is the excision repair system. As pointed out later in this section, other authors (Sedliaková *et al.*, 1971a; Rudé and Doudney, 1973) have found SIRE in an *Hcr*⁻ strain of *E. coli* B/r.

If excision repair is involved in SIRE, one might expect enhanced excision repair in the starved cells with high survival compared to nonstarved cells, which show low survival. Experiments to test this hypothesis were carried out by Sedliaková *et al.* (1970), but no differences were seen even when the survivals of unstarved and starved cells each irradiated at the same fluence were 3% and 52%, respectively. The amount of repair synthesis increases with increasing fluence in log-phase cells (Billen, 1970) although it saturates at high fluences (Achey and Billen, 1969). Strictly speaking, the two situations, log vs. starved cells and high vs. low fluences, are not comparable. In the first situation equal numbers of photoproducts are produced, but in the second, where different doses are used, unequal numbers of photoproducts are produced.

Sedliaková *et al.* (1971a) also showed that enhanced excision cannot

account for the increased survival brought about by preirradiation starvation. In one strain, B/r, excision was just as complete, but in another, 15T-555-7, it was much less complete. The independence of the excision repair system from the SIRE process was shown by the fact that B/r *Hcr*⁻ cells also show SIRE. Mašek *et al.* (1971) measured the degradation of DNA after UV irradiation in both prestarved and unstarved cells; the starvation treatment reduced the amount of degradation to one-half that observed in the unstarved cells. These workers suggested that prestarvation inhibits the activity of exonucleases and prevents large gaps, which are difficult to repair, from forming.

Sedliaková and Bugán (1973) looked at the size distribution of the parental DNA after UV irradiation in amino-acid-prestarved and unstarved cells where survival of the former was 170 times higher than in the unstarved culture. By 30 min after UV irradiation, the size of the single-stranded DNA decreased in both cultures and remained so for 2 h. After 3 h the DNA of the starved cells was slightly larger than that of the unstarved cells, but had not reached the size of that of unirradiated cells. No differences were seen in the velocity sedimentation patterns of the newly synthesized DNA from the two cultures. The growth curve for unstarved cells is similar to that obtained by Swenson and Schenley (1970b) with cells grown on minimal glucose medium in that there is a transient cessation of growth. One might attribute this transient cessation to the irreversible cessation of respiration by cells that are dead. P. A. Swenson and R. L. Schenley (unpublished results) have shown that in both *Hcr*⁺ and *Hcr*⁻ strains, amino acid starvation before UV irradiation allows respiration and growth to resume sooner than what is seen in cells that have not received the preirradiation treatment.

After UV irradiation, the amino-acid-prestarved cells, like the photoprotected cells, behave as though they have received a lower fluence of radiation. The kinetics of DNA synthesis during the first 2 h are also like those of photoprotected cells; in both cases synthesis is delayed for the same time as for unstarved cells or for those that are not photoprotected but, once it resumes, goes more rapidly. These kinetics are consistent with the notion that, without the treatments, cells are dying because of some cause other than faulty or incomplete repair. Such a cause might be related to cessation of respiration. Somehow the photoprotection and amino-acid-prestarvation treatments prevent death. In terms of the model for cell death associated with respiratory failure, these treatments might stabilize the repressor and prevent respiration from being shut off. Sedliaková and Bugán (1973) point out that the more rapid resumption of DNA synthesis by the amino-acid-prestarved cells seems to be a consequence rather than a cause of the high resistance of these cells to UV radiation.

In designing experiments to explain SIRE, Doudney (1971b) and Rudé

and Doudney (1973) placed emphasis on the rates at which cultures, pre-starved and not starved, resume DNA synthesis after initial inhibition by various fluences of UV radiation. Doudney (1971b) found that a semilog plot of relative rate of DNA synthesis vs. UV fluence yielded curves that resembled the survival curves of prestarved (for tryptophan) and unstarved cells. He suggested that inactivation of DNA replication accounts for death of a large fraction of irradiated cells. Doudney pointed out, however, that over half of the irradiated cells die from a fluence of UV radiation that has no measurable effect on DNA replication. But above the critical fluence—that fluence at which the maximum delay in DNA synthesis occurs—inactivation of DNA replication is seen as being an important lethal event, supplemented by events that contribute to lethality at fluences both above and below the critical fluence. One of these events might be damage to complementary strands at closely linked sites (Nishioka and Doudney, 1970) since recombinational repair could not take place.

Rudé and Doudney (1973) continued these SIRE studies and found that *Hcr*⁻ and *rec* cells also showed the effect; these experiments were done with cells grown on arabinose minimal medium and shifted to glucose minimal medium after UV irradiation. The starved and unstarved *Hcr*⁻ cells showed considerable differences in their DNA replication responses after UV irradiation. The unstarved cells stopped synthesizing DNA about 1 h after UV fluences above 20 erg mm⁻², but did not stop synthesizing if starved for tryptophan for 3 h prior to UV irradiation. Rudé and Doudney attribute the inhibition of DNA synthesis in unstarved *Hcr*⁺ and *Hcr*⁻ cells to prevention of reinitiation of replication. Their further interpretation is that starvation favors recovery of viability because reinitiation of DNA synthesis takes place.

As has been pointed out, starvation of unirradiated cells for an essential amino acid causes DNA synthesis to cease after a round of replication is completed (Hanawalt *et al.*, 1961). DNA synthesis also ceases upon addition of cytidine at a concentration of 50 µg/ml. Sedliaková (1971b) starved cells for amino acids and added cytidine at various times during starvation. In addition to stopping DNA synthesis, the riboside arrested any further increase in UV resistance. Thus, DNA synthesis appears to be involved in the generation of resistance to UV radiation during amino acid starvation.

Sedliaková *et al.* (1973) investigated the excision of dimers in UV-irradiated cells under conditions where DNA synthesis prior to UV irradiation was inhibited by thymine deprivation concurrent with starvation for tryptophan. Under these conditions the cells did not undergo thymineless death. After receiving 750 erg mm⁻², the viabilities of the unstarved and starved cultures were the same; but, while the unstarved cells excised nearly

all UV-induced dimers in 90 min, excision was negligible in the prestarved cells (Fig. 20). In the latter case, about 80% of the dimers were present 9 h after UV irradiation. Sedliaková *et al.* (1973) ruled out the possibility that dimers in the prestarved cells might be excised as a large segment of DNA which would be insoluble in the trichloroacetic acid used in the extracting procedure in the measurement of dimers. The dimers were all found in high-molecular-weight DNA, which indicates that they were not excised. Apparently not even the first incision is made in the DNA by the starved cells, because, like the *Hcr*⁻ cells, the single-strand DNA subjected to velocity sedimentation in alkaline sucrose gradients was the same size as that from unirradiated cells. Some repair synthesis did occur in the cells that were starved before UV irradiation although it was much less than in the unstarved cells. This synthesis may have been due to repair of breaks caused by thymine deprivation, since a small amount of synthesis did take place in starved unirradiated cells. These results are remarkable in that the lack of excision in *Hcr*⁻ cells is thought to be the reason for their sensitivity to UV radiation.

Brozmanová *et al.* (1974) were interested in the DNA that was synthesized in B/r *Hcr*⁺ cells starved for thymine and tryptophan prior to

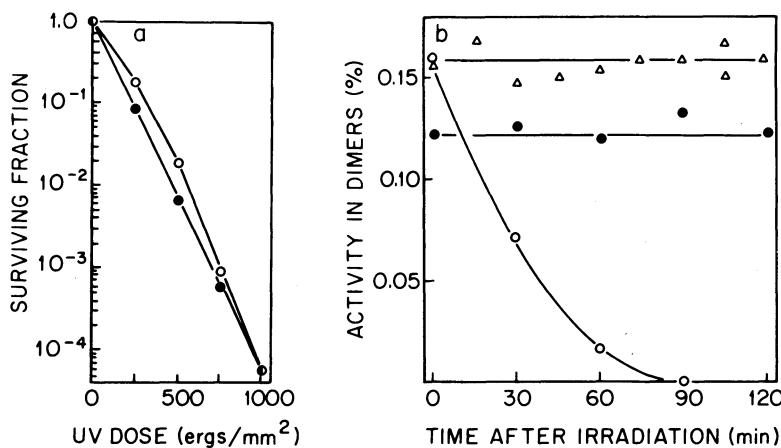


Fig. 20. Influence of 90 min of amino acid and thymine prestarvation on fluence survival curves and thymine dimer excision of UV-irradiated *E. coli* B/r cultures. After starvation the cells were irradiated and plated immediately or incubated in complete liquid medium containing thymidine. (a) Fluence survival curves. Symbols: (○) logarithmically growing cells; (●) prestarved cells. (b) Time course of thymine dimer excision after a UV fluence of 750 erg mm⁻². Symbols: (○) logarithmically growing cells; (●) and (Δ) (for independent experiments), prestarved cells. (Modified from Sedliaková *et al.*, 1973.)

irradiation. By growing the cells in density (^{13}C , ^{15}N) and radioactive ([$2\text{-}^{14}\text{C}$]thymine) label prior to starvation, and by incubating the irradiated (75 erg mm^{-2}) cells in normal medium plus tryptophan and [$6\text{-}^3\text{H}$]thymine, they could follow the density distribution of parental and daughter DNA. Despite the fact that 0.15% of the original thymine was present in the DNA as thymine dimers, normal semiconservative replication of a fraction of the cells occurred between 30 and 120 min after UV irradiation; i.e., some initially heavy (^{14}C) label moved to a hybrid (heavy-light) band, and the light (^3H) label appeared successively in the hybrid and light-light bands. Since the survival was less than 1%, some of the nonsurviving cells must have replicated their DNA. This statement is consistent with the findings of Billen and Bruns (1970a) on the dampening of DNA replication cycles after UV irradiation. Brozmanová *et al.* (1974) suggest that the dimers are repaired by postreplication repair; a copy-choice mechanism in which the opposite daughter strand is copied has also been proposed (Sedliaková *et al.*, 1973).

Recently Masková *et al.* (1974) have shown that when excision-proficient strains such as *E. coli* B/r *thy*⁻ *trp*⁻ are starved successively for tryptophan (90 min) and thymine plus tryptophan (90 min) before irradiation, excision takes place just as in unstarved cells. The amino acid starvation treatment gives the cells protection against the lethal effects of UV radiation, but subsequent thymine plus amino acid starvation gives no further protection. Thus, aligning the chromosome by amino acid starvation prevents the inhibition of excision by amino acid and thymine starvation.

11.4. Introduction of Plasmids

Enteric bacteria such as *E. coli* are capable of harboring DNA entities called plasmids; examples of these are resistance (R) factors and colicin (Col) factors. Because plasmid DNA is not integrated into the host DNA and may be transmitted from one bacterium to another, its introduction into a strain may be considered to be a pre-UV treatment. Bacteria harboring R factors are resistant to, among other things, antibacterial drugs; those cells harboring Col factors produce colicin, which kills other cells of the same or closely related strains. Sensitive cells into which Col factors are introduced are immune to colicin. From the standpoint of UV photobiology, the most interesting point about these plasmids is that, when present, they alter the UV sensitivity of the host. An effect of *col* 1 on *E. coli* was discovered independently by Monk and Devoret (1964) and Howarth (1965); each showed that this plasmid confers resistance in *E. coli* to UV radiation. Howarth (1965) also found that induction of prophage in K-12 (λ) was in-

hibited when *col* 1 was present. All plasmids do not confer protection against UV radiation to *E. coli*; Siccardi (1969) tested 31 plasmids, including R and *Col* factors and found that 15 protected, 11 had negligible effects, and 5 increased the UV sensitivity of their host. When a specific plasmid had a given effect in a UV-irradiated wild-type strain, the same effect was observed in irradiated *uvr* and *recA* hosts.

Marsh and Smith (1969) screened 50 R factors using the radiation-sensitive strain *uvrC* as a host, and selected two R factors that did not produce colicin and that showed protective effects against UV radiation; both of these factors, RE13 and RE1-290, were isolated from other *E. coli* strains. The protective effects were also seen when wild-type *E. coli* K-12 and *uvrA* and *uvrB* derivatives harbored the R factors, but not when the host was *recA* or *uvrA recA*. Thus, the protective effects, which influenced mainly the slope of the survival curves, required functional *recA*⁺ genes.

The influence of R and *Col* factors on the UV sensitivity of *Salmonella typhimurium* has also been tested, and protective effects have been found (Howarth, 1965; Drabble and Stocker, 1968; MacPhee, 1972). It is interesting that an R factor (R-Utrecht) has a protecting effect on wild-type *S. typhimurium* but not on *recA* derivatives (MacPhee, 1973).

The involvement of the *recA* gene product in the protective effects of some R factors against UV radiation suggests that some UV-inducible process may be involved. Mindful of the fact that much remains to be known (including whether the *lex*⁺ gene is also necessary) before drawing that conclusion, we call attention to unpublished work by P. A. Swenson and R. J. Wilkins that seems to link the protective effect to cell respiration. A *col*⁻ strain of *S. typhimurium* grown on minimal medium plus glycerol has its respiration shut off about 1 h after UV irradiation (520 erg mm⁻²), just as takes place in *E. coli* cells (Swenson and Schenley, 1970a), but respiration is maintained in the *col*⁺ strain. As has been pointed out in Section 6.3, the switching off of respiration is dependent upon *recA*⁺ and *exr*⁺ (*lex*⁺) genes.

12. INDUCTION THROUGH DEREPRESSION OF OPERONS

Throughout this review examples of processes in *E. coli* that are dependent upon *recA*⁺ and *lex*⁺ genes have been cited. These processes and the sections in which they are discussed are inhibition of DNA degradation (Section 5.1), cessation of respiration (Section 6.3), UV reactivation and mutagenesis in phage (Section 7.4), prophage induction (Section 7.5), filament formation in *lon* strains (Section 8), and mutagenesis in *E. coli* (Section 4.3). The *rec-lex* responses all occur in *uvr* cells, but at UV fluences of

about one-tenth of those required for *uvr*⁺ cells. All of the processes are thought to be induced by UV radiation in the sense of derepressing an operon and synthesizing special proteins that are normally not present or are only present in small quantities in unirradiated cells. The mutation and other data has led to the attractive hypothesis that an error-prone repair system is induced by UV radiation (Witkin and George, 1973; Radman, 1974). Sedgwick (1975a) has recently found evidence for such a repair system in a tryptophan-requiring *uvr* strain of *E. coli* B/r (WP2_s *uvrA*). Chloramphenicol prevented a small fraction of postreplication repair and also completely prevented fixation of mutations. The antibiotic did not affect strand joining in the *uvrA* strain nor in an *exrA* (*lex*) derivative. Only for the induction of λ prophage has a repressor been identified and the induced synthesis of proteins been demonstrated after derepression (see Hershey, 1971). Increased synthesis of a membrane protein, protein X, is seen in *E. coli* under conditions that inhibit DNA synthesis, e.g., thymidine starvation and treatments with naladixic acid and far-UV radiation (Inouye and Pardee, 1970). Synthesis of protein X does not increase in thymidine-starved *recA* cells (Inouye, 1971). Sedgwick (1975b) has found that a UV-irradiated *uvrA* strain synthesizes a protein not found in the unirradiated strain nor in unirradiated and irradiated *exrA* (*lex*) and *recA* derivatives of that strain.

There is another line of evidence that relates these UV radiation responses and implicates them in control processes. Kirby *et al.* (1967) isolated a thermosensitive mutant T-44 that grows normally at 30°C but forms long nonseptate filaments at 40°C. Presumably the filaments take a long time to form because growth shuts off at about 100 min after the temperature is raised; prophase induction also occurs in T-44(λ) cells at 42°C (Kirby *et al.*, 1967). The gene responsible for the T-44 phenotype has been designated *tif-1* (temperature induced filament) (Castellazzi *et al.*, 1972). The unirradiated *tif-1* mutant is able to reactivate UV-irradiated phage at 42°C (Castellazzi *et al.*, 1972). Likewise, mutation of UV-irradiated λ is more than threefold higher in the *tif-1* mutant at 42°C than at 30°C (Castellazzi *et al.*, 1972). The *tif-1* mutant, after low fluences of UV radiation (0.6 erg mm⁻²) shows a mutation rate 10-fold higher at 42°C than at 30°C, but the differential becomes smaller as the fluence is increased (Witkin, 1974). Chloramphenicol treatment after UV irradiation abolishes these differentials. All these responses of *tif-1* mutants at 42°C are similar to those brought about by UV irradiation at 37°C in *tif-1*⁺ cells, and the results suggest that elevating temperature for *tif-1* and UV irradiating *tif-1*⁺ cells bring about an induced coordinated response involving a cellular control system. The nature of the induction process is not known, but it is interesting that pantoyl lactone, a stimulator of cell division in UV-ir-

radiated *lon* cells (see Section 10.8) also stimulates cell division that is normally inhibited in the T-44 mutant at 42°C (Kirby *et al.*, 1972).

In Section 6.3 evidence was summarized for the involvement of DNA in the cessation of respiration of UV-irradiated cells. The model proposed for stopping respiration of UV-irradiated cells is similar to that proposed for λ induction and filament formation. A gene or operon is under repressor control, and UV irradiation causes derepression, with the formation of a respiratory control protein that stops respiration. The discovery that respiration does not cease in UV-irradiated *recA* or *exrA* (*lex*) cells relates respiration control to the group of *rec-lex* functions and enhances the plausibility of the induction model for the shutoff of respiration.

I should like to develop another line of evidence that the cessation of respiration is an induced phenomenon. In contrast to the situation for *E. coli* cells grown on glycerol where, after receiving a UV fluence of 520 erg mm⁻², the cessation of respiration is virtually complete for several hours, cells grown on glucose show only a transitory cessation of respiration after receiving the same fluence (Swenson and Schenley, 1970a). An interpretation of this result is that less of the respiratory control protein is synthesized after irradiation in the glucose-grown cells. It is well-known that glucose is an inhibitor of the synthesis of certain induced enzymes such as β -galactosidase by catabolite repression (Magasanik, 1961).

Catabolite repression by glucose involves the lowering of intracellular 3',5'-cyclic adenosine monophosphate (cAMP) concentrations either by loss to the medium (Makman and Sutherland, 1965) or by inhibition of the enzyme adenylate cyclase (Peterkofsky and Gazdar, 1974). For normal induction of β -galactosidase, cAMP must form a complex with the cAMP receptor protein (CRP) (Emmer *et al.*, 1970; Zubay *et al.*, 1970); the CRP-cAMP complex presumably binds to the promoter gene of the *lac* operon, thus allowing transcription by RNA polymerase (Riggs *et al.*, 1971). Addition of cAMP to induced cells under catabolite repression (glucose present) causes rapid synthesis of β -galactosidase (Perlman and Pastan, 1968; Ullmann and Monod, 1968). If the inhibition of respiration of UV-irradiated glucose-grown cells is less than that of glycerol-grown cells because of catabolite repression of an operon involved in the synthesis of the respiration inhibitory protein, addition of cAMP to glucose-grown cells should cause as complete inhibition of respiration as with glycerol-grown cells. Recent experiments (Swenson and Schenley, 1975) show that cAMP has such an effect (Fig. 21). The effect is concentration-dependent and is not seen with 5'AMP. This result is taken to support the induction model for stoppage of respiration after UV irradiation. It is, however, possible that cAMP may be acting in a way other than through catabolite repression. Protein kinase activities have been reported to be stimulated by cAMP in *E. coli*.

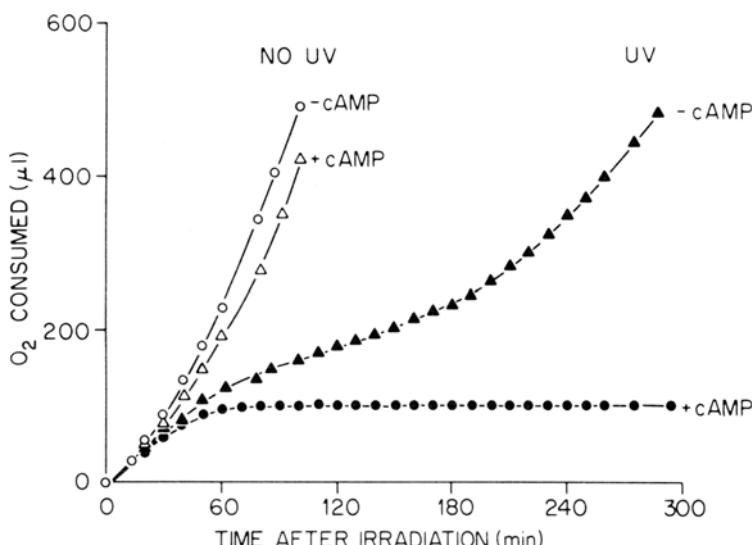


Fig. 21. Effect of 3',5'-cyclic adenosine monophosphate (cAMP) on the respiration of UV-irradiated *E. coli* B/r cells grown on glucose. The UV fluence at 254 nm was 520 erg mm⁻² and the final concentration of cAMP was 5 mM. (Data from Swenson and Schenley, 1975.)

(Powers and Ginsburg, 1974). However, cAMP seems to interact primarily with DNA in prokaryotes, and to date there is no evidence that protein kinases play a role in control processes in these organisms (Rickenberg, 1974). Regardless of the mode of action of cAMP, it is important that the use of this post-UV irradiation treatment expands the ways in which the respiration control system can be manipulated experimentally. Treatment with FUra and elevated temperature (42°C) maintains respiration and increases viability. Treatment with cAMP causes respiration to be shut off at relatively high fluences of UV radiation when cells are grown on glucose, or at relatively low fluences when glycerol is the carbon source (data not shown). Equally important is the point that post-UV irradiation treatment with cAMP causes viability to be lowered (P. A. Swenson, manuscript in preparation) just as FUra and thermal treatments increase viability.

13. THE SHAPES OF SURVIVAL CURVES AND CAUSES OF RADIATION CELL DEATH

Prior to 1960, little was known about the molecular events associated with UV radiation damage to cells. Action spectroscopy of lethal effects

had implicated nucleic acids (see Giese, 1945 for review), and attempts were made to interpret survival curves in terms of inactivation of specific targets in this genetic material (Lea, 1947). Survival curves with simple exponential kinetics were termed *single-hit curves* and those with shoulders were termed *multihit curves*. The interpretations were complicated by the knowledge of modifying genetic and postirradiation treatment effects (see review by Zelle and Hollaender, 1955).

The discovery of pyrimidine dimers and DNA repair processes made possible a new way of thinking about radiation cell death and new interpretations concerning the shoulders on survival curves. Haynes (1966) outlined the known general responses of UV-irradiated bacteria and proposed that the increased cell death which marked the transition from the shoulder to the exponential part of the survival curve was caused by a decrease in the efficiency of repair of the photochemical damage in DNA. Haynes *et al.* (1968) proposed that the position of each point on a survival curve was a function of the lethal hits (unrepaired DNA structural defects) and of the repair of these hits. As we have seen, other mathematical models involving DNA repair have been devised to explain the shape of survival curves (see, for example, Harm, 1968b; Moss and Davis, 1974). Other less quantitative interpretations of survival curves have been made, particularly regarding the shoulder region of the curves. Most, but not all, models regard the shoulder region as one in which the most efficient repair takes place. Alper and Hodgkins (1969) argue that if repair efficiency differs for two pre- or postirradiation treatments and accounts for differences in survival, a dose-modifying effect (see Fig. 16, Section 10.5) should be obtained. Development or loss of a shoulder is not a dose-modifying effect because fluence scales cannot be found for each experimental condition that will cause both sets of points to fall on the same survival curve.

The interpretation of the shape of survival curves demands the understanding of the causes of cell death and survival after irradiation. Haynes *et al.* (1968) suggested several possible causes of lethality including permanent inhibition of DNA synthesis and resumption of DNA replication before complete repair. Some other ideas on the causes of radiation cell death (and the sections in which they are discussed in this review) are listed below:

1. Strand breaks that are formed during excision accumulate at high fluences because of saturation at the excision step of excision repair (Achey and Billen, 1969) (Section 10.1).
2. Dimer excision is inhibited by large UV fluences (Boyle and Setlow, 1970) (Section 10.1).
3. At high fluences the gaps in single strands of DNA formed during excision repair begin to overlap more and more and form double-

- strand breaks (Harm, 1968b; Moss and Davies, 1974; Bonura and Smith, 1975) (Section 10.1).
4. DNA replicates prematurely during DNA repair and leads to double-strand breaks in the new duplex of parental and daughter DNA (Hanawalt, 1966) (Section 11.3).
 5. Abnormal gene dosage and DNA membrane rearrangements arise due to the original replication point not being restored and due to premature reinitiation at new replication sites (Billen, 1969; Billen and Carreira, 1971) (Section 6.1).
 6. Abnormalities arise in controlled production of functionally sequenced genome due to dampening of DNA replication over several postirradiation cycles (Billen and Bruns, 1970a) (Section 6.1).
 7. DNA replication is inactivated and not reinitiated (Doudney, 1968 (Section 6.1), 1971b; Rudé and Doudney, 1973) (Section 11.3).
 8. Derepression of operons are involved in formation of unspecified enzymes (Rudé and Alper, 1972) (Section 10.2).
 9. Derepression of an operon is involved in formation of division-inhibitor protein in *lon* cells (Witkin, 1967a) (Section 8).
 10. Cell wall synthesis in *lon* cells does not keep pace with general cell growth, which leads to lysis of filaments before division takes place (Brown and Gillies, 1972) (Section 10.3).
 11. Derepression of an operon is involved in formation of a protein which turns off respiration (Swenson and Schenley, 1972) (Sections 6.3 and 10.7).
 12. Photochemical formation of DNA-protein cross-links occurs (Smith *et al.*, 1966) (Section 2).

14. THE RELATION BETWEEN REPAIR AND SURVIVAL

It is clear from the majority of the papers covered in this review that the hypothesis upon which much of the work is based is that repair of irradiated DNA must be complete and accurate for the cell to survive. The reasons are not hard to find. DNA plays a central role in the cell, being the repository of genetic information concerning everything that the cell is and the potential for what it can be. Very slight alterations in the base sequences in DNA may cause drastic phenotypic changes. The discovery that the cell has the means of self-repair of its damaged DNA was one of the most important biological discoveries made in this century. At the time of this discovery, mutants were already available that were both radiation-sensitive

and defective in steps in DNA repair. Very soon more mutants were isolated and, since they too showed the striking correlations between repair capability and survival, it was evident that repair and survival were causally related (Howard-Flanders, 1968a). The results of numerous experiments documented in this review have supported this conclusion. One cannot help being impressed, for example, by the finding that very low fluences of UV radiation, sufficient to produce about one dimer in its DNA, are lethal to a *uvrA recA* double mutant (Howard-Flanders and Boyce, 1966).

Repair-deficient mutants have contributed enormously to the understanding of the mechanism of DNA repair. The impression is frequently given that the mastery of the details of repair mechanisms will lead to the understanding of radiation cell death. Perhaps this is true for the repair-deficient cell, but whether the same is true for the repair-competent cell is another matter. The survivors in a culture of repair-competent cells seem to carry out DNA repair in a way that leaves their descendants quite free of imperfections; the mutagenic rate, based on mutants per survivor per fluence, is remarkably low. This fact attests to high-quality repair, at least in the areas of the DNA where it makes a difference as far as survival is concerned. However, there seems to be no good correlation between mutagenesis and survival either in fluence response experiments or in single-fluence experiments with different pre- and postirradiation treatments. Part of the difficulty in this type of experiment is that only one or a few types of mutants are screened for in any given experiment.

While there are many reports on the differences between the repair capabilities of repair-competent and repair-deficient strains, there are relatively few on repair-competent cells under conditions where, after a given UV fluence, survival varies widely depending on pre- and postirradiation conditions. Boyle *et al.* (1971, and unpublished data) found that the rate and extent of excision of pyrimidine dimers was the same (60–70% excised) for glycerol-grown *E. coli* B/r cells with and without post-UV irradiation treatments of FUra or of elevated (42°C) temperature. Each of these treatments given to irradiated cultures in liquid growth medium causes large increases in viability (see Sections 10.7 and 10.9). During these treatments greater closure of single-strand breaks in DNA (that presumably arose during excision repair) was observed than in untreated cells (Boyle *et al.*, 1971, and unpublished data). Photoprotecting treatment that increases survival decreases the rate and extent of dimer excision, but all single-strand breaks appear to become closed (Swenson *et al.*, 1975). The work of Sedliaková and co-workers discussed in Section 11.3 shows that amino acid starvation prior to UV irradiation of *E. coli* repair-competent glucose-grown cells greatly increases their radiation resistance but has little effect on repair processes (Sedliaková *et al.*, 1970, 1971a; Sedliaková and Bugan, 1973).

Furthermore, Sedliaková and co-workers starved repair-competent cells for thymine and amino acids prior to UV irradiation and found that, compared to log-phase cells, their colony-forming ability is unaltered despite the fact that very few dimers are excised (Sedliaková *et al.*, 1973). These studies underline the importance of studying repair in repair-competent cells under a wide variety of cultural and pre- and postirradiation conditions.

Often repair has been assumed to have taken place because of survival increases during post-UV irradiation treatment, or to be complete, because of lack of response to photoreactivation treatment. Repair studies have often involved examination of only one step in a repair process, e.g., excision of dimers or closure of single-strand gaps presumably created as the result of incision in the DNA. Excision measurements give no information on whether there is subsequent closure of the gaps created in the DNA; sedimentation experiments that show complete closure of single-strand gaps (breaks) in DNA brought about through excision, give no indication as to whether unexcised dimers remain in the DNA.

A serious drawback in many repair-type experiments is that they are performed with a mixed population after UV irradiation—cells that have died (or are destined to do so) as well as cells that are destined to survive and give rise to progeny. At low fluences photoproducts are formed at levels too low to be detected, and at high fluences the majority of the events being followed takes place in dead cells. When complete excision is not observed in an irradiated culture, it can be argued that the live cells are repairing and the dead ones are not. Clearly, the resolution of the problem of whether surviving cells carry out complete repair can be achieved only if live and dead cells in an irradiated population can be separated. Fisher *et al.* (1975) have recently achieved this separation. The method is based on the fact that UV irradiation (520 erg mm^{-2}) of *E. coli* B/r cells grown on glycerol causes nearly all of them to stop respiring (Swenson and Schenley, 1970a) and become vulnerable to the detergent Triton X-100 (Swenson and Schenley, 1974b). This vulnerability is indicated by a loss of turbidity of the culture. Khachatourians *et al.* (1974) noted many small cells after detergent treatment and were able to separate pure populations of small dead cells by centrifuging the culture through a 10–20% sucrose gradient. The small cells separated as a distinct band near the top of the gradient. These workers were unsuccessful in gaining a large enrichment of living cells. Fisher *et al.* (1975) used a lower UV fluence (200 erg mm^{-2}) to give about 10% survival and a 1–5% neutral sucrose gradient with the cells uniformly distributed through the gradient. After each centrifugation, the cells at the bottom of the gradient were resuspended in a new gradient and recentrifuged. At the end of three cycles, pure (> 99%) populations of live cells were obtained.

For the first time an answer to the question concerning the extent of excision in live vs. dead cells was attainable. Subsequent experiments showed that 60% of the dimers were excised by unseparated irradiated cells; after separation of pure live and dead cells, equal numbers of dimers were found in each as long as 180 min after UV irradiation (Schenley *et al.*, 1975). The number of dimers remaining were equivalent to that which would be created by a fluence of 75 erg mm⁻². These results are not peculiar to cultures grown on glycerol since nearly identical results were obtained with glucose-grown cells. The conclusion is inescapable that complete excision repair of the DNA of UV-irradiated B/r cells, at least for 4 h after UV irradiation, is not necessary for survival.

The finding that equal numbers of dimers remain in the DNA of live and dead UV-irradiated *E. coli* B/r cells raises a number of important questions. How does a living cell tolerate so many dimers in its DNA? Is there selective or random removal of dimers from the DNA of live and dead cells? What is the ultimate fate of the dimers in the DNA in live cells? If we assume for the moment that the quality as well as the extent of repair is the same in all cells, why do some of them die and some of them live?

15. A HYPOTHESIS FOR RADIATION CELL DEATH

This final section of a review on the effects of UV radiations on *E. coli* is a speculative one in which a hypothesis for radiation cell death is presented. Many other hypotheses have been offered (see Section 13), and this reflects the multiplicity and complexity of the biochemical and physiological responses of repair competent cells. The UV-radiation problem has been studied from many angles, and this reviewer is reminded of the story of the blind men who were touching an elephant. Each man, exploring a different part of its anatomy, gave a different description of the animal. None of the proposed causes of cell death can be dismissed as an unlikely one, but there seems to be no compelling evidence for accepting any of them as a major cause.

The following points are among those that I have considered in developing an hypothesis for radiation cell death. First, repair of UV-damaged DNA is probably quite accurate, as evidenced by the fact that UV-induced mutations are infrequent compared to the number of photoproducts formed. Second, in several cases where repair and survival were studied in repair-competent cells under conditions where survival varied widely after the same fluence of UV radiation, poor correlations between the two were found. Third, pyrimidine dimers have remained in the DNA of surviving cells that

have gone through several cell divisions after UV irradiation. Fourth, the same number of residual dimers has been found in the DNA of surviving and nonsurviving cells. Fifth, many diverse pre- and postirradiation treatments affect survival after UV radiation. Sixth, UV radiation induces, presumably through derepression of operons, a number of secondary effects, including cessation of respiration, that are dependent upon *recA⁺* and *lex⁺* genes. The first four points (see Section 14) suggest that faulty or incomplete repair is not a large factor in radiation cell death. The last point demonstrates that a small number of photoproducts in DNA of *E. coli* can cause widespread biochemical and physiological effects.

A hypothesis for radiation cell death in repair-competent cells based on the above points is that induced biochemical events lead to irreversible physiological changes that ultimately mark the death of the cell. The hypothesis will be referred to as the irreversible physiological change (IPC) hypothesis. It is proposed that following radiation damage to DNA there is set in motion a well-ordered series of repair and recovery events. These events, including the removal of certain critical dimers, take place under a wide variety of cultural conditions. Completion of these events would normally be sufficient to assure the survival of nearly all of the cells were it not for an induced irreversible physiological change. The physiological changes could act in such a way as to cause death without affecting repair processes or they could act by interfering with them. An example of the first case is in *lon* cells where UV radiation is thought to cause induction of a division-inhibitor protein. Hence, some of the irradiated cells die because of irreversible inhibition of cell division (see Section 8) and not because of any repair deficiencies. An example of the second case is cessation of respiration accompanied by loss of pyridine nucleotides in repair-competent cells (see Sections 6.3 and 10.7). The loss of NAD would prevent closure of single-strand breaks arising through excision. Certain pre- or postirradiation treatment would prevent induction and the subsequent irreversible events; thus essential repair could be completed and most of the cells, if irradiated at relatively low fluences, would survive. Closure of single-strand breaks *without* an increase in excision has been observed in photoprotected cells (Boyle *et al.*, 1971) and in FUra-treated cells (Swenson *et al.*, 1975). Although the IPC hypothesis states that death does not occur primarily from incomplete repair or from aberrations in normal repair processes, it is possible that a normal or aberrant repair event may be involved in the induction process. The signal for the UV induction of operons is not known.

We now consider how growth media and various pre- and postirradiation treatments affect survival in terms of the IPC hypothesis. There seem to be three modes of action by these treatments. The first mode involves enhanced or reduced repair of DNA. Enhanced repair is represented by PR

and LHR. PR treatment, which monomerizes pyrimidine dimers (J. K. Setlow, 1967), would, in effect, reduce the extent of the biochemical and physiological changes in the irradiated population. The same situation would apply during liquid-holding treatment in buffer (see Section 10.1), where repair would occur under conditions of limited induction of operons. Repair soon after UV irradiation in repair-competent log-phase cells would also limit the extent of biochemical and physiological changes since all of the *recA*⁺ and *lex*⁺ dependent functions take place in excision-deficient strains at low fluences of UV radiation. The hypothesis is also compatible with the observation that repair inhibitors, such as caffeine, reduce survival of UV-irradiated repair-competent cells.

In the second mode of action, induction takes place normally, but at some step in the formation or functioning of the induced protein there is interference so that respiration is maintained and viability increases. Two examples will be cited from the work on cessation of respiration. FUra is thought to participate in the formation of fraudulent messenger RNA (Swenson and Schenley, 1972), and liquid thermal (42°C) treatment is thought to inactivate the induced protein (Swenson *et al.*, 1974).

The third mode of action is on the induction process itself. Multiple repression of operons in rich media in bacteria is well-known (Schaechter, 1973), and *E. coli* B/r becomes less sensitive to UV radiation as the richness of the medium increases (see Section 10.2). cAMP, known to function in catabolite repression, causes glucose-grown cells to shut off their respiration as completely after UV irradiation as glycerol-grown cells and decreases survival at the same time (see Section 12). Photoprotection of glycerol-grown cells (Swenson *et al.*, 1975) also seems to involve an effect on an induction process because (1) respiration is maintained by more cells than normally at a given fluence; (2) an increase in survival is seen immediately after plating; and (3) the time-course curve for viability is identical with that of cells that are not photoprotected. In both the rich medium effect and photoprotection the high survival may be interpreted as the result of prevention of induction of the operon involving a protein that shuts off respiration irreversibly.

It may be that other pre- and postirradiation treatments that alter survival cause their effects by induction of operons involved in a sequence of events which lead to irreversible physiological changes. In the literature are described many experiments done under special sets of irradiation, growth, and plating conditions. Often, upon finding pre- or postirradiation conditions that give higher survival, the increase has been attributed to enhanced repair or to some special recovery or reactivation process. According to the IPC hypothesis some of these increases are due to prevention of events, associated with induction processes, that lead to death.

The terms "recovery," "recovery events," and "recovery processes" are useful in discussing death as the result of an irreversible physiological change. The meaning of the phrase "recovery from radiation damage" is clear. Any damaged cell that survives has recovered. Recovery events include repair steps and biochemical steps involved in that recovery, for example, resumption of DNA synthesis. It is significant that resumption of DNA synthesis may also be induced since protein synthesis is required (see Section 6.1). In the study of the biochemistry of the irradiated cell it is important to distinguish between the processes leading to recovery and those leading to cell death. The term reactivation is not useful in discussing death in terms of the IPC hypothesis, unless reactivation were defined as a process involved in the prevention of irreversible physiological changes in an irradiated cell.

The discussion in this section has centered around the repair-competent cell. Repair-deficient mutants have additional problems. The *uvr* mutant is subject to the same *recA⁺ lex⁺* induction events as the repair-competent parent strain, but it cannot excise the dimers that cause induction (see Section 12). The causes of cell death of the *uvr* mutant at low fluences of UV radiation may be the same as for repair-competent cells at higher fluences that give comparable survival. Other repair-deficient mutants probably die of entirely different causes. The *recA* and *lex* mutants cannot be induced by UV radiation to stop respiration (Swenson and Schenley, 1974a), but they may also lack the ability to be induced to perform other functions needed for recovery.

One can readily see the significance of certain induced processes such as the formation of an "error prone" repair system (Witkin and George, 1973; Radman, 1974) which presumably allows lesions to pass through the replication fork at the price of making a few mistakes. However, it is difficult to understand why an induced system involving irreversible respiratory failure would be developed during the evolution of *E. coli*. The unirradiated *E. coli* cell has many regulatory systems that may in turn be coordinated by genes such as *recA⁺* and *lex⁺*. In the model proposed for UV-induced cessation of respiration (Swenson and Schenley, 1972), the respiration control system is conceived as functioning under rather tight repression in normal unirradiated cells. If repression of that gene is affected by cAMP levels in irradiated cells, it can be seen how the metabolic rate might be influenced by the richness of the culture medium since glucose in the medium lowers the intracellular cAMP levels (Makman and Sutherland, 1965). When DNA is damaged by UV radiation, the repression is lifted and the system goes out of control; respiration is shut off irreversibly, and the cells are dead. The *recA⁺ lex⁺* system may have evolved for the coordination of a wide variety of cell functions and, under most conditions where environ-

mental stresses are not too great, the system works well. However, in the evolution of such a complex control system, mechanisms were not evolved for withstanding gross insults to DNA like high radiation fluences delivered during a short time period.

In this speculative section on the IPC hypothesis for radiation cell death in repair-competent *E. coli* cells, the irreversible cessation of respiration has been used as an example. Caution must be exercised in closely linking death to cessation of respiration or to any other response since induction by UV radiation may cause a biochemical response that is several steps removed from the physiological effect being studied. The question of whether the irradiated cells die because they stop respiring or stop respiring because they are dead will be answered when the biochemical events between damaged DNA and cessation of respiration are known.

The knowledge concerning induction of operons by UV radiation and ionizing radiation should play an important part in future research in bridging the gap between molecular and cellular radiation biology.

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Note Added in Proof

L. J. Gudas and A. B. Pardee in a 1975 paper entitled "Model for the regulation of *Escherichia coli* DNA repair functions" (*Proc. Natl. Acad. Sci. USA* 72:2330) have proposed a model to explain how the *rec-lex* system functions in the induced synthesis of protein X and DNA repair enzymes. Their model is based on genetic mapping information, the work of others on the behavior of certain *E. coli* mutants, and on their own studies dealing with the synthesis of protein X in *E. coli* cells treated with naladixic acid. In their proposed operon, the *tif* gene is the operator gene, the *lex* gene codes for the repressor, and the *recA* gene codes for an antirepressor; the structural genes code for protein X and for DNA repair enzymes. The steps leading to derepression of the operon include inhibition of DNA synthesis (caused by naladixic acid, UV, and a variety of other treatments) and degradation of DNA by the *recBC* nuclease; degradation products of low molecular weight participate in the inactivation of the repressor. Gudas and Pardee suggest that protein X limits DNA degradation by binding to DNA at the sites of degradation.

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